

Transcription of Two Adjacent Carbohydrate Utilization Gene Clusters in *Bifidobacterium breve* UCC2003 Is Controlled by LacI- and Repressor Open Reading Frame Kinase (ROK)-Type Regulators

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Members of the genus *Bifidobacterium* are commonly found in the gastrointestinal tracts of mammals, including humans, where their growth is presumed to be dependent on various diet- and/or host-derived carbohydrates. To understand transcriptional control of bifidobacterial carbohydrate metabolism, we investigated two genetic carbohydrate utilization clusters dedicated to the metabolism of raffinose-type sugars and melezitose. Transcriptomic and gene inactivation approaches revealed that the raffinose utilization system is positively regulated by an activator protein, designated RafR. The gene cluster associated with melezitose metabolism was shown to be subject to direct negative control by a LacI-type transcriptional regulator, designated MelR1, in addition to apparent indirect negative control by means of a second LacI-type regulator, MelR2. *In silico* analysis, DNA-protein interaction, and primer extension studies revealed the MelR1 and MelR2 operator sequences, each of which is positioned just upstream of or overlapping the correspondingly regulated promoter sequences. Similar analyses identified the RafR binding operator sequence located upstream of the *rafB* promoter. This study indicates that transcriptional control of gene clusters involved in carbohydrate metabolism in bifidobacteria is subject to conserved regulatory systems, representing either positive or negative control.

Bifidobacteria are Gram-positive, anaerobic bacteria that possess a high GC genome content and belong to the phylum *Actinobacteria*. Bifidobacteria typically exhibit a forked (bifid) morphology and are commonly found as anaerobic commensals of the gastrointestinal tract (GIT) (1, 2). Bifidobacteria were first isolated as natural inhabitants of the gastrointestinal tracts of humans and mammals by Tissier (3), who was also the first to recognize their probiotic or health-promoting potential (4). Consistent with Tissier's observations, it is known that members of certain bacterial genera, such as bifidobacteria and lactobacilli, provide protection against certain types of gastrointestinal infections (5, 6).

It is also becoming increasingly apparent that a "normal" (or healthy) colonic microbiota may reduce the risk and/or pathology of gastrointestinal diseases and disorders, such as ulcerative colitis, bowel cancer, and pseudomembranous colitis (7–9). Several recent studies have focused on the use of prebiotics, which are non-digestible food ingredients that beneficially affect the host by selectively stimulating growth and/or activity of one or a limited number of beneficial bacteria in the colon (10), thereby improving host health. Certain carbohydrates, including whole-grain wheat, fructo-oligosaccharides, galacto-oligosaccharides, and lactulose, have been shown to exert prebiotic effects (11–14).

Metagenomic analyses of the human gut microbiota have generated knowledge that may allow the rational selection of novel prebiotics that maintain and/or enhance a healthy gut microbiota (15). Integral to the selection of carbohydrates with prebiotic potential is an in-depth understanding of carbohydrate metabolism by members of the gut microbiota. In this respect, bifidobacteria possess a unique hexose metabolism, the bifid shunt, a metabolic pathway that employs the signature enzyme fructose 6-phosphate

phosphoketolase (F6PPK), which converts carbohydrates to mainly acetic and lactic acids (16).

Our recent work has established that *Bifidobacterium breve* UCC2003, an isolate from nursing stool, can metabolize a remarkable range of mono-, di-, oligo-, and polysaccharides (17–23). Bifidobacterial gene clusters associated with carbohydrate metabolism have in several cases been shown to be regulated by LacI-type repressors, representing LacI/GalR family proteins, which typically consist of an N-terminal helix-turn-helix (HTH) DNA-binding motif and a C-terminal domain for oligomerization and effector binding (24, 25). Furthermore, several observations have been made suggesting that such clusters are subject to carbon catabolite control (26).

Recently, we identified a *raf* gene cluster dedicated to the metabolism of the plant-derived carbohydrates raffinose [α -D-Galp-(1→6)- α -D-Glcp-(1→2) β -D-Fruf], stachyose [α -D-Galp-(1→6)- α -D-Galp-(1→6)- α -D-Glcp-(1→2) α -D-Fruf], and melibiose [α -D-Galp-(1→6)- α -D-Glcp] in *B. breve* UCC2003 (20). The *raf* locus in the *B. breve* UCC2003 genome is located adjacent to the *mel* gene cluster, which allows the strain to metabolize the trisaccharide melezitose [α -D-Glcp-(1→3)- β -D-Fruf-(2→1)- α -

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TABLE 1 Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Reference or source
<i>E. coli</i>		
EC101	Cloning host; <i>repA</i> ⁺ <i>kmr</i>	44
EC101-pNZ-M.Bbrll+Bbr111	EC101 harboring a pNZ8048 derivative containing BbrllM and BbrlllM proteins	40
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacZ</i> ^Δ M15 Tn10 (Tet ^r)]	Stratagene
<i>L. lactis</i>		
NZ9000	MG1363, nisin-inducible overexpression host; <i>pepN::nisRK</i>	62
NZ9700	Nisin-producing strain	62
<i>B. breve</i>		
UCC2003	Isolate from nursing stool	19
UCC2003- <i>melR1</i>	pORI19-tet- <i>melR1</i> insertion mutant of UCC2003	This study
UCC2003- <i>melR2</i>	pORI19-tet- <i>melR2</i> insertion mutant of UCC2003	This study
UCC2003- <i>rafR</i>	pORI19-tet- <i>rafR</i> insertion mutant of UCC2003	This study
Plasmids		
pORI19	Em ^r Δ <i>repA ori</i> ⁺ ; cloning vector	44
pORI19-tet- <i>melR1</i>	Internal 421-bp fragment of <i>melR1</i> and <i>tetW</i> cloned in pORI19	This study
pORI19-tet- <i>melR2</i>	Internal 456-bp fragment of <i>melR2</i> and <i>tetW</i> cloned in pORI19	This study
pORI19-tet- <i>rafR</i>	Internal 331-bp fragment of <i>rafR</i> and <i>tetW</i> cloned in pORI19	This study
pAM5	pBC1-puC19-Tcr	45
pNZ8048	Cm ^r ; nisin-inducible translational-fusion vector	62
pNZ8150	Cm ^r ; nisin-inducible translational-fusion vector	42
PQE30	Amp ^r ; IPTG-inducible vector	Qiagen
pNZ-MelR1-His	<i>melR1</i> with His tag cloned downstream of nisin-inducible promoter on pNZ8048	This study
pNZ-MelR2-His	<i>melR2</i> with His tag cloned downstream of nisin-inducible promoter on pNZ8150	This study
pQE30-RafR	<i>rafR</i> cloned into the IPTG-inducible vector PQE30	This study

D-Glcp], found in honeydew and manna, which are sugar-rich liquid and solid deposits, respectively, associated with leaves and branches of various trees and shrubs (20, 27). It was initially hypothesized that melezitose is an oligosaccharide that is naturally present in various plants (28). However, it was later found that certain insects are responsible for melezitose production, when they use carbohydrates present in tree sap, by itself devoid of melezitose, to form honeydew (27). In the current work, we investigated the transcriptional regulation of the gene clusters associated with the metabolism of the raffinose family sugars and melezitose.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are detailed in Table 1. Bifidobacteria were routinely cultured in either de Mann Rogosa and Sharpe medium (MRS) (Difco, BD, Le Pont de Claix, France) supplemented with 0.05% cysteine-HCl or reinforced clostridial medium (RCM) (Oxoid Ltd.). Carbohydrate utilization by bifidobacterial strains was examined in modified de Mann Rogosa and Sharpe medium (mMRS) prepared from first principles (29) and to which, prior to inoculation, cysteine-HCl (0.05% [wt/vol]) and a particular carbohydrate source (1% [wt/vol]) had been added. The carbohydrates used were raffinose, stachyose, melibiose, melezitose, and lactose (all purchased from Sigma-Aldrich, Steinheim, Germany). Bifidobacterial cultures were incubated at 37°C under anaerobic conditions, which were maintained using an Anaerocult oxygen-depleting system (Merck, Darmstadt, Germany) in an anaerobic chamber with an atmosphere of 5% CO₂, 5% H₂, 90% N₂. *Lactococcus lactis* strains were cultivated in M17 broth containing 0.5% glucose (30) at 30°C. *Escherichia coli* strains were cultured in Luria-Bertani broth (LB) (31) at 37°C with agitation. Where appropriate, growth media contained chloramphenicol (Cm) (5 μ g ml⁻¹ for *L. lactis*, 10 μ g ml⁻¹ for *E. coli*, and 2.5 μ g ml⁻¹ for *B. breve*), erythromycin (Em) (100 μ g ml⁻¹ for *E. coli*), tetracycline (Tet) (10

μ g ml⁻¹ for *E. coli* or *B. breve*), kanamycin (Km) (50 μ g ml⁻¹ for *E. coli*), or ampicillin (Amp) (100 μ g ml⁻¹ for *E. coli*).

In order to determine bacterial growth profiles and final optical densities, 5 ml of freshly prepared mMRS including a particular carbohydrate (see above) was inoculated with 50 μ l (1%) of a stationary-phase culture of a particular *B. breve* strain. Uninoculated mMRS was used as a negative control. The cultures were incubated anaerobically at 37°C for 16 h, and the optical density at 600 nm (OD₆₀₀) was determined at 30-min intervals using a Powerwave microplate spectrophotometer (BioTek Instruments, Inc., USA) in conjunction with Gen5 Microplate software for Windows.

Nucleotide sequence analysis. Sequence data were obtained from the Artemis-mediated (32) genome annotations of *B. breve* UCC2003 (33). Database searches were performed using nonredundant sequences accessible at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) using BLAST (34, 35). Sequence verification and analysis were performed using the Seqman and Seqbuilder programs of the DNASTAR software package v10.1.2 (DNASTAR, Madison, WI, USA). Operator sequences for RafR and MelR1/2 in other bifidobacterial species were detected using MEME (36), and consensus sequences were visualized using Weblogo (37). Regions 250 bp upstream and 50 bp downstream of the translation start site of *B. breve* UCC2003 genes were searched for additional RafR- and LacI-type binding sites using HMMSEARCH (38) with models built from all detected bifidobacterial RafR recognition sites and from known bifidobacterial LacI-type repressor binding motifs (18, 22). Putative binding sites present in the upstream regions of genes in the vicinity of genes coding for predicted LacI-type regulators were extracted and used to build an improved model to find LacI-type repressor binding sites in *B. breve* UCC2003. Genes coding for LacI-type regulators were searched by screening the encoded proteins for the presence of the PF00356 hidden Markov model (HMM). Distances between binding sites were determined using ClustalW.

DNA manipulations. Chromosomal DNA was isolated from bifidobacteria as previously described (39). Minipreparation of plasmid DNA from *E. coli* or *L. lactis* was achieved using the Qiaprep spin plasmid miniprep kit (Qiagen GmbH, Hilden, Germany). For *L. lactis*, an initial lysis step was incorporated into the plasmid isolation procedure by resuspending cells in lysis buffer supplemented with lysozyme (30 mg ml⁻¹), followed by incubation at 37°C for 30 min (31). Procedures for DNA manipulations were performed essentially as described previously. Restriction enzymes and T4 DNA ligase were used according to the supplier's instructions (Roche Diagnostics, East Sussex, United Kingdom). The synthetic single-stranded oligonucleotide primers used in this study, detailed in Table S1 in the supplemental material, were synthesized by Eurofins (Ebersberg, Germany). Standard PCRs were performed using TaqPCR mastermix (Qiagen), while *B. breve* colony PCRs were performed as described previously (40). PCR fragments were purified using the Qiagen PCR purification kit (Qiagen). Electroporation of plasmid DNA into *E. coli* was performed as previously described (31). Electrotransformation of *B. breve* UCC2003 (19) and *L. lactis* (41) was performed according to published protocols. The correct orientation and integrity of all constructs were verified by DNA sequencing, performed by Eurofins (Ebersberg, Germany).

Construction of plasmids pNZ-MelR1-His, pNZ-MelR2-His, and pQE30-RafR. DNA fragments containing the complete coding regions of *Bbr_1864* (here designated *melR1*) and *Bbr_1863* (here designated *melR2*) were generated by PCR amplification employing chromosomal DNA of *B. breve* UCC2003 as a template, employing *PFU* Ultra DNA polymerase (Agilent Technologies) and primer combinations *melR1EcoRV* and *melR1Xba1R*, and *melR2Nco1F* and *melR2Xba1R*, respectively (see Table S1 in the supplemental material). *NcoI* or *EcoRV* and *XbaI* restriction sites were incorporated at the 5' ends of each forward and reverse primer combination, respectively, for the *melR1*- and *melR2*-encompassing primers (see Table S1 in the supplemental material). In addition, an in-frame His₁₀-encoding sequence was incorporated into each of the forward primers to facilitate downstream protein purification using the Ni-nitrilotriacetic acid (NTA) affinity system (Qiagen). The *melR1*- and *melR2*-encompassing amplicons were digested with *NcoI/EcoRV* and *XbaI* and ligated into the *NcoI/SacI* and *XbaI*-digested nisin-inducible translational fusion plasmids pNZ8048 and pNZ8150, respectively (42). The ligation mixtures were introduced into *L. lactis* NZ9000 (Table 1) by electrotransformation, and transformants were then selected based on chloramphenicol resistance (Cm^r) (5 µg ml⁻¹ for *L. lactis*). The plasmid contents of a number of Cm^r transformants were screened by restriction analysis, and the integrity of positively identified recombinant *melR1*- or *melR2*-containing plasmids was verified by sequencing, resulting in constructs pNZ-MelR1-His and pNZ-MelR2-His, respectively.

The coding region of the predicted repressor open reading frame kinase (ROK)-type regulator-encoding gene *Bbr_1868* (here designated *rafR*) was PCR amplified by employing *Taq* DNA polymerase and chromosomal DNA of *B. breve* UCC2003 as a template and the primer combination ROKBglIIF and ROKPst1R, which had *BglII* and *PstI* restriction sites incorporated at the 5' ends of the forward and reverse primers, respectively (see Table S1 in the supplemental material). The *rafR*-encompassing fragment generated was digested with *BglII* and *PstI* and ligated to similarly digested pQE30 (Qiagen), an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible protein expression plasmid, which allows incorporation of a His tag into the N terminus of the expressed protein target. The ligation mixture was introduced into *E. coli* XL1-Blue (Table 1) by electrotransformation, and transformants were selected based on ampicillin and tetracycline resistance. The plasmid contents of a number of Amp^r and Tet^r transformants were screened by restriction analysis, and the integrity of a positively identified recombinant plasmid, designated pQE30-RafR, was verified by sequencing.

Protein (over)production and purification. For the (over)production of MelR1 and MelR2, 25 ml of M17 broth supplemented with 0.5% glucose was inoculated with a 2% inoculum of *L. lactis* NZ9000 harboring

either pNZ-MelR1-His, pNZ-MelR2-His, or the empty vector pNZ8048 (used as a negative control), followed by incubation at 30°C until an OD₆₀₀ of 0.5 was reached, at which point protein expression was induced by the addition of filter-sterilized, nisin-containing, cell-free supernatant of *L. lactis* NZ9700, followed by continued incubation at 30°C for 90 min. Cells were harvested, resuspended in 10 mM Tris-HCl buffer (pH 7.0), and disrupted with glass beads in a mini-bead beater (BioSpec Products, Bartlesville, OK). Cellular debris was removed by centrifugation to produce a crude cell extract. Although protein purification of MelR1-His and MelR2-His was achieved using His tag affinity chromatography, the purification procedure appeared to render the proteins inactive in subsequent electrophoretic mobility shift assays (EMSAs) (results not shown). For this reason, crude cell extracts, prepared in a 10 mM Tris-HCl lysis buffer (pH 7.0), were adopted for the EMSAs (see below).

In the case of RafR, a 300-ml volume of LB supplemented with tetracycline and ampicillin was inoculated with 6 ml of an overnight culture of *E. coli* XL1-Blue cells harboring pQE30-RafR and then incubated at 37°C. At an OD₆₀₀ of 0.5, expression of RafR was induced by the addition of 1 mM IPTG (Roche Diagnostics Ltd., West Sussex, United Kingdom). Following 2 h of incubation in the presence of IPTG, cells were harvested by centrifugation, and crude cell extracts for protein purification were prepared as described above. Subsequent protein purification was performed using a PrepEase kit for His-tagged protein purification (USB, Germany). Elution fractions were analyzed by SDS-polyacrylamide gel electrophoresis, as described previously (43), on a 12.5% polyacrylamide gel. Following electrophoresis, the gels were fixed and stained with Coomassie brilliant blue to identify fractions containing the purified protein. Prestained Rainbow low-molecular-weight protein marker (New England BioLabs, Herefordshire, United Kingdom) was used to estimate the molecular weight of the expressed and/or purified proteins.

Construction of *B. breve* insertion mutant strains. Internal fragments of *rafR* (528 bp, representing codons 88 through 264 out of the 403 codons of the gene), *melR1* (380 bp, representing codons 107 through 234 out of the 340 codons of the gene), and *melR2* (402 bp, representing codons 93 through 227 out of the 343 codons of the gene) were amplified by PCR using *B. breve* UCC2003 chromosomal DNA as the template and the oligonucleotide primer combinations *rafRfHd3in* and *rafRrxba1in*, *melR1fHd3in* and *melR1rxba1in*, and *melR2fHd3in* and *melR2rxba1in*, respectively (see Table S1 in the supplemental material). The PCR products generated were ligated to pORI19, an Ori⁺ RepA⁻ integration plasmid (44), using *HindIII* and *XbaI* restriction sites that were incorporated into the primers for the partial *rafR*-, *melR1*-, and *melR2*-encompassing amplicons, and introduced into *E. coli* EC101 by electroporation. Recombinant *E. coli* EC101 derivatives containing pORI19 constructs were selected on LB agar containing Em and supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside; 40 µg ml⁻¹) and 1 mM IPTG.

The expected genetic structures of the recombinant plasmids pORI19-*rafR* (pORI19 containing an internal 528-bp fragment of the *rafR* gene), pORI19-*melR1* (pORI19 containing an internal 402-bp fragment of the *melR1* gene), and pORI19-*melR2* (pORI19 containing an internal 380-bp fragment of the *melR2* gene) were confirmed by restriction mapping prior to subcloning of the Tet resistance antibiotic cassette, *tetW*, from pAM5 (45) as a *SacI* fragment into the unique *SacI* site present on each of the pORI19 derivatives. The orientation of the tetracycline resistance gene in each of the resulting plasmids, pORI19-tet-*rafR*, pORI19-tet-*melR1*, and pORI19-tet-*melR2*, was determined by restriction analysis. The plasmids were subsequently introduced into *E. coli* EC101 pNZ-MBbrI-MBbrII (40) (transformants were selected based on Cm and Tet resistance) in order to methylate the plasmid constructs before introduction into *B. breve* UCC2003.

Methylation of the plasmid complement of the obtained transformants in EC101 pNZ-MBbrI-MBbrII was confirmed by their observed resistance to *PstI* restriction (40). Plasmid preparations of methylated pORI19-tet-*rafR*, pORI19-tet-*melR1*, and pORI19-tet-*melR2* were intro-

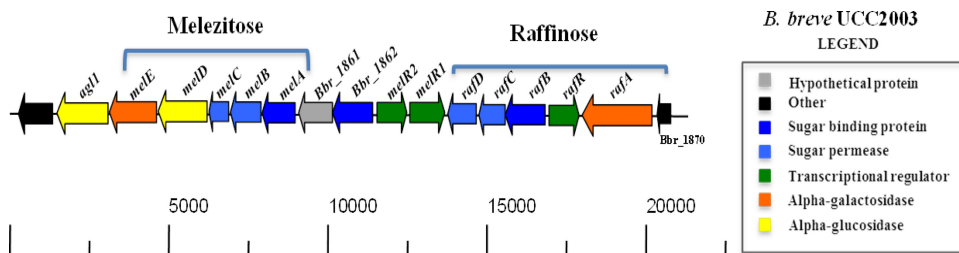


FIG 1 Representation of the raffinose and melizitose utilization operons of *B. breve* UCC2003.

duced by electroporation into *B. breve* UCC2003 with subsequent selection on reinforced clostridial agar (RCA) plates supplemented with Tet. Insertion mutants resulting from site-specific homologous recombination were initially confirmed by colony PCR targeting the tetracycline resistance gene, *tetW*, followed by a second confirmatory PCR adopting a *tetW*-based primer, either forward or reverse, depending on the orientation of *tetW*, in combination with a primer specific for each targeted gene to confirm integration at the correct chromosomal position (see Table S1 in the supplemental material). In this case, a product of a particular size would be obtained only if the correct gene disruption had been achieved. In this manner, mutants were obtained carrying chromosomal insertions in either the *rafR*, *melR1*, or *melR2* gene, resulting in strains *B. breve* UCC2003-*rafR*, *B. breve* UCC2003-*melR1*, and *B. breve* UCC2003-*melR2*, respectively.

Analysis of global gene expression using *B. breve* DNA microarrays.

Global gene transcription was determined by microarray analysis during growth of *B. breve* UCC2003-*rafR*, *B. breve* UCC2003-*melR1*, and *B. breve* UCC2003-*melR2* in mMRS supplemented with ribose, and these transcriptomes were compared to that obtained from *B. breve* UCC2003 wild-type cells that had also been grown on ribose as the sole carbohydrate source. DNA microarrays containing oligonucleotide primers representing each of the 1,864 annotated genes on the genome of *B. breve* UCC2003 were designed by and obtained from Agilent Technologies (Palo Alto, CA, USA). Cell disruption, RNA isolation, RNA quality control, and cDNA synthesis and labeling were performed as described previously (46). The labeled cDNA was hybridized using the Agilent Gene Expression hybridization kit (part number 5188-5242) as described in the Agilent Two-Color Microarray-Based Gene Expression Analysis v4.0 manual (G4140-90050). Following hybridization, the microarrays were washed in accordance with Agilent's standard procedures and scanned using an Agilent DNA microarray scanner (model G2565A). The generated scans were converted to data files with Agilent's Feature Extraction software (v9.5). The DNA microarray data were processed as previously described (47–49). Differential-expression tests were performed with the Cyber-T implementation of a variant of the *t* test (50). A gene was considered to exhibit significantly different expression at a *P* value of <0.001 and an expression ratio of >3 or <0.33 relative to the control.

EMSA. DNA fragments representing different portions of the promoter regions upstream the *rafA*, *rafB*, *Bbr_1862*, and *melA* genes were prepared by PCR using IRD800-labeled primer pairs (MWG Biotech) (see Table S2 in the supplemental material). EMSAs were performed essentially as described previously (51). In all cases, the binding reactions were performed in a final volume of 20 μ l in the presence of poly(dI-dC) in binding buffer (20 mM Tris-HCl, 5 mM MgCl₂, 0.5 mM dithiothreitol [DTT], 1 mM EDTA, 200 mM KCl, 10% glycerol at pH 7.0). Various amounts of a crude cell extract containing either MelR1 or MelR2, or purified RafR, were mixed on ice with a fixed amount of DNA probe (0.1 pmol) and subsequently incubated for 30 min at 37°C. Samples were loaded onto a 6% nondenaturing phosphonoacetic acid (PAA) gel prepared in TAE buffer (40 mM Tris acetate [pH 8.0], 2 mM EDTA) and run in a 0.5 \times to 2.0 \times gradient of TAE at 100 V for 90 min in an Atto Mini PAGE system (Atto Bioscience and Biotechnology, Tokyo, Japan). Signals were detected using an Odyssey Infrared Imaging System (Li-Cor Biosci-

ences United Kingdom Ltd., Cambridge, United Kingdom), and images were captured using the supplied Odyssey software v3.0.

Primer extension analysis. Total RNA was isolated from exponentially growing cells of *B. breve* UCC2003 or *B. breve* UCC2003-*melR2* cultured in mMRS supplemented with 1% raffinose or melizitose for *B. breve* UCC2003 or in mMRS supplemented with 1% ribose for *B. breve* UCC2003-*melR2* (52). Primer extension was performed by annealing 1 pmol of an IRD800-labeled synthetic oligonucleotide to 20 μ g of RNA, as previously described (53), using primers rafAPERP1, rafAPERP2, rafBPERP1, rafBPERP2, melAPERP1, melAPERP2, 1862PERP1, and 1862PERP2 (see Table S2 in the supplemental material). Sequence ladders of the presumed *rafA*, *rafB*, *melA*, and *Bbr_1862* promoter regions were produced using the same primer as in the primer extension reaction and employing a DNA cycle-sequencing kit (Jena Bioscience, Germany) and were run alongside the primer extension products to allow precise alignment of the transcriptional start site with the corresponding DNA sequence. Separation was achieved on a 6.5% Li-Cor Matrix KB Plus acrylamide gel. Signal detection and image capture were performed with a Li-Cor sequencing instrument (Li-Cor Biosciences).

Microarray data accession number. The microarray data obtained were deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO series accession number [GSE50211](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50211).

RESULTS AND DISCUSSION

Transcriptomes of *B. breve* UCC2003 mutants carrying disruptions in *melR1*, *melR2*, or *rafR*. In a previous study (20), we investigated the metabolism of the raffinose-related carbohydrates raffinose, stachyose, and melibiose by *B. breve* UCC2003 and also observed the presence of this cluster in other bifidobacteria. That study identified a gene cluster, comprised of *rafA*, *rafR*, *rafB*, *rafC*, and *rafD*, involved in the utilization of raffinose-related sugars, where *rafA* was shown to encode an α -galactosidase and *rafR* a putative ROK-type transcriptional regulator, while the adjacent *rafBCD* genes specified a putative ABC-type sugar uptake system (Fig. 1). The same study also identified a gene cluster in the *B. breve* UCC2003 genome, located in the immediate vicinity of the *raf* gene cluster, dedicated to melizitose metabolism and comprised of *melA*, *melB*, *melC*, *melD*, and *melE*, with *melABC* specifying a presumed ABC-type sugar uptake system and *melD* and *melE* encoding an α -glucosidase and an α -galactosidase, respectively (Fig. 1), and again also observed the presence of the cluster in other bifidobacteria (20). Located between the aforementioned *raf* and *mel* genes are four genes, two of which, designated *melR1* and *melR2*, encode putative LacI-type transcriptional regulators (Fig. 1).

We previously (20) demonstrated that transcription of the *melABCDE* and *rafABCDE* genes is induced by the presence of melizitose and raffinose (or raffinose-like sugars), indicative of substrate-specific transcriptional regulation. In order to identify and investigate the regulatory players that control the

TABLE 2 Transcriptome analysis of *B. breve* UCC2003-*melR1* and *B. breve* UCC2003-*melR2* compared to *B. breve* UCC2003 grown on 1% ribose

Gene no.	Name	Putative function	Fold upregulation ^a	
			<i>melR2</i>	<i>melR1</i>
<i>Bbr_1862</i>		Solute binding protein of ABC transporter system for sugars	148.62	—
<i>Bbr_1861</i>		Conserved hypothetical protein	59.43	—
<i>Bbr_1860</i>	<i>melA</i>	Solute binding protein of ABC transporter system for sugars	77.64	80.63
<i>Bbr_1859</i>	<i>melB</i>	Permease protein of ABC transporter system for sugars	88.56	67.9
<i>Bbr_1858</i>	<i>melC</i>	Permease protein of ABC transporter system for sugars	86.8	78.1
<i>Bbr_1857</i>	<i>melD</i>	Alpha-1,4-glucosidase	10.24	8.59
<i>Bbr_1856</i>	<i>melE</i>	Alpha-galactosidase	6.6	5.51

^a The cutoff point is 5-fold ($P < 0.001$); values below the cutoff are indicated by a minus.

raffinose and melezitose-induced genes, insertion mutations were made in the *melR1*, *melR2*, and *rafR* genes (see above) (Fig. 1), resulting in strains *B. breve* UCC2003-*melR1*, *B. breve* UCC2003-*melR2*, and UCC2003-*rafR*, respectively (see Materials and Methods). It was hypothesized that if any of the putative regulatory genes encoded a repressor, mutation of the gene would lead to increased transcription even in the absence of the inducing carbohydrate.

Altered gene expression patterns were determined by DNA microarray analysis from two independent biological replicates and employing *B. breve* UCC2003, *B. breve* UCC2003-*melR1*, *B. breve* UCC2003-*melR2*, or UCC2003-*rafR* cultures grown to the exponential growth phase in mMRS supplemented with ribose. The transcriptomes obtained revealed that, compared to *B. breve* UCC2003, the *melABCDE* genes were significantly upregulated (>5.0 -fold change; $P < 0.001$) in *B. breve* UCC2003-*melR1*, while *melABCDE*, as well as its adjacent genes, *Bbr_1861* and *Bbr_1862*, were upregulated (>5.0 -fold change; $P < 0.001$) in *B. breve* UCC2003-*melR2* (Table 2). However, the insertion mutation in *melR2* appears to have a polar effect on the transcription of the downstream gene, *melR1*, as the gene exhibited a 1.7-fold-lower level of transcription in the *B. breve* UCC2003-*melR2* mutant (compared to UCC2003; $P < 0.001$), indicating that *melR1* and *melR2* are cotranscribed. The data obtained are consistent with our prediction that both LacI-type regulators act as transcriptional repressors, since the mutations in *B. breve* UCC2003-*melR1* and UCC2003-*melR2* cause transcriptional upregulation of adjacent genes, several of which have been associated with melezitose metabolism. The transcriptome of *B. breve* UCC2003-*rafR* did not reveal any statistically significant differences from that of *B. breve* UCC2003 that could be linked to the metabolism of raffinose-like sugars (results not shown), which is consistent with the notion that *RafR* is a ROK-type transcriptional activator. ROK regulators were originally described some 20 years ago and include transcriptional repressors, sugar kinases, and as-yet-uncharacterized open reading frames (54). The ROK protein family is characterized by the presence of the PF00480 domain, which belongs to the Actin-ATPase clan in the Pfam database (55).

The three mutants were also investigated for the ability to utilize melezitose or raffinose-like sugars. The *B. breve* UCC2003-*melR1* and UCC2003-*melR2* mutants both retained the ability to utilize melezitose as the sole carbohydrate source (Fig. 2A). Interestingly, *B. breve* UCC2003-*rafR* was incapable of growing to a high optical density in mMRS supplemented with any of the raffinose-like sugars as the sole carbon source (Fig. 2B), while growth of *B. breve* UCC2003-*rafR* in mMRS supplemented with

1% lactose was comparable to that observed for the parent strain, *B. breve* UCC2003. These results demonstrate that *rafR* is required for growth on raffinose-related carbohydrates and suggest that the ROK-type transcriptional regulator *RafR* is required for the activation of the raffinose gene cluster.

MelR1 and MelR2 bind to the *melA* and *Bbr1862* promoter regions, respectively. The transcriptome data obtained for the *B. breve* UCC2003-*melR1* and UCC2003-*melR2* mutants (see above) suggest that *melR1* and *melR2* encode LacI-type regulators that control various genes of the *mel* gene cluster. In order to establish if the MelR1 or MelR2 protein directly and specifically interacts with presumed operator sequences within the promoter region(s) of the *mel* gene cluster, i.e., the *Bbr_1862* and/or *melA* promoter region (see below), EMSAs were performed. For this purpose, we first cloned *melR1* and *melR2* in the nisin-inducible vectors pNZ8048 and pNZ8150, respectively, with the introduction of an N-terminal His tag-encoding sequence to facilitate protein expression and purification in *L. lactis* NZ9000 (generating plasmids pNZ-MelR1-His and pNZ-MelR2-His, respectively).

Although MelR1 and MelR2 could be obtained as purified proteins, they proved unable to form DNA-protein complexes under the conditions used, which had been noted previously for LacI-type regulators in bifidobacteria (18). For this reason, instead of the purified proteins, crude cell extracts of (nisin-induced) *L. lactis* NZ9000 pNZ-MelR1-His or *L. lactis* NZ9000 pNZ-MelR2-His were employed to perform EMSAs. As a negative control for the EMSAs, we used crude cell extract of *L. lactis* NZ9000(pNZ8048) (empty vector) incubated with various DNA fragments, as mentioned below, which, as expected, all failed to alter the electrophoretic behavior of these DNA fragments. The results obtained with a crude cell extract containing MelR1 clearly demonstrate that the protein binds to the IRD800-labeled DNA fragment M1, which encompasses the 444-bp promoter-containing (see below) region preceding *melA*, while MelR1 failed to bind to the IRD800-labeled DNA fragment K1, encompassing the *Bbr_1862* promoter region, or the *rafA* or *rafB* promoter region [Fig. 3A and B(i) and results not shown].

Further dissection of the *melA* promoter region showed that MelR1 binding required an 84-bp DNA segment that is present within fragments M1 to M3 [Fig. 3B(i); see Fig. S1(i) to (iii) in the supplemental material] and that contains an 18-bp imperfect inverted repeat, 5'-TGCATAAGC><GCTTAGCAA-3' (with "><" indicating the center of the inverted repeat), representing the putative MelR1-specific operator sequence. This was further validated by EMSAs using a 46-bp synthetic DNA fragment that contained this MelR1 binding (*melAIR*) [Fig. 3C(i)]. Further-

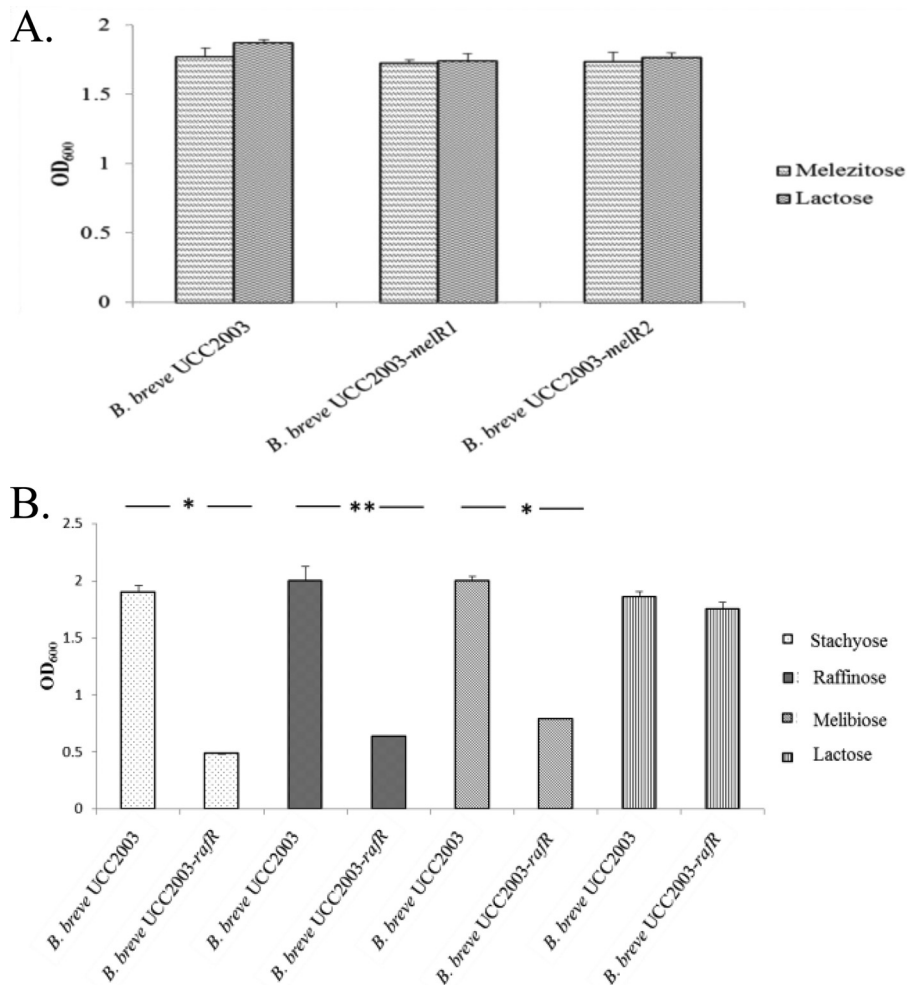


FIG 2 (A) Final OD₆₀₀ values after 16 h of wild-type strain *B. breve* UCC2003 and insertion mutants *B. breve* UCC2003-melR1 and *B. breve* UCC2003-melR2 growth on 1% melezitose or 1% lactose. The results are mean values obtained from three separate experiments. The final optical densities reached by *B. breve* UCC2003 and insertion mutants *B. breve* UCC2003-melR1 and *B. breve* UCC2003-melR2 during growth on lactose or melezitose were not statistically significantly different ($P < 0.1$). (B) Final OD₆₀₀ values after 16 h of growth of wild-type strain *B. breve* UCC2003 and insertion mutant *B. breve* UCC2003-rafR on 1% stachyose, 1% raffinose, 1% melibiose, or 1% lactose. The final optical densities attained by *B. breve* UCC2003 compared to insertion mutant *B. breve* UCC2003-rafR during growth on 1% raffinose, 1% stachyose, or 1% melibiose were statistically significantly different (*, $P < 0.001$; **, $P < 0.01$). No significant difference ($P < 0.1$) was observed in the final optical densities achieved by *B. breve* UCC2003 and *B. breve* UCC2003-rafR during growth on 1% lactose. The error bars indicate standard deviations.

more, introduction of two point mutations in this presumed MelR1 operator sequence (a G-to-T and a C-to-A mutation at positions 8 and 9 of the sequence, as indicated above) were shown to prevent binding of MelR1 (mu-melAIR) [Fig. 3C(iii)]. Other mutations in bases on either side of this motif did not affect the binding of MelR1 (results not shown) (for all wild-type and mutated primer sequences, see Table S3 in the supplemental material). To investigate whether MelR1 interaction with its target DNA sequence is influenced by a carbohydrate effector molecule, as is known to be the case for other LacI-type regulators (reviewed in references 56 and 57), several carbohydrates were tested for their effects on MelR1-DNA complex formation. The results obtained show that the ability of MelR1 to bind to the M1 fragment of the *melA* promoter region is lost in the presence of melezitose at concentrations of >2.5 mM (Fig. 3D), whereas under the same experimental conditions, galactose or lactose did not affect MelR1 binding to its target [see Fig. S2(i) and (ii) in the supplemental material].

When EMSAs were performed using a nisin-induced *L. lactis* NZ9000 pNZ-MelR2-His cell extract, MelR2 was shown to bind to IRD800-labeled DNA fragment K1, which encompasses the 310-bp promoter-containing (see below) region preceding *Bbr_1862*, but not with similarly labeled DNA fragments containing the promoter regions upstream of *melA* (fragment M1), *rafA*, or *rafB* [Fig. 3A and B(ii) and results not shown]. Further EMSAs showed that MelR2 binding required an 85-bp DNA segment present within fragments K1 and K2 and absent in fragments K3 and K4 of the *Bbr_1862* promoter region [Fig. 3B(ii); see Fig. S3 in the supplemental material]. Inspection of this 85-bp fragment revealed the presence of a 18-bp imperfect inverted repeat, 5'-TGC GTAATC>>GATATCGCA-3', representing a putative MelR2 operator sequence. This was validated by EMSAs using a synthetic 50-bp DNA fragment that contained this predicted operator sequence (Bbr1862IR) [Fig. 3C(ii)]. Introduction of two point mutations in the putative 18-bp MelR2 binding motif (an A-to-C and a T-to-G mutation at positions 7 and 8 of the motif) was shown to

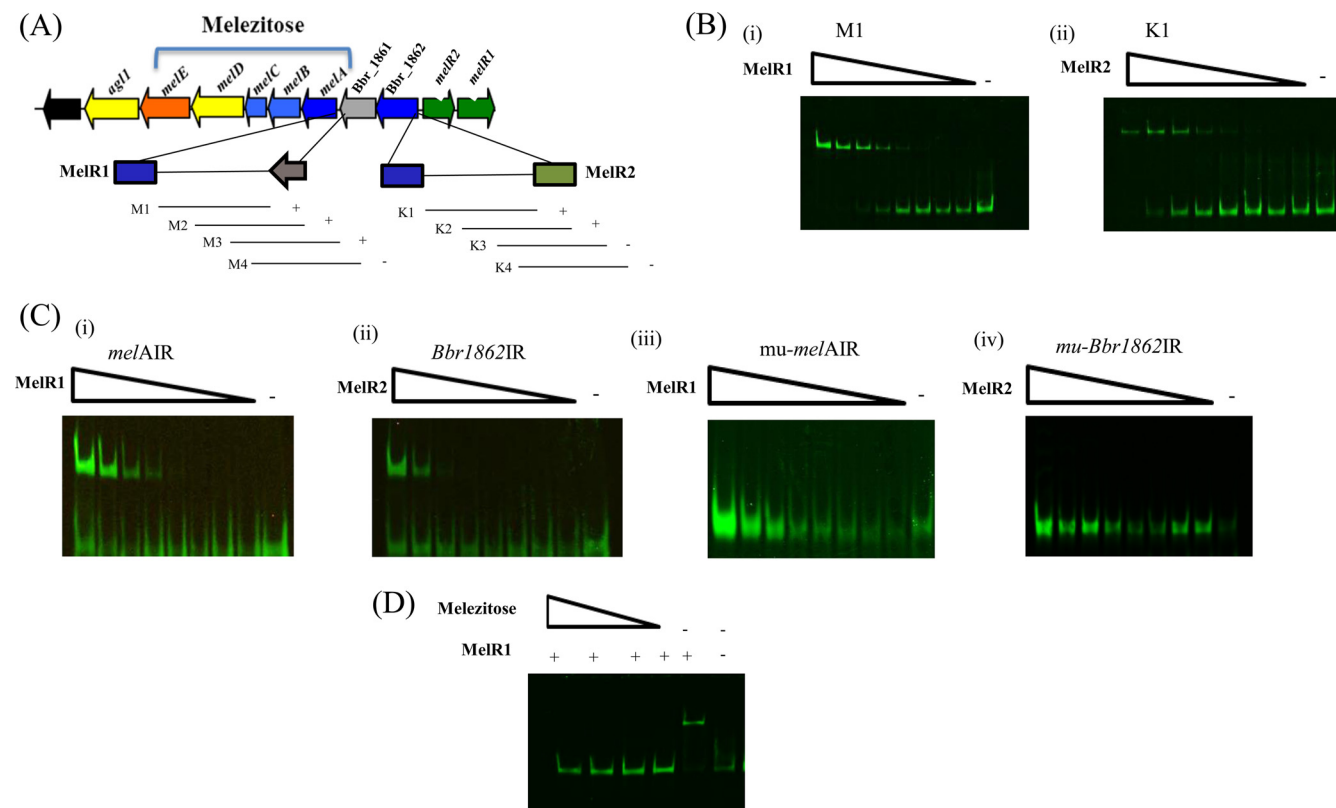


FIG 3 (A) Representation of the melezitose utilization cluster of *B. breve* UCC2003 and DNA fragments used in EMSAs for the *melA* and *Bbr_1862* promoter regions. The plus and minus signs indicate ability or inability of MelR1 or MelR2 to bind to the *melA* or *Bbr_1862* DNA promoter-encompassing fragments, respectively. (B) EMSAs showing MelR1 interaction with DNA fragments encompassing fragment M1 (i) and MelR2 interaction with DNA fragments encompassing fragment K1 (ii). (C) EMSAs illustrating MelR1 interaction with *melAIR* (i) and mutated derivative *mu-melAIR* (iii) and MelR2 interaction with *Bbr1862IR* (ii) and mutated derivative *mu-Bbr1862IR* (iv). The minus signs indicate lanes with binding reactions to which no protein was added, while the remaining lanes represent binding reactions with the respective DNA probes incubated with increasing amounts of protein at concentrations ranging from 0.04 nM to 0.01 μ M. Each successive lane, from right to left, corresponds to a doubling in the amount of protein used for the EMSA. (D) EMSAs showing MelR1 interaction with the DNA fragment M1 with the addition of melezitose at concentrations ranging from 2.5 to 20 mM.

prevent MelR2 binding (see Table S2 in the supplemental material) for IRD800 primer sequences (*mu-Bbr1862IR*) [Fig. 3C(iv)]. Other mutations were also made in bases surrounding this motif, but none of the mutations was shown to affect binding under the conditions tested (results not shown) (see Table S4 in the supplemental material for the relevant primer sequences used). To investigate if MelR2 interaction with its target DNA sequence is influenced by a carbohydrate effector molecule, as shown for MelR1, several carbohydrates were tested for their effects on MelR2-DNA complex formation. However, none of the carbohydrates tested (melezitose, turanose, sucrose, glucose, fructose, α -1,3-galactobiose, or α -1,4-galactobiose) was shown to prevent MelR2 binding to its DNA target [see Fig. S4(i) to (vii) in the supplemental material], suggesting that MelR2 binding is abrogated by an as-yet-unknown molecule (20).

The above-mentioned binding studies, combined with the transcriptome analyses, indicate that MelR1 is the melezitose-responsive repressor of the *melABCDE* gene cluster, while MelR2 is a LacI-type repressor controlling the transcription of *Bbr_1861* and *Bbr_1862*. The observation that the *melR2* mutation leads to an increase in *melABCDE* transcription is most likely an indirect effect due to its negative polar effect on *melR1* transcription, leading to reduced MelR1 expression and, consequently, derepression

of the *melABCDE* gene cluster. Motif searches using a LacI operator consensus model, based on sequences recognized by bifidobacterial LacI-type repressors as determined in this work and reported previously (17, 18, 22), reveal the presence of other LacI-type recognition sites in the genome of *B. breve* UCC2003. These putative LacI-type binding sites are located in intergenic regions upstream of genes involved in carbohydrate metabolism, consistent with experimentally verified LacI-type binding sites, although validation will require EMSAs involving such DNA regions and their cognate LacI-type proteins (see Fig. S5 and Table S5 in the supplemental material). The sequence differences between these putative LacI-type binding motifs are believed to be responsible for target specificity, a finding that has been observed previously in *Lactobacillus plantarum* (58). Interestingly, analysis of the genomes of *Bifidobacterium longum* KACC 91563 and *B. longum* ATCC 55813, which contain homologs of the melezitose locus (20), including two LacI-type regulator-encoding genes, shows the conservation of the deduced MelR1 and MelR2 operator sites (data not shown).

RafR binds to the *rafB* promoter region. The presence of *rafR*, encoding a putative ROK-type regulator within the raffinose gene cluster, suggests that the gene plays a role in the transcriptional regulation of the *raf* gene cluster, a notion that was supported by

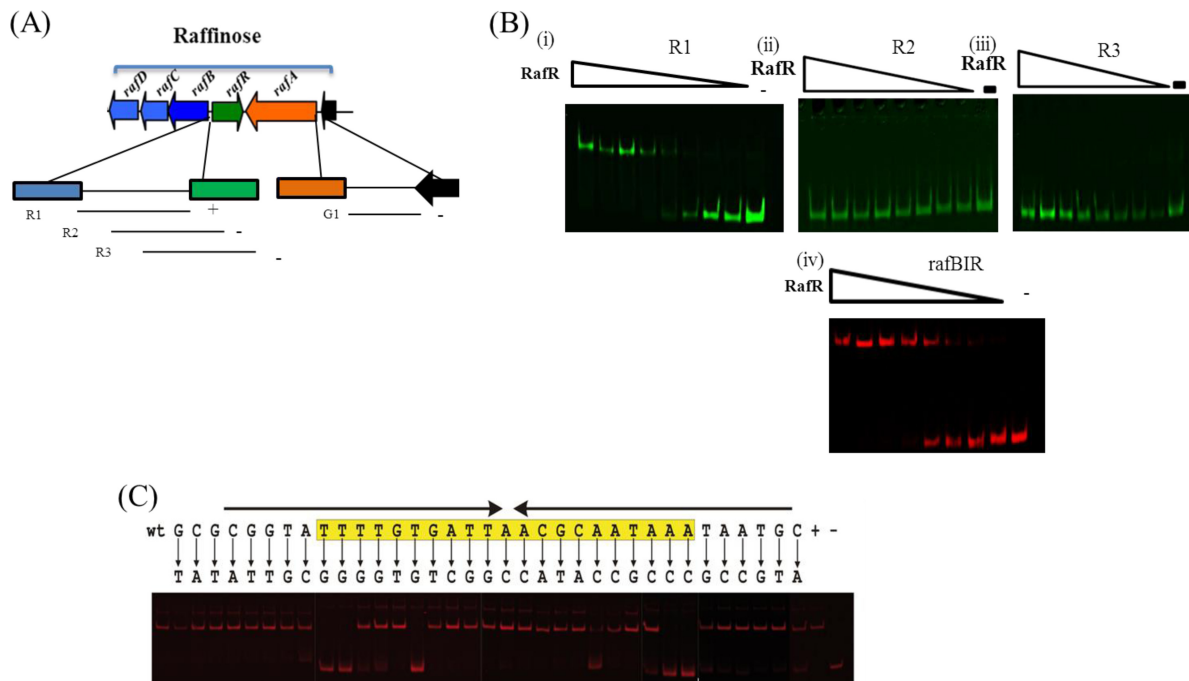


FIG 4 (A) Representation of the raffinose utilization cluster of *B. breve* UCC2003 and DNA fragments used in EMSAs for the *rafB* and *rafA* promoter regions. The plus and minus signs indicate the ability or inability, respectively, of RafA to bind to the *rafB* or *rafA* DNA fragments. (B) EMSAs showing RafR interaction with DNA fragments encompassing fragments R1 (i), R2 (ii), and R3 (iii) and the annealed oligonucleotides representing *rafBIR* (iv). The minus signs indicate lanes with binding reactions to which no protein was added, while the remaining lanes represent binding reactions with the respective DNA probes incubated with increasing amounts of protein at concentrations ranging from 0.04 nM to 0.01 μ M. Each successive lane, from right to left, corresponds to a doubling in the amount of protein used for the EMSA. (C) EMSAs showing RafR interaction with the mutated operator motif of the *rafB* promoter region. DNA fragments were obtained by PCR using IRD700-labeled primers. The operator sequence and incorporated mutations are shown above the image. wt and +, original promoter sequence; –, no added protein. The conserved ROK motif is highlighted in yellow. The arrows indicate the inverted-repeat structure of the motif.

the inability of a *rafR* mutant to support proper growth on raffinose-type sugars (see above). In order to establish if RafR is capable of interacting with specific sequences within the raffinose-induced (20) promoter regions upstream of *rafA* and *rafB* (see below), we overexpressed and purified RafR as a His-tagged protein (see Materials and Methods). RafR was then used to perform EMSAs, the results of which clearly demonstrate that RafR causes a mobility shift of IRD800-labeled DNA fragment R1, which includes the 241-bp promoter region upstream of *rafB* [Fig. 4A and B(i)]. In contrast, under the conditions used, RafR was unable to bind to IRD800-labeled DNA fragment G1, which includes the 223-bp promoter region upstream of *rafA* (results not shown). This suggests that *rafA* is regulated by an as-yet-unknown regulator or molecule.

Further EMSAs involving RafR and fragments R2 and R3 [Fig. 4B(ii) and (iii)] of the *rafB* promoter region showed that RafR binding requires an 81-bp DNA segment that contains an imperfect inverted repeat, 5'-TTTATTGCGTT>A<ATCACAAAATA-3', thus representing the putative RafR operator sequence. This was validated by EMSAs using a 38-bp DNA fragment that contained this sequence (*rafBIR*) [Fig. 4B(iv)]. Introduction of point mutations in a 35-bp sequence encompassing the putative RafR binding motif revealed various positions within the operator sequence that are crucial for RafR binding (Fig. 4C; for the primer sequences used, see Table S6 in the supplemental material). This operator binding sequence is very similar to the consensus operator sequence determined for two other members of the ROK

family proteins, Mlc and NagC, in *E. coli* (54, 59). Analysis of sequenced bifidobacterial genomes, many of which contain a homolog of the *B. breve* *raf* locus, reveals the presence of a single conserved RafR operator site in the *rafB* promoter region, whereas no other sites could be detected. Bases important for binding (as determined above) are conserved between bifidobacterial species (see Fig. S6 in the supplemental material).

RafR interaction with its target DNA sequence was not affected by the presence of raffinose, stachyose, melibiose, galactose, glucose, sucrose, α -1,3-galactobiose, and α -1,4-galactobiose at concentrations ranging from 2.5 mM to 20 mM [see Fig. S7(i) to (viii) in the supplemental material]. Binding of RafR to the *rafA* promoter region was also tested in the presence of these sugars; however, this did not result in binding of RafR to the *rafA* promoter region (results not shown). A summary of all protein interactions is presented in Table S7 in the supplemental material.

Identification of the transcription start sites of *rafA*, *rafB*, *melA*, and *Bbr_1862*. From the genetic organization (Fig. 1) and the expression pattern of the *raf* locus (20) it was deduced that the *raf* cluster contained two raffinose/stachyose/melibiose-inducible promoters, one in front of the *rafA* gene and one in front of the *rafB* gene. In relation to the *mel* cluster, potential promoter regions were identified based on *in silico* analysis of the *mel* locus (Fig. 1) coupled with transcriptomic data obtained during growth on melezitose (20), as well as the transcriptomes of UCC2003-*melR1* and UCC2003-*melR2* (see above). It was reasoned that one melezitose-inducible, MelR1-dependent promoter was located

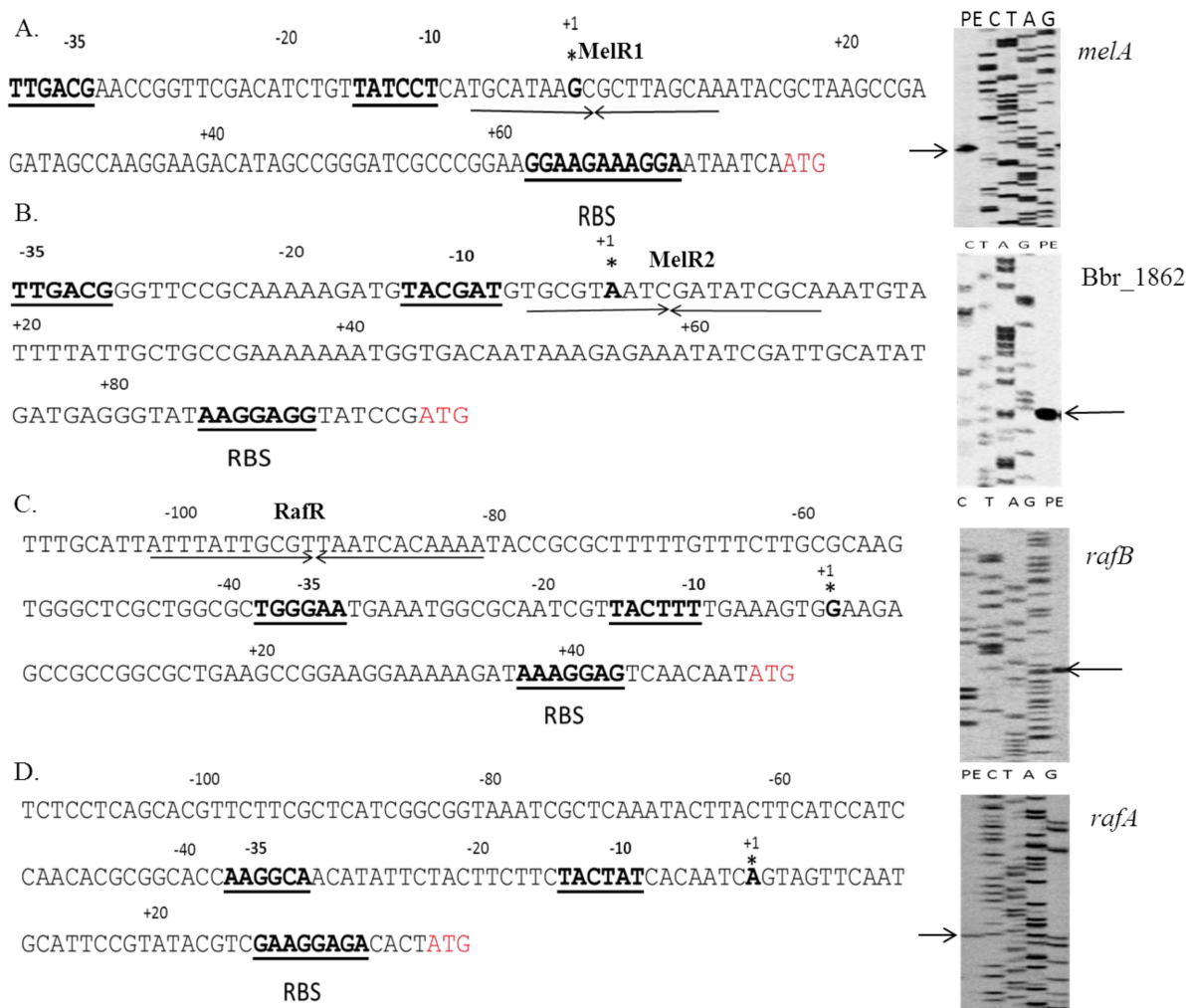


FIG 5 Schematic representation of the *melA* (A), *Bbr_1862* (B), *rafB* (C), and *rafA* (D) promoter regions. Boldface type and underlining indicate the -10 and -35 hexamers deduced from the primer extension results; deduced ribosomal binding sites (RBS) and experimentally determined transcriptional start sites (TSS) are indicated by asterisks. The arrows under sequences with names in boldface indicate the inverted-repeat MelR1, MelR2, and RafR binding sequences displayed above the arrows. The arrows in the right panels indicate the primer extension products.

directly upstream of the *melA* coding sequence and one promoter (transcription of which is subject to MelR2 and an as-yet-unknown inducer molecule) preceded the *Bbr_1862* coding sequence.

In order to determine the transcription start sites of these presumed promoters, primer extension analysis was performed using RNA extracted from *B. brevis* UCC2003 grown in mMRS containing either 1% melezitose or 1% raffinose, as appropriate. To determine the transcription start site of *Bbr_1862*, *B. brevis* UCC2003-*melR2* (whose transcriptome revealed constitutive transcription of *Bbr_1862*) was grown in mMRS supplemented with ribose as the sole carbohydrate source. Single extension products were identified upstream of *melA*, the *Bbr_1862* gene, *rafB*, and *rafA*, and in all cases, potential promoter recognition sequences resembling consensus -10 and -35 hexamers could be identified upstream of these transcription start points (Fig. 5A to D). In the cases of *melA*- and *Bbr_1862*-associated promoters, the MelR1 and MelR2 binding operator sequences overlap the respective -10 sequences, which supports our findings that MelR1 and MelR2 are LacI-type transcrip-

tional repressors. For the *rafB* promoter region, the RafR operator sequence is located upstream of the -35 and -10 promoter recognition sequences, a location that is consistent with the notion that RafR functions as a transcriptional activator.

Concluding remarks. The data assembled in this study provide significant information on the ability of *B. brevis* UCC2003 to regulate two adjacent genetic loci dedicated to the utilization of raffinose-containing carbohydrates and melezitose (20). We conclude that MelR1 is a LacI-type regulator that acts as a melezitose-responsive repressor of the *melABCDE* gene cluster, while MelR2 is also a LacI-type regulator that acts as a repressor of the *Bbr_1861* and *Bbr_1862* genes, the latter encoding a predicted carbohydrate binding protein of an ABC-type sugar transport system. Because of its vicinity and the finding that *melE* encodes an α -galactosidase, we speculate that MelR2 responds to an oligosaccharide containing a melezitose backbone to which one or more α -galactose moieties are attached. This α -galactose-containing oligosaccharide is bound by the product of *Bbr_1862* and internalized by the *melABC* uptake system, after which the α -galactose moiety (or

moieties) is removed by the action of MeIE, followed by further degradation by MeID. Unfortunately, no such substrates are currently commercially available, and this hypothesis therefore remains to be verified.

In a classic example discussed by Irwin and Ptashne (60), it was observed that mutating the catabolite activator protein (CAP) resulted in lack of transcriptional activation of the *gal* promoter. Similarly, in *C. glutamicum* 13032, a deletion in the gene encoding the ROK-type protein CysR rendered the strain incapable of utilizing sulfate or sulfonates as the sole source of sulfur, leading the authors to speculate that CysR functions as a transcriptional activator (61). Based on our experimental findings, we conclude that RafR also acts as a transcriptional activator of the *rafBCD* cluster.

Somewhat to our surprise, RafR did not bind to the *rafa* promoter, at least not under the conditions used here, although the promoter is transcriptionally induced when *B. breve* UCC2003 is grown in raffinose-like sugars (20). It may be that RafR requires an effector for binding to the promoter, and future research will further examine this.

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REFERENCES

- Miyake T, Watanabe K, Watanabe T, Oyaizu H. 1998. Phylogenetic analysis of the genus *Bifidobacterium* and related genera based on 16S rDNA sequences. *Microbiol. Immunol.* 42:661–667.
- Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald GF, Chater KF, van Sinderen D. 2007. Genomics of actinobacteria: tracing the evolutionary history of an ancient phylum. *Microbiol. Mol. Biol. Rev.* 71:495–548. <http://dx.doi.org/10.1128/MMBR.00005-07>.
- Tissier H. 1900. *Recherches sur la flora intestinale normale pathologique du nourrisson*. Thesis, University of Paris, Paris, France.
- Tissier H. 1906. The treatment of intestinal infections by the method of transformation of the bacterial intestinal flora. *C. R. Soc. Biol.* 60:359–361.
- Erdogan O, Tanyeri B, Torun E, Gonullu E, Arslan H, Erenberk U, Oktem F. 2012. The comparison of the efficacy of two different probiotics in rotavirus gastroenteritis in children. *J. Trop. Med.* 2012:787240. <http://dx.doi.org/10.1155/2012/787240>.
- Parker MW, Schaffzin JK, Lo Vecchio A, Yau C, Vonderhaar K, Guiot A, Brinkman WB, White CM, Simmons JM, Gerhardt WE, Kotagal UR, Conway PH. 2013. Rapid adoption of *Lactobacillus rhamnosus* GG for acute gastroenteritis. *Pediatrics* 131(Suppl. 1):S96–S102. <http://dx.doi.org/10.1542/peds.2012-14271>.
- Rohlke F, Stollman N. 2012. Fecal microbiota transplantation in relapsing *Clostridium difficile* infection. *Therap. Adv. Gastroenterol.* 5:403–420. <http://dx.doi.org/10.1177/1756283X12453637>.
- Zhu Q, Gao R, Wu W, Qin H. 2013. The role of gut microbiota in the pathogenesis of colorectal cancer. *Tumour Biol.* 34:1285–1300. <http://dx.doi.org/10.1007/s13277-013-0684-4>.
- Gough E, Shaikh H, Manges AR. 2011. Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. *Clin. Infect. Dis.* 53:994–1002. <http://dx.doi.org/10.1093/cid/cir632>.
- Gibson GR, Roberfroid MB. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.* 125:1401–1412.
- Costabile A, Klinder A, Fava F, Napolitano A, Fogliano V, Leonard C, Gibson GR, Tuohy KM. 2008. Whole-grain wheat breakfast cereal has a prebiotic effect on the human gut microbiota: a double-blind, placebo-controlled, crossover study. *Br. J. Nutr.* 99:110–120. <http://dx.doi.org/10.1017/S0007114507793923>.
- Bouhnik Y, Vahedi K, Achour L, Attar A, Salfati J, Pochart P, Marteau P, Flourie B, Bornet F, Rambaud JC. 1999. Short-chain fructo-oligosaccharide administration dose-dependently increases fecal bifidobacteria in healthy humans. *J. Nutr.* 129:113–116.
- Macfarlane GT, Steed H, Macfarlane S. 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *J. Appl. Microbiol.* 104:305–344. <http://dx.doi.org/10.1111/j.1365-2672.2007.03520.x>.
- Bouhnik Y, Attar A, Joly FA, Riottot M, Dyard F, Flourie B. 2004. Lactulose ingestion increases faecal bifidobacterial counts: a randomised double-blind study in healthy humans. *Eur. J. Clin. Nutr.* 58:462–466. <http://dx.doi.org/10.1038/sj.ejcn.1601829>.
- Cecchini DA, Laville E, Laguerre S, Robe P, Leclerc M, Doré J, Henrissat B, Remaud-Siméon M, Monsan P, Potocki-Véronèse G. 2013. Functional metagenomics reveals novel pathways of prebiotic breakdown by human gut bacteria. *PLoS One* 8:e72766. <http://dx.doi.org/10.1371/journal.pone.0072766>.
- Scardovi V, Trovati ID. 1965. The fructose-6-phosphate shunt as a peculiar pattern of hexose degradation in the genus *Bifidobacterium*. *Ann. Microbiol.* 15:19–29.
- Pokusaeva K, Neves AR, Zomer A, O'Connell-Motherway M, MacSharry J, Curley P, Fitzgerald GF, van Sinderen D. 2010. Ribose utilization by the human commensal *Bifidobacterium breve* UCC2003. *Microb. Biotechnol.* 3:311–323. <http://dx.doi.org/10.1111/j.1751-7915.2009.00152.x>.
- Pokusaeva K, O'Connell-Motherway M, Zomer A, Macsharry J, Fitzgerald GF, van Sinderen D. 2011. Cello-dextrin utilization by *Bifidobacterium breve* UCC2003. *Appl. Environ. Microbiol.* 77:1681–1690. <http://dx.doi.org/10.1128/AEM.01786-10>.
- Maze A, O'Connell-Motherway M, Fitzgerald GF, Deutscher J, van Sinderen D. 2007. Identification and characterization of a fructose phosphotransferase system in *Bifidobacterium breve* UCC2003. *Appl. Environ. Microbiol.* 73:545–553. <http://dx.doi.org/10.1128/AEM.01496-06>.
- O'Connell KJ, O'Connell Motherway M, O'Callaghan J, Fitzgerald GF, Ross RP, Ventura M, Stanton C, van Sinderen D. 2013. Metabolism of four α -glycosidic linkage-containing oligosaccharides by *Bifidobacterium breve* UCC2003. *Appl. Environ. Microbiol.* 79:6280–6292. <http://dx.doi.org/10.1128/AEM.01775-13>.
- Ryan SM, Fitzgerald GF, van Sinderen D. 2005. Transcriptional regulation and characterization of a novel β -fructofuranosidase-encoding gene from *Bifidobacterium breve* UCC2003. *Appl. Environ. Microbiol.* 71:3475–3482. <http://dx.doi.org/10.1128/AEM.71.7.3475-3482.2005>.
- O'Connell Motherway M, Fitzgerald GF, van Sinderen D. 2011. Metabolism of a plant derived galactose-containing polysaccharide by *Bifidobacterium breve* UCC2003. *Microb. Biotechnol.* 4:403–416. <http://dx.doi.org/10.1111/j.1751-7915.2010.00218.x>.
- O'Connell Motherway M, Fitzgerald GF, Neirynck S, Ryan S, Steidler L, van Sinderen D. 2008. Characterization of ApuB, an extracellular type II amylopullulanase from *Bifidobacterium breve* UCC2003. *Appl. Environ. Microbiol.* 74:6271–6279. <http://dx.doi.org/10.1128/AEM.01169-08>.
- Fukami-Kobayashi K, Tateno Y, Nishikawa K. 2003. Parallel evolution of ligand specificity between LacI/GalR family repressors and periplasmic sugar-binding proteins. *Mol. Biol. Evol.* 20:267–277. <http://dx.doi.org/10.1093/molbev/msg038>.
- Weickert MJ, Adhya S. 1992. A family of bacterial regulators homologous to Gal and Lac repressors. *J. Biol. Chem.* 267:15869–15874.
- Parche S, Belet M, Rezzonico E, Jacobs D, Arigoni F, Titgemeyer F, Jankovic I. 2006. Lactose-over-glucose preference in *Bifidobacterium longum* NCC2705: *glcP*, encoding a glucose transporter, is subject to lactose repression. *J. Bacteriol.* 188:1260–1265. <http://dx.doi.org/10.1128/JB.188.4.1260-1265.2006>.
- Bacon JS, Dickinson B. 1957. The origin of melezitose: a biochemical relationship between the lime tree (*Tilia* spp.) and an aphid (*Eucallipterus tiliae* L.). *Biochem. J.* 66:289–297.
- Hudson CS. 1946. Melezitose and turanose, p 1–36. In Pigman MLW, Stanley P (ed), *Advances in carbohydrate chemistry*, vol 2. Academic Press, San Diego, CA.
- De Man JC, Rogosa M, Sharpe ME. 1960. A medium for the cultivation of lactobacilli. *J. Appl. Microbiol.* 23:130–135.
- Terzaghi BE, Sandine WE. 1975. Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* 29:807–813.

31. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32. Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B. 2000. Artemis: sequence visualization and annotation. *Bioinformatics* 16:944–945. <http://dx.doi.org/10.1093/bioinformatics/16.10.944>.
33. O'Connell Motherway M, Zomer A, Leahy SC, Reunanen J, Bottacini F, Claesson MJ, O'Brien F, Flynn K, Casey PG, Munoz JA, Kearney B, Houston AM, O'Mahony C, Higgins DG, Shanahan F, Palva A, de Vos WM, Fitzgerald GF, Ventura M, O'Toole PW, van Sinderen D. 2011. Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. *Proc. Natl. Acad. Sci. U. S. A.* 108:11217–11222. <http://dx.doi.org/10.1073/pnas.1105380108>.
34. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.
35. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389–3402.
36. Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res.* 37:W202–W208. <http://dx.doi.org/10.1093/nar/gkp335>.
37. Crooks GE, Hon G, Chandonia JM, Brenner SE. 2004. WebLogo: a sequence logo generator. *Genome Res.* 14:1188–1190. <http://dx.doi.org/10.1101/gr.849004>.
38. Eddy SR. 2011. Accelerated profile HMM searches. *PLoS Comput. Biol.* 7:e1002195. <http://dx.doi.org/10.1371/journal.pcbi.1002195>.
39. O'Riordan K, Fitzgerald GF. 1999. Molecular characterisation of a 5.75-kb cryptic plasmid from *Bifidobacterium breve* NCFB 2258 and determination of mode of replication. *FEMS Microbiol. Lett.* 174:285–294.
40. O'Connell Motherway M, O'Driscoll J, Fitzgerald GF, Van Sinderen D. 2009. Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in *Bifidobacterium breve* UCC2003. *Microb. Biotechnol.* 2:321–332. <http://dx.doi.org/10.1111/j.1751-7915.2008.00071.x>.
41. Wells JM, Wilson PW, Le Page RW. 1993. Improved cloning vectors and transformation procedure for *Lactococcus lactis*. *J. Appl. Bacteriol.* 74: 629–636.
42. Mierau I, Leij P, van Swam I, Blommestein B, Floris E, Mond J, Smid E. 2005. Industrial-scale production and purification of a heterologous protein in *Lactococcus lactis* using the nisin-controlled gene expression system NICE: the case of lysostaphin. *Microb. Cell Fact.* 4:15. <http://dx.doi.org/10.1186/1475-2859-4-15>.
43. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
44. Law J, Buist G, Haandrikman A, Kok J, Venema G, Leenhouts K. 1995. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. *J. Bacteriol.* 177:7011–7018.
45. Alvarez-Martin P, O'Connell-Motherway M, van Sinderen D, Mayo B. 2007. Functional analysis of the pBC1 replicon from *Bifidobacterium catenulatum* L48. *Appl. Microbiol. Biotechnol.* 76:1395–1402. <http://dx.doi.org/10.1007/s00253-007-1115-5>.
46. Zomer A, Fernandez M, Kearney B, Fitzgerald GF, Ventura M, van Sinderen D. 2009. An interactive regulatory network controls stress response in *Bifidobacterium breve* UCC2003. *J. Bacteriol.* 191:7039–7049. <http://dx.doi.org/10.1128/JB.00897-09>.
47. Garcia De La Nava J, Santaella DF, Alba JC, Carazo JM, Trelles O, Pascual-Montano A. 2003. Engine: the processing and exploratory analysis of gene expression data. *Bioinformatics* 19:657–658. <http://dx.doi.org/10.1093/bioinformatics/btg028>.
48. van Hijum SAFT, Garcia De La Nava J, Trelles O, Kok J, Kuipers OP. 2003. MicroPreP: a cDNA microarray data pre-processing framework. *Appl. Bioinformatics* 2:241–244.
49. van Hijum SAFT, De Jong A, Baerends RJ, Karsens HA, Kramer NE, Larsen R, den Hengst CD, Albers CJ, Kok J, Kuipers OP. 2005. A generally applicable validation scheme for the assessment of factors involved in reproducibility and quality of DNA-microarray data. *BMC Genomics* 6:77. <http://dx.doi.org/10.1186/1471-2164-6-77>.
50. Long AD, Mangalam HJ, Chan BY, Toller L, Hatfield GW, Baldi P. 2001. Improved statistical inference from DNA microarray data using analysis of variance and a Bayesian statistical framework. *Analysis of global gene expression in Escherichia coli K12. J. Biol. Chem.* 276:19937–19944. <http://dx.doi.org/10.1074/jbc.M010192200>.
51. Hellman LM, Fried MG. 2007. Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. *Nat. Protoc.* 2:1849–1861. <http://dx.doi.org/10.1038/nprot.2007.249>.
52. Kuipers OP, Beerthuyzen MM, Siezen RJ, de Vos WM. 1993. Characterization of the nisin gene cluster nisABTCIPR of *Lactococcus lactis*. Requirement of expression of the nisa and nisl genes for development of immunity. *Eur. J. Biochem.* 216:281–291.
53. Ventura M, Zink R, Fitzgerald GF, van Sinderen D. 2005. Gene structure and transcriptional organization of the *dnaK* operon of *Bifidobacterium breve* UCC 2003 and application of the operon in bifidobacterial tracing. *Appl. Environ. Microbiol.* 71:487–500. <http://dx.doi.org/10.1128/AEM.71.1.487-500.2005>.
54. Titgemeyer F, Reizer J, Reizer A, Saier MH. 1994. Evolutionary relationships between sugar kinases and transcriptional repressors in bacteria. *Microbiology* 140:2349–2354.
55. Punta M, Coghill PC, Eberhardt RY, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J, Heger A, Holm L, Sonnhammer EL, Eddy SR, Bateman A, Finn RD. 2012. The Pfam protein families database. *Nucleic Acids Res.* 40:D290–D301. <http://dx.doi.org/10.1093/nar/gkr1065>.
56. Wilson CJ, Zhan H, Swint-Kruse L, Matthews KS. 2007. The lactose repressor system: paradigms for regulation, allosteric behavior and protein folding. *Cell. Mol. Life Sci.* 64:3–16. <http://dx.doi.org/10.1007/s00018-006-6296-z>.
57. Swint-Kruse L, Matthews KS. 2009. Allostery in the LacI/GalR family: variations on a theme. *Curr. Opin. Microbiol.* 12:129–137. <http://dx.doi.org/10.1016/j.mib.2009.01.009>.
58. Francke C, Kerkhoven R, Wels M, Siezen RJ. 2008. A generic approach to identify transcription factor-specific operator motifs; inferences for LacI-family mediated regulation in *Lactobacillus plantarum* WCFS1. *BMC Genomics* 9:145. <http://dx.doi.org/10.1186/1471-2164-9-145>.
59. Plumbbridge J. 2001. Regulation of PTS gene expression by the homologous transcriptional regulators, Mlc and NagC, in *Escherichia coli* (or how two similar repressors can behave differently). *J. Mol. Microbiol. Biotechnol.* 3:371–380.
60. Irwin N, Ptashne M. 1987. Mutants of the catabolite activator protein of *Escherichia coli* that are specifically deficient in the gene-activation function. *Proc. Natl. Acad. Sci. U. S. A.* 84:8315–8319.
61. Ruckert C, Milse J, Albersmeier A, Koch D, Puhler A, Kalinowski J. 2008. The dual transcriptional regulator CysR in *Corynebacterium glutamicum* ATCC 13032 controls a subset of genes of the McbR regulon in response to the availability of sulphide acceptor molecules. *BMC Genomics* 9:483. <http://dx.doi.org/10.1186/1471-2164-9-483>.
62. de Ruyter PG, Kuipers OP, Beerthuyzen MM, van Alen-Boerrigter I, de Vos WM. 1996. Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. *J. Bacteriol.* 178:3434–3439.