Transcriptional and functional analysis of galactooligosaccharide uptake by *lacS* in *Lactobacillus acidophilus*

Joakim M. Andersen[a,b], Rodolphe Barrangou[c], Maher Abou Hachem[a], Sampo Lahtinen[d], Yong Jun Goh[b], Birte Svensson[a], and Todd R. Klaenhammer[b,1]

[a]Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark, DK-2800 Kongens Lyngby, Denmark; [c]Danisco USA Inc., Madison, WI 53716; [d]Health and Nutrition, Danisco BioActives, FI-02460, Kantvik, Finland; and [b]Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, Raleigh, NC 27695

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Probiotic microbes rely on their ability to survive in the gastrointestinal tract, adhere to mucosal surfaces, and metabolize available energy sources from dietary compounds, including prebiotics. Genome sequencing projects have proposed models for understanding prebiotic catabolism, but mechanisms remain to be elucidated for many prebiotic substrates. Although β-galactooligosaccharides (GOS) are documented prebiotic compounds, little is known about their utilization by lactobacilli. This study aimed to identify genetic loci in *Lactobacillus acidophilus* NCFM responsible for the transport and catabolism of GOS. Whole-genome oligonucleotide microarrays were used to survey the differential global transcriptome during logarithmic growth of *L. acidophilus* NCFM using GOS or glucose as a sole source of carbohydrate. Within the 16.6-kbp *gal-lac* gene cluster, *lacS*, a galactoside-pentose-hexuronide permease-encoding gene, was up-regulated 5.1-fold in the presence of GOS. In addition, two β-galactosidases, LacA and LacLM, and enzymes in the Leloir pathway were also encoded by genes within this locus and up-regulated by GOS stimulation. Generation of a *lacS*-deficient mutant enabled phenotypic confirmation of the functional LacS permease not only for the utilization of lactose and GOS but also lactitol, suggesting a prominent role of LacS in the metabolism of a broad range of prebiotic β-galactosides, known to selectively modulate the beneficial gut microbiota.

lactose permease | catabolite repression element

Increased interest in the ability of the human microbiota of the gastrointestinal tract (GIT) and selected probiotic microbes to impact health has been supported by expanded documentation on resistance to allergies (1), respiratory tract infections (2), and various gastrointestinal conditions such as ulcerative colitis, irritable bowel syndrome, and inflammatory bowel disease (3). Research on probiotic bacteria (4) continues to accumulate further knowledge about the biological mechanisms of action and complex interplay between gut microbes and host health.

The functional attributes of gut microbes and those delivered as probiotics rely on their ability to survive in the GIT, adhere to mucosal surfaces, and metabolize available energy sources from nondigestible dietary compounds (5). Notably, the ability to selectively use a broad range of potentially prebiotic carbohydrates (6), ranging from oligosaccharides to polysaccharides, provides a competitive advantage to the beneficial microbiota during colonization of the GIT and to transient probiotic microbes (7). Prebiotic oligosaccharides are not absorbed by the host and resist degradation by intestinal acids, bile acids, and digestive enzymes, allowing them to travel through the small intestine and colon, where they may be selectively used by beneficial microbes. Commercial β-galactooligosaccharides (GOS) are typically produced by enzymatic transglycosylation using lactose as substrate (8), to yield a mixed-length galactosylated product with a degree of polymerization (DP) ranging from 2 to 6. The oligomeric nature and β-galactoside linkages allow GOS to be used as prebiotic supplements, notably for stimulation of particular lactobacilli and bifidobacteria (9, 10). Specifically, GOS supplements have been shown to exert positive impacts on intestinal *Bifidobacterium* and *Lactobacillus* populations in infants (11), to mitigate irritated bowel syndrome (12), and to reduce the severity and duration of travelers' diarrhea (13). GOS has also been shown to inhibit pathogenic *Vibrio cholerae* and *Cronobacter sakazakii* binding to cell surface receptors of epithelial cells (14, 15) and prevent adhesion of *Salmonella enterica* serovar Typhimurium to murine enterocytes (16).

GOS are acquired naturally through the diet from the degradation of galactan side chains of the rhamnogalacturonan I fraction of pectin (17) and from human milk oligosaccharides (HMOs) that are nondigestible by the host (18, 19). HMOs are hypothesized to promote growth of specific beneficial bacteria in the infant's early GIT colonization (20). Marcobal et al. (21) verified that HMOs can support the growth of *Lactobacillus acidophilus* NCFM, although the genetic complement of *L. acidophilus* NCFM reflects a more specific potential for GOS metabolism compared with other adapted GIT bacteria (22). *L. acidophilus* is a widely used probiotic species, originally isolated by Moro in 1900 from infant feces. The *L. acidophilus* NCFM genome was recently sequenced to reveal that the molecular machinery responsible for carbohydrate uptake and catabolism in NCFM accounts for 17% of the genes present in the genome (23). Broad carbohydrate utilization of *L. acidophilus* NCFM was demonstrated and included transporters for trehalose (24), fructooligosaccharides (25), and several other mono-, di-, and trisaccharides (26).

The current understanding of the molecular and genetic basis for uptake and catabolism of GOS by probiotic lactobacilli is limited to *in silico* predictions based on genome sequencing projects (27). The aim of the present study was to functionally identify the genetic loci responsible for GOS transport and catabolism by *L. acidophilus* NCFM.

Results

GOS-Induced Differential Gene Expression. Global changes in gene expression levels across the transcriptome were used to identify genes differentially expressed in *L. acidophilus* NCFM during

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[1]To whom correspondence should be addressed. E-mail: klaenhammer@ncsu.edu.

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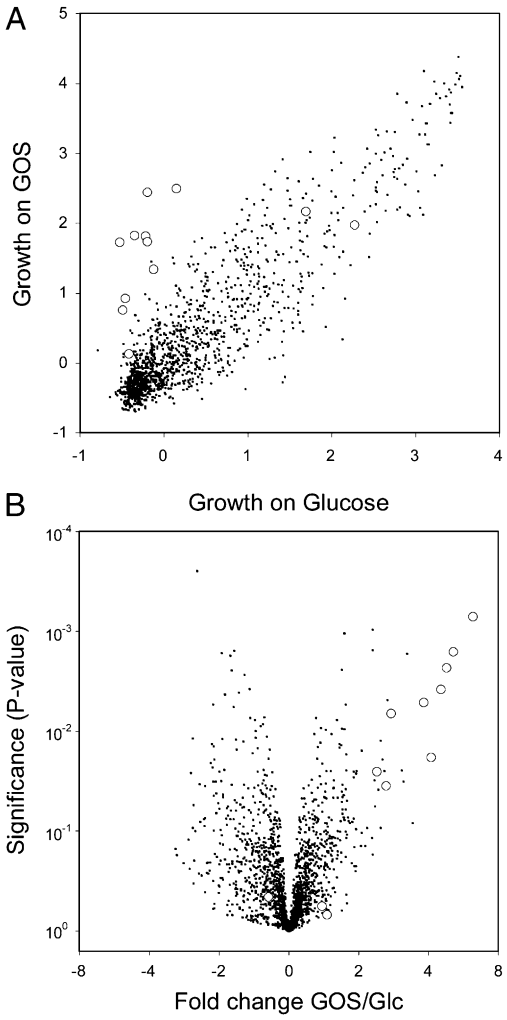

Fig. 1. Differential gene expression profile of GOS vs. glucose utilization by *L. acidophilus* NCFM. Genes involved in lactose metabolism are highlighted by open circles. (*A*) XY scatter plot of the overall normalized logarithmic gene expression profile. (*B*) Comparison of the statistical significance and gene expression differences of GOS (*Right*) vs. glucose (*Left*) depicted as a volcano plot. The *x* axis represents the differential gene induction profile as the ratio of fold difference. The *y* axis indicates statistical significance of expression difference (*P* value from ANOVA).

GOS fermentation in a semisynthetic medium (25). The single differential gene expression profile is depicted as a two-way scatter plot showing an overall linear correlation of GOS and glucose-induced gene expression (Fig. 1*A*). Notably, a subset of genes for lactose metabolism (*lac* genes, shown in white circles) were up-regulated in the presence of GOS, compared with glucose (the full dataset of the *lac* genes are reported in Table S1). Differentially expressed genes of interest were further characterized as statistically relevant ($P < 0.01$ and induction fold >2) in a volcano plot (Fig. 1*B*), confirming GOS induction of the specified *lac* operon (LBA1457–LBA1469). Statistically relevant genes induced by GOS are listed in Table 1 with annotated functions of the up-regulated genes. From Table 1, genes encoded within the 16.6 kbp *lac* operon locus were considered to be potentially involved with GOS metabolism.

The *lac* gene cluster's likely involvement in GOS utilization was consistent with the presence of two β-galactosidase–encoding genes (*lacLM*, LBA1467–1468, and *lacA*, LBA1462) assigned to the glycoside hydrolase family 2 (GH2) and glycoside hydrolase family 42 (GH42), respectively, using the CAZy classification (28).

Table 1. Differentially expressed genes in *L. acidophilus* NCFM identified by DNA microarrays of cells grown in GOS or glucose

Locus tag	Gene annotation	Fold up-regulation	*P* value
GOS-induced genes			
1467	β-galactosidase, large subunit, GH2	6.23	0.0007
1463	Lactose permease	5.10	0.0016
1462	β-galactosidase, GH42	4.79	0.0023
1459	Galactokinase	4.53	0.0038
1469	UDP-galactose-4-epimerase	3.82	0.0051
152	Phosphonate transport system ATP-binding protein	3.23	0.0017
1458	Galactose-1-phosphate uridylyltransferase	2.76	0.0067
1622	S-adenosylmethionine synthetase	2.73	0.0064
965	Hypothetical protein	2.65	0.0049
1952	Putative xanthine-uracil permease	2.29	0.0015
968	30S ribosomal protein	2.29	0.0010
Glucose-induced genes			
1429	Bile efflux transporter	2.50	0.0002
424	Conserved hypothetical protein	2.12	0.0054

Both were predicted to be localized intracellularly using the SignalP tool (29). Enzymatic activity on β-linked galactosides was demonstrated previously for both enzymes when expressed from recombinant constructs in *Escherichia coli* (30, 31). Furthermore, GH2 and GH42 β-galactosidases were proposed by Marcobal et al. (21) to be involved with degradation of HMOs. The identified galactoside-pentose-hexuronide (GPH) permease LacS (LBA1463) showed 83% amino acid sequence identity to the *Lactobacillus helveticus* functionally confirmed lactose permease (32). Two regulatory proteins, LacR (LBA1465), a LacI family regulator, and a noninduced regulator (LBA1461) with an unknown homology association, suggest regulation at the transcriptional level.

No genetic loci involved with carbohydrate metabolism were identified from the list of genes induced by glucose, suggesting that glucose is transported by the constitutively expressed mannose/glucose phospho-enolpyruvate–dependent phosphotransferase system (PEP-PTS) transporter (LBA0452, LBA0454-LBA0456), as suggested previously (26). The transcription analysis indicated that the *lac* operon in *L. acidophilus* NCFM is solely responsible for the metabolism of GOS and potentially other lactose-derived galactosides, because the gene induction profile of GOS is comparable to the lactose-induced *lac* gene expression pattern (26). It also indicates that regulation occurs at the transcriptional level, likely depending upon HPr (*ptsH*, LBA0639), CcpA (*ccpA*, LBA0431), and HPrK/P (*ptsK*, LBA0676), all of which are encoded in the *L. acidophilus* NCFM genome (23) and as previously proposed for carbohydrate utilization in *L. acidophilus* NCFM (26).

Analysis of *lacS* Inactivation. To investigate the potential involvement of the identified GPH permease LacS in GOS uptake, we inactivated the *lacS* gene using a *upp*-based counterselective gene replacement system (33), to create an in-frame deletion of 96% of the *lacS* coding region. The gene deletion had no detectable impact on cell morphology, growth in de Man, Rogosa, and Sharpe medium (MRS) or semi-defined medium (SDM)

using glucose (Fig. 2*A*), sucrose, or galactose as sole carbohydrates, suggesting that the functionality of *lacS* is nonessential for transport of monosaccharides during batch growth. Growth of the *ΔlacS* mutant was significantly impaired on lactose (Fig. 2*B*), confirming the annotation to previously validated *lacS* homologs and the previous findings of lactose induction of *lacS* together with the remaining *lac* genes (26). More significantly, the utilization of GOS (Fig. 2*C*), as well as lactitol, another galactoside prebiotic (34), was also abolished, showing a divergent and broader substrate specificity for GOS, including GOS with a higher degree of polymerization. The identification of a broad specificity transporter combined with the up-regulation of genes encoding two different β-galactosidases based on DNA microarrays illustrates a strong niche adaption by an evolved GPH β-galactosaccharide transporter.

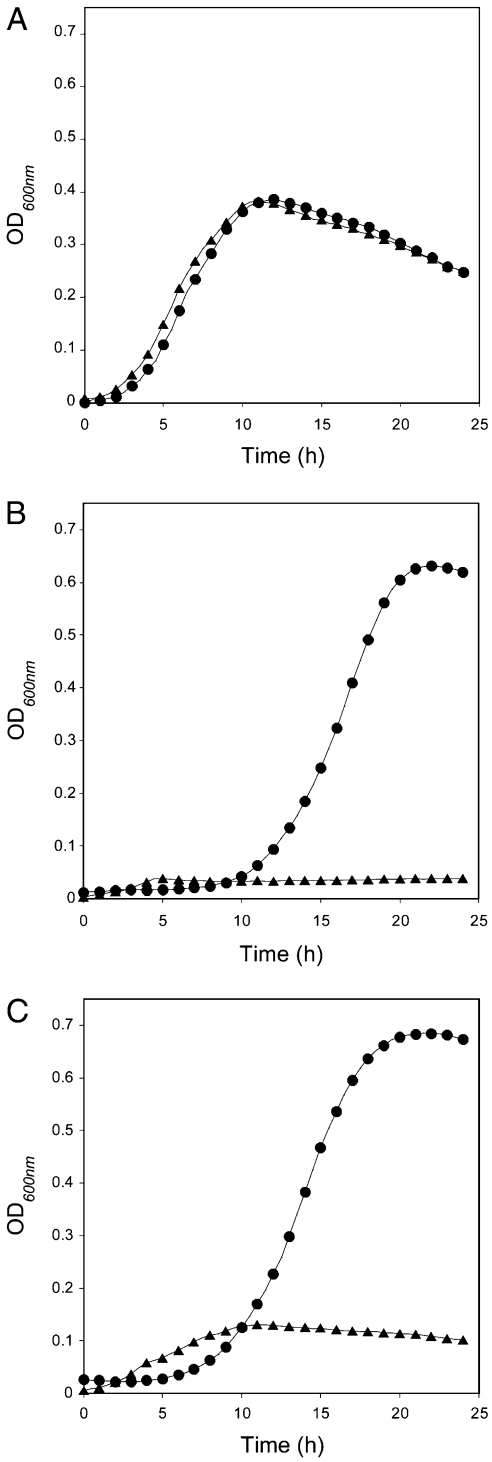

Fig. 2. Phenotype determination of *lacS* deficient mutant (▲) of *L. acidophilus* NCFM compared with wild type (●). (*A*) Growth profile on glucose; (*B*) growth profile on lactose; (*C*) growth profile on GOS.

Complete inhibition of growth by a single gene excision confirmed the hypothesis that the LacS permease was solely responsible for the transport of GOS in *L. acidophilus* NCFM and that no PEP-PTS or ATP-binding cassette (ABC) transporter systems were involved in this process. This finding indicates that the molecular basis for GOS transport and catabolism in other lactobacilli may also rely on GPH transporters and intracellular enzymatic hydrolysis by β-galactosidases from the GH2 and GH42 families before entering the Leloir and glycolysis pathways.

Sequence Analysis of GOS-Induced Gene Cluster. Additional genes surrounding the *lacS* permease and β-galactosidases were annotated in the genome with functions related to lactose and GOS metabolism, indicating a potential polycistronic operon structure for cotranscriptions of 12 genes (Fig. 3). Terminator sites and regulatory catabolite repression element (CRE) sequences were analyzed *in silico*.

The Leloir pathway genes *galM*, *galT*, *galK*, and *galE* were found with putative CRE sites, having palindromic homology to the CRE site of the *L. helveticus* lactose operon (32), yet markedly different from other *lac* CRE sites in *L. acidophilus* NCFM, indicating that these genes can be transcribed independently of the *lac* genes when only galactose is present. The *lacS*, *lacA*, and *lacLM* were all found to be under catabolite repression with two of these CRE sites showing homology to a CRE site found upstream of the *scrB* gene encoding a sucrose hydrolase in *L. acidophilus* NCFM (25). Notably, a CRE site with homology to the *lacR* CRE site was identified upstream of the *mucBP*, indicating cotranscription of *mucBP* simultaneously with the *lac* genes.

Sequence analysis of LacS predicts a two-domain structure with an N-terminal GPH permease and a C-terminal EIIA-like domain, homologous to the enzyme IIA (EIIA) of the PEP-PTS phosphorylation regulation by histidine-containing phosphocarrier protein (HPR) and enzyme I (EI). This indicates rapid regulation of lactose and related galactoside transport by *lacS* on transcriptional level in direct response to a decrease in glucose concentration. The gene locus organization differs from other characterized LacS uptake systems such as in *Lactobacillus bulgaricus* (35), *Leuconostoc lactis* (36), *Streptococcus thermophilus* SMQ-301 (37), and other *Lactobacillus* species (e.g., *Lactobacillus plantarum*, *Lactobacillus johnsonii*, and *Lactobacillus reuteri*) (Fig. S1). The differences in gene arrangement and in the types of encoded glycoside hydrolases reflect a specific adaptation of the varied species of lactic acid bacteria toward a varied β-galactosaccharide metabolism.

Phylogenetic relationships of the above LacS amino acid sequences (Fig. 4*A*) compared with the overall phylogenetic similarity of lactobacilli based on 16S rRNA homologies (38) demonstrates, first, how most *lacS* positive strains are associated with GIT colonization; and second, that the diversity of gene sequences and locus structure follow the evolutionary direction in all but *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 (39).

The gene locus organization and LacS sequence homology suggest that the specific locus originated by recent gene transfer from an unrelated precursor, possibly from within a dairy environment. Interestingly, it is observed that *lacS* genes from lactobacilli are present in the loci together with GH42 β-galactosidases for all but *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, which harbors a *lacZ*-GH2 family enzyme. The phylogenetic tree of identified GH42

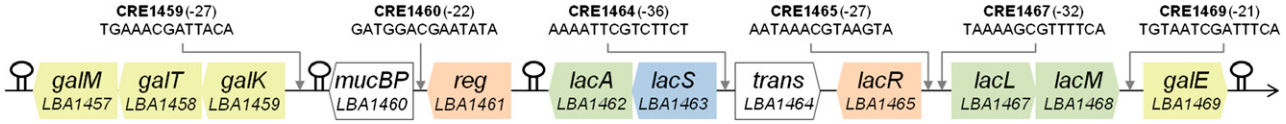

Fig. 3. Gene structure of the GOS-induced genome locus. Predicted ρ-independent transcription terminators (52) are shown as hairpin loops. Regulatory CRE sites are shown above the gene structure, with the upstream base pair distance to the starting codon. Putative functions are indicated by color: carbohydrate permease (blue), transcriptional regulators (red), glycoside hydrolases (green), Leloir pathway (yellow), and genes without a known relationship to carbohydrate metabolism (white boxes). mucBP, mucus-binding domain protein; reg, putative transcriptional regulator; trans, transposase.

β-galactosidases, *lacA*, encoded within *lacS*-containing loci, revealed no marked difference from the tree structure in Fig. 4*A*, indicating the coevolution of LacS with GH42 β-galactosidases (Fig. 4*B*).

Recently available human GIT microbiome sequencing data from the Human Microbiome Project (40) was used to validate the presence of the LacS permease and associated β-galactosidases in the human GIT microbiota by BLAST analysis (41). Both *lacS* and GH42 *lacA* genes were identified with robust statistical significance (threshold e-value $<10^{-15}$) in *L. acidophilus*, *L. helveticus*, *Lactobacillus ultunensis*, and *L. reuteri* strains from a current total of 29 *Lactobacillus* reference genomes.

Discussion

The ability of GOS to selectively promote the growth of selected GIT microbiota further establishes this prebiotic as an attractive nutritional ingredient for foods and dietary supplements. Stimulation of *Bifidobacterium* and *Lactobacillus* species by prebiotic oligosaccharides, including GOS, is well documented by observational studies (13, 42–44). Despite this, only a few studies have confirmed the lactobacilli enrichment by GOS on the strain level (45). In the present study we aimed to identify molecular elements linked to the selective GOS metabolism within *Lactobacillus* to explain in vivo observations of GOS stimulation within the GIT. Whole-genome DNA microarray analysis was performed to differentiate the gene expression pattern of *L. acidophilus* NCFM in the presence of GOS compared with glucose as the sole carbohydrate source. It was found that GOS specifically induced a cluster of genes encoding intracellular proteins involved with galactose and lactose metabolism, notably a LacS permease implicated in GOS transport. The GOS-induced gene cluster was previously identified to be up-regulated by both lactose and bile acids (26, 46), validating a role in metabolism of lactose-derived GOS and suggesting an adaptive combination of GIT-evolved traits for energy metabolism and bile tolerance.

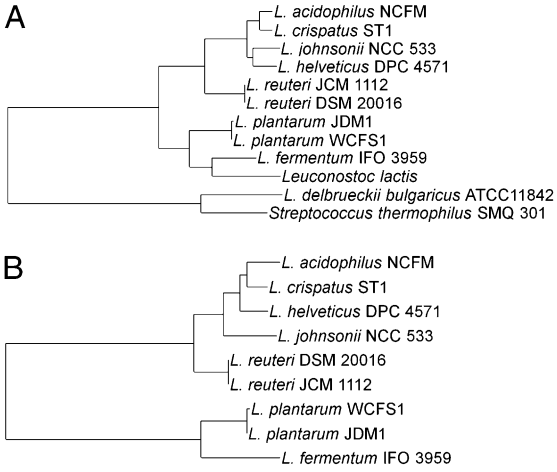

Fig. 4. Unrooted phylogenetic trees of (*A*) LacS and (*B*) LacA.

The environment-adaptive nature of *L. acidophilus* NCFM and the broad specificity of the LacS permease show the potential for delivery of *L. acidophilus* NCFM in dairy-based synbiotic GOS products, whereby a culture prefermented on lactose will rapidly metabolize GOS for increased viability upon exposure in the gut.

This study considered multiple genome sequences of lactose-fermenting lactobacilli to reveal operons encoding either a LacS permease or a PEP-PTS transporter for lactose uptake. Pathway reconstruction positioned these transporters adjacent to β-galactosidases or phospho-β-galactosidases, respectively. However, prediction of potential GOS PEP-PTS transporters was troubled by low sequence similarity to known PEP-PTS transporters families (47). Experimental validation of LacS permease as sole transporter of GOS in *L. acidophilus* NCFM was performed by gene deletion, which eliminated the ability to use lactose, GOS, and lactitol. This serves as the first identified GOS transporter in the *Lactobacillus* genus and is the first evidence that the LacS permease is capable of transporting oligosaccharides such as GOS with a DP of ≥2–6 and modified disaccharides (lactitol).

Bioinformatic identification and analysis of other *lacS* genes and their proximal genetic loci was based on the present study and earlier functional characterization of lactose transport by *lacS* homologs in lactic acid bacteria. Phylogenetic mapping of *lacS* encoding strains revealed that *lacS* is, to date, mainly found in *Lactobacillus* species that are commensals of the human gut. This suggests that transport and metabolism of lactose and complex carbohydrates are important energy sources for intestinal lactobacilli, because GPH permeases compared with ABC and PTS systems do not require ATP for import, allowing an energy-efficient and rapid adaptive transport of GOS. Analysis of the adjacent genes of *lacS* showed three core genes: *lacS*, *lacR*, and β-galactosidase of either GH2 (LacZ or LacLM) or GH42 (LacA) family. Genes without apparent known function for lactose metabolism were also found for some species (e.g., *L. acidophilus* and *L. plantarum*), and interestingly some proximal genes showed putative functional roles for mucin adhesion or rhamno-galactoside metabolism, respectively. This suggests that the base functionality of the *lacS* genetic locus is highly conserved by evolutionary pressure and important for niche survival in the GIT via transport and metabolism of lactose, GOS, and likely fractions of HMOs.

The presence of *lacS* and *lacA* homologs among the intestinal lactobacilli supports the importance of complex galactoside utilization for energy metabolism. The related genetic loci were inclusive within the acidophilus subfamily of lactobacilli (*L. acidophilus*, *L. ultunensis*, and *L. helveticus*), whereas other species (e.g., *Lactobacillus fermentum* and *L. plantarum*) include *lacS*-positive strains and strains that have no homologs of either *lacS* or *lacA*. The retention of *lacS* and *lacA* homologs in *L. helveticus* is consistent with the known genetic lineage and adaptation of these lactobacilli to milk (48). In that process, *L. helveticus* eliminated a number of GIT-related functions (e.g., bile salt hydrolase and mucin binding proteins) but retained the *lac*-related genes while losing most *gal*-related genes except the galactose-1-phosphate uridylyltransferase gene (*galT*).

In conclusion, we identified LacS as the sole transporter for lactose, GOS, and lactitol. A future combination of tran-

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
E. coli strains		
NCK1831	EC101: RepA$^+$ JM101; Km^r; *repA* from pWV01 integrated in chromosome; host for pORI-based plasmids	53
NCK1911	NCK1831 harboring pTRK935	33
NCK2126	NCK1831 harboring pTRK1015	Present study
L. acidophilus strains		
NCFM	Human intestinal isolate	23
NCK1909	NCFM carrying a 315 bp in-frame deletion in the *upp* gene	33
NCK1910	NCK1909 harboring pTRK669, host for pORI-based counterselective integration vector	33
NCK2127	NCK1909 carrying a 1831 bp in-frame deletion in the *lacS* gene	Present study
Plasmids		
pTRK669	Ori (pWV01], Cm^r RepA$^+$	54
pTRK935	pORI28 derived with an inserted *upp* expression cassette and *lacZ´* from pUC19, serves as counterselective integration vector, Em^r	33
pTRK1015	pTRK935 with a mutated copy of *lacS* cloned into BamHI/EcoRI sites	Present study

scriptomics, proteomics, and functional genomics analyses will provide a comprehensive platform for study of the molecular interactions between probiotics and prebiotics.

Materials and Methods

Bacterial Strains and Growth Conditions. All bacterial strains and plasmids used throughout this study are listed in Table 2. *Lactobacillus* broth cultures were cultivated in MRS (Difco Laboratories) or SDM (49), supplemented with 0.5% (wt/vol) glucose (Sigma-Aldrich), lactose, lactitol (Danisco), or GOS (94% GOS, DP 2–6; Danisco; Fig. S2) as carbon source, aerobically at 37 °C or 42 °C. Chloramphenicol (5 μg/mL) and erythromycin (2 μg/mL) were used when necessary for selection. *E. coli* strains were cultivated in Brain Heart Infusion medium (Difco) aerobically at 37 °C with aeration, and erythromycin (150 μg/mL) and/or kanamycin (40 μg/mL) was added for selection. Solid media were prepared by the addition of 1.5% (wt/vol) granulated agar (Difco).

***L. acidophilus* NCFM Microarray Platform.** Whole-genome oligonucleotide microarrays were designed as described by Goh et al. (33) with four replicate spots for each of the 1,824 predicted genes. Hybridization quality was assessed by monitoring the Cy3/Cy5 ratio of labeled cDNA, prepared from total RNA, after slide scan to observe a linear correlation between the two fluorophores. For DNA microarray transcriptome study, semisynthetic media (25) used for cultivation of *L. acidophilus* NCFM were filtered through a 0.22-μm filter, and oxygen was removed by the Hungate method (50). *L. acidophilus* NCFM cultures were propagated in parallel in semisynthetic media with 1% (wt/vol) glucose or GOS as carbon source. Cultures were transferred for four passages on each sugar before being harvested at the early logarithmic phase (OD_{600}= 0.35–0.5) by pelleting at 4 °C (3,000 × *g*, 15 min) and flash freezing the pellets for storage at −80 °C.

cDNA Preparation and Microarray Hybridization. Cells were mechanically disrupted by beadbeating, and total RNA was isolated using TRIzol-chloroform extraction (Invitrogen). Genomic DNA was removed with Turbo DNase (Ambion), followed by RNA purification using an RNeasy Mini Kit (Qiagen) (33).

Reverse transcription of total RNA, fluorescent labeling of cDNA, and hybridizations were done using 20 μg of total RNA for each replicate, as described by Goh et al. (33). Total RNA from each of the two carbohydrate treatments was labeled with both Cy3 and Cy5 for two technical replicates to each growth condition.

Microarray Data Acquisition and Analysis. Hybridized chips were scanned at 10-μm resolution per pixel using a ScanArray Express microarray scanner (Packard BioScience) for 16-bit spot intensity quantification. Fluorescent intensities were quantified and background subtracted using the QuantArray 3.0 software package (Packard BioScience). Median values were calculated for all ORF tetraplicate intensities and $\log_2$-transformed before being imported into SAS JMP Genomics 4.0 (SAS Institute) for data analysis. The full dataset was interquantile normalized and modeled using a mixed-model ANOVA for analysis of the differential gene expression pattern and visualization using heat maps and volcano plots.

Construction and Phenotypic Determination of the *lacS* Deletion Mutant. Genomic DNA of *L. acidophilus* NCFM was isolated by the method of Walker and Klaenhammer (51) or by the Mo Bio Ultraclean microbial DNA isolation kit (Mo Bio Laboratories). Plasmid DNA from *E. coli* was isolated using a QIAprep Spin miniprep kit (Qiagen). Restriction enzymes (Roche Molecular Biochemicals) were applied according to the instructions supplied by the manufacturer. DNA ligation was done using T4 DNA ligase (New England Biolabs) as directed by the manufacturer's recommendations. All PCR primers (Table S2) were synthesized by Integrated DNA Technologies. PCR reactions, preparation and transformation of competent *L. acidophilus* NCFM and *E. coli* cells, analysis by agarose gel electrophoresis, and in-gel purification were done as described by Goh et al. (33).

The construction of an Δ*upp* isogenic mutant with in-frame DNA excision of 96% of the *lacS* (LBA1463) coding region was done according to Goh et al. (33). In short, the upstream and downstream flanking regions (approximate length of 750 bp each) of the deletion target were PCR-amplified with the 1463A/1463B and 1463C/1463D primer pairs, respectively, and fused by splicing by overlap extension PCR (SOE-PCR). The SOE-PCR product was cleaved with EcoRI and BamHI before ligation into pTRK935 linearized with compatible ends and transformed into NCK1831. The resulting recombinant plasmid, pTRK1015, harbored in NCK2126, was transformed into NCK1910 harboring pTRK669, for chromosomal integration of pTRK1015 and following DNA excision to generate the Δ*lacS* genotype. Confirmation of DNA deletion was done by PCR and DNA sequencing using primer pair 1463UP and 1463DN (Table S2).

Lactose and GOS utilization of the *lacS* gene deletion mutant was tested by comparative growth to wild-type *L. acidophilus* NCFM and NCK1909 (*upp* mutant and parent strain of the Δ*lacS* mutant). All strains were grown in SDM supplemented with 1% (wt/vol) glucose before inoculation [1% (vol/vol)] of an overnight culture into SDM supplemented with 0.5% (wt/vol) of the following carbohydrates in separate batches: lactose, GOS, lactitol, galactose, sucrose, and glucose. Growth was monitored by optical density using a Fluostar spectrophotometer in triplicate wells of a 96-well plate (200 μL per well) and covered with an airtight seal. All carbohydrates were at least 95% pure.

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