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Metabolism of Four α-Glycosidic Linkage-Containing Oligosaccharides by *Bifidobacterium breve* UCC2003

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Members of the genus *Bifidobacterium* are common inhabitants of the gastrointestinal tracts of humans and other mammals, where they ferment many diet-derived carbohydrates that cannot be digested by their hosts. To extend our understanding of bifidobacterial carbohydrate utilization, we investigated the molecular mechanisms by which 11 strains of *Bifidobacterium breve* metabolize four distinct α-glucose- and/or α-galactose-containing oligosaccharides, namely, raffinose, stachyose, melibiose, and melezitose. Here we demonstrate that all *B. breve* strains examined possess the ability to utilize raffinose, stachyose, and melibiose. However, the ability to metabolize melezitose was not common to all *B. breve* strains tested. Transcriptomic and functional genomic approaches identified a gene cluster dedicated to the metabolism of α-galactose-containing carbohydrates, while an adjacent gene cluster, dedicated to the metabolism of α-glucose-containing melezitose, was identified in strains that are able to use this carbohydrate.

Bifidobacteria are Gram positive, saccharolytic, nonmotile, nonsporulating anaerobic rods with a high G+C genome content that belong to the phylum *Actinobacteria* and the family *Bifidobacteriaceae* (1, 2). The first representatives of the genus *Bifidobacterium* were isolated more than a century ago (3). These bacteria naturally inhabit the gastrointestinal tracts of humans and other mammals and are particularly abundant in breast-fed infants (4, 5). Claims that certain bifidobacterial strains promote and maintain gastrointestinal health have been advanced (6), and these strains are therefore used as health-promoting or probiotic bacterial ingredients in certain functional foods (7). Their reported beneficial effects on the host include the inhibition of bacterial and viral pathogens, alleviation of lactose intolerance, enhancement of natural immunity, and reduction of serum cholesterol levels (8–10).

As saccharolytic microorganisms, bifidobacteria degrade various oligosaccharides and polysaccharides into their monosaccharide constituents, which are then shuttled into a specific hexose fermentation pathway called the fructose-6-phosphate phosphoketolase pathway, or bifid shunt (11). The proliferation of specific species or strains of commensal bifidobacteria is presumed to be stimulated by particular dietary carbohydrates, which for this reason are termed prebiotic substances (12). The term "prebiotics" was first coined in 1995, when it was defined as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon" (13). As much as 8% of the coding capacity of a bifidobacterial genome is dedicated to carbohydrate metabolism, of which half is considered to be responsible for carbohydrate uptake, mainly via ABC transporters, although proton motive force-driven permeases, proton symporters, and phosphoenolpyruvate-dependent phosphotransferase systems (PEP-PTSs) may also be employed for this purpose (14–16). A range of glycosyl hydrolases (GH), enzymes that hydrolyze a specific glycosidic bond between the monosaccharide moieties of certain oligo- and polysaccharides, allow bifidobacteria to grow on dietary and host-derived carbohydrates present in the gastrointestinal tract (15).

Certain bifidobacterial strains have been shown previously to grow on soymilk-derived α-galacto-oligosaccharides, such as raffinose [α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→2)-β-D-Fru*f*], stachyose [α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→2)-β-D-Fru*f*], and melibiose [α-D-Gal*p*-(1→6)-α-D-Glc*p*] (17–19). Stachyose and raffinose (sugars of the so-called raffinose family, which also includes verbascose) are present in a wide variety of plants (20), while the related sugar melibiose (though not a member of the raffinose family) is also found in many plants and is particularly abundant in soybean roots and stems (21). To metabolize such α-galacto-oligosaccharides, bifidobacteria require α-galactosidase enzyme activity, which has been identified and characterized in five bifidobacterial species or strains: *Bifidobacterium bifidum* JCM 1254 (22), *Bifidobacterium adolescentis* (23, 24), *Bifidobacterium bifidum* NCIMB 41171 (25), *Bifidobacterium breve* 203 (26), and *Bifidobacterium longum* subsp. *longum* (27, 28).

The utilization and transcriptional regulation of raffinose have been characterized in more detail in *Escherichia coli* than in bifidobacteria. In *E. coli*, raffinose is actively transported into the cell by use of a dedicated raffinose permease (encoded by *rafA*) and is then hydrolyzed into sucrose and galactose by an α-galactosidase (specified by *rafB*). The sucrose is then hydrolyzed into glucose and fructose by a sucrose hydrolase, which is encoded by *rafD* (29).

Various α-glucosidases, such as the enzymes encoded by *agl1*

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and *agl2*, which were previously identified and characterized in *B. breve* UCC2003 (30), are produced by *Bifidobacterium* spp. (30, 31). Agl1 and Agl2, both members of GH family 13, which mainly represents enzymes with α-(1→6)-glucosidase activity (EC 3.2.1.10), have been shown to exhibit hydrolytic activity toward panose, isomaltose, and isomaltotriose, as well as toward four sucrose isomers, isomaltulose (Palatinose), trehalulose, turanose, and maltulose. They have also been shown to partially degrade trehalose and nigerose. The preferred substrates for the Agl1 and Agl2 enzymes have been shown to be panose, isomaltose, and trehalulose, carbohydrates that contain either an α-(1→6)-glucosidic bond (present in panose and isomaltose) or an α-(1→1)-glucosidic bond (present in trehalulose) (30).

Melezitose [α-D-Glc*p*-(1→3)-β-D-Fru*f*-(2→1)-α-D-Glc*p*] is an α-glucose-containing trisaccharide found in honeydew and manna, which are sugar-rich liquid and solid deposits, respectively, associated with the leaves and branches of various trees and shrubs (32). Although it was initially believed that melezitose was an oligosaccharide that was naturally present in various plants (33), it was later concluded that certain insects are responsible for melezitose production, since this sugar is absent from the tree sap used by such insects to form honeydew (32). To the best of our knowledge, no information on how (bifido)bacteria metabolize melezitose is available.

In the current study, we describe the identification of two adjacent gene clusters in the genome of *B. breve* UCC2003, *mel* and *raf*, which are involved in the metabolism of melezitose and raffinose family sugars, respectively.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bifidobacteria were routinely cultured in either de Man Rogosa and Sharpe medium (MRS medium; Difco, BD, Le Pont de Claix, France) supplemented with 0.05% cysteine-HCl or reinforced clostridial medium (RCM; Oxoid Ltd., Basingstoke, England). Carbohydrate utilization by bifidobacterial strains was examined in modified de Man Rogosa and Sharpe (mMRS) medium prepared according to first principles (34), though excluding a carbohydrate source. Prior to inoculation, the mMRS medium was supplemented with cysteine-HCl (0.05%, wt/vol) and a particular carbohydrate source (1%, wt/vol). The carbohydrates used were raffinose, stachyose, melezitose, melibiose, and glucose (all purchased from Sigma-Aldrich, Steinheim, Germany). Bifidobacterial cultures were incubated at 37°C under anaerobic conditions, which were maintained by using an Anaerocult oxygen-depleting system (Merck, Darmstadt, Germany) in an anaerobic chamber. *Lactococcus lactis* strains were cultivated in M17 broth containing 0.5% glucose (35) at 30°C. *Escherichia coli* strains were cultured in Luria-Bertani broth (LB) (36) at 37°C with agitation. Where appropriate, growth media contained chloramphenicol (Cm; 5 μg ml^{-1} for *L. lactis*, 10 μg ml^{-1} for *E. coli*, and 2.5 μg ml^{-1} for *B. breve*), erythromycin (Em; 100 μg ml^{-1} for *E. coli*), tetracycline (Tet; 10 μg ml^{-1} for *E. coli* or *B. breve*), or kanamycin (Km; 50 μg ml^{-1} for *E. coli*).

In order to determine bacterial growth profiles and final optical densities, 5 ml of freshly prepared mMRS medium, including a particular carbohydrate (see above), was inoculated with 50 μl (1%) of a stationary-phase culture of a particular *B. breve* strain. Uninoculated mMRS medium was used as a negative control. Cultures were incubated anaerobically at 37°C for 16 h, and the optical density at 600 nm (OD_{600}) was determined during this period 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 (37) genome annotations of *B. breve* UCC2003 (38). Database searches were performed using nonredundant sequences accessible at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the basic local alignment search tool (BLAST) (39, 40). Sequences were verified and analyzed using the SeqMan and SeqBuilder programs of the DNAStar software package (version 10.1.2; DNAStar, Madison, WI, USA).

DNA manipulations. Chromosomal DNA was isolated as described previously (41). Minipreparation of plasmid DNA from *E. coli*, *B. breve*, or *L. lactis* was carried out using the QIAprep Spin plasmid miniprep kit (Qiagen GmBH, Hilden, Germany). For *B. breve* or *L. lactis*, an initial lysis step was incorporated into the plasmid isolation procedure by resuspending cells in a lysis buffer supplemented with lysozyme (30 mg ml^{-1}), followed by incubation at 37°C for 30 min. Procedures for DNA manipulations were performed essentially as described previously (36). 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 are listed in Fig. S1 in the supplemental material and were synthesized by Eurofins (Ebersberg, Germany). Standard PCRs were performed using *Taq* PCR master mix (Qiagen) in a Biometra T3000 thermocycler (Biometra, Göttingen, Germany). PCR products were visualized by ethidium bromide (EtBr) staining following agarose gel electrophoresis (1%). *B. breve* colony PCRs were performed as described previously (42). PCR fragments were purified using the Qiagen PCR purification kit (Qiagen). Plasmid DNA was electroporated into *E. coli* as described previously (36). *B. breve* UCC2003 (14) and *L. lactis* (43) were electrotransformed according to published protocols. The correct orientation and integrity of all plasmid constructs (see also below) were verified by DNA sequencing, performed at Eurofins (Ebersberg, Germany).

Construction of *B. breve* insertion mutant strains. Internal fragments of Bbr_1856 (designated *melE* here) (421 bp, representing codons 242 through 383 of the 620 codons of this gene), Bbr_1857 (designated *melD*) (456 bp, representing codons 92 to 183 of the 556 codons of this gene), Bbr_1860 (designated *melA*) (331 bp, representing codons 230 to 341 of the 441 codons of this gene), Bbr_1867 (designated *rafB*) (394 bp, representing codons 95 to 226 of the 429 codons of this gene), and Bbr_1869 (designated *rafA*) (474 bp, representing codons 319 to 477 of the 771 codons of this gene) were amplified by PCR using *B. breve* UCC2003 chromosomal DNA as the template and the oligonucleotide primer combinations 1856fHd3 and 1856rxba1, 1857fHd3 and 1857rxba1, 1860fHd3 and 1860rxba1, 1867fHd3 and 1867rxba1, and 1869Hd3 and 1869Rxba1, respectively. Each of the PCR products generated was ligated to pORI19, an Ori$^+$ RepA$^-$ integration plasmid (44), using HindIII and XbaI restriction sites that were incorporated into the primers for the *melE*, *melD*, *melA*, *rafB*, and *rafA* fragment-encompassing amplicons, and was 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 g ml^{-1}) and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

The expected genetic structure of each of the resulting recombinant plasmids, pORI19-melE (pORI19 containing an internal 421-bp fragment of the *melE* gene), pORI19-melD (pORI19 containing an internal 456-bp fragment of the *melD* gene), pORI19-melA (pORI19 containing an internal 331-bp fragment of the *melA* gene), pORI19-rafB (pORI19 containing an internal 394-bp fragment of the *rafB* gene), and pORI19-rafA (pORI19 containing an internal 474-bp fragment of the *rafA* gene), was confirmed by restriction mapping and sequencing prior to subcloning of the Tet antibiotic resistance cassette, *tetW*, from pAM5 (45) as a SacI fragment into the unique SacI site in each of the pORI19 derivatives. The orientation of the tetracycline resistance gene in each of the resulting plasmids, pORI19-tet-melE, pORI19-tet-melD, pORI19-tet-melA, pORI19-tet-rafB, and pORI19-tet-rafA (naming is consistent with the names of their predecessor plasmids [see above], to which the "tet" designation was added), was determined by restriction analysis. The plasmids were subse-

TABLE 1 Strains and plasmids used in this study

Strain or plasmid[a]	Relevant characteristic(s)	Reference or source[b]
Strains		
E. coli		
EC101	Cloning host; *repA*$^+$ *kmr*	44
EC101-pNZ-M.Bbrll+Bbr111	EC101 harboring a pNZ8048 derivative containing bbrllM and bbrlllM	
L. lactis		
NZ9000	From MG1363, a nisin-inducible overexpression host; *pepN*::*nisRK*	90
NZ9700	Nisin-producing strain	90
B. breve		
UCC2003	Isolate from nursling stool	14
NCFB 2257	Isolate from infant intestine	NCFB
NCTC 11815	Isolate from infant intestine	NCTC
NCFB 2258	Isolate from infant intestine	NCFB
NCIMB 8815	Isolate from infant feces	NCIMB
JCM 7017	Isolate from human feces	JCM
JCM 7019	Isolate from infant feces	JCM
UCC2005	Isolate from nursling stool	30
Yakult	Isolate from nursling stool	87
Nizo 658	Isolate from nursling stool	Nizo
461	Isolate from infant/adult feces	PRL
689	Isolate from infant/adult feces	PRL
12L	Mother's milk	PRL
UCC2003-MelE	pORI19-tet-1856 insertion mutant of UCC2003	This study
UCC2003-MelD	pORI19-tet-1857 insertion mutant of UCC2003	This study
UCC2003-RafB	pORI19-tet-1867 insertion mutant of UCC2003	This study
UCC2003-RafA	pORI19-tet-1869 insertion mutant of UCC2003	This study
UCC2003-MelA	pORI19-tet-1860 insertion mutant of UCC2003	This study
Plasmids		
pORI19	Em^r RepA$^-$ *ori*$^+$ cloning vector	44
pORI19-tet-MelE	Internal 421-bp fragment of *melE* and *tetW* cloned in pORI19	This study
pORI19-tet-MelD	Internal 456-bp fragment of *melD* and *tetW* cloned in pORI19	This study
pORI19-tet-MelA	Internal 331-bp fragment of *melA* and *tetW* cloned in pORI19	This study
pORI19-tet-RafA	Internal 474-bp fragment of *rafA* and *tetW* cloned in pORI19	This study
pORI19-tet-RafB	Internal 394-bp fragment of *rafB* and *tetW* cloned in pORI19	This study
pAM5	pBC1-puC19-Tcr	45
pNZ8048	Cm^r; nisin-inducible translational fusion vector	90
pNZ8150	Cm^r; nisin-inducible translational fusion vector	52
pNZMelE-His	MelE with His tag cloned downstream of nisin-inducible promoter on pNZ8048	This study
pNZMelD-His	MelD with His tag cloned downstream of nisin-inducible promoter on pNZ8048	This study
pNZRafA-His	RafA with His tag cloned downstream of nisin-inducible promoter on pNZ8150	This study

[a] NCFB, National Collection of Food Bacteria; NCTC, National Collection of Type Cultures; NCIMB, National Collection of Industrial and Marine Bacteria; JCM: Japanese Collection of Microorganisms; UCC, University College Cork culture collection; Nizo, Nizo food research.
[b] PRL, Culture collection of probiogenomics, University of Parma.

quently introduced into *E. coli* EC101 pNZ-MBbrI-MBbrII in order to achieve methylation, and transformants were selected on the basis of Cm and Tet resistance. Methylation of the plasmid complement of the transformants obtained in EC101 pNZ-MBbrI-MBbrII was confirmed by their observed insensitivity to PstI restriction (42). Plasmid preparations of methylated pORI19-tet-melE, pORI19-tet-melD, pORI19-tet-melA, pORI19-tet-rafB, and pORI19-tet-rafA were then introduced into *B. breve* UCC2003 by electroporation, with subsequent selection for transformants 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*. This was followed by a second PCR, employing 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. In this case, a product would be obtained only if the correctly positioned gene disruption had occurred.

Analysis of global gene expression using *B. breve* DNA microarrays. Global gene transcription patterns were determined by microarray analysis during the growth of *B. breve* UCC2003 on raffinose, stachyose, melibiose, melezitose, or sucrose, and these transcriptomes were compared to those obtained from cells that had been grown on ribose as the sole carbohydrate source. All biological replicates were hybridized using a dye swap strategy. 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). Methods for cell disruption, RNA isolation, RNA quality control, cDNA synthesis, and labeling were performed as described previously (46). Labeled cDNA was hybridized using the Agilent Gene Expression hybridization kit (part no. 5188-5242) as described in the Agilent two-color microarray-based gene expression analysis manual, version 4.0 (47). Following hybridization, microarrays were washed in accordance with Agilent standard procedures and were scanned using an Agilent DNA microarray scanner (model G2565A). The scans generated

were converted to data files with Agilent's Feature Extraction software (version 9.5). DNA microarray data were processed as described previously (48–50). Differential expression tests were performed with the Cyber-T implementation of a variant of the *t* test (51). A gene was considered differentially expressed when the *P* value was <0.001 and the expression ratio was >3 or <0.33 relative to the control.

Expression and purification of RafA, MelE, and MelD. DNA fragments containing the complete (predicted) α-galactosidase-encoding genes, *rafA* and *melE*, or the α-glucosidase-encoding gene, *melD*, were generated by PCR amplification from chromosomal DNA of *B. breve* UCC2003 using *Taq* DNA polymerase and the primer combination 1869EcorVF and 1869Xba1R, 1856EcorVF and 1856Xba1R, or 1857Nco1F and 1857Xba1R, respectively (see Fig. S1 in the supplemental material). An NcoI or EcoRV restriction site and an XbaI restriction site were incorporated at the 5′ ends of each forward and reverse primer, respectively (see Fig. S1). In addition, an in-frame His_{10}-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 three amplicons generated were first digested with either NcoI or EcoRV and XbaI and then ligated into the NcoI- or ScaI- and XbaI-digested nisin-inducible translational fusion plasmid pNZ8048 (which contains an NcoI site) or pNZ8150 (which contains a ScaI site), depending on the restriction sites of the amplified fragment (52). The ligation mixtures were introduced into *L. lactis* NZ9000 (Table 1) by electrotransformation, and transformants were then selected on the basis of chloramphenicol resistance. The plasmid contents of a number of Cm^r transformants were screened by restriction analysis, and the integrity of positively identified clones (carrying pNZMelE-His, containing the *melE* gene; pNZMelD-His, containing the *melD* gene; or pNZRafA-His, containing the *rafA* gene) were verified by sequencing.

In order to (over)express and purify proteins, 400 ml of M17 broth supplemented with 0.5% glucose was inoculated with a 2% inoculum of a particular *L. lactis* strain, followed by incubation at 30°C until an OD_{600} of 0.5 was reached. At that point, protein expression was induced by the addition of purified nisin (5 ng ml^{-1}), and incubation was continued at 30°C for 90 min. Cells were harvested by centrifugation, washed, and concentrated 40-fold. Protein was purified using a PrepEase kit specialized for His-tagged protein purification (USB, Germany). Elution fractions were analyzed by SDS-polyacrylamide gel electrophoresis, as described previously (53), on a 12.5% polyacrylamide gel. After electrophoresis, the gels were fixed and stained with Coomassie brilliant blue to identify fractions containing the purified protein. Rainbow prestained low-molecular-weight protein markers (New England BioLabs, Hertfordshire, United Kingdom) were used to estimate the molecular weights of the purified proteins.

Biochemical characterization of MelD, MelE, and RafA. The putative α-glucosidase activity of MelD and the presumed α-galactosidase activities of RafA and MelE were determined essentially as described previously (54). A 50-μl volume of each purified protein (concentration, 0.5 mg ml^{-1}) was incubated with 20 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0). For RafA and MelE, 0.1 mg ml^{-1} (wt/vol) of α-(1→4)-galactobiose, α-(1→3)-galactobiose, raffinose, stachyose, melibiose, melezitose, turanose, or sucrose was added to 20 mM MOPS buffer as the enzyme substrate in a final volume of 1 ml at 37°C. For analysis of the catalytic activities of MelD, 0.1 mg ml^{-1} (wt/vol) of α-(1→4)-galactobiose, α-(1→3)-galactobiose, melezitose, maltulose, isomaltulose, turanose, leucrose, sucrose, raffinose, stachyose, or melibiose was added to 20 mM MOPS buffer at pH 7.0 as the enzyme substrate in a final volume of 1 ml, and the mixture was incubated at 37°C. Following incubation, 200-μl samples were taken at 24-h intervals. Samples were filtered by membrane filtration, using Spin-X centrifuge tube filters (pore size, 0.45 μm; Costar; Corning Inc., NY), and were stored at −20°C prior to high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis (see below).

Kinetic constants for MelD using sucrose or turanose, and for RafA using melibiose, raffinose, or stachyose, were determined by measuring the hydrolysis rates at various substrate concentrations, ranging from 2.5 to 100 mM. Reactions were initiated by the addition of 50 μl of purified protein (concentration, 0.5 mg ml^{-1}) in 20 mM MOPS buffer at the optimum pH and temperature determined for each protein, and the reactions were stopped at different time points (up to 6 min) by heat treatment at 100°C for 15 min. All experiments were performed in duplicate, and the amount of glucose released from each disaccharide substrate, namely, melibiose, sucrose, and turanose, was measured by using the glucose hexokinase assay kit (Sigma) according to the manufacturer's instructions. For raffinose and stachyose hydrolysis, a sucrose assay kit was utilized, in which case enzyme activity is based on the amount of sucrose released as measured according to the manufacturer's instructions (Sigma). Either sucrose, turanose, or melibiose (50 mM) was used as the substrate for the determination of the pH and temperature optima (a pH range of 2.5 to 9.5 and a temperature range of 4°C to 60°C were tested). Reactions were initiated by the addition of 50 μl of purified protein (concentration, 0.5 mg ml^{-1}) in 20 mM MOPS buffer at pH 7.0.

HPAEC-PAD analysis. For HPAEC-PAD analysis, a Dionex (Sunnyvale, CA) ICS-3000 system was used. Carbohydrate fractions (25-μl aliquots) were separated on a CarboPac PA1 analytical-exchange column (dimensions, 250 mm by 4 mm) with a CarboPac PA1 guard column (dimensions, 50 mm by 4 mm) and a pulsed electrochemical detector (ED40) in the PAD mode (all from Dionex). Elution was performed at a constant flow-rate of 1.0 ml min^{-1} at 30°C using the following eluents for the analysis: eluent A, 200 mM NaOH; eluent B, 100 mM NaOH plus 550 mM Na acetate; eluent C, Milli-Q water. The following linear gradient of sodium acetate was used with 100 mM NaOH: from 0 to 50 min, 0 mM; from 50 to 51 min, 16 mM; from 51 to 56 min, 100 mM; from 56 to 61 min, 0 mM. Chromatographic profiles of standard carbohydrates were used for comparison of the results of their breakdown by the MelD, MelE, and RafA proteins. Chromeleon software (version 6.70; Dionex Corporation) was used for the integration and evaluation of the chromatograms obtained. A 10-mg ml^{-1} stock solution of each of the carbohydrates to be used as reference standards was prepared by dissolving the particular sugar in deionized Milli-Q water. The stock solution was then sterilized by membrane filtration using Minisart filters (pore size, 0.45 μm; Sartorius AG, Göttingen, Germany) and was stored at 4°C.

Microarray data accession number. The microarray data obtained in this study have been deposited in NCBI's Gene Expression Omnibus database and are accessible through GEO Series accession number GSE47448.

RESULTS AND DISCUSSION

Carbohydrate-dependent analysis of the growth of various *B. breve* strains. Knowledge of the carbohydrate metabolism of individual bifidobacterial species or strains is important in order to evaluate the prebiotic potential of particular carbohydrates from a dietary perspective. In order to determine whether different strains of *B. breve* possess the ability to utilize the α-glycosidic bond-containing sugars raffinose, stachyose, melibiose, and melezitose as sole carbohydrate sources (all of approximately 99% purity), growth profiles were determined for 11 *B. breve* strains. All *B. breve* strains tested exhibited good growth (final OD_{600}, >1.0) in mMRS medium supplemented with raffinose, stachyose, or melibiose (Fig. 1). Seven strains exhibited good growth in mMRS medium supplemented with melezitose, while five strains, namely, *B. breve* JCM7017, *B. breve* 461, *B. breve* 689, *B. breve* NCFB 2257, *Bifidobacterium longum* subsp. *infantis* ATCC 15697, and *B. breve* NCFB 2258, were not capable of appreciable growth (final OD_{600}, <0.4) on melezitose as a sole carbohydrate source (Fig. 1).

Transcriptome analysis of *B. breve* UCC2003 grown on raffinose, stachyose, or melibiose. In order to identify genes in-

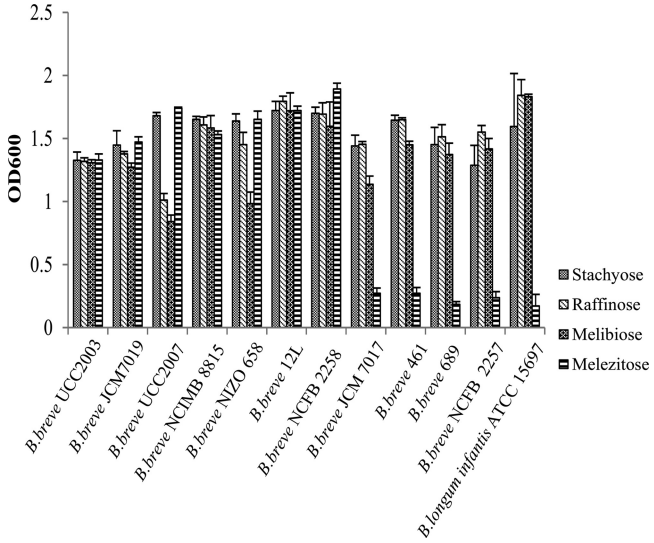

FIG 1 Final OD_{600} values following 16 h of growth of various wild-type *B. breve* strains on 1% raffinose, 1% stachyose, 1% melibiose, or 1% melezitose. The results are mean values obtained from three separate experiments.

volved in the metabolism of raffinose-related carbohydrates, we used DNA microarray analysis (see Materials and Methods) to investigate differences in global gene expression upon the growth of *B. breve* UCC2003 on raffinose, stachyose, or melibiose, compared with growth on ribose (the metabolic pathway for ribose in *B. breve* UCC2003 has been characterized previously, and growth on this sugar has been shown to provide a suitable transcriptomic reference) (55). The resulting transcriptomic data showed that transcription of the contiguous genes Bbr_1869, Bbr_1867, Bbr_1866, and Bbr_1865 (here designated *rafA*, *rafB*, *rafC*, and *rafD*, respectively) was significantly upregulated (fold change, >4.0; *P*, <0.001) in *B. breve* UCC2003 cultures grown on raffinose, stachyose, or melibiose, relative to that for cultures grown on the control carbohydrate ribose, thus implicating this gene cluster in raffinose-related sugar metabolism in *B. breve* UCC2003 (Table 2). Furthermore, transcription of four additional genes was upregulated (relative to the ribose transcriptome) when *B. breve* UCC2003 was grown on raffinose (and on stachyose, although this transcriptional increase was just below the cutoff value of 4-fold for two of these genes) but not when the strain was grown on melibiose: Bbr_0026, Bbr_0027, Bbr_0030, and Bbr_100, which are predicted to encode two ABC-type permeases, a hypothetical protein, and a putative sucrose phosphorylase, respectively. Since these four genes also exhibit increased transcription when *B. breve* UCC2003 is grown on sucrose (relative to growth on ribose) (Table 2), we hypothesize that they are involved in the metabolism of this disaccharide, which is released upon removal of the α-galactose moieties from the nonreducing ends of raffinose and stachyose, in contrast to melibiose hydrolysis, which results in galactose and glucose release.

Our data on the *raf* cluster of *B. breve* UCC2003 suggest that many bifidobacteria metabolize raffinose and the related sugars stachyose and melibiose, by means of a metabolic route somewhat different from that known for *E. coli* (56). Raffinose-type sugar uptake in *B. breve* UCC2003 and other bifidobacteria apparently occurs via an ABC-type transporter system, which is a common way for bifidobacteria to internalize carbohydrates (14, 30, 55, 57–60). Removal of the α-galactose moiety from raffinose and stachyose results in the release of sucrose, which in *E. coli* is further metabolized via a sucrose hydrolase (56), while in *B. breve* UCC2003, sucrose utilization appears to occur by a sucrose phosphorylase, since a gene that is predicted to encode such an activity exhibits increased transcription when UCC2003 is grown on sucrose or sucrose-containing sugars (Table 2). Sucrose metabolism by sucrose phosphorylase has been characterized in other bifidobacteria, for example, *Bifidobacterium adolescentis* DSM20083 (61), *B. longum* (62, 63), and *Bifidobacterium animalis* subsp. *lactis* (64). Based on the ability of all *B. breve* strains examined to utilize raffinose-type oligosaccharides (Fig. 1), corroborated by the comparative genome analysis presented below (see Fig. 2), and on other reports in the literature regarding raffinose utilization by

TABLE 2 Carbohydrate-dependent transcriptional upregulation of specific genes[a]

Gene ID	Gene name	Function	Fold upregulation[b] during growth on:				
			Stachyose	Raffinose	Melibiose	Melezitose	Sucrose
Bbr_0026		Permease protein of ABC transporter system for sugars	—	18.55	—	4.66	23.73
Bbr_0027		Permease protein of ABC transporter system for sugars	—	13.98	—	4.55	44.95
Bbr_0030		Conserved hypothetical protein	28.53	145.75	—	86.54	216.5
Bbr_0100	*SPase*	Sucrose phosphorylase	74.26	74.26	—	128.18	41.71
Bbr_1855	*agl1*	Alpha glucosidase	—	20.43	10.74	31.95	—
Bbr_1856	*melE*	Raffinose synthase or seed inhibition protein	—	—	—	72.76	—
Bbr_1857	*melD*	Alpha glucosidase	—	—	—	94.33	—
Bbr_1858	*melC*	Permease protein of ABC transporter system for sugars	—	—	—	850.82	—
Bbr_1859	*melB*	Permease protein of ABC transporter system for sugars	—	—	—	679.07	—
Bbr_1860	*melA*	Solute binding protein of ABC transporter system for sugars	—	—	—	724.89	—
Bbr_1865	*rafD*	Raffinose transport system permease protein	21.66	98.36	105.07	—	—
Bbr_1866	*rafC*	Raffinose transport system permease protein	49.86	175.51	153.8	—	—
Bbr_1867	*rafB*	Raffinose-binding protein	24.39	86.16	92.5	—	—
Bbr_1868	*rafR*	Transcriptional repressor, ROK family	—	—	—	—	—
Bbr_1869	*rafA*	α-Galactosidase	9.23	10.53	8.07	—	—

[a] Based on comparative transcriptome analysis using *B. breve* UCC2003 grown on 1% raffinose, stachyose, or melibiose compared to growth on ribose. Microarray data were obtained using *B. breve* UCC2003 grown on 1% raffinose, stachyose, or melibiose and were compared with array data obtained when *B. breve* UCC2003 was grown on ribose as a control.

[b] The cutoff point is 4-fold, with a *P* value of <0.001. —, value below the cutoff.

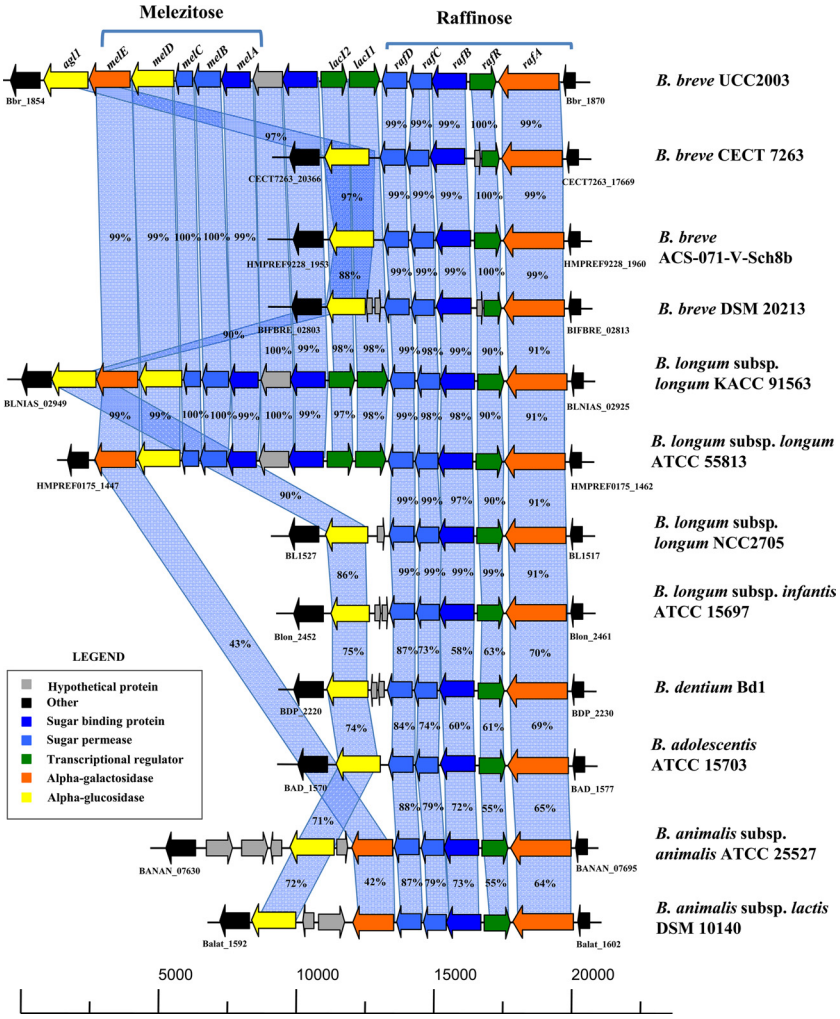

FIG 2 Comparison of the melezitose, raffinose, and stachyose gene clusters of *B. breve* UCC2003 with corresponding putative melezitose, raffinose, and stachyose utilization loci of other bifidobacteria. Each solid arrow represents an ORF. The length of each arrow is proportional to the length of the predicted ORF, and the gene locus name, which is indicative of its putative function, is given at the top. Orthologs are shown in the same color. The amino acid identity of each predicted protein to its equivalent protein encoded by *B. breve* UCC2003, expressed as a percentage, is given above each arrow.

bifidobacteria, raffinose-type sugar utilization appears to be an ubiquitous property of bifidobacteria, supporting previous publications on the prebiotic potential of (some of) these sugars (65–68).

Although transcription of the *raf* gene cluster of *B. breve* is induced in the presence of either raffinose, melibiose, or stachyose, it has been shown that the raffinose system in *Streptococcus pneumoniae* is upregulated in the presence of raffinose but not melibiose, suggesting differential regulatory mechanisms (69).

Transcriptome analysis of *B. breve* UCC2003 grown on melezitose. In order to identify genes involved in the utilization of melezitose by *B. breve* UCC2003, transcriptome analysis was performed for *B. breve* UCC2003 grown on this trisaccharide, and the results were compared with those obtained when the same organism was grown on ribose. Analysis of transcriptome data obtained from two independent biological replicates revealed that transcription of the contiguous genes Bbr1860 through Bbr_1856, here designated *melA*, *melB*, *melC*, *melD*, and *melE*, respectively (see Fig. 2), which are located in close proximity to the *raf* gene cluster, was significantly upregulated (fold change, >4.0; *P*, <0.001) in *B. breve* UCC2003 cultures grown on melezitose relative to that for cultures grown on ribose (Table 2).

These results implicate the *melABCDE* gene cluster in melezitose metabolism in *B. breve* UCC2003. Interestingly, on the basis of previously published comparative genome hybridization data (38), we noticed that *B. breve* strains that did not exhibit growth on melezitose (Fig. 1) lack the *melABCDE* gene cluster. Furthermore, in findings reminiscent of the results obtained for transcriptome analysis when strain UCC2003 was grown on stachyose and raffinose, the transcription of four additional genes, Bbr_0026, Bbr_0027, Bbr_0030, and Bbr_0100, was upregulated when *B. breve* UCC2003 was grown on melezitose (Table 2). Since hydrolysis of melezitose produces sucrose if the α-(1→3)-linked glucose is released, the transcriptional induction of these genes is believed to be correlated to sucrose metabolism.

Genetic organization of the *raf* and *mel* gene clusters and comparison to other available bifidobacterial genomes. Our presumption, based on the microarray results, was that the genes

of the *raf* gene cluster, schematically depicted in Fig. 2, are involved in the metabolism of sugars that contain one or more α-(1→6)-linked galactose moieties (e.g., raffinose, stachyose, and melibiose). The *rafA* gene, which specifies a putative α-galactosidase of GH family 27, is a clear homolog of the *B. breve* CECT 7263 α-galactosidase-encoding gene, with which it shares 99% sequence identity (70). *B. breve* CECT 7263 also contains genes with high sequence similarity (99 to 100% identity) to other genes in the *B. breve* UCC2003 *raf* gene cluster (Fig. 2). The *rafA* gene is presumed to be responsible for the breakdown of raffinose, stachyose, and melibiose via hydrolysis of the α-(1→6)-glycosidic bond that is common to these carbohydrates, thereby releasing galactose. The presumptive *B. breve* UCC2003 α-galactosidase-encoding gene, *rafA*, is located immediately adjacent to a gene, Bbr_1868 (here designated *rafR*), that is predicted to encode a ROK-type transcriptional regulator. Members of the ROK protein family include both transcriptional repressors and sugar kinases (71). ROK kinases possess a conserved N-terminal ATP-binding motif, while ROK repressors contain an N-terminal region that includes a canonical helix-turn-helix motif associated with DNA binding (72). BLAST analysis and Pfam searches revealed the presence of such a helix-turn-helix motif, leading to the prediction that the *rafR* gene product functions as a transcriptional regulator, which may, based on its genomic location, be involved in regulating raffinose (sugar family)-dependent transcription of the other genes of the *raf* gene cluster. Close homologs of *rafA* and *rafR*, and of the neighboring genes *rafB*, *rafC*, and *rafD*, which are predicted to specify a solute binding protein and two permeases, respectively, of a putative ABC-type sugar uptake system, are present in all other publicly available bifidobacterial genomes except for those of *Bifidobacterium asteroides* PRL2011 and *Bifidobacterium bifidum* PRL2010 (68, 73) (Fig. 2; also data not shown). Interestingly, the *raf* gene cluster does not contain a gene with a predicted ATP-binding protein, which is required for providing energy to the ABC-type transport system (74). It is presumed that this activity is encoded by an unconnected gene, whose product can function as an ATP-binding component for multiple ABC transporters, a scenario similar to that suggested for other bifidobacterial ABC-type carbohydrate transport systems (58, 59).

The *melABCDE* gene cluster, whose transcription is specifically induced by growth on melezitose, is predicted to specify a solute binding protein (*melA*) and two permease proteins (*melB* and *melC*) (again, lacking a gene predicted to encode an ATP-binding protein, representing a genetic configuration similar to that observed for the *raf* gene cluster [see above]), an α-glucosidase (*melD*) belonging to GH family 13, and an α-galactosidase/raffinose synthase (*melE*) of GH family 36 (Table 2 and Fig. 2). The DNA region between the *raf* and *mel* gene clusters contains four additional open reading frames (ORFs): Bbr_1863 and Bbr_1864, which encode putative LacI-type transcriptional regulators, here designated *lacI2* and *lacI1*, respectively, and Bbr_1861 and Bbr_1862, which specify a hypothetical protein and a predicted solute binding protein, respectively. The *mel* and *raf* genetic loci, including the genes of the intervening region, share high sequence similarity (99 to 100%) at the amino acid level and a conserved gene organization with corresponding regions in the genomes of *B. longum* subsp. *longum* KACC 91563 and *B. longum* subsp. *longum* ATCC 55813. Interestingly, homologs of the *mel* gene cluster (including Bbr_1861 to Bbr_1864) are lacking in many bifidobacterial genomes, although such genomes do contain clear homologs of the *raf* gene cluster and the *agl1* gene, which in the UCC2003 genome flank the *mel* cluster on either end (Fig. 2), suggesting that those bifidobacterial strains lack the (genetic) ability to metabolize melezitose.

Prevalence and genetic organization of *raf* and *mel* gene clusters in other bacteria. The genetic organization of the bifidobacterial *raf* locus and the raffinose metabolic pathway appears to be quite different from those of various other bacterial species. In *E. coli* (29), *Klebsiella pneumoniae* (75), *Enterobacter cloacae* (76), and *Citrobacter freundii* (77), among others, raffinose uptake is specified by a single permease-encoding gene, which is cotranscribed with two additional genes that specify an α-galactosidase and a sucrose hydrolase. As mentioned above, uptake of raffinose and related sugars in bifidobacteria appears to be performed by a dedicated ABC-type transport system, whose genes are in close proximity to, though not cotranscribed with, the α-galactosidase-specifying gene, due to their opposing genetic orientation (Fig. 2). Furthermore, in bifidobacteria, the metabolic product of this α-galactosidase activity, i.e., sucrose, is apparently metabolized not by a sucrose hydrolase but by a sucrose phosphorylase, encoded by a gene that is not genetically linked to the *raf* locus.

Raffinose metabolism has also been investigated in *Lactobacillus plantarum* ATCC 8014, in which the gene encoding the putative raffinose permease is immediately followed by the α-galactosidase-specifying gene (designated *melA*) and is preceded by *galM*, a putative microbial galactose-1-epimerase (78). Interestingly, two genes located downstream of *melA*, though on the opposite strand, encode the two subunits of a heterodimeric β-galactosidase, which shows that the genetic organization and content of the raffinose utilization cluster of *L. plantarum* differ substantially from those in bifidobacteria. Recently, transcriptome analyses have shown that *B. animalis* subsp. *lactis* Bl-04 possesses a raffinose utilization cluster including three predicted α-glycosidases, exhibiting a transcription profile that is consistent with our observations for *B. breve* (79).

Very little information is available regarding melezitose metabolism in other microorganisms. Melezitose metabolism has been investigated in the yeast *Saccharomyces cerevisiae* (80), where a melezitose-metabolizing system was identified, including an α-glucosidase or melezitase that was shown to be capable of hydrolyzing isomaltulose, turanose, maltose, sucrose, and melezitose.

Construction and phenotypes of mutants carrying individual disruptions in the *rafA*, *rafB*, *melA*, *melD*, and *melE* genes. In order to determine whether disruption of a particular gene of the *raf* gene cluster of *B. breve* UCC2003 affects the resulting strain's ability to metabolize raffinose, stachyose, and/or melibiose, mutants were made with insertions in *rafB*, which is predicted to encode a solute binding protein implicated in the internalization of the α-galactose-containing sugars mentioned above, and in *rafA*, the presumed α-(1→6)-galactosidase-encoding gene. The resulting strains were designated UCC2003-RafB and UCC2003-RafA, respectively (see Materials and Methods). *B. breve* UCC2003-RafA and *B. breve* UCC2003-RafB were analyzed for their abilities to grow on mMRS medium supplemented with either raffinose, stachyose, or melibiose as the sole carbon source. As expected, and in contrast to the findings for the wild type, the *B. breve* insertion mutants were unable to grow on raffinose, stachyose, or melibiose as the sole carbon source. All strains retained their ability to utilize glucose as a sole carbon source (Fig.

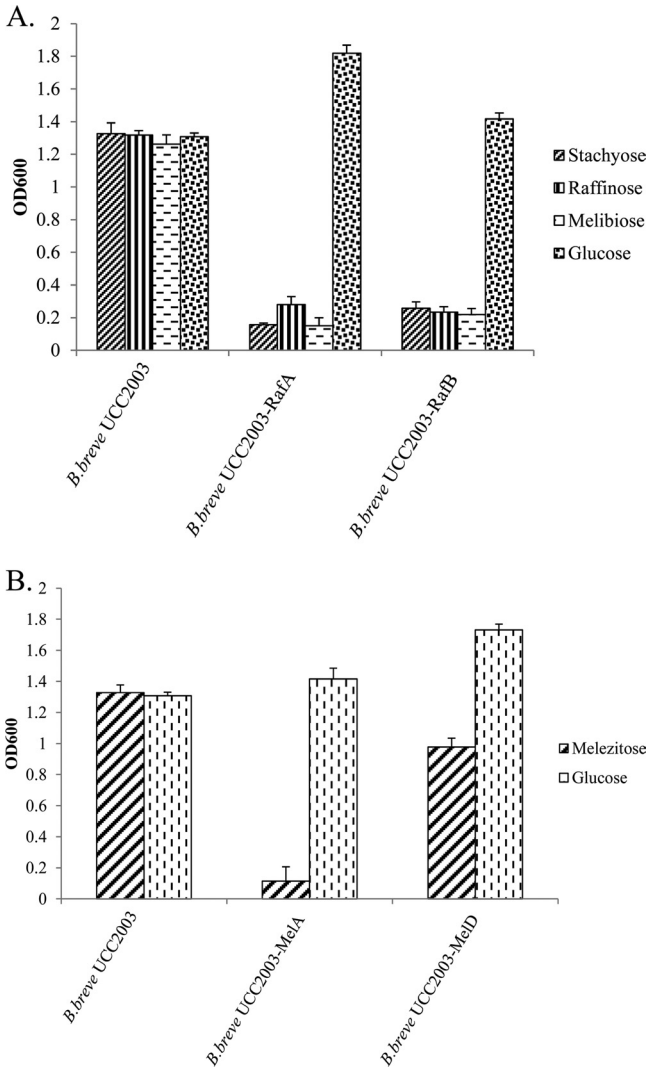

FIG 3 (A) Final OD_{600} after 16 h of growth of UCC2003 and insertion mutants UCC2003-RafB (raffinose binding protein) and UCC2003-RafA (α-galactosidase) on 1% stachyose, raffinose, melibiose, or glucose. (B) Final OD_{600} following 16 h of growth of *B. breve* UCC2003, *B. breve* UCC2003-MelD [α-(1→3)-glucosidase], and *B. breve* UCC2003-MelA (solute binding protein) on 1% melezitose or glucose. In both panels, the results are mean values obtained from three separate experiments.

3A). These results show not only that the *rafA* gene is required for growth on raffinose but also that a mutation in *rafB* causes this growth-deficient phenotype, implying that the *rafBCD* genes encode an ABC-type transport system responsible for the internalization of α-galactose-containing oligosaccharides. Our results corroborate the findings of a recent study in which a deletion in a homolog of the *rafA* gene of *Bifidobacterium longum* 105-A (designated *agl*) was shown to cause a loss of α-galactosidase activity and a growth deficiency on raffinose or melibiose (28).

In order to determine whether disruption of a particular gene from the *mel* cluster results in loss of the ability of *B. breve* UCC2003 to metabolize melezitose, mutants were made with insertions in *melE*, which is predicted to encode an α-galactosidase, resulting in strain *B. breve* UCC2003-MelE; in *melD*, which is predicted to encode an α-glucosidase, resulting in strain *B. breve* UCC2003-MelD; and in *melA*, which encodes a predicted solute binding protein, resulting in strain *B. breve* UCC2003-MelA (see Materials and Methods). These mutants—*B. breve* UCC2003-MelE, UCC2003-MelD, and UCC2003-MelA—were then analyzed for their abilities to grow on mMRS medium supplemented with melezitose as the sole carbon source. As expected, and in contrast to the finding for the wild type, the growth of *B. breve* strain UCC2003-MelA on melezitose was severely reduced from that of the wild-type strain, indicating that *melA* is indeed required for melezitose catabolism. Mutant strain *B. breve* UCC2003-MelD showed impaired growth on melezitose compared to that of the wild-type strain UCC2003, though to a lesser extent than strain UCC2003-MelA, and this growth defect is particularly obvious when the corresponding growth profiles of these three strains on melezitose are compared (Fig. 3B). The less severe growth deficiency of the *melD* insertion mutant than of the *melA* insertion mutant may be due to the presence of other α-glucosidases produced by *B. breve* UCC2003 that partially compensate for the *melD* mutation. Interestingly, the ability of mutant strain UCC2003-MelE to grow on melezitose was not affected, indicating that MelE is not required for growth on this sugar (data not shown). Wild-type UCC2003 and the three *mel* mutants exhibited no differences in their abilities to utilize glucose as a sole carbon source.

Purification, characterization, and substrate specificity of recombinantly produced MelD, MelE, and RafA. In order to analyze the glycosyl hydrolase functions of MelD, MelE, and RafA, we purified and biochemically characterized these three predicted sugar-degrading enzymes. All of the overproduced proteins were purified well and in soluble form, and MelE, MelD, and RafA exhibited molecular masses of approximately 68.2 kDa, 62.4 kDa, and 84.1 kDa (inclusive of the His_6 tag), respectively, when analyzed by SDS-PAGE (results not shown).

Purified recombinant MelD protein was shown to fully hydrolyze melezitose into glucose and fructose (Fig. 4A, graph I), thereby demonstrating that this protein has both α-(1→2)- and α-(1→3)-glycosyl hydrolase activities. This was further confirmed by our findings that both sucrose [α-D-Glc*p*-(1→2)-β-D-Fru*f*] and turanose [α-D-Glc*p*-(1→3)-β-D-Fru*f*] are hydrolyzed by MelD to glucose and fructose (Fig. 4A, graphs II and III, respectively). In contrast, MelD was unable, at least under the conditions tested, to hydrolyze leucrose [α-D-Glc*p*-(1→5)-β-D-Fru*f*], isomaltulose [α-D-Glc*p*-(1→6)-β-D-Fru*f*], or maltulose [α-D-Glc*p*-(1→4)-β-D-Fru*f*] (results not shown). These results, therefore, show that MelD is an α-glucosidase with substrate specificities clearly different from those of the two previously characterized α-glucosidases encoded by *B. breve* UCC2003, Agl1 and Agl2, which cannot hydrolyze leucrose, melezitose, or sucrose but possess hydrolytic activity against turanose, maltulose, and isomaltulose (30). The preferred substrates for Agl1 and Agl2 are thus carbohydrates that contain either an α-(1→6)- or an α-(1→1)-glucosidic bond (30), while MelD is an α-glucosidase with hydrolytic activity against α-(1→2)- and α-(1→3)-glucosidic bonds, which distinguishes it from previously characterized bifidobacterial α-glucosidases (31, 81, 82).

The putative α-galactosidase MelE failed to exhibit hydrolytic activity toward raffinose, stachyose, or melibiose (results not shown), at least under the conditions tested. However, when two synthetic disaccharides, namely, α-(1→4)- and α-(1→3)-galactobiose, were assayed, MelE was shown to be capable of hydrolyzing

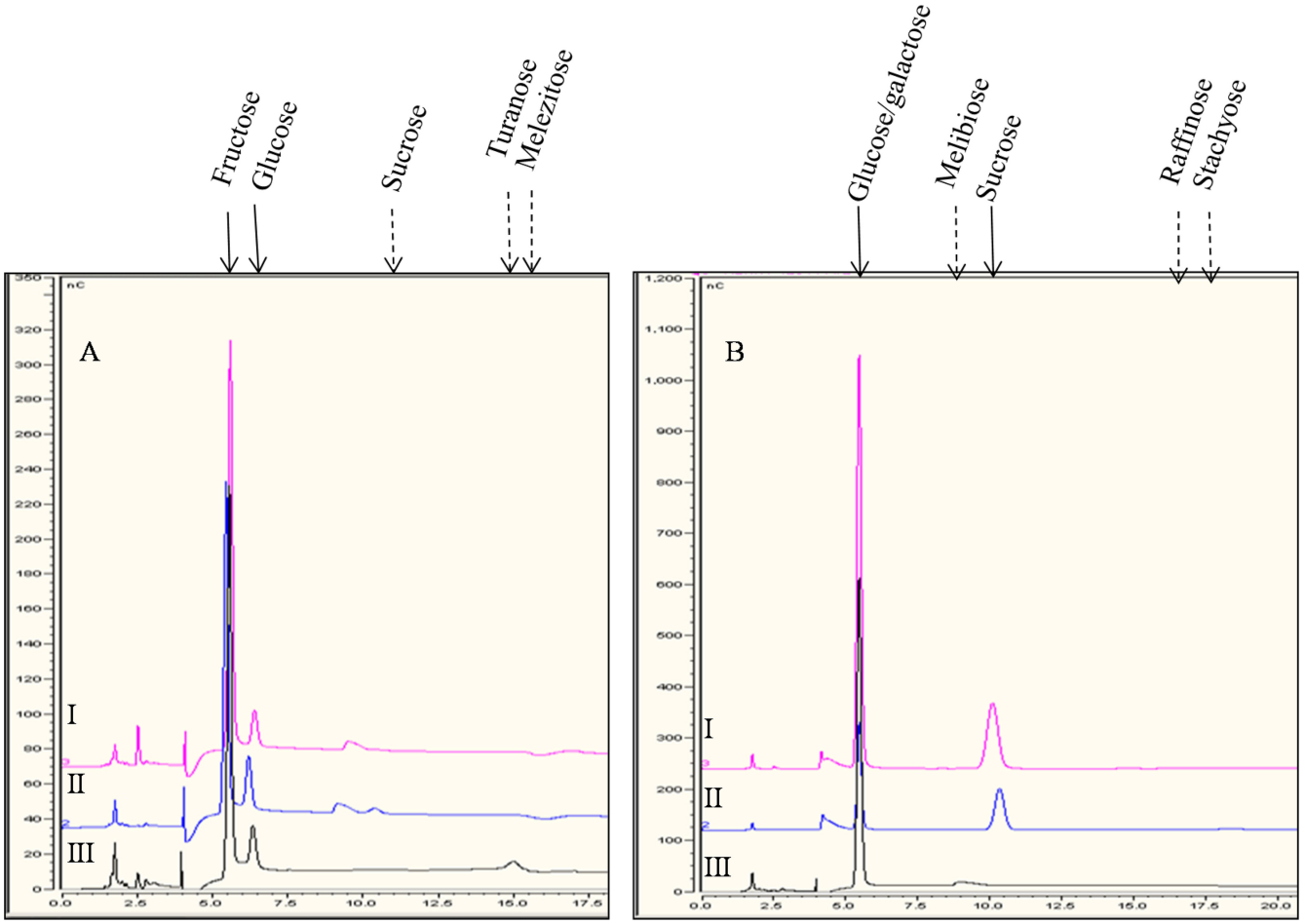

FIG 4 (A) HPAEC-PAD analysis indicating the breakdown of melezitose and turanose (initial concentration, 0.1 mg ml^{-1}) by the purified recombinant protein MelD in 20 mM MOPS buffer (pH 7.0) over 24 h. The chromatogram shows results for melezitose (graph I), sucrose (graph II), and turanose (graph III) incubated with MelD. The liberation of glucose and fructose is visible as chromatographic peaks eluted at 6.25 and 5.5 min, respectively. Breakdown products are indicated by solid arrows. Chromatographic positions of carbohydrate standards are indicated by dashed arrows above the chromatogram. (B) HPAEC-PAD analysis indicating the breakdown of stachyose, raffinose, and melibiose by the purified recombinant protein RafA in 20 mM MOPS buffer (pH 7.0) over 24 h. (Graphs I and II) Stachyose (graph I) and raffinose (graph II) incubated with RafA. The liberation of galactose and sucrose is visible as chromatographic peaks eluted at 5.75 and 10.5 min, respectively. (Graph III) Melibiose incubated with RafA. The hydrolysis of this substrate to glucose and galactose is visible as a single chromatographic peak eluted at 5.75 min. Arrows are as explained for panel A.

both of them (see Fig. S2 in the supplemental material). The finding that the *melE* gene encodes an α-galactosidase and is present and cotranscribed in a melezitose-induced gene cluster suggests that this locus is also involved in the metabolism of a melezitose-related carbohydrate(s) that contains one or more α-galactose moieties linked through α-(1→4)- and/or α-(1→3)-glycosidic bonds. This sugar, like melezitose, may also be present in honeydew, which is secreted by aphids while they feed on the sugar-rich phloem of their host plants to acquire the amino acids they need for growth and reproduction (83). Such honeydew oligosaccharides, which contain large amounts of melezitose and erlose, have been shown to possess prebiotic potential, since they increase the numbers of bifidobacteria and lactobacilli in an *in vitro* fermentation system (84).

Purified RafA was shown to hydrolyze stachyose, raffinose, and melibiose (Fig. 4B, graphs I, II, and III) to produce sucrose and galactose, and to cleave melibiose to its monosaccharide constituents glucose and galactose, confirming that RafA functions as an α-galactosidase. RafA was also tested for its abilities to hydrolyze melezitose, sucrose, and a range of sucrose isomers but failed to exhibit hydrolytic activity against any of these carbohydrates (results not shown). However, it did show hydrolytic activity against synthetic α-(1→4)- and α-(1→3)-galactobiose, demonstrating that RafA has rather broad substrate specificity (see Fig. S2 in the supplemental material). Previously, an α-galactosidase from *B. bifidum* JCM 1254 was shown to be capable of hydrolyzing α-(1→3)-linked galactose in a branched blood group B antigen trisaccharide; however, this α-galactosidase did not possess the ability to hydrolyze α-(1→4)-galactosidic linkages (22). Analysis of the bifidobacterial genomes sequenced to date shows that most of the bifidobacterial strains have at least one gene encoding α-galactosidase (15). Various α-galactosidases, capable of catalyzing the hydrolysis of various α-galacto-oligosaccharides, have been studied in five bifidobacterial species to date (22–27). For example, it was shown that the *B. adolescentis* α-galactosidase hydrolyzes α-(1→6)-galactosidic bonds from raffinose and stachyose,

TABLE 3 Kinetic study of MelD and RafA[a]

Substrate	Protein	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Melibiose	RafA	384.305 ± 0.42	8.15 ± 1.7	542.79 ± 0.63	66.6 ± 0.07
Raffinose	RafA	187.5 ± 2.1	14.55 ± 1.9	264.815 ± 2.9	18.2 ± 2.6
Stachyose	RafA	11.09±.55	3.045 ± 0.51	16.665 ± 0.784	5.47 ± 1.14
Sucrose	MelD	7.75 ± 0.07	19.3 ± 0.28	8.0725 ± 0.07	0.4175 ± 0.007
Turanose	MelD	12.66 ± 1.59	91.93 ± 1.07	13.16 ± 1.61	0.14 ± 0.01

[a] k_{cat}, rate constant; k_{cat}/K_m, catalytic efficiency. All values are means from two experiments ± standard errors.

as well as the α-(1→4) and α-(1→3) bonds of two galactobiose substrates (23, 24), which is consistent with our findings for *B. breve* UCC2003. Interestingly, the α-galactosidase of *B. breve* 203 was shown to have the ability to synthesize a trisaccharide [Gal-α-(1→4)-Gal-α-(1→6)-Glc] using melibiose as a substrate (26). Furthermore, the α-galactosidase encoded by *B. bifidum* JCM 1254 was observed to be capable of hydrolyzing α-(1→3)-linked galactose in a branched blood group B antigen trisaccharide, although this enzyme cannot hydrolyze α-(1→4)-galactosidic linkages (22). The MelD-, MelE-, and RafA-mediated carbohydrate hydrolysis results obtained and the presumed degradation pathways are summarized in Fig. S3 in the supplemental material.

Determination of kinetic parameters of MelD and RafA. In order to determine the kinetic parameters of the MelD and RafA enzymes, we characterized these two glycosyl hydrolases using the substrates against which they had shown hydrolytic activity [except for α-(1→4)- and α-(1→3)-galactobiose, for which we did not have sufficient amounts to perform such studies]. When sucrose and turanose were used as substrates, the optimum temperature and pH values for MelD activity were determined to be 30°C and pH 7.5, respectively, while for RafA in combination with any of the substrates raffinose, stachyose, and melibiose, the optima were 42°C and pH 6.0, respectively.

Kinetic studies were performed to determine V_{max} and K_m values, as well as the rate constants (k_{cat}) and catalytic efficiencies (k_{cat}/K_m), for MelD using sucrose or turanose as a substrate and for RafA using raffinose, stachyose, or melibiose as a substrate (Table 3). MelD, as shown above, exhibits hydrolytic activity against both α-(1→3)- and α-(1→2)-glucosidic linkages. In order to investigate if MelD exhibited any hydrolytic preference for either of these linkages, we looked at the ability of MelD to hydrolyze sucrose and turanose. The data obtained indicate that the preferred bond cleaved by MelD is the α-(1→2) linkage present in sucrose, since MelD hydrolyzes this bond with an efficacy higher than that for the α-(1→3) linkage present in turanose (Table 3). In order to investigate the preferred substrate of RafA, we determined the kinetic parameters of this enzyme related to its hydrolytic activities toward melibiose, raffinose, and stachyose. The preferred substrate was melibiose, followed by raffinose. However, it should be noted that stachyose contains two α-(1→6) linkages, of which one is present between two galactose moieties, while the other is present between galactose and glucose. The hydrolysis of stachyose by RafA is likely to lead to the generation of galactose and raffinose, the latter again representing a substrate for RafA, as demonstrated by us and as reported previously (17). In our kinetic experiments, we assessed stachyose hydrolysis by measuring the release of sucrose, which would be generated directly by the hydrolysis of stachyose to sucrose and a galacto-disaccharide and indirectly by the initial hydrolysis of stachyose to galactose and raffinose, followed by the hydrolysis of the raffinose to sucrose and galactose. The presence of multiple substrates means that the use of standard kinetic techniques to measure stachyose hydrolysis is not absolutely correct. However, the kinetic values calculated provide a reasonably accurate representation of the substrate preference of the enzyme.

A previously published kinetic study of the *E. coli* K-12 α-galactosidase (85) revealed that this enzyme at an optimal pH of 7.2 exhibits K_m values of 3.2 mM for melibiose and 60 mM for raffinose. Since we observed K_m values of 8.15 mM for melibiose and 14.55 mM for raffinose at an optimal pH of 6.0 for the *B. breve* RafA, it is clear that individual α-galactosidases, despite having the same substrate specificities, may still exhibit different kinetic properties.

Concluding remarks. Bifidobacteria are believed to play an important role in the fermentation of nondigestible carbohydrates in the lower gastrointestinal tract. Consistent with this notion is the prediction that a sizable proportion of the average bifidobacterial genome is dedicated to carbohydrate metabolism (2, 86). More than 50 bifidobacterial carbohydrases have been studied to date (for reviews, see references 42 and 7), and various carbohydrate utilization pathways, such as those dedicated to the metabolism of fructose, galactan, starch, ribose, isomaltulose, cellodextrin, and fructo-oligosaccharides, have been characterized in *B. breve* UCC2003 (8, 14, 38, 41, 52, 59, 87).

The data assembled in this study provide significant information on the abilities of various *B. breve* strains to grow on a number of plant-derived α-glucose- and α-galactose-containing oligosaccharides, as well as identifying the genes involved in the metabolism of such sugars. Two adjacent genetic loci dedicated to the utilization of raffinose-containing carbohydrates and melezitose in *B. breve* UCC2003 were identified, encoding a novel α-glucosidase (specified by *melD*) and two α-galactosidases (specified by *rafD* and *melE*), as well as presumed ABC-type uptake systems for their carbohydrate substrates.

Bifidobacteria appear to encode both common carbohydrate utilization pathways (e.g., for the metabolism of raffinose family sugars), as well as strain- and species-specific pathways (e.g., melezitose metabolism). Such pathways may reflect common elements in the diets of the hosts of such bacteria, while it may also allow certain species/strains the capacity to effectively colonize the gut or reach higher numbers when the host's diet contains more specialized carbohydrates. In a recent study (88), levels of *B. animalis* subsp. *lactis* Bl-04 were shown to be selectively increased 10- to 100-fold on melibiose, xylobiose, raffinose, and maltotriose in a model system of the human colon, indicating that these carbohydrates have the potential to serve as prebiotics.

It is possible that the ability of bifidobacteria to lose or acquire a carbohydrate utilization system is similar to that of *L. plantarum*, an organism that appears to acquire, shuffle, substitute, or delete carbohydrate utilization systems in response to niche require-

ments, making it a "natural metabolic engineer" (89). Further investigations will be required to determine how and to what extent specific carbohydrate utilization abilities enable certain bifidobacterial species and strains to colonize and persist in the gastrointestinal tracts of their hosts, and the importance of host diet in this regard.

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