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Analysis of Predicted Carbohydrate Transport Systems Encoded by *Bifidobacterium bifidum* PRL2010

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The *Bifidobacterium bifidum* PRL2010 genome encodes a relatively small set of predicted carbohydrate transporters. Growth experiments and transcriptome analyses of *B. bifidum* PRL2010 revealed that carbohydrate utilization in this microorganism appears to be restricted to a relatively low number of carbohydrates.

Members of the genus *Bifidobacterium* are arguably the most prominent bacterial representatives of the infant gut microbiota (27, 29, 33). Carbohydrates make up a major part of the human diet and are metabolized by both the host and the microbiota residing in the gastrointestinal tract (GIT). Carbohydrate availability is claimed to represent a determining factor that has shaped the genomes of the microbiota that live in the distal part of the intestine (9, 32). Thus, it is important to precisely understand which carbohydrates and how they are metabolized by enteric microorganisms, such as bifidobacteria, and how this capacity may endow bifidobacteria with a competitive edge in this niche.

Although a significant amount of information is available concerning bifidobacterial glycosyl hydrolases, i.e., enzymes involved in the breakdown of carbohydrates (for reviews, see references 14 and 31), little is known about carbohydrate transporters encoded by this group of microorganisms. One relevant finding in this respect came from bifidobacterial genome analysis, which revealed that bifidobacteria mainly perform carbohydrate transport by means of members of the ATP binding cassette (ABC) family but employ relatively few, if any, phosphoenolpyruvate-phosphotransferase (PEP-PTS) systems (10; for a review, see reference 11).

Genomic approaches have in recent times governed investigations regarding the mechanisms by which bifidobacteria colonize the human gut (7, 12, 34). In a recent study, we discovered that the genome of *Bifidobacterium bifidum* PRL2010 harbors a specific set of genes involved in the utilization of host-derived glycans, in particular those present in mucins that represent the outermost layer of the intestinal mucosa (24, 28). These findings therefore highlight *B. bifidum* PRL2010's capacity to colonize the human intestine (24, 28). To further understand how carbohydrate utilization contributes to host colonization, we investigated carbohydrate uptake features encoded by *B. bifidum* PRL2010 and compared such features to those of other bifidobacteria.

Evaluation of the growth profile of *B. bifidum* on different carbohydrates. Bifidobacterial strains used in this study were routinely cultivated in an anaerobic atmosphere (2.99% H_2, 17.01% CO_2, and 80% N_2) in a chamber (Concept 400; Ruskin) at 37°C for 32 h in de Man-Rogosa-Sharpe (MRS) (Scharlau Chemie, Barcelona, Spain) medium supplemented with 0.05% (wt/vol) L-cysteine hydrochloride. Growth curves of bifidobacteria, performed in triplicate, were obtained from cultures grown on a semisynthetic medium supplemented with various carbon sources, which included mono-, oligo-, and polysaccharides. Growth of *B. bifidum* PRL2010 was examined on 32 different carbohydrates, including 20 carbon sources that had previously been tested (24). As shown in Table 1, *B. bifidum* PRL2010 is capable of growth on a variety of carbohydrates, which include various disaccharides, such as lactose, and amino sugars, such as *N*-acetylgalactosamine, as previously shown by Turroni et al. (24). No appreciable growth (i.e., where the final optical density at 600 nm [OD_{600}] was below 0.1) was observed on other sugars, such as mannitol, xylan, or α-cyclodextrin. Notably, the obtained carbohydrate utilization profile demonstrates growth on components of host-derived glycans, such as human milk oligosaccharides (HMOs) or mucin, as represented by lactose, galactose, *N*-acetylgalactosamine, and *N*-acetylglucosamine. These findings may thus be a direct reflection of the ecological niche in which this microorganism predominates, i.e., the infant colon. However, appreciable growth of *B. bifidum* PRL2010 (i.e., a final OD_{600} of >0.6) was also noticed on certain plant-derived carbohydrates such as (iso)maltose, melibiose, sucrose, fructo oligosaccharides (FOS), (iso)maltulose, ribose, fructose, and turanose (Table 1). Compared to other bifidobacteria tested or data available in literature (13), and with the exception of *Bifidobacterium animalis* subsp. *lactis* BB12, it seems that *B. bifidum* PRL2010 is able to metabolize a somewhat lower number of carbohydrates (only eight) that sustain reasonable good growth (i.e., a final OD_{600} of >1.2) (Table 1). However, these data require careful interpretation, as they represent a modest selection of carbohydrates and bifidobacterial strains, and therefore, no solid conclusions can be drawn from this.

Genomics of carbohydrate transport systems. A genome sequence survey of *B. bifidum* PRL2010 employing Artemis software (18) for the presence of genes that encode predicted carbohydrate transporters returned a substantial number of such genes, although this number was lower than that obtained from genome searches of other human enteric bifidobacteria, such as *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium adolescentis*, and *Bifidobacterium breve* (6, 12, 21). In fact, according to the Transporter Classification

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TABLE 1 Growth capacity of *B. bifidum* PRL2010 and other bifidobacteria on various carbohydrates

Type of carbohydrate and carbon source	Growth of bifidobacterial strain[a]						
	B. bifidum PRL2010	*B. longum* subsp. *longum* NCC2705[b]	*B. dentium* Bd1	*B. adolescentis* ATCC 15703	*B. breve* UCC2003	*B. longum* subsp. *infantis* ATCC 15697	*B. animalis* subsp. *lactis* BB12
Monosaccharides							
Arabinose	+	++	++	+	−	−	−
Fructose	++	+	++	+++	+++	+++	+
Fucose	+	ND	−	+	+	++	+
Galactose	++	+++	++	++	+++	+++	+++
Glycerol	+	−	−	+	−	+	−
Glucose	+	+++	+++	+++	+++	+++	+++
Mannitol	−	−	++	+	−	+	−
Mannose	+	−	+++	−	+	++	+
N-Acetylgalactosamine	+++	ND	−	+	−	+	−
N-Acetylglucosamine	++	−	−	+	+	++	−
N-Acetylneuraminic acid	−	−	−	−	+	+	−
Ribose	++	+	+++	−	+++	++	++
Sorbitol	+	−	−	+++	−	+	−
Xylose	+	++	+	−	−	+	+
Di- and trisaccharides							
Isomaltose	++	ND	++	+++	++	++	++
Isomaltulose	++	ND	++	+++	++	+	+
Lactose	+++	+++	+++	++	+++	+++	++
Lactulose	+++	ND	+++	+++	+++	+++	−
Maltose	+++	++	+++	+++	+++	+++	+++
Maltulose	++	ND	++	++	++	+++	−
Melibiose	+++	++	++	+++	+++	+++	+++
Sucrose	+++	++	+++	+++	+++	+++	++
Trehalose	+	−	+	+	−	+	−
Turanose	++	ND	++	++	+++	++	+
Oligosaccharides and polysaccharides[c]							
α-Cyclodextrin	−	ND	−	−	−	−	−
Arabinogalactan	+	+	+	+	−	+	−
Fructo oligosaccharides (FOS)	+++	++	+++	+++	+	+++	+
Galacto oligosaccharides (GOS)	+++	ND	+++	+	+++	+++	++
Inulin	+	ND	+	+++	−	++	−
Maltodextrin	+	ND	+++	+++	++	+++	++
Mucin	++	ND	+	−	−	−	−
Xylan	−	ND	+	−	−	−	−

[a] Symbols: −, no growth as measured by $0.1 \leq OD_{600}$ or $10^5 \leq$ CFU/ml; +, poor growth as measured by $0.1 < OD_{600} \leq 0.6$ or $10^5 <$ CFU/ml $\leq 5 \times 10^6$; ++, good growth as measured by $0.6 < OD_{600} \leq 1.2$ or $5 \times 10^6 <$ CFU/ml $\leq 5 \times 10^7$; +++, very good growth as measured by $OD_{600} > 1.2$ or CFU/ml $> 5 \times 10^7$. ND, not determined.
[b] As indicated in the study of Parche et al. (13).
[c] Carbohydrates were purified by the method of Rada et al. (16).

Database (TCDB; www.tcdb.org), just 25 genes (Fig. 1) are predicted to encode components of transport systems in the *B. bifidum* PRL2010 genome and dedicated to carbohydrate uptake, whereas the other human intestinal bifidobacterial genomes are predicted to contain between 35 and 68 such genes, as represented by *B. adolescentis* ATCC 15703 and *B. longum* subsp. *infantis* ATCC 15697, respectively (see Table S1 in the supplemental material). *B. animalis* subsp. *lactis* BB12 contains just 21 transporter genes, and this rather low number may have been due to genome simplification as a consequence of the continued cultivation of this strain in rather simple substrates during industrial processing. A similar scenario has been noticed for other strains used in the food industry (30).

When the analysis was extended to a nonenteric bifidobacterial strain, such as the oral cavity inhabitant *Bifidobacterium dentium* Bd1, the relatively low number of identified carbohydrate transporters of *B. bifidum* PRL2010 is even more obvious (see Table S1 in the supplemental material). The 25 predicted carbohydrate transport proteins encoded by the PRL2010 genome can be classified into different groups: 8 belong to members of the ABC-type family, 12 are similar to various components of PEP-PTS systems, 1 is deduced to be a major intrinsic protein (MIP), while 4 represent secondary carriers, encompassing 2 members of the major facilitator superfamily (MFS), 1 member of the glycoside-pentoside-hexuronide (GPH):cation symporter family, and finally 1 member of the glucose/ribose porter family (GRP). The genes coding for the components of a given ABC transporter for a carbohydrate are commonly organized in a locus, typically encompassing between two and five genes that specify a sugar-specific solute binding protein (SBP), one or two permeases, and one or

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FIG 1 Genetic maps of the predicted carbohydrate transporter gene clusters identified in the genome of *B. bifidum* PRL2010. Each individual gene is represented by an arrow and is colored or marked according to the predicted function as indicated in the figure. HAD, haloacid dehalogenase.

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TABLE 2 Carbohydrate transporters of *B. bifidum* PRL2010 used in RT-qPCR experiment

			Homologous ORF[b]							
ORF	Predicted function	Family[a]	*B. longum* subsp. *longum* NCC2705	*B. breve* UCC2003	*B. longum* subsp. *infantis* ATCC 15697	*B. longum* subsp. *longum* DJO10A	*B. adolescentis* ATCC 15703	*B. dentium* Bd1	*B. animalis* subsp. *lactis* BB12	Carbohydrate substrate[c]
BBPR_0032	PTS system, subunit IIC	4.A.7: the PTS L-ascorbate (L-Asc) family		Bbr_1892						Degradation product(s) of host glycans (e.g., mucin) and complex carbohydrates (e.g., FOS)
BBPR_0145	Sucrose-like transporter ScrT	2.A.1: the major facilitator superfamily (MFS)	BL_0534	Bbr_0101	Blon_0129	BLD_1330	BAD_0079	BDP_0116	BIF_01422	Disaccharides (e.g., turanose)
BBPR_0240	PTS system, subunit IIBC	4.A.1: the PTS glucose-glucoside (Glc) family		Bbr_1880	Blon_2471					Degradation product(s) of host glycans (e.g., *N*-acetylglucosamine)
BBPR_0366	PTS system, subunit IIC	4.A.5: the PTS galactitol (Gat) family								Pentoses (e.g., ribose)
BBPR_0561	L-Fucose permease	2.A.1.7: the fucose:H^+ symporter (FHS) family								Monosaccharides (e.g., fucose, fructose)
BBPR_1074	Solute binding protein of ABC transporter system	3.A.1.2: the carbohydrate uptake transporter-2 (CUT2) family	BL_1498	Bbr_1111	Blon_1050	BLD_0397	BAD_0775	BDP_1208	BIF_00836	Disaccharides (e.g., isomaltulose, maltulose, and threalose)
BBPR_1056	ABC transporter permease	3.A.1.1: the carbohydrate uptake transporter-1 (CUT1) family	BL_1640	Bbr_1588	Blon_2175	BLD_1764				Multiple carbohydrates (e.g., fructose, glucose, galactose, mucin, and *N*-acetylgalactosamine)
BBPR_1057	ABC transporter permease	3.A.1.1: the carbohydrate uptake transporter-1 (CUT1) family	BL_1639	Bbr_1589	Blon_2176	BLD_1763				Multiple carbohydrates (e.g., fructose, glucose, galactose, mucin, and *N*-acetylgalactosamine)
BBPR_1058	ABC transporter solute binding protein	3.A.1.1: the carbohydrate uptake transporter-1 (CUT1) family	BL_1638	Bbr_1590	Blon_2177	BLD_1762				Multiple carbohydrates (e.g., fructose, glucose, galactose, mucin, and *N*-acetylgalactosamine)
BBPR_1353	Solute binding protein of ABC transporter system	3.A.1.1: the carbohydrate uptake transporter-1 (CUT1) family	BL_1163	Bbr_0531	Blon_2015	BLD_0921	BAD_0476	BDP_0660		Degradation product(s) of host glycans
BBPR_1356	ABC transporter permease	3.A.1.1: the carbohydrate uptake transporter-1 (CUT1) family	BL_1169	Bbr_0528	Blon_0342	BLD_0927	BAD_0427	BDP_0656	BIF_00071	Degradation product(s) of host glycans
BBPR_1399	Glucose uptake protein	2.A.7.5: the glucose/ribose porter (GRP) family							BIF_00684	Monosaccharides (e.g., ribose and glucose)
BBPR_1459	Galactoside symporter	2.A.2: the glycoside-pentoside-hexuronide (GPH):cation symporter family	BL_0976	Bbr_1551	Blon_2331	BLD_0728	BAD_1608	BDP_1646	BIF_00311	Galactose, lactose, and GOS
BBPR_1508	PTS system glucose-specific transporter subunit IIABC PtsG	4.A.1: the PTS glucose-glucoside (Glc) family	BL_1632	Bbr_1594	Blon_2183	BLD_1757	BAD_0366	BDP_0482		Glucose
BBPR_1715	PTS system cellobiose-specific transporter subunit IIA	4.A.3: the PTS lactose-*N*,*N*′-diacetylchitobiose-β-glucoside (Lac) family								Disaccharides (e.g., turanose and trehalose)
BBPR_1717	PTS system cellobiose-specific transporter subunit IIC	4.A.3: the PTS lactose-*N*,*N*′-diacetylchitobiose-β-glucoside (Lac) family								Disaccharides (e.g., turanose and trehalose)

[a] According to the TCDB Database (20).

[b] Similarity with other carbohydrate transporters identified in the currently available bifidobacterial genome sequences (NCBI source). The percentage similarity used to determine whether these transporters are also encoded by other bifidobacteria is above 30% at the amino acid level with an E value below E−4 (0.0001).

[c] The prediction was based on the results achieved by RT-qPCR and global genome transcription profiling. Carbohydrates enhancing expression of the gene were tested.

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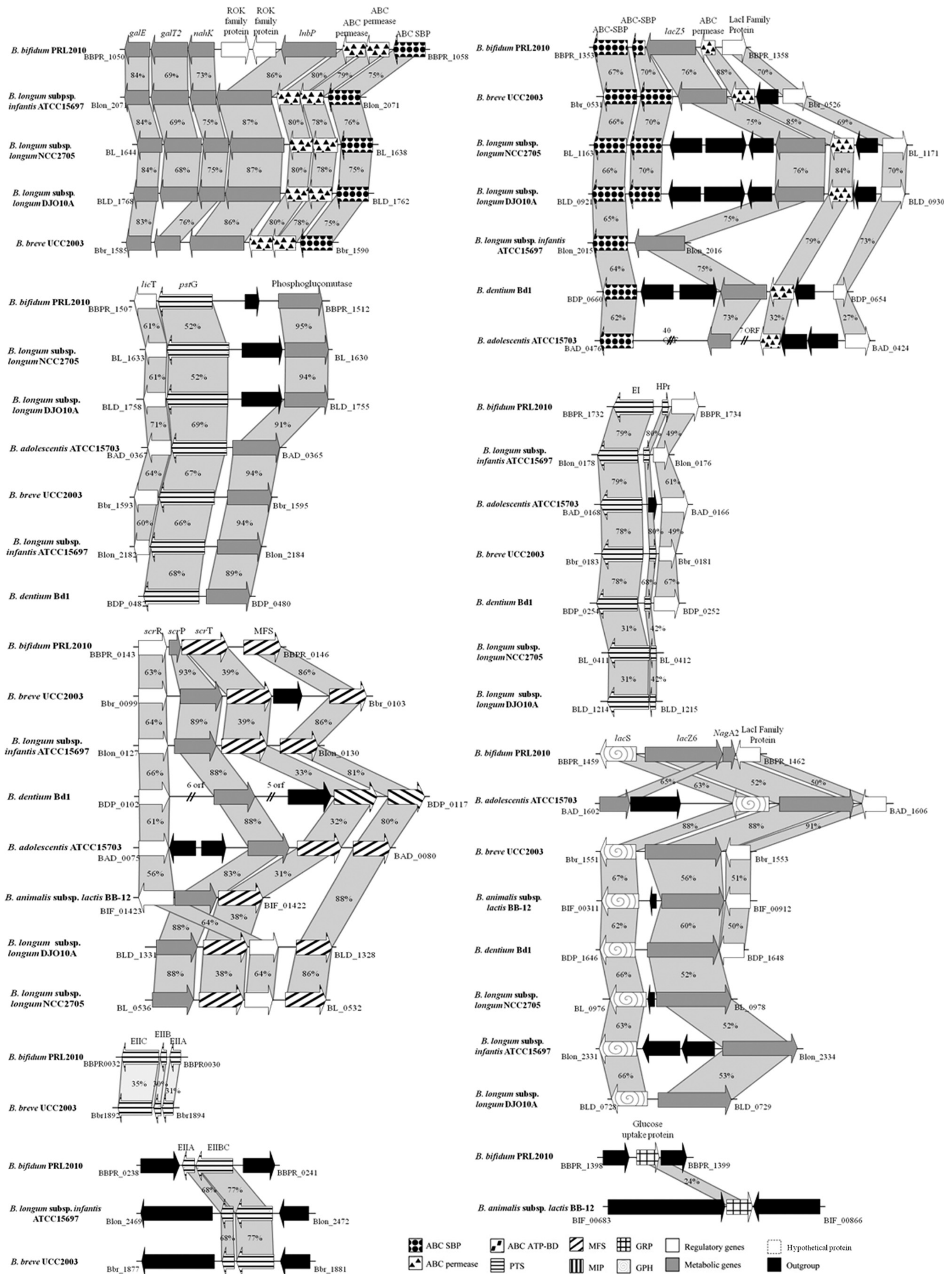

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two predicted ATPase proteins, although such ATPases can be shared among different ABC transporters and the corresponding gene(s) can therefore be absent from such a locus (4). We identified two loci that encode putative carbohydrate uptake ABC-type systems (represented by BBPR_1056 to BBPR_1058, BBPR_1353-BBPR_1354, and BBPR_1356), made up of genes encoding three SBPs (BBPR_1353, BBPR_1354, and BBPR_1058) and three permeases (BBPR_1056, BBPR_1057, and BBPR_1356). Hydropathy profile analysis using the ExPaSy Proteomic Server predicts that PRL2010-encoded permease proteins and SBPs contain six transmembrane domains and one transmembrane domain, respectively (see Fig. S1 in the supplemental material), which is typical for permease and SBP proteins of Gram-positive bacteria (4). Furthermore, the genome of PRL2010 contains a single predicted ATPase-encoding gene (BBPR_1824) as well as an additional SBP-encoding gene (BBPR_1074), neither of which is located next to other genes that specify one or more components of an ABC family carbohydrate uptake system (1, 13). We found a similarity above 60% at the amino acid level (extended to key amino acid residues sustaining enzymatic activities) between the deduced product of BBPR_1824 and that of BL0673 of *B. longum* subsp. *longum* NCC2705 and MsiK of *Streptomyces coelicolor* A3, both of which are believed to encode a universal ATPase for ABC-type carbohydrate transporters identified in these microorganisms (1, 13). These findings suggest that the functionality of the two identified PRL2010 ABC-type carbohydrate uptake systems rely on this single predicted ATPase-encoding gene (Fig. 1). Based on BLAST analysis, the four putative secondary transporters that belong to the MFS, GPH, and GRP families, including proton facilitators and symporters, are expected to transport mono- and disaccharides (Table 2). All these secondary transporters are predicted to consist of a single integral membrane-associated protein, which traverses the membrane 10 to 12 times (Fig. S1).

The genome of PRL2010 also contains four complete PTS systems, represented by the general components histidine protein (HPr) and enzyme I (EI), and four sets of the variable components EIIA, EIIB, and EIIC (see Fig. S1 in the supplemental material).

The organization and location of the carbohydrate transporter-encoding genes identified in the genome of *B. bifidum* PRL2010 and their closest homologs from all other publicly available bifidobacterial genomes are schematically represented in Fig. 2, where the deduced amino acid sequences of the PRL2010 carbohydrate transporter loci are aligned with homologs of other bifidobacteria. This comparative analysis revealed a high level of conservation (>70% identity) between the ABC permeases, SBP, and some of the PTS component proteins found in various bifidobacteria. In contrast, the EIIA, EIIB, and EIIC enzymes encoded by BBPR_1715, BBPR_1716, and BBPR_1717, respectively, and predicted to specify a complete PTS system, and BBPR_0366, which encodes a membrane-embedded EIIC carbohydrate transport component, only appeared to be present in the genomes of strains of *B. bifidum*, thus apparently representing unique genetic features of this species and supporting the notion that such transporters may provide a specific phenotype or particular niche access to *B. bifidum* strains.

Notably, many of the gene clusters involved in carbohydrate transport contain genes encoding putative regulators, which may indicate that such gene clusters are subject to substrate-dependent regulation in a similar fashion as previously shown for other bifidobacterial carbohydrate metabolism loci (14, 23). The genes specifying putative ABC-type permeases as well as PTS systems were indeed flanked by genes encoding such predicted regulators such as carbohydrate kinases belonging to the ROK (repressor, open reading frame, kinase) family (BBPR_1053-BBPR_1054), a transcription antiterminator of the BglG type (BBPR_1507), a DeoR-type regulator (BBPR_0365), and LacI-type regulators (BBPR_0029 and BBPR_1734) (Fig. 1). Similarly, the predicted MFS- and GPH-encoding genes present in the PRL2010 genome are located close to genes specifying putative regulators of the LacI type (BBPR_0143 and BBPR_1462) or belonging to the ROK family (BBPR_0565) (Fig. 1).

Phylogenetic analysis based on SBP protein sequences. In order to assess the distribution of SBP homologs across the *Bifidobacterium* genus, we surveyed available bifidobacterial genomic data for such genes, whose protein products were then aligned using ClustalW to produce an unrooted neighbor-joining phylogenetic tree, which was built using PHYLIP (Phylogeny Inference Package, version 3.5c) (5) (see Fig. S2 in the supplemental material). All analyzed SBPs, except for the *B. bifidum* PRL2010 SBP encoded by BBPR_1074, were shown to be closely related to members of the families Sbp_bac_1 and Sbp_bac_3 (22). Thus, BBPR_1074 might encode a novel solute binding protein never identified previously in bifidobacteria and consequently suggesting the existence of a novel family of SBP.

Transcriptional analysis of carbohydrate transporter loci. In order to determine whether the genes encoding the predicted carbohydrate transporters, including components of the ABC systems, PTS systems, and secondary carriers (MFS, GPH, and GRP), as identified on the *B. bifidum* PRL2010 genome are differentially transcribed when PRL2010 is cultivated on different carbohydrate growth substrates, we evaluated the level of predicted transporter gene-specific mRNAs by quantitative real-time PCR (RT-qPCR) assays according to the guidelines of Bustin et al. (2). Such experiments were performed using mRNA samples extracted from three independent exponentially growing cultures of *B. bifidum* PRL2010, which had been resuspended in prewarmed MRS medium containing one of a varied set of carbohydrates. Aliquots of 20 ml of PRL2010 cultures were centrifuged for 10 min at 4,000 × *g* at 4°C in the presence of RNA-later (Ambion). The pellets were then immediately frozen in liquid nitrogen and submitted to RNA extraction using a previously described method, which includes a DNase treatment (35). The quality and integrity of the RNA were checked by Experion (Bio-Rad) analysis. cDNA was synthesized and purified using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), according to the supplier's instructions. RT-qPCR primers used including those for normalization are described in Table 3. Primers used for normalization were those previously described (25). Criteria for primer design were a desired melting temperature (T_m) value between 58 and 60°C and an amplicon size of approximately 100 to 200 bp. RT-qPCR was performed

FIG 2 Schematic comparative representation of carbohydrate transporter-encoding genes of *B. bifidum* PRL2010 and of various other bifidobacterial strains such as *B. dentium* Bd1, *B. adolescentis* ATCC 15703, *B. breve* UCC2003, *B. longum* subsp. *longum* NCC2705, *B. longum* subsp. *longum* DJO10A, *B. longum* subsp. *infantis* ATCC 15697, and *B. animalis* subsp. *lactis* BB12. Each arrow indicates an open reading frame (ORF), the size of which is proportional to the length of the arrow. Predicted protein function is indicated above each arrow. The corresponding amino acid identity as a percentage is indicated.

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TABLE 3 Primer information related to genes targeted in RT-qPCR experiments

Type of gene targeted and primer[a]	Sequence	Melting temp (°C)	Product size (bp)	Targeted ORF/gene
Target genes				
A1056Fw	CCCCAGTACTCGATCTTCCA	60.07	192	BBPR _1056
A1056Rev	CAGCACGATGGTGAAGAAAA	59.84		BBPR _1056
A1057Fw	GTCGGATTCCAGAACTTCCA	60.05	164	BBPR _1057
A1057Rev	AAACCGGTAACGTGCTTGAC	60.04		BBPR _1057
A1058Fw	AAACGACTGCTTGCTGGTCT	60.05	201	BBPR _1058
A1058Rev	GATGTTCGGGTTGTCCTTGT	59.84		BBPR _1058
A1353Fw	TGACGCCAGCTTCTACAATG	60.01	172	BBPR _1353
A1353Rev	TTTGAGATGGGTGTTGACCA	59.94		BBPR _1353
A1354Fw	GAGACGGTCACCATCAACCT	59.97	168	BBPR _1354
A1354Rev	CTTGACGGTGTAGCCCTGAT	60.13		BBPR _1354
A1356Fw	TCGTCTCCATGCTCATCAAC	59.79	103	BBPR _1356
A1356Rev	GTCACGATGTTGTCCCACAG	60		BBPR _1356
P0030Fw	GATCGAGACGGTCGAGAAAA	60.34	174	BBPR _0030
P0030Rev	AGACCGATGACCAGCGATAC	60.1		BBPR _0030
P0032Fw	ACACCCTCGGCTACATTGTC	60	159	BBPR _0032
P0032Rev	CGGAGAAGACCAGGTACAGC	59.87		BBPR _0032
P0239Fw	AGATTACCACGGTCGCTCAG	60.28	188	BBPR _0239
P0239Rev	CTTGACCACATTCCAGTCCA	59.52		BBPR _0239
P0240Fw	CGATGACGATAACGACGATG	60.1	146	BBPR _0240
P0240Rev	GGGTGATGCAGTTCTCGATT	60.08		BBPR _0240
P1715Fw	ACCCTTGACATGGAAGTGCT	59.58	135	BBPR _1715
P1715Rev	GTCGCCGGACTTCATCTTC	60.76		BBPR _1715
P1717Fw	CTCGTGGGTCTACACCACCT	60.03	152	BBPR _1717
P1717Rev	AGACCTGGCACACCTGGTAG	60.17		BBPR _1717
P1508Fw	GGTGTTCATCAAGGCCATCT	59.93	164	BBPR _1508
P1508Rev	TCCACTTTTCGGAGTCCATC	60.05		BBPR _1508
P0366Fw	TCAACCTGCTGCTTGACAAC	60.03	185	BBPR _0366
P0366Rev	GAGCTTGCAGAACACCATGA	59.99		BBPR _0366
M0561Fw	AAACCACCAAGCCAGTCAAC	60.01	189	BBPR _0561
M0561Rev	GCACGGAGAAGATCAGGAAC	59.81		BBPR _0561
M1459Fw	CTCGTGGACAACACGAACAC	60.2	210	BBPR _1459
M1459Rev	CATGCCCCAGTAGGAGATGT	59.95		BBPR _1459
M0145Fw	ATGATCGGCCTGATTACGAC	59.92	145	BBPR _0145
M0145Rev	GATCCCTCCGAAGTCATCTG	59.61		BBPR _0145
M1399Fw	GGCACGACAATCATCTCCTT	60.08	158	BBPR _1399
M1399Rev	GCCAGACCGTTCATCTCATT	60.08		BBPR _1399
A1074Fw	GCAGATGTGCCTGAAAATCA	59.81	153	BBPR _1074
A1074Rev	ATCAGCGTGCTCTTGAGGTT	60.02		BBPR _1074
Reference genes				
uvrD/Rep-F	ATACTCCGAGAATGCGGATG	60.06	165	*uvrD/rep*
uvrD/Rep-R	ACGACATCCCGCTCATATTC	59.92		*uvrD/rep*
pdxS-F	ATGATCAAGGGCATTCAGGA	60.43	172	*pdxS*
pdxS-R	CGTCGAACTGTGTCTTGTCG	60.5		*pdxS*
gluC-F	CGCATCTCTCCGATATCCTC	59.76	184	*gluC*
gluC-R	ACAGACTCAGTCCCGTCACC	60.16		*gluC*
1653_fw	CTTCTCGTGGTGCTCGGTAT	60.28	171	*atpB*
1653_rev	CGTGAAGAACAACGTCGAAA	59.88		*atpB*
1691_fw	GTGGATATACCCGGTCGATG	60.04	164	*glnD*
1691_rev	TAGTGACGGTCGTCATACCG	59.59		*glnD*
B.bif1	CCACATGATCGCATGTGATTG	63.31	278	16S rRNA
B.bif2	CCGAAGGCTTGCTCCCAAA	65.76		16S rRNA

[a] The direction of the primer is indicated at the end of the primer designation as follows: Fw and F, forward; Rev, R, and rev, reverse.

using the CFX96 system (Bio-Rad, Hercules, CA). PCR products were detected with SYBR green fluorescent dye and amplified according to the following protocol: one cycle of 95°C for 3 min, followed by 39 cycles, with 1 cycle consisting of 95°C for 5 s and 66°C for 20 s. The melting curve was measured from 65°C to 95°C with increments of 0.5°C/s.

Each PCR mix contained 12.5 μl of 2× SYBR SuperMix Green (Bio-Rad, Hercules, CA), 1 μl of cDNA dilution, each of the forward and reverse primers at 0.5 μM, and nuclease-free water to obtain a final volume of 20 μl. In each run, a negative control (no cDNA) for each primer set was included. The expression ratio of the selected genes was calculated and analyzed using the CFX

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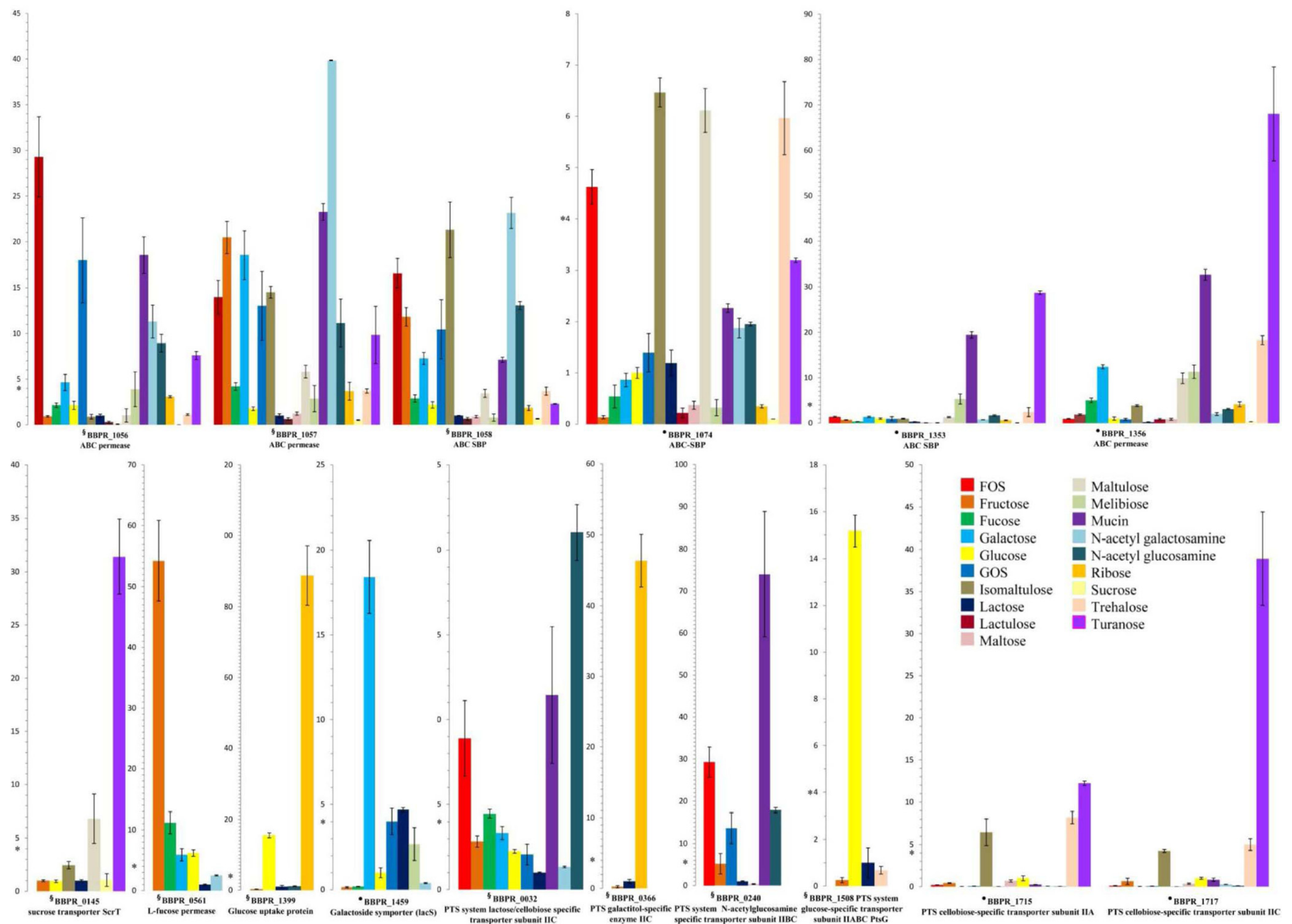

FIG 3 Relative transcription levels of carbohydrate transporter-encoding genes from *B. bifidum* PRL2010 upon cultivation in MRS medium supplemented with various carbon sources as analyzed by quantitative real-time PCR assays. The histograms indicate the relative amounts of the carbohydrate transporter-encoding gene mRNAs for the specific samples. The *y* axis indicates the fold induction of the investigated gene compared to the reference condition (lactose, which is indicated by §, or glucose, which is indicated by ●). The *x* axis represents the different carbohydrates tested for each ORF. In each panel, the ORF numbering indicates the gene code according to Fig. 1. Only those genes/carbohydrate conditions whose expression was significantly changed are shown. An asterisk on the *y* axis indicates that the change in fold induction is considered significant. The error bar for each column represent the standard deviation calculated from three replicates. GOS, galacto oligosaccharides.

Manager expression software (Bio-Rad, Hercules, CA). The cutoff value applied to highlight significant change in the expression was 4.

The mRNA levels corresponding to genes encoding individual components of identified *B. bifidum* PRL2010 carbohydrate transporters were shown to be variable in response to the various carbon sources used for growth (Fig. 3). Interestingly, the transcription of three predicted carbohydrate transport systems of PRL2010, i.e., the ABC locus (BBPR_1353 to BBPR_1356) (this presumed ABC-type transporter also exhibits induction upon growth in turanose [see below]) and two PTS loci (BBPR_0032 and BBPR_0240), was significantly enhanced when PRL2010 was cultivated on host-specific glycans such as mucin and host glycan constituents like *N*-acetylglucosamine (Fig. 3), and this may reflect a specialization of PRL2010 toward the particular ecological niche of the human GIT.

Furthermore, we identified genes that represent predicted carbohydrate transporter components and that were shown to exhibit specific transcriptional induction as a result of growth on particular monosaccharides such as fructose (BBPR_0561), glucose (BBPR_1508), ribose (BBPR_0366 and BBPR_1399), galactose (BBPR_1459) or disaccharides such as the sucrose-like carbohydrate turanose (BBPR_0145 and BBPR_1715, BBPR_1717, and BBPR_1353 to BBPR_1356) (Fig. 4). Each of the genes that specify one of the ABC-type carbohydrate transporters, specified by BBPR_1056 to BBPR_1058, as well as the gene encoding SBP_{BBPR_1074} were shown to be induced when *B. bifidum* PRL2010 was grown on multiple carbon sources, reminiscent of what previously was described for several ABC systems identified in the genome of *B. longum* subsp. *longum* NCC2705 (8, 13). These data suggest that transcription of these transporters is subject to a common control mechanism and thus not necessarily regulated in response to their specific substrate.

Furthermore, relative expression levels between genes that are predicted to encode components of a particular carbohydrate transporter varied quite substantially when *B. bifidum* PRL2010 was grown on a specific carbohydrate. This finding is not unusual

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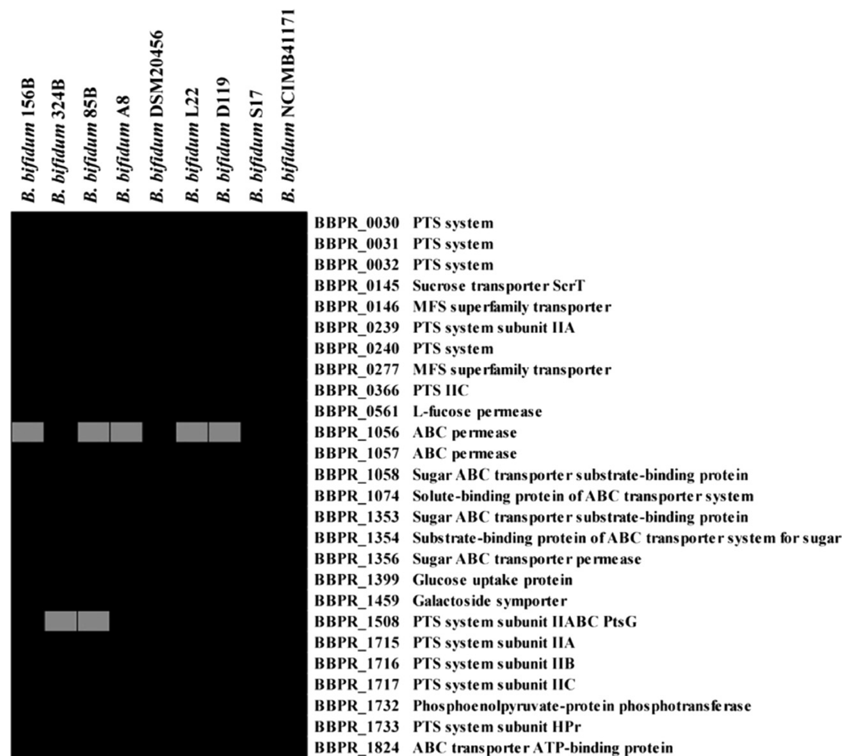

FIG 4 Identification of the conservation of carbohydrate transporter genes within several members of the *B. bifidum* taxon. The presence (black) or absence (gray) of key genes predicted to be involved in carbohydrate transporters in the currently publicly available *B. bifidum* genomes and on the basis of CGH data (24) is shown.

for SBP-encoding genes, because higher transcription is expected to lead to an abundance of SBP molecules (13), thus providing a more efficient substrate scavenging ability and consequent uptake. Since the SBPs of strain PRL2010 are encoded by distinct genes, we may envisage that multiple protein copies of SBP are cooperating with a corresponding carbohydrate permease, in a similar fashion to that described for other bacteria (3, 13, 15). The observation that a range of substrates can induce the expression of predicted SBP-encoding genes in *B. bifidum* PRL2010 may be linked to their low number in this genome with respect to other bifidobacterial genomes (e.g., 16 in *B. breve* UCC2003, 11 in *B. longum* subsp. *longum* NCC2705 and 20 in *B. longum* subsp. *infantis* ATCC 15697) and thus may be pivotal for the colonization strategy of PRL2010. Furthermore, it is tempting to speculate that PRL2010 has specialized itself in the metabolism of a relatively low number of carbohydrates and that the apparently low level of induction specificity of the SBP-encoding genes and other carbohydrate-transporting genes is just a consequence of this adaptation.

Evaluation of genes involved in carbohydrate transport in members of the *B. bifidum* species. In order to investigate the level of conservation of the genetic repertoire involved in carbohydrate transport within the *B. bifidum* taxon, we surveyed the currently publicly available *B. bifidum* genome sequences, i.e., *B. bifidum* S17 (36) and *B. bifidum* NCIMB 41171 (GenBank accession no. NZ_ABQP00000000) as well as previously published comparative genomic hybridization (CGH) data (24). The obtained data demonstrated that a large proportion of the 25 identified PRL2010 carbohydrate transport genes are conserved within these investigated members of the *B. bifidum* taxon. Only BBPR_1056 and BBPR_1508 were found to be variably present in the tested *B. bifidum* strains (Fig. 4). Comparative analyses highlighted a high similarity (higher than 98% at nucleotide level) of these transporter genes with that identified in the genome of *B. bifidum* PRL2010, thus corroborating the CGH findings (Fig. 4).

Conclusions. This study provides the first detailed investigation into the genetic requirements for carbohydrate uptake in *B. bifidum* (Fig. 5). Our analysis of *B. bifidum* PRL2010 genes encoding predicted carbohydrate transporter systems revealed that, compared to other bifidobacterial strains residing in the infant gut, such as *B. longum* subsp. *infantis*, *B. breve*, and *B. longum* subsp. *longum* (17, 26, 27), PRL2010 contains a relatively limited number of such genes. Since this scenario is also found in two other *B. bifidum* strains for which genome sequences are available, it appears that in contrast to other bifidobacteria (for a review, see reference 14), carbohydrate breakdown/uptake in this bifidobacterial taxon is restricted to a comparatively low number of carbohydrates, which may represent an interesting genetic strategy for efficient colonization and survival in its ecological niche. The observed carbohydrate utilization profiles (Table 1) did not appear to reflect this specialization, but the majority of carbohydrates tested were mono- and disaccharides, which may have biased our analysis. Nevertheless, in contrast to other bifidobacteria, *B. bifidum* can utilize complex O-linked glycans associated with mucin, which are not commonly digested by other enteric bacteria, thus highlighting interesting and unique metabolic features of this taxon. The ability to rapidly retrieve a specific carbon source from a particular environment represents an important feature that would endow a bacterium with an undisputed ecological fitness

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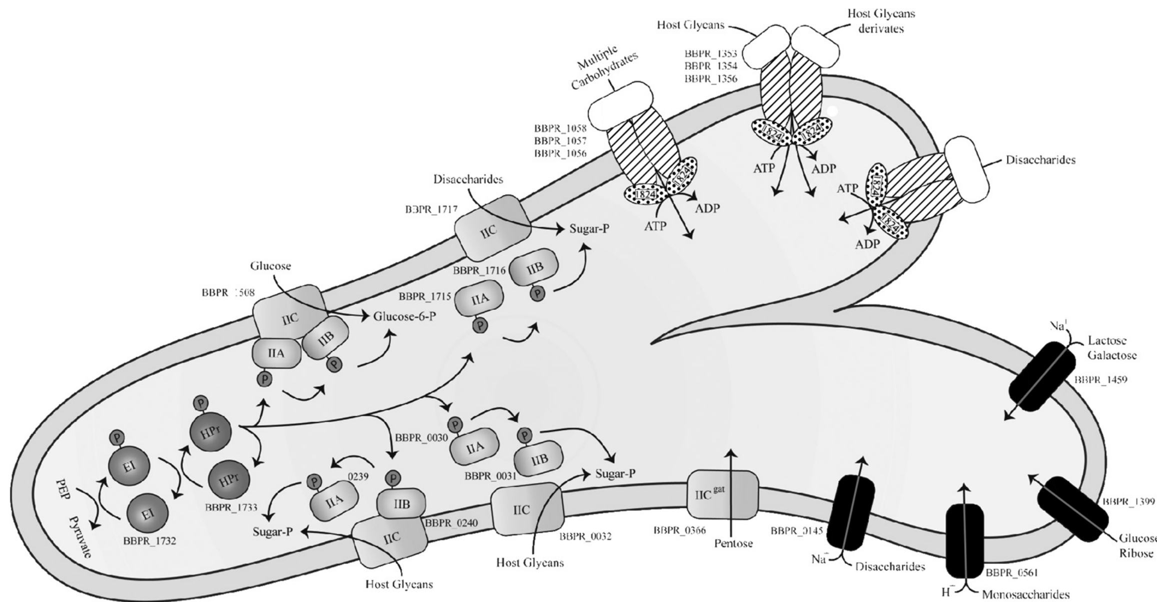

FIG 5 Carbohydrate transport systems of *B. bifidum* PRL2010. The ABC transporters are colored in white (predicted SBP subunit), gray (predicted permease subunit), or with dots (predicted ATPase subunit). The PTS transporters are depicted in gray, and secondary carriers are displayed in black. The derived putative substrates are inferred from *in silico* analyses in combination with experimental data. P, phosphate group.

(31). Furthermore, the main distinction between *B. bifidum* and other bifidobacteria is its capacity to grow on both HMOs and mucin, a capability that apparently provides a very strong selective advantage over other (bifido)bacteria. In fact, one could argue that because of this seemingly unique capability, it may have lost or did not acquire other carbohydrate-metabolizing abilities.

It is worth mentioning that the ABC-type transporters identified in *B. bifidum* genomes are not restricted to the transport of carbohydrates and also include predicted uptake systems for amino acids, peptides, metal ions (such as Mn^{2+}, Zn^{2+}, and Fe^{2+}), phosphate, lipoproteins, teichoic acid, as well as efflux systems for drugs such as nisin, macrolide, and anthracycline. Moreover, relative to other bifidobacterial genomes, a much larger proportion of the genes of the *B. bifidum* genome-encoded ABC-type transporters are dedicated to efflux rather than uptake (24, 28).

This report represents the first step toward understanding the complex nutritional relationships existing between the different members of the infant gut microbiota and toward elucidating the possible synergistic and antagonistic actions exerted by *B. bifidum* species with respect to other bacterial components residing in this ecosystem. Future investigations will be directed to explore how cocultivation of *B. bifidum* PRL2010 with other components of the infant gut microbiota may allow cross-feeding by the provision of unutilized mono- or disaccharides, which are derived from degraded diet or host polysaccharides and which may represent important growth substrates for such cross-feeding (bifido)bacteria.

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