APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2003, p. 24–32 Vol. 69, No. 1
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.1.24–32.2003

Induction of Sucrose Utilization Genes from *Bifidobacterium lactis* by Sucrose and Raffinose

Marla I. Trindade, Valerie R. Abratt, and Sharon J. Reid*

Department of Molecular and Cell Biology, University of Cape Town, Rondebosch 7701, South Africa

Received 1 July 2002/Accepted 3 October 2002

The probiotic organism *Bifidobacterium lactis* was isolated from a yoghurt starter culture with the aim of analyzing its use of carbohydrates for the development of prebiotics. A sucrose utilization gene cluster of *B. lactis* was identified by complementation of a gene library in *Escherichia coli*. 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 the strain hydrolyzed sucrose in a reaction that was dependent on the presence of phosphates. Primer extension experiments with *scrP* performed by using RNA isolated from *B. lactis* identified the transcriptional start site 102 bp upstream of the ATG start codon, immediately adjacent to a palindromic sequence resembling a regulator binding site. In *B. lactis*, total sucrase activity was induced by the presence of sucrose, raffinose, or oligofructose in the culture medium and was repressed by glucose. RNA analysis of the *scrP*, *scrR*, and *scrT* genes in *B. lactis* indicated that expression of these genes was influenced by transcriptional regulation and that all three genes were similarly induced by sucrose and raffinose and repressed by glucose. Analysis of the sucrase activities of deletion constructs in heterologous *E. coli* indicated that ScrR functions as a positive regulator.

Bifidobacteria are gram-positive anaerobes that occur in large numbers in the intestines of humans and other animals (44). Of the several hundred species of bacteria that colonize the large intestine, bifidobacteria are generally considered to be health promoting and beneficial (2, 23). Because of these benefits, considerable research is being directed at promoting the growth of bifidobacteria in the large intestine. One method is to introduce more bifidobacteria into the bowel by the consumption of fermented milk products containing the bacteria. Another approach is to use prebiotics, which are defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or the activity of one or a limited number of colonic bacteria (14). A variety of fructose-containing oligosaccharides, including inulin and oligofructose, have been reported to stimulate the growth of bifidobacteria (15, 16). Other nondigestible oligosaccharides, including raffinose and stachyose, as well as the oligosaccharides containing xylose, galactose, and maltose, have also been shown to have similar effects (35). *Bifidobacterium lactis* has been used industrially in fermented foods, such as yoghurt, cheese, beverages, sausages, infant formulas, and cereals. It is also available in natural remedies and supplemented tablets. *B. lactis* has been reported to be useful for supporting and balancing a healthy intestinal flora, for reducing the incidence of diarrhea and intestinal infections, for promoting the absorption of nutrients, for supporting the immune system, for maintaining cholesterol levels, and for detoxifying the intestine, the blood, and the liver (2, 7, 13, 21, 23). Despite the research to improve bifidobacterial concentrations in the intestine, molecular genetic research with these organisms has been very limited. Only a small number of genes and two plasmids of members of the genus *Bifidobacterium* have been cloned and characterized (25, 45).

Since bifidobacteria are saccharolytic, they play an important role in carbohydrate fermentation in the colon, and inulin, oligofructose, and raffinose have been reported to be important prebiotics. Investigation of the genetics involved in the utilization of carbohydrates is, therefore, of interest. Inulin is a polymer of D-fructose linked by β(2,1) bonds with an α(1,2)-linked D-glucose at the end of the molecule (52). Oligofructose is produced from inulin by partial enzymatic hydrolysis (34). Raffinose, a trisaccharide found in soybeans (9), consists of galactose α(1,6) linked to sucrose. Since the α(1,2) glycosidic linkage of sucrose is found in the oligosaccharides that have been described, screening for the genes involved in sucrose utilization was investigated. *B. lactis* was the organism selected for this study since it is the probiotic most widely used industrially. In this report, cloning of a cluster of three genes from *B. lactis* involved in the utilization of sucrose and characterization of one of these genes, the sucrose phosphorylase gene (*scrP*), are described.

MATERIALS AND METHODS

Plasmids, bacterial strains, and growth conditions. Bifidobacterial cultures were propagated at 37°C under strictly anaerobic conditions in BYG media, as described by Degnan and MacFarlane (10), without peptone water and with 1% (wt/vol) glucose as the carbon source. To obtain growth curves and for sucrase assays, individual carbohydrates at a concentration of 1% (wt/vol) were substituted for glucose. Oligofructose (Raftilose P95) was supplied by Savannah Fine Chemicals (Johannesburg, South Africa), and inulin (from chicory), raffinose, and melezitose were supplied by Sigma.

The *Escherichia coli* strains and plasmids used are listed in Table 1. *E. coli* JM109 (51) was used for all cloning and was routinely cultured aerobically at 37°C in Luria-Bertani (LB) medium (41) containing ampicillin (100 μg ml^{-1}). MacConkey base medium (Difco Laboratories) and M9 minimal medium (27)

* Corresponding author. Mailing address: Department of Molecular and Cell Biology, University of Cape Town, Private Bag Rondebosch, Rondebosch 7701, South Africa. Phone: 27-21-6503257. Fax: 27-21-6897573. E-mail: shez@science.uct.ac.za.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
B. breve NCFB 2257		NCFB[b]
B. lactis		This study
E. coli JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ^- Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}ZΔM15)	41
pEcoR251	Ap^r (*Eco*RI)	53
pMT104	Ap^r, 0.15-kb *B. fragilis* fragment in pEcoR251	49
pSuc1	Ap^r, 4.7-kb *Sau*3A *B. lactis* fragment in pEcoR251	This study
pSuc2	Ap^r, 3.95-kb *Sau*3A *B. lactis* fragment in pEcoR251	This study
pSuc3	Ap^r, 5.9-kb *Sau3*A *B. lactis* fragment in pEcoR251	This study
pΔST8	Ap^r, 3.2-kb *Sma*I-*Stu*I fragment from pSuc3 in pEcoR251	This study
pΔReg2	Ap^r, 3.9-kb *Pst*I fragment from pSuc3 in pEcoR251	This study

[a] Ap^r, resistance to ampicillin.
[b] NCFB, National Collection of Food Bacteria, Reading, United Kingdom.

supplemented with ampicillin and 1% (wt/vol) sucrose were used to assess fermentation of sucrose as a sole carbon source.

Isolation of *B. lactis*. *Bifidobacterium*-specific primers were used to isolate 16S rRNA DNA fragments by PCR from *Bifidobacterium breve* NCFB 2257. A fragment was labeled with digoxigenin according to the manufacturer's instructions (Roche) and was used as a probe for colony hybridization to identify *Bifidobacterium* colonies obtained from a freeze-dried yoghurt starter culture (Chr. Hansen A/S). Colonies showing a positive signal were selected, and the PCR was performed by using *Taq* DNA polymerase (Supertherm) and a GeneAmp 9700 machine (Applied Biosystems). The following primers were used for the *Bifidobacterium* 16S rRNA genes: forward primer 5′-CGC CAG GGT TTT CCC AGT CAC GAC GGG TGG TAA TGC CGG ATG-3′ and reverse primer 5′-CAG GAA ACA GCT ATG ACC CAC CGT TAC ACC GGG AA-3′.

Nucleic acid isolation and manipulation. All DNA manipulations were performed by using standard procedures (41). *B. lactis* chromosomal DNA was prepared as follows. Bacterial cells grown to the late exponential phase in 500 ml of BYG medium containing 0.5% glycine were harvested by centrifugation (6,000 × *g*, 10 min, 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 of a lysozyme solution (25% sucrose, 0.1 M NaCl, 0.05 M Tris-HCl [pH 8.0], 10 mg of lysozyme per ml), and incubated for 2 h 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 gently mixed. Proteinase K (Roche) was added to a final concentration of 100 μg ml^{-1} and was incubated for 1 h at room temperature with gentle mixing. Sodium dodecyl sulfate (final concentration, 2% [wt/vol]) and 0.5 mg of RNase (Sigma) were added, and the mixture was incubated at 37°C for 30 min. The resulting cell lysate was extracted three times with hot phenol (65°C) and then three times with water-saturated ether. The DNA was precipitated with ethanol (41) and redissolved in 500 μl of water. A *B. lactis* chromosomal genomic library was constructed by partially digesting the DNA with *Sau*3A restriction endonuclease. DNA fragments between 5 and 10 kb long were ligated into the *Bgl*II restriction site of plasmid pEcoR251 (Table 1). Plasmid DNA was introduced into *E. coli* JM109 by transformation of competent cells (41) for routine cloning and manipulation procedures. All restriction endonucleases were purchased from Amersham or Roche and were used as specified by the manufacturer. Electrophoresis was performed on 0.8 to 1.0% agarose gels by using standard procedures (41). DNA fragments were purified from the agarose gels with a High Pure PCR product purification kit (Roche). For Southern hybridization and colony hybridization, DNA was transferred to a nylon membrane (Hybond-N; Amersham), and hybridization and detection procedures were carried out as recommended by Roche. DNA fragments that were used as probes were generated by random-primed labeling by using a nonradioactive digoxigenin labeling and detection kit (Roche). DNA sequencing was performed by the dideoxy chain termination method (42) by using a Thermo-Sequenase sequencing kit (U.S. Biochemicals) and universal and reverse primers fluorescently labeled with Cy5 as recommended by the manufacturers. The sequencing reaction products were separated with an AlfExpress DNA sequencer (Pharmacia), and the nucleotide sequence obtained was analyzed by using the DNAMAN software package. Nucleotide and amino acid homology searches were carried out by using the databases at the National Center for Biotechnology Information and the BLAST program (1).

Enzyme and protein assays. Cell extracts were prepared from 100-ml cultures grown to the required growth stage and harvested by centrifugation (6,000 × *g*, 10 min, 4°C). Cells were washed twice and resuspended in 5 ml of TA buffer (100 mM Tris, 100 mM acetate; pH 6) or TAP buffer (100 mM Tris, 100 mM acetate, 64.2 mM Na_2HPO_4; pH 6). The cells were disrupted by sonication at 4°C and 95 W for 3 min (*E. coli*) or 6 min (*B. lactis*) with 30-s cooling intervals (VirSonic Digital 475 cell disrupter) and then centrifuged (15,000 × *g*, 20 min, 4°C). Sucrase activity in the cell extract was measured by incubating 15 μl of 0.88 M sucrose with 35 μl of the appropriate enzyme dilution for 30 min at 60°C. Dinitrosalicylic acid was used to determine the amount of reaction product formed, and glucose was used as the standard (26). Sucrose phosphorylase activity was expressed in micromoles of reducing sugar produced per minute per milligram of protein. The substrates used for specificity assays (sucrose, raffinose, melezitose, oligofructose, and inulin) were each supplied at a concentration of 1% (wt/vol). Protein concentrations were determined by the method of Bradford (5), and bovine serum albumin was used as the standard.

RNA analysis and primer extension. Total RNA was isolated from 50 ml of a mid-exponential-phase culture of *B. lactis* as described by Beg (3), except that the following DNase procedure was performed after isolation. 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 precipitated DNA and incubated for 30 min at room temperature. Twenty microliters of 0.25 M EDTA was added to inactivate the enzyme, and this was followed by extraction with phenol-chloroform and with chloroform-isoamyl alcohol (24:1). RNA was precipitated by standard procedures (41). RNA slot blot analyses were performed in duplicate by using a Hoeffer Scientific apparatus. For the *B. lactis* studies, a 16S rRNA probe was used as an internal control. RNA was transferred to nylon membranes (Roche) and hybridized according to the manufacturer's instructions by using digoxigenin-labeled DNA probes specific for the transcripts of the *scrP*, *scrR*, and *scrT* genes. The following fragments were used as probes: 1.4-kb *Hin*dIII-*Bam*HI fragment, 0.35-kb *Pvu*I-*Pvu*I fragment, and 0.85-kb *Pvu*I-*Pvu*I fragment, respectively. Signal intensities were measured with a Macbeth TD 109 transmission densitometer. Results were expressed as the ratio of the gene-specific signal to the internal control signal.

Primer extension was carried out with total RNA from *B. lactis* by using the protocol described by Eikmanns et al. (12). Primer Sp (5′-GCA GAA TGT CGG TCA TCG AAG C-3′) that was fluorescently labeled with Cy5 was used for the *scrP* gene.

Nucleotide sequence accession numbers. The DNA sequences of the *Bifidobacterium* 16S RNA gene (521 bp) and the *scrPRT* gene cluster have been deposited in the GenBank database under accession numbers AY151397 and AF441242, respectively.

RESULTS

Isolation of *B. lactis*. *Bifidobacterium* colonies were isolated from a freeze-dried yoghurt starter culture by colony hybridization by using a 0.6-kb PCR fragment from *B. breve*, which was generated with genus-specific primers for *Bifidobacterium*. PCR was performed with colonies that hybridized to the probe by using the same *Bifidobacterium*-specific primers. Sequence analysis with a BLAST search revealed sequence identity (99%) to *B. lactis* 16S rRNA (accession no. AB050136). How-

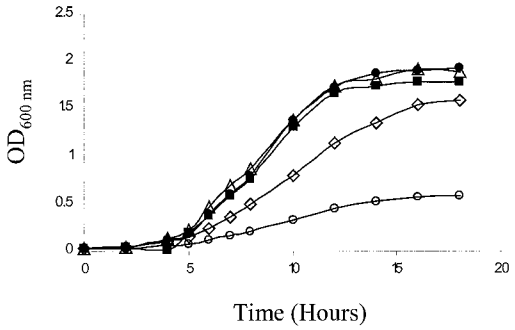


FIG. 1. Growth curves for *B. lactis* grown in media containing of each of the various sugars used at a concentration of 1% (wt/vol). An overnight culture of *B. lactis* in BYG medium was diluted 10^{-2} into the relevant media, and the growth was monitored by measuring the optical density at 600 nm ($OD_{600\ nm}$) at the times indicated. Symbols: ◇, glucose; ■, sucrose; △, glucose plus sucrose; ●, raffinose; ○, oligofructose.

ever, 99% sequence identity to *Bifidobacterium animalis* rRNA (accession no. AB050138) was also observed, and it has been suggested based on a DNA-DNA hybridization analysis that *B. lactis* is a subjective synonym of *B. animalis* (6).

Growth curves for *B. lactis*. Growth curves for *B. lactis* grown in BY media containing various carbohydrates (fructose, glucose, sucrose, glucose plus sucrose, raffinose, oligofructose, and inulin) were determined (Fig. 1). Of the carbohydrates that have been shown to be bifidogenic (namely, raffinose, oligofructose, and inulin), only raffinose was found to support better growth than glucose. *B. lactis* grew very weakly in oligofructose, while no growth was observed in inulin and in fructose.

Total sucrase activity in *B. lactis*. Sucrase activity was assayed in *B. lactis* cultures grown to the mid- and late-logarithmic phases in BY medium containing sucrose, glucose, sucrose and glucose, raffinose, or oligofructose (Table 2). The sucrase activities under the different conditions did not differ greatly between mid- and late-log-phase cultures. Similar activities were recorded for cultures grown in glucose and in glucose plus sucrose, and these activities were considered the basal level. Sucrase activity was induced 2-fold in the presence of sucrose or raffinose and 1.4-fold in the presence of oligofructose compared to the basal level.

Isolation of the *scr* gene cluster of *B. lactis*. A library of *B. lactis* DNA was established in *E. coli* JM109 by insertional inactivation of the *Eco*RI gene of pEcoR251 and was screened on minimal medium containing sucrose. Six colonies able to utilize sucrose as the sole carbon source were isolated and were found to contain inserts that were different sizes but had a 1.9-kb *Bam*HI-*Pst*I fragment in common. The plasmid containing the largest insert was designated pSuc1, and analysis of this plasmid revealed a 4.6-kb insert (Fig. 2). DNA sequencing of pSuc1 revealed two open reading frames (ORFs) that were read divergently (ORF1 and -2) and a third truncated ORF (ORF3) (Fig. 2). ORF2 and -3 were oriented in the direction opposite that of the λ promoter on the plasmid vector. The origin of the insert in pSuc1 was confirmed by Southern hybridization of the 1.4-kb *Bam*HI-*Hin*dIII fragment to *B. lactis* chromosomal DNA. Complete ORF3 was isolated by colony hybridization by using the 0.45-kb *Pvu*I-*Sal*I DNA fragment from pSuc1 as a probe. One clone was selected for further analysis and designated pSuc2 (Fig. 2).

Sequence analysis. DNA sequencing of pSuc1 revealed that ORF2 consisted of 1,518 bp encoding 506 amino acids with a calculated molecular mass of 55,660 Da. At the protein level, the ORF2 product showed identity to sucrose phosphorylases of *Agrobacterium vitis* (level of identity, 56%; accession no. P33910), *Pseudomonas saccharophila* (level of identity, 53%; accession no. AF158367), *Streptococcus mutans* (level of identity, 41%; accession no. P10249), and *Leuconostoc mesenteroides* (level of identity, 39%; accession no. Q59495). On the basis of sequence identity and functionality (see below), ORF2 was designated *scrP*. Sucrose phosphorylases reversibly catalyze the following reaction: sucrose + phosphate ⇆ α-D-glucose-1-phosphate + D-fructose (36, 48).

Several possible in-frame ATG start codons were identified for ORF3, none of which had a typical Shine-Dalgarno sequence preceding it. BLAST searches showed amino acid sequence identity from the ATG at nucleotide position 2,072 bp. The levels of amino acid sequence identity to transporters belonging to the sugar transporter family of prokaryotes and eukaryotes were 20 to 28%, and the highest overall identity was that to a putative membrane protein from *Caulobacter crescentus* (accession no. AE005901). The N terminus of the ORF3 product showed 29 to 33% identity to plant sucrose symporters, including those of *Vitis vinifera* (level of identity, 33%; accession no. AF182445), *Daucus carota* (level of identity, 29%; accession no. T14339), *Solanum tuberosum* (level of identity, 31%; accession no. AF237780), and *Lycopersicon esculentum* (level of identity, 30%; accession no. AF176950), suggesting that this ORF product might be involved in the transport of sucrose. A hydropathy plot of the predicted protein revealed a hydrophilic N terminus with a number of highly hydrophobic regions, which were interspersed with regions of hydrophilicity (data not shown). This secondary structure is common in proteins that belong to the sugar transport family (22) and generally have 11 or 12 discrete hydrophobic domains which are

TABLE 2. Total sucrase activities in *B. lactis* grown in the presence of various carbohydrates[a]

Growth phase	Enzyme in the presence of[b]:				
	Glucose	Glucose + Sucrose	Oligofructose	Sucrose	Raffinose
Mid-log	1.35 (0.3)	1.32 (0.3)	1.93 (0.2)	2.74 (0.3)	2.65 (0.3)
Late log	1.03 (0.2)	1.08 (0.2)	1.98 (0.2)	2.16 (0.6)	2.95 (0.6)

[a] Sucrase assays were performed in TAP buffer.
[b] Specific activity is expressed in micromoles of μmol reducing sugar per minute per milligram of protein. The values are means of two experiments, and standard deviations are indicated in parentheses.

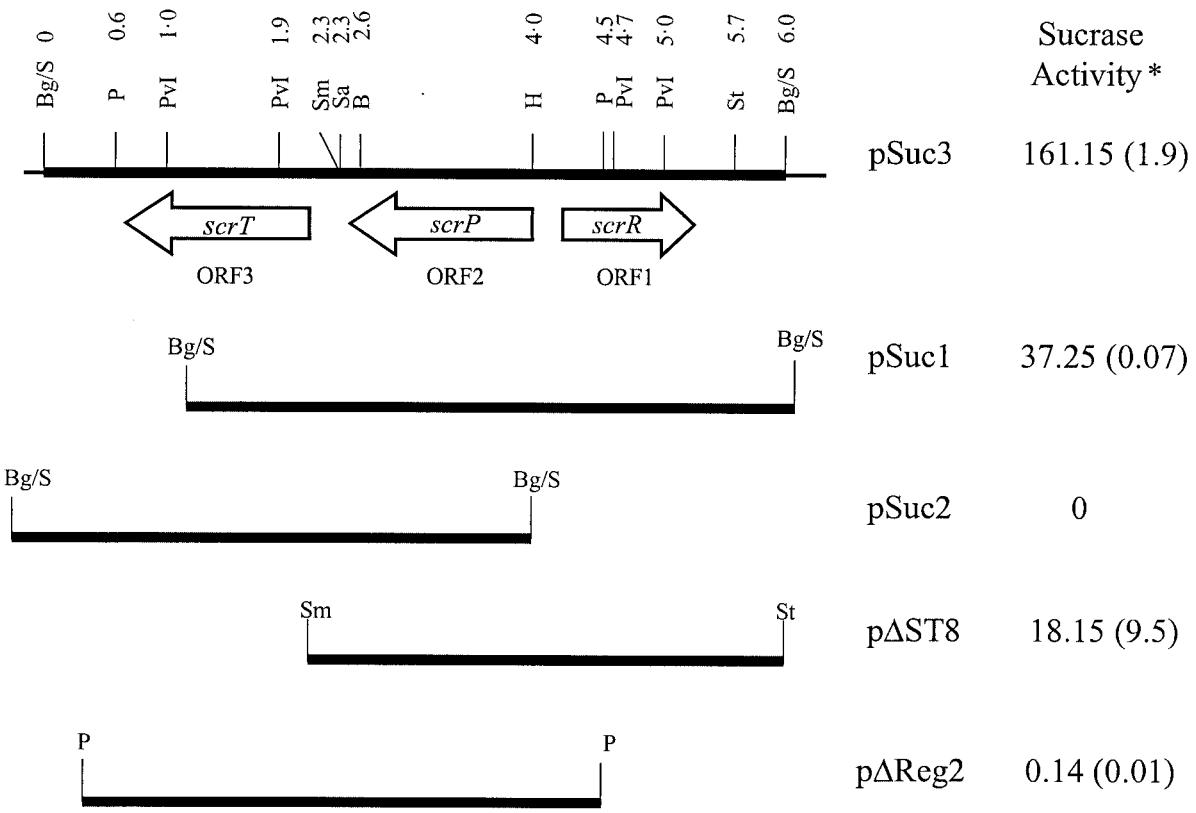


FIG. 2. Genetic organization of the *scr* genes of the *B. lactis* sucrose utilization system. Transcriptional polarities are indicated by arrows. The thick and thin lines represent insert and vector, respectively. Plasmids pSuc1 and pSuc2 originated from the *Sau*3A gene bank constructed in the *Bgl*II site of the vector pEcoR251. Plasmid pSuc3 was constructed from pSuc1 and pSuc2, and pΔST8 and pΔReg2 were subcloned from pSuc3 into pEcoR251. The sucrase activity conferred on *E. coli* by the constructs was measured in cell extracts and was expressed in micromoles of reducing sugar per minute per milligram of protein. Assays were performed in duplicate, and standard deviations are indicated in parentheses. Sizes (in kilobases) are indicated after the restriction enzyme abbreviations. 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.

potential transmembrane α-helices. ORF3 appeared to encode 11 domains and was designated *scrT*.

ORF1, which read divergently from ORF2, consisted of 978 bp encoding 326 amino acids with a calculated molecular mass of 35,860 Da. At the protein level, sequence identity was observed between the ORF1 product and the N-terminal regions of members of the GalR-LacI family of bacterial transcriptional regulators (29). Since this region of the GalR-LacI family has been implicated in DNA binding due to a helix-turn-helix motif (Fig. 3), these results suggested that the ORF1 product might also be involved in such interactions. Moreover, the ORF1 product also showed identity throughout the amino acid sequence to other sucrose regulators which also belong to the GalR-LacI family, including those of *Lactococcus lactis* (level of identity, 37%; accession no. Q04939), *S. mutans* (level of identity, 31%; accession no. Q54430), and *Pediococcus pentosaceus* (level of identity, 29%; accession no. P43472). A multiple-sequence alignment of these sucrose regulators with the ORF1 product is shown in Fig. 3, in which conserved regions other than the N-terminal region are shown. These conserved regions may be involved in sugar binding (50). Therefore, ORF1 was designated *scrR*. The alignment revealed a possible start codon, ATG, which is preceded by a potential Shine-Dalgarno sequence (AGGAGG) at positions −6 to −11 relative to the ATG (Fig. 4) (46). A stem-loop structure was detected 73 bp downstream of the stop codon, which might function as a rho-independent terminator; however, the T stretch is missing. Two direct repeats were identified (Fig. 4), which may be involved in promoter activity or could serve as potential protein binding sites for regulators (30).

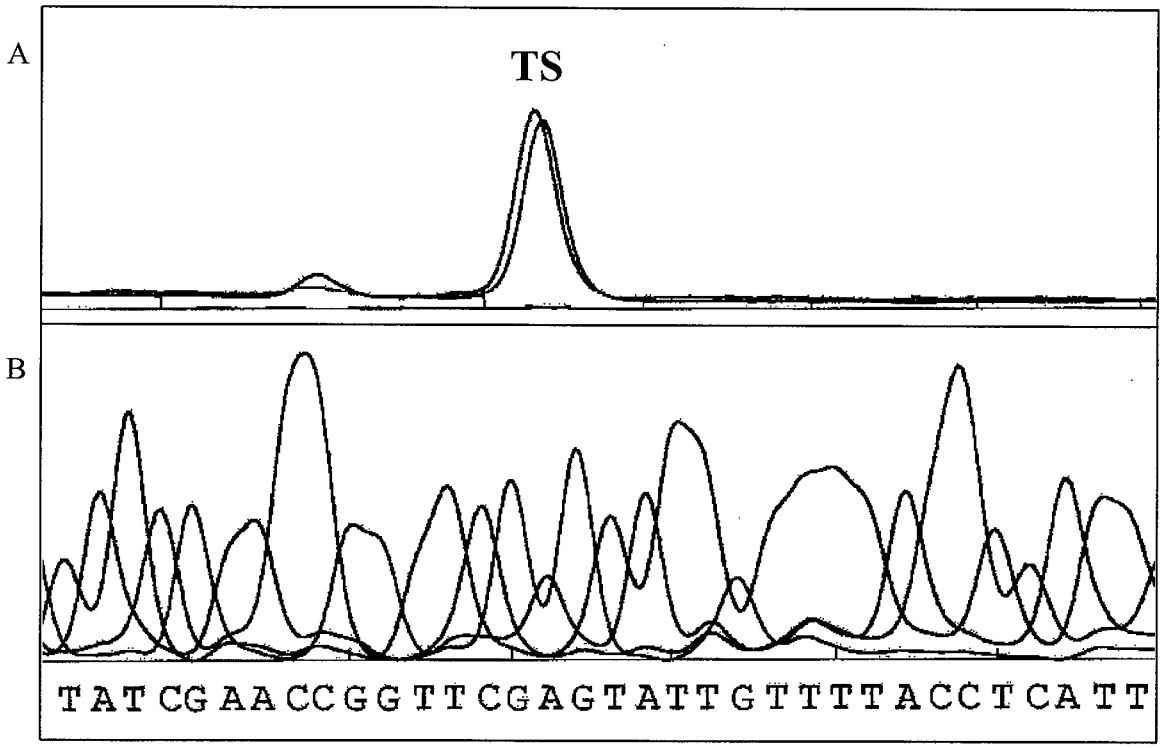
Transcriptional regulation of *scrP*. To determine the transcriptional start point, primer extension analyses were performed with RNA extracted from *B. lactis* cells grown in raffinose. A transcription start site was assigned to a T at position 102 bp in front of the *scrP* ATG start codon (Fig. 4). Screening for promoter consensus sequences did not reveal any typical *E. coli* −10 and −35 sequences. Examination of the adjacent sequence revealed a GGTAAA sequence 13 bp from the transcription start site. In *Corynebacterium glutamicum*, a gram-positive and high-G+C-content organism closely related to *Bifidobacterium*, this sequence has been shown to be a probable −10 sequence (31). A TTGCAT sequence in the −35 region was found, which might be involved in transcription initiation. An inverted repeat was identified at positions 1 to 14 relative to the transcriptional start site; this repeat could serve as a potential operator site for the ScrR regulator (50). This

FIG. 3. Multiple-sequence alignment of ScrR of *B. lactis* (BlscrR) and other sucrose regulators, including those of *S. mutans* (SmscrR) (accession no. Q54430), *L. lactis* (LlsacR) (accession no. Q04939), and *P. pentosaceus* (PpscrR) (accession no. P43472). Identical amino acids are indicated by asterisks, and similar amino acids are indicated by dots. The helix-turn-helix motif (enclosed in boxes) is conserved in all these proteins, which belong to the GalR-LacI family of regulators.

inverted repeat sequence also showed weak homology to the consensus sequence of catabolite response elements which control catabolite repression in gram-positive bacteria (20). This potential catabolite response element differed from the consensus sequence at the 2nd, 3rd, 12th, and 13th positions. A canonical gram-positive Shine-Dalgarno sequence, AGGAGG (46), was found 4 bp from the *scrP* ATG start codon (Fig. 4).

Sucrose phosphorylase activity conferred on *E. coli* by pSuc1. Analysis of enzyme activities in cell extracts prepared from *E. coli* cells carrying pSuc1 confirmed the presence of constitutive sucrose phosphorylase activity. It was found that if phosphate was present in the buffer (TAP buffer), the enzyme activity increased 30-fold (from 1.3 to 41.3 μmol min^{-1} mg^{-1}), suggesting that the phosphate component of the TAP buffer plays a role in the activity of this enzyme. This phosphate dependence is a property of such proteins (36, 47, 48), and a similar result was obtained for the *gtfA* product of *S. mutans* (37). Together with the amino acid homology search results, this confirms that the *scrP* product is a sucrose phosphorylase. Our investigation of the substrate specificity indicated that the sucrose phosphorylase could catalyze hydrolysis of sucrose but not hydrolysis of raffinose, melezitose, or inulin.

Regulation of expression of the *scrP*, *scrR*, and *scrT* genes in *B. lactis*. RNA slot blot analysis was performed to investigate the transcriptional regulation of the *scrP*, *scrR*, and *scrT* genes by various carbohydrates. RNA was extracted from *B. lactis* cells grown in BY medium containing one of the following: sucrose, raffinose, oligofructose, glucose, or glucose plus sucrose. Then the RNA was hybridized to probes specific for each gene (Fig. 5). Compared to the *scrP* mRNA levels in



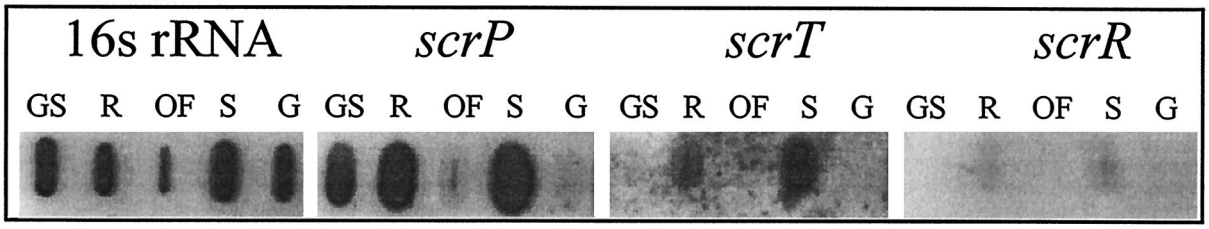
C

```
                                                    Primer Sp
                                                5'----------------
1                                                 GCAGAATGTCGGTCAT  16
                                                  CGTCTTACAGCCAGTA
      ------ 3'
 17   CGAAGCAAGGTTGCCGTCACCCAAGCGATCCGCATAGGTGATCAGCTGAACTTTGTTCTT  76
      GCTTCGTTCCAACGGCAGTGGGTTCGCTAGGCGTATCCACTAGTCGACTTGAAACAAGAA

                                                 EcoRV
 77   CATGGCTCCTCCTGGATTCGTCTATCACGTCTTCGGGATATCTCCGGGGCGATGCGTCTG  136
      GTACCGAGGAGGACCTAAGCAGATAGTGCAGAAGCCCTATAGAGGCCCCGCTACGCAGAC
← scrP      SD
        HindIII
137   CCTGAAGCTTCATCGTTGCGCGAGTGCCGTATCGAACCGGTTCGAGTATTGTTTTACCTC  196
      GGACTTCGAAGTAGCAACGCGCTCACGGCATAGCTTGGCCAAGCTCATAACAAAATGGAG
                                                      TS      -10
197   ATTCTTCACGCGCATGCAAGCGCTCTGCTCTCGGCGTATCAAACCGGTTCGATATTCGAC  256
      TAAGAAGTGCGCGTACGTTCGCGAGACGAGAGCCGCATAGTTTGGCCAAGCTATAAGCTG
                  -35
257   GATTCACCGCGCTCTGCCGAAACGCGGTTGCCGGAGGACGAAATCTGCACATACAATCGG  316
      CTAAGTGGCGCGAGACGGCTTTGCGCCAACGGCCTCCTGCTTTAGACGTGTATGTTAGCC
                                                                SD
317   ATAGTGCTCGTCAAACCGGTTCGATACCGCGGGCGATAGCGCACGGAGCAGAAAATGAGG  376
      TATCACGAGCAGTTTGGCCAAGCTATGGCGCCCGCTATCGCGTGCCTCGTCTTTTACTCC
            scrR →
377   AGGCGAACATG
      TCCGCTTGTAC
```

FIG. 4. Mapping of the transcription start site of the *B. lactis scrP* gene by primer extension analysis. (A) Primer extension products obtained by using RNA extracted from *B. lactis* grown in BY medium containing sucrose. (B) DNA sequencing fluorogram corresponding to the region analyzed. The Cy5-labeled SP primer was used for both the primer extension and sequencing reactions. (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 indicated by boldface type and underlining. The transcriptional start site (TS) of *scrP*, as determined by primer extension analysis, is indicated by boldface type. The arrows indicate the directions of the direct and indirect repeat sequences. The indirect repeat is a perfect palindrome which may act as an operator site for a GalR-LacI family regulatory protein.

A



B

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

FIG. 5. RNA slot blot analysis of *scrP*, *scrT*, and *scrR* mRNA in mid-logarithmic-phase cells of *B. lactis* grown in different carbon sources. (A) Gene-specific mRNA and 16S rRNA signals detected by slot blot analysis. (B) mRNA levels expressed as ratios of the gene-specific hybridization signal to the 16S rRNA hybridization signal. Experiments were performed in duplicate, and standard deviations are indicated in parentheses. GS, glucose plus sucrose; R, raffinose; OF, oligofructose; S, sucrose; G, glucose; ND, no gene-specific signal detected.

glucose-grown cultures, the *scrP* mRNA levels were approximately 6-fold greater in the presence of sucrose or raffinose and 1.25-fold greater in the presence of oligofructose. In the presence of glucose and sucrose, the activity was increased only approximately fourfold, indicating that there was glucose repression. For *scrR* and *scrT*, mRNA signals were present only under inducing conditions in the presence of sucrose or raffinose.

Regulation of *scrP* by *scrR* in *E. coli*. In order to assign a regulatory function to *scrR*, the following constructs were designed (Fig. 2). *B. lactis* DNA from pSuc1 and pSuc2 was ligated at the *Bam*HI site to obtain pSuc3. The 3.2-kb *Sma*I-*Stu*I and 3.9-kb *Pst*I fragments from pSuc3 were cloned into the *Bgl*II site of pEcoR251, resulting in plasmids pΔST8 and pΔReg2, respectively, which had the *scrT* gene and a portion of the *scrR* gene encoding 245 amino acids deleted, respectively (Fig. 2). Growth of *E. coli* harboring constructs pSuc1, pSuc3, and pΔST8 resulted in loss of the plasmid when the organisms were cultured in minimal media and during late-logarithmic-phase growth in LB broth. *E. coli*(pSuc3) was unable to grow in LB broth containing 1% sucrose. Sucrase activity was, therefore, determined by using mid-logarithmic-phase cells cultured in LB broth, which had been confirmed to contain the plasmid. The results are shown in Fig. 2. Vector plasmid pMT104 did not confer sucrase activity in *E. coli. E. coli* cells carrying pSuc2 were unable to hydrolyze sucrose since the *scrP* promoter and the ATG start codon were not present on the insert. The activity was highest with pSuc3, when all three genes were present. Activity was reduced 1,000-fold with pΔReg2, in which the *scrR* gene was truncated, and was reduced 4- and 8-fold with pSuc1 (*scrT* truncated) and pΔST8 (*scrT* deleted), respectively.

DISCUSSION

Bifidobacteria play a large role in the fermentation of carbohydrates in the colon, and inulin, oligofructose, and raffinose have been implicated as bifidogenic factors (15, 16, 19). Our aim was to investigate the genetic systems involved in the utilization of carbohydrate substrates in order to understand the growth and survival of bifidobacteria in the intestine. Screening for genes involved in the utilization of sucrose, which contains the α(1,2) bond between glucose and fructose and is common in the three bifidogenic carbohydrates, resulted in isolation of a cluster of sucrose utilization genes. Nucleotide sequence analysis indicated that three genes were cloned from *B. lactis*; these genes, *scrP*, *scrR*, and *scrT*, encode a sucrose phosphorylase, a sucrose regulator, and a transport protein, respectively. To our knowledge, this cluster of sucrose utilization genes is the first cluster of sucrose utilization genes isolated from *Bifidobacterium*.

For the majority of gram-positive bacteria, the predominant mechanism that facilitates sucrose uptake is the phosphoenolpyruvate-dependent phosphotransferase system (39), which results in intracellular accumulation of sucrose 6-phosphate. The sucrose 6-phosphate is metabolized further, yielding glucose 6-phosphate and fructose. In organisms that do not possess active phosphotransferase systems, sucrose hydrolysis could imply that there is either an invertase or a sucrose phosphorylase (11). Catabolism of sucrose by sucrose phosphorylases

results in the products α-D-glucose 1-phosphate and D-fructose (47, 48). The deduced amino acid sequence of the *scrP* gene product of *B. lactis* exhibits identity to the sequences of sucrose phosphorylases from both gram-positive and gram-negative bacteria. It is likely, therefore, that glucose 1-phosphate is generated by the ScrP protein, which is then converted by glucose-6-phosphate isomerase for utilization in the D-fructose 6-phosphate shunt typical of bifidobacteria (4). Further analysis of the products generated from ScrP activity is being undertaken to confirm the mode of action.

Expression of sucrose catabolic genes is in most cases regulated at the transcriptional level (24). Analysis of the *scrP* mRNA levels indicated that there was sixfold induction in the presence of sucrose or raffinose compared to the mRNA levels in the presence of glucose (Fig. 5). This was reduced to fourfold induction in cells grown in glucose plus sucrose. Transcription of the *scrR* and *scrT* genes was induced similarly by sucrose and raffinose and could not be detected in glucose-grown cells (Fig. 5). Total sucrase activity in *B. lactis* was also induced by sucrose and raffinose and repressed by glucose (Table 2). These results indicate that transcriptional regulation plays a role in the control of *scrP*, *scrT*, and *scrR* expression and that these genes are subject to glucose repression.

The development of cloning vectors and transformation techniques has not yet progressed sufficiently to enable genetic manipulations, such as site-directed mutagenesis, to be performed with *B. lactis*. The regulation of *scrP* by *scrR* was, therefore, determined in *E. coli* by using plasmid deletion constructs. The activity was highest in cells with construct pSuc3 (Fig. 2), in which *scrP*, *scrT*, and *scrR* were all present. Since *scrP* is oriented in the direction opposite that of the λ promoter of the vector, it is likely that the genes are expressed from their own promoters in *E. coli*. Phosphorylase activity was almost eliminated in cells with pΔReg2, which contained the entire *scrR-scrP* intergenic region and the truncated *scrR* gene, indicating that ScrR could function as a positive regulator. Although cells with pSuc1 and pΔST8 exhibited significant sucrase activities, the levels were greatly reduced compared to the level in cells containing pSuc3. This suggests that ScrT, at least in the heterologous system of *E. coli*, might also contribute to the activity of ScrP.

Therefore, by analogy with other GalR-LacI-like regulatory proteins, the mode of action of ScrR could be as follows: in the presence of sucrose or raffinose, transcription is induced by binding to palindromic operator sites. A potential operator site was identified next to the transcription start site of *scrP* (Fig. 4). It is possible that this operator sequence, which has similarity to the consensus sequence of catabolite response element sites, might also be a weak target for regulatory protein CcpA, which mediates catabolite repression in gram-positive bacteria (17, 20, 38).

It is interesting that the amino acid sequence encoded by *scrR* exhibits significant homology with the sequences of sucrose regulators of *L. lactis*, *S. mutans*, and *P. pentosaceus*. In a phylogenetic analysis of sucrose regulators (33), it was reported that within the GalR-LacI family, proteins involved in the regulation of sucrose catabolic genes have evolved separately at least four times. The *B. lactis* ScrR protein, therefore, falls in the cluster consisting of the *S. mutans* and *P. pentosaceus* proteins. However, unlike the genes in these organisms, the *scrR* gene of *B. lactis* is not associated with sucrose phosphotransferase system and hydrolase genes. In *P. pentosaceus*, *L. lactis*, and *S. mutans*, the *scrR* gene is cotranscribed with the *scrB* gene (sucrose-6-phosphate hydrolase), and the *scrA* gene (enzyme II^{Scr}) is transcribed from the opposite DNA strand (18, 24, 28, 32, 43). In *L. lactis* and *P. pentosaceus* these sucrose utilization genes are located on plasmids or transposons (28, 32), and therefore horizontal gene transfer from these organisms to *B. lactis* could have occurred.

The rate at which an organism can grow on a particular carbon source influences its ability to compete with other bacteria in the colon and, therefore, determines whether a particular substrate can be used as a prebiotic (19). Although inulin and oligofructose are considered good prebiotics for stimulation of bifidobacteria in the intestine, these substrates were unable to support significant growth of the *B. lactis* strain isolated in this study. It has been reported, however, that *B. lactis* Lafti B94 is able to utilize inulin and fructooligosaccharides (8). The carbohydrate utilization patterns within a bifidobacterial strain have been shown to vary considerably (19), which also seems to be the case with the species *B. lactis*. These results indicate that raffinose-containing substrates, such as soybean oligosaccharides (9, 40), might be better candidates for stimulation of *B. lactis* growth.

ACKNOWLEDGMENTS

We thank Darleon Distribution for providing the yoghurt starter cultures and D. M. James for performing DNA sequencing.

This work was supported by a research grant from the National Research Foundation, South Africa.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Ballongue, J.** 1998. Bifidobacteria and probiotic action, p. 519–587. *In* S. Salminen and A. Von Wright (ed.), Lactic acid bacteria. Microbiology and functional aspects. Marcel Dekker, Inc., New York, N.Y.
3. **Beg, O. U.** 1995. Extraction of RNA from Gram-positive bacteria. BioTechniques **19:**880–884.
4. **Bezkorovainy, A.** 1989. Classification of bifidobacteria, p. 1–28. *In* R. Miller-Catchpole and A. Bezkorovainy (ed.), Biochemistry and physiology of bifidobacteria. CRC Press, Boca Raton, Fla.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Cai, Y., M. Matsumoto, and Y. Benno.** 2000. *Bifidobacterium lactis* Meile et al. 1997 is a subjective synonym of *Bifidobacterium animalis* (Mitsuoka 1969) Scardovi and Trovatelli 1974. Microbiol. Immunol. **44:**815–820.
7. **Chiang, B. L., Y. H. Sheih, L. H. Wang, C. K. Liao, and H. S. Gill.** 2000. Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (*Bifidobacterium lactis* HN019): optimisation and definition of cellular immune responses. Eur. J. Clin. Nutr. **54:**849–855.
8. **Crittenden, R. G., L. F. Morris, M. L. Harvey, L. T. Tran, H. L. Mitchell, and M. J. Playne.** 2001. Selection of a *Bifidobacterium* strain to complement resistant starch in a synbiotic yoghurt. J. Appl. Microbiol. **90:**268–278.
9. **Crittenden, R. G., and M. J. Playne.** 1996. Production, properties and applications of food-grade oligosaccharides. Trends Food Sci. Technol. **7:**353–361.
10. **Degnan, B. A., and G. T. Macfarlane.** 1993. Transport and metabolism of glucose and arabinose in *Bifidobacterium breve.* Arch. Microbiol. **160:**144–151.
11. **Dols, M., W. Chraibi, M. Remaud-Simeon, N. D. Lindley, and P. F. Monsan.** 1997. Growth and energetics of *Leuconostoc mesenteroides* NRRL B-1299 during metabolism of various sugars and their consequences for dextransucrase production. Appl. Environ. Microbiol. **63:**2159–2165.
12. **Eikmanns, B. J., N. Thumb-Shmitz, L. Eggeling, K. U. Lüdtke, and H. Sahm.** 1994. Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. Microbiology **140:**1817–1828.
13. **Fukushima, Y., S.-T. Li, H. Hara, A. Terada, and T. Mitsuaoka.** 1997. Effect

of follow-up formula containing bifidobacteria (NAN BF) on fecal flora and fecal metabolites in healthy children. Biosci. Microflora **16:**65–72.
14. **Gibson, G. R., and M. B. Roberfroid.** 1995. Dietary modulation of the colonic microbiota: introducing the concept of prebiotics. J. Nutr. **125:**1401–1412.
15. **Gibson, G. R., and X. Wang.** 1994. Bifidogenic properties of different types of fructooligosaccharides. Food Microbiol. **11:**491–498.
16. **Gibson, G. R., and X. Wang.** 1994. Selective enrichment of bifidobacteria from human gut contents by oligofructose using continuous culture. FEMS Lett. **118:**121–128.
17. **Henkin, T. M., F. J. Grundy, W. L. Nicholson, and G. H. Chambliss.** 1991. Catabolite repression of the α-amylase gene expression in *Bacillus subtilis* involves a trans-acting gene product homologous to the *Escherichia coli lacI* and *galR* repressors. Mol. Microbiol. **5:**575–584.
18. **Hiratsuka, K., B. Wang, Y. Sato, and H. Kuramitsu.** 1998. Regulation of sucrose-6-phosphate hydrolase activity in *Streptococcus mutans*: characterization of the *scrR* gene. Infect. Immun. **66:**3736–3743.
19. **Hopkins, M. J., J. H. Cummings, and G. T. Macfarlane.** 1998. Inter-species differences in maximum specific growth rates and cell yields of bifidobacteria cultured on oligosaccharides and other simple carbohydrate sources. J. Appl. Microbiol. **85:**381–386.
20. **Hueck, C. J., W. Hillen, and M. H. Saier, Jr.** 1994. Analysis of a *cis*-active sequence mediating catabolite repression in gram-positive bacteria. Res. Microbiol. **145:**503–518.
21. **Klaver, F. A., and R. van der Meer.** 1993. The assumed assimilation of cholesterol by lactobacilli and *Bifidobacterium bifidum* is due to their bile salt-deconjugating activity. Appl. Environ. Microbiol. **59:**1120–1124.
22. **Kruckeberg, A. L.** 1996. The hexose transporter family of *Saccharomyces cerevisiae*. Arch. Microbiol. **166:**283–292.
23. **Kurmann, J. A., and J. L. Rasic.** 1991. The health potential of products containing bifidobacteria, p. 117–157. *In* R. Robinson (ed.), Therapeutic properties of fermented milks. Elsevier Applied Science, London, United Kingdom.
24. **Luesink, E. J., J. D. Marugg, O. P. Kuipers, and W. M. de Vos.** 1999. Characterization of the divergent *sacBK* and *sacAR* operons, involved in sucrose utilization by *Lactococcus lactis*. J. Bacteriology. **181:**1924–1926.
25. **Meile, L., L. M. Rohr, T. A. Geissmann, M. Herensperger, and M. Teuber.** 2001. Characterization of the D-xylulose 5-phosphate/D-fructose-6-phosphate phosphoketolase gene (*xfp*) from *Bifidobacterium lactis.* J. Bacteriol. **183:**2929–2936.
26. **Miller. G. L.** 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31:**426–428.
27. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Naumoff, D. G., and V. A. Livshits.** 2001. Molecular structure of the *Lactobacillus plantarum* sucrose utilization locus: comparison with *Pediococcus pentosaceus*. Mol. Biol. **35:**15–22.
29. **Nguyen, C. C., and M. H. Saier, Jr.** 1995. Phylogenetic, structural and functional analyses of the LacI-GalR family of bacterial transcription factors. FEBS Lett. **377:**98–102.
30. **Ouyang, S., S. Sau, and C. Y. Lee.** 1999. Promoter analysis of the *cap8* operon, involved in type 8 capsular polysaccharide production in *Staphylococcus aureus.* J. Bacteriol. **181:**2492–2500.
31. **Patek, M., B. J. Eikmanns, J. Patek, and H. Sahm.** 1996. Promoters from *Corynebacterium glutamicum*: cloning, molecular analysis and search for a consensus motif. Microbiology **142:**1297–1309.
32. **Rauch, P. J. G., and W. M. de Vos.** 1992. Transcriptional regulation of the Tn5276-located *Lactococcus lactis* sucrose operon and characterisation of the *sacA* gene encoding sucrose-6-phosphate hydrolase. Gene **121:**55–61.
33. **Reid, S. J., M. S. Rafudeen, and N. G. Leat.** 1999. The genes controlling sucrose utilization in *Clostridium beijerinckii* NCIMB 8052 constitute an operon. Microbiology **145:**1461–1471.
34. **Roberfroid, M.** 1999. Concepts in functional foods: the case of inulin and oligofructose. J. Nutr. **129:**1398S-1401S.
35. **Rumney, C. J., and I. R. Rowland.** 1995. Non-digestible oligosaccharides—potential anti-cancer agents? Br. Nutr. Found. Nutr. Bull. **20:**194–203.
36. **Russell, R. R. B., J. Aduse-Opoku, I. C. Sutcliffe, L. Tao, and J. J. Ferretti.** 1992. A binding protein-dependant transport system in *Streptococcus mutans* responsible for multiple sugar metabolism. J. Biol. Chem. **267:**4631–4637.
37. **Russell, R. R. B., H. Mukasa, A. Shimamura, and J. J. Ferretti.** 1988. *Streptococcus mutans gtfA* gene specifies sucrose phosphorylase. Infect. Immun. **56:**2763–2765.
38. **Saier, M. H., Jr., S. Chauvaux, G. M. Cook, J. Deutscher, I. T. Paulsen, J. Reizer, and J.-J. Ye.** 1996. Catabolite repression and inducer control in gram-positive bacteria. Trends Biochem Sci **20:**267–271.
39. **Saier, M. H., Jr., and J. Reizer.** 1992. Proposed uniform nomenclature for the proteins and protein domains of the bacterial phosphoenolpyruvate: sugar phosphotransferase system. J. Bacteriol. **174:**1433–1438.
40. **Saito, Y., T. Takano, and I. Rowland.** 1992. Effects of soybean oligosaccharides on the human gut microflora in *in vitro* culture. Microb. Ecol. Health Dis. **5:**105–110.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
43. **Sato, Y., F. Poy, G. R. Jacobson, and H. K. Kuramitsu.** 1989. Characterization and sequence analysis of *scrA* gene encoding enzyme II^{Scr} of the *Streptococcus mutans* phosphoenolpyruvate-dependant sucrose phosphotransferase system. J. Bacteriol. **171:**263–271.
44. **Scardovi, V.** 1986. Genus *Bifidobacterium*, p. 1418–1434. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. Williams and Wilkins, Baltimore, Md.
45. **Tanaka, H., H. Hashiba, J. Kok, and I. Mierau.** 2000. Bile salt hydrolase of *Bifidobacterium longum*—biochemical and genetic characterization. Appl. Environ. Microbiol. **66:**2502–2512.
46. **Vellanoweth, R. L.** 1993. Translation and its regulation, p. 699–711. *In* A. Sonenshein, J. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
47. **Voet, J. G., and R. H. Abeles.** 1966. The occurrence of a glucose-enzyme complex in the reaction catalysed by sucrose phosphorylase. J. Biol. Chem. **241:**2731–2736.
48. **Voet, J. G., and R. H. Abeles.** 1970. The mechanism of action of sucrose phosphorylase. J. Biol. Chem. **245:**1020–1031.
49. **Wehnert, G. U., V. R. Abratt, H. J. K. Goodman, and D. R. Woods.** 1990. Cloning of *Bacteroides fragilis* plasmid genes affecting metronidazole resistance and ultraviolet survival in *Escherichia coli*. Plasmid **23:**155–158.
50. **Weickert, M. J., and S. Adhya.** 1992. A family of bacterial regulators homologues to Gal and Lac repressors. J. Biol. Chem. **267:**15869–15874.
51. **Yanisch-Peron, D., J. Viera, and J. Messing.** 1985. Improved M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
52. **Yazawa, K., and Z. Tamura.** 1982. Search for sugar sources for the selective increase of bifidobacteria. Bifidobacteria Microflora **1:**39–44.
53. **Zabeau, M., and K. K. Stanley.** 1982. Enhanced expression of cro-β-galactosidase fusion proteins under the control of the P_R promoter of the bacteriophage lambda. EMBO J. **1:**1217–1224.