STUDIES ON CARBOHYDRATE METABOLISM IN *BIFIDOBACTERIUM*: ISOLATION, CHARACTERISATION AND REGULATION OF A SUCROSE-UTILISATION GENE CLUSTER IN *BIFIDOBACTERIUM LACTIS*

by

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CERTIFICATION OF SUPERVISOR

In terms of paragraph GP 8 of "General Rules for the degree of Doctor of Philosophy (Ph.D.)" we, as supervisors of the candidate Marla Trindade, certify that we approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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CONTENTS

ABSTRACT

Bifidobacteria are numerically important components of the human intestinal microflora and they are considered to be health promoting and beneficial. Their saccharolytic nature enables them to be involved in a direct interaction with host metabolism. For these reasons, there is currently much interest in determining ways to increase the numbers of these bacteria in the human colon. This is being achieved by introducing the bifidobacteria by oral consumption (probiotics), stimulating the growth of resident bifidobacteria using non-digestible oligosaccharides (prebiotics), or a combination of these (synbiotics). Several prebiotic substrates have been identified which selectively stimulate the growth of bifidobacteria, however further information is required with regards to the metabolism of these substrates by individual bifidobacterial species. The primary aim of the project was, therefore, to analyse carbohydrate metabolism for the identification of and/or the development of prebiotic substrates, and to provide a molecular characterisation for their utilisation.

Several carbohydrates were tested for their ability to support the growth of bifidobacteria as a sole carbohydrate source. The four bifidobacterial strains, *B. breve, B. bifidum, B. longum* and *B. lactis* were able to utilise a wide variety of substrates. Fructose-containing oligosaccharides, such as inulin, oligofructose and raffinose have been identified as bifidogenic, and all three have a sucrose bond in common. Growth experiments with *B. lactis* indicated that raffinose stimulated cell growth in comparison to glucose, while growth in oligofructose was poor, and no growth occurred with inulin as a sole carbohydrate source. To better understand the genetic mechanisms by which *B. lactis* utilises fructose-containing oligosaccharides, the isolation of the genetic systems for the utilisation of raffinose and sucrose was undertaken. Several strategies were employed. For the raffinose utilisation genes, these included screening two *B. lactis* genebanks in *Escherichia coli* on raffinose and the histochemical substrate ρ-nitrophenyl-α-D-galactopyranoside, amplification of the α-galactosidase gene by PCR using bifidobacterial gene-specific primers, construction and the subsequent screening of a genebank prepared with DNA which hybridised to a *B. longum* α-galactosidase gene probe. A clone was identified which produced weak α-galactosidase activity in *E. coli*. Although several open reading frames were identified, none showed any sequence identity to an α-galactosidase or a related protein. Transcription of *B. lactis* mRNA hybridising to the insert DNA was induced when cells were grown in the presence of raffinose and could not be detected when cells were grown in the presence of glucose. However, a distinct transcript could not be identified. Furthermore, a distinct protein band could not be identified by SDS-PAGE analysis.

A sucrose utilisation gene cluster of *B. lactis* was identified by complementation of a gene library in *E. coli*. Three genes, encoding a sucrose phosphorylase (*scrP*), a sugar transporter (*scrT*) downstream of *scrP*, and a GalR-LacI transcriptional regulator (*scrR*) transcribed divergently from *scrP*, were identified by sequence analysis. The *scrP* gene was expressed constitutively from its own promoter in *E. coli*, and hydrolysed sucrose in a reaction that was dependent on the presence of phosphates. In *B. lactis*, total sucrase activity was induced by growth in the presence of sucrose, raffinose or oligofructose, and was repressed by glucose. RNA analysis of the *scrP, scrR* and *scrT* genes in *B. lactis* indicated that their expression was influenced by transcriptional regulation, and that all three genes were induced by growth in sucrose or raffinose and repressed by growth in glucose. As well as being affected by catabolite repression, the *scr* genes, or at least *scrP*, were also regulated by ScrR. Analysis of ScrP activity using *scrR* deletion constructs in *E. coli* indicated that ScrR functioned as a positive regulator, where ScrP activity was abolished when *scrR* was deleted.

Primer extension analyses of the *scrP-scrR* divergent promoter region identified transcriptional start sites upstream of the *scrP* and *scrR* ATG start codons, immediately adjacent to palindromic sequences resembling regulator binding sites. These sequences also showed homology to the CRE (catabolite response element) consensus sequence to which the catabolite control protein CcpA binds. These binding sites are well positioned for the binding of repressors. A third palindromic sequence was identified adjacent to the *scrP* -35, and is well positioned for the binding of a positive regulator, possibly ScrR. Gel binding analyses were performed using *B. lactis* crude protein extract from cells grown in the presence of sucrose or glucose and the *scrP-scrR* promoter DNA fragment. A shift was observed with both extracts, which was inhibited when unlabelled promoter DNA was added as competitor. This could suggest that ScrP bound to the promoter fragment when sucrose-induced extract was used, and that another unidentified regulator protein bound to the promoter fragment when glucose-induced extract was used.

From the results in this dissertation, and by analogy with previously studied sucrose utilisation systems, it is proposed that sucrose is not metabolised via a PTS mechanism in *B. lactis*. Rather, sucrose is transported into the cell in its native form by the ScrT permease and is hydrolysed by a sucrose phosphorylase (ScrP), yielding glucose-1-phosphate and fructose. The glucose-1-phosphate would be converted by glucose-6-phosphate isomerase for its utilisation by the bifidus pathway of glucose fermentation. It was proposed that transcription of the *scr* genes is positively regulated by the *scrR* gene product.

ABBREVIATIONS

A_{600}	: absorbance measured at a wavelength of 600 nm
aa	: amino acids
Amp^R	: ampicillin resistance
Ap	: ampicillin
ADP	: adenosine diphosphate
ATP	: adenosine triphosphate
CcpA	: catabolite control protein
cDNA	: complementary DNA
.CFE	: cell free extract
CFU	: colony forming units
CHCA	: α-cyano-4-hydroxycinnamic acid
CL	: crude lysate
Cm^R	: chloramphenicol resistance
CRE	: catabolite response element
Da	: daltons
DDT	: dithiotheitol
ddUTP	: dideoxy uridyl triphosphate
DIG	: digoxygenin
DNA	: deoxyribonucleic acid
DNAse	: deoxyribonuclease
DNS	: dinitrosalicylic acid
dNTP	: deoxynucleotide triphosphate
EDTA	: ethylenediaminetetra-acetic acid
EI	: enzyme I of the PTS
EII	: enzyme II of the PTS
EIIA	: enzyme IIA domain of an enzymeII^{Sugar} protein of the PTS
EIIB	: enzyme IIB domain of an enzymeII^{Sugar} protein of the PTS
EIIC	: enzyme IIC domain of an enzymeII^{Sugar} protein of the PTS
F6PPK	: fructose-6-phosphate phosphoketolase
g	: gram
g	: standard gravitational acceleration
Glc	: glucose
h	: hour(s)
His	: histidine
HPr	: histidine phosphorylatable protein

H-T-H : helix-turn-helix
IPTG : isopropyl β-D-thiogalactopyranoside
IR : inverted repeat, palindrome
kb : kilobase pair(s)
kDa : kilodalton(s)
Km^R : kanamycin resistance
LB : Luria-Bertani medium
log : logarithmic
M : molar
MALDI-TOF : matrix-assisted laser desorption ionisation – time of flight
Mb : mega bases
MBP : maltose-binding protein
MCS : multiple cloning site
MF : membrane fraction
mg : milligram
MIC : minimum inhibitory concentration
min : minute(s)
mM : millimolar
mRNA : messenger RNA
MW : molecular weight
NCBI : National Centre for Biotechnology Information
NCIMB : National Centre for Marine Bacteria
nm : nanometres
No. : number
NpGal : ρ-nitrophenyl-α-D-galactopyranoside
nt : nucleotide
Nx^R : nalidixic acid resistance
OD_{600} : optical density measured at a wavelength of 600 nm
O/N : overnight
ORF : open reading frame
p : plasmid
PCR : polymerase chain reaction
PEP : phosphoenolpyruvate
P_λ : lambda promoter
P_i : inorganic phosphate
pmol : pica mol
PTS : phosphotransferase system

rDNA	: ribosomal DNA
RNA	: ribonucleic acid
RNAse	: ribonuclease
RNasin	: ribonuclease inhibitor
rRNA	: ribosomal RNA
ROK	: repressor, orf, kinase
RT	: room temperature
RT-PCR	: reverse transcriptase PCR
s	: second(s)
SD	: Shine-Dalgarno
SDS	: sodium dodecyl sulphate
SDS-PAGE	: SDS polyacrylamide gel electrophoresis
Ser	: serine
Scr	: sucrose
spp.	: species
TMG	: methyl-1-thio-β-D-galactopyranoside
Tris	: tris(hydroxymethyl)aminomethane
TS	: transcription start
U	: units
v/v	: volume per volume (in ml per 100ml)
W	: watts
w/v	: weight per volume (in grams per 100ml)
wt	: wild type
X-gal	: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

α	: alpha
β	: beta
Δ	: deletion
λ	: lambda
Ω	: ohms
ρ	: para
μ	: micro

CHAPTER 1

GENERAL INTRODUCTION

Contents

1.1. INTRODUCTION TO BIFIDOBACTERIA

Bifidobacteria are Gram-positive rods, non-spore forming, non-motile, anaerobic organisms (212, 260, 261). They are heterofermentative, producing both lactic and acetic acid as major end-products of fermentation, and, therefore, many microbiologists consider them to be lactic acid bacteria, albeit a special case (284). This genus is among the three most prevalent bacterial genera in the human colon (198), constituting up to 25% of the total population in the intestinal tracts in adults and 95% in newborns (342). There is general agreement on the important role of the gastrointestinal microflora in the health status of humans and animals (118). In accordance with this, bifidobacteria are considered to be probiotic organisms due to the health benefits that they provide to the human host.

A brief introduction to the biology and general characteristics of bifidobacteria will be presented. This will be followed by an introduction to the intestinal flora and the role that they play in the human gut, with the focus on the health benefits associated with bifidobacteria. Carbohydrate metabolism in bifidobacteria and how this relates to their function in the gut will be reviewed, and will be followed by an introduction to bacterial raffinose and sucrose utilisation systems.

1.1.1. Biology and General Characteristics of the Bifidobacteria

The classification of the genus *Bifidobacterium,* a member of the family *Actinomycetaceae*, has been a source of controversy in the scientific community since its discovery. A strain isolated from faeces of breast-fed infants in 1899 by Tissier was named *Bacillus bifidus* (18). Later, in the early 1920s, it was classified as a member of the family *Lactobacteriaceae* by Orla-Jensen based on its morphology and physiology. This work was challenged in 1934 by Weiss and Rettger, who named it a variant of *Lactobacillus acidophilus* based on its serological and fermentative patterns (331). However, in 1936, Orla-Jensen's work showed that these bacteria were a unique species and called them *Bacterium bifidum*, although they were termed *L. bifidus* in the fifth edition of Bergey's Manual in 1939 (18). Today the genus is classified into 32 different species, and they are not only part of the human microbial flora but also of various animals, including honeybees. Twelve of the species are of human origin (174). These organisms are also isolated from sewage or sewage-polluted surface waters and clinical material (314).

Bifidobacteria are anaerobic microorganisms in that they are unable to grow on plates under aerobic conditions (261). However, the sensitivity to O_2 is different among different strains and species. The principal reason for anaerobiosis is different for different strains of bifidobacteria

(212). In some strains, accumulation of H_2O_2 was found to be the principal reason for the requirement for anaerobic conditions. H_2O_2 causes a block in the fermentation pathway by inactivation of fructose-6-phosphate phosphoketolase, which is a key enzyme in the carbohydrate metabolism of this organism (discussed further in 1.3.2). Other strains of *Bifidobacterium* do not accumulate H_2O_2, and the presence of O_2 is not lethal for these strains. It is concluded that O_2 prevents growth and fermentation of these strains by establishing too high an oxidation-reduction potential.

1.1.1.1. Morphology

The colony morphology of *Bifidobacterium* is characterised as smooth, glistening, convex elevation, entire edges, circular form, soft consistency and cream to white colour (261, 314). Originally, Tissier described bifidobacteria as curved rods and rods with ends split to give the characteristic Y-shape (Figure 1.1), which led to the designation of "bifid" (298). In addition, bizarre small branches and bulbous or swollen ends were seen. Hayward *et al.* (109) noted that in unfavourable culture media the organisms were so greatly branched that resemblance to rods was lost. Other organisms were unbranched but were swollen and irregular. As the strains become adapted to artificial culture by transfers, the branched and swollen forms become less frequent until rods predominate. Although bifidobacteria have a tendency to show morphological variation when grown *in vitro*, they are mostly rod-shaped in the natural habitat. As an explanation, it has been suggested that bifidobacteria probably have a more complicated pathway of cell wall synthesis compared to other organisms (212).

Although the mechanism of induction of pleomorphism (branching) is not fully known, it appears that the presence of Ca^{2+} ions plays a principle role in its prevention (212). Whereas Ca^{2+} induces the bacilloid form, NaCl has been shown to induce the branched form. The addition of the amino acids alanine, aspartic acid, glutamic acid and serine, enables the organisms to convert to their bacilloid form. Therefore, by manipulating these amino acids and the Ca^{2+} and NaCl contents in the media, the strain of bifidobacteria can be maintained indefinitely in either a bifid or highly branched form.

A comparison of the cell morphology of large numbers of strains grown anaerobically in stabs of trypticase phytone-yeast extract medium (TPY), showed that some of the bifidobacterial species had distinct cell shapes or rearrangements which might be of help in their recognition (261). Those of *B. longum, B. breve, B. bifidum* and *B. animalis* are shown in Figure 1.2.

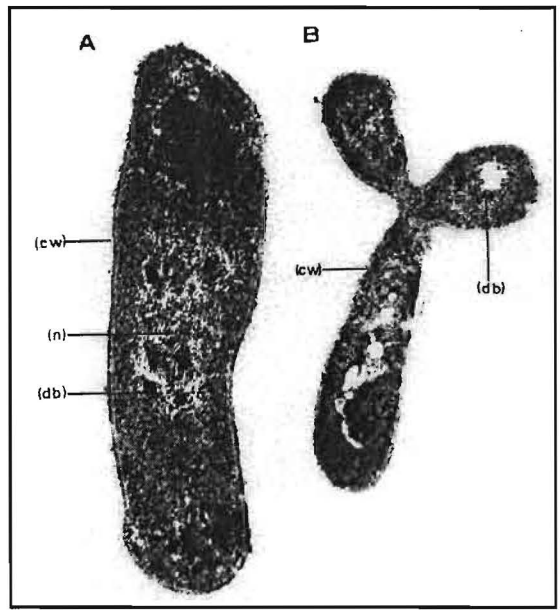


Figure 1.1. Electron micrographs of *Bifidobacterium bifidum*. A: an unbranched phase. B: the bifid phase. Cells were fixed with glutaralydehyde-osmium tetroxide and stained with uranyl acetate-lead citrate. Abbreviations: (n): nuclear area, (db) dense bodies, (cw): a distinct cell wall (212).

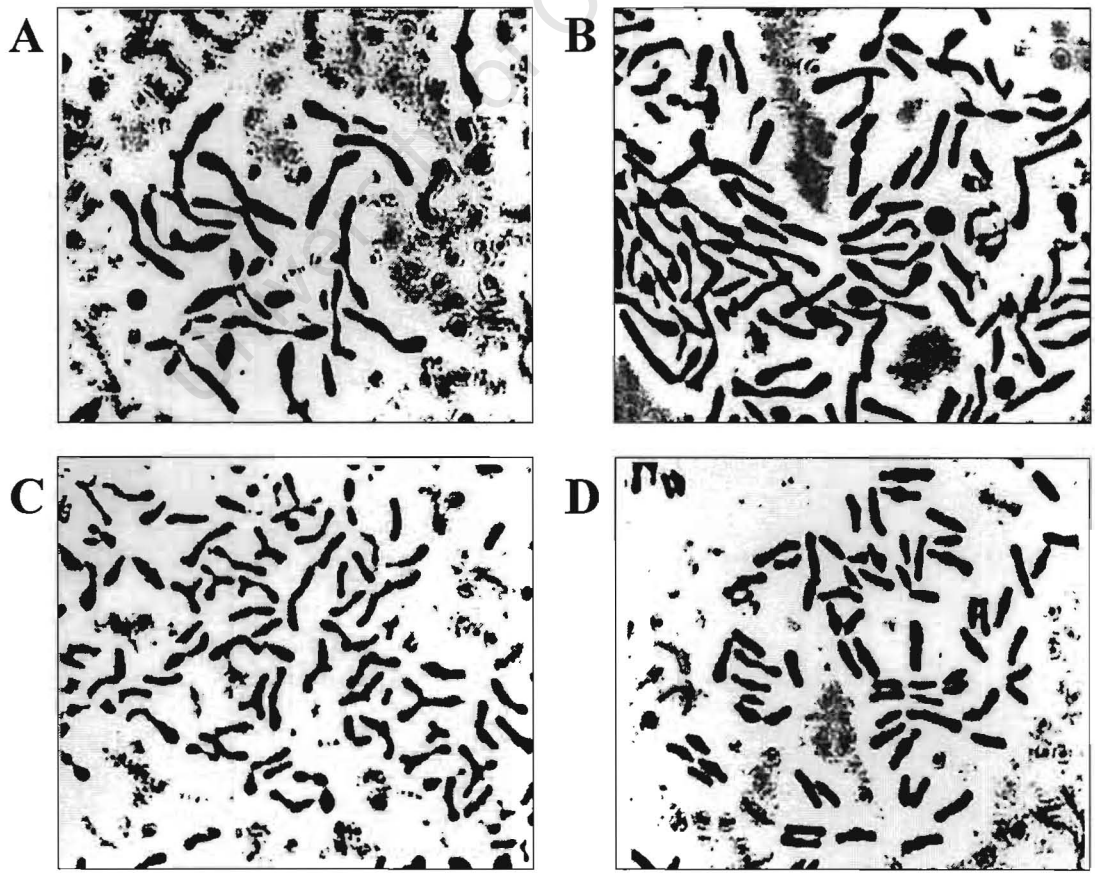


Figure 1.2. Morphology of *Bifidobacterium*. A: *B. bifidum*. B: *B. longum*. C: *B. breve*. D: *B. animalis*. Cells were grown in TPY medium (261).

1.1.1.2. Cell Wall Structure

The principle cell wall component is peptidoglycan (murein) (290). This complex material consists of linear chains of a polysaccharide composed of molecules of *N*-acetylmuramic acid and *N*-acetylglucosamine alternating along the length of the chain. These chains are crosslinked by tetrapeptides consisting of alanine, glutamic acid, and ornithine or lysine, while links between adjacent tetrapeptides may consist of one or more of the amino acids glycine, serine, aspartic acid and threonine. The amino acid composition of the basic tetrapeptide can vary between species and /or strains of the same species, as can their sequence in the chain (Table 1.1). Strain differences can also arise from the substitution of ornithine by lysine, and the extent of crosslinkage between chains, i.e. crosslinks may arise between one, two or three amino acids in adjacent sequences.

Table 1.1. Cell wall components of some bifidobacteria (290).

Species	**Type of peptidoglycan or murein**	**Polysaccharides**		
		Glucose	**Galactose**	**Rhamnose**
B. adolescentis	Lys or Orn-D-Asp	+	+	-
B. bifidum	Orn or Lys-D-Ser-D-Asp	+	+	+
B. breve	Lys-Gly	+	+	+
B. infantis	Orn or Lys-Ser-Ala-Thr-Ala	+	+	+
B. longum	Orn or Lys-Ser-Ala-Thr-Ala	+	+	+

Lys: lysine Orn: ornithine Asp: aspartic acid Ser: serine
Gly: glycine Ala: alanine Thr: threonine

Glucose, galactose and rhamnose are usual components of the cell wall structure, with qualitative and quantitative differences being observed with respect to species, strain and growth medium (Table 1.1) (290). The principal fatty acids are myristic, palmitic, palmitoleic and oleic, but the precise composition varies considerably with growth medium, the presence or absence of human milk and growth temperature. The phospholipids, such as phosphatidylglycerol and diphosphatidylglycerol are well represented, along with derivatives like alanylphosphatidylglycerol which are specific to bifidobacteria. Lipoteichoic acids, which appear to be essential for cell adhesion to the wall of the intestine, form links with the polysaccharide chains but, once again, the precise nature of these acids is not constant.

1.1.1.3. Genetic Aspects of Bifidobacteria

The G+C content of *Bifidobacterium* DNA varies from 55-67 mol%, and, therefore, these organisms are classified as having a high G+C content (212). The genome sizes for eight *Bifidobacterium* species were estimated by restriction analysis and pulse field gel electrophoresis to be an average of 1.8 Mb, and ranged from 1.5 to 2.1 Mb (198).

The presence of plasmids has been demonstrated in the following bifidobacterial strains (125, 275, 276, 277): *B. breve* and *B. longum,* which are found in the human intestine; *B. asteroides,* and *B. indicum*, found exclusively in the intestines of honeybees; and *B. globosum*, the most common in animals. Most of the plasmids remain cryptic.

Most of the plasmid studies have been done on the *B. longum* B2577 plasmid pMB1, and it was the first to be sequenced (170, 234). Plasmid pMB1 is approximately 1.9 kb in size, and contains two antibiotic resistance genes: Amp^R and Cm^R. Sequence analysis revealed high degree of homology with plasmids from the Gram-positive, high GC organisms *Corynebacterium glutamicum* and *Mycobacterium fortuitum*, which are also members of the *Actinomycetaceae* family. Furthermore, two tandem genes present on pMB1 were highly homologous to those found in *C. glutamicum*, *M. fortuitum*, and *Neisseria gonorrhoeae* plasmids. This suggests that this two-gene operon is shared by a wide family of plasmids belonging not only to phylogenetically related bacteria, but also to unrelated species such as the gram-negative *N. gonorrhoeae*. One of the ORFs identified showed significant similarity to ColE-type plasmid Rep proteins, and may be involved in plasmid replication.

A second plasmid from *B. longum*, pKJ50, has been isolated and sequenced (205). Analysis of plasmid replication indicated that this occurs by a rolling-circle mechanism. pKJ50 and a chloramphenicol resistance gene were cloned into pBR322, resulting in the *Bifidobacterium-E. coli* shuttle vectors, pBKJ50F and pBKJ50R, capable of transforming *B. animalis*. Another *B. longum-E. coli* shuttle vector, pRM2, has been constructed and successfully introduced into *B. longum* (179). This vector is based on the pMB1 replicon discussed above, and confers spectinomycin resistance.

Up to now, no conjugation or transfection systems have been available for bifidobacteria. Transformation of bifidobacteria is particularly difficult because of the cell wall composition, and the physical conditions are also affected by the medium composition and growth phase (235). However, several reports of successful transformation by electroporation of *Bifidobacterium*

strains have been published (8, 179, 235). Despite these advancements, there is still a shortage of genetic tools available for this genus, particularly cloning vectors, for genetic manipulations to be performed in bifidobacteria. However, due to the large industrial and medical importance of this organism, there is no doubt that this field will advance greatly in the next few years, specially with the recent sequencing of the *B. longum* NCC2705 genome (262). Suggestions have already been made as to how site-directed gene knockout strategies can be facilitated.

1.2. FUNCTION OF BIFIDOBACTERIA AS PROBIOTICS

1.2.1. The Intestinal Flora

The intestinal tracts of all mammalian species that have been studied, appropriately harbour a complex collection of microorganisms, most of which are anaerobic bacterial species (292). These organisms are known as the intestinal microflora. While the upper intestinal tract contains relatively few bacteria, numbers increase enormously in the colon (Figure 1.3) (118). The gastrointestinal tract of an adult human is estimated to harbour about 10^{14} viable bacteria/g of intestinal contents (158), this is 10 times the total number of eukaryotic cells in all tissues in a human body. About 400 bacterial species belonging to more than 190 genera have been detected in human faeces, but 30 to 40 species constitute 99% of the collection in any one human subject (118, 292). Only a few major groups dominate, all of which are strict anaerobes, such as *Bacteroides, Eubacterium, Bifidobacterium* and *Peptostreptococcus* (118).

There is a wide variation of bacteria in the microflora among individuals, but, globally, the number of species and the population of bacteria are relatively stable in healthy adults. In spite of its stability, the intestinal microflora can be affected by a variety of factors. Host factors such as gastric acid, bile salts, and mucus in the intestinal wall can affect the composition of the microflora (183). In addition, diet, medication, infections, stress, ageing, and climate also contribute to the microflora composition. Bacterial interactions such as antagonism or symbiosis can also influence the contents of the microflora (65).

Bifidobacteria are normal inhabitants of the human gastrointestinal tract throughout life, starting just days after birth (251). The digestive tract of newly-born infants rapidly becomes colonised by bacteria as the result of oral contamination from the vagina during birth (288). In breast-fed infants, this mixed population rapidly becomes dominated over the next 4 to 7 days by species of *Bifidobacterium*, with *B. bifidum* being one of the most frequently isolated species (212, 290). As

food is introduced into the diet, the intestinal microflora becomes more complex and diversified (344). By two years of age, the composition and metabolism of the child's microflora resembles those of adults in many ways, and is more stable (100). Characteristic shifts in composition and functioning of the microflora may occur with ageing (181). It has been reported that a small decrease in bifidobacteria, and an increase in clostridia, lactobacilli, streptococci, and enterobacteriaceae seem to be associated with ageing.

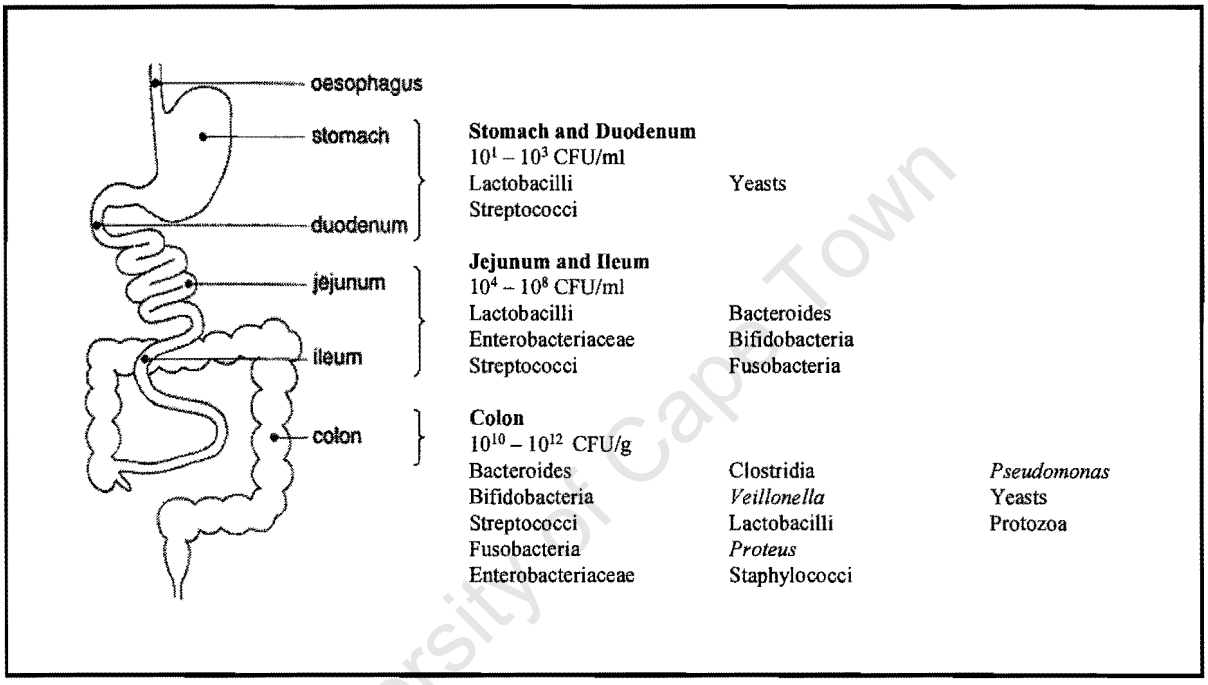


Figure 1.3. Microbial colonisation of the human gastrointestinal tract (118, 292).

1.2.2. Bifidobacteria as Probiotics

The importance of bacteria for human health and longevity was first hypothesised by Elie Metchnikoff at the beginning of the 20th century, who believed that the desirable effects to human health would only be expected if the gut microbes were substituted for yoghurt bacteria (cited in the review article by Holzapfel *et al*, 118). Since then attempts have been made to modulate the indigenous intestinal flora by the use of live microbial adjuncts, now called probiotics. By definition, a probiotic is a live microbial feed supplement which beneficially affects the host animal by improving the properties of the indigenous microflora (252).

Tissier's hypothesis almost 100 years ago that bifidobacteria might have health benefits was based on the observations that bifidobacteria are normal inhabitants of the human intestinal tract

throughout the life cycle, and that they are often the predominant microorganism in the gut of breast-fed infants. It has since been shown that breast-fed babies are less at risk for diarrhoeal disease than formula-fed infants (110).

For a probiotic effect to be exerted, numerous ingested bifidobacteria must reach the site of action in the gut. A minimum of 10^6-10^7 viable organisms/g intestinal contents is recommended. *In vivo* studies in adults and in infants have confirmed that some strains of bifidobacteria are able to survive passage through the gastrointestinal tract (1, 146). Marked strain differences exist in their ability to tolerate acid and bile salts, thus making survival an important criterion of selection (141). Once live bifidobacteria reach the site of action, they must be able to exert the desired effect; function is essential (254).

Over 70 products are currently offered by which bifidobacteria can be administered. These include fermented milks, cottage cheese, buttermilk, frozen desserts, candy, beverages, cereals, infant formulas and pharmaceutical preparations (290). Products often contain *B. bifidum, B. longum, B. lactis, B. breve,* or *B. infantis*; some use *Bifidobacterium* spp. (i.e. not yet classified) or *B. animalis.* Bifidobacteria may be fermented alone, but are commonly used in association with lactic acid bacteria for organoleptic or technological reasons, in particular, with *Lactobacillus acidophilus* and/or yoghurt cultures (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*).

The health benefits that are offered by bifidobacteria will be discussed briefly.

1.2.2.1. Improvement of Lactose Digestion

Dairy foods are an important source of high quality protein, riboflavin and calcium (297). However, 20 to 25% of the US population maldigest milk because of its lactose content (258). Lactose maldigestion is caused by a reduction in lactase (β-galactosidase) activity in the small intestine sometime after weaning. In these "lactase nonpersistent" individuals, unhydrolysed lactose passes into the large intestine, where it is fermented by the indigenous microflora into gases (H_2, CH_4 and CO_2) and short-chain fatty acids. The excessive gas production and the osmotic effects of excessive undigested lactose can cause gastrointestinal symptoms, including flatulence, abdominal pain, and diarrhoea. Lactose maldigesters may avoid milk and other dairy foods because of these symptoms, and thus potentially reduce their intake of some essential nutrients. When bifidobacteria are included in non-fermented milk, their consumption improves lactose digestion in humans (297), measured by the reduction in breath hydrogen and in reduced

symptoms of flatulence. Bifidobacteria have a relatively high level of β-galactosidase activity (122, 167), which enables them to exert their positive effect.

1.2.2.2. Improvement in Gastrointestinal Transit

In elderly people, mild constipation can be partially corrected through the consumption of milk fermented by bifidobacteria (274). The colonic transit times of woman have been shown to be significantly accelerated in the sigmoid colon after consuming fermented milk, particularly in those woman with slow initial time. This effect is not observed with traditional yoghurt (no bifidobacteria), thus showing the specificity of bifidobacteria for the increased colonic motility (99).

1.2.2.3. Reduction of Risk of Cancer

Indirect evidence in humans shows that consuming milk fermented by bifidobacteria leads to reduced levels of certain colonic enzymes (β-glucuronidase, nitroreductase, azoreductase, and glycoholic acid hydrolase), which are implicated in the conversion of procarcinogens to carcinogens such a nitrosamines or secondary bile salts (31). In addition, faecal putrefactive metabolites such as p-cresol, indole and ammonia were shown to be reduced when subjects consumed milk fermented by *B. longum* and *S. thermophilus* (288). Bifidobacteria inhibit the procarcinogens, nitrites and nitrosamines, through non-enzymatic and intracellular mechanisms (98). They also bind to heterocyclic amines (carcinogens formed during cooking of red meat), which, when bound, can then be eliminated in the faeces (199).

Epidemiological studies have found that colon cancer risk is inversely related to the consumption of diets that include fermented milks (208). Research in rats indicated a significant decrease in colon tumour incidence and reduced number of tumours by the ingestion of bifidobacteria. (279). Interest has more recently focused on a possible reduction of the risk of breast cancer. A recent *in vitro* report showed inhibition of growth of a human breast cancer cell line by milk fermented by bifidobacteria, thus demonstrating possible anticancer effects (19).

1.2.2.4. Antimicrobial Activity

The intestinal mucosa is an important organ of defence, providing a barrier against the antigens encountered by the enteric route, and most foreign antigens are excluded by the intestine's mucosal barrier (252). It has been suggested that the lactic acid bacteria contribute to this barrier effect.

Infants and young children are particularly susceptible to the ill effects of diarrhoea. Gastroenteritis can be caused by a variety of pathogens including, rotavirus, *E. coli* and *Campylobacter* (250). Studies have shown that children aged between 3-36 months with persistent diarrhoea had decreased symptoms and increased weight gain when given a yoghurt diet consisting of *Bifidobacterium* and other lactic acid bacteria (30). Furthermore, it was found that infants fed a formula with added *B. bifidum* and *S. thermophilus* had less incidence of hospital-induced diarrhoea and lower rates of rotavirus shedding compared to infants taking a standard formula (241). The antagonistic effects against various pathogens, including *E. coli* (82, 93), *Shigella dysenteriae* (178), and *Yersinia enterocolitica* (203) have also been investigated.

Two possible mechanisms by which bifidobacteria could be exerting an effect are plausible, both involving the production of antimicrobial substances. The first is the production of acetic and lactic acid. These are toxic to certain pathogens and also create an acidic environment which prevents the growth of certain organisms. The second is the excretion of bacteriocins and peptides which have a broad spectrum of activity. *B. bifidum* produces a bacteriocin, bifidocin B, which shows inhibitory activity against *Bacillus cereus, Enterococcus faecalis, Listeria innocua, Leuconostoc oenos, Micrococcus roseus* and many others (342). Several *Bifidobacterium* strains also produce a proteinaceous factor which inhibits the adherence of some *E. coli* and other strains to the intestinal epithelium (82). Bifidobacteria and *E. coli* have high affinities for the GA1 receptor, a neutral sphingoglycolipid found in the intestinal epithelium. Therefore, bifidobacteria not only competitively exclude *E. coli* by competing for the same binding sites, but also inhibit their binding by the production of a proteinaceous factor. Since the adhesion of pathogenic bacteria to host tissues is regarded as a prerequisite for colonisation-based infection, inhibition by bifidobacteria may help to prevent infection in the early stage of colonisation.

1.2.2.5. Immunomodulating Effects

Bifidobacteria can modulate various parameters of the immune system. In one report, blood samples of subjects consuming milk fermented by various cultures with added bifidobacteria and *L. acidophilus*, showed greater increase in humoral IgA response when subjects were given attenuated *Salmonella typhi* compared to the sera of subjects with no fermented milk intake (156). In a second study, *B. bifidum* added to fermented milk led to an increase in overall phagocytic activity against *E. coli* in peripheral blood. This increase was greater than that shown with fermented milk containing *L. acidophilus* but no *Bifidobacterium* (263). In addition, *in vitro* studies have demonstrated the production of interleukin (IL)-6, IL1b, and g-interferon (molecules

which serve as signals between cells during immune response) in blood mononuclear cells in the presence of bifidobacteria (1).

1.2.2.6. Contribution to Nutrition

The diversity of bacteria permits the breakdown of essentially all substrates that enter the colon. Degradation of non-digested substrates by the microflora, particularly of dietary carbohydrates (e.g. fibre, resistant starch, unabsorbed sugars) and endogenous substances such as mucus, lead to the production of gases (H_2, CO_2, CH_4), short-chain fatty acids (SCFA), lactic acid, branched chain fatty acids, ethanol, and ammonia (230). Products of degradation have nutritive functions, as in the case of SCFA, especially butyrate and lactic acid, which serve as energy substrates for colonocytes, liver cells, and peripheral tissues. Bifidobacteria are also capable of synthesising some B group vitamins (290) and a variety of amino acids (171) which can be beneficial to the consumer (181).

The saccharolytic nature of bifidobacteria has induced much research interest in the science community for a number of reasons, one of which is their contribution to the host nutrition. The main focus is now on the development of pre- and synbiotics which will be discussed in further detail in the next section, which will deal with carbohydrate metabolism in bifidobacteria.

1.3. CARBOHYDRATE METABOLISM IN BIFIDOBACTERIA

Dietary carbohydrates that escape digestion by the host and microflora in the upper gastrointestinal tract form the predominant substrates for bacterial growth in the colon (231). The environment in which bifidobacteria find themselves is, therefore, poor in mono- and disaccharides, and consists mainly of non-digestible oligosaccharides. These include oligosaccharides that contain fructose, glucose, xylose and galactose; resistant starch, which is not hydrolysed by pancreatic amylases but can be metabolised by bacterial enzymes; and non-starch polysaccharides, such as celluloses, hemicelluloses, pectins and gums (231). Host mucopolysaccharides and various proteins and peptides are also present (52).

Bifidobacteria have been shown to utilise a great variety of carbohydrates as a carbon source (18). The substrate preferences are not only highly variable between different bifidobacterial species, but also between strains (119, 162). They are able to utilise a variety of complex carbohydrates

(50, 249), including plant-derived dietary fibres, such as arabinogalactans and gums; and meat glycoproteins which originate from sloughed epithelial cells of the intestinal tract. Several glycosidases have been extracted and purified from bifidobacteria. These include neuraminidases, β-glucosidases, α-galactosidases, and β-galactosidase (18). In view of these organisms' ecological significance, it is surprising that virtually nothing is known about carbohydrate transport and metabolism in these organisms.

1.3.1. Carbohydrate Transport Mechanisms

Bacteria can potentially assimilate carbohydrates by a variety of mechanisms, either passively or by active mechanisms (27). Passive diffusion does not require any energy because bacterial membranes are selectively permeable to the solutes. Here, solute size is important in regulating the uptake of nutrients. Transmembrane protein complexes may facilitate diffusion by selectively recognising molecules that may be accumulated intracellularly, and this often occurs by the formation of a pore to effect uptake.

Active transport systems are energy-dependant and are usually ATP-driven (244). These include the periplasmic binding protein dependent system (PBPDS), where solute binding proteins in the periplasm transport carbohydrates in an unmodified state; the phosphoenolpyruvate (PEP)-dependant group translocation (this will be discussed further in section 1.5.1.), which primarily operates in bacteria that use the glycolytic pathway of metabolism; and ion-linked transport systems (162). Three classes of ion-linked transport systems exist: symports, antiports, and uniports. Symports consist of a solute and coupling ion traversing the membrane in the same direction. Solute accumulation is energised by a transmembrane proton gradient. Antiports consist of the solute and coupling ion moving in opposite directions. The formation of a transmembrane gradient may then be used to drive the uptake of other solutes by symports. Uniport systems do not have a coupling ion and the driving force for uptake is provided by the charge on the substrate molecule itself (162). Many of these uptake processes are subject to control by either catabolite repression, catabolite inhibition or inducer exclusion mechanisms.

Although the sugar uptake mechanisms in bifidobacteria are largely unknown, it has been shown that several *Bifidobacterium* species exhibit substrate preference with respect to sugar uptake (57, 58). In one study (57), five monosaccharides were tested, and it was found that some of the substrates were co-utilised, whereas others were sequentially transported into the cells. The sequential uptake of the substrates suggested that catabolite regulatory mechanisms were involved. In support of this, it was shown that in *B. breve*, glucose repressed the assimilation of

galactose and mannose. However, the mechanisms involved have not been identified. With respect to transport mechanisms, the experiments with *B. breve* showed that glucose was not assimilated by facilitated diffusion, and that phosphorylation was involved in its transport (58). However, ATP rather than PEP was the source of the phosphate, and, therefore, it remains unclear if this was mediated by a PEP:PTS. On the other hand, arabinose uptake appeared to occur by facilitated diffusion.

1.3.2. Glucose Fermentation

Bifidobacteria degrade hexoses by a fructose-6-phosphate shunt rather than a glucose-6-phosphate shunt, as for the heterolactic lactobacilli, (18, 290), and this is referred to as the bifidus pathway. This alternative phophoketolase pathway (Figure 1.4) appears to be specific to the genus *Bifidobacterium* and is characterised by the presence of fructose-6-phosphate phosphoketolase (F6PPK). F6PPK is present in all bifidobacterial species, and is used as a marker in the identification of this genus. Bifidobacterial cell extracts do not possess the glycolysis pathway or the hexosemonophosphate shunt pathway. This was established by the virtual absence of aldolase, an enzyme unique to glycolysis, and glucose-6-phosphate dehydrogenase, an enzyme associated with the hexosemonophosphate shunt. Some bifidobacterial species, however, do possess aldolase, but the physiological importance of this is not clear. There is no carbon dioxide production, confirming the absence of the hexosemonophosphate shunt.

The end products of bifidobacterial glucose fermentation are acetate, lactate, formate and ethanol (18, 290). Ideally, the overall reaction would occur as follows:

$$2\ \text{Glucose} + 5\ \text{ADP} + 5\ P_i \rightarrow 3\ \text{acetate} + 2\ \text{lactate} + 5\ \text{ATP}$$

However, bifidobacteria have an alternative pathway, the phosphoroclastic reaction, that can convert pyruvate to ethanol and formate, and so some pyruvate is diverted from forming lactate to forming ethanol and formate (18). Different bifidobacterial species yield different relative amounts of acetate, lactate, ethanol, and formate under the same conditions. Moreover, the quantities of the fermentation products can be varied by varying the growth conditions, such as type and quantity of the carbon source.

1.3.3. Galactose Fermentation

There are two pathways for the catabolism of galactose in bifidobacteria: the classical Leloir pathway and the pyrophosphorylase pathway (Figure 1.5) (18). It is thought that the glucose-1-phosphate generated can be converted to glucose-6-phosphate by a phosphoglucomutase, which could then be metabolised via the bifidus pathway described above (Figure 1.4). All the enzymes

in the two pathways have been isolated and characterised. Surprisingly, these enzymes are constitutively expressed. The enzyme that creates an alternative pathway for galactose metabolism is the UDP-galactose (glucose) pyrophosphorylase, catalysing the pyrophosphorolysis of both UDP-galactose and UDP-glucose. The two substrates are competitive inhibitors of each other. There is no information concerning possible mechanisms for which galactose-utilisation pathway is chosen in a given situation. Furthermore, it is not understood what the advantage is for the presence of two galactose-metabolising pathways.

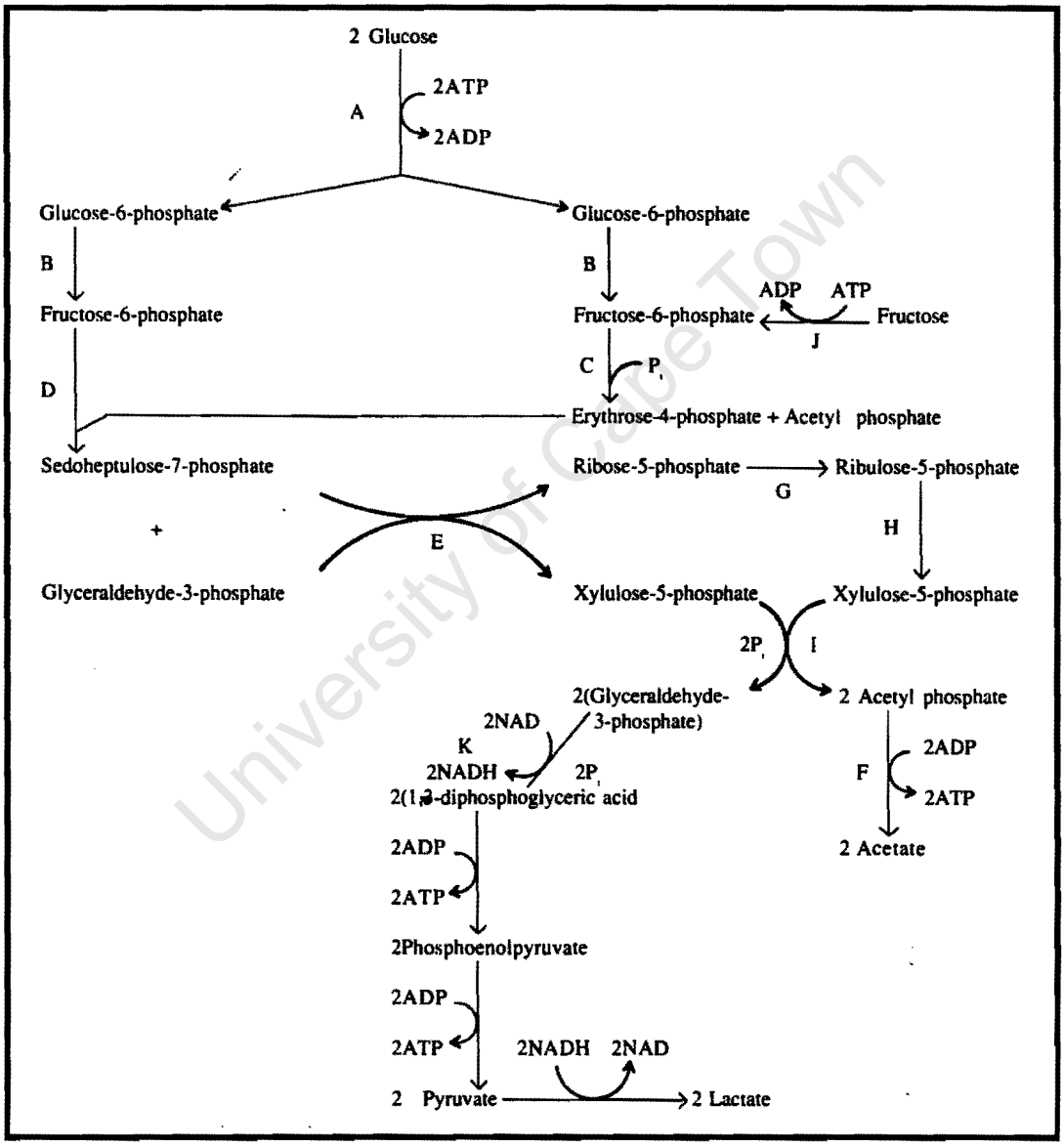


Figure 1.4. The bifidus pathway for glucose fermentation. Enzymes involved are as follows: A: hexokinase, B: glucose-6-phosphate isomerase, C: fructose-6-phosphate phosphoketolase, D: transaldolase, E: transketolase, F: acetate kinase, G: ribose-5-phosphate isomerase, H: ribulose-5-phosphate epimerase, I: xylulose-5-phosphate phosphoketolase, J: fructokinase, K: glyceraldehyde-3-phosphate dehydrogenase. Enzymes not identified are the usual glycolysis pathway enzymes. All sugars are D-isomers (18).

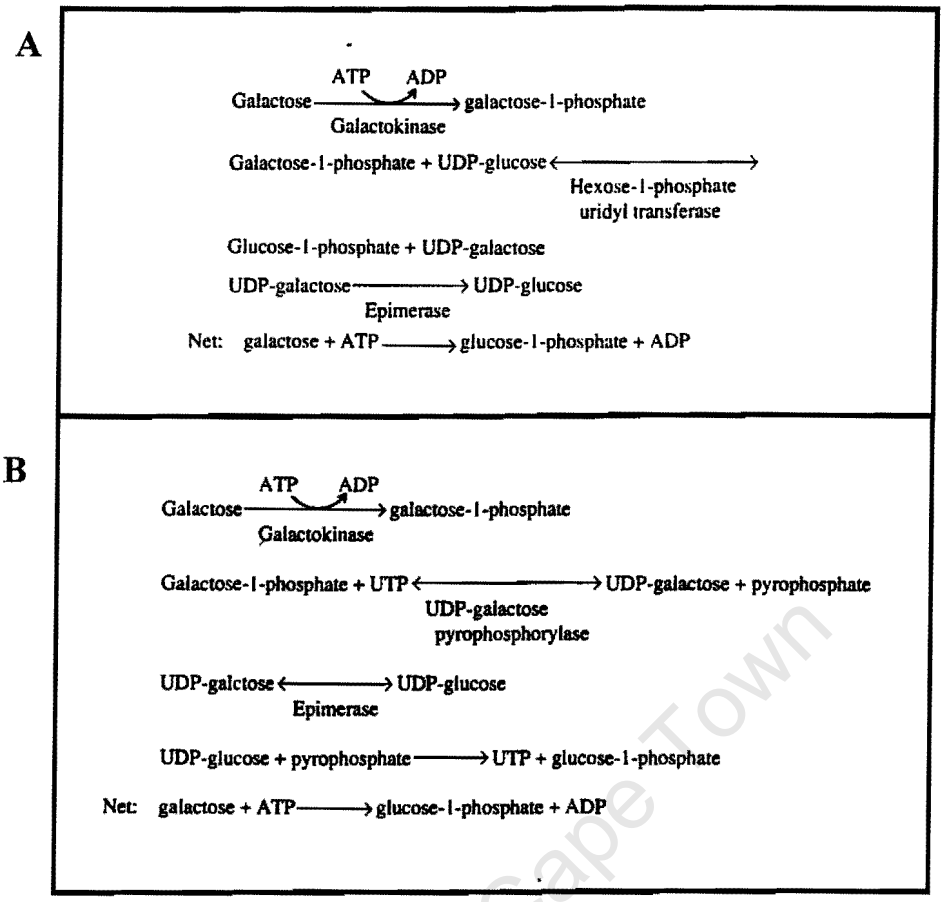


Figure 1.5. The Leloir (A) and Pyrophosphorylase (B) pathways of galactose metabolism in bifidobacteria (18).

1.3.4. Other Metabolic Pathways

It is generally accepted that bifidobacteria metabolise sugars via the bifidus shunt, and that glycolysis and the hexosemonophosphate shunt is not used (18, 290). However, claims have been made that the glycolytic pathway exists in some bifidobacterial species, since hexokinase, glucokinase, and pyruvate kinase were detected in cell extracts (18). However, these enzymes have a function in the bifidus pathway (Figure 1.4), and therefore, the hypothesis that glycolysis is used by bifidobacteria is not warranted on the basis of the evidence presented.

It has been reported that the glycolytic enzyme aldolase and the hexosemonophosphate shunt enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were absent from bifidobacteria. However, all three of these enzymes are present in the rumen bacteria *B. globosum* and *B. thermophilum* (18). In the human species *B. angulatum, B. catenulatum*, and *B. longum*, the aldolase and 6-phosphogluconate dehydrogenase have been identified. The physiological importance of these enzymes is unclear.

1.3.5. *B. longum* Genome Analysis and Carbohydrate Metabolism

The full genome sequence of *B. longum* NCC2705 was recently published, and a bioinformatics analysis was performed with respect to carbohydrate metabolism (262). This analysis revealed several physiological traits with respect to carbohydrate metabolism, which could explain firstly, the bacteria's ability to utilise a variety of complex carbohydrates, and secondly, how this might contribute to it being a probiotic organism. Some of the important points made which bear relevance to this study will be discussed briefly.

It was found that >8.5% of the total predicted proteins were dedicated to carbohydrate transport and metabolism. This is 30% higher than what was found for *E. coli, Enterococcus faecium, Lactococcus lactis, Bacillus halodurans,* and *B. subtilis*, thus indicating the important role that bifidobacteria play in carbohydrate metabolism in the human gut. More than 40 predicted glycosyl hydrolases were identified whose predicted substrates cover a wide range of di-, tri-, and higher order oligosaccharides. Based on sequence identity the following homologues were identified: 2 xylanases, 9 arabinosidases, 2 α-galactosidases, neopullanase, isomaltase, maltase, inulinase, 4 β-galactosidases, 3 β-glucosidases, 3 hexoaminidases and 3 α-mannosidases. Homologues of all enzymes needed for the fermentation of glucose, fructose or gluconate to lactate and acetate were shown to be present. This includes the characteristic F6PPK (section 1.3.2) and all the components of the fructose-6-phosphate shunt. Furthermore, homologues of enzymes needed to feed fructose, galactose, *N*-acetyl-glucosamine, *N*-acetyl-galactosamine, arabinose, xylose, ribose, sucrose, lactose, cellobiose, melibiose, gentobiose, maltose, isomaltase, raffinose and mannose into the fructose-6-phosphate shunt were identified. This substantiates the findings which reported the ability of *B. longum* to ferment a wide variety of carbohydrates (18, 50, 249, 290). Many of the glycosyl hydrolases were found to be organised in 7 clusters. Each cluster consisting of a LacI-type sugar-responsive repressor; an ABC-type oligosaccharide transporter; and 1-6 genes encoding various types of glycosyl hydrolases.

Several unusual glycosyl hydrolases were identified, many showing identity to those from eukaryotes, strongly suggesting a special role in the adaptation of *B. longum* to its gastrointestinal niche (262). In further support of this, one of the carbohydrate clusters was found to contain genes which are more commonly found in eukaryotes, and are involved in the utilisation of glycoproteins. Glycoproteins are produced by epithelial cells of the colon, and its utilisation could possibly enhance colonisation of *B. longum* to the gastrointestinal tract. A significant number of oligosaccharides that pass intact into the colon have uncommon structures, such as those found in

human milk (74) and in plant polymers. They could be selective substrates for *B. longum* and its collection of unusual glycosyl hydrolases (262).

Several of the clusters were found to have recently duplicated genes and adjacent insertion (IS) elements (262). This suggests that *B. longum* is under strong pressure to evolve the diversity of its metabolic capabilities so that it can cope with nutritional competition of varied substrates in the gastrointestinal tract (262). Furthermore, the carbohydrate utilisation gene clusters identified indicate that *B. longum* is well equipped with the machinery necessary for the utilisation of a diverse spectrum of substrates, either from the host's diet or perhaps from the host itself.

1.4. PREBIOTICS AND SYNBIOTICS

As was discussed in section 1.2, bifidobacteria have been identified as probiotic organisms, and considerable research has been aimed at the delivery of these organisms to the human host. Bifidobacteria administered in foodstuffs have been shown to be able to pass the terminal ileum and are detected in the faeces, despite adverse physiochemical barriers in the gastrointestinal tract such as gastric acidity, bile production and rapid small intestinal transit (92, 211). However, they rapidly disappear from faeces when the oral dosing stops, indicating that they do not colonise the colon (32). With the realisation that bifidobacteria utilise a diverse range, as well as unusual carbohydrate substrates, and that diet could significantly alter the colonic flora, an alternative approach to modify colonic bacteria was developed which could overcome the difficulties associated with probiotics. In this approach, substrates which are indigestible by humans are used to selectively stimulate the growth and/or the activity of one or a limited number of colonic bacteria already resident in the gut (31). These non-digestible substrates, usually a carbohydrate, are termed prebiotics.

Due to their health-promoting benefits, bifidobacteria have been identified as preferred target microorganisms for prebiotics (91). A number of oligosaccharides have been shown to selectively stimulate bifidobacteria, which when fed to humans, resulted in a significant increase in faecal bifidobacterial counts (Figure 1.6) (231). In addition, this increase in bifidobacteria was concomitant with the reduction of *Bacteroides* spp., *E. coli* and clostridia (231, 322).

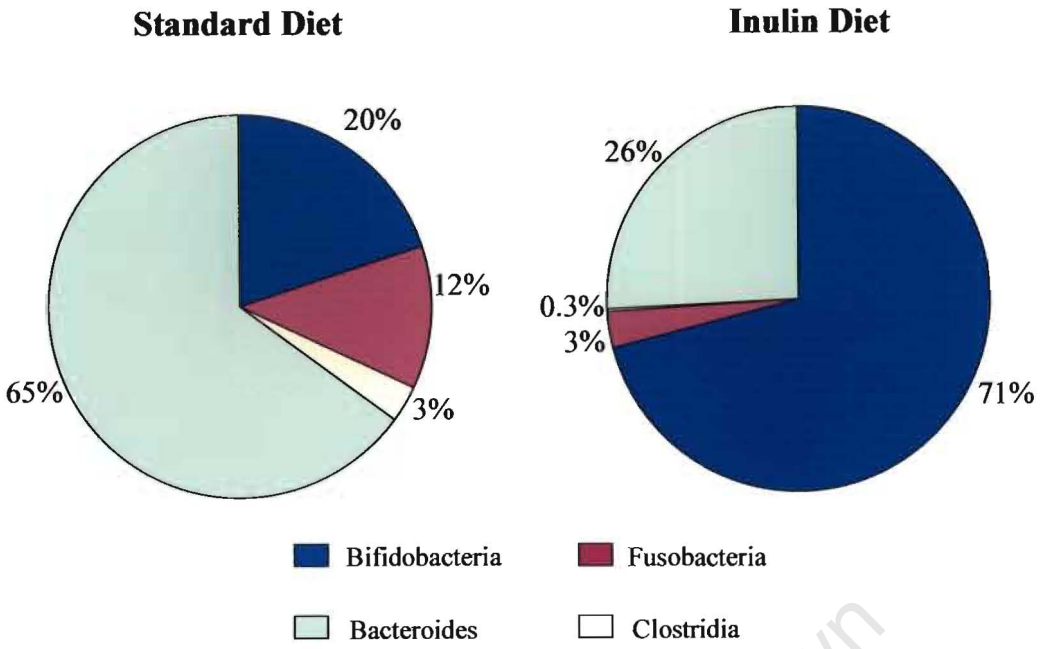
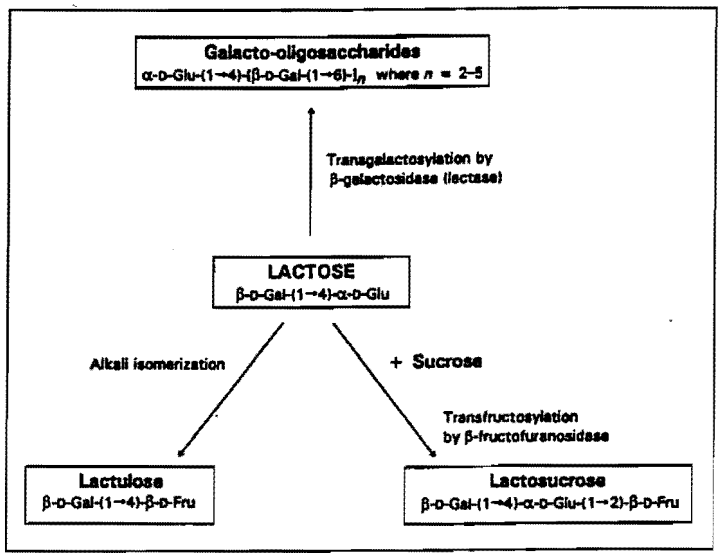


Figure 1.6. Shifts in the distribution of faecal microflora in humans provided a diet with and without supplemental inulin (193).

For a food to be classified as a prebiotic, certain criteria have to be met (231). Firstly, it must not be hydrolysed or absorbed in the upper part of the gastrointestinal tract. Secondly, it must be a selective substrate for one or a limited number of potentially beneficial bacteria commensal to the colon. Therefore, they must not be utilised by potentially harmful residents of the colon such as the enterobacteriaceae and clostridia. Thirdly, it must be able to alter the colonic flora, and as a consequence exert health benefits on the host.

Several prebiotics have been identified which stimulate bifidobacteria, including the fructo-oligosaccharides (46, 49), galacto-oligosaccharides (46, 49), soybean oligosaccharides (49, 247), oat β-glucan and xylan (129). A schematic representation of these is shown in Figure 1.7. With regards to bifidobacteria, the most studied of these oligosaccharides are the fructo-oligosaccharides inulin and oligofructose (92, 93, 94, 193, 228, 229, 231). These will be discussed further in chapter 2.

A.



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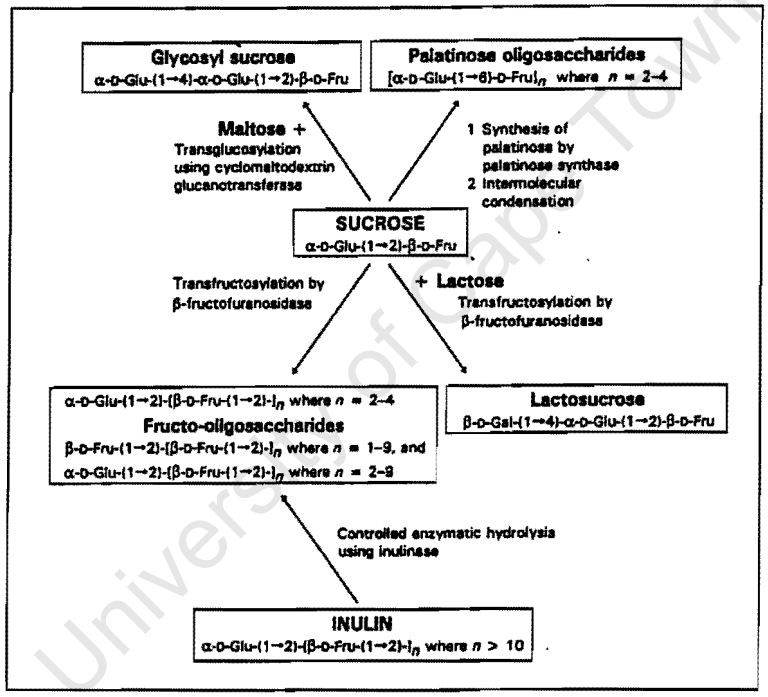

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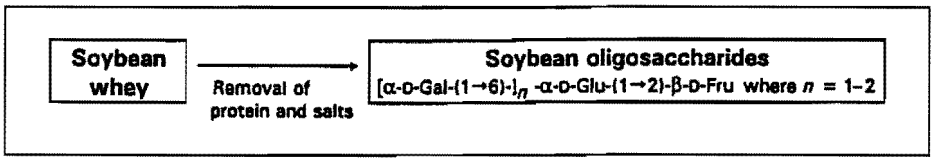

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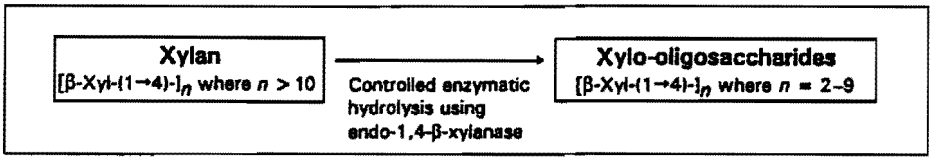

Figure 1.7. Properties of the classes of oligosaccharides that have been identified as prebiotics, showing those manufactured from A: lactose, B: sucrose, C: soybean, D: xylan. Abbreviations: Gal, galactose; Glu, glucose; Fru, fructose; Xyl, xylose (49).

As can be seen in Figure 1.7, oligosaccharides can be manufactured enzymatically using mono- and disaccharides by transglycosylation activity (49). Some bifidobacterial enzymes have been isolated, such as a β-galactosidase (216), α-galactosidase (310) and a β-glucosidase (194), which have been shown to carry out transglycosylation reactions. The possibility of synthesising bifidogenic prebiotic substrates using bifidobacterial enzymes is, therefore, receiving some interest. Studies thus far report the synthesis of oligosaccharides by transglycosylation activity and have looked at expression in *E. coli* for the possibility of mass production (194, 216, 310). Although some indication was given as to whether these synthesised oligosaccharides were able to support bifidobacterial growth (194, 216), suitable mixed culture fermentations and feeding trials have not been performed to indicate that they are bifidogenic.

Recently, research has been aimed at the development of food products which contain combinations of the probiotic organism with prebiotic compounds. These products have been termed synbiotics (228). This combines the stimulatory effects by the body's own bacteria and new ones (135). Furthermore, the prebiotic acts to improve the survival, implantation, and growth of the newly added probiotic strains (193). Commercially available synbiotics include a yoghurt consisting of three probiotic strains and inulin; skimmed milk, enriched vitamins and micronutrients with chicory-fructo-oligosaccharides; and a product containing two probiotic strains and oligofructose (7). Recently, a synbiotic yoghurt containing resistant starch as the prebiotic and *B. lactis* Lafti™ B94 has been investigated (48).

Due to the importance of fructose-containing oligosaccharides for the stimulation of bifidobacteria in the human gut, knowledge about the mechanisms by which these substrates are utilised by bifidobacteria is important. This would facilitate the development of novel prebiotic substrates and synbiotic products for biotechnological application. Bacterial mechanisms involved in the uptake and catabolism of the fructose-containing carbohydrates, raffinose and sucrose, will be reviewed, as this pertains to the research that was conducted and which will be presented in this dissertation. Firstly, raffinose utilisation will be discussed and this will be followed with an analysis of sucrose utilisation. However, before the two systems are described, some general aspects to carbohydrate utilisation, which are relevant to the discussion for both the sugars, will be considered in the next section.

1.5. GENERAL ASPECTS OF CARBOHYDRATE METABOLISM IN BACTERIA

The general aspects to carbohydrate utilisation which will be discussed include: the bacterial phosphoenolpyruvate-dependant (PEP) carbohydrate:phosphotransferase system (PTS) and its regulation of non-PTS systems, in particular the involvement of HPrSer in regulation in Gram-positive bacteria. This will be followed by a discussion on catabolite regulation in Gram-positive bacteria.

1.5.1. The Carbohydrate Phosphotransferase System (PTS)

The principle route for the transportation of carbohydrates in both Gram-positive and gram-negative bacteria is the high-affinity, multi-component (PEP)-dependant PTS (210, 246). Concomitant with the translocation of the carbohydrate across the membrane is its phosphorylation. Regardless of the organism or the carbohydrate, all PTSs that have been identified catalyse the following overall process:

$$\text{Phosphoenolpyruvate}_{(in)} + \text{Carbohydrate}_{(out)} \rightarrow \text{Pyruvate}_{(in)} + \text{Carbohydrate-P}_{(in)}$$

The PTS is composed of two general energy-coupling proteins, Enzyme I, and HPr (histidine-phosphorylatable protein) and several sugar-specific Enzyme II proteins (210, 223, 246). Enzyme II proteins typically consist of up to four protein domains EIIA, EIIB, EIIC, and EIID, at least one of which is membrane-bound. Transport and phosphorylation of PTS sugars occurs as follows: a phosphoryl group donated by PEP is passed via EI, HPr, $EIIA^{Sugar}$, $EIIB^{Sugar}$ to the incoming sugar (Figure 1.8). The translocation of the sugar through the membrane is facilitated by the integral membrane domain, $EIIC^{Sugar}$.

EI proteins autophosphorylate a conserved histidyl residue with PEP as the phosphoryl donor. HPr proteins are phosphorylated by the phosphorylated form of EI at the conserved histidyl (His15) residue. In low-GC Gram-positive bacteria, HPr can also be phosphorylated at a conserved serine (Ser46) residue. The phospho group of HPrHis-P can be transferred to an EII, whereas the phospho group of HPrSer-P is removed by a phosphoprotein phosphatase and has been implicated in the regulation of several systems, and will discussed in section 1.5.2.1. The organisation of EII protein domains varies, and may consist of a single fused protein or fused and unfused domains.

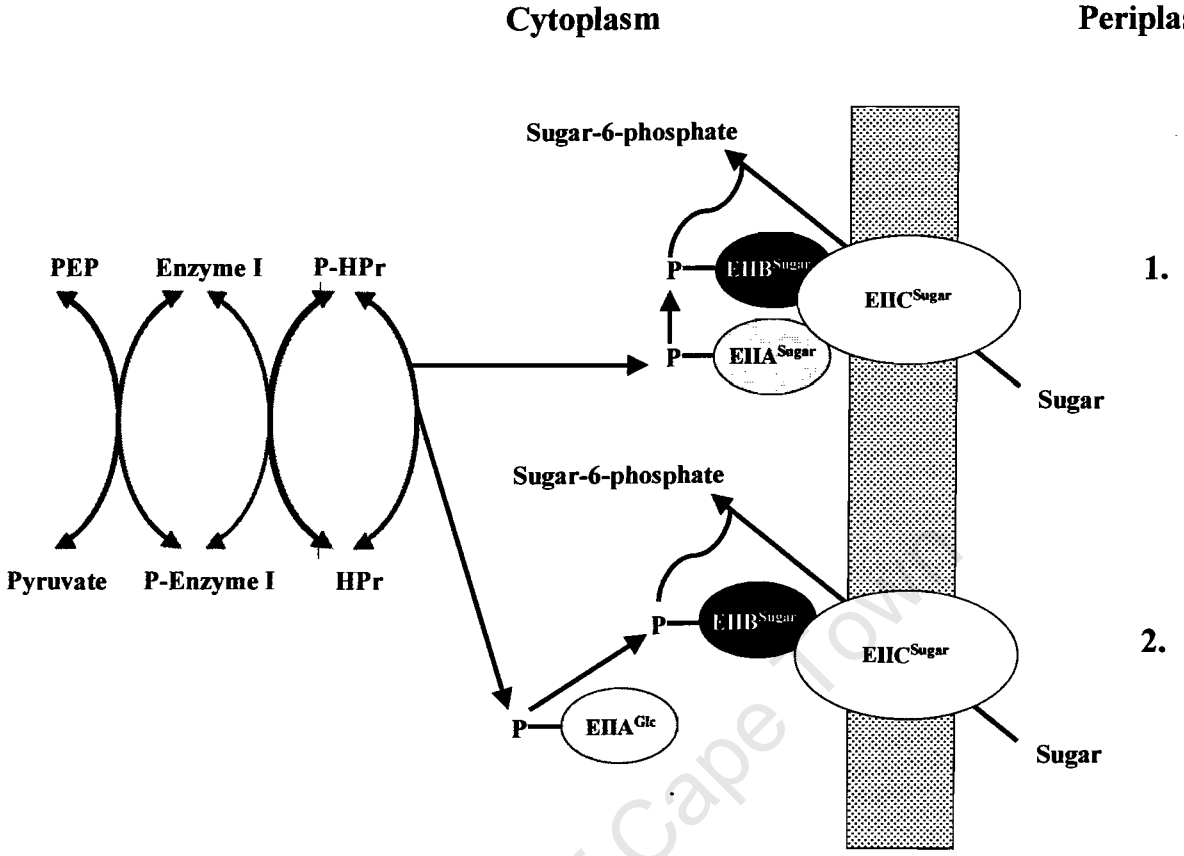

Figure 1.8. Organisation of the PTS components. Enzyme I and HPr are the general proteins for all PTSs. EIIs contain two hydrophilic domains, IIA containing the first phosphorylation site (P-His), and IIB containing the second phosphorylation site. The hydrophobic IIC domain is membrane-bound. Two varieties exist for sucrose-specific EII proteins corresponding to (1) those found in *Streptococcus mutans, Pediococcus pentosaceus,* and *Lactococcus lactis* where the EIIA EIIB and EIIC domains are fused to form $EIIBCA^{Scr}$ proteins and (2) those found in *Bacillus subtilis, Staphylococcus xylosus, Vibrio alginolyticus, Clostridium beijerinckii* and *Klebsiella pneumoniae* where only the EIIB and EIIC domains are fused to form $EIIBC^{Scr}$ proteins. It should be noted that this is a simplified representation of PTS components and that different combinations of EII protein domains specific for other sugars exist. Abbreviations: P-, phosphorylated form of the various proteins; Glc, glucose; PEP, phosphoenolpyruvate. (After 149 and 210).

1.5.2. Regulation by PTS

The PTS also regulates the transport and metabolism of non-PTS carbohydrates (210, 243). This is triggered by the presence of a preferred carbon source in the growth medium, usually glucose, which inhibits the expression of proteins involved in the utilisation of alternative carbon sources. In Gram-positive bacteria, glucose has been shown to inhibit the uptake of both PTS and non-PTS sugars (inducer exclusion), and stimulates the dephosphorylation of intracellular sugar-phosphates and/or the efflux of the dephosphorylated sugar (inducer expulsion). These regulatory mechanisms by PTS occur by direct phosphorylation of target proteins of non-PTS systems or by

protein-protein interactions between proteins/components of the PTS with non-PTS proteins (210, 226). These have been studied extensively and for a review see references (210) and (243). For the purpose of this study, the mechanisms by which PTS components regulate non-PTS systems in Gram-positive bacteria will be discussed briefly, and all involve the HPr protein.

1.5.2.1. Regulation Involving HPrSer in Gram-positive Bacteria

As already mentioned, Gram-positive bacteria phosphorylate serine-46 in addition to histidyl-15 on HPr. This is performed by an ATP-dependant HPr kinase and by phosphorylated-EI respectively (210). Whereas the phospho group of HPrHis-P can be transferred to the various EIIs, the phospho group of HPrSer-P is removed by a phosphoprotein phosphatase. HPrSer phosphorylation regulates different functions in different bacteria. In *Lactobacillus brevis*, HPrSer-P inhibits sugar:H^+ symport permeases such as those specific for lactose and glucose (339, 341,). More specifically, HPrSer-P binds to the cytoplasmic surface of the permease to uncouple sugar transport from H^+ cotransport. This converts the transport from an active system to a facilitated diffusion system (i.e. from a sugar:H^+ symporter to a sugar uniporter). It is thought that other permeases are probably also targets of this type of regulation (341).

In *Lactococcus lactis*, sugars are taken up by the PTS. It appears that HPrSer-P inhibits the PTS and stimulates the activity of, and possibly interacts with, a cytoplasmic sugar-P phosphatase that initiates the process of inducer expulsion (210, 224, 340). The detailed mechanisms involved remain to be established.

HPrSer-P is also involved in catabolite repression in Gram-positive bacteria. Catabolite repression is mediated by the CcpA protein, and this will be discussed in the next section. HPrSer-P is involved in CcpA-repression of both PTS and non-PTS genes for catabolic and transport proteins (62, 121, 226). The mechanism described thus far is as follows (243): in the presence of glucose, the HPrSer kinase is activated and phosphorylates HPr on ser-46. HPrSer-P, possibly together with a cytoplasmic metabolite, binds to and activates the CcpA protein, inducing a conformation that possesses high affinity for the CRE in the regulatory regions of affected genes (see 1.5.3.) This nucleoprotein complex, possibly with other effector molecules, retards or blocks transcription initiation of catabolite-repressible operons. In some cases, the interaction between CcpA and HPrSer-P has been found to be stronger in the presence of glycolytic intermediates such as fructose-1-6-bisphosphate (FBP) (61).

Heterofermentative lactic acid bacteria and bacteria that use carbohydrate pathways other than glycolysis seem to lack an intact PTS, and transport their carbohydrates by active transport energised by the proton-motive force (210, 232, 243, 246). In some of these organisms, such as *L. brevis,* and *L. buchneri*, HPr and an ATP-dependant protein kinase that phosphorylates HPr are present, but EI and EII are absent (225). Furthermore, *L. brevis* exhibits metabolite-activated sugar expulsion that is independent of sugar-P hydrolysis, but involves HPrSer-P. It has since been revealed that incomplete PTSs may be characteristic of several prokaryotic genera (210).

1.5.3. Catabolite Repression in Gram-positive Bacteria

In gram-negative bacteria catabolite repression is mediated by cyclic AMP (cAMP) and the cAMP receptor protein (CRP) (163). CRP in complex with cAMP binds to a specific site in the promoter region of the affected genes, thereby activating transcription. In the low GC Gram-positive bacteria, however, catabolite repression is mediated via a negative regulatory mechanism (120).

Catabolite repression in the low GC Gram-positive bacteria is thought to involve the catabolite control protein (CcpA) (111, 120). CcpA is a transcriptional regulator belonging to the GalR-LacI family (see 1.8.4), and binds to a *cis*-active palindromic DNA sequence in the vicinity of, or overlapping, the transcriptional start site of the affected gene (192, 329). This sequence is called the catabolite responsive element (CRE), and the consensus sequence ((T/A)GNAA(C/G)CGN (T/A)(T/A)NCA) has been proposed (121). Binding of CcpA to the CRE is stimulated by HPr that has been phosphorylated at the serine residue (226). In *Bacillus subtilis*, Crh, an HPr-like protein has also been shown to stimulate CcpA binding (84). Besides its repressor function, CcpA also operates as an activator, and this is also mediated by binding to a CRE (160).

The mechanisms involved in catabolite repression in the high GC Gram-positive bacteria are poorly understood. Catabolite repression is best characterised in *Streptomyces coelicolor*, and it has been suggested that serine phosphorylated HPr does not play a role in the high GC Gram-positive bacteria (207). This was proposed since it appears that they lack both HPr serine kinase and HPr serine phosphatase. Several *cis*-acting regions containing either direct or indirect repeat sequences have been identified in *Streptomyces* which are involved in the negative regulation of catabolite repression-controlled promoters. None of these *cis*-acting regulatory sequences share any obvious sequence or structural similarity. Some *trans*-acting factors have also been implicated in catabolite repression in *Streptomyces*, however the precise roles of these proteins in mediating catabolite repression have not been clearly defined.

One of the *trans*-acting proteins that is receiving more attention with regards to its role in catabolite repression is the glucose kinase GlkA, an enzyme which catalyses glucose phosphorylation and is essential for glucose metabolism in *S. coelicolor* (5, 6, 143). It has been proposed that GlkA has a regulatory role that is distinct from its catalytic function, since complementation of a *glkA* mutant with a heterologous glucose kinase, or activation of a cryptic glucose kinase, could restore glucose metabolism but not catabolite repression (5, 143). Sequence analysis of the *glkA* gene revealed that its product is a member of the ROK (repressor, orf, kinase) family of proteins, and is closely related to sugar kinases (6, 301). GlkA and other sugar kinases lack the N-terminal helix-turn-helix DNA binding domain found in transcriptional repressors, and therefore, their similarity likely reflects their sugar binding property. It has been suggested that kinases of this family would exert their regulatory function by the interaction with transcriptional factors (35, 301). A regulatory role for the glucose kinase in the low-GC Gram-positive bacteria, *Bacillus megaterium* and *Staphylococcus xylosus,* has also been proposed (319, 320). Furthermore, the *S. xylosus* GlkA contributed to catabolite repression as part of a non-PTS glucose utilisation system (75, 128). However, in *S. xylosus*, catabolite repression is mediated by CcpA and HPr (69, 123), and although GlkA participated in activation of CcpA, it was not able to trigger CcpA-mediated regulation independently from HPr kinase (128). Therefore, the role of GlkA in catabolite repression, particularly its involvement in the high GC Gram-positive bacteria, is yet to be clearly defined, and other proteins involved have probably yet to be discovered (207).

Carbon catabolite repression is generally considered to be a phenomenon whereby a preferred carbon source in the medium, usually glucose, is sequentially metabolised before an alternative carbon source. This generally results in the repression of genes and operons, whose gene products are involved with the utilisation of the alternative carbon source. More recently, however, it has been suggested that catabolite repression serves as an autoregulatory device to keep sugar utilisation at a certain level, rather than to establish preferential utilisation of carbon sources (35). Autoregulation, therefore, enables bacteria to adjust sugar utilisation to their metabolic capacities, protecting cells from adverse effects which would result by the uptake of excess carbohydrate. The mechanisms involved allow bacteria to establish a hierarchy of sugar utilisation for the economical use of carbon and energy sources.

1.6. GENERAL ASPECTS OF BACTERIAL RAFFINOSE METABOLISM

Raffinose (galactose-α-1,6-sucrose) can be hydrolysed by a β-fructosidase giving rise to fructose and melibiose (galactose-α-1,6-glucose), or by an α-galactosidase resulting in galactose and sucrose (glucose-α-1,2-fructose). Melibiose and sucrose can then be further hydrolysed by α-galactosidases and sucrases respectively. Generally, the latter hydrolysis which results in sucrose, is preferred, however, in the ethanologenic strains *Klebsiella oxytoca* M5A1, *Erwinia chrysanthemi* EC16 and *E. coli* KO11, the fructose is cleaved first (185). In this section, the focus will be on the uptake of raffinose and on the hydrolysis of the galactose bond to sucrose (in raffinose) and to glucose (in melibiose).

1.6.1. Bacterial α-Galactosidase Enzymes

The enzyme α-galactosidase catalyses the hydrolysis of α-1,6 linked α-galactoside residues from simple oligosaccharides such as melibiose, raffinose, stachyose (galactose-α-1,6-raffinose), and from polymeric galactomannans (α-galactosides α-1,6-linked to the backbone of β-1,4-linked D-mannopyranose units). α-Galactosidases are used in industrial applications for the hydrolysis of raffinose from beet sugar syrups (136), hydrolysis of raffinose and stachyose from soybean milk (294), hydrolysis of hemicelluloses for the pulp and paper industry (166), and for the modification of galactomannan to improve its gelling properties (37). High temperatures used during these processes have led, primarily, to the isolation of thermostable α-galactosidases from thermophilic bacteria (80, 177). α-Galactosidases also catalyse transgalactosylation, and therefore, are being isolated for the synthesis of novel compounds, particularly for the use as prebiotics (150, 310).

Based on similarities in primary structure and hydrophobic cluster analyses, α-galactosidases have been grouped into three well conserved families in the general classification of glycosyl hydrolases (113). Those from bacteria have been grouped into families 4 and 36, while those from eukaryotes into family 27. The bacterial α-galactosidases from family 36 are larger than those in families 4 and 27, having a molecular weight of ~80 kDa vs. the ~50 kDa for the enzymes in the latter families (81). Only a limited degree of amino acid sequence identity occurs between the enzymes in families 36 and 27. The only shared consensus pattern [LIVMFY]-x(2)-[LIVMFY]-x-[LIVM]-D-[DS]-x-[WY], is near the N-terminal end of family 27 enzymes and within the central region of family 36 enzymes. Its presence indicates a similar reaction mechanism or a substrate binding site. The *E. coli* melibiase (*melA*) represents family 4-type α-galactosidases, and is structurally related to neither family 27 nor family 36 enzymes, and the consensus pattern

described is missing (154). The *E. coli* melibiase requires NAD^+ and manganese ions as a cofactor (38). Family 4-type α-galactosidases have also been identified in *Klebsiella pneumoniae* (103), *Salmonella typhimurium* (182), *Enterobacter cloacae* (196), *Enterobacter aerogenes* (197) and *Rhizobium meliloti* (83).

Both intracellular and extracellular α-galactosidases have been isolated from bacteria. Some organisms, such as *Bacteroides ovatus* (90) and *Trichoderma reesei* (166) have been shown to possess more than one α-galactosidase. α-Galactosidase substrate specificities can vary, and can decrease with the number of sugar units present in the substrate (105, 106). In organisms where multiple enzymes exist, the type of α-galactosidase produced is dependent on the type of galactoside present in the medium (90, 166).

1.6.2. Bacterial Uptake of Raffinose and Melibiose

Unlike for sucrose transport (will be discussed in section 1.8.1.), raffinose transport systems isolated thus far do not depend on group translocation by PTS. Raffinose and its hydrolysis product, melibiose, can be taken up by bacterial cells via various membrane-associated permeases that function via ion-linked transportation. These permeases can be grouped into two different types of mechanisms. The first type involves ABC transporters, while the second uptake system involves cation coupling.

Periplasmic transport systems functioning as ABC (ATP-binding cassette) transporters are utilised by *Streptococcus mutans* (*msmEFGK*) (238), *S. pneumoniae* (*rafEFG*) (233) and *R. meliloti* (*agpA*) (83) for the transportation of raffinose and melibiose. These transporters are clustered together with other α-galactosidase genes. Periplasmic transport systems typically consist of a ligand-binding protein which is in the periplasm of gram-negative bacteria or tethered to the outer face of the cytoplasmic membrane of Gram-positive bacteria by covalently attached lipid, together with two different transmembrane proteins and at least one protein capable of binding and hydrolysing ATP (289). For *R. meliloti*, only the periplasmic ligand-binding protein has been identified (*agpA*), although an ORF (*agpB*) downstream of *agpA* could turn out to be another transport component (34, 83). In both the streptococci, the *msmE/rafE* genes code for proteins that are likely to be anchored in the membrane and which have an ATP/GTP-binding site motif (233, 238). They also have the *msmFG/rafFG* genes encoding for sugar transporters, and *S. mutans* but not *S. pneumoniae*, encodes a cytoplasmic protein (MsmK) which shows homology to members of the family of ATP-binding proteins (238). The streptococci raffinose utilisation systems will be discussed in more detail in section 1.7.2.

The other three systems of raffinose transport involve cation-coupled symporters. These are the raffinose utilisation RafB permease of *E. coli* (12), the LacY permease of the *E. coli lac* operon (236), and the melibiose utilisation MelB permeases from *E. coli* (215), *K. pneumoniae* (103), *S. typhimurium* (182), *E. cloacae* (196) and *E. aerogenes* (197). The secondary structures of these proteins, as predicted by hydropathy profiles, contain 12 hydrophobic segments thought to represent transmembrane α-helical domains (Figure 1.9) (12, 29, 76, 103, 144, 182). The N- and C-termini are on the cytoplasmic side of the cytoplasmic membrane. Although the LacY permease is primarily involved in the uptake of β-galactosides, it has been shown to transport raffinose and melibiose as well (45, 236). The RafB permease transports raffinose, melibiose and lactose (12). The melibiose permeases mediate transport of mono-, di- and trisaccharides (TMG and galactose, melibiose and lactose, and raffinose respectively) (153, 236, 268).

In many of the bacterial cotransport systems, H^+ is the sole coupling cation. This has been shown for the *E. coli* LacY protein (332). The *E. coli* RafB permease exhibits striking similarities to the LacY protein (12). Furthermore, amino acids shown to participate in lactose-proton symport in the LacY are well conserved in RafB, and transport via RafB depends on the proton motive force. Interestingly, the melibiose permease of *E. coli* utilises either H^+, Na^+ or Li^+ as a coupling cation, depending on the substrate being transported (304). For melibiose transport, Na^+ or H^+ is utilised as a coupling ion, whereas Na^+ or Li^+ is used for TMG (methyl-1-thio-β-D-galactopyranoside) transport and Na^+, H^+ or Li^+ is utilised for methyl-α-galactoside transport. The *S. typhimurium* melibiose permease utilises either Na^+ or Li^+, but not H^+ (182), while in *K. pneumoniae* and *E. aerogenes* melibiose transport is not stimulated by Na^+ at all, but is stimulated by Li^+ (103, 197). In *E. cloacae*, neither Na^+ nor Li^+ stimulate transport, and only H^+ can be used as the coupling cation (196). For the *E. coli* melibiose permease, four and eighteen amino acid residues have been identified which are involved in cation and substrate recognition respectively (132, 338). All except one of the identified residues are also conserved in the *S. typhimurium* melibiose permease (Figure 1.9) (182). The carboxyl tail of the *E. coli* melibiose permease plays no direct role in substrate recognition or energy transduction, but rather the sugar/cation binding sites are formed by the interaction of the transmembrane helices 3, 4, 6, 9 and 10 (29).

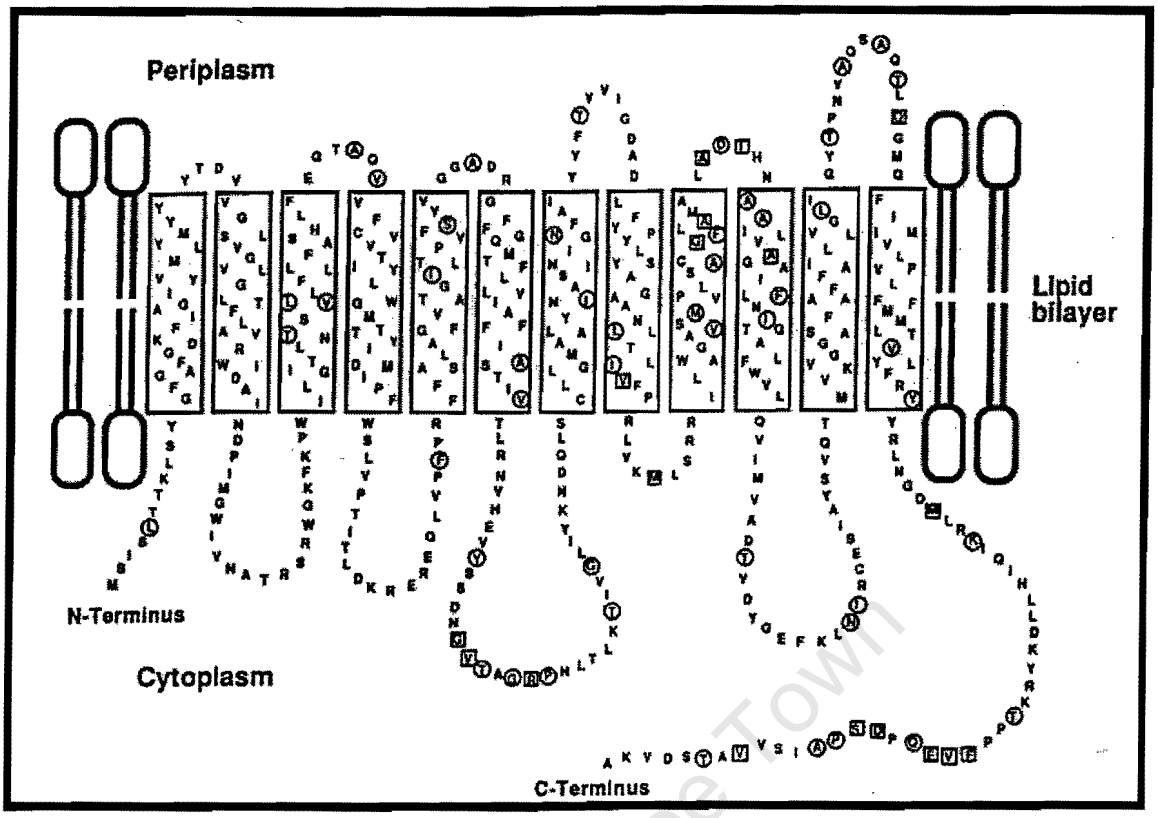

Figure 1.9. A membrane topology model of the melibiose permease (MelB) of *Salmonella typhimurium*. Amino acid residues with no mark are identical in the *S. typhimurium* and *E. coli* permeases, encircled residues are conserved, and boxed residues are non-conserved (182).

1.7. RAFFINOSE UTILSATION SYSTEMS IN BACTERIA

1.7.1. *Escherichia coli*

Raffinose and melibiose utilisation in *E. coli* is mediated via two different mechanisms: an inducible, plasmid-borne *raf* operon consisting of 4 genes, or by an inducible chromosomally-linked *mel* operon consisting of 3 genes. Both of these systems will be discussed briefly.

1.7.1.1. The E. coli raf Operon

Among the 39 Raf plasmids that have been independently isolated from *E. coli*, one, pRSD2, has been sequenced and analysed (12, 13). Originally, a 6.1 kb *raf* operon consisting of four genes was identified. These were, *rafA*, encoding an α-galactosidase; *rafB*, encoding a raffinose permease; *rafD*, encoding a sucrose hydrolase; and *rafR*, encoding a repressor (Figure 1.10). The first three genes, *rafABD*, are thought to be transcribed from a single promoter, while *rafR* is

located upstream of *rafA* and is transcribed from its own promoter. More recently, a fifth gene, *rafY*, encoding an outer membrane protein, has been identified 599 bp downstream from the 3' end of *rafD* (Figure 1.10) (306). The *raf* operon closely resembles the *E. coli lac* operon in overall organisation and mode of control. The differences pertain to substrate specificities of α- versus β-galactosidase and to sucrose hydrolase versus transacetylase (12). The closest resemblance is between the two permeases, suggesting a common origin of the two transport systems.

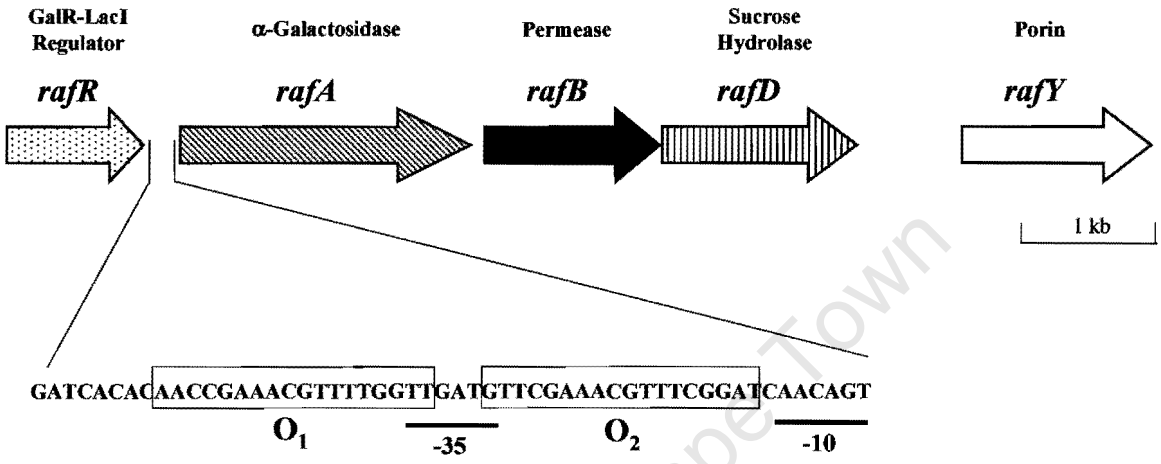

Figure 1.10. Genetic organisation of the *Escherichia coli raf* operon. The *rafABD* promoter region is enlarged showing operators O_1 and O_2 (boxed), and the -10 and -35 sequences (underlined) (12, 13, 186, 306).

RafA, α-galactosidase, functions as a tetramer of identical 82 kDa subunits (12). It is distinct from the *E. coli melA* analogue (189) by cofactor requirement, stability and primary structure, but shows identity to two eukaryotic α-galactosidases (12, 266). RafD, sucrose hydrolase, is a homodimer of 55 kDa subunits, and has no counterpart in *E. coli* K12 (12). The cleavage of sucrose by RafD is an indispensable step in the catabolism of raffinose. Mutants in *rafD* grown in 1% raffinose accumulate sucrose, causing the cells to lyse after 3 divisions. Other *E. coli* K12 hosts harbouring the same mutation could cope with excessive sucrose by exporting it via an unknown transport system. The *rafB* and *rafD* ORFs overlap by 1 bp, a configuration frequently accompanied by translation coupling. It is thought that this ensures sufficient synthesis of sucrose hydrolase for immediate breakdown of sucrose in order to avoid the detrimental effects on the cells.

The membrane-bound RafB permease transports raffinose, melibiose and lactose. Although uptake is via a non-PTS, RafB is subject to regulation by the PTS. This PTS-mediated regulation involves binding of $EII^{Glucose}$ (300). It is likely that this binding occurs at the central loop of RafB between transmembrane helices 6 and 7 by analogy to binding to the *E. coli* LacY (300, 333). The RafY glycoporin downstream from *rafD*, was found to increase the uptake rates for maltose, sucrose and raffinose, and also permitted the diffusion of stachyose and maltodextrins (306). However, more recently, it has been shown that RafY does not contain a binding site for carbohydrates, and it has been suggested that it forms a general diffusion pore as apposed to a carbohydrate-specific porin (4).

The *raf* operon transcription is negatively regulated by RafR and this repression is reversed by the addition of inducer, melibiose (13, 186). An N-terminal helix-turn-helix domain interacts with two nearly identical 18 bp palindromic operator sites, O_1 and O_2, which flank the -35 promoter box, with O_2 located between the -35 and -10 boxes (Figure 1.10) (186). Although both operators bind RafR with equal affinities, O_2 is more efficient in regulating promoter activity *in vivo*: RafR bound to O_1O_2 results in a 1200-fold repression, when bound to O_2 alone results in 45% repression, whereas binding to O_1 results in a 6% repression (183). Therefore, O_2 is the dominant site crucial for controlling transcription initiation by RNA polymerase, whereas O_1 has an accessory function. The affinity of RafR for an operator site is lowered approximately 13-fold if the adjacent site has already been occupied by RafR. This is likely due to the steric hindrance between the RafR dimers bound to O_1 and O_2 which are only 3 bp apart. It has been suggested that this arrangement facilitates a single repressor molecule to oscillate between sites.

1.7.1.2. The E. coli K-12 mel Operon

The *E. coli mel* operon consists of three genes, *melR, melA* and *melB*, which encode for a positive regulator of the AraC/XylS family of transcriptional regulators, an α-galactosidase and a permease respectively (104, 325). The *melA* and *melB* genes are co-transcribed, and *melR* is transcribed divergently from its own promoter (Figure 1.11).

Expression of *melR* and *melAB* is induced by melibiose and other α-D-galactosides (195), and this induction requires that *melR* be present either in *cis* or in *trans* (325). Regulator proteins belonging to the AraC/XylS family have conserved DNA-binding domains (85). These domains are about 100 amino acids long, and are usually located at the C-terminus of the protein. In some proteins, amino acids outside of the DNA-binding domain may be involved in dimerisation, ligand-binding or environmental sensing. By analogy to AraC/XylS proteins, it has been

suggested that MelR binds to the *melAB* promoter to initiate transcription (326). In support of this, the *melAB* promoter contains a poor -10 hexamer sequence, and lacks a -35 consensus sequence, which is typical of a promoter requiring an activator (202, 217). However, experimental evidence is required to prove DNA-binding of MelR.

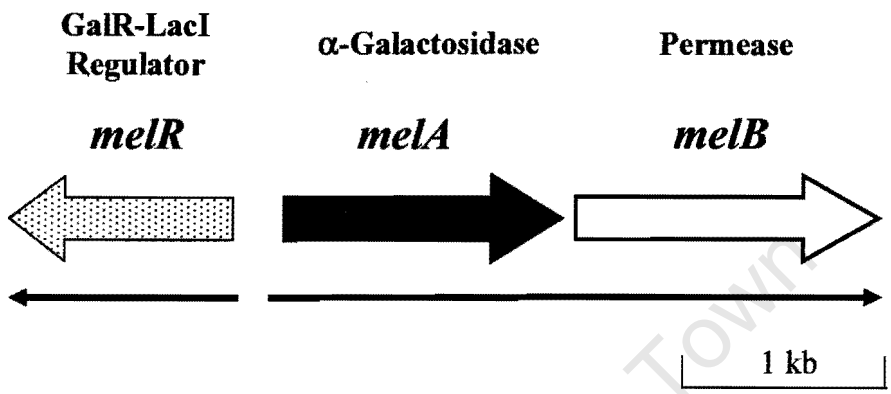

Figure 1.11. Genetic organisation of the *Escherichia coli* K-12 *mel* operon. Boxed arrows indicate the gene, and thin arrows indicate transcriptional polarity and co-transcription of the *melA* and *melB* genes.

In addition to MelR regulation, cAMP also seems to be involved in transcriptional regulation. Transcription from the *melR* promoter, but not the *melAB* promoter, is dependant on cAMP-CRP. A sequence showing homology to the cAMP-CRP binding consensus sequence (56) was identified from position -52 to -31 upstream of the *melR* transcription site, where 10 out of the 14 positions are homologous (325). cAMP-CRP is only able to bind to the *melR* promoter in the presence of RNA polymerase. It has been suggested that cAMP-CRP and RNA polymerase make direct contact in open complexes and mutually tighten binding. This low affinity that the *melR* promoter has for cAMP ensures that MelR is made only under extreme conditions, suggesting that melibiose is one of the "least preferred" substrates for *E. coli*. Although several *melAB* genes have been isolated from other organisms, the regulatory gene has only been identified in *E. coli, R. meliloti* and *K. pneumoniae* (34, 103, 325). Regulatory analysis has not been conducted in *K. pneumoniae*, while in *R. meliloti* it has been shown that the regulator (*agpT*) also functions as an AraC-like transcriptional activator (34).

1.7.2. *Streptococcus pneumoniae and S. mutans*

These two organisms each have a cluster of genes bearing significant structural similarity to each other, with some of the genes showing high amino acid homology (233, 238). The *S. mutans msm* (multiple sugar metabolism) system, an 11 kb gene region containing 8 contiguous genes, is

involved in the metabolism of multiple sugars (Figure 1.16) (238). It is responsible for the uptake of melibiose, raffinose and isomaltotriose and for the metabolism of raffinose, melibiose, sucrose and isomaltosaccharides. In the *S. pneumoniae raf* system, a 10.2 kb gene region also containing 8 genes is required for the regulation and metabolism of raffinose alone (Figure 1.12) (233). Besides the substrate utilisation specificities, the differences between these two systems is that in the *S. pneumoniae* cluster the *dexB* (dextran glucosidase) and the *msmK* (ATP-binding protein) genes are absent, while the two genes, *rafS* and *rafX*, are only found in the pneumococcal gene cluster. Due to the similarities between the two systems, and considering that the *S. pneumoniae* system is specific for raffinose utilisation, only the *S. pneumoniae* system will be discussed further here. It should be noted however, that the utilisation of additional substrates by *S. mutans* could be due to the absence of the *msmK* and *dexB* genes in *S. pneumoniae*, and that besides this, the two systems possibly function by very similar mechanisms. The *S. mutans msm* system will be discussed further in section 1.9.3 with regards to sucrose utilisation.

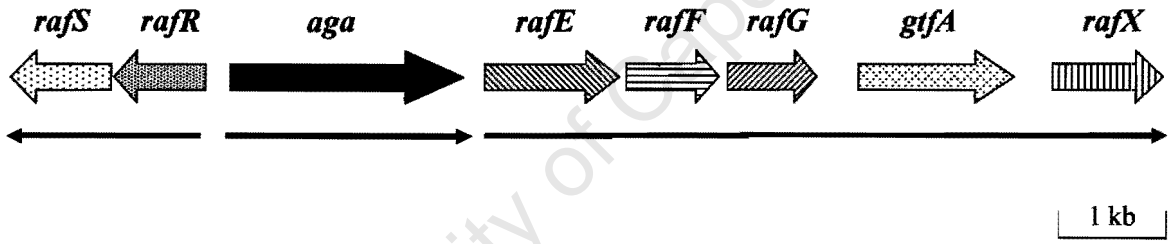

Figure 1.12. Genetic organisation of the *Streptococcus pneumoniae raf* operon. Thin arrows indicate operons and transcriptional polarities.

In the *S. pneumoniae raf* system, three transcripts are produced. The first transcript contains the *rafS* and *rafR* genes, encoding proteins which find homology to the *B. subtilis* biotin repressor protein (BirR) and to an AraC/XylS family transcriptional regulator, respectively. Reading divergently from these is the *aga* gene encoding an α-galactosidase (Figure 1.12). Downstream of the *aga* is an operon of 5 genes: *rafE, rafF, rafG, gtfA* and *rafX*. The first three genes encode proteins with high homology to the *S. mutans* MsmE, MsmF and MsmG proteins respectively, and are likely to be involved in sugar binding and transportation, specifically of raffinose. The *gtfA* gene encodes a sucrose phosphorylase and is, therefore, likely to be involved in the catabolism of sucrose, the product from raffinose hydrolysis by the α-galactosidase. The last gene in the cluster, *rafX*, does not show any significant homology to any known protein.

α-Galactosidase activity is low in the presence of glucose and sucrose as a carbon source, and raffinose is the only sugar that induces activity. However, only sucrose, and not glucose, catabolite-represses *aga* induction when present in media containing raffinose. Furthermore, this repression is mediated by the sucrose PTS either directly or indirectly. With a CRE located upstream of the *aga* translational initiation codon, and the identification of a CcpA homologue in the *S. pneumoniae* genome, it is postulated that catabolite repression is mediated by the CcpA protein. However, inactivation of the gene has no effect on the regulation of *aga* expression. Therefore, catabolite repression is either mediated by the PTS directly, or by another regulatory protein. The two repressors identified, RafR and RafS, function as a positive and a negative regulator of the *aga* promoter respectively. An inverted repeat identified within the *aga* promoter shows high homology to the AraC binding region and could, therefore, function as the site for RafR binding. However, the mechanisms by which RafR and RafS regulation occurs, and the identity of the triggers for induction, remain unknown.

1.7.3. *Pediococcus pentosaceus*

Raffinose utilisation genes have been located on a plasmid, pSRQ1, adjacent to a gene cluster containing genes for sucrose metabolism (see 1.9.5) (151). The raffinose regulon is thought to consist of 2 transcriptional units with opposite polarities (Figure 1.18) (149). The first contains *rafP* and *agaR* encoding a putative raffinose permease and an α-galactosidase. The second contains the *rafR* gene which has homology to the *S. mutans msmR* activator located in the multiple sugar metabolism system (238). Further research will be required in order to discuss this system in more detail.

1.8. GENERAL ASPECTS OF BACTERIAL SUCROSE METABOLISM

It is thought that bacterial sucrose utilisation systems have evolved by modular evolution, where individual genes sharing a common origin became independently associated into regulons and operons (299). In most organisms the genes mediating sucrose uptake and hydrolysis are clustered together on the chromosome, forming operons or regulons. Many of the sucrose utilisation systems that have been isolated thus far have certain features or components in common. These include: (i) a sucrose transport system, typically involving the PTS; (ii) enzymes that hydrolyse sucrose; (iii) ATP-dependent fructokinases and (iv) regulatory proteins, typically of the GalR-LacI family of transcriptional regulators. These components will be addressed individually, and

will be followed by a more detailed analysis of bacterial sucrose utilisation systems that have been characterised. Examples of non-PTS sucrose uptake and utilisation systems will also be presented.

1.8.1. Sucrose Transport and the Phosphotransferase System

A general introduction to the PEP-dependent PTS was given in section 1.5.1. The vast majority of bacteria take up the disaccharide via the sucrose-specific PTS. Sucrose-specific PTS genes have been isolated from several Gram-positive and gram-negative organisms, including *Bacillus subtilis* (77, 78, 88, 283), *Streptococcus mutans* (285, 286), *Streptococcus sobrinus* (43), *Lactococcus lactis* (295), *Pediococcus pentosaceus* (151), *Klebsiella pneumoniae* (14, 66, 127, 299), *Salmonella typhimurium, Staphylococcus xylosus* (36, 89, 318), *Vibrio alginolyticus* (21, 22, 269), *Clostridium beijerinckii* (222) and *Erwinia amylovora* (24). Typically, sucrose transported by the enzyme II proteins of the PTS yields intracellular sucrose-6-phosphate, which is cleaved by an intracellular invertase to yield glucose-6-phosphate and fructose (210). Fructose is further phosphorylated by an ATP-dependent fructokinase for metabolism via the glycolytic pathway.

The organisation of Enzyme II protein domains varies, consisting of a single fused protein or fused and unfused domains. In the case of sucrose-specific Enzyme II proteins, two varieties exist. Those of *S. mutans, P. pentosaceus,* and *S. sobrinus*, the $EIIA^{Scr}$ $EIIB^{Scr}$ and $EIIC^{Scr}$ domains are fused to form $EIIBCA^{Scr}$ proteins. Those of *B. subtilis, V. alginolyticus, S. typhimurium, K. pneumoniae, Clostridium beijerinckii* and *S. xylosus*, only the $EIIB^{Scr}$ and $EIIC^{Scr}$ domains are fused, forming $EIIBC^{Scr}$ proteins. In enteric bacteria, the $EIIA^{Glc}$ protein allosterically controls the activities of target permeases, possibly by phosphorylation (209, 246). In cases where the $EIIB^{Scr}$ proteins exist, evidence suggests that the $EIIB^{Scr}$ subunit is phosphorylated by the $EIIA^{Glc}$ domain (21, 152, 280, 287).

Non-PTS sucrose uptake pathways involve facilitated diffusion or active ion-symport transport systems, followed by intracellular hydrolysis of unphosphorylated sucrose. Such systems have been described for *Zymomonas mobilis* (101), *V. alginolyticus* (130, 269), *S. mutans* (238) and *E. coli* (23).

1.8.2. Enzymatic Sucrose Hydrolysis

Sucrose hydrolysis is carried out by several glycosyl hydrolases belonging to different families, and in some bacteria more than one sucrose-hydrolysing activity is present. The β-fructofuranosidases belonging to family 32 are the largest group of these (112, 113, 114). They cleave the terminal fructose from fructose-containing carbohydrates with varying specificity.

Some levanases and inulinases have increased specificity towards high molecular weight fructose polymers (β(2,6)-linked levans and β(2,1)-linked inulins). Some hydrolyse the low molecular weight fructose containing sugars such as raffinose and sucrose. In this group are the sucrose-6-phosphate hydrolases, which hydrolyse both sucrose and sucrose-6-phosphate, but have a lower K_m for the latter substrate (161, 267, 296).

Many of the lactic acid bacteria employ glucosyltransferases (GTFs) and fructosyltransferases (FTFs) for the synthesis of high molecular weight glucans and fructans respectively from sucrose. These GTFs and FTFs also catalyse the hydrolysis of sucrose. The insoluble glucans and fructans produced are resistant to microbial degradation and are thought to provide an extracellular energy reserve. In the oral streptococci, they are believed to play a role in the colonisation of smooth dental surfaces, and therefore, in the formation of dental caries (188). *Lactobacillus reuteri* has also been found to produce large amounts of glucans and fructans from sucrose (311). A glucosyltransferase has recently been isolated from this organism (139), and interestingly, a levansucrase (311) and an inulosucrase (312) have also recently been isolated for the synthesis of two types of fructans, levan and inulin respectively. *L. reuteri* has been designated a probiotic and the glucans and fructans produced are considered to be prebiotics which contribute to human health (40). They also have been implicated in antitumoral (60), immunomodulating (263) and cholesterol-lowering activity (227).

Sucrose phosphorylases catalyse the simultaneous hydrolysis and phosphorylation of sucrose using inorganic phosphate, and yields glucose-1-phosphate and fructose. Sucrose phosphorylases have been identified in only a few bacteria, and include *S. mutans* (238), *Leuconostoc mesenteroides* (137), *Pseudomonas saccharophila* (330), *Pseudomonas putrefaciens* and *Agrobacterium vitis* (79).

1.8.3. Fructokinases Associated with Sucrose Metabolism

Fructose resulting from sucrose hydrolysis must first be phosphorylated by kinases to be metabolised by glycolysis. Two families of kinases phosphorylate fructose, namely the ROK/hexokinases and the ribokinase/*pfkB* (28, 301). Fructokinases from both families catalyse the same reaction: ATP + fructose → ADP + fructose-6-phosphate. Both types of fructokinases are associated with sucrose-PTS regulons, as well as being independently encoded.

1.8.4. GalR-LacI-Family Transcriptional Regulators

Sucrose catabolic genes are predominantly regulated by the GalR-LacI family transcriptional regulators. GalR-LacI proteins have an N-terminal helix-turn-helix DNA binding motif, and the remainder of the protein is involved in ligand binding, dimerisation, and in some cases, tetramerisation (191, 328). These proteins typically bind to operator sites containing palindromic nucleotide sequences in the promoter regions of the affected genes. The central bases ($A_4A_3N_2C_1/G_1'N_2'T_3'T_4'$) are more highly conserved than the outer bases. The regulatory proteins bind to the operators as dimers, with each subunit binding a half site.

Regulation of gene expression is typically achieved by transcriptional repression where unliganded regulator dimers bind in the promoter regions. Ligand binding changes the conformation of the helix-turn-helix domain of the regulator and ultimately results in its dissociation from the DNA. In some cases however, the liganded form of the regulator binds its operator site, such as the PurR protein of *E. coli* (273). In addition, the catabolite repressor/activator (Cra) protein of enteric bacteria and the catabolite control protein (CcpA) of low-GC Gram-positive bacteria are able to effect both transcriptional repression and activation (160, 245). In these cases, the position of the operator site relative to the respective promoter appears to determine whether the regulator protein acts as a repressor or activator.

1.9. SUCROSE UTILISATION SYSTEMS IN BACTERIA

1.9.1. *Bacillus subtilis*

Three pathways exist in *B. subtilis* which are involved in sucrose metabolism. The first, involves a non-specific levanase, SacC, which is encoded as the distal gene in a fructose inducible fructose-PTS operon, and is, therefore, likely to be involved primarily in the metabolism of fructose polymers (142, 323). The second two are regulated by antitermination and these will be discussed further.

The first pathway regulated by antitermination involves an extracellular levansucrase, SacB, which catalyses both the hydrolysis of sucrose and the synthesis of levans (283). The *sacB* gene was thought to be transcribed as a single unit since the region downstream of the gene contains a 13 nt long dyad symmetry thought to be a strong rho-independent transcription terminator. It was recently shown, however, that the dyad symmetry is an antiterminator of a second gene, *levB*,

downstream of the *sacB* gene (Figure 1.13) (204). The *levB* gene is in turn followed by another antiterminator structure and the *yveA* gene. *sacB, levB* and *yveA* are part of the same transcription unit. *levB* encodes a protein with similarity to the *B. subtilis* SacC described above, and is associated with the cell membrane acting in conjunction with SacB to degrade levan. The protein encoded by *yveA* displays all the features of a membrane protein, but its role remains unknown.

The second pathway regulated by antitermination involves an $EIIBC^{Scr}$ protein (SacP) and a sucrose-6-phosphate hydrolase (SacA) and they are encoded by the *sacPA* operon (77, 78, 282). Expression of the *sacPA* operon is induced by sucrose concentrations lower than 1mM, while expression of *sacB* is fully induced by concentrations of sucrose higher than 30mM (282). *sacPA* expression is also subject to repression by glucose (11).

Regulation in response to sucrose occurs predominantly by transcriptional antitermination at ribonucleotide antiterminator (RAT) sequences located in the leader regions of the *sacB* and *sacPA* operons (Figure 1.13) (51, 55). The transcriptional antiterminators SacT and SacY, are required for readthrough of the *sacB* and *sacPA* operons, respectively, by binding to the RAT sequences. SacY and SacT share strong identity to each other and to the *E. coli* BglG antiterminator protein (55). The *sacT* gene lies upstream of the *sacPA* operon (Figure 1.13). The *sacY* gene is encoded as part of the *sacXY* operon where the predicted *sacX* protein finds identity to $EIIBC^{Scr}$-like proteins and to SacP (346).

The RAT elements overlap the terminator component in the leader regions (Figure 1.14) (15). Both the RAT and adjacent terminator are predicted to form stem-loop structures. An activated form of SacT or SacY binds to the RNA chain preventing the formation of the terminator, in which case full length transcripts are produced. In the absence of an activated terminator protein, the terminator structure forms, resulting in early transcription termination.

The regulation of SacY and SacT antitermination has not been completely characterised. Analogy with antitermination by the *E. coli* BglG suggests that phosphorylation/dephosphorylation may play a role (240). This was supported by the finding that SacY can substitute for BglG in antiterminating transcription of the *bgl* operon in *E. coli* (124). Furthermore, phosphorylated and nonphosphorylated forms of SacY have been identified, and the ratio between them was dependent on the external level of sucrose and the cellular levels of SacX. The phosphorylation of SacY is carried out by the SacX PTS permease at a conserved His99 residue in the absence of sucrose (302). SacX, therefore, functions as a sucrose sensor, and is phosphorylated by the general

PTS proteins (51). Therefore, in the presence of sucrose, the dephosphorylated form of SacY is active and facilitates antitermination at the *sacB* RAT sequence. In the absence of sucrose, SacX phosphorylates SacY, thereby inactivating it and early termination of the *sacB* occurs.

Unlike SacY, an intact PTS is essential for SacT activity (11). It is thought that SacT is phosphorylated at two sites (11, 240). The one would be as for SacY, and would occur in response to sucrose concentration. The other site would act as an "activation" site and although the phosphoryl donor has not been clearly identified, it is likely to be phosphorylated by the PTS proteins, EI and HPr. In support of this, SacT has been shown to be phosphorylated by EI and HPr (10). However, the phosphorylation state of SacT did not affect its activity. Therefore, the precise role of EI and HPr mediated phosphorylation remains unclear.

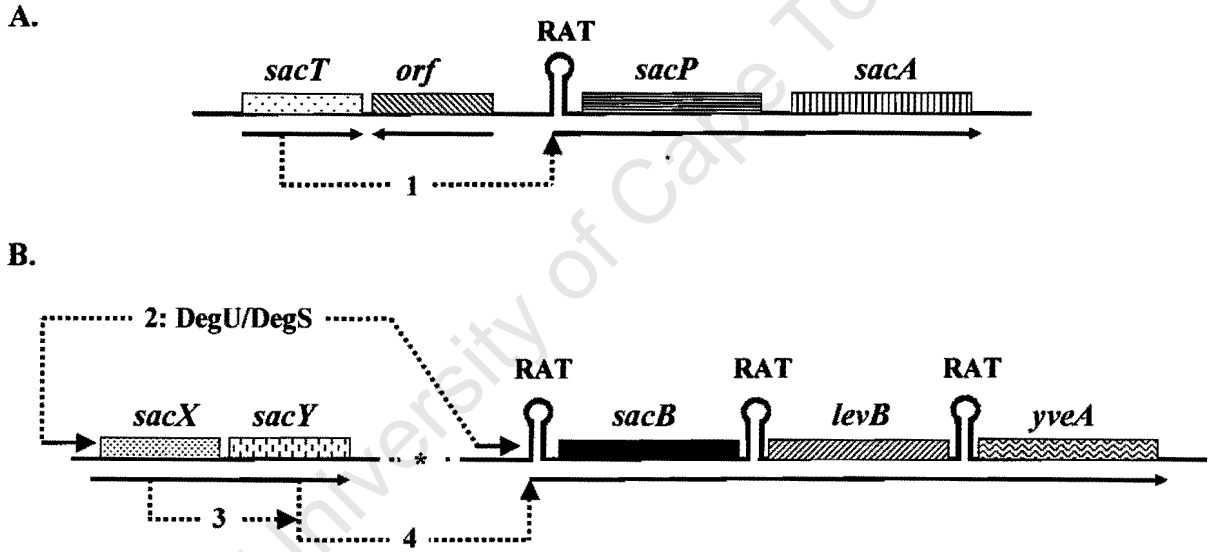

Figure 1.13. Genetic organisation of the *Bacillus subtilis sacPA* operon (A) and the unlinked *sacB-levB-yveA* and *sacXT* operons (B). Solid arrows indicate transcriptional polarity, and where it extends over more than 1 gene, co-transcription occurs. Dashed lines and arrows indicate regulatory relationships: (1): SacT as an antiterminator active at the *sacPA* RAT, (2): the DegU/DegS signalling system which increases transcription off the *sacXY* and *sacB-levB-yveA* promoters, (3): SacX as a negative regulator of SacY and (4): SacY as an antiterminator of the *sacB* RAT. SacY and SacT also regulate *sacXY* transcription in response to sucrose. RAT: ribonucleotide antiterminator/terminator sequence. *: Indicates that the *sacXY* and *sacB-levB-yveA* operons are not linked. Not drawn to scale. (Adapted from 149, 204, 281).

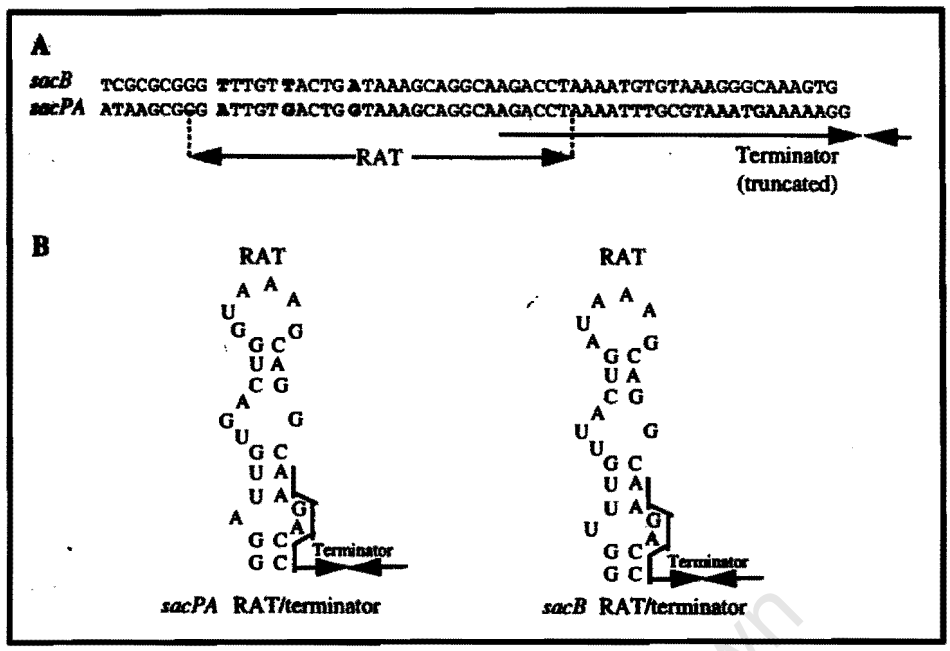

Figure 1.14. Sequence (A) and structure (B) of the *sacB* and *sacPA* RAT elements and terminators. In both cases the terminator overlaps the RAT sequence. The *sacB* and *sacPA* RAT sequences differ in 3 positions, indicated in bold. Adjacent terminators are indicated by opposing arrows. (After 15 and 149).

SacT and SacY are also involved in the induction of the *sacXY* operon in response to sucrose (51). The mechanisms involved are not clear since the RAT sequence identified ahead of the *scaXY* transcriptional start is 100 bp from the nearest transcriptional terminator.

Transcriptional termination is not the only mechanism involved in the regulation of sucrose metabolism in *B. subtilis*. The DegU/DegS signalling system is involved in the induction of *sacB* and *sacXY* expression (Figure 1.13) (51, 278). Sucrose uptake is also regulated by inducer exclusion, where the $EIIB^{Scr}$ domain of SacP is phosphorylated by the $EIIA^{Glucose}$ domain of the $EIICBA^{Glucose}$ protein (287).

1.9.2. *Staphylococcus xylosus*

S. xylosus is a low-GC, Gram-positive, non-pathogenic bacterium isolated from human skin (265). Two genetic systems have been identified which are involved in sucrose metabolism. The first, consists of the *malA* gene, encoding an α-glucosidase that is needed for maltose-maltotriose catabolism (68). The second, involves a sucrose PTS (36, 89, 318). MalA however, plays a minor role in sucrose metabolism in *S. xylosus*, probably because sucrose-6-phosphate is yielded intracellularly, which is not likely to be a substrate for MalA hydrolysis (68).

The PTS genes involved in sucrose metabolism include: *scrA*, encoding a EIIBC^{Scr}; *scrR*, encoding a GalR-LacI family regulatory protein; and *scrB*, encoding a sucrose-6-phosphate hydrolase (Figure 1.15) (36, 89, 318). The *scrR* and *scrB* genes are clustered, with the *scrR* ORF termination codon 84 bp from the start codon of the *scrB* ORF (36, 89). Despite their proximity, the two genes are independently transcribed. The *scrA* gene is not associated with the *scrR* and *scr* genes, and no additional sucrose utilisation genes are encoded close to the *scrA* (318).

There are 137 bp downstream of the *scrB* gene in the published nucleotide sequence (accession no. X67744). Sequence analysis of this region indicated the presence of a truncated, 47 amino acid ORF. The deduced sequence showed identity to the N-terminal regions of ribokinase/*pfkB* family fructokinases, with the highest identity (45%) to the *Clostridium acetobutylicum* ScrK (accession no. AAF35840.1). Furthermore, two domains conserved in the N-terminal regions of ribokinase/*pfkB* family fructokinases, (GEALID) and (GGAPCNVA) (28, 334), were identified. It is, therefore, likely that a *scrK* gene lies immediately downstream of *scrB*, it would follow that the *scr* gene cluster consists of three, and not two, genes, namely *scrR, scrB* and *scrK*.

The *scrA*, *scrR* and *scrB* genes have been disrupted in the *S. xylosus* genome (36, 89, 318). The *scrA* and *scrB* mutants were deficient in sucrose uptake and sucrose hydrolase activity respectively. The *scrR* mutant expressed these activities constitutively, suggesting that ScrR functions as a negative regulator of *scrA* and *scrB* production (89).

An imperfect palindromic sequence, O_B, was identified +6 to +21 bp relative to the *scrB* transcription start site, which was proposed to be the ScrR binding site (89). A 4 bp deletion of this site resulted in the constitutive expression of sucrose hydrolase activity. Furthermore, gel shift analyses demonstrated that a fusion of ScrR to the maltose-binding protein (MBP-ScrR) specifically bound to DNA fragments containing O_B. This suggests that in the absence of sucrose, ScrR is bound to O_B, preventing transcription of *scrB*. Loss of binding in the O_B mutant indicates that ScrR binds as a dimer. Neither glucose, fructose nor any of their metabolic intermediates affected the binding of ScrR. Sucrose also failed to have an effect. However, it was suggested that sucrose-6-phosphate would likely be the inducer molecule, given that sucrose would be present in this form as a result of PTS-dependent transport.

Evidence from the strain carrying the 4 bp deletion in O_B suggests that this operator site also plays a role in glucose-mediated repression of *scrB*, since glucose was unable to repress sucrose hydrolase activity in this mutant (89). It was noted that the O_B found identity to the CRE

consensus sequence (discussed in section 1.5.3), raising the possibility that CcpA might also exert an effect at O_B. Certainly, the CcpA has been cloned and characterised from *S. xylosus* and has been shown to mediate catabolite repression in this organism (69).

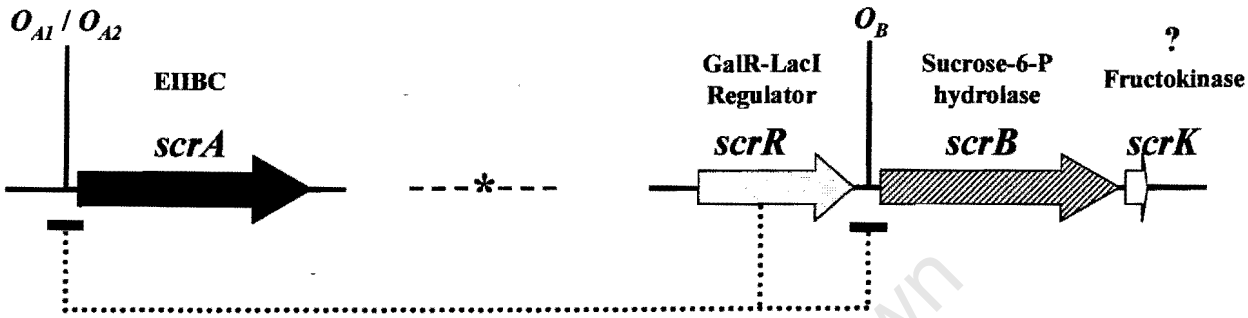

Figure 1.15. Molecular organisation of the *Staphylococcus xylosus* sucrose-PTS utilisation system. The *scrA, scrR* and *scrB* genes are independently transcribed and transcriptional polarity is indicated by the arrows. The dotted lines indicate the repression of the *scrA* and *scrB* transcription by ScrR at the O_{A2}/O_{A1} and O_B operators respectively. In the presence of sucrose ScrR is released from the operator sites and transcription proceeds. The 137 bp sequence after the published *scrB* nucleotide sequence (Accession no. X67744) was analysed. A truncated ORF showing identity to ribokinase/*pfkB* family fructokinases was observed, suggesting that a *scrK* gene could be located downstream of *scrB* gene. ---*---, indicates that the *scrA* gene is not linked to the *scrR,* and *scrB* genes.

The *scrA* transcription is almost certainly also regulated by ScrR. Two 24 bp O_B-like palindromes were identified in the *scrA* promoter region (318). The one, O_{A1}, has three mismatches compared with O_B, and overlaps the *scrA* -10 promoter. The second, O_{A2}, differs at two positions compared to O_B and is located between positions +11 and +24 downstream of the major transcriptional start. Gel mobility shift assays demonstrated that ScrR bound to the *scrA* promoter region, but it was not established whether O_{A1} or O_{A2} or both operator sites were involved (89).

1.9.3. *Streptococcus mutans and S. sobrinus*

The oral bacterium *S. mutans* has been implicated as the organism of greatest significance in the causation of dental caries, and the role of dietary sucrose in this is well documented (157). *S. mutans* possess a large number of enzymes which can act on sucrose (42). Extracellularly there are several glucosyltransferases (GTF) and fructosyltransferases which form polymers from one half of the disaccharide and release free monosaccharides (239). Under certain conditions, GTF can demonstrate invertase-like activity. Less than 10% of sucrose is metabolised extracellularly

while the remainder is metabolised intracellularly (157). Intracellular sucrose metabolism is mediated by at least two systems: a high affinity PTS-dependent system (285) and a low affinity multiple sugar metabolism (msm) system (238). In addition, a trehalose-PTS system has been shown to transport sucrose (213). Only the msm system and PTS-dependent system will be reviewed in the following discussion.

The msm system is involved in the uptake and metabolism of several sugars, including raffinose, melibiose and isomaltosaccharides, and this is carried out by eight genes which are located on an 11 kb region (Figure 1.16 A) (238). These are: *msmR*, encoding a positive effector protein; *aga*, encoding an α-galactosidase; *msmE*, encoding a sugar-binding lipoprotein; *msmF* and *msmG*, encoding integral membrane proteins; *gtfA*, encoding a sucrose phosphorylase; *msmK*, encoding an ATP-binding protein and *dexB*, encoding a dextran glucosidase. All the genes, except for *msmR*, are present in the same orientation and are co-transcribed (173). Expression of the genes is induced by raffinose and melibiose but not by isomaltose and isomaltotriose (238).

The MsmE lipoprotein is thought to reside on the cell surface, where it binds target sugars, which are then transported by the membrane components MsmF and MsmG (173, 238). Transport and metabolism by msm is regulated by the PTS (54). As was discussed in section 1.5.2, PTS-mediated regulation of non-PTS systems in Gram-positive bacteria involves the phosphorylation of non-PTS target proteins. Although the precise details have not been determined, it seems likely that a phosphoprotein specific to msm substrate-grown cells is involved and is phosphorylated by the PTS. At first, it was thought that sucrose was only generated intracellularly as a result of α-galactosidase activity on raffinose (293). It would then be metabolised by the sucrose phosphorylase (GtfA). However, sucrose was found to block 70% of melibiose transport. This was further validated when sucrose uptake was increased in a sucrose PTS-deficient strain. However, the msm system only transports sucrose with a low affinity, and only plays a role in the presence of high concentrations of sucrose.

The sucrose PTS on the other hand, is likely to facilitate the uptake of sucrose in low concentrations. The sucrose PTS regulon is encoded by the *scrK, scrA, scrB* and *scrR* genes (Figure 1.16 B) (107, 116, 161, 256, 257). The *scr* genes encode: a ROK/hexokinase family fructokinase (ScrK); and EIIBCA^{Scr} protein (ScrA); a sucrose-6-phosphate hydrolase (ScrB) and a GalR-LacI family regulatory protein (ScrR). The *scrA* and *scrK* genes are independently transcribed divergently from the co-transcribed *scrB* and *scrR* genes (Figure 1.16 B). A

phosphomannose isomerase gene (*pmi*) is located downstream of the *scrK*, and disruption of the *scrK* reduced *pmi* activity suggesting that these two genes are co-transcribed (257).

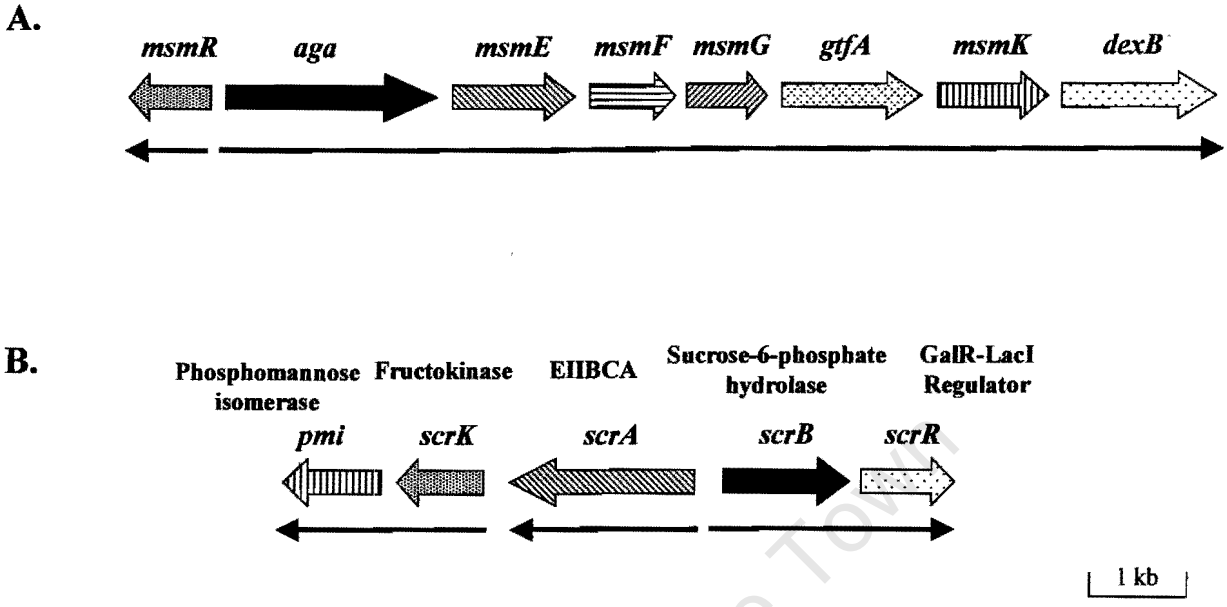

Figure 1.16. Genetic organisation of two sucrose utilisation clusters of *Streptococcus mutans*. A: the msm (multiple sugar metabolism) system, B: the sucrose-PTS. Thin arrows indicate transcriptional polarities and where it extends over more than one gene co-transcription has been inferred.

Expression of *scrA* and *scrB* is induced by sucrose and repressed by glucose and fructose (116). In addition, maltose and sorbitol repressed *scrA* activity to levels similar to what was observed for fructose. However, for *scrB*, contradicting results have been reported for the effect of sorbitol and mannitol on expression (116, 286). Expression of the *scrA* and *scrB* genes was reduced under acidic growth conditions (pH 5.6) relative to neutral pH (116). Plaque bacteria are subjected to fluctuations in pH (157), and the fermentation of sugars would lead to lactic acid formation thereby decreasing the environmental pH. Therefore, this possibly plays a role as a feedback repression system, however further experimental evidence is required.

The presence of the *scrR* gene suggests an involvement of its gene product in the regulation of the *scr* genes. Inactivation of *scrR* resulted in the constitutive expression of *scrB*, suggesting that ScrR functions as a negative regulator (116). Due to its identity to GalR-LacI-like proteins and the presence of a helix-turn-helix domain in the N-terminus, ScrR was investigated for its ability to bind to the *scrA-scrB* promoter region. A gel shift was observed with crude extract from cells expressing *scrR* which was absent when crude extract from *scrR*-mutant cells was used, and this binding was shown to be specific. However, specific binding of purified ScrR fused to maltose-

binding protein or to transcarboxylase to the *scrB* promoter could not be obtained. This could, however, be due to the fusion. Further experimental evidence is required to establish the exact mechanism by which ScrR interacts with the *scrA-scrB* promoter.

A truncated sucrose PTS operon has also been cloned from *S. sobrinus*, and it is similar to the *S. mutans* system (43, 44). The *scrA* and *scrB* genes were identified, encoding an EIIBCA^{Scr} protein and a sucrose-6-phosphate hydrolase respectively, and they are arranged identically as in *S. mutans*. A truncated ORF was identified immediately downstream of the *scrB* gene, encoding a helix-turn-helix motif similar to that of the *S. mutans* ScrR. This suggests that as in *S. mutans*, a gene encoding a regulatory protein lies adjacent to the *scrB* gene in *S. sobrinus*.

1.9.4. *Lactococcus lactis*

Sucrose utilisation in *L. lactis* is associated with resistance to and production of the antibiotic nisin (87). These traits are encoded by conjugative transposons, all of which are approximately 70 kb in size and show a similar organisation (219). They differ with regard to the variant of nisin produced, either nisin A or nisin Z, and in their ability to conjugate to other *L. lactis* strains. Due to the 30% GC content of the transposons as opposed to the 38% GC content of the *L. lactis* genome, it has been proposed that the transposons originated outside of the genus *Lactococcus*. Sucrose utilisation genes have been isolated and studied from *L. lactis* NIZO R5 and *L. lactis* K1, where they are encoded on Tn5276 and Tn5306 respectively. The majority of the analyses have been performed on the genes occurring on TN5276, and this will be discussed further.

Sucrose utilisation by *L. lactis* occurs via a typical sucrose PTS pathway (295). The product of translocation is sucrose-6-phosphate, which is cleaved by a sucrose-6-phosphate hydrolase to glucose-6-phosphate and fructose. Fructose is then converted to fructose-6-phosphate by an ATP-dependent fructokinase. Four genes are involved in the utilisation of sucrose and are clustered together forming two transcriptional operons: *sacAR* and *sacBK* (Figure 1.17) (159, 221). A third transcript produced from a *sacR* promoter is also identified under sucrose-inducing conditions (159). *sacA* and *sacR* encode the sucrose-6-phosphate hydrolase and a GalR-LacI family regulator respectively. Reading in the opposite direction to *sacAR* are the *sacB* and *sacK* genes encoding a PTS enzymeII and a fructokinase respectively. *sacAR, sacBK* and *sacR* transcripts are only identified in cells grown in sucrose, and are not detected when glucose is used for growth, indicating firstly, that regulation occurs at the transcriptional level, and secondly, that glucose repression also plays a role in regulation.

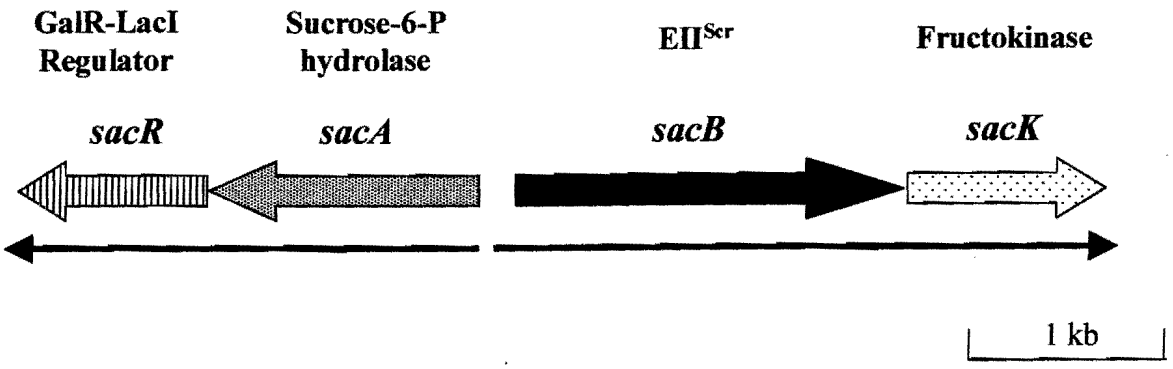

Figure 1.17. Genetic and transcriptional organisation of the Tn5276-located sucrose gene cluster of *Lactococcus lactis*. Thin arrows indicate transcriptional polarities and where it extends over more than one gene co-transcription has been inferred (159).

Inverted repeats were identified in the promoter regions of the *sacA, sacB* and *sacR* genes which could possibly function as the binding sites for factors involved in sucrose-specific regulation (159). Together with the presence of the *sacR* gene, it was postulated that *sacAR* and *sacBK* could be regulated by SacR, possibly by binding to the inverted repeats identified. In order to investigate the role of SacR in transcriptional control the *sacR* gene was disrupted. Northern analysis indicated that in the presence of glucose, *sacAR* and *sacBK* transcription became constitutive, and therefore, SacR functions as a repressor of transcription. Furthermore, the same mRNA levels of *sacAR* and *sacBK* were obtained from sucrose-grown cells indicating that SacR also mediates glucose repression. SacR-mediated substrate induction and negative autoregulation of *sacR* results in the efficient transcriptional control of the *sac* genes in response to variations in sucrose concentrations. Binding of SacR to the inverted repeats, however, still needs to be shown experimentally.

1.9.5. *Pediococcus pentosaceus and Lactobacillus plantarum*

Bacteria of the genus *Pediococcus* do not utilise sucrose, with the exception of several strains which carry plasmids (96, 97, 187). One of these plasmids, pSRQ1 in *P. pentosaceus* NRRL B-11465, contains the genes required for the utilisation of sucrose, raffinose and melibiose (151). There are thought to be two transcriptional units with opposite polarities (Figure 1.18) (149). The first contains the *scrB, scrR* and *agl* genes, encoding a sucrose hydrolase, a GalR-LacI family regulator and an α-glucosidase respectively. The second transcriptional unit is thought to involve the *scrA, agaS* and *scrK* genes, encoding a PTS EIIBCA^{Scr}, a putative α-galactosidase and a fructokinase respectively. Putative transcriptional terminators flank the two transcriptional units,

and these in turn, are flanked by IS elements similar to the *E. coli* IS30 and *L. lactis* IS981. This could suggest that the regulon is located on a transposon. Transcription of the two units is repressed by fructose but not by glucose.

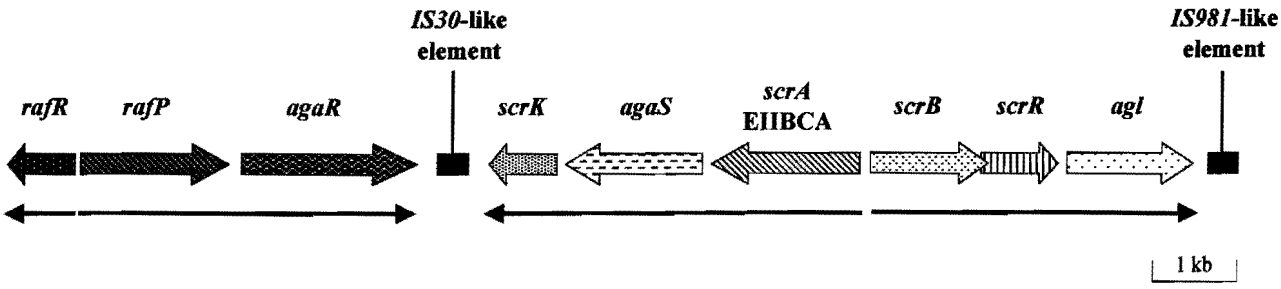

Figure 1.18. Organisation of the adjacent sucrose- and raffinose-utilisation genes from *Pediococcus pentosaceus*. Thin arrows indicate transcriptional polarities and where it extends over more than one gene co-transcription has been inferred (149, 151).

Most *Lactobacillus* species utilise sucrose and they transport the sugar via the PTS (148). The sucrose utilisation locus in *L. plantarum* was analysed using PCR, Southern hybridisation and restriction mapping, revealing high similarity to the sucrose utilisation locus of *P. pentosaceus* pSRQ1 (190). It contained the oppositely orientated *scrA* and *scrBRagl* operon, but not the *agaS* gene. The presence of the *scrK* gene was not determined. Sequencing of the *scrB* gene revealed 98.6% identity to that of *P. pentosaceus*, whereas only 94.4% homology was observed between their 16S rRNA genes. This suggests horizontal transfer of the sucrose utilisation locus between the lactic acid bacteria. This will be discussed further in the next section.

1.9.5.1. Similarities between the Sucrose PTS Regulons of Streptococci, L. lactis, P. pentosaceus and L. plantarum

The PTS regulons of *S. mutans, S. sobrinus, L. lactis, P. pentosaceus* and *L. plantarum* share several features and these similarities suggest a common origin (190, 220). The shared features are discussed briefly below. For simplicity, the gene nomenclature, *scr*, will be used throughout, where *scrA* is the PTS EII, *scrB* is the sucrose hydrolase, *scrR* is the regulator, and *scrK* is the fructokinase.

In each system, the *scrA* and *scrB* genes are divergently transcribed and are separated by between 180 and 256 bp. A *scrR* gene is present for all the organisms and is located downstream of and co-

transcribed with *scrB*. This is not the case in *L. lactis* however, where the *scrR* is co-transcribed with *scrA*. The *scrK* is located downstream of, and in the same orientation as, *scrA*, except for in *L. lactis*. For *P. pentosaceus* and possibly *L. plantarum*, the *scrK* gene is not immediately downstream of *scrA* as in *S. mutans*, and in *S. sobrinus* a *scrK* gene has not been identified.

In all the cases the *scrA* gene encodes an $EIIBCA^{Scr}$ protein. This differs from other PTS systems which are characterised by $EIIBC^{Scr}$ proteins, such as those found in *B. subtilis, S. xylosus, C. beijerinckii, S. typhimurium, K. pneumoniae* and *V. alginolyticus*. In all the cases, the *scrK* encodes a fructokinase belonging to the ROK/hexokinase family. In other bacterial sucrose utilisation systems, the fructokinase is typically of the ribokinase/*pfkB* family.

The fact that the *L. lactis* sucrose utilisation systems are located on conjugative transposons which have a GC content different to the *L. lactis* genome, and that the *P. pentosaceus* system is flanked by insertion sequences, makes it tempting to propose that the PTS regulons of *S. mutans, S. sobrinus L. lactis, P. pentosaceus* and *L. plantarum* share a common origin. However, for the streptococci it has not yet been determined if the sucrose utilisation system is encoded on a transposon. Further investigation is required to clearly establish whether these systems do in fact share a common origin.

1.9.6. *Escherichia coli*

Less than 50% of wild-type isolates of *E. coli* are sucrose positive (67), however, several sucrose-metabolising strains have been isolated. In *E. coli* EC3132 and *E. coli* B-62, the sucrose catabolic genes are chromosomally encoded (23, 127, 242), while in a third clinical isolate they are present on a transposon, Tn2555 (64). The most studied of these is the *csc* gene cluster (mnemonic for *c*hromosomally encoded *su*crose) in *E. coli* EC3132, which will be the focus in this discussion.

In *E. coli* EC3132 sucrose is taken up by a non-PTS mechanism and is hydrolysed by an invertase/sucrose hydrolase and the released fructose is phosphorylated by a fructokinase (23, 126). Four genes have been cloned and characterised (Figure 1.19). The *cscK* and *cscB* genes, encoding a ribokinase/*pfkB* family fructokinase and sucrose/H^+ symporter respectively are co-transcribed. Reading divergently from *cscKB* is the *cscA* gene encoding the sucrose hydrolase. Downstream from and in the opposite direction to the *cscA* is *cscR*, encoding a GalR-LacI-type regulatory protein.

This sucrose utilisation system in *E. coli* EC3132 is unique in that it involves a sucrose/H^+ symporter (CscB) (23, 126). CscB resembles permeases of the cluster 5 of the major facilitator superfamily (MFS), particularly with the lactose permease (LacY) from *E. coli* K-12. However, the transport activities of CscB are significantly lower compared to LacY (126). Furthermore, the *csc* genes are poorly expressed and it seems to be, at least in part, due to the inefficient transport of sucrose via CscB.

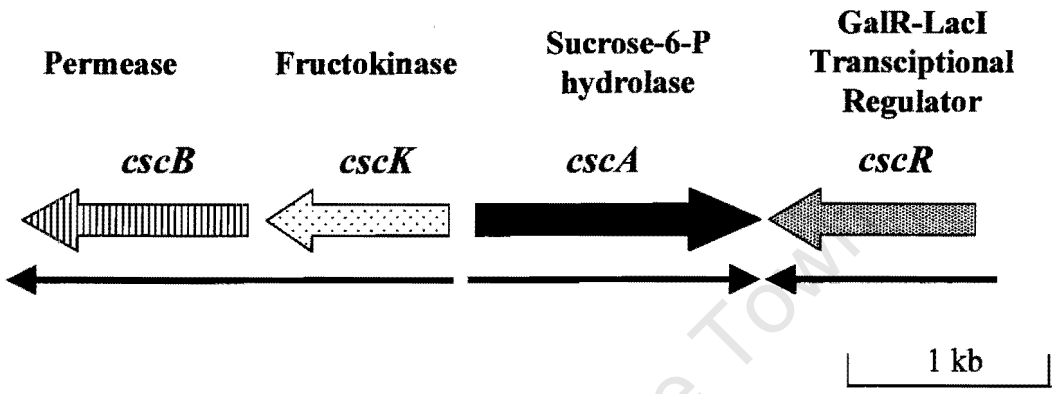

Figure 1.19. Organisation of the chromosomally encoded sucrose utilisation regulon of *Escherichia coli* EC3132. Thin arrows indicate transcriptional polarities and where it extends over more than one gene co-transcription has been inferred (23, 126).

CscR functions as a negative regulator of *cscKB* and *cscA* expression, and the presence of a Helix-turn-Helix motif in the N-terminus suggests that it binds DNA. In the *cscK* and *cscA* promoter region, a palindromic motif ($G_6T_5T_4A_3A_2C_1/G'_1T'_2T'_3A'_4A'_5C'_6$) is present twice, most likely corresponding to the operators of the two genes (126). Analysis of a mutant exhibiting enhanced sucrose utilisation indicated that a T_5-to-C_5 transition in the left half of the putative *cscK* operator had occurred, further suggesting the involvement of CscR in regulation. However, further analyses need to be performed to characterise the interaction between CscR and the putative operators. In the *csc* promoter-operator region two segments sharing high identity with the 22-bp palindromic consensus sequence for a cAMP-CrpA binding site were identified. Expression of the *csc* genes in a Cya^- mutant confirmed that both the *cscK* and *cscA* promoters were under the global control of cAMP-CrpA.

The *csc* genes are integrated into the *dsd* gene cluster on the *E. coli* chromosome (126). A comparison of the *csc* coding region and adjacent genes from *E. coli* EC3132 with the genomic sequences of the *E. coli* K-12 and the sucrose-positive uropathogenic *E. coli* O157:H7, revealed a pronounced diversity in this region of the chromosome (126). This diversity was caused by the

presence of the *argW* gene, coding for an arginine-specific tRNA, which served as an integration site for mobile genetic elements. A 21 bp repeat sequence was identified at the 3' end of *argW* which is a typical indicator for a site-specific integration of phage-like elements via tRNA-encoding genes. The repeat identified in O157:H7 was not annotated in a previous analysis of the O157:H7 sequence, suggesting that the horizontal gene transfer and stable integration must have occurred relatively recently in evolutionary terms. This is further supported by the fact that the *csc* genes in EC3132 are poorly expressed and, therefore, have not yet optimally adapted. The B-62 strain has a doubling time of 48 min on 0.2% sucrose (305), compared to the 20 hour doubling time for EC3132 (126). The *cscKB* operator of B-62 is almost identical to the sequence of EC3132 except that there is a C'_6-to-T'_6 transition in the right half of the palindrome. It has, therefore, been suggested that B-12 acquired an adaptation mutation to enable the cells to grow fast on relatively low concentrations of sucrose, and it therefore, likely inherited its *csc* genes before EC3132. Furthermore, it has been suggested that the *csc* genes do not belong to the set of housekeeping or backbone genes but rather belong to a group of optional genes that are highly mobile among the enteric bacteria.

1.10. AIMS OF THIS STUDY

Since bifidobacteria are saccharolytic, they play an important role in carbohydrate fermentation in the human colon. Taken together with the health benefits that they provide the human host, they are target organisms for the development of more successful probiotics and prebiotics. A variety of fructose-containing oligosaccharides, including inulin, oligofructose and raffinose, has been reported to selectively stimulate the growth of bifidobacteria, and several are currently added to foodstuffs. Despite the considerable commercial and research interests in oligosaccharides and probiotic bacteria however, the genetic mechanisms by which these organisms metabolise carbohydrates remain virtually unexplored, and relatively little is known about which strains actually metabolise the prebiotic substrates that have been identified. Bifidobacteria are very diverse in their ability to utilise a wide variety of carbohydrates, and this diversity exists not only between species, but also between strains. Therefore, for the purpose of selecting suitable probiotic organisms, prebiotic substrates and possible synbiotic applications, it is important to establish the substrate requirements, specificities and carbohydrate utilisation mechanisms of individual bifidobacterial strains and species. This knowledge will enable the development of novel oligosaccharides with improved prebiotic, physiological and technological properties.

The main objective of this study was, therefore, to perform a molecular analysis of carbohydrate metabolism in these bacteria for the development of a suitable probiotic strain, and for the identification of prebiotic compounds which could selectively stimulate their growth in the human colon. Comparative studies were to be performed in the three type culture strains, *B. breve, B. bifidum* and *B. longum* and in the industrial strain, *B. lactis*. This would involve, firstly, the examination of the carbohydrate fermentation patterns of the four bifidobacteria. A molecular analysis of carbohydrate metabolism would be conducted for those carbohydrates identified as having the potential for the development as a prebiotic from the fermentation experiments performed. This would entail the cloning and characterisation of the genes involved in the utilisation of the carbohydrate, and examining their role in carbohydrate metabolism. The regulatory mechanisms involved would be analysed, both at the physiological and genetic level. In this study, these analyses were performed in the industrial strain *B. lactis*, focussing on the carbohydrates raffinose and sucrose.

Considerable insight into carbohydrate metabolism has been achieved by the recent release of the *B. longum* genome sequence, which will undoubtedly catalyse research in this field in bifidobacteria. It should be noted, however, that this information was not available at the time that the work presented in this thesis was initiated. Besides many 16S rRNA sequences, only six genes and two plasmids of the members of *Bifidobacterium* had been cloned and sequenced. In this report, the findings of the functional and regulatory studies will be placed in the context of the sequence information offered by the *B. longum* genomic analysis.

CHAPTER 2

PHYSIOLOGICAL ANALYSIS OF CARBOHYDRATE UTILISATION IN *BIFIDOBACTERIUM*

Contents

2.1. SUMMARY

The industrially-used *B. lactis* strain was isolated from a yoghurt starter culture using a genus-specific 16S rRNA-targeted probe by colony hybridisation and PCR. Carbohydrate utilisation patterns of *B. lactis, B. breve, B. longum,* and *B. bifidum* were determined, where a variety of substrates were analysed for their ability to support bacterial growth as a sole carbohydrate source. *B. lactis* fermented the second most number of the carbohydrates tested, and it was selected for further analyses. Growth curves performed with *B. lactis* in media containing the prebiotic compounds raffinose and oligofructose as well as sucrose, revealed that raffinose was the preferred substrate, and that growth in oligofructose was weak.

Sucrase and α-galactosidase activities expressed in *B. lactis* were also investigated. Raffinose induced both sucrase and α-galactosidase activities, and sucrase activity was additionally induced by sucrose, and to a lesser extent by oligofructose. Both sucrase and α-galactosidase activities were repressed by the presence of glucose in the media, however this was more deleterious to α-galactosidase production than it was for sucrase activity. Partial repression of both activities was observed in the presence of glucose plus sucrose.

2.2. INTRODUCTION

Bifidobacteria play a very important role in carbohydrate fermentation in the colon since their saccharolytic nature enables them to utilise the different types of carbohydrate substrates that are potentially available to them in the intestinal environment. Since bifidobacteria provide the human host with health benefits (discussed in chapter 1), the prebiotic and synbiotic research approaches are receiving a great deal of attention.

The effectiveness of a probiotic is determined by its ability to survive and compete in the colon for food (53), therefore, understanding carbohydrate utilisation by bifidobacteria is extremely important. Furthermore, the prebiotic and synbiotic approaches cannot be implemented until carbohydrate utilisation patterns of the organisms of interest are determined.

A variety of fructose containing oligosaccharides, including inulin and oligofructose, have been reported to stimulate the growth of bifidobacteria (92, 94). Inulin is commonly found in plants such as artichoke, chicory, onion, leek, garlic and asparagus, with smaller amounts in cereal (91). It is a polymer of D-fructose linked by β(2,1) bonds with an α(1,2) linked D-glucose at the terminal end of the molecule, and has a degree of polymerization of 2-60+ (229, 231, 322). Oligofructose is produced commercially in one of two ways: either by partial enzymatic hydrolysis from chicory inulin using endoglycosidases (91), or by synthesis from sucrose using fungal fructofuranosidase (115). Its degree of polymerization is between 2 and 7 (229, 231, 322). Soybean is one of the genetically modified crops being adopted, particularly in developing countries where one-and-a-half million people suffer from hunger and malnutrition. South African farmers have recently begun planting transgenic soy. Soybean oligosaccharides could, therefore, become an important component of the African diet. Soybean oligosaccharides have also been shown to be prebiotics for bifidobacteria (49), and contain the oligosaccharides raffinose and stachyose, as well as sucrose, fructose and glucose. Raffinose and stachyose are both indigestible by humans and reach the colon intact. These substrates contain α(1,6)-linked galactose residues, and in raffinose it is linked to sucrose.

The primary aim of the work presented in this chapter was to compare the fermentation patterns of the commercial *B. lactis* strain to various other *Bifidobacterium* species with a view to its further development for biotechnological applications. In particular, the utilisation of inulin, oligofructose and raffinose by the bifidobacteria was of interest due to their prebiotic effects, and therefore, the utilisation of these substrates was analysed further. In order to understand the genetic and

enzymatic mechanisms required to utilise these carbohydrates, the production and regulation of sucrase and α-galactosidase activity in response to different growth conditions was investigated in *B. lactis*.

2.3. MATERIALS AND METHODS

2.3.1. Bacterial Strains, Plasmids and Culture Conditions

Bifidobacterium bifidum NCFB 2203, *B. breve* NCFB 2257, and *B. longum* NCFB 2259 were obtained from the NCIMB culture collection, UK. Bifidobacterial cultures were propagated anaerobically at 37°C in an anaerobic chamber (Forma Scientific, Model 1024), in an atmosphere of 5% H_2, 10% CO_2, and 85% N_2. A comparative analysis of various growth media (Table 2.1) was performed (2.4.3) and BYG medium (Appendix B.1.3.) was selected for the culturing of the bifidobacteria. However, glycerol stocks were prepared from cells grown in BHI broth (Appendix B.1.1.) as previously described (253), and were stored at -70°C under the same anaerobic gas mixture as for growth. For the growth curves, and the sucrase assays, 1% (w/v) of each carbohydrate was substituted for glucose.

Table 2.1. Various basal media used for the growth analysis of the bifidobacteria

	BG Medium	**BYG Medium**	**BCG Medium**	**BSG Medium**
Basic media components[a] (grams/litre)	Tryptone 10 Yeast extract 2.5 Glucose 5 Tween 80 1 Salts[b]	BG medium + Yeast extract 2.5	BG medium + Casamino acids 0.2	BG medium + $ZnSO_4.7H_2O$ 0.025 $CaCl_2$ 0.015
Reference	57, without peptone water	58	-	261

[a] A complete description of the media components is described in the appendix.

[b] The following salts were added in gram/Litre: NaCl, 4.5; KCl, 0.25; $MgCl_2.6H_2O$, 0.15; KH_2PO_4, 0.4; K_2HPO_4, 0.2; NH_4Cl, 0.4.

Escherichia coli JM109 (336) was used for all cloning purposes and was routinely cultured aerobically at 37°C in Luria-Bertani (LB) medium (253), containing ampicillin (Ap) (100 μg/ml) when plasmid was present. The plasmid vector pBluescript SK (pSK) (Stratagene, La Jolla, California, USA) was used for cloning purposes. Further strain and plasmid details are supplied in Appendix A.

2.3.2. Isolation of *Bifidobacterium lactis*

One gram of a freeze-dried yoghurt starter culture (Darleon Distribution), containing a mixture of *Bifidobacterium lactis* together with *Lactobacillus acidophilus, Lactobacillus casei,* and *Streptococcus thermophilus*, was dissolved in 10 ml of BHI broth and incubated anaerobically for 18 hours. Serial dilutions were performed and plated in duplicate onto BHI agar and incubated aerobically and anaerobically. Single, well separated colonies growing only anaerobically were selected as putative *B. lactis* cells and were analysed further by colony hybridization. *Bifidobacterium* genus-specific primers were used to PCR a 16S rRNA DNA fragment from *B. breve*. The PCR fragment was DIG-labelled according to manufacturers' instructions (Boehringer Mannheim), and was used as a probe for colony hybridization (Boehringer Mannheim). Colonies showing a positive signal were analysed by PCR. PCR was performed as follows (50 μl): 5 μl buffer (supplied with the enzyme); 2.5 mM $MgCl_2$; 0.5 μM of each primer; 200 μM of each dNTP; 50 ng chromosomal DNA; and 1 U of *Taq* DNA polymerase (Supertherm). The PCR was carried out in a GeneAmp 9700 machine (Applied Biosystems). The amplification program consisted of one cycle of 96°C for 5 min, then 25 cycles of 96°C for 1 min, 66°C for 45 s, and 72°C for 1 min, and finally one cycle of 72°C for 5 min. The reaction was cooled down to 4°C. The *Bifidobacterium* genus-specific 16S rRNA gene primers for PCR were: Forward 5'-CGC CAG GGT TTT CCC AGT CAC GAC GGG TGG TAA TGC CGG ATG-3' and Reverse 5'-CAG GAA ACA GCT ATG ACC CAC CGT TAC ACC GGG AA-3'. The underlined sequence represents the *Bifidobacterium* genus-specific 16S rDNA sequence (146). The sequence in the 5' region is pSK-derived M13 forward and reverse primers respectively to facilitate sequencing.

2.3.3. General DNA Manipulations

Competent *E. coli* JM109 cells were prepared using the rubidium chloride method (9). Plasmid DNA was extracted using the Nucleobond® AX KIT (Macherey-Nagel, Germany). T4 ligase and restriction endonucleases were purchased from Amersham or Boehringer Mannheim and were used as specified by the manufacturer. DNA ligations were performed as previously described (253). Gel electrophoresis was conducted in 0.8% w/v agarose gels in Tris-Acetate-EDTA buffer

as described (253). DNA fragments were purified from the agarose gels using the High Pure PCR Product Purification kit (Roche).

2.3.4. Nucleotide Sequence and Analysis

DNA sequencing was done by the dideoxy-chain termination method (255), using the Thermo-Sequenase Sequencing kit (USB) and Cy5 fluorescently labelled universal and reverse primers, as per manufacturers' instructions. The sequencing reaction products were separated using the AlfExpress DNA sequencer (Pharmacia) and the nucleotide sequence obtained was analysed using the DNAMAN software package. Nucleotide and amino acid homology searches were carried out against the databases at NCBI, USA, using the BLAST programme (3). The *B. lactis* 16S rRNA DNA sequence was submitted to Genbank and has been assigned the accession No. AY151397.

2.3.5. Oxygen Tolerance Analyses

Overnight BYG cultures of the bifidobacteria were diluted and plated onto BYG agar plates. Plates were incubated for 0, 1, 3, 4, 5, and 24 hours aerobically and then transferred to the anaerobic incubator for 2 days. The number of colony forming units after 2 days growth was recorded.

2.3.6. Carbohydrate Fermentation Analysis of the Bifidobacteria

BY agar containing 0.5% (w/v) of the sugars (arabinose, xylose, ribose, glucose, fructose, galactose, sorbitol, sucrose, lactose, maltose, cellobiose, melezitose, raffinose, trehalose, melibiose, mannitol, glucosamine, amylose, starch, glycogen, xylan, carboxymethyl cellulose (CMC), oligofructose, and inulin) was used. Phenol red (5% v/v of a 0.2% w/v solution) was added as a colourimetric pH indicator, the resazurin was omitted, and the media was adjusted to pH 7.6 with 6 N NaOH. A change of the colour of the media from orange to yellow indicated that acid had been produced via fermentation of the sugar. The utilisation of starch, xylan and cellulose was also tested in complete media (final concentrations of 0.1%, 0.5% or 1% w/v in BHI), and zones of utilisation were determined by staining with Gram's iodine (117) for starch, and Congo red (0.1% w/v) for xylan and cellulose. Oligofructose (Raftilose P95) was supplied by Savannah Fine Chemicals (South Africa), soluble starch was supplied by BDH, and xylan by Aldrich, all other substrates were Sigma products.

2.3.7. *Bifidobacterium* Chromosomal Extraction

Bacterial cells grown to late-exponential-phase in 500 ml BYG medium containing 0.5% glycine were harvested by centrifugation (6 000 x *g*, 10 min at 4°C). The cells were washed twice in buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 1 mM EDTA), resuspended in 10 ml lysozyme solution (25% sucrose, 0.1 M NaCl, 0.05 M Tris-HCl pH 8.0, 10 mg/ml lysozyme) and incubated for two hours at room temperature. After incubation, 10 ml of buffer (10 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 7.5) was added and the suspension gently mixed. Proteinase K (Boehringer Mannheim) was added to a final concentration of 100 µg/ml, and was incubated for one hour at room temperature with gentle mixing. SDS (final concentration of 2% w/v) and 0.5 mg of RNAse (Sigma) were added and the mixture incubated at 37°C for 30 minutes. The resulting cell lysate was extracted three times with hot phenol (65°C), followed by three extractions with water-saturated ether. The DNA was precipitated with ethanol (253) and resuspended in 500 µl water.

2.3.8. Processing of *B. lactis* Cells for Sucrase and α-Galactosidase Assays

For sucrase assays, cell free extracts (CFEs) were prepared from 100 ml cultures grown to mid- and late-logarithmic phase in BY medium containing the various carbohydrates, and were harvested by centrifugation (6 000 x *g*, 10 min, 4°C). Cells were washed twice and resuspended in 5 ml TAP buffer (100 mM Tris, 100 mM acetate, 64.2 mM Na_2HPO_4, pH 6). Cells were disrupted by sonication (4°C) at 95 W for 6 min with 30 s cooling intervals (VirSonic Digital 475 Cell Disruptor), and cell debris was removed by centrifugation (15 000 x *g*, 20 min at 4°C). The supernatant was used as the CFE.

α-Galactosidase assays were performed on three different preparations of the cells: crude lysate (total protein after sonication) (CL), cell free extract (CFE), and membrane fraction (MF) and were prepared as follows: mid-logarithmic phase cultures (100 ml) were harvested by centrifugation (6 000 x *g*, 10 min, 4°C), and were washed, and resuspended in 400 µl of 20 mM sodium phosphate buffer (pH 6.5). Cells were sonicated as above, resulting in the CL fraction. The CL fraction was then centrifuged (14 000 x *g*, 20 min at 4°C), where the supernatant was used as the CFE. The remaining pellet which consisted of cell debris and the membrane fraction (MF) was washed and resuspended in 200 µl of buffer.

2.3.9. Enzyme Assays

2.3.9.1. Sucrase Assays

Sucrase assay conditions were determined from the characterization of ScrP activity experiments reported in Chapter 4. Sucrase activity was assayed in the following way: 15 µl of 0.88 M sucrose was incubated with 35 µl of the appropriate CFE dilution for 30 min at 60°C. The reaction was terminated with 150 µl dinitrosalicylic acid reagent (DNS) (175), and boiled for 5 minutes. The amount of reaction product formed, was determined spectrophotometrically at 510 nm, using glucose (concentration range of 0.5-10 mM) as a standard. Sucrase activity was expressed as µmol reducing sugar produced per min per mg protein.

2.3.9.2. α-Galactosidase Assays

For the α-galactosidase assays, activity was determined by the rate of hydrolysis of ρ-nitrophenyl-α-D-galactopyranoside (NpGal). A suitable enzyme assay was developed from four different protocols (81, 166, 233, 310) and was performed as follows: 20 µl of sample was incubated with 180 µl of NpGal (2.66 mM in 20 mM sodium phosphate buffer pH 6.5) at 37°C for 5 mins. The reaction was stopped by adding an equal volume of stop buffer (0.5 M glycine/NaOH pH 9, 2 mM EDTA), and the volume was made up to 1 ml with water. The liberated ρ-nitrophenol was measured spectrophotometrically at 405 nm, which was also used as the standard (concentration range of 100 µM to 1 mM). Protein concentrations were standardized Activity was expressed as µmol ρ-nitrophenol released per min per mg protein.

2.3.10. Protein Assays

Protein concentrations were determined using the method of Bradford (33), with bovine serum albumin as the standard (concentrations of 0.025 mg/ml to 0.1 mg/ml). The protein concentration in the CFE and the various fractions was standardized prior to its use in the sucrase and α-galactosidase assays.

2.4. RESULTS AND DISCUSSION

2.4.1. Isolation of *B. lactis*

The comparison of 16S rRNA sequences has been a reliable method for the classification and identification of bifidobacteria (169), even down to 1-nucleotide difference (335). This technique is more rapid, simple and sensitive than previously used methods based on phenotypic characteristics and DNA-DNA homology. On this basis, genus-specific primers for *Bifidobacterium* were used for the isolation of *B. lactis* from a yoghurt starter culture, which also contained *Lactobacillus acidophilus, Lactobacillus casei,* and *Streptococcus thermophilus.*

Since *B. lactis* was the only obligate anaerobe species present, cells were incubated aerobically and anaerobically for the primary isolation of potential *B. lactis* cells (those cells which only grew anaerobically). Using the *Bifidobacterium* primers, a 0.55 kb rDNA fragment from *B. breve* was generated by PCR and was used as a probe to perform colony hybridizations on the putative *B. lactis* cells isolated. Colonies which hybridised to the probe were analysed by PCR using the same primers (Figure 2.1). PCR fragments were subcloned into the *Eco*RV restriction enzyme site of pSK yielding pLac2. The insert was sequenced using T7 and T3 universal primers and analysed using the BLAST search (3). Sequence analysis revealed sequence identity to bifidobacterial 16S rRNA (Table 2.2). A high sequence identity was also found to the *B. animalis* 16S rRNA gene. A report by Cai *et al* (39), where DNA-DNA hybridization between the type strains of *B. lactis* and *B. animalis* was done, concluded that they represent a single species. Furthermore, they concluded that the name *B. lactis* has no taxonomic standing and that it should be reclassified as *B. animalis* (39). However, since this report, other publications have used the name *B. lactis*. Furthermore, *B. animalis* is associated more with animals than with humans (261), and due to the human application aspects of this study, the name *B. lactis* was selected for future use in this work.

2.4.2. Bifidobacterial Oxygen Tolerance

One of the properties of a successful probiotic strain is that it maintains viability and activity in the carrier food before consumption (95). For an anaerobic organism, its tolerance to oxygen is of importance considering that the majority of food preparations containing probiotics are stored and/or prepared aerobically. Bifidobacteria, although anaerobic, have varying degrees of tolerance towards oxygen (18, 39). We were interested in determining this in the four bifidobacterial strains, which in addition to facilitating the selection of a probiotic strain, it would also provide insight into the culturing and handling of the organisms in the laboratory. All the strains tested survived a 24 hour aerobic incubation and could be cultured thereafter (Figure 2.2). This was repeated three

times, and with each experiment the cell numbers for *B. longum, B. lactis* and *B. bifidum* did not decrease more than 10-fold, while *B. breve* cell numbers were reduced $4x10^4$-fold. From this type of experiment it appears that *B. longum, B. lactis* and *B. bifidum* can tolerate prolonged periods of exposure to oxygen, and would therefore make good probiotic candidates with respect to maintaining viability in the carrier foods.

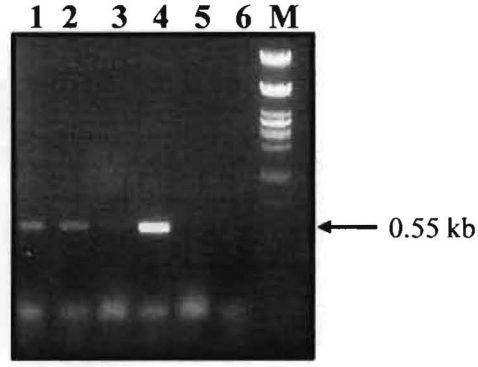

Figure 2.1. PCR analysis of putative *B. lactis* colonies using *Bifidobacterium* 16S rRNA primers. Lanes 1-3: Putative *B. lactis* isolates, Lane 4: *B. breve* 2257 positive control, Lane 5: *E. coli* JM109 negative control, Lane 6: no DNA, M: λ DNA digested with *Pst*I as molecular weight marker.

Table 2.2. Sequence similarities of the *B. lactis* 16s rRNA DNA sequence in pLac2 to sequences in the database.

Organism to which sequence similarity was found	% Identity
Bifidobacterium sp. (Accession no. X89111)	99% (523/524)
B. lactis (Accession no. AB050136)	99% (523/524)
B. lactis (Accession no. X89513)	99% (522/524)
B. animalis (Accession no. AB050138)	99% (523/524)
B. animalis (Accession no. D86185)	98% (520/526)
B. infantis (Accession no. BIN311604)	98% (517/527)

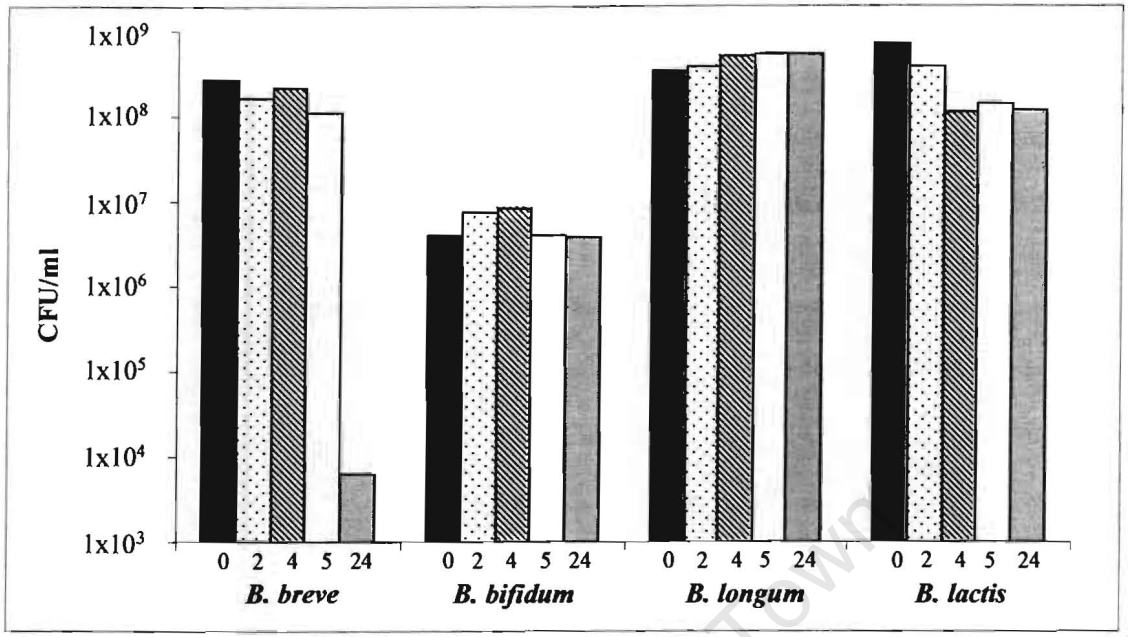

Figure 2.2. Bifidobacterial tolerance to aerobic incubation. Cells on BYG agar plates were exposed to aerobic incubation for the hours indicated and then incubated anaerobically for 2 days. The experiment was repeated 3 times and the results for one of these is reported. The same trend was observed for each of the experiments. CFU: colony forming units.

2.4.3. Analysis of the Bifidobacterial Carbohydrate Fermentation Patterns

In order to analyse the fermentation patterns of the four bifidobacterial strains on various carbohydrates, a suitable basal medium containing a fermentation indicator was required. BG medium, a modified version of that used by Degnan *et al* (57), was selected. Growth was compared with BG medium containing either additional yeast extract (BYG), or casamino acids (BCG), or additional salts (BSG) (Table 2.1). Growth curves were performed with the four bifidobacterial strains in the media described and growth in BHI was used as a control (Figure 2.3). Although there were no major differences in the growth of the bifidobacterial strains with the various media, BYG was preferred by most, and was, therefore, selected as the media to be used throughout this study. This medium was also selected for the fermentation analyses, where the carbohydrates were substituted for the glucose (see 2.3.1). Phenol red was selected as the fermentation indicator (Figure 2.4).

Fermentation of a variety of carbohydrates as a sole carbohydrate source was tested (Table 2.3). *B. breve* was the most versatile in its utilisation, followed by *B. lactis*, then *B. longum*, and finally,

B. bifidum. *B. bifidum* would perhaps not be the ideal target for industrial application due to its limited use of carbon sources, particularly the prebiotic substrates inulin, oligofructose and raffinose. However, carbohydrate utilisation patterns between strains of a particular bifidobacterial species have been shown to vary considerably, particularly in *B. bifidum* (119).

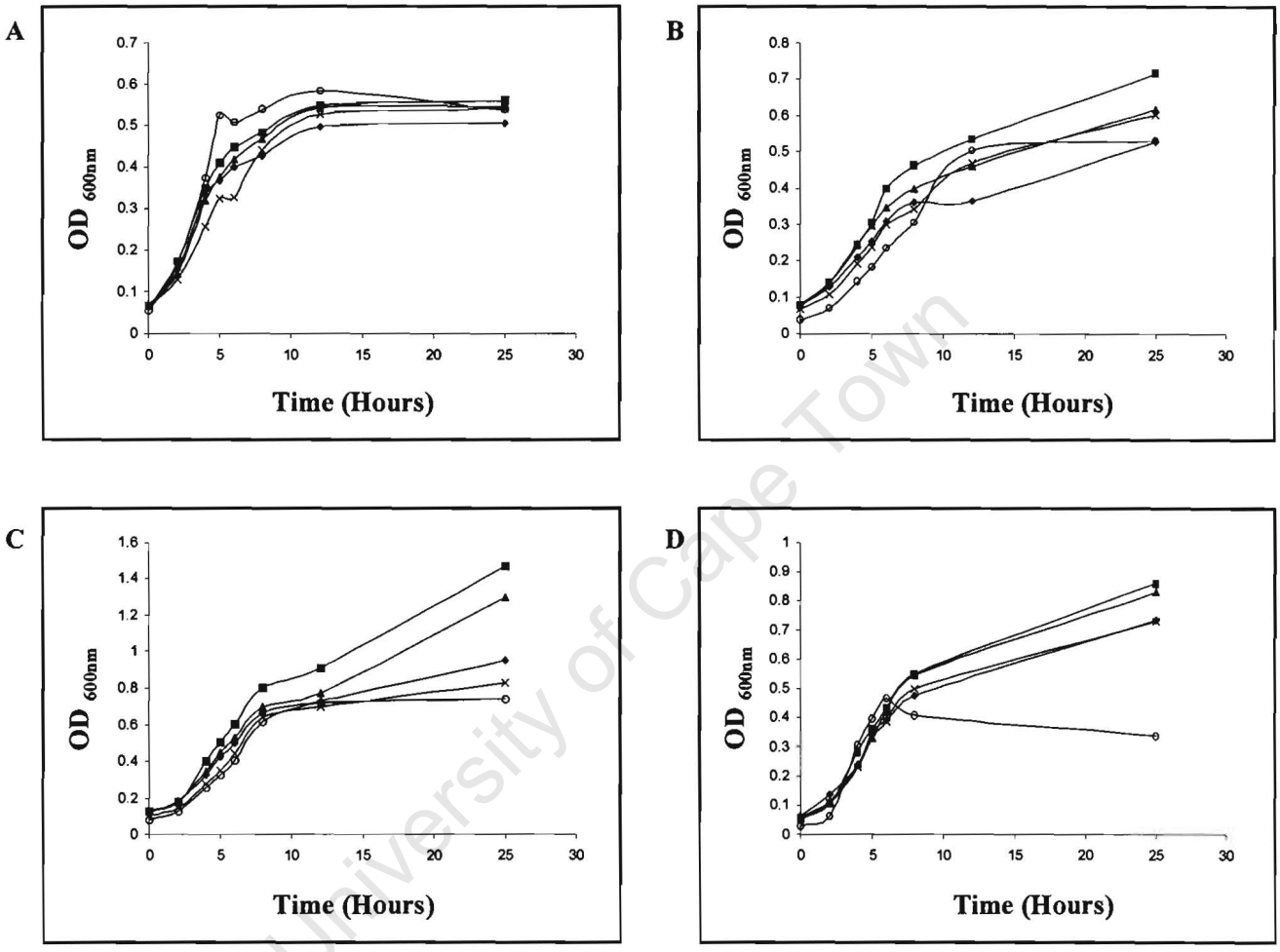

Figure 2.3. Growth curves of the bifidobacteria grown in the media described in Table 2.1. and in BHI as a control medium. Growth was monitored by measuring absorbance at 600 nm ($OD_{600\ nm}$) at the times indicated. The values are the mean of three experiments. A: *B. bifidum*, B: *B. breve*, C: *B. lactis*, D: *B. longum*. Symbols: (♦) BG, (■) BYG, (▲) BCG, (x) BSG, (○) BHI.

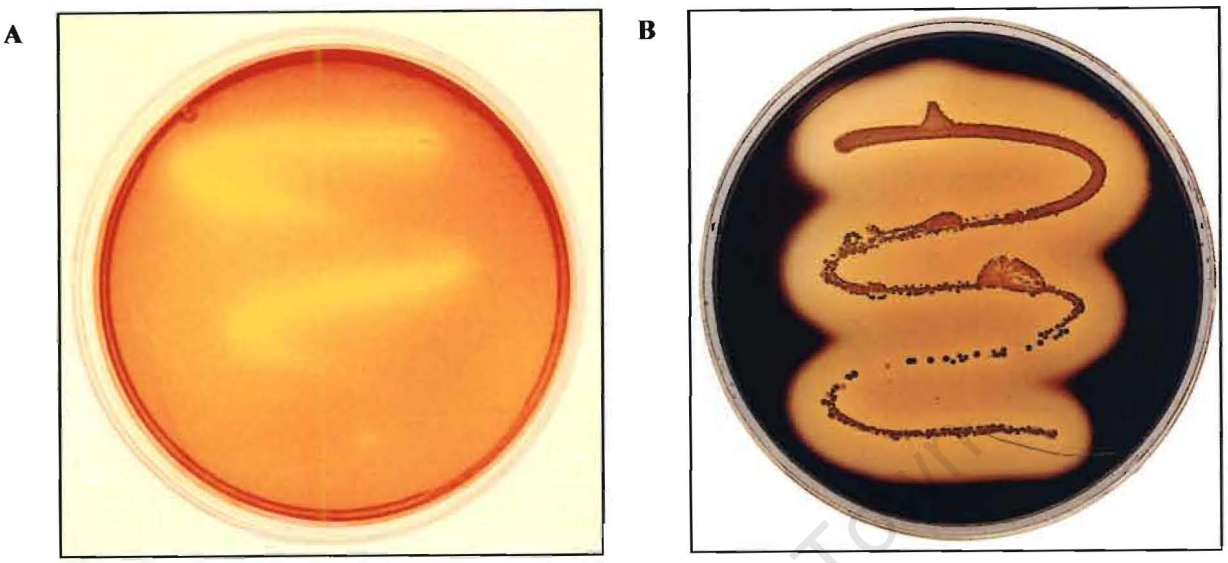

Figure 2.4. Positive carbohydrate fermentations by *B. breve* grown on two different media. A. BY medium containing 0.5% glucose. Acid production as a result of fermentation reduces the pH to below 6.7 causing a change in the phenol red indicator from orange to yellow. B. Complete media (BHI) with 0.1% starch. A zone of starch utilisation is detected when stained with Gram's iodine.

It is interesting that some of the simple sugars, such as fructose, galactose could not be utilised by some of the strains, even though they were able to ferment the more complex substrates composed of these moieties, such as oligofructose and raffinose. This phenomenon appears to be common in many of the bifidobacterial strains (48, 119). Similarly, the thermophilic yoghurt bacteria *S. thermophilus* and *Lactobacillus bulgaricus* efficiently transport and hydrolyse lactose intracellularly, however, they only ferment the glucose portion of lactose, while the galactose is excreted (313). Simple sugars are metabolised by the human host, while the more complex carbohydrates reach the colon intact (249), thus, the latter probably represent the major source of carbon and energy for the gut bacteria. It would seem likely, therefore, that the substrate transport mechanisms of these organisms would be more efficient for the complex carbohydrates.

Table 2.3. Carbohydrate fermentation patterns of the bifidobacteria.

Substrate	*B. bifidum*	*B. breve*	*B. lactis*	*B. longum*
Arabinose	-	-	+	-
Xylose	-	-	-	+++
Ribose	(+)	-	+	-
Glucose	+++	+++	+++	+++
Fructose	++	++	-	++
Galactose	-	++	(+)	-
Sorbitol	-	+	-	-
Mannitol	-	++	-	-
Glucosamine	++	+++	-	-
Sucrose	-	++	+++	+
Lactose	++	+++	+++	+++
Melibiose	+	++	+	++
Maltose	-	++	++	++
Cellobiose	-	+++	-	-
Melezitose	-	-	++	-
Raffinose	-	+++	+++	++
Trehalose	-	-	-	-
Amylose	-	+	-	-
Starch	++[ab]	+++[b]	+[a]	+[a]
Glycogen	-	++	-	-
Xylan	-	(+)[c]	-	-
Cellulose	-	-	-	-
Oligofructose	-	++	++	++
Inulin	-	-	-	-

- No fermentation + Fermentation after 24 hours (+) Ferments slowly

+[a] Was not fermented as a sole carbohydrate source, but was weakly utilised in complete media at 0.1% (w/v)

+[b] Was fermented as a sole carbohydrate source, and was utilised in complete medium at all concentrations

+[ab] Was not fermented as a sole carbohydrate source, but was utilised in complete media at all concentrations

+[c] Was fermented as a sole carbohydrate source, but was not utilised in complete media

As discussed in section 2.2, oligofructose has a DP below 7, while that of inulin is between 2 and 60+ (229, 322). Therefore, the fact that *B. lactis*, *B. breve* and *B. longum* could utilise the former but not the latter substrate, could indicate that the degree of polymerization affects the bacterial ability to utilise the prebiotic substrates. The *B. longum* and *B. breve* strains used in this study have previously been shown in the literature to utilise Raftiline, which is 92-99% inulin with an average DP of 10-12 (92, 119, 322). Therefore, their inability to show growth on the inulin substrates used in this study was puzzling. Since the DP of inulin can vary significantly, it may be possible that our source of inulin has a DP greater than that of Raftiline, and thus could be too complex for the organisms to utilise. In support of this, it has been reported that inulin with a DP > 10 was fermented half as quickly as molecules with a DP < 10 (231).

Starch has recently been investigated for its ability to complement a bifidobacterial strain in a synbiotic yoghurt (48), and for its use in microencapsulation (47). In our analyses, only *B. breve* was able to utilise soluble starch as a sole carbohydrate source, however, the other strains were able to utilise starch to some extent when it was supplied in complete medium. Starch is hydrolysed by the combined action of several enzymes such as α-amylases, β-amylases, glucoamylases, and other exo-1,4-glucanases (324). There are a few organisms from which activity of more than one of the enzymes has been detected, including from *B. bifidum* and *B. pseudolongum* (321). It is possible that *B. breve* similarly possesses more than one, considering that it was able to utilise soluble starch, amylose and glycogen as a sole carbohydrate source. In comparison, the other strains could only utilise the soluble starch, suggesting a less diverse genetic ability to hydrolyse this substrate. Interestingly, *B. longum* showed weak amylase activity when grown in complete media containing soluble starch (Table 2.3). Sequence analysis of the *B. longum* NCC2705 genome indicated that β- and α-amylases are absent (262). Furthermore, it is generally considered that *B. longum* cannot utilise this substrate (260, 261, 290). However, some strains have been shown to hydrolyse Hi-Maize™ (48), and therefore must possess the required enzymes. It appears, therefore, that the *B. longum* strain used in this study similarly possesses, albeit weak, enzymatic activity towards starch, once again illustrating the variation in carbohydrate utilisation patterns between bifidobacterial strains.

2.4.4. Sucrase and α-Galactosidase Activity in *B. lactis*

B. lactis was selected for further analyses for a number of reasons. It is the probiotic used most industrially and, unlike many of the bifidobacterial strains tested, it has been shown to survive well in the conditions stimulating the human stomach (48), an important factor for the selection of a probiotic. In addition, very few carbohydrate utilisation studies have been conducted in this

organism due to its recent discovery (174), and, therefore, a greater knowledge is required for this organism to be developed further for biotechnological applications. It was also the second best utiliser of the carbohydrates tested (Table 2.3), including oligofructose and raffinose. Due to the importance of these substrates as prebiotics, we were interested in isolating and studying the genetic mechanisms by which *B. lactis* utilised them. Three key enzyme activities are required for their utilisation: sucrase, fructanase, and α-galactosidase. Since *B. lactis* could not utilize fructose, only α-galactosidase and sucrase activity was investigated in response to growth in the presence of various carbohydrates. The isolation of these genetic systems was undertaken and will be presented in chapters 3 and 4 respectively.

2.4.4.1. B. lactis Growth Curves

Growth curves were performed to determine an appropriate growth stage and time point at which sucrase and α-galactosidase enzyme activities could be assayed. In order to establish how these activities were regulated, *B. lactis* growth curves were performed in the presence of 1% (w/v) of the following carbohydrates: glucose, sucrose, glucose plus sucrose, raffinose and oligofructose (Figure 2.5). Raffinose and sucrose were the carbon sources most preferred by *B. lactis*, giving maximum cell yields as well as maximum growth rates, in comparison to glucose. Growth in oligofructose was poor. These results should be of particular interest in the viewpoint of future prebiotic development. It appears that raffinose-containing substrates might be better candidates for the growth stimulation of *B. lactis*. Soybean oligosaccharides (SBO) have been shown to stimulate the growth of bifidobacteria while being poorly fermented by other genera of the microflora (108, 247). Furthermore, raffinose has been shown to be more rapidly and efficiently assimilated by bifidobacteria compared to other non-digestible oligosaccharides, including fructose-oligomers (129). However, the raffinose and stachyose present in SBO may be responsible for flatulence after fermentation (259), and therefore, their use as prebiotics would need to take this into consideration.

2.4.4.2. Regulation of Sucrase Activity in B. lactis

Sucrase activity was assayed in *B. lactis* cultures grown to mid- and late-logarithmic phase in BY medium containing sucrose, glucose, glucose plus sucrose, raffinose or oligofructose (Figure 2.6). Sucrase activity in the different conditions did not differ greatly between mid- and late-log cultures. Similar activities were recorded for cultures grown in glucose and in glucose plus sucrose. Taking activity in glucose as the basal level, sucrase activity was induced 2-fold in the presence of sucrose or raffinose, and 1.4-fold in oligofructose in comparison with it. It is clear that there is some form of glucose repression, however, activity is not completely repressed. From the

growth curves performed for *B. lactis* (see 2.4.4.1 and Figure 2.5), it was suggested that growth in the presence of sucrose was preferred in comparison to glucose. It is, therefore, surprising that glucose reduced sucrose utilisation. Perhaps glucose was in fact the preferred carbon source, and that growth in the presence of glucose was inhibited by strong acidification. The fact that sucrase activity did not increase in late-log cultures grown in glucose plus sucrose, suggests that the sucrose was not co-utilised by the cells. However, the growth curves suggest the contrast, since growth was higher in the presence of glucose plus sucrose compared to in glucose alone (Figure 2.5), indicating that sucrose was co-utilised. It could be argued that in the presence of glucose plus sucrose, constitutive systems would utilise the sucrose, thereby allowing the simultaneous utilisation of both sugars, and the resulting activity would be the same as that seen when grown in glucose. Future experiments would need to be performed where the concentration of the sugars in the medium would be monitored as the growth progressed.

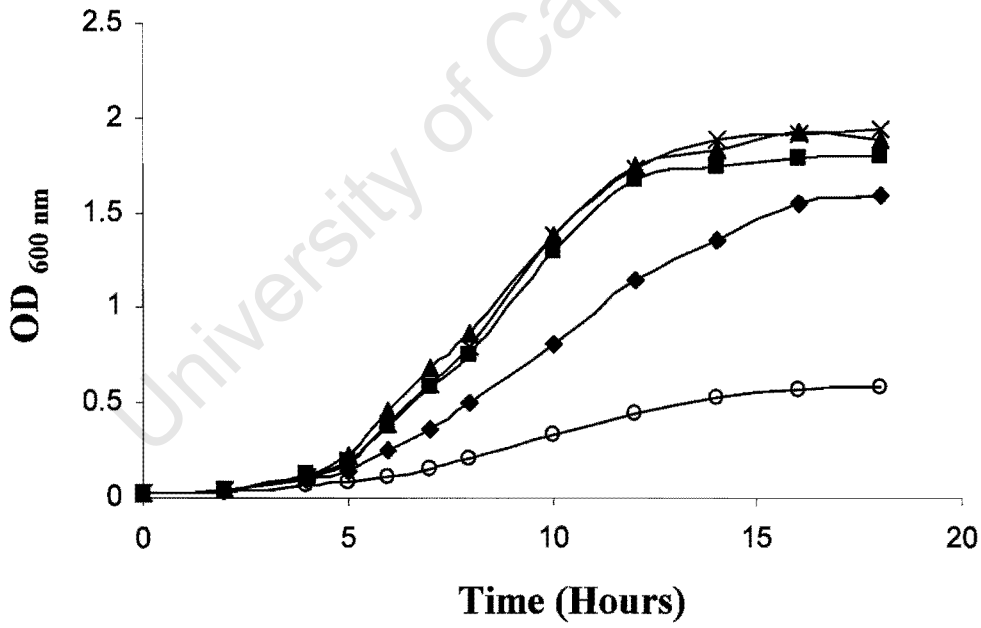

Figure 2.5. Growth curves of *B. lactis* grown in BY medium containing 1% (w/v) of each of the various sugars. Growth was monitored by measuring the absorbance at 600 nm (OD_{600}) at the times indicated. Symbols: (♦) Glucose, (■) Sucrose, (▲) Glucose plus sucrose, (x) Raffinose, (○) Oligofructose.

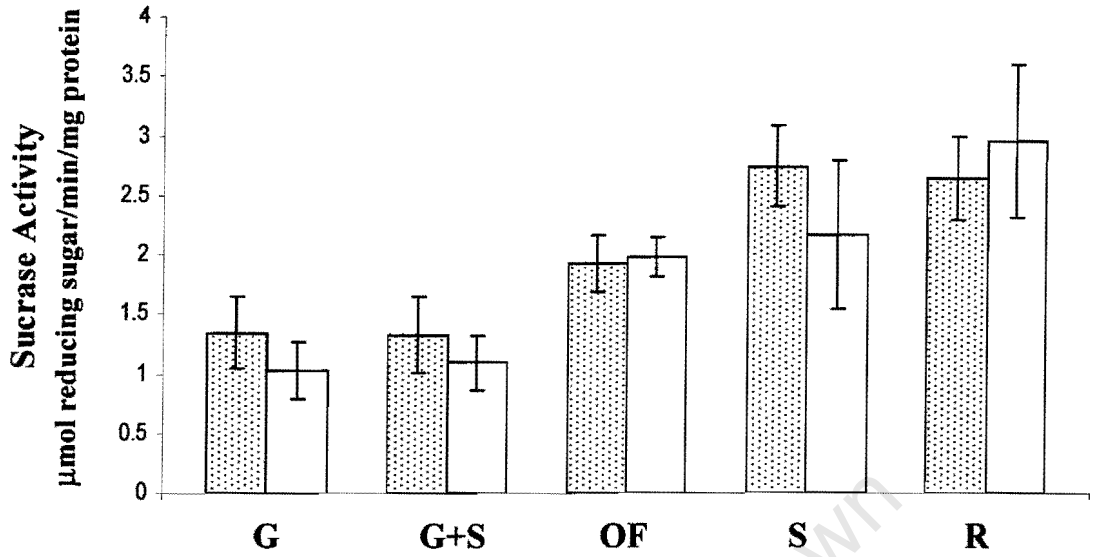

Figure 2.6. Sucrase activity from *B. lactis* cells grown in the presence of various carbohydrates to mid-log (▒) and late-log (□). Results are an average of three experiments, with the bars indicating the standard deviation from the mean.

2.4.4.3. Regulation of α-Galactosidase Activity in B. lactis

α-Galactosidase activity was measured from *B. lactis* cells grown to mid-logarithmic phase in BY medium containing glucose, raffinose, or glucose plus raffinose. Growth in galactose was very poor, reaching a maximum OD_{600} of 0.15, and was, therefore, not included in the study. The assays were initially conducted separately on the CFE and MF fractions (see 2.3.8), but the activity was found to be approximately the same for both. It could be possible that more than one enzyme conferring α-galactosidase activity exists in *B. lactis*. Although in *B. adolescentis* and *B. longum* CRL849 only one α-galactosidase was purified (86, 150, 310), some organisms are known to produce several, such as *Bacteroides ovatus* (90) and *Trichoderma reesei* (166). In fact, sequence analysis of the *B. longum* NCC2705 genome revealed the presence of two α-galactosidase genes (BL0177 and BL1518) (262). Alternatively, the enzyme might non-specifically associate with the membrane. In *B. ovatus* for example, it was found that after sonication and centrifugation, only 49% of total α-galactosidase activity remained in the CFE, compared with 95% of the marker enzyme (90). Although activity was released from the pelleted membrane fraction by washing with two different buffers, some activity still remained with the

membrane. It is possible that this may also be the case in *B. lactis*. Nonetheless, activity was assayed on crude lysate, representing a combination of the CFE and MF fractions. The results (Figure 2.7) showed a 20-fold induction in the presence of raffinose in comparison to glucose, while in glucose plus raffinose this induction was reduced to 16-fold. The reduced activity in the presence of glucose plus raffinose indicates that α-galactosidase activity is regulated by catabolite repression, and has similarly been reported for *Bifidobacterium adolescentis* (150). Raffinose was suggested to be the carbon source preferred by *B. lactis* for growth compared to glucose (see section 2.4.4.1 and Figure 2.5), therefore, repression of raffinose utilisation by glucose would not be expected. As was discussed in section 2.4.4.2, perhaps glucose is the preferred carbon source. The significant induction seen in the presence of glucose plus raffinose implies that both sugars were utilised by *B. lactis*, however, the mechanisms involved are not known.

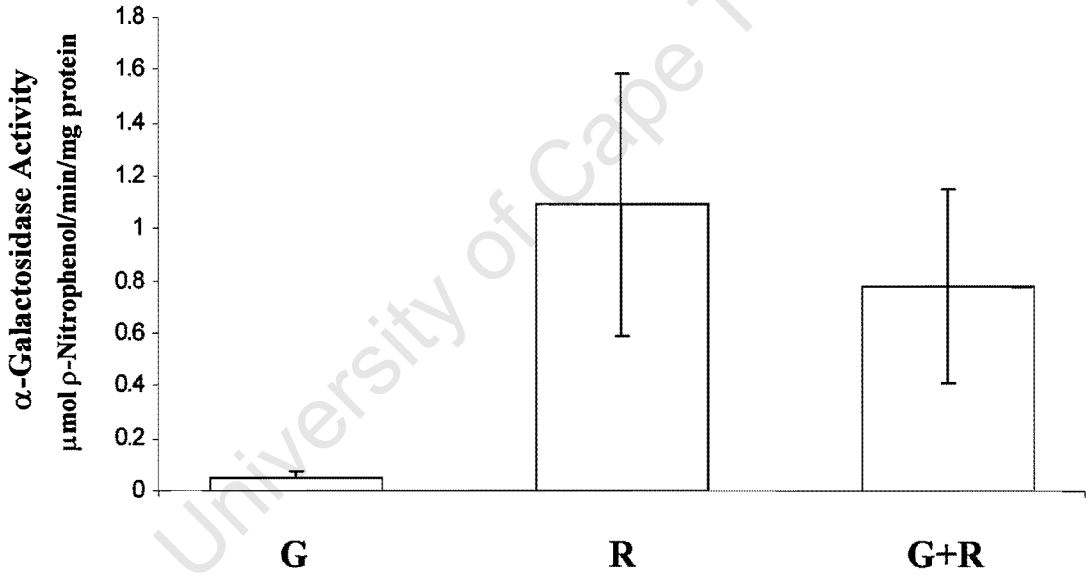

Figure 2.7. α-Galactosidase activity measured from *B. lactis* cells grown to mid-log in the presence of various carbohydrates. G: glucose, R: raffinose. Activity is represented as the mean of three experiments, with the bars indicating the standard deviation from the mean.

2.5. CONCLUSIONS

The carbohydrate utilisation patterns of *B. lactis, B. breve, B. longum,* and *B. bifidum* were determined, where a variety of substrates were analysed for their ability to support bacterial growth as a sole carbon source. These kind of analyses have been used for the identification of

bifidobacteria (261), but are being replaced by more rapid and reliable methods such as ribotyping (172), pulse-gel electrophoresis and 16s rRNA sequencing (169, 335).

This fermentation study has given a useful indication of the carbohydrate utilisation patterns of these bifidobacteria, and has certainly illustrated the importance of such information for the selection of suitable probiotics and prebiotics for future biotechnological applications. However, considering that these conditions do not mimic those encountered in the colon, it is important that these interpretations be made in conjunction with growth rate and overall cell yield analyses. Hopkins *et al* (119) showed that high maximum specific growth rates occurred with some bifidobacterial strains on certain carbon sources, even though the overall growth was poor. The rate at which an organism can grow on a particular carbon source will influence its ability to compete with other bacteria in the colon (119). Furthermore, bacteria divert a greater proportion of energy into the maintenance of cellular functions as the residence time in the gut progresses. Time is, therefore, an important factor for the production of bacterial cell mass from carbohydrates, indicating a substrate's efficacy if its utilisation results in a high growth rate. Another disadvantage to this type of analysis is that the organism's inability to ferment a particular substrate does not necessarily mean that they do not possess the machinery to do so. In a previous study, *B. breve* was found that although it could not utilise arabinose as a sole carbon source, the sugar was co-utilised with glucose when present as a mixture (58). The enzymes needed for its metabolism were present and the sugar could be used to provide energy. This further strengthens why *in vitro* analyses are not strongly supported, since the conditions that the organisms encounter in the colon are different, and therefore, different profiles could be obtained in "the real world".

However, these carbohydrate utilisation analyses could be used to supply the groundwork from which *in vivo* experiments can then be developed. One can get an idea of which strains are the most diverse in their ability to utilise a range of carbohydrates. One can also determine the class of carbohydrate that is preferred, for example, starches or fructan-containing substrates. Detecting diet-induced changes in bacterial types and numbers *in vivo* has been difficult however, and sometimes, contradictory (31, 247). An alternative method is to measure metabolic products and activities related to the microflora in order to assess diet-related effects (247). An analysis of fermentation patterns, could therefore, aid in selecting appropriate enzymatic markers to allow conclusions to be made as to the likely consequences for the health of the host. With so many aspects of fermentation analyses to consider, the rate at which prebiotics are being developed and applied is quite surprising.

The bifidobacterial population present in the microflora of adult humans has been shown to vary between individuals, and undergoes fluctuations with time (172). Taken together with the variable fermentation patterns within a bifidobacterial strain, one can understand why the synbiotic approach is the latest concept being researched and developed. In this way, a particular probiotic strain (or combination of strains) could be selected for its/their specific health benefits, and be administered in its/their specific prebiotic to enable the passage through the gut and the colonisation thereafter. This method combines the advantages offered by pro- and prebiotics, whilst overcoming some of the disadvantages associated with them.

Growth curves performed with *B. lactis* in raffinose, oligofructose and sucrose, revealed that raffinose was the preferred substrate, and that growth in oligofructose was weak (Figure 2.5). Taken with the failure to utilise inulin as a sole carbon source (Table 2.3), the fructo-oligosaccharides would not make choice prebiotics if *B. lactis* was to be considered for the synbiotic application, whereas soybean oligosaccharides could provide a more suitable alternative.

Sucrase activity in *B. lactis* was found to be partially repressed when cultured in the presence of glucose (Figure 2.6), while activity in glucose plus sucrose did not improve, even though it supported better growth (Figure 2.5). The activity detected possibly represents the action of a number of systems regulated at different levels and possibly by different mechanisms. Although catabolite repression appears to regulate sucrase activity to some extent in *B. lactis*, it has never been addressed in this genus, and therefore, the mechanisms involved remain completely unknown. A more detailed analysis of sucrose utilisation by *B. lactis* is discussed in chapter 5, where the genetic regulation of the sucrase system isolated was analysed. α-Galactosidase activity in *B. lactis* appears to be similarly regulated by catabolite repression. Activity was induced by growth in the presence of raffinose, while being minimally produced in the presence of glucose (Figure 2.7). The induction that occurred during growth in glucose plus raffinose possibly indicates that the sugars are co-utilised, however conclusions to the mechanisms involved are not known.

In order to further understand the genetic mechanisms employed by *B. lactis* for the utilisation of the prebiotics oligofructose and raffinose, the isolation of these genetic systems was undertaken. To achieve this, the cloning and characterisation of the α-galactosidase and sucrase genes was performed and is presented in chapters 3 and 4 respectively.

CHAPTER 3

ISOLATION OF A PUTATIVE α-GALACTOSIDASE FROM *BIFIDOBACTERIUM LACTIS*

Contents

3.1. SUMMARY

The isolation of the *B. lactis* raffinose utilisation genes was attempted using several strategies: (i) screening of two *B. lactis* genebanks on raffinose and the histochemical substrate ρ-nitrophenyl-α-D-galactopyranoside, (ii) amplification of the α-galactosidase from *B. lactis* by PCR using primers designed to conserved regions of the *B. longum* and *B. adolescentis* genes, (iii) construction and the subsequent screening of a *B. lactis* genebank prepared with DNA which hybridised to a *B. longum* α-galactosidase gene probe. Screening of the genebanks on the substrate ρ-nitrophenyl-α-D-galactopyranoside resulted in the isolation of three clones which displayed weak α-galactosidase activity. From restriction analysis, only one of these clones, p4B11, could contain a functional gene, and it was analysed further. Sequence analysis revealed 7 putative ORFs, none of which showed identity to other α-galactosidases or related proteins. Insert DNA from p4B11 was hybridised to RNA from *B. lactis* cells in a slot blot experiment, and a signal could only be detected from cells which had been grown in the presence of raffinose and not glucose. However, a transcript could not be identified. Protein from *E. coli* cells with p4B11 was analysed by SDS-PAGE, but a distinct protein band which could be responsible for the activity was not identified. This was not unexpected however, since the clone displayed very weak α-galactosidase activity.

3.2. INTRODUCTION

Although inulin and oligofructose are the carbohydrates considered to be the most effective prebiotics for bifidobacteria (92, 94), galactose- and raffinose-containing oligosaccharides also selectively stimulate the growth of bifidobacteria (49, 247). As was discussed in chapter 2, soya could become a significant component in the African diet, and its high content of raffinose, which is indigestible by humans, could play an important role in the stimulation of bifidobacteria in the gut. Certainly for *B. lactis*, raffinose was the preferred carbon source in comparison to glucose and oligofructose (Figure 2.5).

Raffinose contains α(1,6)-linked galactose residues linked to sucrose, requiring two catabolic enzymes for its hydrolysis: an α-galactosidase and a sucrase. As was reviewed in chapter 1, the two raffinose catabolic genes are organised in close proximity on the chromosome in *Escherichia coli, Streptococcus mutans*, *S. pneumoniae* and in *Pediococcus pentosaceus* (12, 151, 233, 238). In both the streptococci the α-galactosidase is associated with, and found in, close proximity to a sucrose phosphorylase. Besides hydrolytic activity, the α-galactosidase from *B. adolescentis* was shown to have transglycosylation activity, where α-galacto-oligosaccharides were produced from melibiose and stachyose (310). These oligosaccharides could potentially be used as bifidobacterial growth promoting factors.

The fact that raffinose was the carbon source preferred by *B. lactis*, together with the prebiotic potential of this carbohydrate, its utilisation by *B. lactis* was of interest to us. Furthermore, the bifidobacterial enzymatic synthesis of growth promoting factors for biotechnological applications could be investigated if an α-galactosidase with transglycosylation activity was isolated. In order to achieve this, the raffinose-utilisation genes were to be isolated and characterised.

Raffinose utilisation genes have been isolated by screening gene libraries on raffinose, sucrose and histochemical substrates for clones conferring a positive phenotype on *E. coli* (12, 81, 166, 238, 310). Raffinose utilisation genes have also been isolated by PCR (80, 233), and with the *B. adolescentis* and *B. longum* α-galactosidase sequences available (83, 310), this could be an alternative strategy. This approach, however, would require "chromosome walking" to obtain the entire gene sequence and to determine the presence of additional genes. Considering that in *E. coli* and *S. pneumoniae* the raffinose utilisation genes are organised in clusters comprising five and eight genes respectively (12, 233), screening a genebank would more likely result in the isolation

of several genes. The steps taken to isolate the raffinose utilisation genes from *B. lactis* are presented and discussed in this chapter.

3.3. MATERIALS AND METHODS

3.3.1. Bacterial Strains, Plasmids and Culture Conditions

Bifidobacterium lactis, isolated in chapter 2, was propagated in BYG medium as described in 2.3.1. Where applicable, 1% (w/v) of raffinose was substituted for glucose.

E. coli JM109 (336) was used for all cloning purposes and was routinely cultured aerobically at 37°C in Luria-Bertani (LB) medium (253). Ampicillin (Ap) (100 µg/ml) was added to media for the growth of cells containing plasmid. MacConkey base medium (Difco Laboratories) and M9 minimal medium (176) supplemented with Ap and 1% (w/v) raffinose, was used to assess carbohydrate fermentation phenotypes.

The following plasmids were used for subcloning and sequencing: M13-derived Bluescript SK plasmid (pSK) (Stratagene, La Jolla, California, USA), pEcoR251 (343), pMT104 (327), and pGem®-T Easy (Promega). Further strain and plasmid details are given in Appendix A.

3.3.2. General DNA Manipulations

Extraction of plasmid DNA, preparation of *E. coli* JM109 cells, DNA ligations, gel electrophoresis and other general manipulations are described in section 2.3.3.

3.3.3. *B. lactis* Chromosomal DNA Extraction

B. lactis chromosomal DNA was extracted as described in section 2.3.7.

3.3.4. Construction of Genebanks

Two size-selected *B. lactis* genebanks were prepared. The first was prepared with 5-10 kb *Sau*3A-digested DNA fragments, and will be referred to as the *Sau*3A genebank. The second was prepared with 6-9 kb *Bam*HI-digested DNA fragments which hybridised to the 1.4 kb α-galactosidase probe from *B. longum*, and will be referred to as the *Bam*HI genebank.

The *Sau*3A genebank was prepared as follows. *B. lactis* chromosomal DNA was partially digested with *Sau*3A restriction endonuclease and subjected to agarose gel electrophoresis. DNA fragments of between 5 and 10 kb were excised from the gel and recovered using the High Pure PCR Product Purification kit (Roche). The fragments were ligated into the *Bgl*II restriction site of the positive selection vector pEcoR251 and transformed into *E. coli* JM109. The genebank consisted of approximately 3 500 clones divided into 7 pools.

The *Bam*HI genebank was prepared as follows. *B. lactis* chromosomal DNA was digested to completion with *Bam*HI restriction endonuclease. Two-thirds of the digested DNA was electrophoresed on one half of an agarose gel, while the other one-third was run on the second half for Southern hybridisation (see 3.3.7.). The DNA fragments (7-8 kb) which hybridised to the *B. longum* α-galactosidase probe were purified from the gel and ligated into pEcoR251 that had been digested with *Bgl*II restriction enzyme. Ligated DNA was used to transform *E. coli* JM109 competent cells.

3.3.5. Screening of the *B. lactis* Genebanks

E. coli JM109 cells transformed with the *B. lactis* genebanks were screened for two different phenotypes: raffinose utilisation and α-galactosidase enzyme production. For raffinose utilisation, transformants were incubated on M9 minimal and MacConkey agar plates containing raffinose. *E. coli* transformed with pMT104 was plated on the same medium to ensure that it in fact was unable to utilise raffinose. *E. coli* cells harbouring the control plasmid did not give growth on the raffinose minimal medium, however, on raffinose MacConkey agar all colonies were slightly pink, suggesting that some background activity could be detected from *E. coli*.

Transformants were screened for α-galactosidase activity following a method described by Fridjonsson *et al* (81) which uses ρ-nitrophenyl-α-D-galactopyranoside (NpGal) as the substrate. *E. coli* JM109 was transformed with 2 µl DNA from each pool of the *Sau*3A and *Bam*HI genebanks and 200 µl aliquots of the transformation mix (each containing approximately 100 recombinants) were grown in 5 ml of LB medium containing ampicillin. Once turbid, 4 ml of cells were washed and resuspended in 100 µl lysis buffer (lysozyme 4 mg/ml, 25 mM EDTA, 0.1% Triton X-100, pH 8) and incubated for 30 min at 37°C. To this, 350 µl of 0.1 mM potassium phosphate buffer (pH 6.5) containing the substrate ρ-nitrophenyl-α-D-galactopyranoside (NpGal) (0.8 mg/ml) was added and the suspension was incubated for 15 h at 55°C. The enzyme reaction was stopped with 700 µl of borate buffer (0.4 M, pH 9.3), and liberated ρ-nitrophenol was

measured spectrophotometrically at 405 nm. This procedure was also conducted on *E. coli* cells transformed with pMT104 plasmid DNA as a negative control. The culture belonging to the aliquot displaying the highest hydrolysing activity was divided into 13 sub-pools and each part was again grown in 5 ml of LB medium containing ampicillin, and the same procedure was repeated. The culture exhibiting the highest α-galactosidase activity after the second screening was diluted and plated on Luria agar. Single colonies were selected and analysed for α-galactosidase activity in the same way.

3.3.6. PCR of the α-Galactosidase from Bifidobacteria

PCR amplification of the α-galactosidase gene was performed on *B. lactis* and *B. longum* chromosomal DNA in the following reaction (50 µl): 5 µl buffer; 2.5 mM $MgCl_2$; 0.5 µM of each primer; 200 µM of each dNTP; 50 ng chromosomal DNA; and 1 U of *Taq* DNA polymerase (Supertherm). The PCR was carried out in a GeneAmp 9700 machine (Applied Biosystems). The amplification program consisted of one cycle of 96°C for 5 min, then 25 cycles of 96°C for 1 min, 54°C for 45 s, and 72°C for 1 min, and finally one cycle of 72°C for 5 min. The reaction was cooled down to 4°C. The primers were designed to a conserved region of the *B. longum* and *B. adolescentis* α-galactosidase genes (Figure 3.4): GalF 5'-GAG ATC CTC ACC ACC ACC G-3' and GalR 5'-CTG CGT GAA GCG GTA GAT CG-3'.

3.3.7. Southern and Colony Hybridisation

For Southern hybridisation, DNA was digested with *Bam*HI restriction enzyme, subjected to agarose gel electrophoresis and transferred via the capillary alkali transfer procedure to a Hybond-N^+ nylon membrane (253). For colony hybridisation, DNA from colonies was transferred to nylon membrane according to the manufacturers' instructions (Roche). Fragments to be used as probes were non-radioactively labelled by the random-primed method using the Digoxigenin labelling and detection kit (Roche). Hybridisation and detection procedures were performed according to the manufacturers' instructions. Hybridisations performed with the 1.4 kb α-galactosidase fragment from *B. longum* were done using low stringency conditions described by the manufacturer and temperatures between 48°C and 55°C were used.

3.3.8. Nucleotide Sequence and Analysis

DNA sequencing was performed and analysed as described in section 2.3.4.

3.3.9. α-Galactosidase Assays

α-Galactosidase assays were performed on *E. coli* JM109 crude lysate (CL) as described in section 2.3.9.2.

3.3.10. Protein Assays

Protein concentrations were determined as described in section 2.3.10.

3.3.11. Total RNA Extraction

Total RNA was isolated from 50 ml mid-logarithmic phase cultures of *B. lactis* and *E. coli* based on a protocol for Gram-positive bacteria (17). Cells were harvested by centrifugation (6 000 *g*, 10 min, 4°C) and immediately resuspended in 2 ml of buffer (20 mM sodium acetate pH 5.5, 1 mM EDTA). Two hundred microliters of 10% SDS and 2 ml of water-equilibrated phenol/chloroform (1:1) were added and tubes were incubated for 10 min at 70°C. The phases were separated by centrifugation and the aqueous phase was extracted twice more with water-equilibrated phenol/chloroform. RNA was then precipitated from the aqueous phase with 1/10 volume of 3 M sodium acetate (pH 5.5), and 2.5 volumes of ethanol. After 30 min at –70°C, RNA was pelleted (14 000 *g*, 15 min, 4°C), and resuspended in 180 µl of water. A DNAse step was performed as follows. RNAse-free DNAse (30 U) and 20 µl of DNAse buffer (200 mM sodium acetate pH 4.5, 100 mM $MgCl_2$, 100 mM NaCl) were added to the resuspended RNA and incubated for 30 min at room temperature. Twenty microliters of 0.25 mM EDTA was added to inactivate the enzyme, followed by an extraction with phenol/chloroform and then with an equal volume of chloroform/isoamyl alcohol (24:1). RNA was precipitated as before and resuspended in 40 µl of water.

3.3.12. Northern Hybridisation and RNA Slot Blot Analysis

For Northern hybridisation, RNA was separated on formaldehyde gels (253) and was capillary transferred to positively charged nylon membrane (Roche), using 10X SSC as a transfer medium (253). RNA slot blots were performed in duplicate as described (253) using the Hoeffer Scientific apparatus. Hybridisation and detection of the DIG-labelled DNA probes was performed according to the manufacturers' instructions. The 4.2 kb *Hind*III-*Bam*HI fragment from p4B11 was used as the experimental probe and the *B. lactis* 16S rDNA probe constructed in chapter 2 (2.3.2) was used as an internal control.

3.3.13. SDS Polyacrylamide Gel Electrophoresis

E. coli proteins to be analysed by SDS-PAGE were isolated as follows. Overnight cultures (2 ml) were microfuged and the resulting cell pellet resuspended in disruption mix (0.125 M Tris pH 7.6, 10% β-mercaptoethanol, 10% SDS, 10% sucrose, 0.01 % bromophenol blue). The suspension was vortexed for 5 mins and boiled for 10 mins. This procedure was repeated, and the protein suspension was then placed on ice. For electrophoresis, 10-20 µl of protein was separated on 10% SDS polyacrylamide gels as described previously (145) using the Mighty Small apparatus (Hoeffer Scientific Instruments). Molecular weight markers were obtained from Amersham Pharmacia.

3.4. RESULTS

3.4.1. Screening for Raffinose Metabolic Genes by Complementation.

Raffinose utilisation genes have been isolated by screening on raffinose or sucrose (1, 12). Screening on sucrose was performed and is presented in chapter 4, while the screening on raffinose is described here. *E. coli* JM109 cells are unable to grow in minimal medium containing raffinose, and were, therefore, transformed with the *B. lactis Sau*3A genebank, and the transformants were screened on minimal medium and MacConkey agar containing raffinose. Raffinose-positive colonies were not obtained. Failure to isolate genes involved in the metabolism of raffinose in this way could be due to size limitation of the genebank. The *E. coli raf* operon consists of 5 structural genes containing approximately 8.4 kb of nucleotide sequence (12), while the *S. pneumoniae* system is comprised of 8 structural genes present on a 10.2 kb region (233). It is, therefore, possible that the *B. lactis* genes are similarly organised on a large region of the chromosome, and that they are not all present on a single DNA fragment in the genebank. Alternatively, it could be possible that the genes involved in raffinose utilisation are not clustered together in *B. lactis*. It was, therefore, decided to isolate a gene coding for a single, assayable enzyme, the α-galactosidase.

3.4.2. Isolation of the α-Galactosidase Gene by PCR

The α-galactosidase gene has previously been cloned and sequenced from *B. longum* and *B. adolescentis* (150, 310) and primers (GalF and GalR) were designed to conserved regions in order to isolate the *B. lactis* α-galactosidase by PCR. PCR performed on *B. lactis* chromosomal

DNA did not yield the expected 1.4 kb product, even with variations in annealing temperatures, concentration of $MgCl_2$, and DNA *Taq* polymerase. The high GC DNA content of these organisms likely impairs the ability of *Taq* DNA polymerase to efficiently amplify DNA fragments due to formation of secondary structures. Difficulty with PCR was encountered throughout the work reported in this thesis, and has similarly been experienced with *Corynebacterium glutamicum* as well (J. Kensley, personal communication). Products specific for the amplification of high GC DNA are available, such as the High GC PCR Kit (Roche), which could be used in future amplifications.

Since *B. longum* was a strain that we had in our collection, it was decided to isolate the α-galactosidase gene from it by PCR with the primers designed, and the resulting DNA fragment would serve as a probe for hybridisation to *B. lactis* chromosomal DNA. A 1.4 kb fragment was

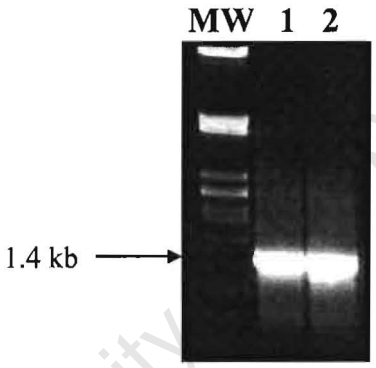

Figure 3.1. Amplification of the α-galactosidase gene from *B. longum* NCFB 2259 with the GalF and GalR primers. Lane 1: PCR from undigested chromosomal DNA, Lane 2: PCR from chromosomal DNA digested with *Sac*I, *Stu*I, and *Kpn*I.

obtained by PCR (Figure 3.1) and it was subcloned into the *Sma*I restriction site of the pSK plasmid vector. A 0.65 kb *Eco*RI restriction fragment present in the published sequence of the *B. longum* α-galactosidase gene (accession no. AF160969) was used to identify clones which potentially contained the expected sequence. Sequence analysis of one of these clones (pLacZ8, previously named pLonGal8) revealed that a 1.4 kb region of the β-galactosidase gene had been subcloned instead of the α-galactosidase gene, also indicating that 6 out of 19 and 6 out of 20 bases of the GalF and GalR primers respectively annealed to the *B. longum* β-galactosidase DNA sequence in the PCR reaction (Figure 3.2). The partially cloned *B. longum* β-galactosidase showed 55% sequence identity to the *B. longum* MB219 (accession no. AJ242596) and *B. bifidum*

(accession no. AJ224434) protein sequences, and the alignment of these proteins is shown in Figure 3.3. β-galactosidases from *B. bifidum* and *B. infantis* have been shown to have transgalactosylase activity (184), and therefore these proteins could also be applied for the production of novel prebiotics for bifidobacteria. It would, therefore, be interesting to determine if the cloned β-galactosidase from *B. longum* could similarly be able to carry out this activity.

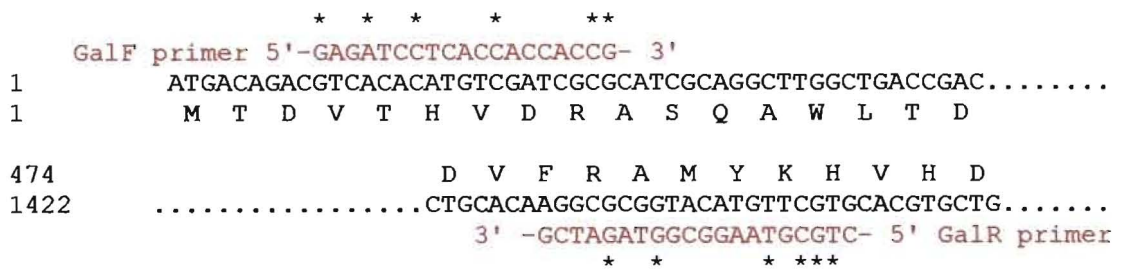

Figure 3.2. Alignment of the α-galactosidase PCR primers with the cloned *B. longum* NCBF 2257 β-galactosidase DNA sequence from pLacZ8. The GalF and GalR primer sequences are shown in red. Primer bases which could have annealed are indicated by an asterisk. The top row shows the coding sequence, while the anti-sense sequence is shown on the bottom row.

Although it was not certain that the α-galactosidase gene was being amplified, a strategy was employed to eliminate the β-galactosidase gene product from the PCR reaction. The chromosomal DNA to be used as template was digested with restriction enzymes present within the β-galactosidase gene of *B. longum*, which would not digest within the 1.4 kb fragment of the *B. longum* α-galactosidase gene to be amplified. From the analysis of the preliminary sequence obtained from the cloned β-galactosidase (pLacZ8), the enzymes *Sac*I, *Stu*I, and *Kpn*I were selected. PCR performed on digested DNA resulted in the same 1.4 kb fragment as for undigested DNA (Figure 3.1). The PCR products were subcloned in pGem-T Easy vector. One clone, pGal8, containing the expected 0.65 kb *Eco*RI fragment was analysed further. Sequence analysis of the insert DNA revealed that the α-galactosidase gene had been successfully cloned. Figure 3.4 illustrates the amino acid sequence alignment of the bifidobacterial α-galactosidases that have been isolated. The cloned α-galactosidase from *B. longum* found 99% sequence identity at the protein level to the published *B. longum* VMKB44 α-galactosidase sequence (accession no. AF160969). Furthermore, an α-galactosidase (BL1518) from the *B. longum* NCC2705 sequenced genome was found and it was 100% identical to that of *B. longum* VMKB44. The four non-identical amino acids for the partially cloned *B. longum* α-galactosidase, marked by asterisks in

Figure 3.4, are most likely a result of *Taq* DNA polymerase errors. Although such mismatch mutations are commonly associated with PCR, the frequency with which it occurred within the pGal8 sequence is alarmingly higher than the expected error rate of 10^{-5} to 10^{-4} per nucleotide synthesised (345). Alternatively, they could be genuinely different, representing strain variation.

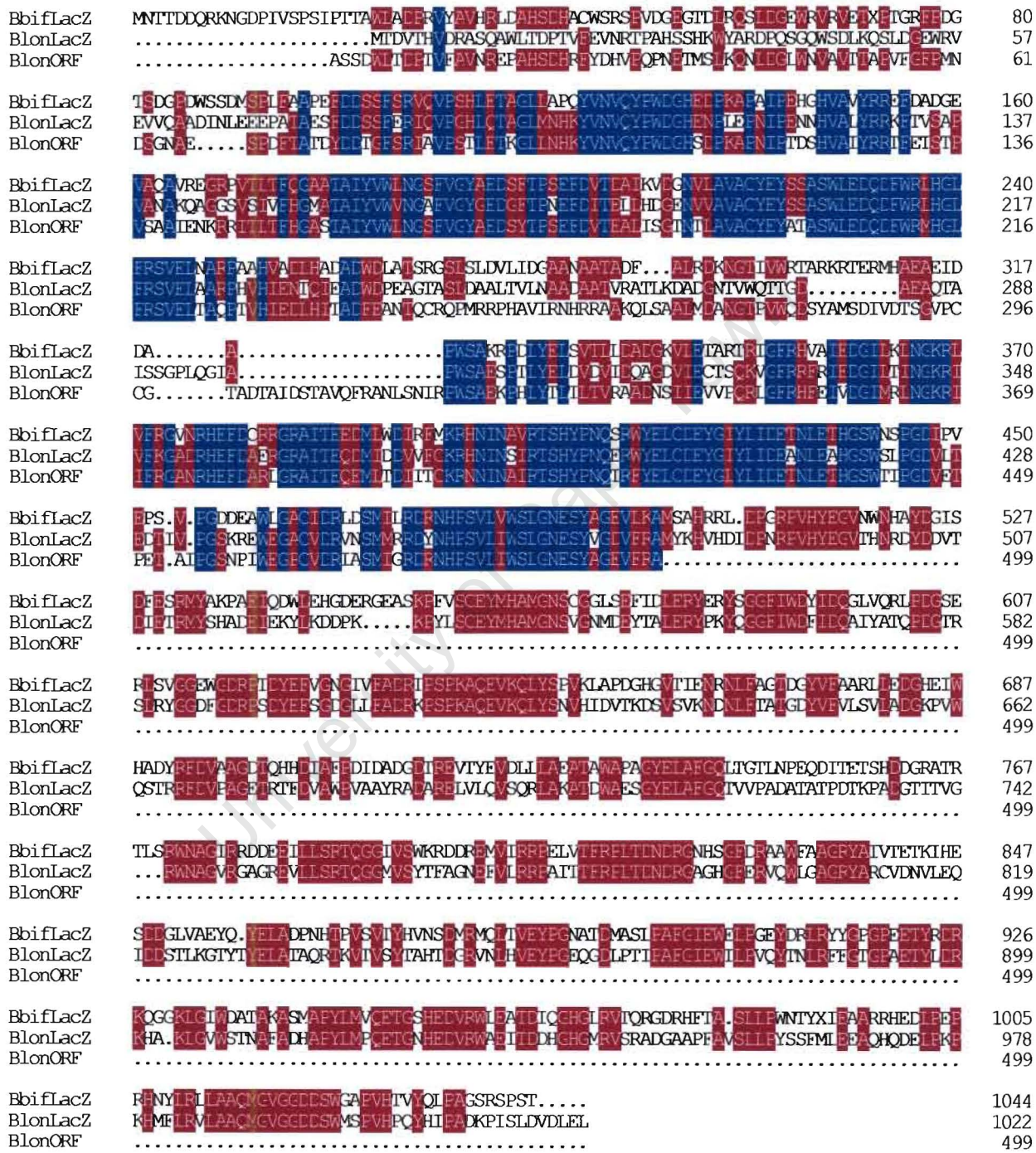

Figure 3.3. Multiple sequence alignment of the cloned *B. longum* β-galactosidase (BlonORF) to bifidobacterial LacZ proteins. *B. bifidum* DSM 20215 (BbifLacZ) (accession no. AJ224434), *B. longum* MB219 (BlonLacZ) (accession no. AJ242596).

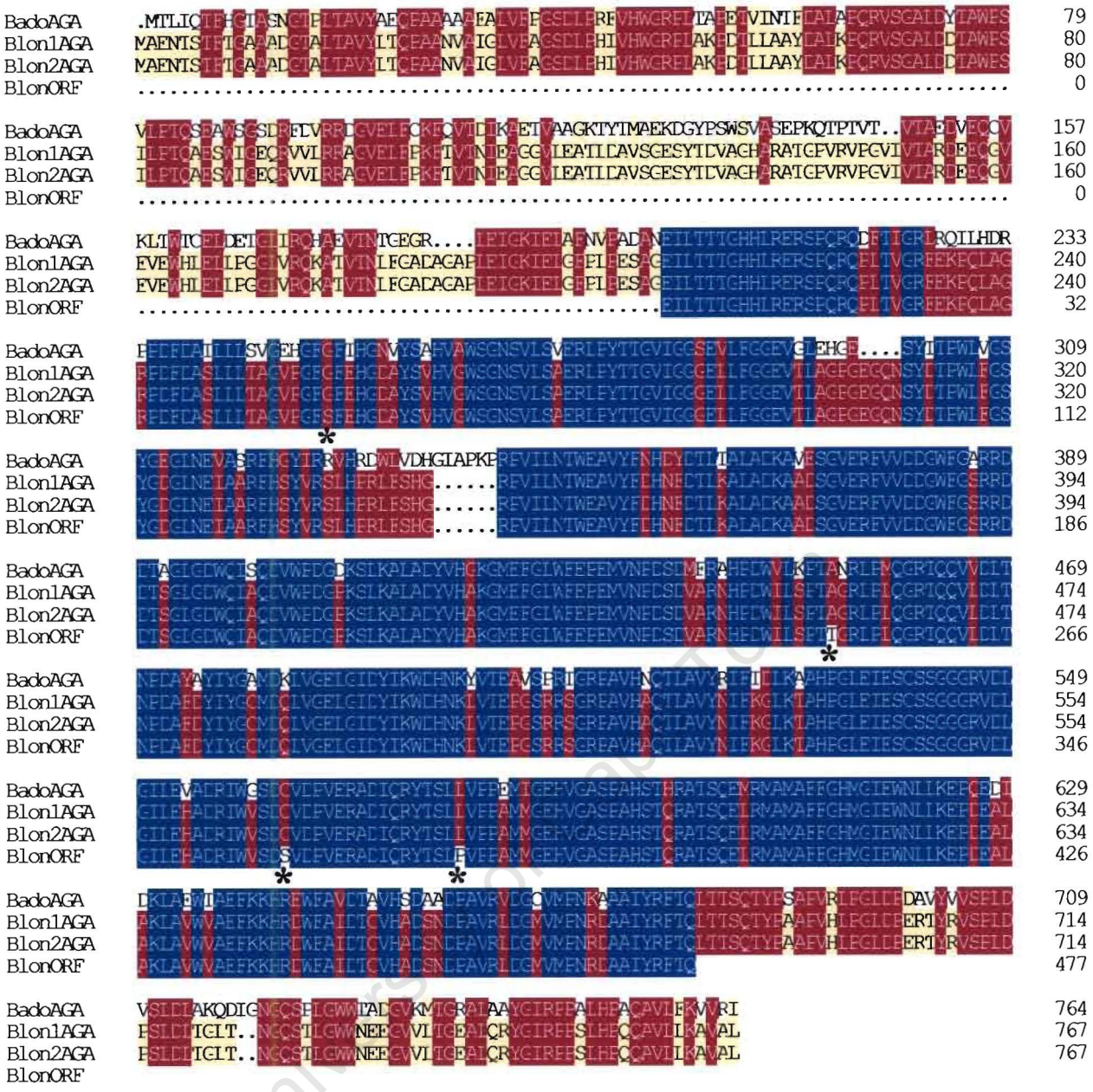

Figure 3.4. Multiple sequence alignment of the cloned *B. longum* NCFB 2257 α-galactosidase (BlonORF) to others isolated from bifidobacteria. BadoAGA: *B. adolescentis* (accession no. AF124596), Blon1AGA: *B. longum* VMKB44 (accession no. AF160969), Blon2AGA: *B. longum* NCC2705 (BL1518). The asterisks indicate the amino acids that are different for BlonORF in comparison to the Blon1AGA and Blon2AGA protein sequences.

3.4.3. Southern Hybridisation of the *B. longum* α-Galactosidase Gene to *B. lactis*

The 1.4 kb α-galactosidase DNA fragment isolated from *B. longum* could now be used to isolate the gene from *B. lactis* by hybridisation and the subsequent construction of a mini-genebank. The 1.4 kb *Apa*I-*Sac*I fragment from pGal8 was prepared for southern hybridisation to *B. lactis* and *B. longum* chromosomal DNA digested with *Bam*HI. *B. lactis* shares very little DNA homology to

B.longum (122), therefore, hybridisation was performed under low stringency conditions. The 1.4 kb probe hybridised to a 4 kb fragment of *B. longum*, and to a 7-8 kb fragment of *B. lactis* (Figure 3.5).

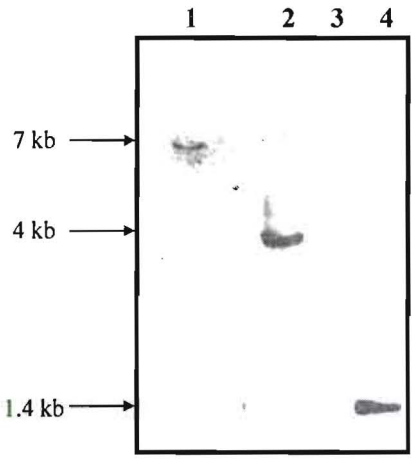

Figure 3.5. Southern hybridisation of the *B. longum* α-galactosidase probe. Lane 1: *B. lactis* chromosomal DNA digested with *Bam*HI, Lane 2: *B. longum* chromosomal DNA digested with *Bam*HI, Lane 3: pMT104 plasmid DNA, Lane 4: pGal8 plasmid DNA digested with *Apa*I and *Sac*I. The 1.4 kb *Apa*I-*Sac*I fragment from pGal8 was used as the probe.

A *B. lactis* genebank was constructed in *E. coli* with *Bam*HI-digested DNA fragments of 6-9 kb in size which included the DNA fragment that hybridised to the α-galactosidase probe. Colony hybridisation was performed using the same probe for the identification of *E. coli* transformants containing the *B. lactis* α-galactosidase gene. Of the 850 colonies screened, several hybridised weakly, but only one contained an insert of approximately 7 kb. This clone however, failed to give α-galactosidase activity.

3.4.4. Isolation of a Putative α-Galactosidase from *B. lactis* by Complementation

The two *B. lactis* genebanks were screened for α-galactosidase activity by assaying for the liberation of ρ-nitrophenol as described in the methods (3.3.5.). Of the 13 pools, 7 showed a yellow colour development distinguishable from the other pools and from the cells transformed with the control plasmid pMT104. The pool displaying the highest activity was selected for a new round of screening. This time, 10 portions of 13 developed the yellow colour. In the next round, single colonies were analysed for the release of the ρ-nitrophenol and 3 gave a positive phenotype. These were designated p4B11, pH1 and pH2. Restriction analysis of these plasmids

revealed that they contained different inserts of 3.7, 1.8, and 0.2 kb respectively. The isolation of 3 plasmids containing inserts different from each other could suggest that the α-galactosidase activity in *E. coli* was not as a result of a cloned α-galactosidase gene. This is discussed further in the conclusions.

Since p4B11 displayed the highest activity in the screening process, and since the insert sizes in pH1 and pH2 were too small to contain a full length α-galactosidase gene, further analyses were only performed on p4B11. α-Galactosidase assays performed on cell lysate from *E. coli* JM109 cells with p4B11 were found to have an activity of 0.0215 ± 0.0025 μmol/min/mg protein. In *B. lactis*, an activity of 1.145 ± 0.48 μmol/min/mg protein was obtained when cells were grown in the presence of raffinose, and was reduced to 0.048 ± 0.028 μmol/min/mg protein in the presence of glucose (section 2.4.4.3, chapter 2). The activity in *E. coli* with p4B11 was 2-fold lower than the repressed activity obtained from *B. lactis* cells, indicating a very weak, and probably also repressed expression of the *B. lactis* gene in *E. coli* in LB medium. Especially considering that the construct is in multicopy in *E. coli*. In contrast, the *B. adolescentis* α-galactosidase, which also showed catabolite repression in the presence of glucose, gave activity in *E. coli* cultured in LB medium comparable to that in *B. adolescentis* (310).

In order to locate the activity on the insert, the 2.6 kb *Pst*I and 2.3 kb *Hind*III-*Eco*RI fragments were subcloned from p4B11 into the *Bgl*II site of pEcoR251, in both orientations for each of the fragments, yielding pTsp1, pTsp2 and pHE3, pHE5 respectively (Figure 3.6). The four clones were analysed for α-galactosidase activity, but none was detected. Since the α-galactosidase genes can vary between 2.1 to 2.4 kb in size, it was possible that the gene responsible for the activity had been truncated when the two fragments were subcloned. The sequence of p4B11 was, therefore analysed.

3.4.5. Southern Blot Analysis of the p4B11 Insert

Before continuing with the analysis of this clone, a Southern blot was performed to confirm that *B. lactis* DNA had indeed been cloned. The 0.8 kb *Eco*RI fragment from p4B11 was hybridised to *B. lactis* chromosomal DNA digested with either *Eco*RI or *Bam*HI restriction enzymes (Figure 3.7). The probe hybridised to a 0.8 kb *Eco*RI genomic fragment confirming that the p4B11 insert DNA did originate from *B. lactis*. The probe also hybridised to an approximately 11.5 kb genomic fragment digested with *Bam*HI. Since the *Bam*HI genebank was constructed with fragments of 6-9

kb in size (3.3.4.), it was anticipated that the insert DNA from p4B11 would not be present in this genebank.

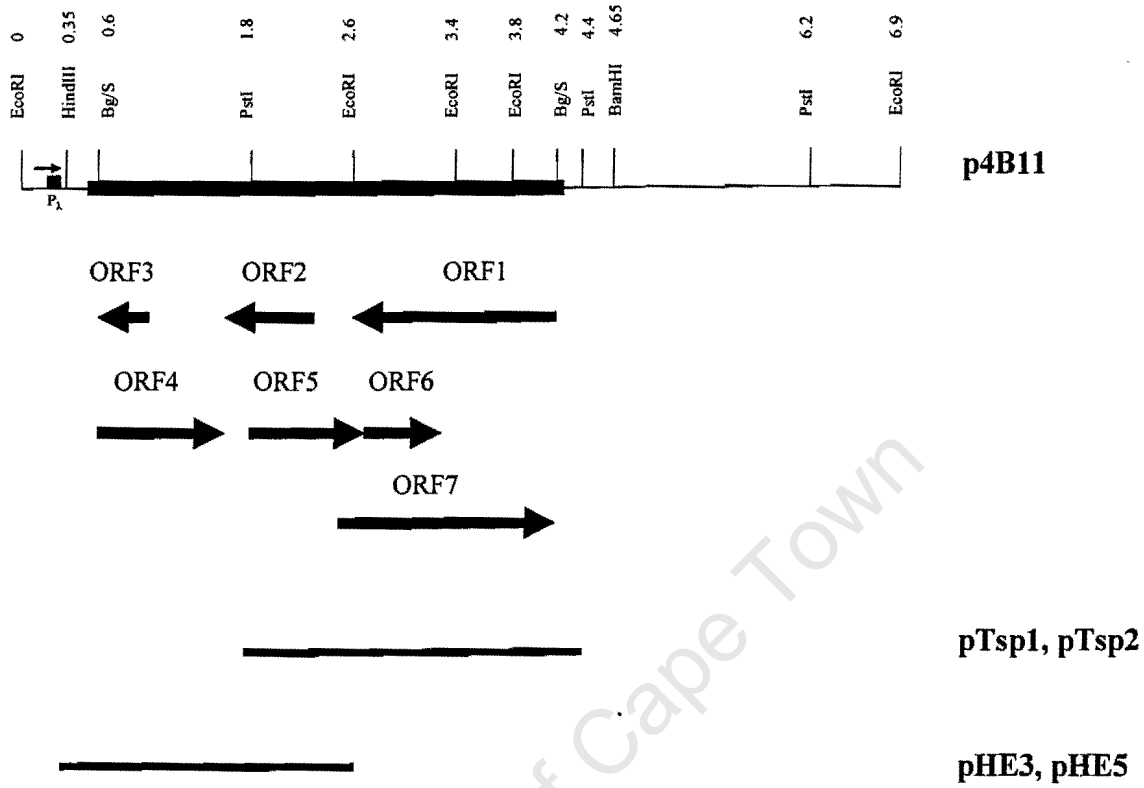

Figure 3.6. Restriction map of p4B11 showing the ORFs that were located by sequence analysis. The 2.6 kb *Pst*I and 2.3 kb *Hin*dIII-*Eco*RI fragments were subcloned in both orientations in pEcoR251 in the *Bgl*II restriction site. The filled box and arrow on p4B11 indicate the position and direction of the λ promoter (P_λ) present on the pEcoR251 vector. Sizes are shown in kb. Bg/S: *Bgl*II/*Sau*3A.

3.4.6. Sequence Analysis of p4B11

Since the activity could not be localised to a smaller fragment of the insert DNA, the entire 3.7 kb insert was sequenced. Several open reading frames were located on the insert (Figure 3.6), but only ORF3 showed any significant sequence identity to sequences in the database. This truncated ORF showed 35-38% sequence identity at the protein level to putative methylases from various bacteria (Table 3.1). The DNA sequence for ORF3 was used to search the *B. longum* sequenced genome, and found identity to a hypothetical protein with a possible methylase domain (BL0328), and an alignment of the protein sequences is shown in Figure 3.8. Analysis of the sequence adjacent to the *B. longum* putative methylase did not reveal a similar genetic arrangement as for the *B. lactis* insert in the construct p4B11. Furthermore, similarity between the sequences of the

other ORFs located on p4B11 and the *B. longum* genome was not identified. The subclone pTsp1 contains ORFs 5, 6 and 7 in the same orientation relative to the λ promoter present on the vector (Figure 3.6), while on pTsp2 ORF 1 is in the same orientation as the promoter. In the subclone pHE3, ORF 4 is in the same orientation as the promoter, while on pHE5 ORFs 2 and 3 are in the same orientation as the promoter. Since none of the ORFs are truncated in these subclones, if activity was being generated by one of these ORFs, it should have been detected in cells harbouring one of the four subclones. It is possible that due to sequencing errors the correct ORF was not identified. In this case, it could be possible that the ORF responsible for the α-galactosidase activity was truncated when the insert from p4B11 was subcloned, and this would explain the loss of activity observed.

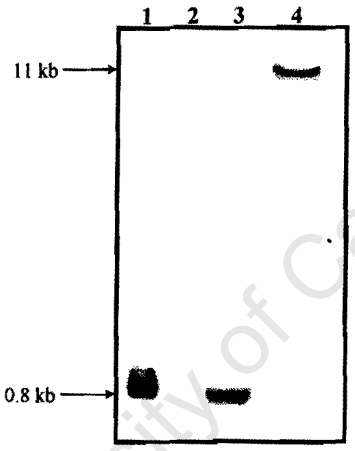

Figure 3.7. Southern hybridisation of the 0.8 kb *Eco*RI fragment from p4B11 to *B. lactis* chromosomal DNA. Lane 1: p4B11 DNA digested with *Eco*RI, Lane 2: pEcoR251 DNA digested with *Eco*RI, Lane 3: *B. lactis* chromosomal DNA digested with *Eco*RI, Lane 4: *B. lactis* chromosomal DNA digested with *Bam*HI.

Table 3.1. Percent identity and similarity of the deduced *B. lactis* ORF3 amino acid sequence to bacterial methylases.

Homologous Protein	Accession Number	% Identity	% Similarity
Streptomyces coelicolor putative DNA methylase	AL034447	38	54
Bacillus anthracis putative RNA methylase	NC_003995	36	51
Chlamydia trachomatis methylase	AE001322	35	55

3.4.7. Transcriptional Regulation of *B. lactis* RNA Coding for Insert DNA from p4B11

Since the activity generated by p4B11 could not be assigned to one of the ORFs by sequence identity or by localisation, and since it was confirmed by southern blot analysis that the insert DNA did originate from *B. lactis*, RNA analysis was undertaken. We wanted to determine if a transcript relating to the insert DNA from p4B11 was produced in *B. lactis*, and determine if it was similarly regulated by catabolite repression, as was observed for α-galactosidase activity (chapter 2). Furthermore, by determining the transcript size, the ORF responsible for the activity could possibly be identified. Since there was no indication as to where the activity was localised, the 4.3 kb *Hind*III-*Bam*HI fragment from p4B11 was used as a probe. RNA slot blots were performed using RNA extracted from *B. lactis* cultures grown in the presence of 1% (w/v) raffinose or glucose (Figure 3.9). RNA from *E. coli* JM109 with p4B11 and pMT104 was also included. For *B. lactis*, a signal was detected from cultures grown in the presence of raffinose, but was absent when grown in the presence of glucose. This suggests firstly, that whatever gene is present on p4B11, is regulated by catabolite repression in *B. lactis*, and secondly, that the activity generated by the clone in *E. coli* is not likely to be spurious. A positive signal was also detected with RNA from *E. coli* with p4B11. Although the probe contains 0.7 kb of vector sequence, RNA from *E. coli* with pMT104 did not hybridise to the probe, suggesting that the signal obtained was due to mRNA transcribed from the insert DNA. In order to determine the transcript sizes, Northern hybridisation was performed with RNA as for the slot blots, however, transcripts were not detected from *B. lactis* or *E. coli*.


```
BL0328    MKAMRVISGRFKGVALAIPKTGTRPTTDRTKEAIFSHLDSWGVLDDARVLDLFAGTGALG   60
ORF3      ...MRVITGRFKGVPLTIPRSETRPTTDRTKEAMFSRLDSTGILSGARVLDLFGGTGALG   57

BL0328    IEALSRGARELVAVESSRPAAALLTKTLAQLQKNRSWDASLKARVLVKKAEQVAG..GFG   118
ORF3      IEALSRGANELVWVEASGPAAKLIAHTLTALRRNPAWEESMNARIVHAKAERYAAKAKVG   117

BL0328    EPFDVIFIDPPYAYETAECNQLLSDLAAGSATNENTVIIMLERSVRSDDPIAPGGWQITES   178
ORF3      EPFDVVLIDPPYAFSTADCERLIGDLITARGLVARDGMIVLERSVRTERPEAPSD......   171

BL0328    RNYGETAVFYIESADDDSDIERSDDSAA                                    206
ORF3      ............................                                    171
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Figure 3.8. Multiple sequence alignment of the *B. lactis* ORF3 from p4B11 with a probable methylase from *B. longum* NCC2705 (BL0328).

3.4.8. Analysis of Protein Production from *E. coli* (p4B11)

Since the transcript responsible for α-galactosidase activity could not be identified, it was decided to analyse the proteins produced by *E. coli* cells with p4B11. If a distinct band could be identified, MALDI-TOF (matrix assisted laser desorption ionisation-time of flight) mass spectrometry could be performed to facilitate the identification of the protein. SDS-PAGE was performed using total protein from *E. coli* JM109 containing the plasmids p4B11 and pMT104 (Figure 3.10), however there was no significant difference between them. Considering that cells with p4B11 give a maximum activity of 0.025 μmoles/min/mg protein, it should not be surprising that a distinct band could not be detected.

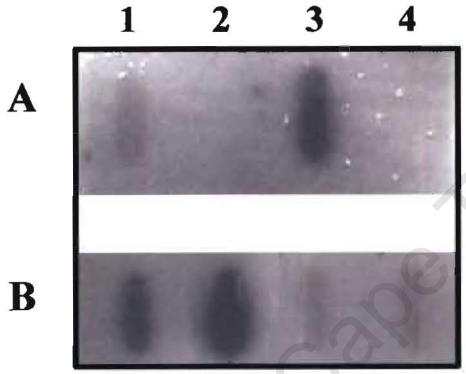

Figure 3.9. *B. lactis* mRNA from mid-logarithmic cultures grown in different carbon sources probed with (A) the 4.3 kb *Hin*dIII-*Bam*HI fragment from p4B11, and (B) the *B. lactis* 16s rDNA probe (prepared in chapter 2). Lane 1: mRNA from cells grown in raffinose, Lane 2: mRNA from cells grown in glucose, Lane 3: mRNA from *E. coli* with p4B11, Lane 4: mRNA from *E. coli* with pMT104.

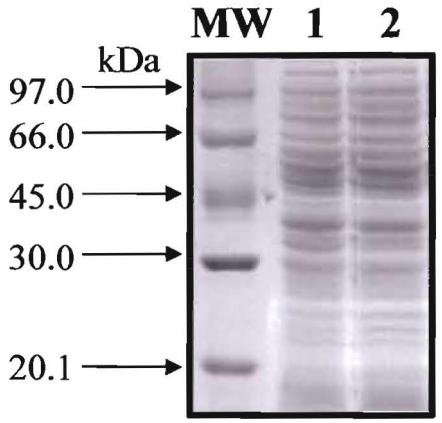

Figure 3.10. SDS PAGE analysis of protein produced by *E. coli* JM109 cells containing p4B11 (lane 1) and pMT104 (lane 2).

3.5. CONCLUSIONS

Soya oligosaccharides, of which raffinose is a major component, have been implicated as bifidogenic factors (49, 247). Furthermore, we have shown that raffinose was the preferred substrate for the growth of *B. lactis* in comparison to glucose and oligofructose. Also, α-galactosidases have transglycosylation activity which could be applied for the development of novel prebiotics (310). The characterisation of the metabolism of raffinose by *B. lactis*, was therefore, investigated.

Several strategies were employed for the isolation of the genes required for the metabolism of raffinose. These were (i) screening the *B. lactis* genebanks on raffinose and the histochemical substrate ρ-nitrophenyl-α-D-galactopyranoside; (ii) amplification of the α-galactosidase from *B. lactis* by PCR; (iii) construction and the subsequent screening of a *B. lactis* genebank prepared with DNA which hybridised to the *B. longum* α-galactosidase gene probe. Three clones displaying α-galactosidase activity were isolated as a result. Restriction analysis revealed that each of the clones contained inserts ranging from 3.7-0.2 kb in size, and that they were not related to each other. This was the first indication that the activity was spuriously being expressed by these clones in *E. coli*. Furthermore, since α-galactosidase genes range from 2.1-2.4 kb in size, it was not possible that a functional protein was being expressed from, at least, one of the clones. However, *E. coli* transformed with the vector plasmid, pMT104, did not display any α-galactosidase activity, indicating that the cloned *B. lactis* DNA was somehow involved in the activity produced in *E. coli*. Future attempts to isolate the α-galactosidase from this organism should possibly be conducted in the *E. coli* strain M2508, which has a mutated *melA* gene (268), coding for α-galactosidase. This would possibly reduce the occurrence of false positives as was experienced in this investigation.

Since one of the clones isolated, containing the plasmid p4B11, had an insert large enough for a full length α-galactosidase gene to be present, it was investigated further. Sequence analysis revealed 7 putative ORFs, none of which showed identity to other α-galactosidases or related proteins. Hybridisation of the insert DNA from p4B11 to RNA from *B. lactis* cells could only be detected when grown in the presence of raffinose and not glucose, thereby suggesting the production of a transcript that was regulated by catabolite repression in *B. lactis*. Furthermore, this supported the possibility that the activity detected in *E. coli* containing p4B11 was genuine. However, SDS-PAGE analysis of protein from *E. coli* with p4B11 did not reveal a distinct protein

band which could be responsible for the activity. This, however, could be expected since the enzyme activity measured from the clone was very low in comparison to that measured in *B. lactis*, even in repressive conditions.

There are several strategies that could be employed for the isolation of the *B. lactis* α-galactosidase gene. The PCR-amplification of the gene could be performed using the High GC PCR kit, which facilitates the denaturation of the GC-rich DNA regions, thereby "opening up" the DNA to allow the specific hybridisation of the primers and to improve the *Taq* polymerase processivity. Another approach is to purify the *B. lactis* α-galactosidase using the protocols developed for the purification of the *B. longum* and *B. adolescentis* proteins (83, 150). The protein could be end sequenced, from which PCR primers specific to the *B. lactis* gene could be designed. However, the GalF and GalR primer sequences only differed in 2 bp when the *B. adolescentis* and *B. longum* sequences were aligned, indicating that the sequences are very conserved, and therefore, the *B. lactis* sequence will probably also not be significantly different. This strategy would also identify whether the *B. lactis* α-galactosidase is homologous to those of *B. longum* and *B. adolescentis*. Considering that sequence analysis of the *B. longum* NCC2705 genome revealed the presence of two different α-galactosidase genes, it could be possible that *B. lactis* gene is different to the one that was being amplified. Another consideration is that *B. lactis* and *B. longum* do not share very high DNA sequence homology (261). Perhaps the α-galactosidase gene could, therefore, be amplified from another *Bifidobacterium* strain that is more closely related to *B. lactis*, such as *B. breve*, and then used to hybridise to the *B. lactis* DNA.

As was mentioned before, raffinose hydrolysis requires the action of two catabolic enzymes. The first, α-galactosidase, was investigated in this chapter and could not be isolated from *B. lactis*. The second, a sucrase, was also to be investigated, specially considering that these two genes are clustered together in several organisms (12, 233, 238). The following chapter, therefore, reports the screening of a *B. lactis* genebank for sucrose utilisation genes. The isolation, sequence analysis and characterisation of a sucrose gene cluster are presented.

CHAPTER 4

CLONING, SEQUENCING AND CHARACTERISATION OF A SUCROSE UTILISATION SYSTEM FROM *BIFIDOBACTERIUM LACTIS*

Contents

4.1. SUMMARY

A sucrose utilisation system of *B. lactis* was cloned in two stages. In the first stage a *B. lactis* gene library was screened for plasmids conferring a sucrose-positive phenotype on *E. coli*. A plasmid, pSuc1, was isolated and contained an insert of 4.6 kb of *B. lactis* chromosomal DNA. Preliminary sequence analysis indicated that the insert contained a truncated sucrose utilisation gene cluster. The remainder of the gene cluster was cloned by chromosome walking.

Nucleotide sequence analysis revealed that this *B. lactis* sucrose utilisation gene cluster contained 3 closely linked genes, designated *scrP*, *scrT* and *scrR*, encoding for a sucrose phosphorylase, a GalR-LacI family sucrose regulator and a transporter protein of the sugar transporter family. The *scrP* and *scrT* genes did not find sequence identity to the equivalent PTS components, namely a sucrose-6-phosphate hydrolase and an enzyme II protein respectively, suggesting that sucrose is not transported via the PTS in *B. lactis*. The presence of the *scrR* gene implied that sucrose metabolism would be regulated.

4.2. INTRODUCTION

The carbohydrates inulin, oligofructose and raffinose are some of the major bifidobacterial prebiotic compounds, the first two being the most extensively studied (92, 94, 237). The key aim of this study was, therefore, to investigate and characterise the utilisation of these carbohydrates by *B. lactis* at the molecular level.

The approach was two-pronged, involving the isolation of, firstly, the inulinase and secondly, the raffinose utilisation genes by complementation of a *B. lactis* genebank in *E. coli*. The second approach was undertaken and was reported in chapter 3. The isolation of an inulinase was considered, however, inulin was not utilised as a sole carbon source by the four bifidobacterial strains analysed (chapter 2), and, although *B. lactis* was able to ferment oligofructose, it supported very poor growth. Since it was not clear whether the inability to utilise inulin was due to the absence of the catabolic gene or due to transport difficulties, it did not seem feasible to screen on inulin. Therefore, another approach was undertaken.

Inulin is a fructose polymer with an α(1,2) linked glucose at the terminal end of the molecule, and since oligofructose is an enzymatic hydrolysis of inulin, a proportion of the polymers would also have the terminal α(1,2)-glucose (229, 337). Raffinose also contains the α(1,2)-glucose-fructose bond. Since the α(1,2) glycosidic linkage of sucrose is found in all three of the oligosaccharides, it was decided to screen for the genes involved in sucrose utilisation.

The work presented in this chapter describes the steps taken to isolate the *B. lactis* sucrose utilisation (*scr*) gene cluster by screening the *B. lactis Sau*3A genebank in *E. coli*. The subsequent sequence analysis of all the genes isolated, and the characterisation of the sucrase is described.

4.3. MATERIALS AND METHODS

4.3.1. Bacterial Strains, Plasmids and Culture Conditions

Bifidobacterium lactis was propagated in BYG medium as described in 2.3.1. Where applicable, 1% (w/v) of the carbohydrate was substituted for glucose.

E. coli JM109 (336) was used for all cloning purposes and was cultured as described in 2.3.1. MacConkey base medium (Difco Laboratories) and M9 minimal medium (176) supplemented with ampicillin (Ap) and 1% (w/v) sucrose, was used to assess fermentation of sucrose as a sole carbohydrate source.

The following plasmids were used for subcloning and sequencing: M13-derived Bluescript SK plasmid (pSK) (Stratagene, La Jolla, California, USA), pEcoR251 (343), and pMT104 (327). Further strain and plasmid details are given in Appendices A and B.

4.3.2. General DNA Manipulations

Extraction of plasmid DNA, preparation of *E. coli* JM109 cells, DNA ligations, gel electrophoresis and other general manipulations are described in section 2.3.3.

4.3.3. Screening of the *B. lactis* Genomic Library

The *B. lactis Sau*3A genebank (3.3.4) was transformed into *E. coli* JM109. Transformants were screened for sucrose-positive phenotypes on M9 minimal medium containing sucrose and Ap and were confirmed to be sucrose-positive on MacConkey agar containing sucrose and Ap.

4.3.4. Southern Blot and Colony Hybridisation

Southern blot and colony hybridisation procedures were conducted as described in section 3.3.7. The 1.4 kb *scrP*-specific *Bam*HI-*Hin*dIII and 0.45 kb *scrT*-specific *Pvu*I-*Sal*I fragments from pSuc1 were used as probes and were prepared as described in section 3.3.7.

4.3.5. Sucrase Assays

E. coli cell free extracts (CFEs) were prepared as described in section 2.3.8. For the temperature optimisation preparations, the cells were washed and resuspended in TA buffer (100 mM Tris, 100 mM acetate, pH 7), whereas for the pH optimisation assays, TAP buffer (100 mM Tris, 100 mM acetate, 64.2 mM Na_2HPO_4, pH 4-pH 9) was used. Sucrase activity was assayed in the CFEs as described in section 2.3.9.1. For the temperature optimisation assays, the reactions were incubated at temperatures between 22°C and 100°C. For the substrate specificity assays the substrates (sucrose, raffinose, melezitose, oligofructose, and inulin) were supplied at a concentration of 1% (w/v).

4.3.6. Protein Assays

Protein concentrations were determined as described in section 2.3.10.

4.3.7. Nucleotide Sequence and Analysis

DNA sequencing was performed and analysed as described in section 2.3.4.

4.4. RESULTS

4.4.1. Isolation and Preliminary Sequence Analysis of the Truncated *scr* Gene Cluster

The *Sau*3A gene library of *B. lactis* established in *E. coli* JM109 was screened on minimal medium containing sucrose. From approximately 3 500 colonies that were screened, six were able to utilise sucrose as the sole carbon source. Plasmid restriction analysis of these clones indicated that they all had a 1.9 kb *Bam*HI-*Pst*I fragment in common. The plasmid containing the largest insert was designated pSuc1 and its analysis revealed an insert of 4.6 kb (Figure 4.1). In order to localise the sucrase activity to a smaller fragment and to facilitate sequence analysis, the insert in pSuc1 was digested into several fragments and subcloned into pSK. The plasmid pHind1 (Figure 4.1) containing a 2.9 kb *Hin*dIII insert fragment was found to confer a sucrose-positive phenotype on *E. coli*. However, when sucrase assays were performed on *E. coli* cells containing pHind1, the activity was reduced 200-fold relative to cells with pSuc1, suggesting that sequence downstream of the *Hin*dIII site was critical for activity.

The insert of pSuc1 was sequenced so that the gene expressing sucrase activity could be characterised, and to determine if other related genes were present. This revealed the presence of two open reading frames (ORFs) reading divergently (ORFs 1 and 2), and a third truncated ORF (Figure 4.1). ORF1 showed sequence identity to regulator proteins of the GalR-LacI family (full details are supplied in section 4.4.4.2 and in Table 4.2) and was designated *scrR*. ORF2, reading divergently from *scrR*, showed sequence identity to sucrose phosphorylases (full details are supplied in section 4.4.4.1 and in Table 4.1) and was designated *scrP*. The truncated ORF3, upstream from and reading in the same direction as *scrP*, found weak sequence identity to transporters of the sugar transporter family of pro- and eukaryotes (details are supplied in section 4.4.4.3 and Table 4.3), and was designated *scrT*. It appeared, therefore, that a truncated sucrose gene cluster had been cloned, consisting of three genes: a *scrR*, *scrP*, and a truncated *scrT*.

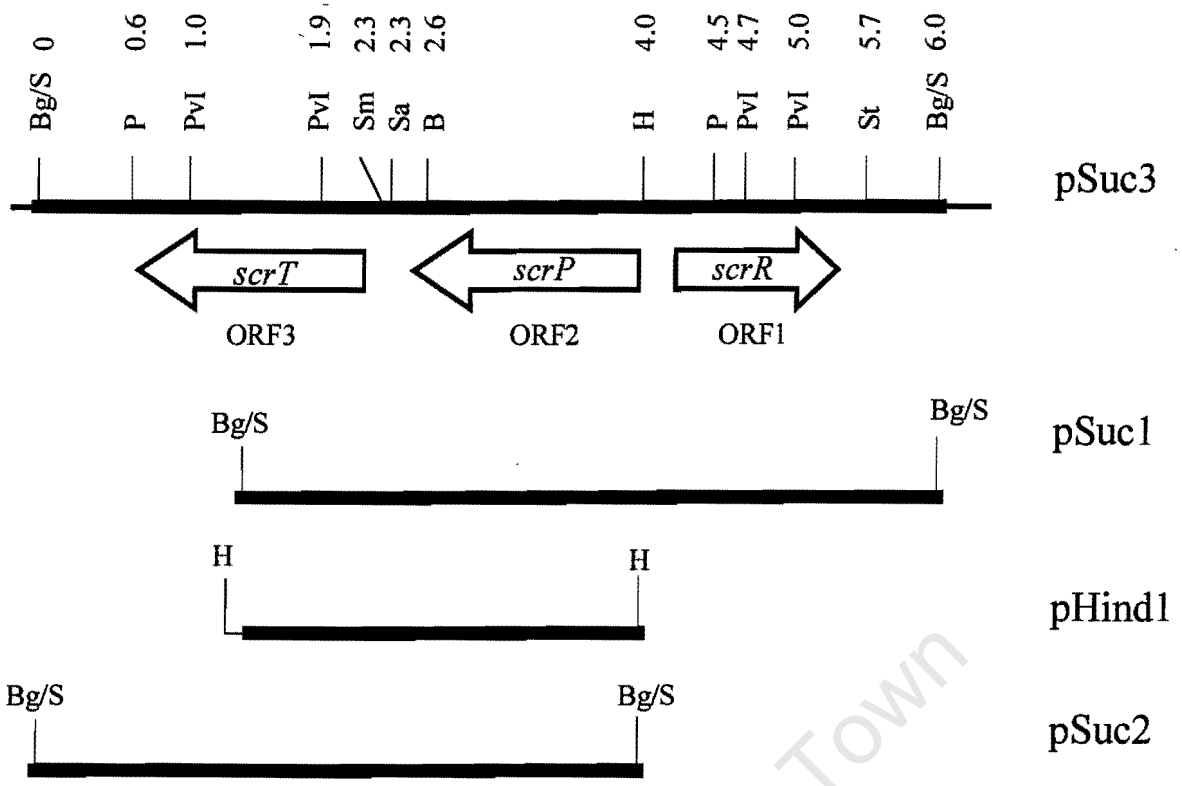

Figure 4.1. Genetic organisation of the *scr* genes of the *B. lactis* sucrose utilisation system. Transcriptional polarities are indicated by arrows. Thick and thin lines represent insert and vector respectively. Plasmids pSuc1 and pSuc2 originate from the *Sau*3A genebank constructed in the *Bgl*II site of the vector pEcoR251. Plasmid pSuc3 was constructed from pSuc1 and pSuc2 (see text for details). Plasmid pHind1 contains a 2.9 kb *Hin*dIII fragment from pSuc1 subcloned in pSK. Sizes are indicated in kb. B, *Bam*HI; Bg/S, *Bgl*II/*Sau*3A; H, *Hin*dIII; P, *Pst*I; PvI, *Pvu*I; Sa, *Sal*I; Sm, *Sma*I; St, *Stu*I.

4.4.2. Southern Blot Analysis of the Sucrose Cluster

Before continuing with further analyses, a Southern hybridisation was performed to confirm that the cloned insert DNA from pSuc1 originated from *B. lactis*. The 1.4 kb *Bam*HI-*Hin*dIII was DIG-labelled and hybridised to *B. lactis* chromosomal DNA digested with *Bam*HI, *Hin*dIII, and *Bam*HI-*Hin*dIII (Figure 4.2). The probe hybridised to an approximately 7.8 kb *Bam*HI fragment, 14 kb *Hin*dIII fragment, and a 1.4 kb *Bam*HI-*Hin*dIII fragment, confirming that *B. lactis* chromosomal DNA had been cloned.

4.4.3. Isolation of the Complete *scr* Gene Cluster

The plasmid pSuc1 isolated from the genebank contained a sucrose cluster which was truncated at the *scrT* gene. To isolate the remainder of the gene, colony hybridisation of the *B. lactis* genebank was performed using the *scrT* 0.45 kb *Pvu*I-*Sal*I DNA fragment from pSuc1 as a probe. Seven

colonies gave a positive signal, two of which appeared to have the same construct from restriction analysis of their plasmids. A Southern hybridisation of the digested plasmids was performed with the same probe (not shown), and together with the comparison of their restriction patterns to that of pSuc1, one clone was selected which was thought to contain the full *scrT* gene. This was designated pSuc2 (Figure 4.1). Sequence analysis of the insert in pSuc2 indicated that the complete *scrT* gene had been isolated. This clone was unable to confer a sucrose-positive phenotype on *E. coli.* Sequence analysis revealed that the *scrP* ATG start codon was not present.

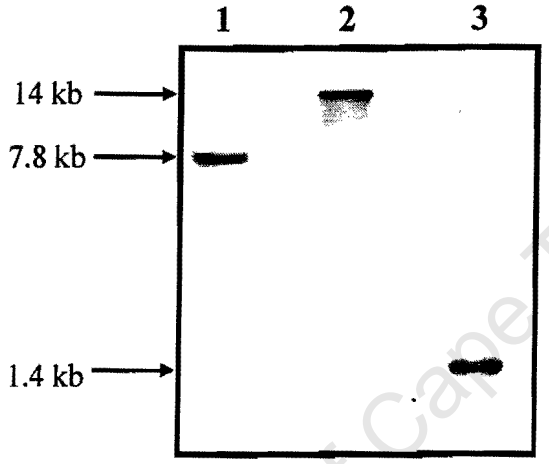

Figure 4.2. Southern hybridisation of the *scrP* gene. The 1.4 kb *Bam*HI-*Hin*dIII fragment was used as DIG-labelled probe and was hybridised to *B. lactis* chromosomal DNA. Lane 1: *B. lactis* chromosomal DNA digested with *Bam*HI, Lane 2: *B. lactis* chromosomal DNA digested with *Hin*dIII, Lane 3: *B. lactis* chromosomal DNA digested with *Bam*HI-*Hin*dIII. No hybridisation was detected with plasmid DNA from pSK (not shown).

Since the three genes were present on two different clones, a cloning strategy was devised to ligate the inserts from pSuc1 and pSuc2 so that the genes would be assembled on one construct. The 2.8 kb *Hin*dIII-*Bam*HI fragment from pSuc2 was cloned into pSuc1 replacing the 1.5 kb *Hin*dIII-*Bam*HI, and the resultant clone was designated pSuc3 (Figure 4.1). Analysis of the sequence across the *Bam*HI site in the insert of pSuc3 confirmed, together with restriction digests, that the subclone was successful. Fermentation on MacConkey agar containing sucrose indicated that this clone was able to confer sucrase activity on *E. coli.*

4.4.4. Sequence Analysis of the *B. lactis scr* Genes and their Translation Products

A detailed analysis of the DNA and amino acid sequences of the three *B. lactis scr* genes was performed and is described below.

4.4.4.1. B. lactis scrP

The *scrP* gene consists of 1518 bp encoding 506 amino acids with a calculated molecular mass of 55,660 Da, and specifies sequence identity to sucrose phosphorylases of various bacteria (Table 4.1). A multiple sequence alignment of these proteins illustrates several very conserved regions (Figure 4.4), however, these domains have not yet been identified or assigned catalytic function. A canonical gram-positive Shine-Dalgarno (SD) sequence AGGAGG (315) was located 4 bp away from the proposed ATG start codon (Figure 4.3). A detailed analysis of the promoter region will be presented in chapter 5.

Sucrose phosphorylases reversibly catalyse the reaction sucrose + phosphate ⇆ α-D-glucose-1-phosphate + D-fructose. This is catalysed in a double replacement reaction, where a glucose-enzyme complex intermediate is formed (238, 317). Interestingly, sucrose phosphorylases also catalyse transglucosylation in addition to phosphorolysis (133) and therefore, can be used for the synthesis of novel glucooligosaccharides. This could possibly be applied for the synthesis of prebiotic substrates specific for the stimulation of *B. lactis*. The sucrose phosphorylase from *L. mesenteroides* was able to synthesise glucosyl-xylitol (133). This oligosaccharide is being analysed as a preventative for dental caries because it reduced the synthesis of water-insoluble glucan which is produced by *S. mutans* from sucrose. Therefore, this enzyme has potential for biotechnological applications.

Table 4.1. Percent identity and similarity of the deduced *B. lactis* ScrP to known protein sequences.

Homologous Protein	Accession Number	% Identity	% Similarity
Agrobacterium tumefaciens SucP	AF0652465	57	71
Agrobacterium vitis SucP	P33910	56	69
Pseudomonas saccharophila sucrose phosphorylase	AF158367	53	67
Streptococcus mutans GtfA	P10249	41	58
Leuconostoc mesenteroides SucP	Q59495	39	56
Escherichia coli SucP	P76041	28	45

4.4.4.2. B. lactis scrR

The *scrR* gene consists of 978 bp encoding 326 amino acids with a calculated molecular mass of 35,860 Da. A possible ATG start codon preceded by a potential Shine-Dalgarno sequence (AGGAGG) at positions –6 to –11 relative to the ATG was identified (Figure 4.3). The promoter region was analysed and will be presented in chapter 5. Two stem-loop structures were detected 47 and 73 bp downstream of the stop codon, which might function as rho-independent terminators, however, the T-stretch is missing.

At the protein level, sequence identity was observed to the N-terminal regions of the GalR-LacI family of bacterial transcriptional regulators (191). Since this region of the GalR-LacI family has been implicated in DNA binding due to a helix-turn-helix motif (Figure 4.5), this identity suggested that ScrR might also be involved in such interactions. Further investigations were performed and will be presented in chapter 5. Moreover, ScrR also showed identity throughout the amino acid sequence to other sucrose regulators belonging to the GalR-LacI family (Table 4.2). These conserved regions were, at first, proposed to be involved in sugar-binding (328). However, when the inducer binding domains of these proteins were aligned (by comparison to the regions identified for the *E. coli* LacI protein) (Figure 4.5), it could be seen that the *B. lactis* ScrR lacked identity in those regions, suggesting that the *B. lactis* ScrR perhaps responds to a different ligand. In the case of *K. pneumoniae* and *S. typhimurium* ScrR proteins, the inducer molecule has been demonstrated to be fructose or fructose-1-phosphate (127), but for other ScrR proteins, the molecules may be sucrose or one of its metabolites, such as sucrose-6-phosphate (116). The metabolites produced when sucrose is metabolised by a sucrose phosphorylase would differ to those produced by a sucrose hydrolase. Moreover, in *B. lactis* sucrose would enter the cell in its native form for hydrolysis by ScrP, whereas for organisms transporting sucrose via a PTS, the product of transport would be sucrose-6-phosphate. Therefore, the *B. lactis* ScrR might recognise a different ligand molecule, which could explain the lack of identity in the inducer binding regions.

Table 4.2. Percent identity and similarity of the deduced *B. lactis* ScrR to known protein sequences.

Homologous Protein	Accession Number	% Identity	% Similarity
Lactococcus lactis SacR	Q04939	37	57
Streptococcus mutans ScrR	Q54430	31	49
Pediococcus pentosaceus ScrR	P43472	29	45

It is interesting to note that the *B. lactis* ScrR shares significant homology to the sucrose regulators of *L. lactis*, *S. mutans*, and *P. pentosaceus*. In a phylogenetic analysis of sucrose regulators (222), it was reported that within the GalR-LacI family, proteins involved in the regulation of sucrose-catabolic genes had evolved separately at least four times. The *B. lactis* ScrR protein would, therefore, fall within the cluster consisting of the *S. mutans* and *P. pentosaceus* genes. However, unlike in these organisms, the *scrR* gene of *B. lactis* is not associated with sucrose PTS and hydrolase genes (see chapter 1 for the genetic arrangement of the *P. pentosaceus, L. lactis* and *S. mutans scrR* gene). In *L. lactis* and *P. pentosaceus* these sucrose utilisation genes are located on plasmids or transposons (190, 221), and, therefore, horizontal gene transfer from these organisms to *B. lactis*, or perhaps *Bifidobacterium* (see 4.4.5), could have occurred.

4.4.4.3. B. lactis scrT

Several possible in-frame ATG start codons were identified for *scrT* (Figure 4.3), none of which have a typical Shine-Dalgarno sequence preceding it. However, an alignment of the Shine-Dalgarno regions of 6 bifidobacterial genes revealed that only the AGG of the canonical GGAGG sequence was conserved (291). On this basis, the sequence GAGG located 11 bp from one of the ATG start codons was identified (Figure 4.3). Although this sequence has also been identified as the Shine-Dalgarno for the *B. longum ldh* gene (291), BLAST searches of the *scrT* amino acid sequence only showed identity from the third ATG (Figure 4.3, Figure 4.6). An intergenic region of 350 bp separates the *scrP* stop codon and the first ATG codon of the *scrT* gene. In the analysis of this region, transcription terminator sequences (such as a stem loop) or promoter sequences could not be identified, suggesting that these two genes could possibly be co-transcribed as an operon. This was investigated and is reported in chapter 5. Two stem-loop structures were identified 22 and 73 bp from the *scrT* stop codon which could function as rho-independent terminators, however, the T stretch is missing.

The *scrT* sequence specified weak identity (20-28%) to a variety of transporters of the sugar transporter family of prokaryotes and eukaryotes, with the highest overall identity to a putative membrane protein from *Caulobacter crescentus* (accession no. AE005901) (Figure 4.6). When only the N-terminus (200 bp) sequence was used to search the databases, identity to plant sucrose symporters was detected (Table 4.3), which did not extend beyond the N-terminus. This suggests that the *scrT* gene product might be involved in the transport of sucrose. The functionality and possible action of the *scrT* gene product will be discussed further in chapter 5 (section 5.4.3).

The hydropathy plot of the *scrT* predicted protein revealed a hydrophilic N-terminus with a number of highly hydrophobic regions, interspersed with regions of hydrophilicity (Figure 4.7). This secondary structure is common in proteins belonging to the sugar transport family (140), and generally have 11 or 12 discrete hydrophobic domains which are potential transmembrane α-helices. Therefore the *scrT* gene likely codes for a membrane-bound sucrose transporter protein. The *B. lactis* ScrT appears to have 11 domains (Figure 4.7). Typically, the carboxyl termini of sugar transport proteins are hydrophilic and are predicted to be on the cytosolic side of the membrane. However, ScrT appears to have a hydrophobic C-terminus.

Table 4.3. Plant sucrose symporters to which the *B. lactis* ScrT N-terminus finds identity.

Homologous Protein	Accession Number	% Identity	% Similarity
Vitis vinifera	AF182445	33	48
Solanum tuberosum	AF237780	31	47
Lycopersicon esculentum	AF176950	30	44
Daucus carota	T14339	29	42

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1       CGGGCTCGTGGTGCGGGACATGGCACAGGCCGCCTTAGCACGCCTTCTTCATCAGCAACT
61      CTGGGCTCTTGCGCCACCTACACCGCGGGCTGCTCTAGTAGCCGTGGACGCTGCTGCAGT
121     GGACGAACTTGCACAAGAAGTGGTGGCTCGAGCTGCCGCCGGGCTGGTGCTGGCTGCCGA
181     GGGACCGCTCGTGGCTGACGCGGCTGCTGCACAAGACCAGGCGCACCAGCCGCAGGCTCC
241     ACAGCCGGCTCTGCTATAGCTACAGGAACGGCTCGTGCGGCGGCCGGCGCAACTACAGCA
301     AGACACGCTCGAAGTAGCAGCGCTACAAGCGGCCAGGGTTTATACGGTACCTCGGCCACT
361     CGCCCTCCTCGGGCCGGCGGCACTTGCACCGGAGCAGCCGCAGGCGGAACGAAAGGCGCG
421     GGCTCGGCGTCGGGCTGTCGCACTCGTAGCGGCGGCGCCGGTAACGGCAGCGGCACTGGT
481     AGGTACTGAACTAAAAGCGTAACAAGTAACGTTAGTGGCAAAGGAGAGAGACTAGAGAAC
541     GGGCGCGCTTAGACGCTTACGTTGAGAAGCGTTCGTCCGCTTGCCACCAAAGCTAAGTGT
601     AGGGATCTGAAGAACGCACTTAGACAGACGTCTAATGCACGCGGTGTTTGGCTCGCGCAC
661     TCGGCGCAATGCGAGCGTACTTGCGCCTTTTCGCAAGCTTTGTGTTCCACTATACCTTGT
                                                    scrT Stop
                                                      *  K  V
721     CCGGCCGTTGCGCTTGCGACGGCCGGACGCCAGACCTCTCTCCATTCCCTAATGAACTGC
        N  R  I  R  M  I  L  A  A  A  A  L  V  I  V  I  A  V  G  F
781     AACGCCTACGCGTACTACTCACGCCGGCGCCGCTCGTGTTAGTGCTAGCGCTGCGGCTTG
        V  W  A  Y  A  V  A  P  T  T  S  V  H  M  S  S  K  V  I  S
841     TGGGTACGCATCCGCTGCCGCCCGCACCACCTCTGCACGTAGCTCGAGAACTGCTACCTC
        V  V  L  S  T  M  A  T  G  I  V  T  S  L  T  N  A  L  N  L
901     TGCTGCTCGCTCCAGTAACGGCACGGCTAGTGACACCTTTCGCACAACCGCTCCAACTCT
        I  G  L  D  K  G  A  E  K  P  N  P  L  V  A  V  N  L  A  Q
961     TATGGGTCTAGGAACGGCCGAAGGAAGCCTAAGCCGTCGTGCCGGTGCAACTCGCGGACC
        D  I  A  N  Y  V  G  Y  G  L  G  A  V  A  A  F  L  Y  M  G
1021    AGCTAGCGCAACATCTGCGGCATTGGCTCCGGCCGCTGCCGCCGCTTGTCTATGTACGGG
        M  P  S  R  F  F  L  P  M  A  A  G  V  A  F  L  C  S  A  L
1081    TAGCCGCTCGCCTTCTTGTCGCCGTAGCGCCGTGGCTGCCGCTTGTCCGTCGAACGCTCG
        A  V  P  L  K  R  R  G  L  R  D  T  I  P  G  A  T  V  A  A
1141    CGGTGGCCGTCGAACGCGGACGGCTCTGCCAGCCACTAGCCCGGACGGCACTGCCGCCGC
        I  L  S  V  V  L  T  I  V  G  M  T  A  I  I  T  A  A  K  D
1201    TAGTCGCTCTGCTGCTCGCACTAGTGCGGGTACCACCGCTACTAGCATCGGCGGAACAGG
        K  A  N  P  D  G  A  Y  I  Y  D  Q  A  I  Y  L  Q  Y  T  T
1261    AAGCGTAAGCCTAGCGGCCGCATCTACATCAGGACGCGTTACATGTCGACCATCCAGCAC
        I  M  W  Y  G  G  M  M  L  T  R  G  V  L  A  Y  Y  F  D  P
1321    TAGTAGGTCATCGGCGGGTAGTACTCGCACGCCGGCTGCTCGCGCATCATCTTCAGCCCC
        A  H  R  P  P  R  F  N  L  L  V  S  K  V  T  V  H  E  S  Q
1381    CGCACCGCGCCGCCGGCCTTCAAGTCCTCGTGCCTGAAGTGCCAGTGTACGAGCGAGACG
        E  E  F  R  S  S  K  E  R  P  W  M  A  V  V  F  I  G  T  L
1441    AGGAGCTTCGCCGAGCTGAAGAGTGCGCCGGTGTACCGCTGCTGCTTCTACGGCCACTCT
        S  F  I  G  L  G  M  M  W  A  S  F  L  G  S  Q  G  Q  R  L
1501    GACTTCTACGGGTCTGGGTAGTAGGTACGCCTCTTCTCAGGGCTGACGGGGACTGCGTCC
        F  A  A  G  V  V  S  G  L  T  Q  G  V  A  I  G  A  G  Y  F
1561    TTGCGCCGTGGGTGCTGGCTCGGCTCGCAGACCGGCTGCCGCTACGGCCGCGGCATCTTC
        G  S  I  T  G  R  V  K  D  P  V  R  D  S  M  V  A  V  Y  P
1621    GGCCTCTAGCATGGCGCGTGGAACAGCCCGTGTGCCAGCCTGTAGTGCCGGTGCATACCG
                                                                5'- G
        A  L  M  M  N  Y  G  M  Q  A  V  C  W  L  F  I  I  L  A  E
1681    CGCTCGTAGTACAACATCGGGTAGACCCGCTGCGTGGTGTCCTTCTACTAGTTGCGGAGC
        CGCTCGTAGTACAACATCG- 3'
            Primer B
        N  R  T  F  A  I  A  G  I  A  L  G  T  I  I  G  G  G  V  I
1741    AACGCGCACTTACGCTACCGGGGCTACCGTTCTGGCCACTACTACGGCGGCGGGTGCTAG
        W  P  T  R  K  G  F  R  S  R  T  M  D  S  L  T  G  F  I  I
1801    GTGCCGCACGCGAACGGCTTCGCCCTCGCGCAGTACAGTCTGTCGCACGGCTTCTACTAC
```

```
      N  A  L  L  A  V  I  A  G  I  S  N  V  L  G  L  M  A  E  K
1861  AAGCGGTCGTTACGGTGTTAGCGCGGCTAGCTCAACTGGTTCGGCTCGTACCGAAGGAAC

      S  S  N  D  I  L  E  F  V  R  P  L  I  V  T  S  L  A  V  W
1921  GAGCTCAACAGTTAGTCGAGCTTGTGTGCGCCGTCTTACTGCCACGAGTCGCGGTGGGTG
                                3' -GCAGAATGACGGTGCTCAG- 5'
                                      Primer C2

      P  I  A  C  A  V  A  S  V  T  F  G  I  G  L  R  V  L  E  A
1981  CCTTAGCGCGTCCGCTGACGCCTTTGCCACTTCGGCTACGGGTTCGCGTGCTCAAGCCGG
                      5' -CGGAAACGGTGAAGCCGAT- 3'
                            Primer St
                                                      ◄··· scrT
      K  T  P  R  H  G  D  A  S  V  M  P  D  R  M  D  M  F  A  S
2041  AACCAGCCCGCCACCGGCAGCCGGCTCTGGTAGCCTAGCGCGTACAGGTACTTACGCCTG

      A  E  D  P  V  A  T  D  V  A  A  L  A  A  M  G  S  G  V  A
2101  CGGAGCAGGCCGTGGCGGCACAGGTGGCGCCGGTCGCGGCGGTACGGCCTCGGCTGCCGG
      SD
      Q  S  D  T  A  L  K  T  G  Q  V  Q  E  D  G  V  S  S  D  A
2161  ACGCTTAGCCACCGCTCAAACCACGGGACGTGGACGAGCAGCGGGTGTGACGACAGCCGC

      A  R  K  K  P  M  P  A  T  L  R  S  I  N  E  K  D  E  Q  S
2221  CGAGCGAAGAAGCCGTAGCCGCGCCACTCTGCCCTCTACAAGAGGAACAGAAGGACCGAG

      L  V  K  D  F  D  P  R  D  D  I  G  P  R  I  Y  G  N  S  G
2281  TTATGGAACAGCTTCAGCCCCGCCAGCAGCTACGGGCCCGCTTACATCGGCAAGCTCGGG

      T  S  P  A  S  G  G  H  T  D  Q  E  D  V  M
2341  CAGCTGCCGCGCGAGGGAGGCACCCACAGAACAAGCAGTTGGTACTAAAGAGAAAAGGCC
                                                  scrP Stop
                                                  *  S  A  Q  M  V  P
2401  CTTAGACTTTTATAGACCTTAGAGATAGCCCTTGGCGCGTAGTCGAGCGGACGTACTGCC

        P  N  A  I  L  D  D  T  R  H  E  G  T  S  D  T  W  V  L  T
2461  CGCCCAAGCGTTACTCCAGCAGCCACGCCACGAGCGGTCACCTTAGGCAGGTCTGCTCGC
    3' -GGTTCGCAATGAGGTCGTC- 5'
          Primer A

        A  V  S  Q  P  N  E  V  G  M  G  K  S  P  T  F  T  L  T  P
2521  ACCGCTGCCTGACACCCAAGAGCTGTGGGTACGGGAACCTGCCGCACTTGCACTCGCATC

        F  S  G  F  G  N  W  S  F  A  I  S  E  D  D  G  V  S  Y  S
2581  CCTTCCTAGGCTTTGGCAAGGTCCTCTTGCGTTAGCTGAGCAGTAGCGGGTGGCTCATCG

        F  D  G  D  F  A  P  L  E  N  R  F  R  A  L  A  N  L  A  K
2641  ACTTCAGCGGCAGCTTGCGGCCGTCGAGCAACGCCTTAGACCGCTCGCGCAAGTCCCGGA

        V  V  P  R  E  L  N  K  D  I  E  S  T  T  Y  Y  H  R  N  I
2701  ACTGGTGGCCTGCAAGCTCCAAGAACAGCTAGAGCCTCCAGCACATTATCACCGCCAACT

        D  R  G  V  N  T  R  K  L  L  E  M  D  N  E  G  A  L  A  G
2761  ACAGCGCCGGGTGTAAGCACGCGAACTCGTCGAGGTACAGCAAGAGCGGCCGCTCGCGCG

        V  Y  Y  V  Q  P  V  G  P  L  F  F  Q  V  A  R  A  A  I  Y
2821  GGTGTATCATGTGGACGCCTTGCGGGCCGTCCTTCTTGACGTGCCGCGCACGCCGTTACA

        H  Q  D  N  C  G  L  A  S  Y  Y  T  S  N  V  Q  Y  L  D  L
2881  TTACGACCAGTAACGTCGGCTCGCGGCTTATTATACACCTCAAGTGGACCATCTCCAGCT

        N  S  A  A  A  G  T  A  A  K  S  E  G  H  T  N  K  A  I  T
2941  CCAAGCTTCGCCGCCGCGGCCAACGCCGGAAGCTGAGCGGTACCCACAAGAAGCGCTAGC

        E  V  M  N  D  V  D  A  D  P  V  L  G  K  L  S  R  D  L  Q
3001  AGAGCTGGTACAACAGCTGCAGGCGCAGCCCGTGGTCTGGGAACTCGCTTGCCAGCTCGA

        D  S  G  I  D  I  V  G  I  G  D  H  T  D  L  V  T  V  A  N
3061  CCAGCCTCGGCTACAGCTACTGCGGCTACGGCAGCACGCACAGCTCGTGCCACTGGCGTA

        N  P  R  I  E  T  W  H  A  L  A  D  V  K  G  T  F  L  S  H
3121  ACAAGCCCGCTTAGAGCCAGGTCACGCGCTCGCGCAGGTGGAACGGCCACTTGTCGCTCA

        L  L  L  P  P  L  A  F  D  Y  V  R  D  V  K  A  A  I  E  V
3181  CGTCGTCGTTACCGCCGTCGCGCTTCAGTATCTGCGCTAGGTGGAACCGCCGCTAGAGGT

        Q  K  K  Y  Y  S  H  V  E  I  L  I  E  L  G  R  K  A  G  E
3241  GGACGAAGAACATCATGCTCACGTGGAGCTACTCTTAGAGGTCTGGCGCGAACCGTGGGA
```

```
          E  R  L  R  S  I  L  E  F  T  K  P  T  M  F  C  S  T  G  A
3301  GGAGCGCGTCAGCGCTCTACTCAAGCTTCCAGAACCCCCAGTACTTCGTCGACCACGGCC

          E  K  A  G  Y  G  V  A  D  L  R  I  Y  K  V  H  S  K  S  M
3361  GGAGGAACCGCGGCATCGGCTGCCGCAGCTCCGCTTACATGAACTGCACGCTGAACGAGT

          Q  D  F  I  S  M  L  Y  E  W  G  K  A  S  D  T  D  I  D  V
3421  AGACCAGCTTCTAGCTGTAGTCCATAAGGGTCGGGAACCGCCTCAGCCACAGTTACAGCT

          Q  Q  P  T  F  T  V  W  V  L  R  T  K  G  A  F  S  Y  H  T
3481  GGACGACGCCGCACTTGCACTGGGTGTGGTTCGCCCAGAACGGCCGCTTCGACATCACCC

          F  P  L  G  P  R  P  R  Y  I  G  A  L  E  E  E  T  A  G  D
3541  ACTTGCCGTCCGGGCCCGCGCCCGCCATCTACGGCCGCTCGAGGAGGAGCCAGCGCGGCA

          P  F  V  S  S  M  T  L  F  M  P  Y  Y  E  S  D  E  G  N  A
3601  GCCCCTTGTGCCTCGAGTAGCACTCCTTGTAGCCCATCATGAGCCTTAGGAGCGGCAAGC

          L  V  D  Q  F  Q  K  S  Q  W  S  M  H  N  V  I  A  D  V  M
3661  GCTCGTGTAGGACCTTGACGAACCTGACGGTCGAGTACACCAACTGCTACCGTAGCTGGT

          I  D  H  T  K  A  L  E  A  I  D  D  W  D  G  L  R  A  D  V
3721  ACTACAGTACGCAGAACCGCTCGAGCCGCTACAGTAGGGTCAGTGGCTCCGCCCGCAGCT

          K  T  H  D  I  P  D  F  G  A  D  A  G  D  F  P  T  F  F  P
3781  GGAACCACACCAGCTAGCCCAGCTTCGGACGCAGGCGCGGTAGCTTGCCCCACTTCTTGC

          L  I  H  V  G  E  Y  V  G  D  F  R  T  R  L  I  D  T  M  S
3841  CGTCTTACACGTGCGGGAGCATGTGCGGCAGCTTAGCGCACGCGTCTTACAGCCAGTAGC
                                                  5'-GCAGAATGTCGGTCATCG
                                                        Primer Sp

          A  L  N  G  D  G  L  R  D  A  Y  T  I  L  Q  V  K  N  K  M
3901  TTCGTTCCAACGGCAGTGGGTTCGCTAGGCGTATCCACTAGTCGACTTGAAACAAGAAGT
      AAGC- 3'                                                  ···scrP

3961   GGCTCCTCCTGGATTCGTCTATCACGTCTTCGGGATATCTCCGGGGCGATGCGTCTGCC
      ACCGAGGAGGACCTAAGCAGATAGTGCAGAAGCCCTATAGAGGCCCCGCTACGCAGACGG
           SD
                                                        Primer DIR
                                              5' -CCCAAGCTTGTTTTACCTCAT
4021  TGAAGCTTCATCGTTGCGCGAGTGCCGTATCGAACCGGTTCGAGTATTGTTTTACCTCAT
      ACTTCGAAGTAGCAACGCGCTCACGGCATAGCTTGGCCAAGCTCATAACAAAATGGAGTA
                                    IR1     TS        -10

      TCTTCACG- 3'                          IR2
4081  TCTTCACGCGCATGCAAGCGCTCTGCTCTCGGCGTATCAAACCGGTTCGATATTCGACGA
      AGAAGTGCGCGTACGTTCGCGAGACGAGAGCCGCATAGTTTGGCCAAGCTATAAGCTGCT
                -35          -35                    -10
4141  TTCACCGCGCTCTGCCGAAACGCGGTTGCCGGAGGACGAAATCTGCACATACAATCGGAT
      AAGTGGCGCGAGACGGCTTTGCGCCAACGGCCTCCTGCTTTAGACGTGTATGTTAGCCTA
      TS              IR3                                      SD
4201  AGTGCTCGTCAAACCGGTTCGATACCGCGGGCGATAGCGCACGGAGCAGAAAATGAGGAG
      TCACGAGCAGTTTGGCCAAGCTATGGCGCCCGCTATCGCGTGCCTCGTCTTTTACTCCTC
                                                        Primer Sr
          scrR···▶                                3' -GCAGAGCCACTCGTCACAG
4261  GCGAACATGGTCGGCATGCGCGATGTCGCGAAACGGGCGGGCGTCTCGGTGAGCAGTGTC
      CGCTTG M  V  G  M  R  D  V  A  K  R  A  G  V  S  V  S  S  V

      AGC- 5'
4321  TCGCTGGTGATCAACGGCACCGGCTATGTCTCACGCGATATGCGCGAGAAGGTCGAGCAG
       S  L  V  I  N  G  T  G  Y  V  S  R  D  M  R  E  K  V  E  Q

4381  GCCATGCGCGAGCTCGACTATGTGCCCAACGAGCTCGCGCGCAATTTCTACCACGGCAAG
       A  M  R  E  L  D  Y  V  P  N  E  L  A  R  N  F  Y  H  G  K

4441  ACTGACATCATCGGCGTCATCGTGCCCACCATCCAGCACCCGTTCTTCGCCACGCTCACC
       T  D  I  I  G  V  I  V  P  T  I  Q  H  P  F  F  A  T  L  T

4501  GCGCACCTGCAGCACGAGTTCGCGGACCGCGGTCTGCAGACGATGCTGTGCTCCACCGCC
       A  H  L  Q  H  E  F  A  D  R  G  L  Q  T  M  L  C  S  T  A
```

```
4561  GACTCCGCGAACGGCGAGGCGCAGTACGTCGACATGCTGCGTCGGCACAGTCTGGACGCG
        D  S  A  N  G  E  A  Q  Y  V  D  M  L  R  R  H  S  L  D  A

4621  CTGGTCGTCGCCGCGCACACCACCCACGACCCGCAGTATTGGCGCGCGATCGACCGCCCG
        L  V  V  A  A  A  H  T  T  H  D  P  Q  Y  W  R  A  I  D  R  P

4681  GTGGTCGCCTTCGACCGCAATCTGGGGGCGGGGATCACTCAGGTGAGTTCAGACCACGTG
        V  V  A  F  D  R  N  L  G  A  G  I  T  Q  V  S  S  D  H  V

4741  CACGGCGGCGAGCTCATCGCAGAACTCCTCGTGCGCACCGGCGCGCACCATGTGGTCATG
        H  G  G  E  L  I  A  E  L  L  V  R  T  G  A  H  H  V  V  M

4801  GTGGGCGGCCCGCGCGCGCAATTCACCGACCTCGGCGACCACTCCACCTTCCCCACCGTC
        V  G  G  P  R  A  Q  F  T  D  L  G  D  H  S  T  F  P  T  V

4861  CGCTATGTACAGACGTTGGAAGACCGCCTTTCCCAAGCGCGGATTGCGCACACGTACATC
        R  Y  V  Q  T  L  E  D  R  L  S  Q  A  R  I  A  H  T  Y  I

4921  GAGTCGGGCGAGGTGTTCGACATCGCCGGCGTGCGGCGTGCGGTTCGCGAGGCATTCGAC
        E  S  G  E  V  F  D  I  A  G  V  R  R  A  V  R  E  A  F  D

4981  ACATACCCGGACATGGATGCGTTCGTGGGGGCAGATCTCGCCGCCGCATTCGCCGTGCAG
        T  Y  P  D  M  D  A  F  V  G  A  D  L  A  A  A  F  A  V  Q

5041  GAGGCGGTCTCGCGCTCGATCGCGGTGCCCCGTGATGTGCAGATCATCGCCTACGACGGC
        E  A  V  S  R  S  I  A  V  P  R  D  V  Q  I  I  A  Y  D  G

5101  ACACTCGCCGCGGATTGCGCCGGCATGCCGCTCACCACGGTCGCCCAGGATTTCAGGCAG
        T  L  A  A  D  C  A  G  M  P  L  T  T  V  A  Q  D  F  R  Q

5161  ATCGCGCACACCATCGCCGCGTGCGTGGAACACGGGATCGAGTCTTCGCCGCAACGCCCG
        I  A  H  T  I  A  A  C  V  E  H  G  I  E  S  S  P  Q  R  P

5221  GATGCCACGGACGCGATGCCAACATGACGATCATCCCGGTGACGCTGCACGAGCGCGCCA
        D  A  T  D  A  M  P  T  *
                              scrR Stop

5281  CCACCCGTCGCTGAGGTCGGCGCCCGACCCGCAAAAACAGCGAAGGGCCGCCTTGTGGGC
5341  AGCCCTTCGTATAGAGGAGAGGGATGGTAAGTCACTCGGTCGGCACGTCGCCGCTCTGCA
5401  TGTCCTGGAAGACGTTGACGTTGTTGCCGTCGTACTCGAAGATGCCGATGTTCGCCGAGC
5461  TCGGGTCGTTGTTCTTGTTGAAGGGCCCGATGCCGGTCTTGCCGAAGTACTGGATCTTCT
5521  TCTTGTCTTTGATGAGCGCCTTGCAGGCCTCGAAGCTCTCGCACTTCTCCGCCGTCGGCG
5581  CCGGAGACCGAGGAGAGGTTCTTCGCGACCGTCTCGCCGCTGGTGCTCGCCCTGTTCGGC
5641  GGCGAGCGCGGCGAGGATCACTGCGTCGTAGGTTTCGGCGGCGTATGTGGTGTCGGAGAT
5701  GTCGGTGCCGGTGCCCTTGAGGTCGGACTTGAACTTGTCGTCGACATGGGTGCCGGGGAT
5761  GGTGCCCTTCGAGCCCTTGAGCAGGCCGGCGTCGTAGGTCTTCGAGTAGTCGACGGTGTT
5821  GCCGTCGACTAGATAGAGCTTGTGCGTGTCCACGCCGGCGGAGCCAGCGCCTTGATCAAG
5881  CGCGTCGCCTGATCTCATAA
```

Figure 4.3. Nucleotide and deduced amino acid sequence of the *B. lactis scr* gene cluster. Deduced amino acid sequences are shown in single-letter code with the coding sequences. Putative initiation and termination codons for the *scrR, scrP* and *scrT* genes are shown in bold type. Putative promoters (-35 and -10 regions) and ribosome-binding sites (SD) are in bold type and underlined. Putative transcription start sites (TS) for the *scrP* and the *scrR* genes are indicated in bold type. Three indirect repeats (IR1-3) indicated by facing arrows may act as operator sites for GalR-LacI family regulator proteins. A more detailed illustration of the *scrP-scrR* promoter region is given in Figure 5. 7. Inverted repeat sequences representing potential transcriptional terminators are indicated by facing arrows after the *scrR* and *scrT* transcription stop codons. The positions and sequences of primers Sp, Sr and St used for the primer extension experiments are indicated in bold type with the corresponding nucleotide sequence. The positions and sequences of primers A, B and C2 used for the reverse transcription experiments are similarly indicated. The position and sequence of the DIR primer used for the gel binding analyses is also shown.

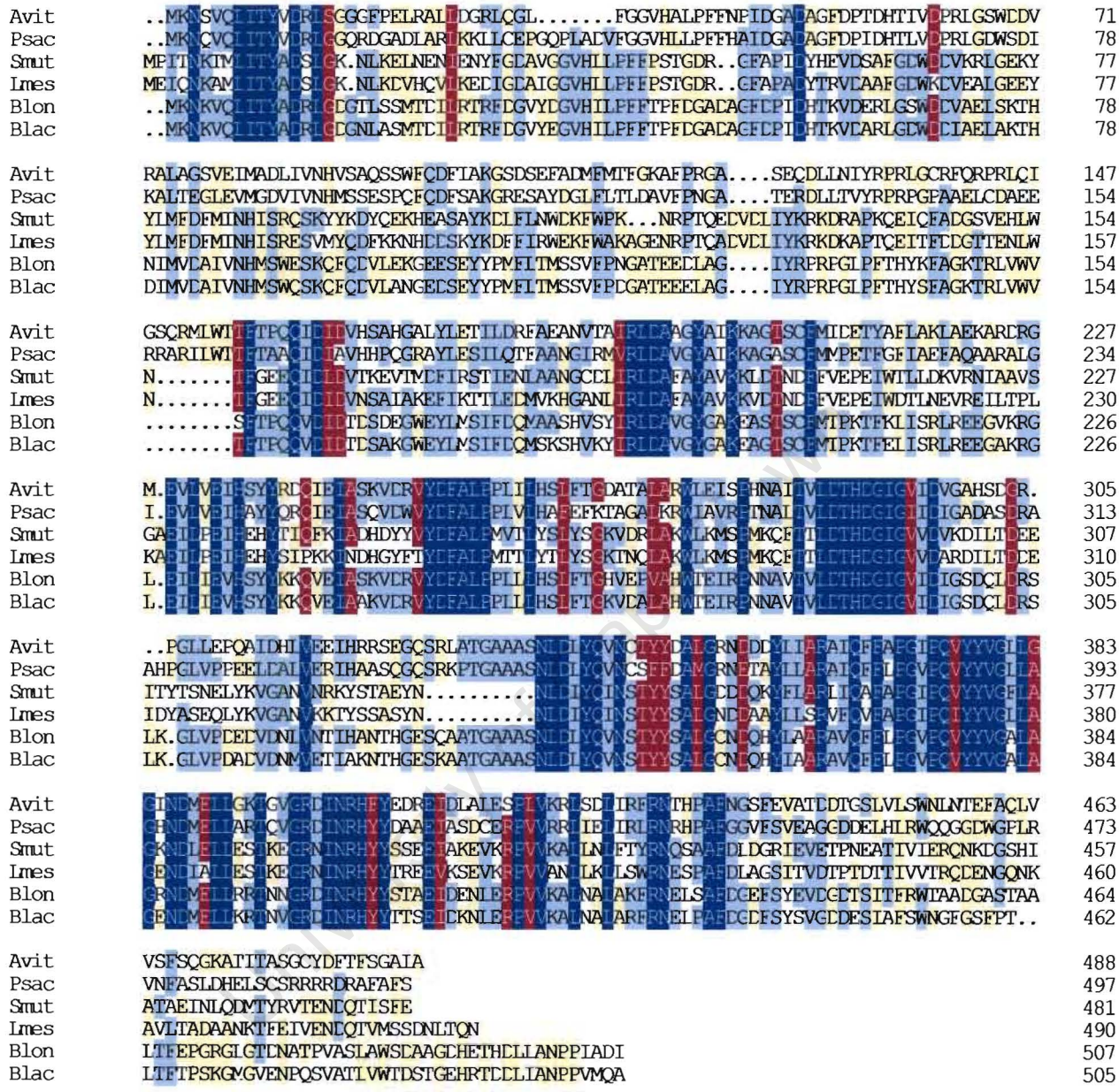


Figure 4.4. Multiple sequence alignment of bacterial ScrP proteins. Sequences included are: *A. vitis* SucP (Avit), *P. saccharophila* sucrose phosphorylase (Psac), *S. mutans* GtfA (Smut), *L. mesenteroides* SucP (Lmes), *B. longum* Spl (Blon) and *B. lactis* ScrP (Blac). Sequence accession numbers are supplied in Table 4.1. Identical amino acids are shaded in dark blue, and similar amino acids are shaded in red, yellow and light blue.

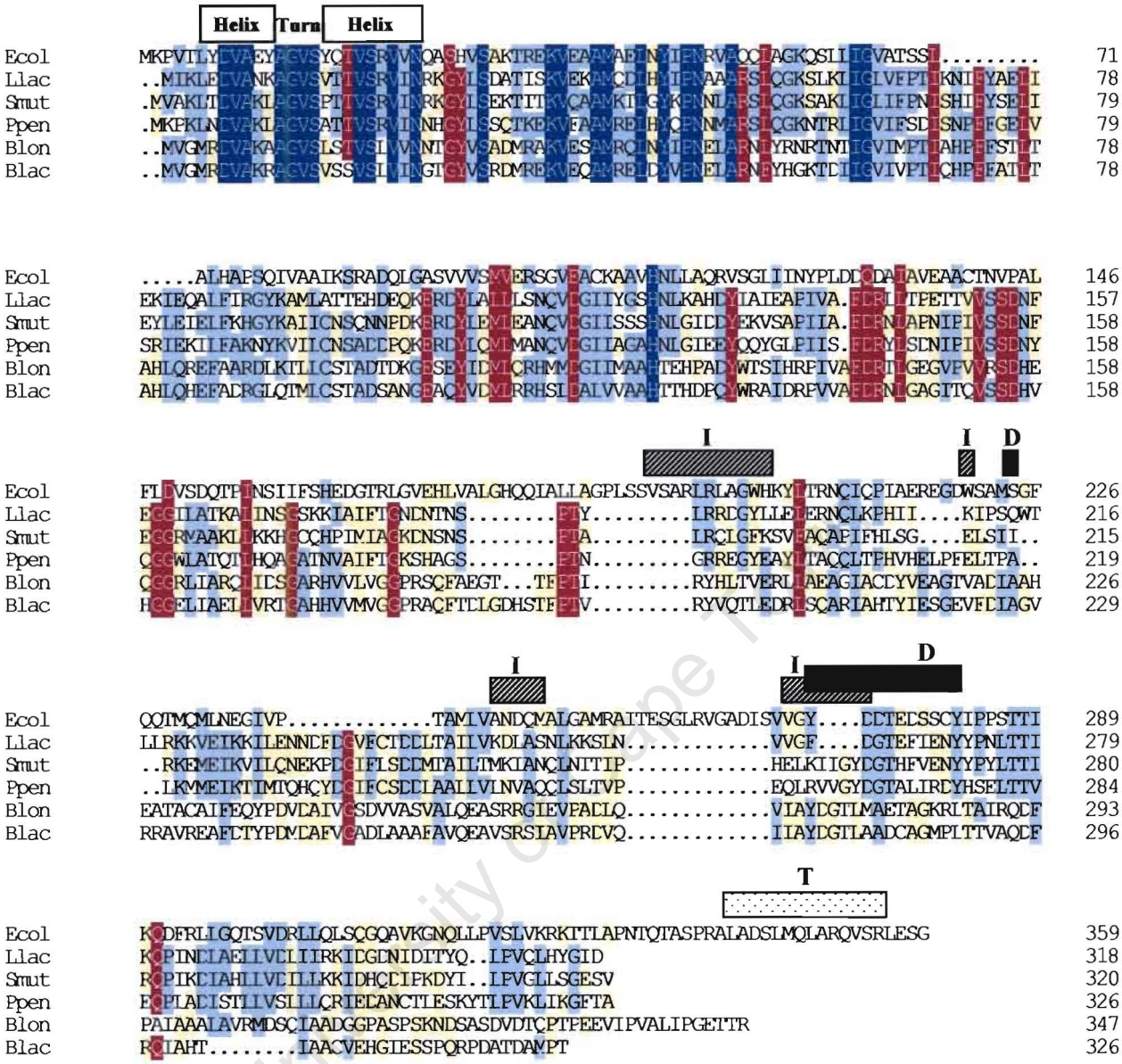


Figure 4.5. Multiple sequence alignment of bacterial ScrR proteins. Sequences included are: *E. coli* LacI (Ecol), *L. lactis* ScrR (Llac), *S. mutans* ScrR (Smut), *P. pentosaceus* ScrR (Ppen), *B. longum* ScrR (Blon) and *B. lactis* ScrR (Blac). Sequence accession numbers are supplied in Table 4.2. *E. coli* LacI was included to orientate the helix-turn-helix DNA-binding domain; inducer binding (I, cross-hatched); dimerisation (D, dark filled box) and tetramerisation (T, dotted box) domains of LacI (328). Shaded areas represent similar amino acids.

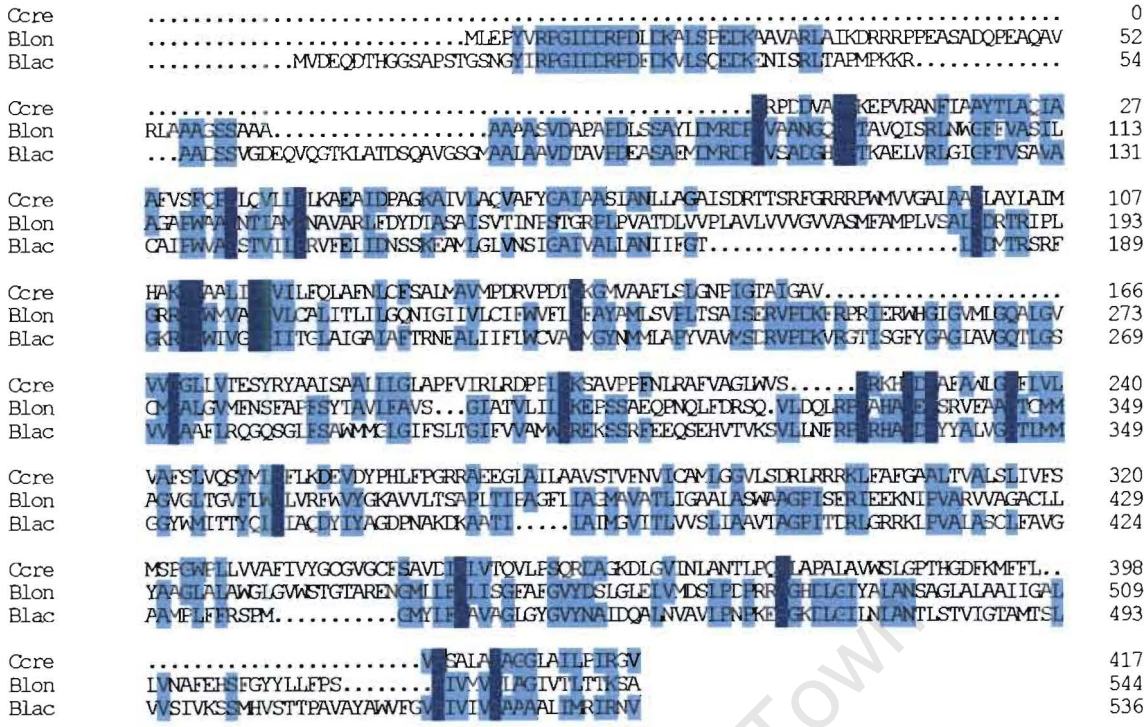


Figure 4.6. Multiple sequence alignment of the deduced *B. lactis* ScrT protein (Blac) with putative membrane proteins from *C. crescentus* (Ccre) (accession no. AE005901) and *B. longum* (Blon) (accession no. NC004307, ORF no. BL0534). Identical and similar amino acids are shaded in dark blue and light blue respectively.

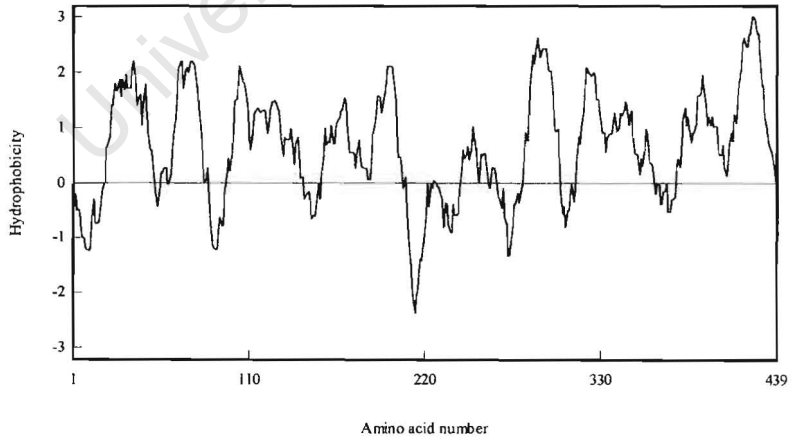


Figure 4.7. Hydropathy plot of the deduced *B. lactis* ScrT protein. The calculation was performed according to Kyte and Doolittle (144) using DNAMan with a window of 15 amino acids. Hydrophobic regions are given a positive hydropathy index.

4.4.5. Analysis of the *B. longum* Sequenced Genome for Sucrose-Utilising Genes

The *B. longum* sequenced genome (accession no. NC004307) was explored for the presence of sucrase genes. Two clusters were identified. The first cluster (Figure 4.8) consists of 3 genes namely, *cscA* (BL0105), encoding a β-fructofuranosidase/inulinase; *cscB* (BL0106), encoding a sucrose transport protein; and the third gene (BL0107) encodes for a LacI-type transcriptional regulator. The second cluster, 540 kb from the first, has genes similar to the *B. lactis scr* genes (Figure 4.8). It consists of the *spl* gene (BL0536), encoding a sucrose phosphorylase; *scrR* (BL0533) encoding a LacI-type transcriptional regulator; and the third gene (BL0534), which will hereafter be referred to as *scrT*, encodes a putative transmembrane protein (Figure 4.8).

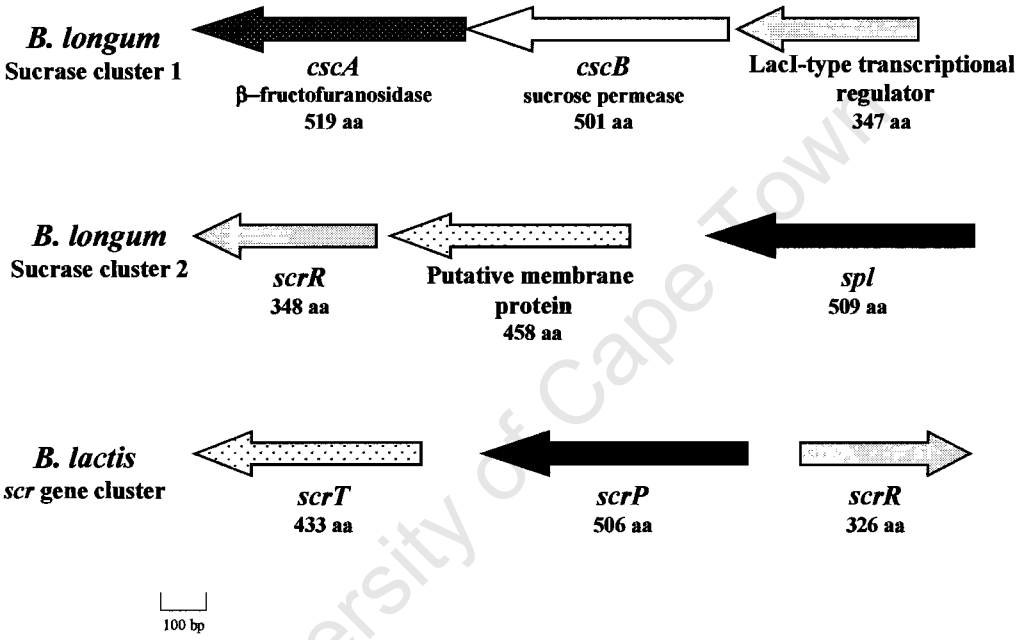


Figure 4.8. Comparison of the genetic organisation of the *B. lactis* and *B. longum* sucrase gene clusters. The *B. longum* cluster 1 is found at positions 126784 to 130943 bp and cluster 2 at 671691 to 676166 bp on the genome map (accession no. NC 004307). The deduced amino acid (aa) sizes for each gene is indicated. For the *B. lactis* ScrT and the *B. longum* putative membrane protein, the number of amino acids was calculated from the ATG start codon at which homology to the *C. crescentus* protein begins (Figure 4.6).

The *B. longum spl* deduced amino acid sequence shows 83.7% identity to the *B. lactis* ScrP (Figure 4.4). The *scrT* follows the *spl* by 465 bp, reading in the same direction, and shows 27.9% amino acid identity to the *B. lactis* ScrT (Figure 4.6). The *scrR* follows from the *scrT* by 64 bp and shows 53.1% amino acid identity to the *B. lactis* ScrR (Figure 4.5).

The genetic organisation of the *B. longum scr* gene cluster, therefore, differs from the *B. lactis* one in the position and orientation of the *scrR* gene, as can be seen in Figure 4.8. It was previously

suggested (section 4.4.4.2) that the unusual organisation of the *B. lactis scrR* with the *scrP* and *scrT* genes, instead of with sucrose PTS genes, was the result of horizontal gene transfer events from another organism to *B. lactis*. The fact that in *B. longum* the *scrR* gene is organised with similar genes and in a different orientation to that in *B. lactis*, suggests that the horizontal gene transfer occurred at the genus, and not species level.

The *cscA* gene present in the first gene cluster in *B. longum* (Figure 4.8) has also been identified in *B. lactis* and has been preliminarily characterised (71). The deduced protein sequence of the *B. longum cscA* gene also finds homology to sucrose-6-phosphate hydrolases from other bacteria. However, the *cscB* gene, encoding the sucrose permease, does not find any identity to sucrose PTS transporters. Furthermore, other sucrose PTS type genes were not identified upon searching the *B. longum* sequenced genome. It therefore, appears that in *B. longum* the PTS system is not the mechanism by which sucrose is assimilated, and this possibly extends to all species belonging to *Bifidobacterium* (see conclusions).

4.4.6. Characterisation of the Recombinant Sucrose Phosphorylase Enzyme Activity

The aim of this study was to determine the optimum conditions under which the cloned sucrose phosphorylase activity could be assayed. The reaction catalysed by sucrose phosphorylase is a double replacement reaction (316, 317). In the initial phase the reaction involves the formation of a glucose-enzyme complex by interaction with sucrose with the release of D-fructose. In the second phase the glucose is transferred to an acceptor, such as P_i, which would result in the formation of glucose-1-phosphate. Sucrose phosphorylase activity is measured by the rate at which sucrose is converted to glucose-1-phosphate, which in turn is assayed enzymatically with the enzymes glucose-6-phosphate dehydrogenase and phosphoglucomutase (308, 316). Alternatively, the sucrose catabolism activity of sucrose phosphorylase can be measured by the rate at which reducing sugar is formed, using dinitrosalicylic acid as a colourimetric indicator (175), and this was the method used. However, this method would not distinguish between sucrose phosphorylase and hydrolase activity.

Analysis of enzyme activities in cell-free extracts prepared from *E. coli* JM109 cells carrying pSuc1 confirmed the presence of constitutive sucrose phosphorylase activity, and was absent in cells carrying the negative control plasmid pMT104. The pH and temperature optimum for the recombinant enzyme were determined. The activity was highest at pH 6 and at 60°C (Figure 4.9). A previous analysis of three bacterial sucrose phosphorylases from *P. saccharophila*, *P. putrefaciens* and *L. mesenteroides* reported an optimum pH of 6.6 to 6.8 (330). The *B. lactis*

ScrP, therefore, is active under more acidic conditions. The temperature optimum of 60°C is interesting for two reasons. Firstly, *B. lactis* has a growth optimum of 37°C, and secondly, the sucrose phosphorylase from *L. mesenteroides* has been shown to be destroyed by heating to 60°C for 1 minute (330). It was found that if the phosphate component of the buffer was eliminated (TA buffer), the enzyme activity decreased by 30-fold (from 41.3 to 1.3 μmol/min/mg protein), suggesting that the phosphates in the TAP buffer play a role in the activity of this enzyme. This is a biochemical property of these proteins, as without acceptor, in this case P_i, the glucose complexed with enzyme cannot be released (316, 238, 317). A similar result was obtained for the *gtfA* of *S. mutans* (239). This result supports the sequence homology results which indicated that the *scrP* gene encodes a sucrose phosphorylase. This could be confirmed by measuring the formation of glucose-1-phosphate using the method discussed above. The investigation of the substrate specificity indicated that ScrP could catalyse the hydrolysis of sucrose but not raffinose, melezitose or inulin, which is in agreement with previous reports (330).

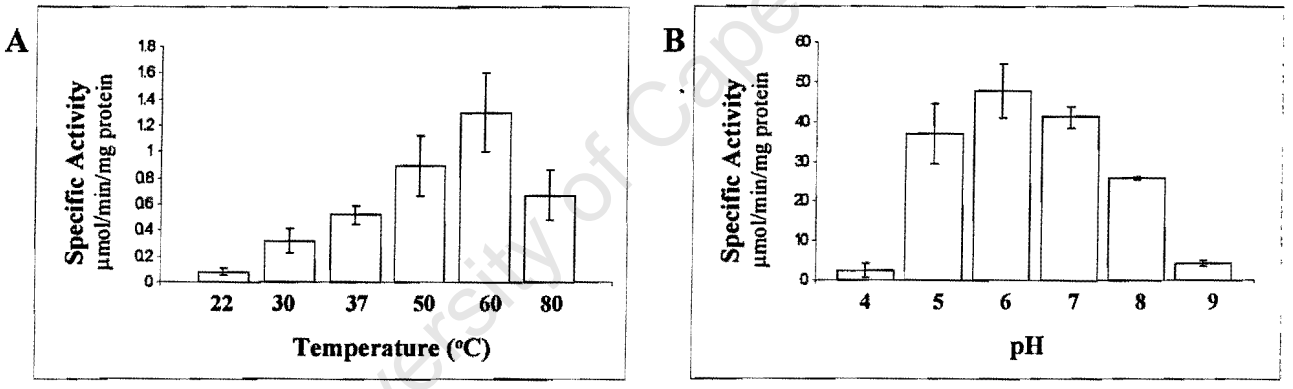


Figure 4.9. Temperature (A) and pH (B) optima of sucrase activity conferred on *E. coli* JM109 by pSuc1. Results are an average of three experiments with the error bars indicating the standard deviation from the mean.

4.5. CONCLUSIONS

Screening for genes involved in the utilisation of sucrose resulted in the isolation of a cluster of 3 genes. Nucleotide sequence analysis indicated that a sucrose phosphorylase (*scrP*), a putative sucrose regulator (*scrR*), and a possible sucrose transporter (*scrT*) had been isolated. Analysis of the *B. longum* sequenced genome revealed the presence of a cluster of genes similar to these isolated from *B. lactis*, as well as a second cluster of genes which are likely to also be involved in sucrose utilisation. The catabolic gene in this second cluster, *cscA*, encodes for a protein which

possibly functions as an inulinase, or a sucrase. There is evidence that this gene is also present in *B. lactis* (71), and it is therefore, surprising that it was not isolated when the *B. lactis* genebank in *E. coli* was screened on sucrose, particularly since the *B. lactis cscA* gene has been shown to be expressed in *E. coli* (71). Perhaps the combined expression of the three genes present in this cluster which is required for a sucrose-positive phenotype to be conferred on *E. coli* did not occur, either due to the nature of the heterologous system, or due to a different organisation of these genes in *B. lactis*. It is also possible that these genes are not present on a single clone in the *B. lactis Sau*3A genebank.

As was reviewed in chapter 1, the predominant mechanism facilitating sucrose uptake in Gram-positive bacteria is the phosphoenolpyruvate-dependent phosphotransferase system (PTS), which results in the intracellular accumulation of sucrose-6-phosphate. Transport and phosphorylation of the carbohydrate is mediated by the sucrose-specific Enzyme II (EII^{Scr}). The sucrose-6-P is further metabolised by sucrose-6-P hydrolases, yielding glucose-6-P and fructose.

The catabolism of sucrose by sucrose phosphorylases results in the products α-D-glucose-1-P and D-fructose, and not glucose-6-P as with the PTS system. Since the deduced amino acid sequence of the *B. lactis scrP* gene demonstrates sequence identity to sucrose phosphorylases, it is likely that glucose-1-P is generated by this enzyme. Furthermore, although *scrT* of *B. lactis* does not show a high degree of sequence identity to any protein in the databases, it does not show any identity to PTS permeases. Taken together, these findings suggest that the cloned sucrose cluster of *B. lactis* is not a PTS system. The glucose-1-phosphate generated by the *B. lactis scrP* could be converted by glucose-6-phosphate isomerase for its utilisation by the bifidus pathway of glucose fermentation (18).

It is generally believed that heterofermentative lactic acid bacteria do not possess active phosphotransferase systems (232). Sucrose hydrolysis could imply either an invertase or a sucrose phosphorylase (63). In *L. mesenteroides*, for example, it is postulated that sucrose is taken up by a permease and is then concomitantly phosphorylated and hydrolysed to yield glucose-1-P and fructose intracellularly. Glucose-1-P is then converted by phosphoglucomutase to glucose-6-P, which then enters the phosphoketolase pathway (63). This reaction by sucrose phosphorylase employs P_i and not a high-energy phosphate bond, and, therefore, offers a significant energy gain for the cell. *B. longum* and *B. lactis* seem to have both an invertase (*cscA*) and a sucrose phosphorylase (*scrP/spl*) (Figure 4.8). Taken together with the absence of sucrose PTS genes in

the *B. longum* genome, the idea that PTS systems do not exist in heterofermentative lactic acid bacteria is supported, and may apply to *Bifidobacterium* in general.

The genetic organisation of the sucrose phosphorylases that have been identified is not clearly understood and has only been analysed in *Agrobacterium vitis* and *S. mutans*. In *S. mutans*, the sucrose phosphorylase (*gtfA*) is located in a cluster of multiple sugar metabolism genes and is thought to be involved in the utilisation of raffinose (238). The *gtfA* is regulated by the product of the *msmR* gene, which is thought to be a DNA-binding protein that functions as a positive effector. In *A. vitis*, the sucrose phosphorylase gene is situated on the Ti (tumour-inducing) plasmid upstream of a *traR*-like sequence (79). TraR functions as a transcriptional activator of the *tra* genes, and is thought to be part of the same operon as the sucrose-phosphorylase. However, it has not been shown whether the sucrose phosphorylase is regulated by TraR. The sucrose phosphorylase is thought to be involved in the utilisation of agrocinopine A, an opine that contains a phosphorylated sugar moiety. Analysis of the *B. longum* genome revealed that the sucrose phosphorylase (*spl*) was also associated with genes which could encode for a membrane transporter protein and regulator as in *B. lactis*. Furthermore, the deduced protein sequences of these genes found identity to the *B. lactis* ScrP, ScrT and ScrR proteins respectively. The *scrP* and *scrT* genes, but not the *scrR* gene, are similarly organised in the two bifidobacteria. This genetic organisation of *scrP* with *scrR* and *scrT* is, so far, unique to these bifidobacteria. Furthermore, *Bifidobacterium* is the first genus where the *scrR* is not associated with PTS genes.

The molecular analysis of the *B. lactis scr* gene cluster and the physiological analysis of *B. lactis* sucrase activity performed in chapter 2, have given insight into how sucrose is metabolised and how this metabolism may be regulated. This primary characterisation provided the background from which an analysis of the genetic regulation of sucrose metabolism could be conducted. The transcriptional regulation of the *B. lactis scr* gene cluster and the role of the ScrR in the regulation of these genes was investigated, and the work is reported in the following chapter.

CHAPTER 5

TRANSCRIPTIONAL AND REGULATORY ANALYSIS OF THE *SCR* GENE CLUSTER

Contents

5.1. SUMMARY

RNA analysis indicated that the *scrP, scrR* and *scrT* genes were regulated at the transcriptional level, and that their expression was induced by the presence of sucrose and raffinose, and was repressed by glucose. *scrR*-deletion constructs in *E. coli* revealed that ScrP activity was only produced if the *scrR* gene was present. Deletion of the *scrR* gene abolished ScrP activity, suggesting that the ScrR protein functions as a positive regulator. Analysis of the divergent *scrP-scrR* promoter region revealed the presence of three inverted repeats which showed sequence identity to operator sequences of the GalR-LacI family proteins, and could, therefore, function as ScrR binding sites. Furthermore these inverted repeat sequences also showed identity to the CcpA-CRE consensus sequence suggesting that the *scrP* and *scrR* genes might also be regulated by the catabolite control protein CcpA. The *scrP* and *scrR* transcriptional start sites were identified by primer extension analyses and were found to be adjacent to two of the palindromic sequences identified. These two sequences could, therefore, be binding sites for a transcriptional repressor, such as CcpA. The third palindromic sequence was found adjacent to the *scrP* -35 region, and could, therefore, function as a binding site for a positive regulator protein, such as ScrP. Gel shift assays were performed to determine the role of the ScrR protein in DNA-binding. Purification of ScrR-His fusion protein did not yield a soluble product, therefore, the gel shifts were performed using *B. lactis* crude extract from sucrose- or glucose-grown cells, and the *scrP-scrR* promoter DNA fragment. A shift was observed with both extracts, which was inhibited when unlabelled promoter DNA was added as competition. This could suggest that ScrR was bound to the promoter fragment when extract from sucrose-grown cells was used, and that another regulatory protein was bound to the promoter fragment when extract from glucose-grown cells was used.

5.2. INTRODUCTION

The expression of sucrose catabolic genes has in most cases been shown to be regulated at the transcriptional level. Transcription is generally negatively controlled by a sucrose regulator. This has been demonstrated for *Salmonella typhimurium* (127), *Klebsiella pneumoniae* (127), *Vibrio alginolyticus* (22), *Staphylococcus xylosus* (89), *Lactococcus lactis* (159), and *Clostridium beijerinckii* (222). Furthermore, the sucrase genes of these systems are all under glucose regulation as well.

In the systems mentioned above, the sucrose regulator belongs to the GalR-LacI family of regulators, and contains a DNA binding helix-turn-helix domain on the N-terminus. These proteins bind to palindromic operator sequences, thereby blocking transcription (328). In the presence of inducer, usually sucrose or sucrose derivatives, the regulator is no longer able to bind, and transcription continues. The *B. lactis scrR* was shown to have sequence identity to GalR-LacI-like sucrose regulatory proteins (chapter 4). Furthermore, as with *L. lactis* (221), *S. mutans* (256), *Lactobacillus plantarum* (190) and *Pediococcus pentosaceus* (151), the *B. lactis scrR* gene is clustered with sucrose catabolic genes, suggesting an involvement in their regulation.

Evidence has already been presented in preceding chapters indicating that sucrase activity in *B. lactis* is induced by sucrose and repressed by glucose. The main objective of the work described here was, therefore, to determine how this regulation was facilitated. For this, it was necessary firstly, to identify whether the *scrP*, *scrR* and *scrT* genes were transcriptionally regulated, and secondly, to establish whether the *scrP* and *scrT* genes were transcriptionally coupled. These aspects were investigated by northern blot analysis. Thirdly, it was necessary to determine the regulatory role of the *scrR* gene product, and this was analysed in *E. coli* with *scrR* deletion constructs. ScrR was also investigated for its ability to bind to the *scrP-scrR* promoter region by gel binding assays.

5.3. MATERIALS AND METHODS

5.3.1. Bacterial Strains, Plasmids and Culture Conditions

Bifidobacterium lactis was propagated anaerobically in BYG medium as described in section 2.3.1. Where applicable, 1% (w/v) of the carbohydrate was substituted for glucose.

Corynebacterium glutamicum 13032 was propagated aerobically in LM media (appendix B.2.) containing 50 μg/ml nalidixic acid at 30°C with vigorous shaking. Where indicated, 5 μg/ml chloramphenicol, or 50 μg/ml kanamycin was added to media.

E. coli JM109 was cultured as described in section 2.3.1. Where indicated, M9 minimal medium (176) was supplemented with Ap (100 μg/ml) and 1% (w/v) sucrose or glucose. Growth curves were performed by inoculating from an overnight LB culture of *E. coli* transformed with the relevant plasmid into minimal medium (10^{-2} dilution) and monitoring growth by measuring A_{600} at various time intervals. *E. coli* cells transformed with *C. glutamicum* plasmid DNA were plated onto Luria agar (253) containing 5 μg/ml chloramphenicol or 50 μg/ml kanamycin.

Plasmids used for subcloning and sequencing are listed in section 3.3.1. In addition, the *C. glutamicum* plasmids pEKplCm (Figure 5.1), pGS and pΔGS2 were also used in this study. The construct pGS has the *C. glutamicum* glutamine synthetase promoter cloned into the pEKplCm multiple cloning site, and confers CAT activity on *C. glutamicum* (272). pΔGS2 was constructed by digesting pGS plasmid DNA with *Xho*I and *Mlu*I restriction enzymes (Figure 5.1), and the resulting 5.8 kb fragment ends were blunted and re-ligated.

5.3.2. General DNA Manipulations

Extraction of plasmid DNA from *E. coli*, preparation of *E. coli* JM109 cells, DNA ligations, gel electrophoresis and other general manipulations are described in section 2.3.3.

5.3.3. Extraction of Plasmid DNA from *C. glutamicum*

pGS and pΔGS2 plasmid DNA was extracted from *C. glutamicum* as follows. Single colonies were inoculated into 10 ml LM medium containing 0.5% glycine and incubated O/N. Cells were harvested, resuspended in 200 μl ice-cold solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8, 15 mg/ml lysozyme) and incubated for 1 hour at 37 °C. Freshly prepared solution 2 (400 μl) (0.2 N NaOH, 1% (w/v) SDS) was added and tubes were inverted gently to mix and

incubated on ice for no longer than 5 minutes. Ice-cold potassium acetate solution (300 µl) was added to the cell suspension and incubated for 5 min on ice. Potassium acetate solution was prepared as follows: 11.5 ml glacial acetic acid and 28.5 ml H_2O was added to 60 ml 5 M K-acetate. The cell suspension was microfuged, and 0.9 ml of the supernatant was transferred to a fresh tube. DNA was extracted with an equal volume of phenol/chloroform (1:1), followed by an extraction with chloroform/isoamyl alcohol (24:1). Plasmid DNA was precipitated according to standard procedures (253), and was resuspended in 20 µl buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 1 mg/ml RNAse A). For plasmid digestions, 5-10 µl DNA was used.

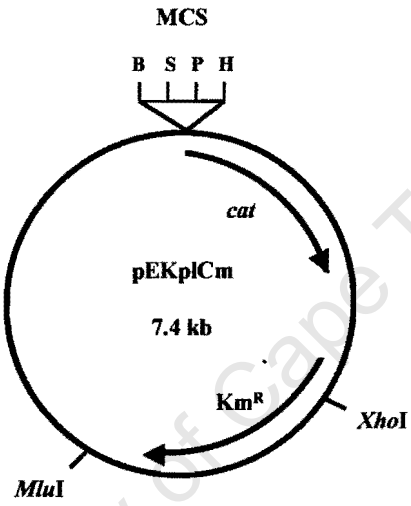


Figure 5.1. Partial restriction enzyme map of the *Corynebacterium glutamicum* promoter probe plasmid vector pEKplCm. For full pEKplCm details please see reference 72. Abbreviations: MCS, multiple cloning site; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I. Not drawn to scale. (Adapted from reference 72).

5.3.4. Extraction of Total RNA

Total RNA was isolated from mid-logarithmic phase cultures of *B. lactis* and *E. coli* as described in section 3.3.11.

5.3.5. Northern and RNA Slot Blot Analysis

Northern hybridisation and RNA slot blot analysis were performed as described in section 3.3.12. As internal controls, a 16S rDNA probe and a β-lactamase probe were used for the *B. lactis* and *E. coli* studies respectively. The gene specific fragments used as probes were: 1.4 kb *Hin*dIII-*Bam*HI (*scrP*), 0.45 kb *Pvu*I-*Pvu*I (*scrR*), 0.85 kb *Pvu*I-*Pvu*I (*scrT*), and were prepared from pSuc1 plasmid DNA. The 0.45 kb *Xmn*I-*Bgl*I (β-lactamase) fragment was prepared from pEcoR251 plasmid DNA. Preparation of the *B. lactis* 16S rDNA probe is described in section

2.3.2. Signal intensities were measured using a MacBeth® Transmission densitometer TD 109. Results were expressed as a ratio of the gene specific signal to the internal control signal.

5.3.6. Primer Extension Analyses

Primer extension was carried out as described (206) with some modifications. RNA (100-120 µg) was precipitated and the pellet dissolved in 100 µl HP buffer (40 mM PIPES pH 6.4, 1 mM EDTA pH 8.0, 400 mM NaCl, 80% deionised formamide). Fluorescently labelled Cy^5 primer (4 pmol) was added and incubated for 10 min at 95°C to denature, and then hybridised overnight at the required temperature for each gene primer. RNA hybridised to primer was precipitated with 300 µl of ice-cold water and 800 µl ethanol, incubated at –20°C for 30 min and then centrifuged (14 000 *g*, 12 min, 4°C). The pellet was dried, dissolved in 20 µl RTB buffer (4 µl AMV reverse transcriptase buffer, 1 µl 10 mM dNTP mix, 1 µl 40 U RNasin, 2 µl 1 mg/ml actinomycin D, 16 µl water), and incubated for 2 min at 42°C. Two microliters (20 U) of AMV reverse transcriptase (Promega) was added and the reaction was incubated for 2 hours at 42°C. The reaction was stopped with 1 µl of 0.5 M EDTA (pH 8.0). RNA was digested by incubation for 30 min at 37°C with 1 µl RNAse (10 mg/ml). After digestion, 150 µl TES buffer (10 mM Tris-HCl pH 7.6, 1mM EDTA, 100 mM NaCl) and 500 µl of cold ethanol was added, and the cDNA was precipitated. The pellet was dissolved in 5 µl TE (10 mM Tris, 1 mM EDTA, pH 7.6). Five microliters of STOP buffer was added and the solution heated for 5 min at 95°C, and was then sequenced. The gene-specific primers used were: Primer Sp (*scrP*) 5'-GCA GAA TGT CGG TCA TCG AAG C- 3'), Primer Sr (*scrR*) 5'-CGA GAC ACT GCT CAC CGA GAC G- 3'), and Primer St (*scrT*) 5' -CGG AAA CGG TGA AGC CGA T- 3').

5.3.7. PCR Amplification of the *scrP-scrT* Intergenic Region

The PCR reaction was as follows (50 µl): 5 µl buffer (supplied with the enzyme); 2.5 mM $MgCl_2$; 0.5 µM of each primer; 200 µM of each dNTP; 50 ng DNA; and 1 U of *Taq* DNA polymerase (Supertherm). The amplification program consisted of one cycle of 96°C for 5 min, then 25 cycles of 96°C for 1 min, 60.5°C for 45 s, and 72°C for 1 min, and finally one cycle of 72°C for 5 min. The reaction was cooled down to 4°C. Primers A (5' –CTGCTGGAGTAACGCT TGG- 3') and B (5' -GCGCTCGTAGTACAACATCG- 3') were used (Figure 4.3.).

5.3.8. Antimicrobial Susceptibility Tests

Minimum inhibitory concentrations (MIC) were determined by a two-fold macrodilution broth method (138). A standard inoculum of an overnight BYG *B. lactis* culture was added to the 2-fold

dilution series of BYG medium containing the relevant antibiotics. This was incubated at 37°C and visually examined for turbidity after 48 hours. The lowest concentration of antimicrobial agent that resulted in complete inhibition of visible growth represented the MIC. The following antibiotic concentration ranges were tested: ampicillin 0.8-50 μg/ml, erythromycin 0.15-5 μg/ml, chloramphenicol 0.25-15 μg/ml.

5.3.9. *C. glutamicum* Electroporation

Electrocompetent *C. glutamicum* 13032 cells were prepared as follows. A 10 ml LM overnight culture was added to 400 ml LM containing 0.5% glycine and nalidixic acid and incubated until an OD_{578} 0.4 was reached. Cells were chilled on ice for 10 min, and then harvested (10 000 *g*, 10 min, 4°C). Cells were resuspended in 100 ml ice-cold buffer (10% (w/v) glycerol, 8 mM Tris-HCl, pH 7.4) and were harvested as before. The washing was repeated twice more, and cells were resuspended in 0.4 ml ice-cold 10% (w/v) glycerol. Cells were aliquoted (100 μl) into pre-cooled Eppendorf tubes and stored at -70°C. *C. glutamicum* cells were electroporated as follows. Cells were thawed on ice, washed twice with 20 volumes of ice-cold 10% (w/v) glycerol, and resuspended in 98 μl 10% glycerol. Plasmid DNA (500 ng in 2 μl dH_2O) was added to cells, mixed and dispensed into cold electroporation cuvettes (Bio-Rad, 2 mm electrode gap). A high voltage electric pulse was delivered with a Gene Pulser apparatus (Bio-Rad) by using the 25 μF capacitor, applying a voltage of 2.5 kV at a resistance of 400 Ω. One ml of BHIS medium (37 g/L BHI (Difco), 0.5 M sucrose) was added to cells and incubated at 30°C for 1-2 hours. Cells were plated onto LM medium containing 0.5 M sucrose, nalidixic acid, and chloramphenicol.

5.3.10. Overexpression of ScrR in *E. coli*

The *B. lactis scrR* gene was amplified using the ScrRF (5'-GGA TCC ACA TGG TCG GCA TGC GCG ATG TCG- 3') and ScrRR (5'-AAG CTT TCG TCA TGT TGG CAT CGC GTC C-3') primers, introducing the *Bam*HI and *Hin*dIII restriction sites (underlined) respectively, in the following PCR reaction (50 μl): 5 μl buffer (supplied with the enzyme); 2.5 mM $MgCl_2$; 0.5 μM of each primer; 200 μM of each dNTP; 50 ng chromosomal DNA; and 1 U of High Fidelity *Taq* DNA polymerase (Roche). The amplification program consisted of one cycle of 96°C for 5 min, then 25 cycles of 96°C for 1 min, 60°C for 45 s, and 72°C for 1 min, and finally one cycle of 72°C for 5 min. The reaction was cooled down to 4°C. The 0.9 kb DNA fragment ends were blunted using T4 DNA Polymerase, were purified using the PCR Product Purification Kit (Roche), and were ligated into pEcoR251 plasmid vector and used to transform *E. coli* JM109 cells. Plasmid DNA was isolated from all transformants and was digested with *Bam*HI and *Hin*dIII restriction enzymes. The 0.9 kb *scrR*-containing DNA fragments were purified and ligated into the *Bam*HI

and *Hin*dIII restriction sites of the expression vector pProEx HTc (Life Technologies) (see Figure A.4. in appendix for plasmid map), downstream of, and in frame with, the 6 histidine residues and the ATG translational start. A small scale induction of *E. coli* JM109 cells transformed with the resulting plasmids was performed and the proteins were analysed by SDS-PAGE as described by the supplier (Life technologies). One clone, pExScrR4, was sequenced to confirm the presence of the *scrR* gene and was selected for the purification of ScrR-His fusion protein.

For the production and purification of ScrR-His fusion protein *E. coli* (pExScrR4) was cultured in LB medium to an OD_{600} 0.5. Expression of the His_6-*scrR* fusion was induced by the addition of IPTG to a final concentration of 0.6 mM. After 2 hours, cells were harvested by centrifugation (6 000 *g*, 5 min, 4°C), resuspended in 2 ml buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 10 mg/ml lysozyme, pH 8.0) and incubated for 30 min, 4°C. The suspension was sonicated (4°C) at 95 W for 3 min with 30 s cooling intervals (Virsonic Digital 475 Cell Disruptor), and then 10 ml of buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 8.0) was added. After a 1 h incubation at room temperature (RT) the suspension was centrifuged (10 000 *g*, 25 min, RT) to pellet the cellular debris, and the lysate was bound to the Ni-NTA resin. Purification steps were carried out as recommended by the supplier (Qiagen Inc.). The eluted protein was dialysed in a stepwise manner in ScrR buffer (20 mM Tris-HCl pH 7.5, 0.4 M NaCl, 0.1 mM EDTA) containing 10% (w/v) glycerol and decreasing concentrations of urea (2 M - 0 M), until all the urea had been dialysed out. Precipitation of the protein resulted if the urea was not dialysed gradually. Protein fractions were analysed by SDS-PAGE (145).

For the isolation of inclusion bodies, bacterial cells were resuspended in lysis buffer 2 (50 mM Tris-HCl pH 8, 5 mM β-mercaptoethanol, 10 mM EDTA, 0.5 % triton) and were incubated for 5 min at room temperature. Protein was centrifuged (14 000 *g*, 15 min, 4°C) and was resuspended in ScrR buffer.

5.3.11. Western Hybridisation

Proteins from SDS-PAGE gels were transferred onto nylon membrane as described previously (303). Hybridisation and detection of mouse Penta·His antibody (Qiagen) was performed according to the manufacturer's instructions.

5.3.12. MALDI-TOF Mass-Spectrometry

Purified ScrR-His protein was digested with trypsin as described previously (70). Molecular weights of the digested peptides were analysed by mass spectrometry using the PerSeptive

Biosystems Voyager-DE™ PRO Biospectrometry MALDI mass spectrometer using CHCA (α-cyano-4-hydroxycinnamic acid) matrix. Bovine carbonic anhydrase II was used as a standard control. Using the MS-Fit fingerprinting tool (16) the peptide digest sizes were used to perform homology searches against the NCBI database.

5.3.13. Processing of *B. lactis* Cells for Gel Shift Assays

B. lactis cells grown to mid-logarithmic stage in BY medium containing 1% (w/v) glucose or sucrose were harvested (6 000 *g*, 10 min, 4°C), and the cell pellet was washed and resuspended in buffer (20 mM Tris-HCl pH 7.5, 0.4 M NaCl, 0.1 mM EDTA). Cells were disrupted by sonication (95 W for 3 min with 30 s cooling intervals, 4°C), and the cell debris was removed by centrifugation (14 000 *g*, 25 min, 4°C). The supernatant containing protein was stored at -20°C.

5.3.14. Amplification of Promoter Fragments for Gel Shift Assays

Four *scrP-scrR* promoter fragments were constructed as described below and are referred to by their sizes, and were prepared as follows. The **450 bp** fragment was amplified by PCR (amplification details are described below) using primers Sp2 and Sr2 (Figure 5.7 C). Primers Sp and Sp2 are the same except that the latter primer has the *Bam*HI restriction site incorporated into the sequence. Similarly for primers Sr and Sr2, except that the restriction site is *Hin*dIII. After amplification, the 450 bp promoter fragment was purified and then digested with *Hin*dIII restriction enzyme to yield the **300 bp** and **120 bp** fragments. The **270 bp** fragment was amplified using the primers Sr2 and DIR (Figure 5.7 C). Purified fragments were non-radioactively 3'end-labelled with DIG-11-ddUTP using terminal transferase as per manufacturer's instructions (Roche). The PCR reactions were as follows (50 µl): 5 µl buffer (supplied with the enzyme); 2.5 mM $MgCl_2$; 0.5 µM of each primer; 200 µM of each dNTP; 50 ng chromosomal DNA; and 1 U of *Taq* DNA polymerase (Supertherm). The amplification program consisted of one cycle of 96°C for 5 min, then 25 cycles of 96°C for 1 min, 54°C for 45 s, and 72°C for 1 min, and finally one cycle of 72°C for 5 min. The reaction was cooled down to 4°C. Primers are shown in Figure 5.7 C. Primers: Sp2 5'-CGC GGA TCC GCA GAA TGT CGG TCA TCG AAG C-3', Sr2 5'-CCC AAG CTT CGA GAC ACT GCT CAC CGA GAC G- 3', DIR 5' –CCC AA GCT TGT TTT ACC TCA TTC TTCA CG- 3'. Underlined sequences indicate the *Bam*HI and *Hin*dIII restriction enzyme sequences incorporated to facilitate cloning of the PCR fragments.

5.3.15. Gel Shift Assays

Besides the protein and target DNA fragments, all assay components were supplied in the Bandshift Kit (Pharmacia, Cat. # 27-9100-01). A typical assay mix contained in 20 µl: 10 mM

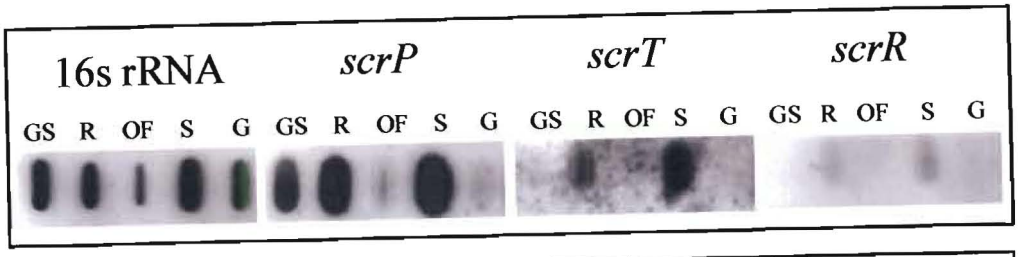
Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM DDT, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.05% NP-40, 1 μg poly (d(I-C)), 7.5 ng labelled DNA probe, 230 ng unlabelled DNA probe (cold target), 7 μg purified ScrR-His or 75 μg of *B. lactis* crude extract. Sucrose or glucose (final concentration of 1 mM) was added when indicated. After 20 min incubation at room temperature, samples were loaded onto an agarose gel (2%), prepared with low-ionic-strength buffer (7 mM Tris-HCl, pH 7.5, 3 mM NaAc, 1 mM EDTA), and were electrophoresed at room temperature in the same buffer with constant circulation. After electrophoresis, the DNA was transferred overnight to Hybond N^+ nylon membrane via the capillary transfer procedure (253) using the electrophoresis buffer for transfer. Detection of the DIG-labelled DNA was performed as described by the manufacturer (Roche).

5.4. RESULTS

5.4.1. Regulation of Expression of the *scrP, scrR* and *scrT* genes in *B. lactis*

The presence of *scrR*, specifying a GalR-LacI-type regulatory protein, suggested that the *scr* gene expression might be regulated at the transcriptional level. RNA slot blot analysis was, therefore, performed to investigate the transcriptional regulation of the *scrP, scrR* and *scrT* genes by growth in various carbohydrates. *B. lactis* RNA extracted from cells grown on glucose, sucrose, glucose and sucrose, raffinose, or oligofructose was hybridised to probes specific for the *scrP, scrR* and *scrT* transcripts (Figure 5.2). RNA from *E. coli* cells with pSuc1 (positive control) and pMT104 (negative control) was included in the analysis. In comparison to glucose-grown cultures, the *scrP* mRNA levels were increased approximately 6-fold in the presence of sucrose or raffinose, and 1.25-fold in the presence of oligofructose. In the presence of glucose and sucrose, *scrP* expression was only increased approximately 4-fold. For *scrR* and *scrT*, mRNA signals were only present under inducing conditions, in the presence of sucrose or raffinose. Therefore, all three of the *scr* genes are repressed by glucose and are thus subject to catabolite repression.

A.



B.

	scrP	*scrT*	*scrR*
GS	0.55 (0.16)	ND	ND
R	0.83 (0.09)	0.14 (0.03)	0.16 (0.09)
OF	0.16 (0.08)	ND	ND
S	0.78 (0.02)	0.27 (0.007)	0.16 (0.04)
G	0.13 (0.07)	ND	ND

Figure 5.2. RNA slot blot analysis of the *scrP*, *scrR* and *scrT* mRNA from mid-logarithmic phase cells of *B. lactis* grown in different carbon sources. (A) Gene-specific mRNA and 16s rRNA signals detected on slot blot analysis. (B) mRNA levels expressed as a ratio of the gene specific hybridisation signal to the 16s rRNA hybridisation signal. Experiments were performed in duplicate and standard deviations are shown in parentheses. GS, glucose plus sucrose; R, raffinose; OF, oligofructose; S, sucrose; G, glucose; ND, no gene specific signal detected.

These findings also correlate with the observations made at the physiological level, where sucrase activity was induced by sucrose and raffinose, and repressed by glucose (chapter 2). However, sucrase activity was repressed 2-fold by glucose, whereas *scrP* mRNA production was repressed 6-fold. Furthermore, sucrase activity was the same for cells grown in glucose or in glucose plus sucrose, whereas *scrP* mRNA was significantly induced when grown in the presence of the two sugars. Since the sucrase assays were performed on total protein, other enzymes such as invertases, fructosyltransferases, and fructanases could have contributed to the activity. Assuming that this was the case, it could be possible that more than one sucrose catabolite system exists in *B. lactis*: (i) constitutively expressed systems which are responsible for the basal sucrase activity seen in the presence of glucose, and (ii) systems which are induced by sucrose and raffinose and repressed by glucose, as seen with *scrP*. As was discussed in chapter 4, analysis of the *B. longum* genome revealed the presence of a β-fructofuranosidase (*cscA*), suggesting sucrase or invertase activity. Furthermore, this gene has been cloned from *B. lactis* (71). However, the genetic organisation of the *B. longum cscA* gene with a GalR-LacI-type regulator, suggests that its activity would be regulated, and therefore, would not contribute to the constitutive activity in the presence of glucose that has been suggested.

mRNA transcripts were detected for all three genes in *E. coli* cells harbouring the plasmid pSuc1, but were absent from cells with pMT104. The *scrP* and *scrT* genes are inversely orientated to the λ promoter on the plasmid pEcoR251 (Figure 5.4). It would follow therefore, that they are expressed from a *B. lactis* sequence recognised in *E. coli*. The *scrR* gene could however, be expressed from a *B. lactis* promoter or the λ promoter.

5.4.2. Investigation of *scrP-scrT* Co-transcription

The possibility that the *scrP* and the *scrT* genes were transcribed as an operon was suggested by the fact that the *scrT* gene was clustered with, and present in, the same orientation as the *scrP* gene, and that the two genes were similarly regulated at the transcriptional level (Figure 5.2). Furthermore, *scrT* promoter sequences could not be identified in the intergenic region between the two genes. Three strategies were employed to determine whether these genes were transcriptionally coupled. These were northern hybridisation, *scrT* primer extension analysis, and reverse transcriptase PCR.

5.4.2.1. Northern Blot Analysis

B. lactis RNA extracted from cells grown on sucrose or glucose plus sucrose was hybridised to probes specific for the *scrP* and *scrT* genes (Figure 5.3 B). If these two genes were transcriptionally coupled, a transcript size of 3290 bp (from the transcription start and termination codon of the *scrP* and *scrT* genes respectively) would be expected. A distinct band could not be identified from *B. lactis* RNA with either of the gene probes. Rather, a smear starting at 3.3 kb was detected, probably representing *scrP* or *scrT* mRNA co-migrating with high concentrations of ribosomal and degraded RNA. Two regions where signal was not detected presumably represent 23S and 16S ribosomal RNA shielding due to high concentrations. Although it cannot be excluded that the smears represent non-specific hybridisation, the difference in signal intensities obtained between sucrose- versus glucose plus sucrose-grown samples suggests that hybridisation was gene-specific. Relatively good quality RNA was achieved (Figure 5.3 A) and therefore, the inability to obtain a distinct mRNA signal cannot be accounted for. Variations in RNA and probe concentrations, and RNA transfer times did not improve the hybridisation signals.

Hybridisation of RNA from *E. coli* cells with pSuc3 to the *scrT* probe resulted in several distinct bands with sizes of 3.3 kb, 1.8 kb, 1.4 kb, and 0.9 kb (Figure 5.3 C). This suggested that, at least in *E. coli*, the *scrP* and *scrT* genes were co-transcribed as an operon. The other bands detected for *scrT* could represent processed derivatives of the *scrP-scrT* transcript, or could represent other genes with homology to *scrT*. The 1.4 kb band detected could represent another *scrT* transcript,

transcribed from its own or another promoter-like sequence, since the first putative *scrT* initiation codon is separated by 1318 bp from the termination codon (Figure 4.3.). Although promoter sequences were not identified for *scrT*, there may be spurious sequences which are recognised and used by *E. coli*.

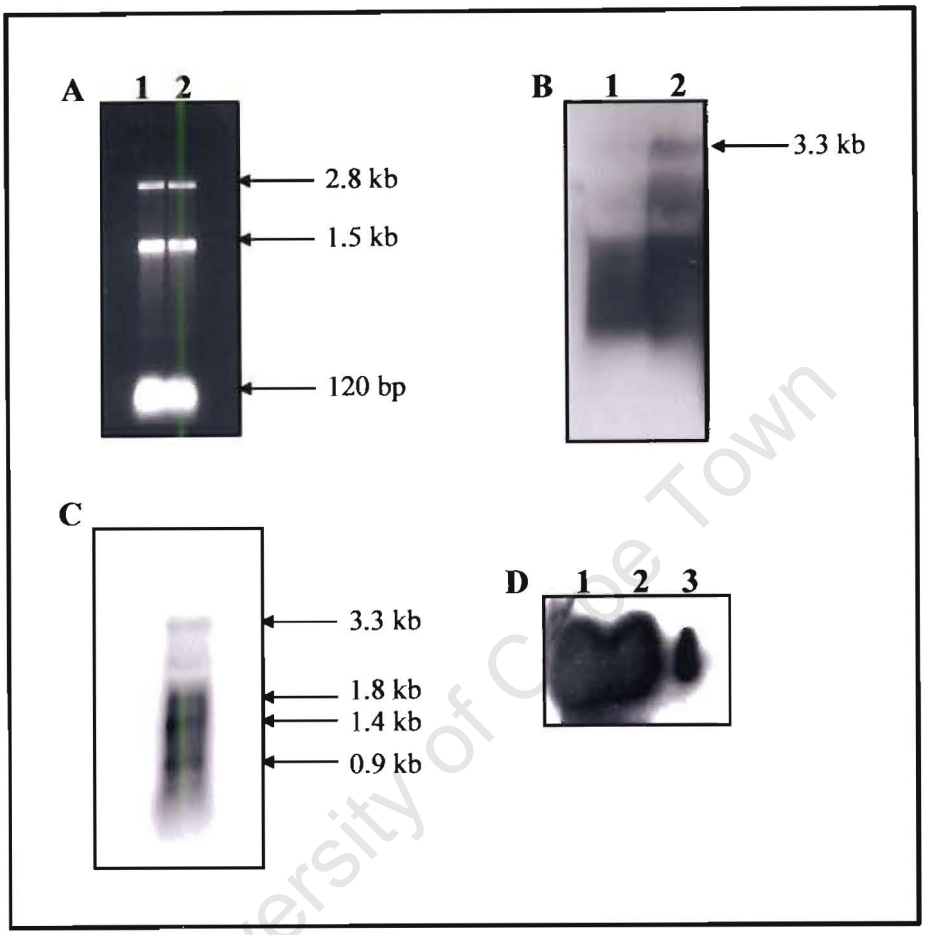


Figure 5.3. RNA analysis of the *scrP* and *scrT* genes. A: RNA extracted from *B. lactis* cells grown on glucose plus sucrose (lane 1) and sucrose (lane 2), electrophoresed on a formaldehyde gel (253). B: Northern blot conducted with the *scrP* specific probe hybridised to *B. lactis* RNA from cells grown on glucose plus sucrose (lane 1) and sucrose (lane 2). C: Northern blot conducted with the *scrT* specific probe hybridised to RNA from *E. coli* cells with the construct pSuc3. D: RNA slot blot conducted with the *scrT* specific probe hybridised to RNA from *E. coli* cells with the constructs pSuc1 (lane 1), pΔReg2 (lane 2), or pSuc2 (lane 3).

The construct pSuc2 does not confer sucrase activity on *E. coli* cells since the *scrP* promoter and ATG codon is truncated. Therefore, if *scrT* transcripts could be detected from this clone, it would confirm the *scrP*-independent transcription of *scrT* that was suggested from the Northern blot results. RNA slot blots were performed with RNA from *E. coli* cells with pSuc1, pΔReg2 and pSuc2 and RNA was hybridised to the *scrT* probe (Figure 5.3 D). *scrT* transcripts could be

detected from cells with pSuc2, but the reduced level compared to that obtained from cells with pSuc1 and pΔReg2, suggest that it is not the major *scrT* transcript produced in *E. coli*, further supporting the co-transcription of *scrT* with *scrP*.

5.4.2.2. Primer Extension Analysis of scrT

Primer extension analyses were undertaken to investigate whether transcription from a *scrT* promoter occurred in *B. lactis* as was seen in *E. coli.* No transcription start signals could be detected. This is further evidence to support the co-transcription of the *scrP* and *scrT* genes, and that the *scrT* transcript identified in *E. coli* must be due to the recognition of spurious promoter sequences in the intergenic region between the two genes.

5.4.2.3. Reverse Transcriptase PCR (RT-PCR)

Since co-transcription of the *scrP* and *scrT* genes could not be shown in *B. lactis* by Northern hybridisation, analysis by RT-PCR was attempted. Primers A and B (Figure 4.3) designed to sequence within the *scrP* and *scrT* genes respectively, would only give a RT-PCR product of 782 bp if the two genes were co-transcribed. As a positive control, primers B and C2 (Figure 4.3) specific for *scrT* were designed which would yield a 290 bp product. PCR performed with primers A and B and pSuc1 plasmid DNA resulted in a 680 bp fragment. Since this was smaller than expected, the fragment was cloned and sequence-analysed. The sequence indicated that a different region of the pSuc1 insert had been amplified. The PCR conditions were varied but did not result in the amplification of the correct DNA fragment, therefore due to the non-specificity of the primers, the RT-PCR could not be performed.

5.4.3. Regulation of *scrP* by *scrR* in *E. coli*

The majority of GalR-LacI proteins function as negative regulators (328). Certainly, all the ScrR proteins to which the *B. lactis* ScrR found sequence identity to are reported to function in this way (Table 4.2). Transcriptional analyses of *scrR* showed that mRNA production was induced in the presence of sucrose and raffinose, and could not be detected in the presence of glucose (5.4.1). This would be typical of a positive regulator. However, the possibility of it being a negative regulator could not be excluded.

Targeted disruption of the *scrR* gene in *B. lactis* would be the ideal route to investigate the role of ScrR in transcriptional regulation. However, due to the limited molecular genetic tools available for *Bifidobacterium*, the development of cloning vectors and transformation techniques has not yet progressed sufficiently for such genetic manipulations to be performed. The analysis was,

therefore, conducted using deletion constructs in *E. coli* JM109 by measuring sucrase activity produced by the cells transformed with each of the constructs. The constructs designed are illustrated in Figure 5.4 and are described in the legend. They are as follows: pSuc3, containing the *scrP, scrR,* and *scrT* genes; pSuc1, which has 195 of 438 amino acids of the *scrT* gene truncated; pΔST8, which has the *scrT* gene deleted; pΔReg2, which has 245 of 326 amino acids of the *scrR* gene truncated; and pΔSTR1, which has the *scrR* gene truncated and the *scrT* gene deleted.

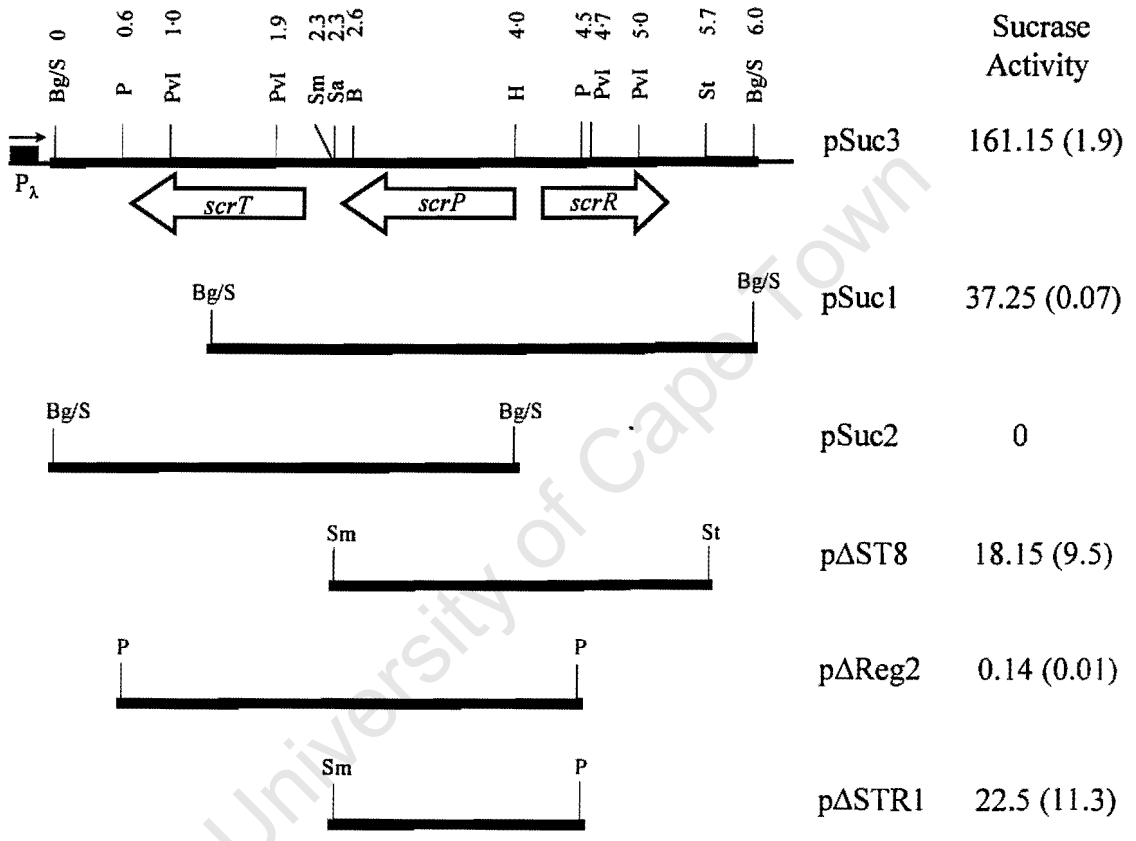


Figure 5.4. Analysis of sucrase activity produced in *E. coli* JM109 cells containing *scr* deletion constructs grown in LB. The plasmids pSuc1 and pSuc2 originate from the *B. lactis* genebank, and were used to construct plasmid pSuc3 (see chapter 4 for details). From pSuc3, the 3.4 kb *Sma*I-*Stu*I and 3.9 kb *Pst*I fragments were cloned into the *Bgl*II site of pEcoR251 resulting in plasmids pΔST8 and pΔReg2 respectively, which have the *scrT* gene and 245 amino acids of the *scrR* gene deleted respectively. The 2.2 kb *Sma*I-*Pst*I fragment from pSuc3 was cloned into pEcoR251 resulting in plasmid pΔSTR1, which has the *scrT* gene deleted and the *scrR* gene truncated. Sucrase activity conferred on *E. coli* by the constructs was measured in cell extracts and expressed as μmol reducing sugar/min/mg protein. Assays were performed in duplicate and standard deviations are shown in parentheses. Cells used for the assays were also analysed for the presence of plasmid (Table 5.1). The filled in box and arrow on pSuc3 indicate the position and direction of the λ promoter (P_λ) present on the pEcoR251 vector. Sizes are indicated in kb. B, *Bam*HI; Bg/S, *Bgl*II/*Sau*3A; H, *Hin*dIII; P, *Pst*I; PvI, *Pvu*I; Sa, *Sal*I; Sm, *Sma*I; St, *Stu*I.

The analyses were initially performed in minimal medium containing glucose or sucrose. Growth curves were performed so that suitable sampling time points could be determined (Figure 5.5). RNA extracted from mid-logarithmic (OD_{600} 0.7) *E. coli* cells harbouring the various deletion constructs, was hybridised to probes specific for the *scrR, scrP, scrT* and β-lactamase genes. Probing for the β-lactamase gene served as an indication of the presence of the plasmid in each strain. It became apparent that, although cells were cultured in ampicillin and reached OD_{600} values of 1.4, transcription of the β-lactamase gene could not be detected for some of the samples (Figure 5.6), suggesting that the cells did not contain plasmid. To confirm this, plasmid-containing cells were cultured in glucose and sucrose minimal medium broth until mid-logarithmic growth was attained, and were then plated on Luria agar with and without ampicillin so that the percentage cells containing plasmid could be numerically determined (Table 5.1). It was found that after the growth in minimal media, cells which should have contained the constructs pSuc3 and pΔSTR1, were plasmid-less, confirming what was seen in the RNA blots.

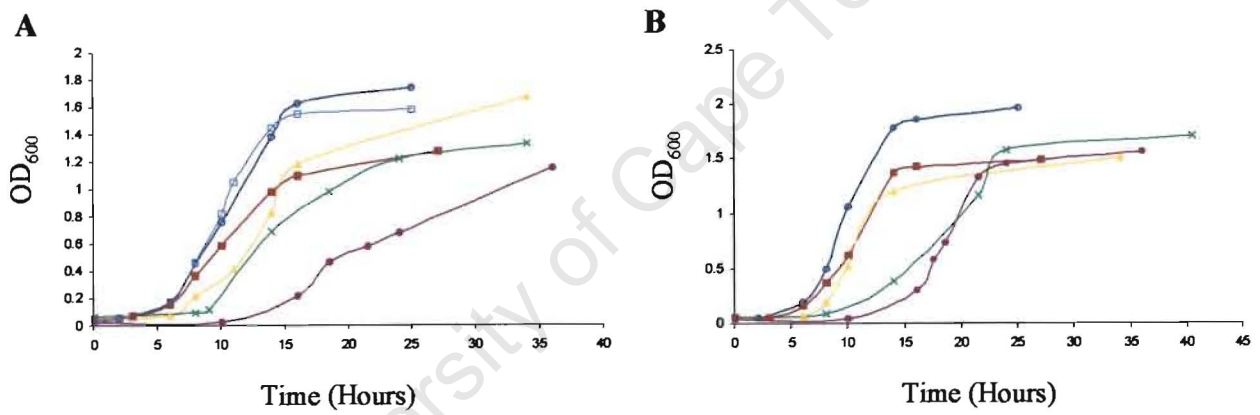


Figure 5.5. Growth curves of *E. coli* with the *scr* deletion constructs grown in minimal medium containing (A) glucose or (B) sucrose. Growth was monitored by measuring absorbance at 600 nm (OD_{600}) at the times indicated. Symbols: (○) pSuc3; (■) pSuc1; (▲) pΔST8; (x) pΔReg2; (●) pΔSTR1; (□) pMT104.

Growth periods of 15-25 hours were required for the cells to reach mid-logarithmic growth in minimal medium (Figure 5.5). In order to reduce the growth period and thereby reduce plasmid loss/cell death, cells with the various constructs were cultured in LB until an OD_{600} 0.5 was reached. The percentage of cells containing plasmid was determined as before (Table 5.1). The different constructs were found to be more stable in LB, with the majority of strains containing plasmid, however, this percentage varied each time it was performed, and is reflected by the standard deviations calculated for the two experiments (Table 5.1). It is interesting that growth in minimal medium containing sucrose reached OD_{600} levels as high as 1.4 in the absence of the sucrose-utilising genes after plasmid loss (Figure 5.5). However, the fact that this also occurred in

minimal medium containing glucose, and that the percentage of cells which did contain plasmid varied considerably in LB, suggests that the system is not a stable one, and that, therefore, the results in minimal medium should be treated with reserve.

Table 5.1. Analysis of plasmid levels and sucrase activity in *E. coli* JM109 cells with the *scr* deletion constructs.

Construct	Medium	% Cells containing plasmid (B)	Sucrase Activity (A)	A / B
pSuc3	MM + G	0.0077		
	MM + S	0.0001		
	LB	100 (0)	161.15 (1.9)	1.61
pSuc1	MM + G	92		
	MM + S	100		
	LB	74.6 (14.8)	37.25 (0.07)	0.50
pΔST8	MM + G	74		
	MM + S	1.5		
	LB	54.9 (22.9)	18.15 (9.5)	0.33
pΔReg2	MM + G	100		
	MM + S	100		
	LB	97.35 (3.75)	0.14 (0.01)	0.0014
pΔSTR1	MM + G	0.00074		
	MM + S	0.0056		
	LB	81.6 (26.0)	22.5 (11.3)	0.28
pMT104	MM + G	100		

Experiments in LB were conducted in duplicate and the standard deviation is shown in parentheses.
(A): Sucrase assays were performed on the same cells that were used to analyse plasmid (B) (Figure 5.4).
(A/B): Ratio of sucrase activity (A) vs. % of cells containing plasmid (B).

Sucrase assays were performed, and due to the plasmid variability, the same cell samples that were analysed for plasmid described above were used for the assays. Sucrase activity was highest with pSuc3 (Figure 5.4), Table 5.1), where all three genes are present. Activity was reduced 1000-fold with pΔReg2, where the *scrR* gene is truncated, and was reduced 4-, 8- and 7-fold with pSuc1

(*scrT* truncated), pΔST8 (*scrT* deleted) and pΔSTR1 (*scrT* deleted and *scrR* truncated) respectively. In Table 5.1 the sucrase activity obtained was normalised to the % number of cells containing plasmid. This calculation was to determine if the reduced activity obtained by cells with pSuc1, pΔST8 and pΔSTR1 was only due to the fact that fewer cells contained plasmid. This ratio reflected the same trend as the sucrase assay results did. Comparing the activity between cells with pSuc3 and pΔReg2, it appears that ScrR functions as a positive regulator, even in the absence of sucrose. The reduced activity in cells with pSuc1 and pΔST8, where the *scrR* gene is present, suggests that the *scrT* gene product contributes to the expression of *scrP*, also in the absence of sucrose. Cells with pΔSTR1 and pΔST8 gave similar activity regardless of whether *scrR* was present or not, confirming the requirement of *scrT* for activity. Furthermore, cells with pSuc1, where part of the *scrT* gene is present, displayed a 2-fold increase in sucrase activity compared to cells with pΔST8 or pΔSTR1 which have the *scrT* gene completely deleted. This could suggest that the truncated *scrT* produces a partially functional protein which is able to contribute slightly to the activity.

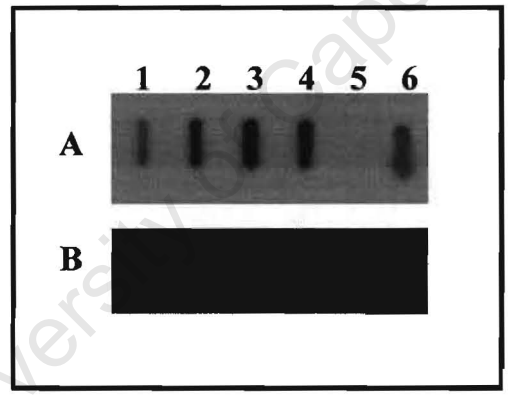


Figure 5.6. RNA slot blot analysis of β-lactamase transcription in *E. coli* cells containing the *scr* deletion constructs grown in minimal medium containing (A) sucrose or (B) glucose. Lane 1: pSuc3, Lane 2: pSuc1, Lane 3: pΔST8, Lane 4: pΔReg2, Lane 5: pΔSTR1, Lane 6: pMT104.

Some proteins have been shown to engage in multiple functions. In particular, certain transporter proteins possess regulatory components. In *Saccharomyces cerevisiae* the *SNF3* gene, required for high-affinity glucose transport, belongs to the family of sugar transporters to which the *B. lactis scrT* gene product found identity (20, 140). A number of observations, however, suggest that SNF3 might not be a functional transporter, but rather acts by regulating the expression or activity of other hexose transporter proteins. The *E. coli* MalK maltose transporter has been shown to interact with the positive regulator MalT, thereby abolishing MalT-dependent transcription (25, 26). The *E. coli* K-12 *bglS* gene specifying a transport protein also functions as a negative

regulator of the *bgl* operon involved in the catabolism of aromatic β-glucosides (164). Studies on the *put* operon of *S. typhimurium* involved in the catabolism of proline have indicated that the *putA* gene product is both a negative regulator of the *put* operon expression, and a membrane-bound bifunctional oxidase-dehydrogenase (165). The *glnA* gene product of *B. subtilis*, which codes for the enzyme glutamine synthetase, has been implicated in the autoregulation of the *glnA* gene (270). Hence, the presence of proteins with dual functions may be a more common feature among prokaryotes than is currently understood. It is, therefore, possible that the *B. lactis scrT* gene product might similarly have a regulatory component in addition to its transport function.

Alternatively, the perceived regulatory role of *scrT* could be a result of counter-transcription from the lambda promoter (P_λ) present on the pEcoR251 vector (Figure 5.4). Two possible terminators were identified 22 and 73 bp from the *scrT* stop codon (see section 4.4.4.3). With construct pSuc1, the absence of these terminators could possibly allow counter-transcription to run into *scrP*, thereby reducing its transcription. Similarly with constructs pΔST8 and pΔSTR1, by moving *scrP* closer to P_λ the negative effect is enhanced.

Interestingly, cells with pSuc3 were unable to grow in LB containing sucrose. Initially, this together with the cell death/loss of plasmid discussed above, gave the impression that the overexpression of *scrP* might be lethal, as has been seen with the levansucrase (*sacB*) gene from *Bacillus subtilis* (88). However, the plasmid pΔSTR1 had more of an effect than pSuc3, even though sucrase activity was reduced 7-fold in cells with pΔSTR1. Alternatively, if sucrose was not hydrolysed efficiently by cells with pSuc3, the accumulation of sucrose in the cells could have caused cell lysis. This has also been reported for *E. coli rafD* mutants (see section 1.7.1.) (12). Nevertheless, it is possible to conclude that sucrose has a detrimental effect only when the full *scrT* gene is present, since cells containing pSuc1, where the *scrT* gene is truncated (Figure 5.4), were culturable in LB containing sucrose, and they displayed the same activity in the presence or absence of sucrose. This could possibly indicate that the *scrT* gene product is involved in the transport of sucrose in *E. coli*. Furthermore, cells with pΔReg2 displayed an 8-fold increase in sucrase activity when cultured in minimal medium containing sucrose (1.37 μmol/min/mg protein) compared to in glucose (0.17 μmol/min/mg protein), clearly indicating that *scrP* is induced by sucrose, but that the major induction occurs in the presence of *scrR*.. Due to the heterologous nature of this study and the numerous interactions possible, it is difficult to analyse exactly how these genes function and interact, and it is, therefore, impossible to understand every aspect of this system.

5.4.4. Analysis of the Divergent *scrP-scrR* Promoter Region

An intergenic region of 320 bp between the ATG codons of the divergent *scrP* and *scrR* genes (Figure 5.7) was analysed for promoter and regulatory sequences, and primer extension experiments were performed to identify the transcriptional start for the two genes. Such an analysis could give some insight as to how these genes might be regulated.

Primer extension analyses were performed on RNA extracted from *B. lactis* cells grown in raffinose or glucose plus sucrose using *scrP*- and *scrR*-specific primers (Figure 5.7). For *scrP*, a transcription start site was assigned to a T at position 102 bp in front of the *scrP* ATG start codon. The signal intensities (determined by height of the peaks) did not reflect the transcriptional levels between raffinose or glucose plus sucrose samples as was expected. If however, the primers were not present in excess, such determinations would not be possible. Screening for promoter consensus sequences did not reveal any typical *E. coli* –10 and –35 sequences. In *C. glutamicum*, a Gram-positive and high GC organism closely related to *Bifidobacterium*, an analysis of 33 promoters revealed the consensus sequences to be (TA.aaT) for the -10 region, and (ttGcca) for the -35 region (206). For the *B. lactis scrP*, a GGTAAA sequence 13 bp from the transcription start site was identified (Figure 5.7). In the *C. glutamicum* promoter study, this sequence was shown to be a probable –10 sequence (206). A TTGCAT sequence in the –35 region, which was also identified in *C. glutamicum*, was found which might be involved in transcription initiation.

For the *scrR* gene, several possible transcription starts were identified, but were not consistently obtained with repeated primer extension experiments. However, only for one of these, an A situated 68 bp in front of the *scrR* ATG, could a potential promoter consensus sequence be identified: TACAAT in the -10 region and TTGCCG in the -35 region (Figure 5.7). As for the promoter of the *scrP* gene, these sequences share closer homology to the *C. glutamicum* than the typical *E. coli* promoter sequences. Neither *C. glutamicum* nor *E. coli* type promoter sequences could be identified for the other transcription starts identified. Further primer extension experiments performed with a different primer might help resolve which of the *scrR* transcription starts is the correct one. However, in three of the four primer extension experiments performed, the transcripts terminated in a GC-rich region (Figure 5.7 C). This was probably more the case of the reverse transcriptase enzyme prematurely falling off due to a GC-compression, rather than genuine mRNA termination. Since this region is -35 bp from the *scrR* initiation codon, it would be difficult to design a more suitable primer.

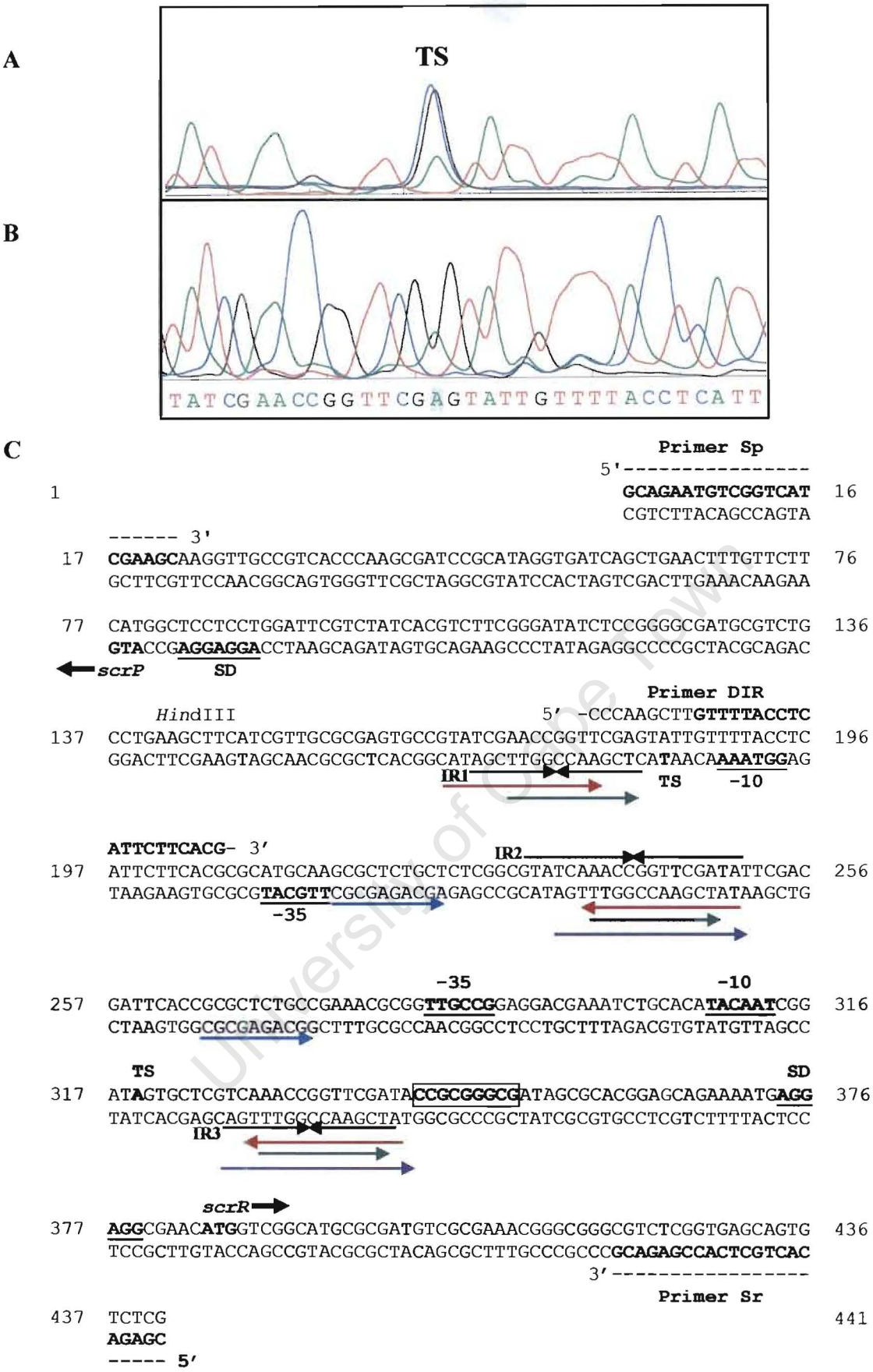


Figure 5.7. Mapping of the transcription start site of the *B. lactis scrP* and *scrR* genes by primer extension analysis. A: Primer extension product obtained for the *scrP* gene using RNA extracted from *B. lactis* grown in BY medium containing sucrose (blue curve) or glucose plus sucrose (black curve). A and T sequencing reactions (green and red curves respectively) were used as tracking lanes. B: The DNA sequencing fluorogram corresponding to the region

analysed. The Cy^5-labelled Sp and Sr primers were used for both the primer extension and sequencing reactions of the *scrP* and *scrR* genes respectively. C: Nucleotide sequence of the promoter regions preceding the *scrP* and *scrR* genes. Putative promoters (-35 and –10 regions) and ribosome-binding sites (SD) are in bold type and underlined. The transcriptional start site (TS) of *scrP*, as determined by primer extension analysis, is indicated in bold. A possible transcription start for *scrR* is also shown in bold. The compression at which the majority of the *scrR* transcripts terminated are boxed (see text for details). Primers Sp, Sr and DIR shown in bold type were used to generate the fragments for gel binding analyses. The coloured arrows indicate directions of the direct and indirect repeat sequences. The palindromic sequences, IR1, IR2 and IR3 discussed in the text which may act as operator sites for GalR-LacI family regulatory proteins are shown in black. Other palindromic sequences which might also be involved are shown in different colours.

Table 5.2. Comparison of the inverted repeat sequences identified in the *scrP-scrR* promoter region to the CRE consensus sequence and the GalR-LacI operator consensus sequence.

Element	Sequence	Position in Figure 5.7
GalR-LacI operator consensus	$A_4A_3N_2C_1/G'_1N'_2T'_3T'_4$	
CRE consensus	$(T/A)_1G_2N_3A_4A_5(C/G)_6C_7/G_8N_9(T/A)_{10}(T/A)_{11}N_{12}C_{13}A_{14}$	
Inverted repeat 1 (IR1)	$T_7\mathbf{C}_6G_5A_4A_3C_2C_1/G'_1G'_2T'_3T'_4C'_5\mathbf{G}'_6A'_7$	168 bp - 181 bp
Inverted repeat 2 (IR2)	$T_9A_8T_7\mathbf{C}_6A_5A_4A_3C_2C_1/G'_1G'_2T'_3T'_4C'_5\mathbf{G}'_6A'_7T'_8A'_9$	233 bp – 250 bp
Inverted repeat 3 (IR3)	$T_7\mathbf{C}_6A_5A_4A_3C_2C_1/G'_1G'_2T'_3T'_4C'_5\mathbf{G}'_6A'_7$	327 bp – 340 bp

The bases that differ from the CRE consensus described in the text are shown in bold type.

Numbering: although the CRE consensus is also an inverted repeat homologous to the GalR-LacI operator consensus sequence, it is numbered from left to right, whereas the GalR-LacI sequence is numbered from the centre of the palindrome.

GalR-LacI family regulators typically act at palindromic operator sequences centred around a ($A_4A_3N_2C_1/G'_1N'_2T'_3T'_4$) consensus (328). Three palindromic repeats (IR1, IR2, IR3), the first perfect and the second two imperfect, were identified (Figure 5.7, Table 5.2). These sequences could serve as a potential operator sites for the ScrR regulator (328). They also show homology to the consensus sequence of catabolite response elements (CREs) (Table 5.2) which control catabolite repression in Gram-positive bacteria (120). All three of the palindromic sequences differ from the CRE consensus sequence at the 2nd and 13th positions (Table 5.2). It has been shown that mutation of the first nucleotide in the *B. subtilis* amylase CRE has little effect on catabolite repression. Notably, mutation of position 13 of the amylase CRE largely alleviated catabolite repression, and therefore, identity to CRE sequences might be coincidental. Several direct repeats, some of which form part of the inverted repeat sequences, were also identified

which could be involved in promoter activity or could serve as potential protein sites for the ScrR and other regulators (Figure 5.7 C) (201). In order to establish the possible role of these sequences in gene regulation, gel binding analyses between the ScrR and the promoter region were performed and will be presented in section 5.4.6.

5.4.5. Development of a Promoter Expression Vector for *B. lactis*

Although a *Bifidobacterium-E. coli* shuttle vector (pBKJ50F/R) expressing the chloramphenicol resistance gene has been developed (205), expression and promoter probe vectors have yet to be constructed for genetic manipulations in *Bifidobacterium*. However, *Corynebacterium* vectors have been shown to be able to replicate in *Bifidobacterium* (8), and a family of *C. glutamicum-E. coli* shuttle vectors for cloning, controlled gene expression, and promoter probing have been constructed (72). We were, therefore, interested in investigating the possibility of using these *Corynebacterium* vectors for genetic analyses in *B. lactis*. Specifically, we wanted to investigate *scrP* and *scrR* promoter activities in response to growth in different carbon sources, and to determine firstly, the promoter-active sequences required for gene expression and secondly, the elements required for transcriptional regulation.

The *C. glutamicum-E. coli* promoter probe vector pEKplCm, carries a promoter-less *cat* reporter gene followed by transcription termination sequences (Figure 5.1). A multiple cloning site (MCS) in front of the *cat* gene allows for the cloning of promoter fragments. The region between the MCS and the reporter gene contains translational stop codons in all three reading frames followed by a ribosome-binding site. Promoter activity is determined by measuring CAT activity (72). The kanamycin-resistance-encoding gene is present for the selection of transformants. Bifidobacteria however, are resistant to this antibiotic (155), and *B. lactis* was highly resistant to kanamycin when it was plated on agar containing a filter disc delivering 1 mg of the antibiotic (C. Price, personal communication). Bifidobacteria are, however, susceptible to erythromycin and the β-lactam antibiotics (155). We, therefore, investigated the possibility of replacing kanamycin resistance with ampicillin or erythromycin resistance for the selection of pEKplCm *B. lactis* transformants. The minimum inhibitory concentrations (MIC) of these antibiotics, including chloramphenicol, were determined in *B. lactis*, and the cells were found to be susceptible to ≤0.8 μg/ml of ampicillin, 1.25 μg/ml of erythromycin, and between 1.875-3.75 μg/ml of chloramphenicol. Due to the extreme sensitivity of *B. lactis* to ampicillin, this antibiotic was chosen for the selection of pEKplCm transformants.

Analysis of the pEKplCm restriction map revealed the restriction sites *Xho*I and *Mlu*I (Figure 5.1), and that digestion with these enzymes would result in the deletion of a 1.6 kb fragment containing a large portion of the kanamycin-resistance gene, which could then be replaced with the ampicillin-resistance gene. However, a 300 bp fragment flanking the kanamycin-resistance gene would also be deleted which could be detrimental to the plasmid's function. The 1.6 kb *Xho*I-*Mlu*I fragment was deleted from pGS2, resulting in the construct pΔGS2 (see 5.3.1 for details). *E. coli* cells transformed with pΔGS2 were sensitive to kanamycin, whereas cells with pGS were resistant, indicating that the kanamycin-resistance had been successfully deleted. In order to determine whether the 300 bp fragment was required, *C. glutamicum* cells were transformed with pGS and pΔGS2 plasmid DNA and were plated onto agar containing chloramphenicol and nalidixic acid. Transformants were obtained for both plasmids, however, plasmid DNA could only be extracted from cells that had been transformed with pGS DNA. This suggested that the pΔGS2 plasmid was unable to survive in *C. glutamicum*, and that the 300 bp fragment was essential for plasmid function. It would, therefore, be impossible to replace kanamycin-resistance with ampicillin-resistance as was intended. Although it could have been possible to select for transformants on chloramphenicol, this would only have been possible if a functional promoter was cloned, and this could have been investigated with the *B. lactis scrP* and *scrR* promoters. However, it would not have been possible to obtain a negative control to determine the basal CAT activity in *B. lactis*. We were, therefore, unable to perform promoter analyses in *B. lactis* using the *Corynebacterium* promoter vector.

5.4.6. Binding of ScrR to the *scrP-scrR* Promoter DNA

Sequence identity of the *B. lactis* ScrR to GalR-LacI-like proteins suggested that it might bind DNA at palindromic operator sequences in promoter regions. Three such sites were identified in the *scrP-scrR* promoter region (Figure 5.7). To confirm this function and to determine the sequences to which it binds, gel shift assays were performed using different protein preparations.

Ideally, DNA-protein interactions are performed with purified recombinant fusion protein. This facilitates the acquisition of large amounts of protein free of nucleases, phosphatases and proteases which may be detrimental to the formation of the DNA-protein interaction being investigated. The *B. lactis* ScrR was, therefore, N-terminally fused to a 6 histidine sequence (pExScrR4) and was overexpressed in *E. coli* JM109 (see 5.3.10 for details). Analysis of the expression revealed that the protein was associated with the insoluble pellet, forming inclusion bodies in *E. coli*. Aggregation of proteins is favoured at high temperatures (180), and it has been shown that by incubating the cells at 28°C instead of 37°C, the proteins remain soluble (131, 180).

However, even at this lower temperature, together with reduced concentrations of IPTG, the protein remained insoluble. Therefore, ScrR was purified by affinity chromatography using denaturing conditions. SDS-PAGE analysis indicated that a protein of approximately 40 kDa had been purified (Figure 5.8). From the DNA sequence, the *B. lactis* ScrR is predicted to have a molecular weight of 37 kDa. The extra 3 kDa are contributed by the additional 27 amino acids of vector sequence which are translated before the *scrR* gene. Attempts to renature the purified protein resulted in precipitation. However, stepwise dialysis in the presence of buffer containing 10% glycerol greatly assisted in the solubilisation of the protein. Similar difficulties with the purification of the *Erwinia amylovora* and *Staphylococcus xylosus* ScrR proteins linked to a His-tag were reportedly experienced, and they could only be purified by fusion to the maltose binding protein (24, 89).

A Western hybridisation of the purified ScrR fusion protein probed with mouse antibodies to the histidine tag was performed to confirm that the protein isolated was a histidine fusion protein (Figure 5.9). An approximately 37 kDa protein band was detected, as well as a band larger than 106 kDa for the purified ScrR fusion protein. The larger band presumably represents aggregated ScrR protein, indicating that dialysis in the presence of 10% glycerol was not sufficient to prevent the protein from aggregating as was initially thought. To confirm that the protein purified was the ScrR, MALDI-TOF (matrix-assisted laser desorption ionisation-time of flight) analysis was performed. MS-Fit homology searches of the peptide masses identified the *B. lactis* ScrR sequence (Table 5.3).

Table 5.3. MS-Fit peptide search results of the ScrR-His protein MALDI-TOF mass spectrometry data.

Protein	Accession No.	MOWSE Score	Masses Matched (%)	Protein MW (kDa)
B. lactis ScrR	AF441242	5.532e+006	54	35.55
C. crescentus ATP-binding protein	NP_419007.1	1.149e+006	54	36.13
S. coelicolor LacI-family regulator	NP_627024.1	4.883e+005	50	37.59

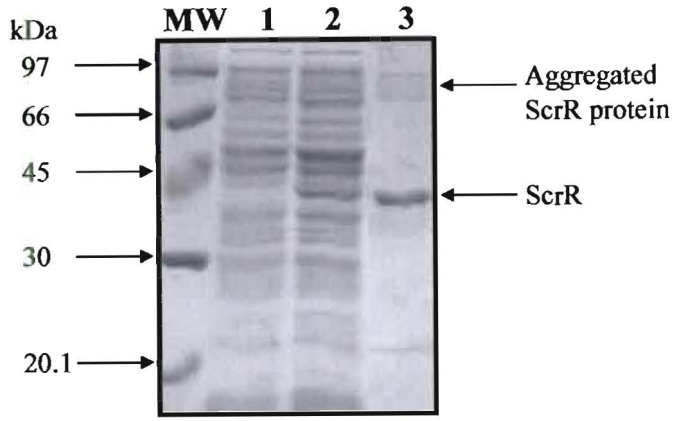


Figure 5.8. Purification of the *B. lactis scrR*-histidine fusion protein expressed in *E. coli*. Lane 1: non-induced cells, Lane 2: cells induced with IPTG, Lane 3: eluate under denaturing conditions.

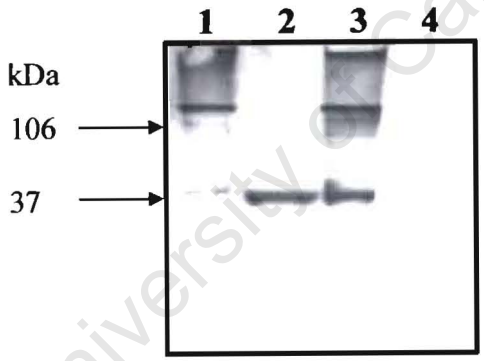


Figure 5.9. Western hybridisation of mouse histidine antibodies to purified ScrR-His fusion protein. Lane 1: Purified ScrR-His fusion protein dialysed in buffer containing 10% glycerol, Lane 2: Denatured protein from IPTG-induced *E. coli* cells with pExScrR4, Lane 3: Inclusion bodies from IPTG-induced *E. coli* cells with pExScrR4, Lane 4: Denatured protein from IPTG-induced *E. coli* cells with the control vector plasmid pProEx HTc. Preparation of Scr-His fusion protein and inclusion bodies is described in 5.3.10.

Gel shift assays were performed non-radioactively and were optimised using 2% agarose gels. DNA-protein complexes are commonly resolved using 4-8% polyacrylamide gels. However, for electrophoresis of DNA fragments larger than 300 bp, agarose gels are recommended. Four DNA fragments were analysed: 450 bp *scrP-scrR* promoter fragment (positions 1 to 441, Figure 5.7 C); 300 bp fragment (position 141 to 441, Figure 5.7 C) which is shortened on the *scrP* side; 270 bp

fragment (positions 187 to 441, Figure 5.7 C) which has one of the palindromic sequences deleted; 150 bp fragment (positions 1 to 146, Figure 5.7 C) which does not have any identified promoter or protein binding sites. Gel shifts were attempted with the purified ScrR and the 450 bp DNA fragment but a shift was not detected. Although sucrose has not been shown to be the ligand for the *B. lactis* ScrR, it was included in the binding reaction, but it did not have an effect on the binding. The DNA-binding domain of ScrR is located in the N-terminus, 4 amino acids from the ATG start codon (Figure 4.4). The lack of binding could, therefore, be due to the interference of the leader amino acids (containing the His-tag) with the binding or proper folding of the protein. It is possible to remove the histidine residues from the purified protein by protease cleavage and this could be investigated in order to determine whether the binding properties of ScrR would be restored. It is also possible to fuse the histidine tag on the C-terminus of the protein so as to avoid possible interference of the His-tag with the binding of the protein, however, this vector was not available to us. In light of the *S. xylosus* and *E. amylovora* studies however, further analyses using a His-fusion protein will probably be futile, and therefore, the fusion of ScrR to the maltose-binding protein will rather be investigated. As was seen in the Western hybridisation, the majority of the purified protein was in an aggregated form, even in the presence of SDS (Figure 5.9), a common occurrence for DNA-binding proteins when highly expressed in *E. coli* (89, 116). This further suggests that the protein is not folded in its native conformation and could also, therefore, be why the purified ScrR was unable to bind to the *scrP-scrR* promoter DNA.

Since binding was not achieved with the purified ScrR, gel binding assays were performed using crude extract prepared from *B. lactis* cells cultured in media containing either glucose or sucrose. When the 450 bp promoter fragment was used, a shift was achieved with protein from both glucose- and sucrose-grown cells, which was inhibited when unlabelled DNA probe was added as competition (Figure 5.10).

In the transcriptional analysis of *scrR*, transcripts could not be detected from cells which had been grown in glucose (section 5.4.1, Figure 5.2). Therefore, sufficient regulator protein would not be expected from glucose-grown cells to result in a shift. In Gram-positive organisms, catabolite repression is mediated by a transcriptional regulator, the catabolite control protein (CcpA) (243). CcpA proteins exhibit similarities to the GalR-LacI transcriptional repressors, and bind to palindromic operator sequences called catabolite-responsive elements (CRE) (120, 243). The palindromic sequences in the *scrP* promoter region to which ScrR was thought to bind to, also exhibit similarity to the CRE of CcpA proteins (see section 5.4.4). Since it was shown that the *scrP* and *scrR* genes were under catabolite regulation, it is possible that the shift obtained with

extract from glucose-grown cells could be due to CcpA binding. Certainly, the binding of a protein to the proposed CRE overlapping the *scrP* transcriptional start site would result in transcription inhibition, consistent with the CcpA mode of action (41, 120).

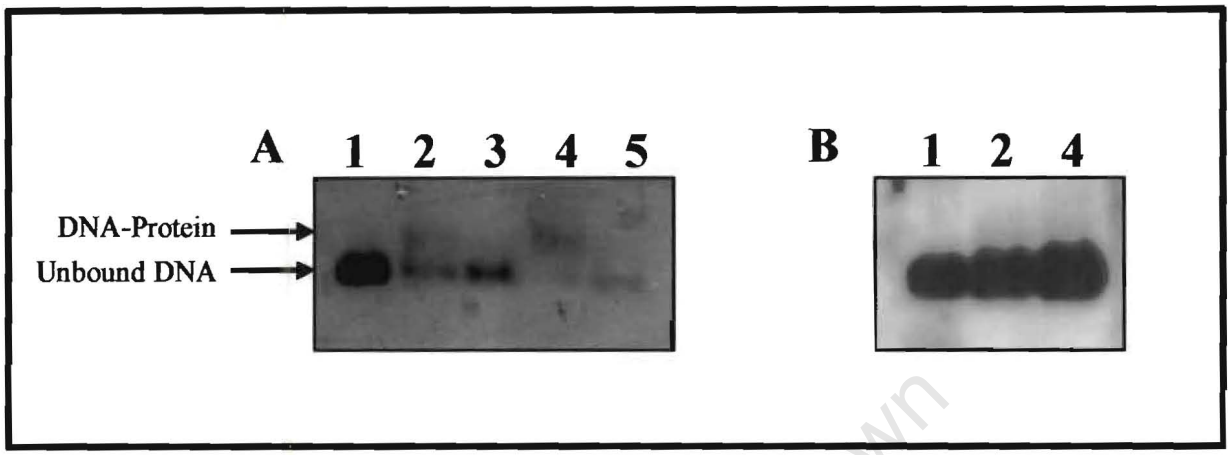


Figure 5.10. Gel mobility shift assay with *B. lactis* crude extract and the *scrP-scrR* promoter DNA fragments. A: 450 bp promoter fragment, B: 150 bp promoter fragment. Lanes 1: no protein, lanes 2: protein from sucrose-grown cells, lane 3: protein from sucrose-grown cells and cold target DNA, lanes 4: protein from glucose-grown cells, lane 5: protein from glucose-grown cells and cold target DNA. After a 20 min incubation at room temperature, samples were loaded onto a 2% agarose gel. The autoradiographs of the gels are shown.

The *ccpA* gene has been identified in *B. adolescentis* (Accession no. AF411186, unpublished) in close proximity to an alpha-glucosidase (*aglB*) gene. An alignment of the *B. longum* genome sequence (Accession no. NC004307) with the *B. adolescentis ccpA* sequence identified a gene, which had similarity to GalR-LacI transcriptional regulators, with an alpha-glucosidase gene next to it (ORFs BL0528 and BL0529 respectively). As was reviewed in chapter 1, CcpA binding is stimulated by the HPr protein of the PTS, phosphorylated at serine-46 (226). Consequently, sequence analysis of the *B. longum* NCC2705 genome revealed the presence of a gene encoding HPr (BL0412). These analyses, at first, suggested that catabolite repression in *Bifidobacterium* could also be mediated by CcpA. However, the putative CcpA proteins of *B. adolescentis* and *B. longum* did not show significant identity (only 25% at the protein level) to CcpAs of *B. subtilis* (Accession no. NP_390852), *S. xylosus* (Accession no. CAA64713), *L. lactis* (Accession no. AAK29739) and *S. mutans* (Accession no. O07329). The CcpAs of *B. lactis, S. xylosus, L. lactis* and *S. mutans*, however, showed 60% sequence identity at the protein level. Furthermore, the *B. longum* HPr protein lacks serine at position 46, and the genome lacks HPr kinase which is required for the phosphorylation of the HPr protein. It has previously been suggested that in the

high GC Gram-positive bacteria serine phosphorylated HPr, and CcpA, do not play a role in catabolite repression (207). Based on these findings, it is possible that glucose repression is also mediated via a different mechanism in bifidobacteria.

The gel shift obtained with protein extract from glucose-grown cells was, therefore, more likely due to some other regulatory protein involved in catabolite repression. Catabolite regulation mechanisms have not yet been clearly identified in Gram-positive bacteria, much less in bifidobacteria. As was discussed in chapter 4, it appears that *B. longum* has a second sucrose-utilisation gene cluster, and evidence suggests that it too exists in *B. lactis*. This cluster also contains a GalR-LacI family regulatory protein. Perhaps this protein functions as a negative regulator of sucrose-utilisation genes, binding in the presence of glucose to inhibit transcription.

The finding that ScrR functions as a positive regulator suggested that by binding DNA it would result in initiation of transcription of, at least, the *scrP* gene in the presence of sucrose or one of its metabolites. Activator sites usually occur in the -40 to -35 promoter region, just upstream from the RNA polymerase recognition site (134, 217). Consistent with this, an imperfect palindrome (IR2) site was identified 18 bp upstream of the proposed *scrP* -35 promoter region (Figure 5.7). Taken together with the fact that both *scrP* and *scrR* transcription was induced by sucrose, it is possible that the shift obtained with protein from sucrose-grown cells could be as a result of ScrR binding to the *scrP*, and possibly even the *scrR*, promoter.

Although it has been postulated that the gel shifts obtained were as a result of ScrP binding, it is entirely possible that another protein, such as RNA polymerase, or a complex of proteins, caused the shifts observed. A shift was not seen when the 150 bp DNA fragment was used as a target (Figure 5.10 B), suggesting that the 450 bp fragment shifts with *B. lactis* protein extract was due to sequence-specific binding. Gel shifts performed with the 300 and 270 bp fragments were inconclusive as smears were obtained which could not be resolved. We were, therefore, unable to determine the role of the palindromic sequences in binding. However, this may indicate that either, binding occurs at several sites other than the three palindromic sequences that have been discussed, or, that the regulatory proteins simultaneously bind to a number of sites, as with the AraC and MalT positive regulators in *E. coli* (264). Several direct repeat sequences were identified which could be involved in protein binding (Figure 5.7C) (201), as has been shown for the AraC protein (264). Other invert repeat sequences, spaced distantly from each other were also identified which could be involved in DNA-looping to facilitate protein-protein interactions, as with the positive regulators LevR and AraC from *B. subtilis* and *E. coli* respectively (168, 264).

Further investigations using purified ScrR protein will be required to confirm the mechanism by which ScrR specifically acts.

5.5. CONCLUSIONS

RNA slot blot analysis demonstrated that the *scrP, scrR* and *scrT* genes were similarly regulated at the transcriptional level, and were induced by growth in sucrose and raffinose, but were repressed in glucose. Identity of the *scrR* gene product to members of the GalR-LacI family of transcriptional regulators suggested that it was involved in the regulation of the *scr* genes. The role of the *scrR* gene product was investigated in *E. coli* with plasmid deletion constructs. Even under non-inducing conditions, it was found that when the *scrR* gene was truncated, sucrase activity was reduced 1000-fold. This suggested that the *scrR* gene product played a role in regulation, of at least the *scrP* gene, by a positive regulatory mechanism. Analogy with other GalR-LacI-like regulatory proteins suggests that the *B. lactis* ScrR binds to an imperfect palindromic operator site in the vicinity of the *scrP* promoter. Such a site was identified ($T_9A_8T_7C_6A_5A_4A_3C_2C_1$/G'$_1G'_2T'_3T'_4C'_5$ G'$_6$A'$_7$T'$_8$A'$_9$) (Table 5.2) 18 bp upstream of the *scrP* -35 promoter region (Figure 5.7C), a likely position for the binding of an activator protein (134, 217). The possible ScrR mode of action is discussed in the final chapter. Although the majority of GalR-LacI members function as negative regulators, including the ScrR of *S. typhimurium* (127), *K. pneumoniae* (127), *V. alginolyticus* (22), *S. xylosus* (89), *C. beijerinckii* (222), and the SacR of *L. lactis* (159), some positive regulators also belong to this family, while some perform both functions (160, 214, 218, 243, 309, 313). Certainly, this is the first sucrose-regulator identified that positively regulates the transcription of sucrose-catabolic genes.

In order to determine whether ScrR did in fact bind to the proposed imperfect palindromic sequence, gel binding assays were performed. A shift of the *scrP-scrR* promoter fragment was obtained with crude protein from *B. lactis* cells grown in sucrose-containing medium and, binding was found to be sequence specific. However, it is not possible to conclude, however likely, that the shift was as a result of ScrR binding.

Analysis of sucrase activity in *E. coli* with the deletion constructs indicated that ScrR could only positively regulate ScrP activity in the presence of a full length *scrT* gene. Although very weak sequence identity of the proposed *scrT* gene product to sucrose symporters was observed, there

was no evidence to suggest that it could be involved in the regulation or stabilisation of sucrase activity. Since sucrose was not present in the media for these experiments, the positive effect cannot be attributed to the transport of sucrose into the cell, which would result in the induction of sucrase activity. Perhaps the *scrT* gene product has a dual function, having both transport and regulatory functions as for the *bglS, glnA, putA, malK,* and *SNF3* gene products discussed in section 5.4.3. Alternatively, the deletion of a possible terminator upstream of the *scrT* stop codon, allowing counter-transcription of *scrP* from P_{λ}, could have resulted in the reduced ScrP activity observed.

In low-GC Gram-positive bacteria catabolite repression is mediated by the binding of the CcpA protein (belonging to the GalR-LacI family of transcriptional regulators) to the *cis*-acting CRE, present near the promoter of genes affected by catabolite repression (328). In high-GC Gram-positive organisms, this is thought to be mediated via a different mechanism (207). Several observations indicated that the *B. lactis scr* genes are also controlled by catabolite repression. Firstly, sucrase activity in *B. lactis* was reduced in glucose-grown cells (chapter 2). Secondly, RNA slot blot analyses indicated that the *scrP, scrR* and *scrT* genes were repressed by glucose at the transcriptional level (Figure 5.2). Thirdly, the 450 bp *scrP-scrR* DNA promoter fragment was shifted when incubated with protein from glucose-grown cells. Although there was some evidence to suggest that catabolite repression is also mediated by CcpA in *B. lactis,* two important findings suggest that this is unlikely, at least in *B. longum*, and possibly also in bifidobacteria in general: (i) the *B. longum* HPr protein lacks a serine at position 46, (ii) *B. longum* lacks HPr kinase. The mechanisms involved in glucose-mediated repression, therefore, remain to be discovered.

Collectively, the experimental data presented in this chapter suggest that, at least in *E. coli*, the *B. lactis scr* gene cluster is regulated by several mechanisms: positively by ScrR, sucrose, and the *scrT* gene product; and negatively by glucose and an as yet unidentified regulatory protein. While the precise details of the regulatory mechanisms remain unclear, particularly the involvement of the *scrT* gene product, a preliminary model can be hypothesised. This model will be discussed in the general conclusions chapter.

CHAPTER 6

GENERAL CONCLUSION

The probiotic organism, *Bifidobacterium lactis*, was isolated from a yoghurt starter culture with the aim of analysing its utilisation of carbohydrates for the identification of prebiotics. The development of prebiotic substrates for bifidobacteria to be included in food products and for synbiotic applications is advancing rapidly. Despite this, relatively little research has been conducted to show which bifidobacterial strains actually metabolise the prebiotic substrates that have been identified. More importantly, the genetic mechanisms by which prebiotics are metabolised by the bifidobacteria have not been identified. The prebiotic oligosaccharides raffinose and oligofructose, have been identified as bifidogenic, however, *B. lactis* growth was stimulated most in the presence of the former substrate. In order to establish the mechanisms required for bifidobacteria to utilise these substrates, the physiological and genetic utilisation of raffinose and sucrose was analysed. These analyses were performed in the industrial *B. lactis* strain, since it already is an established biotechnologically valuable organism and is currently included for the fermentation of food products and in pharmaceutical supplements.

The isolation of the raffinose utilisation genes, in particular the α-galactosidase was attempted by several strategies. These included amplification by PCR, hybridisation using a *B. longum* α-galactosidase DNA fragment, and the screening of two genebanks on raffinose, sucrose, and the histochemical substrate ρ-nitrophenyl-α–D-galactopyranoside. A construct conferring very weak α-galactosidase activity on *E. coli*, p4B11, was isolated. Sequence analysis of the p4B11 insert identified several ORFs, none of which showed identity to an α-galactosidase or related protein, and activity could not be assigned to any of them. At the physiological level, α-galactosidase activity was repressed in the presence of glucose, and induced in raffinose in *B. lactis* cells. RNA slot blot analyses using the cloned insert fragment from p4B11 as a probe revealed similarly that *B. lactis* mRNA was induced by growth in raffinose, and could not be detected in glucose. This suggested that the activity was not spuriously produced in *E. coli*. However, the identity of the transcript which hybridised to the probe could not be determined. It was also not possible to identify a protein which could be responsible for the activity by SDS-PAGE analysis.

In order to identify the genetic element responsible for the activity produced by the clone, the translation products of p4B11 could be analysed by an *E. coli* DNA-directed cell-free

transcription/translation system. However, when the insert was subcloned, the activity was lost, and could not be attributed to the truncation of any of the ORFs identified nor due to their direction relative to the vector λ promoter. It is possible that due to sequencing errors, the correct open reading frame was not identified, and that it was truncated in the preparation of the subclones. Analysis using the transcription/translation system, and re-sequencing of the insert, would facilitate in determining if this was the case.

Further attempts to isolate the *B. lactis* α-galactosidase gene will be considered. The *B. lactis* genebanks could be re-screened, possibly using the *E. coli* M2508 mutant in the *melA* gene. A different histochemical substrate, 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (similar to X-Gal, but with the α- instead of the β- link) could be used, which has been shown to be effective in the isolation of three α-galactosidases from *Trichoderma reesei* by complementation and by plating the transformants on agar plates containing the substrate (166). The PCR using the bifidobacterial α-galactosidase gene primers could be repeated using techniques/additives that would reduce the difficulties encountered with amplification of high GC DNA. The most successful, however, would perhaps be to purify the protein from *B. lactis* first, and end-sequence it to determine if it is homologous to the other bifidobacterial α-galactosidase proteins. From the amino acid sequence determined from the purified protein, appropriate primers could then be designed to isolate the gene by PCR. It is entirely possible that the *B. lactis* enzyme possessing α-galactosidase activity is different to the *B. adolescentis* and *B. longum* enzymes from which the PCR primers were designed. Incidentally, sequence analysis of the *B. longum* genome revealed that this organism possesses more than one α-galactosidase gene (262). The *B. lactis* enzyme, might therefore, be similar to the other *B. longum* α-galactosidase. This could explain the failure to isolate the gene by PCR. Hybridisation experiments using probes prepared from the two *B. longum* α-galactosidase genes could be performed to determine if *B. lactis* also possesses two such α-galactosidases.

Complementation of the *B. lactis* gene library in *E. coli* on sucrose resulted in the isolation of a sucrose utilisation gene cluster. Three genes, encoding a sucrose phosphorylase (ScrP), a GalR-LacI type transcriptional regulator (ScrR), and a sucrose transporter (ScrT), were identified by sequence analysis. The *scrP* gene was expressed constitutively from its own promoter in *E. coli* grown in complete medium, and hydrolysed sucrose in a reaction that was dependent on the presence of phosphates. This supported the proposal made on the basis of sequence analysis that the *scrP* gene encoded a sucrose phosphorylase. This suggests that glucose-1-phosphate would be

the product of sucrose hydrolysis, and not glucose-6-phosphate as with hydrolysis by the sucrose-6-phosphate hydrolases generally associated with sucrose PTS genes. The *B. lactis scrR* gene found identity to sucrose regulators that are all associated with PTS-dependant genes. However, this *B. lactis* sucrose cluster is not likely to be involved in PTS-dependent utilisation of sucrose.

With regards to regulation of the sucrose utilisation genes isolated, equivalent effects were observed at the molecular and physiological levels. The *scrP*, *scrR* and *scrT* genes were transcriptionally induced in cells grown in the presence of sucrose or raffinose, but not in glucose. Similarly, at the physiological level, sucrase activity in *B. lactis* was induced in sucrose- or raffinose-grown cells. The observed regulation is possibly mediated by at least two mechanisms. The *scrR* gene product almost certainly mediates the positive regulation, since deletion of the *scrR* gene in plasmid constructs in *E. coli* resulted in the complete loss of sucrase activity. Furthermore, the negative glucose effect on the transcription of the *scr* genes suggests that they are also regulated by catabolite repression. In support of the presence of these two regulatory mechanisms, gel shifts were observed with *B. lactis* protein extract from both sucrose- and glucose-grown cells.

A possible mechanism by which these *scr* genes are regulated at the molecular level is proposed from the experimental evidence and from the identification of three palindromic sequences in the *scrP-scrR* promoter region. From their positions relative to the transcriptional starts, identified by primer extension, and to the potential promoters of the two genes, one can speculate as to how both positive and negative regulation could occur. This mechanism is illustrated in Figure 6.1. In the presence of glucose, an unidentified negative regulatory protein, would bind to the inverted repeat sequences proximal to the *scrP* transcription start and to the proposed *scrR* transcription start. This would prevent RNA polymerase from initiating transcription at the two promoters. In the presence of sucrose, the repressor would no longer be activated and would not be able to bind to the invert repeat sequences. Either sucrose, or a sucrose metabolite, would bind to ScrR, changing its conformation to allow it to bind the inverted repeat situated between the proposed -35 promoters of the *scrP* and *scrR* genes. As a result, the binding of RNA polymerase and/or the activation of transcription would be facilitated.

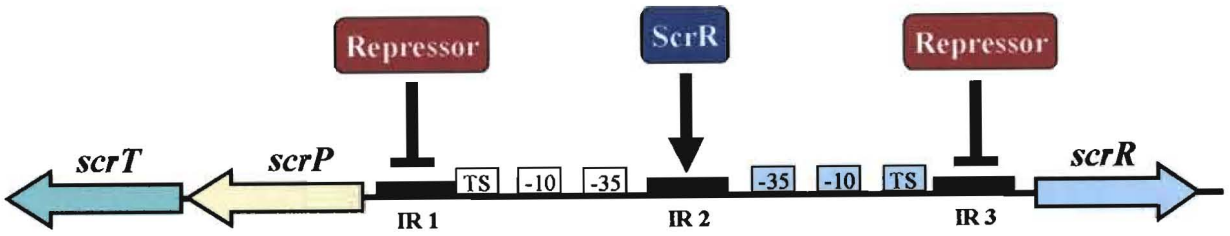


Figure 6.1. Illustration of the proposed mechanism by which the *B. lactis scr* genes are regulated. The mechanisms are discussed in the text. Coloured arrows indicate the direction of transcription of the *scrP, scrR* and *scrT* genes. The black arrow and perpendicular arrows indicate positive and negative controls respectively. TS, transcription start site; IR, inverted repeat; -10 and -13, putative promoters for the *scrP* and *scrR* genes.

This model needs to be proved experimentally. Gel mobility shift assays using purified ScrR, possibly fused to the maltose-binding protein would need to be performed. Furthermore, the operator sites to which ScrR binds need to be confirmed. This could be analysed by gel shift assays with DNA fragments containing the target sites, and DNA footprinting experiments. Promoter fusions performed in *B. lactis* would also identify these binding sites, once the genetics have been sufficiently developed for such analyses to be carried out in *B. lactis*. However, similar fusion analyses could be performed in *E. coli* by providing the *scrR in trans*. The binding of ScrR in a liganded state needs to be shown, and sucrose, glucose, fructose and their phosphorylated derivatives need to be tested for their ability to either prevent, or induce, ScrR interaction with its operator sites. With regards to the gel shift assays performed with crude extract: antibodies produced to the ScrR-His fusion protein could be used to perform a Western hybridisation to identify whether the shift obtained with extract from sucrose-grown cells was due to ScrR binding. The involvement of another protein in the negative regulation of these genes in the presence of glucose would need to be proved by similar experimental procedures.

The finding that *E. coli* cells with the construct pSuc3 were unable to grow in the presence of sucrose, suggests that regulation in response to carbon source could occur in *E. coli*. Therefore, promoter fusions could be used to further investigate this. Evidence was presented to suggest that the *scrT* gene product might also function in the production of ScrP activity. This involvement of ScrT could be investigated in *E. coli* by introducing the *scrT* gene *in trans* to establish whether its presence in this way would have an effect on the activity of ScrP. Also, uptake studies in *E. coli* with the *scrT* could be performed so that its function as a sucrose transporter could be determined.

In this study, three genes coding for proteins with hydrolytic activity were isolated, which might possibly also have transglycosylation activity. These were the *B. longum* α- and β-galactosidases and the *B. lactis* sucrose phosphorylase. Certainly, transglycosylation activity has been demonstrated for α- and β-galactosidases isolated from bifidobacteria (216). These enzymes could be investigated for the enzymatic synthesis of novel prebiotic oligosaccharides, which could specifically be used for promoting the growth of the industrially used *B. lactis* strain, or for synbiotic applications. Once transferase activity has been determined, the reaction products obtained would need to be analysed and identified. Several methods, such as thin-layer chromatography, high performance anion-exchange chromatography, or MALDI-TOF could be used for their analysis. Fermentation tests of the oligosaccharides would then be performed to determine their effects on bifidobacterial growth rates.

Two sucrose utilisation clusters were identified in the *B. longum* genome. One of these is the *B. lactis scr* cluster identified in this study. There is evidence to support that *B. lactis* also contains the second *csc* sucrose cluster. In both gene clusters there are genes coding for a sucrose-hydrolysing protein, a transporter protein and a GalR-LacI family regulator. It would be interesting to determine if both of these clusters are indispensable for sucrose utilisation. Perhaps the *csc* gene cluster is more specific for the utilisation of inulin-type substrates, considering that the sucrase gene shows identity to inulinases and invertases. It would also be interesting to determine if the two regulator proteins show cross-reactivity: are they functionally homologous, and do they both bind to the promoter regions of the two sucrase genes to mediate regulation? Many possibilities could be investigated that would facilitate a better understanding of the mechanisms involved in, not only sucrose, but of also other fructose-containing carbohydrates. This knowledge, together with the identification of several enzymes which could possibly catalyse transglycosylation reactions, would certainly contribute greatly to the development of species-specific prebiotic substrates for biotechnological applications.

APPENDIX A

Bacterial Strains and Plasmids Used in this Study

Strain	Genetic characteristic(s)	Reference
Escherichia coli		
JM109	*RecA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ⁻ Δ(lac-proAB)* (F', *traD36 proAB lacI^{q}ZΔ*M15)	329
Bifidobacterium		
B. lactis	Industrial strain used in yoghurt	This study
B. breve NCFB 2257		NCFB, UK
B. bifidum NCFB 2203		NCFB, UK
B. longum NCFB 2259		NCFB, UK
Corynebacterium glutamicum		
ATCC 13032	Nx^R, type strain	AECI, South Africa
Plasmids		
pEcoR251	Amp^R (EcoRI)	336
pBluescript SK (pSK)	Amp^R, *lacZ'*	Stratagene
pMT104	Amp^R, 0.15 kb *B. fragilis* fragment in pEcoR251	320
pGem®-T Easy	Amp^R, *lacZ'*, T-tailed	Promega
pEKplCm	Km^R, promoterless *cat*	70
pGS	*C. glutamicum* glutamine synthetase promoter in pEKplCm	267

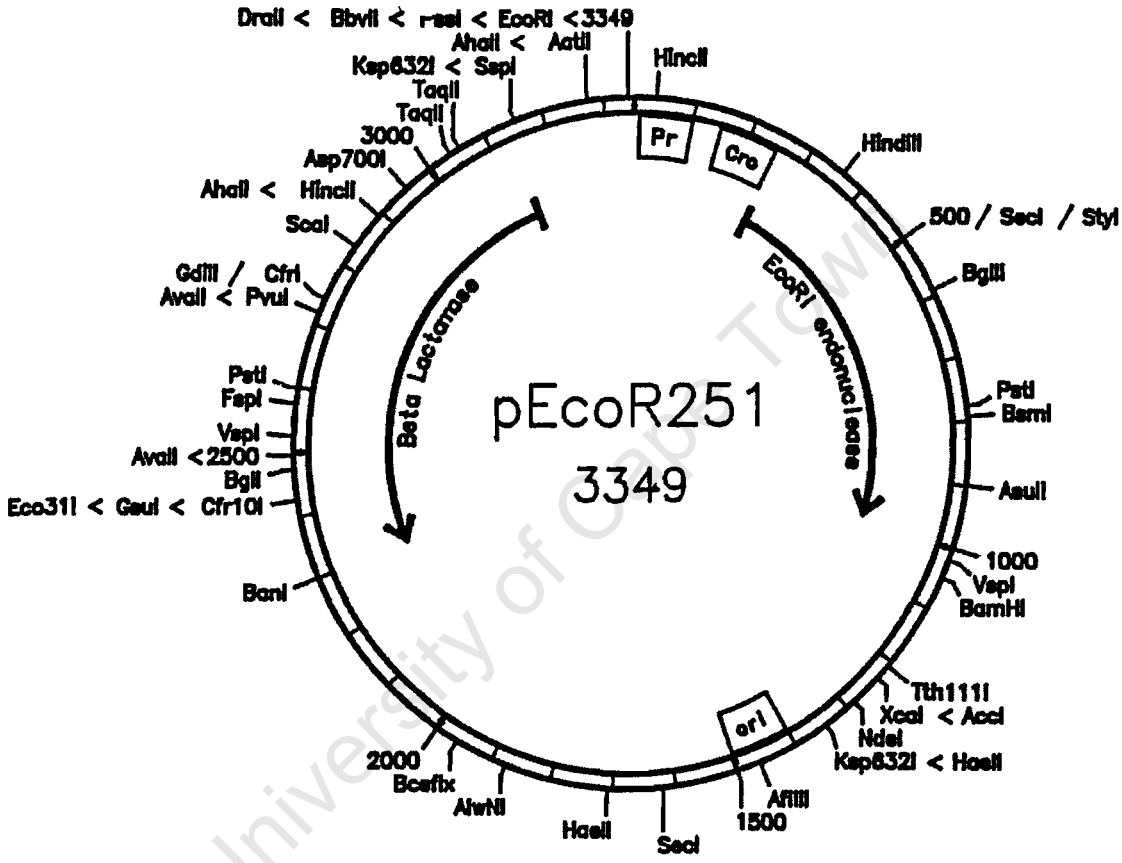


Figure A.1. Restriction map of pEcoR251 (336).

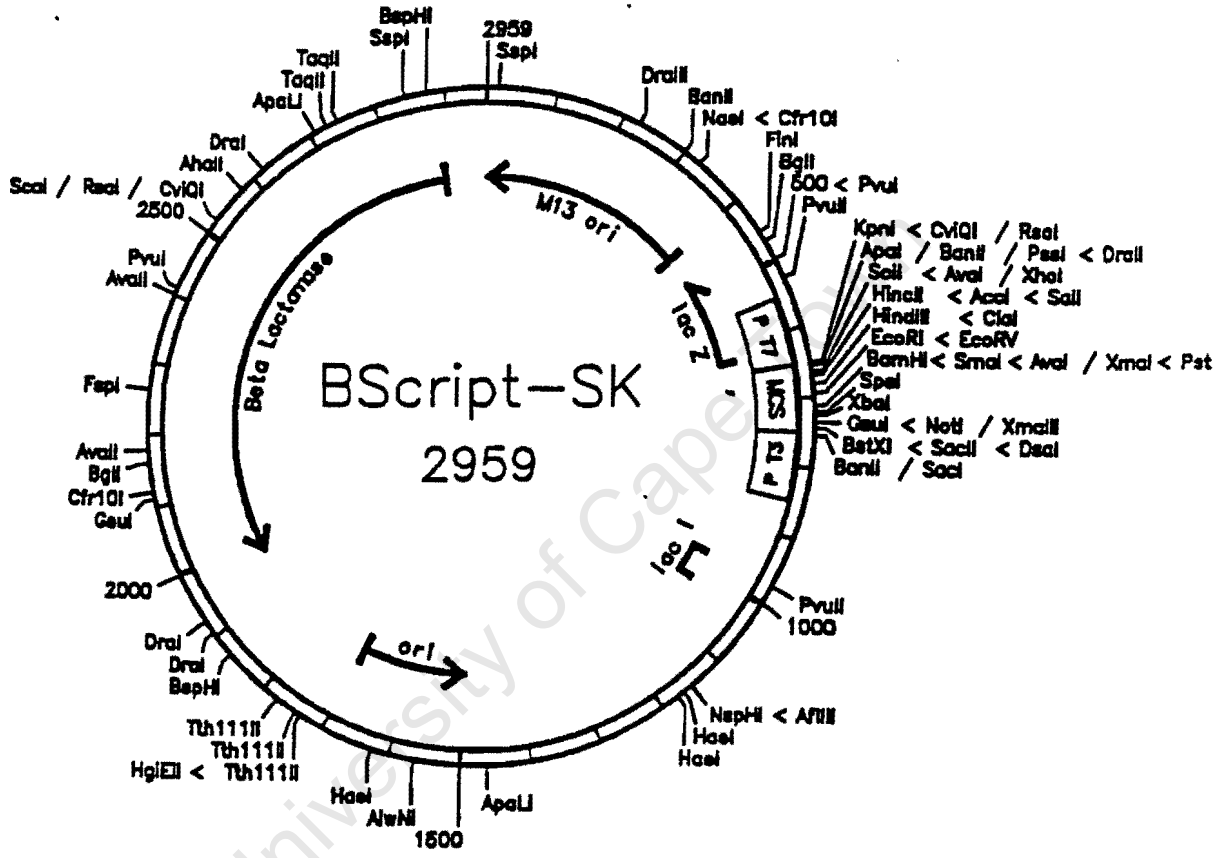


Figure A.2. Restriction map of pBluescript SK (Stratagene, San Diego)

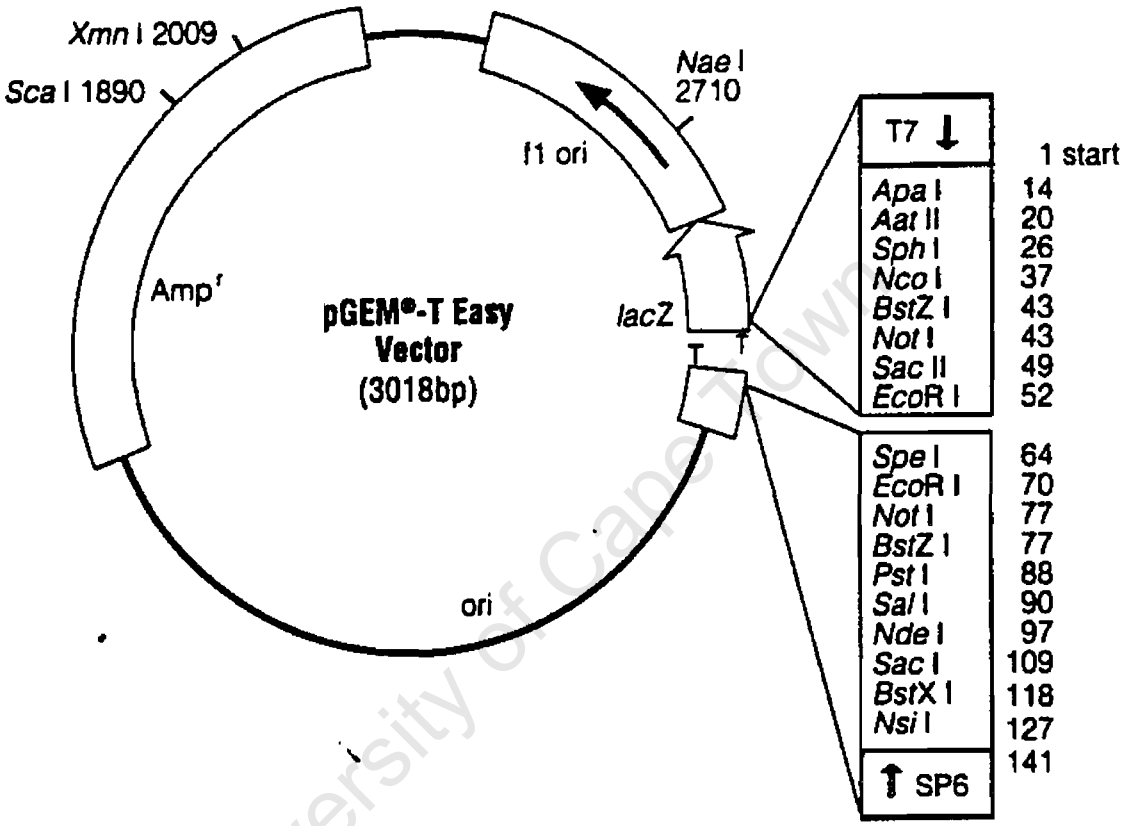
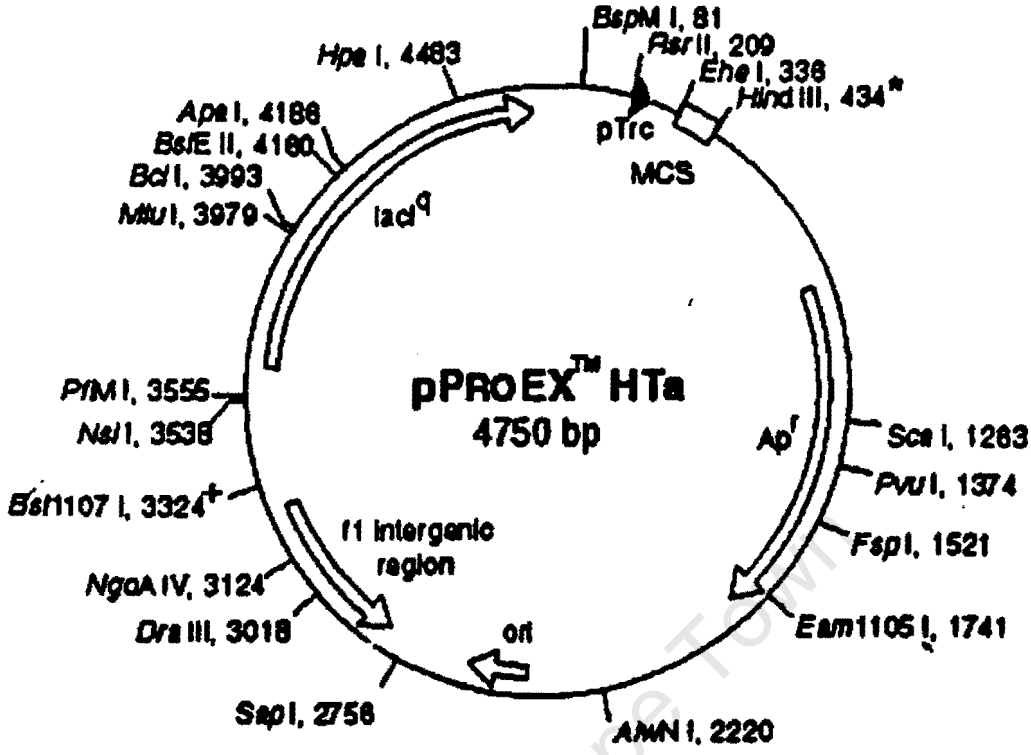


Figure A.3. Restriction map of pGem®-T Easy (Promega, USA).

A.



B.

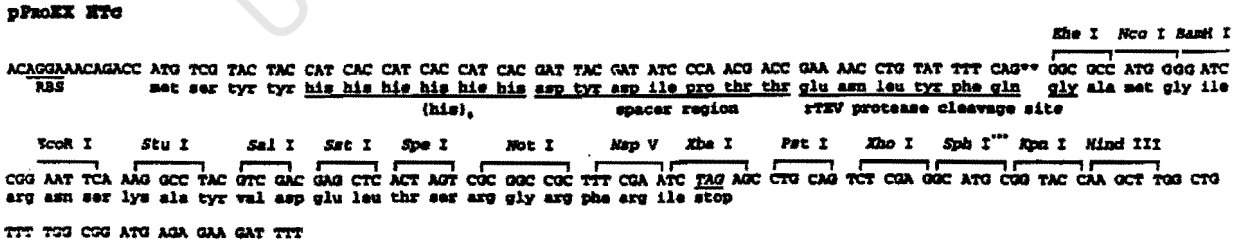


Figure A.4. Restriction map of pProEx™ expression vector (A), and multiple cloning site of pProEx HTc (B). The sequence for the His-tag, spacer region and recombinant TEV protease cleavage site are underlined (Life Technologies).

APPENDIX B

B.1. Preparation of Bifidobacterial Media

B.1.1. Brain Heart Infusion Medium (BHI)

In grams per litre

Brain heart infusion (Difco).....37
Yeast extract (Difco).....5
Cysteine HCl.....0.5
Rezasurin.....0.001
Agar.....15

B.1.2. Basal Medium (BY)

In grams per litre:

Tryptone.....10
Yeast extract (Difco).....2.5
Tween 80.....1
NaCl.....4.5
KCl.....0.25
$MgCl_2.6H_2O$.....0.15
KH_2PO_4.....0.4
K_2HPO_4.....0.2
NH_4Cl.....0.4
Cysteine HCl.....0.5
Rezasurin.....0.001
Agar.....15

B.1.3. BYG Medium (Basal + yeast + glucose)

Basal medium (BY) containing the following in grams per litre:

Yeast extract (Difco)……..2.5
Glucose……...5

B.2. *C. glutamicum* LM Medium

In grams per litre:

Tryptone……..10
Yeast Extract……...10
NaCl……5

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