Global analysis of carbohydrate utilization by *Lactobacillus acidophilus* using cDNA microarrays

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The transport and catabolic machinery involved in carbohydrate utilization by *Lactobacillus acidophilus* was characterized genetically by using whole-genome cDNA microarrays. Global transcriptional profiles were determined for growth on glucose, fructose, sucrose, lactose, galactose, trehalose, raffinose, and fructooligosaccharides. Hybridizations were carried out by using a round-robin design, and microarray data were analyzed with a two-stage mixed model ANOVA. Differentially expressed genes were visualized by hierarchical clustering, volcano plots, and contour plots. Overall, only 63 genes (3% of the genome) showed a >4-fold induction. Specifically, transporters of the phosphoenolpyruvate:sugar transferase system were identified for uptake of glucose, fructose, sucrose, and trehalose, whereas ATP-binding cassette transporters were identified for uptake of raffinose and fructooligosaccharides. A member of the LacS subfamily of galactoside-pentose hexuronide translocators was identified for uptake of galactose and lactose. Saccharolytic enzymes likely involved in the metabolism of monosaccharides, disaccharides, and polysaccharides into substrates of glycolysis were also found, including enzymatic machinery of the Leloir pathway. The transcriptome appeared to be regulated by carbon catabolite repression. Although substrate-specific carbohydrate transporters and hydrolases were regulated at the transcriptional level, genes encoding regulatory proteins CcpA, Hpr, HprK/P, and El were consistently highly expressed. Genes central to glycolysis were among the most highly expressed in the genome. Collectively, microarray data revealed that coordinated and regulated transcription of genes involved in sugar uptake and metabolism is based on the specific carbohydrate provided. *L. acidophilus*'s adaptability to environmental conditions likely contributes to its competitive ability for limited carbohydrate sources available in the human gastrointestinal tract.

ATP-binding cassette | carbon catabolite repression |
fructooligosaccharide | galactoside-pentose hexuronide

A large, diverse, and dynamic microbial community resides in the human gastrointestinal tract (GIT) (1). In particular, the complex intestinal microbial population includes beneficial bacteria, such as bifidobacteria and lactobacilli (2). Among species considered important for human health, a number of documented lactobacilli have been characterized as probiotics (3), which are defined as “live microorganisms which, when administered in adequate amounts confer a health benefit on the host” (4). For such microbes, survival and residence in the GIT relies partially on their ability to survive gastric passage and use available nutrients.

Lactobacillus acidophilus NCFM (North Carolina Food Microbiology) is a Gram-positive lactic acid bacterium that has the ability to survive in the GIT (5, 6), adhere to human epithelial cells *in vitro* (5, 7), modify fecal flora (6), modulate the host immune response (8), and prevent microbial gastroenteritis (8). Additionally, *L. acidophilus* NCFM has the ability to use non-

digestible oligosaccharides, which may also contribute to the organism's ability to compete in the human GIT (9).

Undigested carbohydrates are a primary source of energy for intestinal microbes residing in the large intestine. Nondigestible oligosaccharides (NDO) consist primarily of plant carbohydrates that are resistant to enzymatic degradation and are not absorbed in the upper intestinal tract. Such dietary compounds eventually reach the large intestine, whereby they are hydrolyzed by a limited range of organisms. As a result, NDO have the ability to selectively modulate the composition of the intestinal microflora (6). NDO, such as raffinose and fructooligosaccharides (FOS), have been shown to selectively promote the growth of probiotic species and are therefore considered prebiotic compounds (10, 11). Prebiotics are defined as nondigestible substances that provide a beneficial physiological effect on the host by selectively stimulating the favorable growth or activity of a limited number of indigenous bacteria (4). Although considerable attention has been devoted to studying modulation of the intestinal flora by prebiotics, the molecular mechanisms involved in uptake and metabolism of those compounds by desirable intestinal microbes remains mostly uncharacterized.

Lactic acid bacteria can use a variety of nutrients. Specifically, the genomes of lactobacilli and streptococci encode specialized saccharolytic machinery that reflects the nutrient availability in their respective environments (12–15). In particular, the versatile saccharolytic potential of *L. acidophilus* likely reflects its ability to efficiently use energy sources available in the intestinal environment. Although the *L. acidophilus* NCFM genome encodes numerous putative genes potentially involved in the uptake and metabolism of a variety of carbohydrates (16), little information is available regarding their biological functions and expression profiles.

The objective of this study was to identify genes involved in carbohydrate utilization by *L. acidophilus*. Global gene transcription profiles were used to identify uptake systems, catabolic machinery, and regulatory networks involved in the utilization of eight carbohydrates. This work is a comparative global transcriptional analysis of a lactic acid bacterium over a range of carbohydrates.

Results

Differentially Expressed Genes. Global gene expression patterns obtained from growth on eight different carbohydrates were

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Abbreviations: ABC, ATP binding cassette; CCR, carbon catabolite repression; FOS, fructooligosaccharide; GIT, gastrointestinal tract; GPH, galactoside-pentose hexuronide; PTS, phosphoenolpyruvate:sugar transferase system.

Data deposition: The microarray data reported in this paper have been deposited in the GenBank database [accession nos. GPL1401 (platform) and GSE2577 (data series)].

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into fructose-1 phosphate, which the phosphofructokinase FruK phosphorylates into fructose-1,6 biphosphate.

In the presence of sucrose, the three genes of the sucrose locus were differentially expressed (Figs. 1 and 7), namely, La399 (ScrR, transcription regulator), La400 (ScrB, sucrose-6 phosphate hydrolase), and La401 (ScrA, sucrose PTS transporter EII^{BCA}^{Suc}). Compared with glucose, induction levels were up to 3.1, 2.8, and 17.2 for *scrR*, *scrB*, and *scrA*, respectively. La401 in particular showed high induction levels between 8.0 and 17.2 compared with monosaccharides and disaccharides. These results indicate that sucrose could be transported into the cell by a PTS transporter into sucrose-6 phosphate, which is subsequently hydrolyzed into glucose-6 phosphate and fructose by ScrB.

The six genes of the FOS operon were differentially expressed (Figs. 1–3 and 7), namely La502, La503, La504, La506 (MsmEFGK, ABC transporter), La505 (BfrA, β -fructosidase), and La507 (GtfA, sucrose phosphorylase). Induction levels varied between 15.1 and 40.6 when compared with monosaccharides and disaccharides and between 5.5 and 8.9 when compared with raffinose. These results suggest that FOS is transported into the cell by an ABC transporter and subsequently hydrolyzed into fructose and sucrose by the fructosidase. Sucrose is likely subsequently hydrolyzed into fructose and glucose-1 phosphate by the sucrose phosphorylase. In addition to the FOS operon, FOS also induced the fructose operon, the sucrose PTS transporter, the trehalose operon, and an ABC transporter (La1679–La1680).

In the presence of raffinose, the six genes of the raffinose operon were specifically induced (Figs. 1–3 and 7). The raffinose locus consists of La1442, La1441, La1440, La1439 (MsmEFGK₂, ABC transporter), La1438 (Mela, α -galactosidase), and La1437 (GtfA₂, sucrose phosphorylase). Induction levels varied between 15.1 and 45.6 compared with all other conditions. Additionally, La1433–La1434 (dihydroxyacetone kinase) and La1436 (glycerol uptake facilitator) were induced between 1.9- and 24.7-fold compared with other conditions.

In the presence of lactose and galactose, 10 genes distributed in two loci were differentially expressed, namely La1463 [LacS, permease of the galactoside-pentose hexuronide (GPH) translocator family], La1462 (LacZ, β -galactosidase), La1461 (conserved hypothetical protein), La1460 (surface protein), La1459 (GalK, galactokinase), La1458 (GalT, galactose-1 phosphate uridylyl transferase), La1457 (GalM, galactose epimerase), La1467–La1468 (LacLM, β -galactosidase large and small subunits), and La1469 (GalE, UDP-glucose epimerase). LacS is similar to GPH permeases previously identified in lactic acid bacteria (18–20). Although LacS contains a EIIA at the carboxy terminus, it is not a PTS transporter. In addition, LacS includes a His at position 560, which might be involved in interaction with HPr, as shown in *Streptococcus salivarius* (19). In the presence of lactose and galactose, *galKTM* was induced between 3.7- and 17.6-fold; *lacSZ* was induced between 2.8- and 17.6-fold; *lacL* and *galE* were induced between 2.7 and 29.5 when compared with other carbohydrates not containing galactose, i.e., glucose, fructose, sucrose, trehalose, and FOS. These results suggest that lactose is transported into the cell via the LacS permease. Inside the cell, lactose is hydrolyzed into glucose and galactose by LacZ. Galactose is then phosphorylated by GalK into galactose-1 phosphate and further transformed into UDP-galactose by GalT. UDP-galactose is subsequently epimerized to UDP-glucose by GalE. UDP-glucose is likely turned into glucose-1 phosphate by La1719 (GalU), which encodes a UDP-glucose phosphorylase that is consistently highly expressed. Finally, the phosphoglucosyltransferase (La687), also highly expressed, likely acts on glucose-1 phosphate to yield glucose-6 phosphate.

The three genes of the putative trehalose locus were also differentially expressed (Figs. 1, 3, and 7). The trehalose locus consists of La1012 (encoding the TreB trehalose PTS trans-

porter EIIABC^{Tre}), La1013 (TreR, trehalose regulator) and La1014 (TreC, trehalose-6 phosphate hydrolase). Induction levels were between 4.3 and 18.6 for *treB*, between 2.3 and 7.3 for *treR*, and between 2.7 and 18.5 for *treC* compared with glucose, sucrose, raffinose, and galactose. These results suggest that trehalose is transported into the cell via a PTS transporter, phosphorylated to trehalose-6 phosphate, and hydrolyzed into glucose and glucose-6 phosphate by TreC.

In addition, genes showing differential expression included hypothetical genes La457, La466, La1006, La1008, La1010, La1011, and La1206; sugar- and energy- related genes La874 (β -galactosidase), La910 (L-lactate dehydrogenase), La1007 (pyridoxal kinase), La1812 (α -glucosidase), La1632 (aldehyde dehydrogenase), La1401 (NADH peroxidase), and La1974 (pyruvate oxidase); adherence genes La555, La649, La1019; aminopeptidase La911 and La1086; and amino acid permease, La1102, La1783 (ABC transporter), and La1879 (pyrimidine kinase).

Real-Time Quantitative RT-PCR (QRT-PCR). The induction levels measured by microarrays were plotted against induction levels measured by QRT-PCR for the five selected genes (Fig. 8, which is published as supporting information on the PNAS web site). Individual R^2 values ranged between 0.64 and 0.88 for each of the tested genes (between 0.65 and 0.98 with data in a $\log_2$ scale). When the data were combined, the global R^2 value was 0.78 (0.88 with data in a $\log_2$ scale). A correlation analysis was run in SAS (SAS Institute, Cary, NC), and showed a correlation between the two methods ($P < 0.001$, Spearman, Hoeffding, and Kendall tests). Additionally, a regression analysis was run in EXCEL (Microsoft), and it showed a statistically highly significant correlation ($P < 1.02 \times 10^{-25}$) between microarray data and QRT-PCR results. Nevertheless, QRT-PCR measurements revealed larger induction levels, notably at the higher fold-expression range, which is likely because the microarray scanner has a smaller dynamic range compared with that of the QRT-PCR cyclor.

Discussion

Comparative analyses of global transcription profiles determined for growth on eight carbohydrates identified the genetic basis for carbohydrate transport and catabolism in *L. acidophilus*. Specifically, three different types of carbohydrate transporters were differentially expressed, illustrating the diversity of carbohydrate transporters used by *L. acidophilus*. Transcription profiles suggested that monosaccharides and disaccharides were transported by members of the PTS family, whereas polysaccharides were transported by members of the ABC family, and galactosides were transported by a GPH translocator. Globally, microarray results allowed reconstruction of carbohydrate transport and catabolism pathways (Fig. 4). Although transcription of most carbohydrate transporters and hydrolases was specifically induced by their respective substrates, glycolysis genes were among the most consistently highly expressed genes throughout the genome (Fig. 9, which is published as supporting information on the PNAS web site).

Microarray results indicated that fructose, sucrose, and trehalose are transported by PTS transporters EIIABC^{Fru} (La1777), EII^{BCA}^{Suc} (La401), and EIIABC^{Tre} (La1012), respectively. These genes are encoded on typical PTS loci (Fig. 4) along with regulators and enzymes that have been well characterized in other organisms. In contrast, FOS and raffinose are transported by ABC transporters of the MsmEFGK family, La502–La505 and La1437–La1442, respectively. In the case of trehalose and FOS, microarray results correlate well with functional studies in which knock out of transporters and hydrolases modified the saccharolytic potential of *L. acidophilus* (9, 21). Differential expression of the EIIABC^{Tre} is consistent with recent work in *L. acidophilus*, indicating that La1012 is involved in trehalose uptake, because knock out of the trehalose PTS

Fig. 4. Carbohydrate utilization in *L. acidophilus*. (A) Pathway reconstruction as predicted by transcriptional profiles. PTS, red; GPH, yellow; ABC, green. (B) Genetic loci of interest: *man*, glucose-mannose; *Fru*, fructose; *Suc*, sucrose; *raff*, raffinose; *Lac*, lactose-galactose loci; *tre*, trehalose; *CCR*, carbon catabolite repression loci.

transporter resulted in a loss of the ability to grow on trehalose (21). Similarly, differential expression of the *fos* operon is consistent with previous work in *L. acidophilus* indicating that those genes are involved in uptake and catabolism of FOS, induced in the presence of FOS, and repressed in the presence of glucose (9). Additionally, induction of the raffinose *msm* locus is consistent with previous work in *Streptococcus mutans* (22) and *Streptococcus pneumoniae* (23).

A number of lactic acid bacteria take up glucose via a PTS transporter. The EII^{Man} PTS transporter has the ability to import mannose and glucose (24). The *L. acidophilus* mannose PTS system is similar to that of *Streptococcus thermophilus* (24). Specifically, the EII^{Man} is composed of three proteins, IIA^{Man}, IIC^{Man}, and IID^{Man}, encoded by La452 (*manL*), La455 (*manM*), and La456 (*manN*), respectively (Fig. 4). Whereas most of the carbohydrates examined here specifically induced genes involved in their own transport and hydrolysis, glucose did not. Analysis of the mannose PTS revealed that the genes encoding the EIIABCD^{Man} were consistently highly expressed, regardless of the carbohydrate source (Fig. 1), which is consistent with the important regulatory roles in Gram-positive bacteria established for EII^{Man} (24, 25). Similarly, EIIABC^{Fru} seemed consistently highly expressed (Fig. 1). These expression profiles suggest that glucose and fructose are preferred carbohydrates and that *L. acidophilus* also is designed for efficient utilization of different carbohydrate sources, as was suggested previously for *Lactobacillus plantarum* (14).

The genes differentially expressed in the presence of galactose and lactose included a permease (LacS) and the enzymatic machinery of the Leloir pathway. Members of the LacS subfamily of GPH translocators have been described in a variety of lactic acid bacteria, including *Leuconostoc lactis* (26), *S. thermophilus* (27), *S. salivarius* (19), *Lactobacillus delbrueckii* (28), and *Lactobacillus helveticus* (20). LacS has been reported to have the ability to import both galactose and lactose in select organisms (26, 27). Although the combination of a LacS lactose permease with two β -galactosidase subunits (LacL and LacM) has been described in *L. plantarum* (14), *L. helveticus* (20), and *Leuconostoc lactis* (26), it has never been reported in *L. acidophilus*. Although constitutive expression of *lacS* and *lacLM* has been reported (20, 26), our current results indicate specific induction of the genes involved in uptake and catabolism of both galactose and lactose. Operon organization for galactoside utilization is variable and unstable among

Gram-positive bacteria (20, 28–32). Interestingly, even among closely related *Lactobacillus* species, namely *L. johnsonii*, *L. gasseri*, and *L. acidophilus*, the lactose–galactose locus is not well conserved (Fig. 10, which is published as supporting information on the PNAS web site) (15). It was also recently shown that, within the *L. helveticus* species, the *lac* operon is not well conserved (20). Perhaps the presence of mobile elements in the vicinity of those genes is responsible for the instability of this locus (16, 20).

Although it was previously suggested that PTS is the primary sugar transport system of Gram-positive bacteria (12, 25), current microarray data indicate that ABC transport systems also are important. Although PTS transporters are involved in uptake of monosaccharides and disaccharides, those carbohydrates are digested in the upper GIT. In contrast, oligosaccharides reach the lower intestine whereby commensals are likely to compete for more complex and scarce nutrients. Perhaps under such conditions ABC transporters are even more crucial than the PTS, given their apparent roles in the transport of oligosaccharides like FOS and raffinose. In this regard, the ability to use nutrients that are nondigestible by the host has been associated with competitiveness and persistence of beneficial intestinal flora in the colon (13).

Transcription profiles of genes differentially expressed in the conditions tested indicated that most carbohydrate uptake systems and their respective sugar hydrolases were specifically induced by their substrate. Moreover, genes within those inducible loci were down-regulated in the presence of glucose, and *cre* sequences (33) were identified in their promoter-operator regions (Fig. 11, which is published as supporting information on the PNAS web site). Together, these results indicate regulation of carbohydrate uptake and metabolism at the transcription level and implicate the involvement of a global regulatory system compatible with carbon catabolite repression (CCR), which controls transcription of proteins involved in the transport and catabolism of carbohydrates. CCR is a mechanism widely distributed amongst Gram-positive bacteria, including lactobacilli (34); mediated in *cis* by catabolite responsive elements (33, 35); and in *trans* by repressors of the LacI family, which is responsible for transcriptional repression of genes encoding unnecessary saccharolytic components in the presence of preferred substrates (25, 35–37). This regulatory mechanism allows cells to coordinate carbohydrate utilization and focus primarily on preferred energy sources. CCR is based on several key proteins, namely HPr (La639, *ptsH*), EI (La640, *ptsI*), CcpA (La431, *ccpA*),

and HPrK/P (La676, *ptsK*), all of which are encoded in *L. acidophilus* (16).

The PTS is characterized by a phosphate transfer cascade involving PEP, EI, HPr, and EIIABC, whereby a phosphate is ultimately transferred to the carbohydrate substrate (25, 38). HPr, an important component of CCR, is regulated via phosphorylation by EI and HPrK/P (39). Although the phosphorylation cascade suggests regulation at the protein level, several studies report transcriptional modulation of *ccpA* and *ptsHI*. In *S. thermophilus*, CcpA production is induced by glucose (27). In several bacteria, the carbohydrate source modulates *ptsHI* transcription levels (40). In contrast, expression levels of *ccpA*, *ptsH*, *ptsI*, and *ptsK* did not vary widely in the presence of different carbohydrates in *L. acidophilus* (Fig. 1). These results are consistent with regulation via phosphorylation at the protein level. Similar results have been reported for *ccpA* expression levels in *Lactobacillus pentosus* (34) and for *ptsHI* transcription in *S. thermophilus* (24).

In summary, a variety of carbohydrate uptake systems were identified and characterized, including PTS, ABC, and GHP transporters. The uptake and catabolic machinery is highly regulated at the transcription level, suggesting that the *L. acidophilus* transcriptome is flexible, dynamic, and designed for efficient carbohydrate utilization. Differential gene expression indicated the presence of a global CCR regulatory network, where key genes were consistently highly expressed, suggesting regulation at the protein level rather than the transcriptional level. Collectively, *L. acidophilus* appears able to adapt its metabolic machinery to fluctuating carbohydrate sources available in the environment. In particular, ABC transporters of the MsmEFGK family involved in uptake of FOS and raffinose likely play an important role in the ability of *L. acidophilus* to withdraw energy sources from the intestinal environment and compete with intestinal commensals for sugars that are not digested by the human host.

Materials and Methods

Bacterial Strains and Media. The strain used in this study is *L. acidophilus* NCFM (NCK56) (16). Cultures were aerobically propagated at 37°C in a semisynthetic medium described in ref. 9. The carbohydrates added were glucose (dextrose) (Sigma), fructose (Sigma), sucrose (Sigma), FOS (raffinose P95; Orafiti, Tienen, Belgium), raffinose (Sigma), lactose (Fisher), galactose (Sigma), or trehalose (Sigma). Cells underwent at least five passages on each sugar before RNA isolation to minimize carryover between substrates (41). The various carbohydrates used did not affect the growth pattern of *L. acidophilus*.

RNA Isolation. Total RNA was isolated using TRIzol (GIBCO/BRL) by following the manufacturer's instructions. *L. acidophilus* cells were inoculated into semisynthetic medium supplemented with 1% (wt/vol) select sugars and propagated to mid-log phase ($OD_{600} \approx 0.6$). Cells were harvested and RNA was isolated as described in ref. 42.

Microarray Fabrication. A whole-genome cDNA microarray was used for global gene expression analysis (42). The microarray contained triplicate spots of 1,889 cDNA PCR products amplified from genomic DNA, as described in ref. 42. PCR amplicons were spotted on GAPPS II aminosilane-coated glass slides (Corning) by using an Affymetrix 417 Arrayer, and slides were processed as described in refs. 42 and 43. Details for the microarray platform are available at the National Center for Biotechnology Information Gene Expression Omnibus database (GEO platform GPL1401).

cDNA Preparation and Microarray Hybridization. For each hybridization, two total RNA samples (25 μ g each) were amino-allyl-labeled by reverse transcription using random hexamers (Invitrogen) as

primers in the presence of amino-allyl dUTP (Sigma), by a SuperScript II reverse transcriptase (Invitrogen), as described in refs. 41 and 42. Labeled cDNA samples were subsequently coupled with either Cy3 or Cy5 *N*-hydroxysuccinimide-dyes (Amersham Pharmacia Biosciences), and purified by using a PCR purification kit (Qiagen). The resulting samples were hybridized onto microarray slides and further processed as described in ref. 42 and according to The Institute for Genomic Research protocol (43). Hybridizations were performed according to a single round-robin design, so that all possible direct pair-wise comparisons were conducted. With eight different sugars, a total of 28 hybridizations were performed (see Fig. 12, which is published as supporting information on the PNAS web site). Each treatment was labeled seven times, and every other treatment was labeled with either Cy3 or Cy5 four and three times, alternatively. Microarray data details are available at the National Center for Biotechnology Information (GEO series GSE2577).

Microarray Data Collection and Analysis. Microarray images were acquired with a ScanArray 4000 microarray scanner (Packard Bioscience, which is now PerkinElmer). Signal fluorescence, including spot and background intensities was subsequently quantified and assigned to genomic ORFs using QUANTARRAY 3.0 (PerkinElmer). Raw data were imported into SAS (SAS Institute), compiled, background-corrected, log₂-transformed, and subjected to a mixed model of analysis of variance (SAS proc mixed) with two sequential linear models (44) outlined in the *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site. ANOVA mixed models have proven successful at analyzing microarray data (41, 44–50). The array and spot effects were treated as random effects, whereas dye and treatment effect were considered fixed effects. The resulting difference between least-square estimates for two different treatments is analogous to a log₂-transformed ratio of gene expression between those two treatments. Differences were calculated between all pairs of treatments for each gene, and a measure of statistical significance was obtained from a *t* test using these differences and their associated standard errors. A Bonferroni correction was applied to account for bias due to multiple tests by dividing the desired level of significance ($\alpha = 0.05$) by the total number of comparisons performed (54,781), as used in refs. 49 and 50. Thus, the corrected false-positive rate was $\alpha = 9.12 \times 10^{-7}$ corresponding to a $-\log_{10}(P \text{ value}) = 6.04$ ($10^{-6.04}$). All *P* values that fell below α were considered statistically significant. Volcano plots of log₂-transformed fold changes (induction ratios) versus log₁₀-transformed *P* values (statistical significance) and three-way plots (contour plots) of individual treatment effects were used to visualize contrasts between treatments and statistical significance of the results. Global transcriptional patterns were visualized by using Ward's method of hierarchical clustering in JMP 5.0 (SAS Institute) with least-squares mean estimates and their standardized counterparts as input.

Real-Time QRT-PCR. Five genes differentially expressed in microarray experiments were selected for real-time QRT-PCR experiments to validate the induction levels measured. These genes were selected for their broad expression range (least-squares mean between -1.52 and $+3.87$) and their induction levels between sugars (fold induction up to 35). Also, the selected genes were correlated functionally with carbohydrate utilization. The selected genes were β -fructosidase (La505), trehalose PTS (La1012), glycerol uptake facilitator (La1436), β -galactosidase (La1467), and ABC transporter (La1679). Experiments were conducted with a QRT-PCR thermal cycler (I-cycler; Bio-Rad) in combination with a QuantiTect SYBR Green PCR kit (Qiagen). Six carbohydrate samples were included, namely glucose, fructose, sucrose, FOS, lactose, and galactose. Each set of samples was analyzed in triplicate. The RNA samples used in QRT-PCR experiments were identical to those used in microarray experiments.

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