Genetic and phenotypic analysis of carbohydrate metabolism and transport in *Lactobacillus reuteri*

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Abstract.

Lactobacilli derive metabolic energy mainly from carbohydrate fermentation. Homofermentative and heterofermentative lactobacilli exhibit characteristic differences in carbohydrate transport and regulation of metabolism, however, enzymes for carbohydrate transport in heterofermentative lactobacilli are poorly characterized. This study aimed to identify carbohydrate active enzymes in the *L. reuteri* strains LTH2584, LTH5448, TMW1.656, TMW1.112, 100-23, mlc3, and lpuph by phenotypic analysis and comparative genomics. Sourdough and intestinal isolates of *L. reuteri* displayed no difference in the number and type of carbohydrate-active enzymes encoded in the genome. Predicted sugar transporters encoded by genomes of *L. reuteri* strains were secondary carriers and most belong to the major facilitator superfamily. The quantification of gene expression during growth in sourdough and in chemically defined media corresponded to the predicted function of the transporters MalT, ScrT and LacS as carriers for maltose, sucrose, and lactose or raffinose, respectively. The genotype for sugar utilization matched the fermentation profile of 39 sugars for *L. reuteri* strains, and indicated preference for maltose, sucrose, raffinose and (iso)-malto-oligosaccharides, which are available in sourdough and in the upper intestine of rodents. Pentose utilization in *L. reuteri* species was strain-specific but independent of the origin or phylogenetic position of isolates. Two glycosyl hydrolases, licheninase (EC 3.2.1.73) and endo-1, 4-β-galactosidase (EC 3.2.1.89) were identified based on conserved domains. In conclusion, the study identified the lack of PTS systems, preference for secondary carriers for carbohydrate transport, and absence of carbon catabolite repression as characteristic features of the carbohydrate metabolism in the heterofermentative *L. reuteri.*

Keywords: *Lactobacillus reuteri*, sourdough, carbohydrate transport, maltose metabolism, sucrose metabolism, heterofermentative lactobacilli

Introduction.

Production of a majority of food fermentations involves lactobacilli as abundant members of fermentation microbiota, and the conversion of carbohydrates to lactic acid is a major contributor to the quality and safety of these fermented foods (Gänzle, 2015). Carbohydrate metabolism also provides the main source of metabolic energy in lactobacilli and thus contributes to their ecological fitness (Gänzle, 2015). Lactobacilli preferentially metabolize monosaccharides and oligosaccharides; enzymes for extracellular hydrolysis of polysaccharides are exceptional (Gänzle and Follador, 2012). The genus *Lactobacillus* includes a large and diverse number of species; physiological, ecological, and phylogenetic properties separate *Lactobacillus* spp. in two major clades comprising homofermentative and heterofermentative lactobacilli (Duar et al., 2017b; Zheng et al., 2015a). Homofermentative lactobacilli metabolize glucose by glycolysis; heterofermentative lactobacilli metabolize glucose by the phosphoketolase pathway; pentoses are metabolized by the phosphoketolase pathway or the pentose phosphate pathway by organisms in both groups (Gänzle, 2015; Zheng et al., 2015a). The regulation of carbohydrate metabolism differs between homofermentative and heterofermentative lactobacilli. Homofermentative lactobacilli preferentially metabolize glucose; the use of alternative carbon sources is generally repressed by carbon catabolite repression if glucose is available (Andersson et al., 2005; Gänzle et al., 2007; Monedero et al., 2008). Carbohydrate transport is mediated by members of the ATP-binding cassette (ABC) superfamily of ABC-transporters, secondary transporters of the Major Facilitator Superfamily (MFS), or phospho-transferase systems (PTS). PTS systems, which mediate phosphorylation and transport of mono- and

disaccharides, directly or indirectly mediate carbon catabolite repression through interaction with the catabolite control protein A (Andersson et al., 2005; Galinier and Deutscher, 2017; Monedero et al., 2008). Genomes of heterofermentative lactobacilli code for fewer genes for PTS systems when compared to homofermentative lactobacilli (Zheng et al., 2015a). In contrast to homofermentative lactobacilli, glucose transport and metabolism is not constitutive but induced by the substrate in heterofermentative lactobacilli (Neubauer et al., 1994; Ye and Saier, 1995) and the utilization of maltose, sucrose, and pentoses is not repressed by glucose (Ehrmann and Vogel, 1998; Teixeira et al., 2013). The few carbohydrate transport enzymes that were characterized in heterofermentative lactobacilli include MFS permeases but no ABC-transporters or PTS systems (Chaillou et al., 1998; Djordjevic et al., 2001; Neubauer et al., 1994).

Homofermentative and heterofermentative lactobacilli co-exist in many of their natural and man-made habitats including sourdough, rice vinegar fermentations, and fermented vegetables (De Vuyst et al., 2014; Duar et al., 2017a; Wu et al., 2012; Zheng et al., 2015a). This co-existence has been described as complementary rather than competitive (Andreevskaya, 2017; Tannock et al., 2012) and may reflect resource partitioning by means of preferential utilization of different carbohydrates. However, only few studies describe mechanisms and regulation of carbohydrate transport and metabolism in heterofermentative lactobacilli as a prerequisite to understand the molecular basis of resource partitioning between homo- and heterofermentative lactobacilli (Gänzle and Follador, 2012). It was therefore the aim of this study to provide a genome-wide assessment of carbohydrate transport and metabolism in *Lactobacillus reuteri*, and to complement bioinformatic

analyses by phenotypic characterization of carbohydrate utilization and quantification of gene expression. *L. reuteri* was used as model organism. The species *L. reuteri* represents the *L. reuteri* group in the heterofermentative clade of lactobacilli (Duar et al., 2017b; Zheng et al., 2015a). Owing to its occurrence in the upper intestine of animals (Frese et al., 2011) and in fermented cereals (Su et al., 2012), *L. reuteri* is well characterized genetically and physiologically and metabolic traits that contribute to its ecological fitness in cereal and intestinal ecosystems are well understood (Frese et al., 2011; Gänzle et al., 2007; Krumbeck et al., 2016; Lin et al., 2015; Lin and Gänzle, 2014). The study employed 7 strains of *L. reuteri* with known genome sequence (Zheng et al., 2015b).

Materials and Methods

Strains and growth conditions. The sourdough isolates *L. reuteri* LTH2584, LTH5448, TMW1.112 and TMW1.656, and rodent isolates *L. reuteri* 100-23, mlc3 and lpuph were routinely grown on mMRS medium. The sourdough isolates belong to the rodent-adapted lineages I and III (Zheng et al., 2015b); rodent isolates were selected from the same lineages. Working cultures were prepared by streaking cultures on mMRS agar from the -80 °C glycerol stocks, followed by two subcultures in mMRS broth. The cultures were grown anaerobically at 37 °C. The mMRS broth medium contained the following ingredients per litre: beef extract (5 g), yeast extract (5 g), peptone (10 g), malt extract (10 g), NH_4Cl (3 g), K_2HPO_4 (4 g), KH_2PO_4 (2.6 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), $MnSO_4 \cdot 4H_2O$ (0.05 g), L-cysteine·HCl (0.5 g), Tween-80 (1 g), glucose (5 g), fructose (5 g) and maltose (10 g). All chemicals were obtained from Sigma Aldrich (Oakville, Ontario, Canada) unless otherwise specified. The pH was adjusted to 6.2 before autoclaving; solid media were

prepared by adding 2 % agar.

Carbohydrate metabolism was analysed in chemically defined medium (CDM) (Hüfner et al., 2008) that contained the following ingredients per litre: sodium acetate (6 g), KH_2PO_4 (3 g), K_2HPO_4 (3 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), Tween-80 (1 g), L-alanine (0.1 g), L-arginine (0.1 g), L-asparagine (0.2 g), L-asparic acid (0.2 g), L-cysteine (0.2 g), L-glutamine (0.2 g), L-glutamic acid (0.2 g), glycine (0.1 g), L-histidine (0.1 g), L-isoleucine (0.1 g), L-leucine (0.1 g), L-lysine (0.1 g), L-methionine (0.1 g), L-phenylalanine (0.1 g), L-proline (0.06 g), L-serine (0.1 g), L-threonine (0.1 g), L-tryptophan (0.1 g), L-tyrosine (0.1 g), L-valine (0.1 g), nicotinic acid (0.001 g), calcium pantothenate (0.001 g), pyridoxal (0.002 g), riboflavin (0.001 g), uracil (0.001 g), di-ammonium hydrogen citrate (2 g), NaCl (0.02 g), ascorbic acid (0.5 g), guanine (0.1 g), cytidine (0.1 g), 2'-deoxyadenosine (0.1 g), 2'-deoxyuridine (0.1 g), xanthine (0.1 g), inosine (0.1 g), $MnSO_4 \cdot H_2O$ (0.02 g), $CoCl_2$ (0.0046 g), cyanocobalamin (0.004 g), para-aminobenzoic aicd (0.0006 g), myo-inositol (0.005 g), D-biotin (0.001 g), folic acid (0.0005 g), thymine (0.004 g), and sugars (at 2 g or 20 g). The pH of stock solutions was adjusted to 6.3 prior to sterilization. Of above compounds, sodium acetate, KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and Tween-80 were prepared as stock solution which was autoclaved at 121 ^oC for 15 min. Stock solutions of sugars were sterilized with 0.22 μm filters; remaining ingredients were prepared as a concentrated stock solution, filter sterilized, and added to the final medium.

CAZyme annotation. Carbohydrate active enzymes in the genomes of 7 strains of *L. retueri* were annotated in two platforms, the CAZymes Analysis Toolkit

(http:/gg/mothra.ornl.gov/cgi-bin/cat.cgi) (Park et al., 2010) and the dbCAN prediction web server (http://csbl.bmb.uga.edu/dbCAN/) (Yin et al., 2012). Sequence-similarity-based method was used in both analysis platforms with default threshold of e-value. Protein sequences were assigned to the respective CAZyme families if the annotation in the CAZymes Analysis and dbCAN prediction platforms was consistent.

Determination of carbohydrate utilization. Growth of *L. reuteri* in chemically defined media with different carbon sources was assayed by incubation in microtitre plates (Lin et al., 2015). In brief, overnight cultures were washed twice in saline and re-suspended to the same volume sterile saline; 20 µL of this cell suspension was inoculated into 96-well microtitre plates containing 180 µL of CDM with different carbon sources at 2 g L^{-1}. Plates were sealed with 50 µL paraffin oil after inoculation to exclude oxygen. Plates were incubated at 37 °C for 24 h in a mictotitre plate photometer and the optical density at 600 nm was measured every 0.5 h. The assay was performed in three biological replicates. The following carbon sources were evaluated: L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-lactulose, L-rhamnose, D-cellobiose, D-trehalose, D-gentiobiose, xylobiose, palatinose, D-raffinose, D-glucotriose, D- mannotriose, D-maltotriose, D-melezitose, isomalto-oligosaccharides (IMO), fructo-oligosaccharides (FOS), D-mannose, D-sorbitol, D-mannitol, inositol, glucuronic, amygdalin, dulcitol, D-gluconate, inulin, xylan, pectin, cellulose, starch, dextran, and galactan.

The carbohydrate fermentation profile was also assessed by using the API 50 CH system (bioMérieux, Inc., Marcy l'Etoile, France), according to the manufacturer's instructions.

Enzyme profile was assayed with API ZYM system (bioMérieux, Inc., Marcy l'Etoile, France).

Bioinformatics analysis of sugar transporters. Genomes of the 7 strains of *L. reuteri* were downloaded from NCBI FTP website (ftp://ftp.ncbi.nlm.nih.gov/genomes/), and built as local databases using standalone BLAST+, 2.2.30 (Camacho et al., 2009). Sequences of prokaryotic transporter enzymes were retrieved from the Transporter Classification Database (TCDB, http://www.tcdb.org/) (Saier et al., 2014). TCDB transporter sequences were used as query sequences for blastp search of the *L. reuteri* genomes with standalone BLAST+, 2.2.30 with a cutoff of e^{-10}. Initial hits were used as query sequences for blastP search of the TCDB database for further confirmation or exclusion. Genome-wide annotation of membrane transporters, including sugar transporters and non-sugar transporters, was performed using Transporter Automatic Annotation Pipeline, TransAAP in Transporter Database (Ren et al., 2007). The predicted, substrate-specific, sugar transports above would be further verified if their corresponding properties are consistent with the annotation or supporting evidence of TransAAP. Characterized proteins with homologies to the predicted sugar transporters were retrieved from Uniprot database (http://www.uniprot.org/blast/) to support predictions of substrate specificity.

Quantification of gene expression during growth of *L. reuteri* in sourdough. To determine which sugar transporters are expressed during growth in sourdough, mRNA was quantified by reverse transcription-quantitative PCR (RT-qPCR). Whole wheat sourdough was prepared by mixing 10 g of whole wheat flour with 10 mL or a cell suspension of *L. reuteri* LTH5448, 100-23, or TMW1.656 in tap water to achieve an initial cell count of

about 10^7 cfu / g (Lin et al., 2014). Dough fermented with *L. reuteri* LTH5448 was fermented with and without addition of 2 % baker's yeast. The dough was fermented at 37°C until the pH was reduced to 4.5, corresponding to the exponential phase of growth. Cells were isolated from sourdoughs as described (Teixeira et al., 2014) and RNA was extracted using RNAprotect Bacteria Reagent and RNeasy Minikit (Qiagen, USA) prior to DNAase treatment with RQ1 RNase-Free DNase Kit (Promega, Madison, USA) to eliminate residual DNA. RNA quality and quantity were assessed spectroscopically (Nanodrop 2000, Thermo Fisher Scientific, Waltham, Massachusetts, USA) prior to reverse transcription to cDNA using QuantiTect Reverse Transcription Kit (Qiagen, USA). Quantitative PCR was performed with the QuantiFast SYBR Green master mix (Qiagen) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Burlington ON) with primers and annealing temperatures shown in Table 1. DNase-treated RNA samples served as negative controls. Relative gene expression was calculated as

$$\frac{(E_{target})^{\Delta C_T(reference-sample)}}{(E_{reference})^{\Delta C_T(reference-sample)}}$$ (Pfaffl, 2001),

where E_{target} is the PCR efficiency for the target gene, $E_{reference}$ is the PCR efficiency for the housekeeping gene, and $\triangle C_T$ is the threshold cycle for samples obtained at sample and reference conditions. Exponentially growing cultures (OD_{600nm} 0.4 – 0.6) in CDM-glucose (10 g L^{-1}) were used as reference conditions and *pho* coding for phosphoketolase was used as housekeeping gene. The experiment was performed in triplicate independent experiments, each analyzed in duplicate PCR reactions.

Effect of the carbohydrate source on expression of sugar transporters. To determine the effect of the carbohydrate source on gene expression in *L. reuteri* 100-23 and LTH5448,

strains were grown in CDM broth containing 20 g L^{-1} raffinose, maltose, sucrose, melibiose, or lactose, or 10 g L^{-1} maltose and 10 g L^{-1} xylose. Cultures were grown to an OD_{600nm} of 0.4; cells were harvested for isolation of RNA and quantification of mRNA by RT-qPCR as described above. Results were indicated as means ± standard deviations for 5 technical replicates from two biological replicates.

Global reconstruction of metabolism pathway. Carbohydrates metabolism pathways were visualized through KEGG Automatic Annotation Server (KAAS) (http://www.genome.jp/kaas-bin/kaas_main) (Moriya et al., 2007) and manually curated to match metabolic pathways in heterofermentative lactobacilli (Zheng et al., 2015a). For KAAS analysis, proteins encoded by the genomes were searched against defined gene data sets using bi-directional best hit (BBH)-based GHOSTX program.

Multiple alignment for genomes or protein domains. Multiple alignment of genomes was performed using standalone BLAST+, 2.2.30. Genome sequences were concatenated with Geneious (6.1.6) prior to alignment. Target protein domains (Finn et al., 2014) of glycosyl hydrolase family 53 proteins and glycosyl hydrolase family 8 proteins were aligned with in MEGA 6.

Statistical analysis. The one-way ANOVA (SigmaPlot, version 12.5) was used for statistical analysis. Statistical analysis was performed using Student's t-test (SigmaPlot, version 12.5) and significant differences in gene expression were evaluated with a 5 % probability of error ($p < 0.05$).

Results

Genomic and phenotypic characteristics of carbohydrate metabolism of *L. reuteri*. Genome scale annotation of carbohydrate active enzymes (CAZymes) was conducted for

223 sourdough- and rodent-isolates of *L. reuteri* (Table 2). The genome size or the number of
224 protein coding sequences was not significantly different between strains of different
225 lineages; the number of carbohydrate active enzymes relative to the total number of
226 predicted proteins ranged from 1.2 to 1.6 %. CAZymes in *L. reuteri* consisted mainly of
227 glycoside hydrolases (GH) and glycosyl transferases (GT). Only few carbohydrate
228 esterases (CE) and carbohydrate-binding modules (CBM) were present; polysaccharide
229 lyases and auxiliary activity family proteins were absent (Table 2).
230 The presence of glycosyl hydrolases largely overlapped between the 7 strains of *L. reuteri*
231 (Table 2). Strain-specific differences related to levansucrases and reuteransucrases in the
232 GH68 and GH70 families, and a predicted GH53 family endo-β(1→4)-galactanase that was
233 present in *L. reuteri* TMW1.112 and lpuph (Table 2). A CBM50 family protein which
234 attaches cell-wall associated hydrolases to peptidoglycan was present in all strains; a
235 CBM37 family protein was identified only in the genome of *L. reuteri* 100-23. This protein
236 was previously considered to be unique to the cellulose-degrading *Ruminococcus albus*
237 (Ezer et al., 2008).
238 The phenotype of carbohydrate utilization in the 7 *L. reuteri* strains was evaluated by API
239 50CH profiling of strains, and by assessment of growth in chemically defined medium
240 (Table 3, Table S1 of the online supplementary material). Not all of the carbohydrates that
241 were fermented in the API 50CH assay supported growth in chemically defined medium,
242 which may reflect that the chemically defined medium is a poor substrate for growth of *L.*
243 *reuteri*. The capacity to metabolize arabinose and xylose differed between strains of *L.*
244 *reuteri*. Comparison of the genotype of xylose and arabinose-utilizing strains with the

genotype of *L. reuteri* LTH2584 and LTH5448, which were unable to ferment arabinose and xylose, respectively, demonstrated that the lack of pentose utilization corresponded to the loss of operons coding for enzymes involved in pentose utilization (Fig. 1).

L. reuteri grow on isomalto-oligosaccharides (IMO) with a degree of polymerization (DP) of 2 – 6 (Hu et al., 2013) as well as raffinose (Teixeira et al., 2012) but enzymes for extracellular hydrolysis of polysaccharides were absent. In keeping with the genotype and the fermentation patterns, the API ZYM assay identified esterase, α-glucosidase, α-galactosidase and β-galactosidase activity (Table S2 on the online supplementary material).

***In silico* analysis of sugar transporters.** Transport enzymes for carbohydrates were initially predicted *in silico* (Table 4). Transporters predominantly belonged to the major facilitator superfamily and few transporters belonged to the drug/metabolite transporter (DMT) superfamily (Table 4). Members of other transporter families, particularly PTS and ABC-type transporters, were absent. The substrate for the transporters was inferred from the protein homology to experimentally characterized transport enzymes, and by analysis whether the genes are part of a sugar utilization operon (Table 4 and Fig. S1 of the online supplementary material). The strain-specific absence of genes coding for L-arabinose and D-xylose transporters corresponded to the inability of the strains to ferment the corresponding sugars (Fig. 1, Table 3 and 4, Table S1 of the online supplementary material).

Analysis of the expression of genes coding for sugar transporters in sourdough and during growth on defined carbohydrate sources. Quantification of the expression of 17

predicted transporter genes aimed to determine whether particular transporters are over-expressed in response to the respective substrates. Expression was initially quantified in *L. reuteri* growing in sourdough, a carbohydrate-rich substrate which represents the origin of the strains or resembles the rodent forestomach with respect to the carbohydrate availability (Schwab et al., 2014; Tannock et al., 2012). Two sourdough isolates and one rodent isolate were selected for analysis of gene expression; gene expression of *L. reuteri* LTH5448 was additionally analysed after addition of baker's yeast. All 17 genes were expressed in at least one of the strains (Fig. 2); the addition of baker's yeast, which rapidly depletes glucose during growth in sourdough and thus alleviates carbon catabolite repression, did not alter expression of genes coding for carbohydrate transporters. Relative to growth with glucose as sole carbon source, genes coding for transporters ScrT, LacS, MalT, MalT1 and MalT2, which code for transporters specific for sucrose, raffinose and lactose, and maltose or malto-oligosaccharides (Table 4), were significantly overexpressed during growth of one or several strains in sourdough (Fig. 2). Conversely, the expression of genes coding for enzymes transporting glucose, gluconate and ribose was significantly reduced in sourdough (Fig. 2).

Gene expression of 7 genes was analysed during growth of two strains in chemically defined medium with different carbohydrate sources (Fig. 3). Transport genes and substrates were selected to include genes with uncertain substrate assignment, or genes that were highly expressed during growth in sourdough. Overexpression of *malT* was only observed in presence of maltose but not in response to other sugars. Sucrose and raffinose induced expression of *sucT*. Induction by raffinose corresponds to intracellular sucrose

release when raffinose is metabolized intracellularly by α-galactosidases (Teixeira et al., 2012), however, in *L. reuteri* LTH5448, *sucT* expression was also induced by melibiose (Fig. 3). The gene coding for the lactose transporter LacS was induced by lactose in both strains but also by raffinose and melibiose in *L. reuteri* 100-23, suggesting a broad substrate specificity of this transporter (Fig. 3). Expression of *sotB1, sotB2, xylT* and *xynT* was below the detection limit, or did not respond to the predicted substrates. Overall, the quantification of gene expression conforms to the assignment of *malT*, *scrT* and *lacS* as transporters for D-maltose, D-sucrose and D-lactose, respectively. D-raffinose and melibiose are likely additional substrates of LacS.

Protein sequence analysis for two novel glycoside hydrolases involved in oligosaccharide utilization. The CAZyme annotation identified two novel glycoside hydrolases which are not characterized biochemically in lactic acid bacteria, a licheninase (EC 3.2.1.73) and an endo-(1→4)-β-galactosidase (EC 3.2.1.89) (Table 2). The functions were predicted by identifying conserved functional domains as well as signature patterns in the deduced protein sequences (data not shown) and by alignment of the proteins with homologous proteins (Fig. S2 of the online supplementary material). The putative licheninase in *L. reuteri* TMW1.112 is a GH53 family protein with 419 amino acids. A phylogenetical tree based on multiple protein alignments (Fig. S2 of the online supplementary material) revealed that the proteins are homologous to licheninases and endo-β-galactosidases in bacilli and clostridia, respectively, and more distantly related to enzymes in Gram-negative bacteria and fungi (Fig. S2 of the online supplementary material). The major signature patterns as well as key active sites were conserved in the

311 catalytic domains of the predicted enzyme (data not shown). *In silico* analysis of the
312 cellular location indicated that the licheninase includes a transmembrane helix and may be
313 membrane bound. The protein sequence of the endo-β-galactosidase includes a signal
314 peptide and is thus likely an extracellular enzyme.

315 **Global pathway for carbohydrate utilization in *L. reuteri*.** Genomic data, information on
316 the effect of substrate on gene expression, and phenotypic and genomic data provided in
317 this and previous studies (Table 3 and 4, Fig. 3, Table S1 and S2 of the online
318 supplementary material, Cardelle-Cobas et al., 2011; Gänzle, 2015; Hu et al., 2013;
319 Teixeira et al., 2012; Zheng et al., 2015a) was used to depict carbohydrate transport and
320 metabolism (Fig. 4). Pending characterization of the putative membrane bound or
321 extracellular licheninase and endo-β-galactosidase, fructansucrases and reuteransucrases
322 are the only extracellular glycosyl hydrolases (Fig. 4). Fructansucrases use sucrose or
323 raffinose-family oligosaccharides as substrate (Teixeira et al., 2012) while glucansucrases
324 including reuteransucrase use only sucrose. Oligosaccharides, the preferred substrate for
325 growth of *L. reuteri* (Gänzle et al., 2007), are transported by ScrT, LacS, MalT, MalT1 and
326 MalT2. Analysis of gene expression provided here and elsewhere suggests that melibiose
327 and raffinose-family oligosaccharides are transported by LacS and / or ScrT (Fig. 3 and 4).
328 The putative transporters SotB1 and SotB2 have only low homology to biochemically
329 characterized enzymes, are not highly expressed, and their expression is not altered in
330 response to carbohydrates present in the substrate (Table 4, Fig. 2 and 3).

331 Genomes of *L. reuteri* code for sucrose- and maltose phosphorylase, intracellular
332 α-glucosidase and intracellular α- and β-galactosidases (Fig. 4). Other intracellular glycosyl

hydrolases are absent, in keeping with the fermentation pattern of the strains. The phosphoketolase pathway is the only pathway for conversion of hexoses and pentoses; galactose and pentoses are shunted in to the phosphoketolase pathway with substrate-specific enzymes (Fig. 4).

Discussion.

This study matched carbohydrate fermentation in *L. reuteri* with a genome-wide analysis of carbohydrate active enzymes and carbohydrate transporters and the quantification of genes coding for transport enzymes during growth in sourdough. This analysis revealed that carbohydrate transport in the heterofermentative *L. reuteri* differs substantially from the homofermentative model organisms *L. plantarum* and *L. casei*, and thus contributes to the molecular understanding of co-existence and resource partitioning of homofermentative and heterofermentative lactobacilli in food fermentations.

Genotypes of *L. reuteri* match phenotype of carbohydrate utilization. The sugar fermentation profile of *L. reuteri* strains matches the genotype. The lack of extracellular polysaccharide degrading enzymes reflects the adaptation *L. reuteri* to nutrient rich segments of the upper intestine of animals (Duar et al., 2017a; Frese et al., 2011; Walter, 2008), which contains high concentrations of fermentable carbohydrates including maltose, sucrose, and raffinose family oligosaccharides (Schwab et al., 2014; Tannock et al., 2012). These oligosaccharides are also the main carbohydrate sources in wheat and rye sourdoughs (Gänzle, 2014). The success of host-adapted *L. reuteri* in cereal fermentations (Su et al., 2012; Zheng et al., 2015b) is thus also explained by substrate profiles that match intestinal ecosystems, and the highly efficient metabolism of maltose, sucrose and raffinose

(Gänzle et al., 2007). Strains of *L. reuteri* match to host adapted lineages (Duar et al., 2017a); maintenance of lactose metabolism in *L. reuteri* likely reflects the availability of lactose in the intestine of neonate mammals, contrasting the adaptation of *L. delbrueckii* subsp. *bulgaricus* to lactose-rich dairy environments (van de Guchte et al., 2006). Strain specific differences were observed for pentose utilization; phenotypic observations were confirmed by matching deletions of the corresponding pentose utilizing genes. The carbohydrate fermentation patterns did not differentiate strains of *L. reuteri* based on their origin or lineage. Likewise, sourdough and intestinal isolates were not differentiated based on their carbohydrate metabolism (Zheng et al., 2015b) and carbohydrate catabolic enzymes were not identified among genes that are specific to host adapted lineages of *L. reuteri* (Frese et al., 2011). Propanediol and glycerol metabolism, however, is specific to human and chicken lineage strains of *L. reuteri*. In humans, *L. reuteri* are found in the carbohydrate-restricted lower intestine (Flint et al., 2008; Walter, 2008). In the human colon, propanediol is available as bacterial metabolite of fucose or rhamnose and supports trophic relationships between propanediol producing and propanediol utilizing bacteria (Schwab et al., 2017).

Extracellular or cell wall-associated polysaccharide hydrolases in lactobacilli are limited to the exceptional and strain-specific occurrence of amylases or β-fructosidases (Gänzle and Follador, 2012; Goh et al., 2007); extracellular pentosanases or β-glucosidases have not been characterized biochemically in lactobacilli. Extracellular fructansucrases contribute to metabolism of raffinose-family oligosaccharides but not to polysaccharide degradation (Teixeira et al., 2012). Two strains of *L. reuteri* harbored genes coding for an extracellular

licheninase and an endo-(1→4)-β-galactosidase. The biochemical characterization of these enzymes and their implications for ecological fitness, however, remain subject to future investigations.

Identification of enzymes for carbohydrate transport. Sugar transport in bacteria is catalyzed by ABC-binding cassette (ABC) transporters, secondary carriers and phosphotransferase systems (PTS) (Saier, 2000). All sugar transporters identified in *L. reuteri* were secondary carriers and belonged predominantly to the major facilitator superfamily (MFS) (Pao et al., 1998). Members of the MFS use the proton motive force as energy source for transport (Konings, 2002; Pao et al., 1998). A striking feature of the carbohydrate metabolism in *L. reuteri* is the complete absence of ABC transporters or PTS systems (Table 4), which are the mainstay of carbohydrate transport in homofermentative lactobacilli (Andersson et al., 2005; Monedero et al., 2008). The preferential use of secondary carriers over PTS systems is shared by other heterofermentative lactobacilli (Zheng et al., 2015a) and the heterofermentative *Oenococcus* and *Leuconostoc* (Kim et al., 2011; Zaunmüller and Unden, 2009), however, the complete absence of ABC-transporters or PTS systems is unprecedented. Heterofermentative hexose metabolism via the phosphoketolase pathway yields only one mole of ATP per mole of glucose (Gänzle, 2015). Monosaccharide transport via ABC transporters or PTS systems thus consumes all of the metabolic energy yield that is generated through subsequent catabolism while oligosaccharide transport through secondary carriers is more efficient, particularly when coupled to disaccharide phosphorylases and the use of external electron acceptors (Gänzle, 2015; Fig. 4). Studies determining the specificity of oligosaccharide carriers remain scarce

399 but current evidence suggests that oligosaccharide transport remains limited to di-, tri- and
400 tetrasaccharides (Gänzle and Follador, 2012; Hachem et al., 2013).
401 We identified the substrate specificity of the secondary transporters by a multi-pronged
402 approach including bioinformatic analyses, comparison to biochemically characterized
403 homologues, the genetic organization, and the effect of putative substrates on gene
404 expression. A comparable approach previously identified genes coding for carbohydrate
405 transport in *Bifidobacterium bifidum* (Turroni et al., 2012). Carriers for maltose, maltose-
406 and isomaltose-oligosaccharides, sucrose, and lactose/raffinose were highly expressed
407 during growth of *L. reuteri* in sourdough and in the rodent forestomach (Fig. 2; Schwab et
408 al., 2014). LacS-mediated lactose transport in *Streptococcus thermophilus* is inhibited by
409 melibiose, suggesting that α-GOS and raffinose-family oligosaccharides are an additional
410 substrate for LacS (Gänzle and Follador, 2012; Poolman et al., 1992); this conforms to the
411 pattern of gene expression observed in this study (Fig. 3). The sucrose transporter ScrT in *L.*
412 *reuteri* is overexpressed by sucrose and raffinose, and is part of a sucrose utilization operon
413 (Fig. 3 and Fig. S1; Teixeira et al., 2013). The maltose transporter MalT in *L. reuteri* is
414 induced by maltose. The maltose-utilization operon also encodes for maltose phosphorylase,
415 phosphoglucomutase, and the regulator *malR* in addition to Mal T.
416 **Homofermentative and heterofermentative lifestyles – complement or competition?**
417 Homofermentative and heterofermentative lactobacilli differ fundamentally with respect to
418 metabolic pathways for carbohydrate uptake and metabolism, and with respect to the
419 regulation of sugar metabolism (this study; Gänzle, 2015; Gänzle et al., 2007).
420 Homofermentative and heterofermentative lactobacilli, however, co-exist in many intestinal

421 and man-made habitats including the upper intestine of rodents, birds, and swine, insect
422 intestinal microbiota, and cereal and dairy fermentations (Duar et al., 2017b; Gänzle et al.,
423 2007; Zheng et al., 2015a). For example, microbiota of back-slopped sourdoughs contain
424 homofermentative and heterofermentative lactobacilli (De Vuyst et al., 2014). In rice
425 vinegar microbiota, heterofermentative *Lactobacillus fermentum* is associated with the
426 homofermentative *Lactobacillus plantarum* and *Lactobacillus casei* during the alcoholic
427 fermentation stage (Wu et al., 2012). Co-existence of homofermentative and
428 heterofermentative lactic acid bacteria was described as "resource partitioning" where one
429 group of organisms preferentially utilizes glucose while the other preferentially utilizes
430 oligosaccharides including maltose, sucrose, and raffinose (Gänzle et al., 2007; Schwab et
431 al., 2014; Tannock et al., 2012). Our study on carbohydrate transport and metabolism in *L.*
432 *reuteri* provides a molecular explanation for the differential preference for glucose and
433 oligosaccharides in homofermentative and heterofermentative lactobacilli, and hence the
434 co-existence of these organisms in many of their natural habitats and in food fermentations.
435 This study also adds to the body of evidence that a differentiation of the diverse genus
436 *Lactobacillus* is required to appreciate its contribution to intestinal and food-associated
437 ecosystems (Duar et al., 2017b; Zheng et al., 2015a).
438 In conclusion, this study provides a detailed physiological and genetic analysis of
439 carbohydrate metabolism in the heterofermentative model species *L. reuteri.* The
440 specificity of several predicted secondary carriers for carbohydrates was validated by
441 quantification of gene expression in sourdough and in chemically defined media. Our
442 analysis revealed significant differences between *L. reuteri* and homofermentative

lactobacilli, representing general differences between homofermentative and heterofermentative lactobacilli. The study contributes to our understanding of the co-existence of different lactobacilli in food fermentations on the molecular level and may be used to improve the assessment of the impact of lactobacilli on food quality.

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Figure legends

Figure 1. Comparison of the pentose utilization gene cluster and surrounding genomic regions in pentose fermenting and pentose negative strains. **Panel A.** Cluster for xylose utilization *L. reuteri* LTH2584, 100-23, and LTH5448. **Panel B.** Cluster for arabinose utilization in *L. reuteri* 100-23, LTH5448, and LTH2584. Gray areas connect genomic regions with high (>98%) nucleotide identity. Gene names are provided in Figure S1.

Figure 2. Expression of predicted transporter genes by *L. reuteri* during growth in sourdough. White bars, *L. reuteri* LTH5448, white, hatched bars, *L. reuteri* LTH5448 with baker's yeast, gray bars, *L. reuteri* 100-23, gray, hatched bars, *L. reuteri* TMW1.656. Sourdoughs were fermented at 37°C until the dough pH reached pH 4.5, corresponding to the exponential phase of growth. Gene expression was quantified relative to the expression in chemically defined medium with glucose as sole carbon source; the horizontal line represents unity (gene expression equivalent to gene expression at the reference conditions). Results are shown as means ± standard error of triplicate biological repeats, each sample was analyzed in technical duplicates. Genes that were differentially expressed ($P<0.05$) relative to expression by the same strain at reference conditions are marked with an asterisk. Genes coding for XylT and XynT are present only in *L. reuteri* 100-23.

Figure 3. Gene expression of predicted sugar transporters in *L. reuteri* 100-23 (Panel A) and LTH5448 (Panel B). Gene expression was quantified in cultures growing in chemically defined media with different carbon sources relative to expression in medium with glucose. Data represent mean ± standard deviations of 5 replicates. Genes that were differentially expressed ($P<0.05$) relative to expression by the same strain at reference conditions are

663 marked with an asterisk. The mRNA levels of *sotB1* or *sotB2* in samples of *L. reuteri*
664 100-23 were below the detection limit.

665 **Figure 4.** Overview of carbohydrate transport and metabolism in *L. reuteri*. Putative transport
666 proteins which are located in an operon specific for the predicted substrate are printed in bold.
667 Putative transport proteins which were overexpressed during growth on the predicted substrate
668 are highlighted in grey. Note that several transport proteins have no known function or are
669 redundant while at least two substrates, melibiose and fructose, could not be assigned to
670 transport proteins. Enzymes and transporters are annotated as follows:

671 **Sugar transporters**: **ScrT**: D-sucrase transporter; **LacS**: D-lactose/ D-raffinose transporter;
672 **SotB1**/**SotB2**: putative D-melibiose transporter; **GalP**: D-galactose transporter; **MalT**:
673 D-maltose transporter; **MalT1**/**MalT2**: putative (iso)-malto-oligosaccharides transporter;
674 **GlucU**/**GlucU1**/**GlucU2**: D-glucose transporters; **GntP**: D-gluconate transporter; **RbsD2**:
675 D-ribose transporter; **XylT/XynT**: D-xylose transporter; **AraE1**/**AraE**/**AraE2**: (putative)
676 L-arabinose transporters.

677 **Sucrose/fructose metabolism: ScrP**: sucrose phosphorylase; **Tdh**: mannitol dehydrogenase;
678 **FtfA**: levansucrase, **GtfA**: reuteransucrase.

679 **Galactose/lactose/GOS/raffinose metabolism**: **GalA**: α-galactosidase; **LacZ**:
680 β-galactosidase; **GalM**: aldose 1-epimerase; **GalK**: galactokinase; **GalT**:
681 galactose-1-phosphate uridylyltransferase; **GalE**: UDP-glucose 4-epimerase; **GalU**:
682 UDP-glucose pyrophosphorylase

683 **Glucose/gluconate/maltose/IMO metabolism and phosphoketolase pathway: DexB**:
684 α(1→6)-glucosidase; **MalP**: maltose phosphorylase; **PgmB**: β-phosphoglucomutase; **GluK**:

glucokinase; **G6PD**: glucose-6-phosphate 1-dehydrogenase; **GlcN6P**: 6-phosphogluconolactonase; **Pgd**: 6-phosphogluconate dehydrogenase; **Rpe**: ribulose-phosphate 3-epimerase; **GlnK**: gluconokinase.

Pentose metabolism: **Rbsk**: ribokinase; **RpiA**: ribose 5-phosphate isomerase A; **XylA**: xylose isomerase; **XylB**: xylulose kinase; **AraA**: L-arabinose isomerase; **AraB**: ribulokinase; **AraD**: L-ribulose-5-phosphate 4-epimerase.

Table 1. Sequence of primers used to quantify mRNA levels of putative sugar transporters

Gene	Primers (5'-3')		Annealing temp. (ºC)	Amplicon size (bp)	PCR efficiency
*rbs*D2	rbsD2_F/R	AACAATTCAAGGACGGGTATCA / GGTGCTCAGTCCAGAAGTAAAT	62	107	2.04
*ara*E1	araE1_F/R	CACTTGGCTGGCTCCTATTT / CCGCTTGTCCATTGGTGTAA	62	107	2.08
*ara*E	araE_F/R	GCTTCTCTCGTTGGTTGGATTA / TTCCGCCGACCAAACTTATC	62	98	2.00
*ara*E2	araE2_F/R	CCAGTGGAGTTGGTACTTGTAT / GACCGCCAAGATGAGTTAAGA	62	90	1.97
*xyl*T	xylT_F/R	GTCTCATTAACATTCCCTCCTCTAC / TGGAGTGGACGAACCAAATAG	60	103	2.01
*xyn*C	xynC_F/R	GTCTTTCTTTGGCCGCTTATTC / AAATGCTGGGAAAGACCAAATC	60	117	2.24
*glc*U	glcU_F/R	CCGACAAACGACGTCATAACTA / TTGGACAGGTTGGTCAGTTC	62	100	2.05
*glc*U1	glcU1_F/R	GATCAGAGCAGCGGAAAGAA / CATCTTTGGGAATGCTGAGTAAAC	62	96	2.00
*glc*U2	glcU2_F/R	GCACGCTGTCTTTCTTGTTTAT / TCACTGGTTGGACGGATTTAG	62	143	2.04
*gal*P	galP_F/R	CTTCTCTACTCGTCACGCAATC / ATCCAGTACCACGAAGCTTAAC	62	98	2.07
malT	mal_F/R	CCTTGGCTGGTTCTTCATCT / GCCCATGTACGGTCTGAATAA	60	84	1.93
*mal*T1	malT1_F/R	GCAGTGAGAAAGCCATGTTTATT / CGAAGCAGGTTGATCTGGATAC	62	102	1.99
*mal*T2	malT2_F/R	CTGAGAACTCTGCAGTGAGAAA / GGTTGATCTGGATACAGGGATG	62	106	1.91
sucT	suc_F/R	TTGCCTTCCTCTTGGTTGTAG / CAGTATAGCTGCTGCCCTTAAT	60	87	1.98
*lac*S	lacY_F/R	GGGTTGATTACTGGGTTGATTG / CCACCGGGTCTTCGTATTATC	60	96	2.10
*sot*B1	sotB1_F/R	GCTGATCGGGAATATCCAGAAG / TACTGATCGATGCCGTCAAAG	60	103	1.95
*sot*B2	sotB2_F/R	CCGTTATCAGCACTACCCTTAC / TGGGACGAGCCAAATCAAG	60	95	2.11
*gnt*P	gntP_F/R	CGCTAACCTTGGACACGTATTA / ACGGTAAACACGCGGATAAA	62	115	1.99
phok	phk_F/R	GTCCAGACCTCGTTAAGGAATAC / CGTGGGTGCTTAGAAGTTACA	60	118	2.04

Table 2. Strain characteristics, genome features, and carbohydrate active enzymes (CAZy) of strains of *L. reuteri*.

	LTH2584	LTH5448	TMW1.656	TMW1.112	100-23	mlc3	lpuph
Source	sourdough	sourdough	sourdough	sourdough	Rodent intestine	Rodent intestine	Rodent intestine
Lineage[a]	III	I	III	III	III	III	I
Genome size	2.07Mb	1.90Mb	1.94Mb	2.03Mb	2.31Mb	2.02Mb	2.12Mb
Protein number	1803	1699	1632	1745	2049	1805	1918
GH[b]	13	12	13	13	16	15	14
GT[b]	6	12	9	9	13	12	12
Esterase	1	1	1	2	1	0	1
Carbohydrate-binding module	2	2	2	3	3	3	2
CAZyme count[b] **(% total protein)**	22 (1.2%)	27 (1.6%)	25 (1.5%)	27 (1.5%)	33 (1.6%)	30 (1.6%)	29 (1.5%)
GH families (number proteins in one family)	GH2(2), GH8, GH13(2), GH31, GH36, GH42, GH65	GH2, GH8, GH13(2), GH31, GH36, GH42, GH65, GH68	GH2, GH8, GH13(2), GH31, GH36, GH42, GH65, GH68, GH70	GH2, GH8, GH13(2), GH31, GH36, GH42, GH53, GH65, GH68, GH70	GH2(2), GH8, GH13(2), GH31, GH36, GH42, GH65, GH68, GH70	GH2(2), GH8, GH13(2), GH31, GH36, GH42, GH65, GH68, GH70	GH2(2), GH13(2), GH31, GH36, GH42, GH53, GH65, GH68, GH70
Genome accession# in NCBI	NZ_JOSX01000020.1	NZ_JOOG01000004.1	NZ_JOSW02000004.1	NZ_JOKX02000004.1	NZ_AAPZ02000001.1	NZ_AEAW01000043.1	NZ_AEAX01000045.1

[a.] Lineages were assigned based on core-genome alignment (Zheng et al., 2015b).

[b] GH, glycosyl hydrolase; GT, glycosyl transferase. None of the genomes contained genes annotated as members of the polysaccharide lyase (PL) family or the auxiliary activity (AA) family.

Table 3. Carbohydrate metabolism and growth on defined substrates by strains of *L. reuteri*

Sugars	LTH2584		TMW1.112		TMW1.656		LTH5448		100-23		lpuph		mlc3	
	API	**Growth**	**API**	**Growth**	**API**	**Growth**	**API**	**Growth**	**API**	**Growth**	**API**	**Growth**	**API**	**Growth**
L-arabinose	-	-	+	-	-	-	+	+	+	+	+		+	
D-ribose	+	n/d	+	n/d	+	n/d	+	n/d	+	n/d	+		+	
D-xylose	+	-	-	-	-	-	-	-	+	-	-		+	
D-galactose	+	+	+	+	+	+	+	+	+	+	+		+	
D-glucose	+	+	+	+	+	+	+	+	+	+	+		+	
Methyl-αD-Glucopyranoside	+	n/d	-	n/d	-	n/d	-	n/d	-	n/d	-		-	
D-maltose	+	+	+	+	+	+	+	+	+	+	+		+	
D-lactose	+	+	+	+	+	+	+	+	+	+	+	n/d	+	n/a
D-melibiose	+	+	+	+	+	+	+	+	+	+	+		+	
D-sucrose	+	-	+	+	+	+	+	+	+	+	+		+	
D-raffinose	+	+	+	+	+	+	+	+	+	+	+		+	
Potassium gluconate	+	-	+	-	+	-	+	+	+	-	-		+	
Isomalto-oligosaccharides (IMO)	n/d	+	n/d	+	n/d	+	n/d	+	n/d	+	n/d		n/d	
D-lactulose	n/d	-	n/d	-	n/d	+	n/d	+	n/d	+	n/d		n/d	
Palatinose hydrate	n/d	+	n/d	+	n/d	+	n/d	+	n/d	+	n/d		n/d	

Carbohydrates with negative result for all strains in the API 50ch assay: glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-Mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, ferric citrate, salicin, D-cellobiose, D-trehalose, inulin, D-melezitose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate, potassium 5-ketogluconate.

Carbohydrates with negative result for all strains in the growth test: D-xylose, L-rhamnose, D-cellobiose, D-trehalose, D-maltotriose, D-fructose, D-mannose, D-sorbitol, D-mannitol, inositol, gentiobiose, D-melezitose monohydrate, glucuronic, amygdalin, dulcitol, inulin, xylan, pectin, cellulose, starch, dextran, glucotriose, xylobiose, mannotriose, galactan and fructo-oligosaccharides (FOS).

+: growth; -: no growth; n/d: not determined.

Table 4. *In silico* identification of putative sugar transporters in strains of *L. reuteri*

Substrates	Protein symbols[b]	# of TMSs in queries	Query length (aa)	Accession # (UniProt)	Identity (%)	Refs	Transp. families[c]	Locus tag[d]						
								LTH2584	**TMW1.112**	**TMW1.1656**	**LTH5448**	**100-23**	**mlc3**	**lpuph**
D-ribose	**RbsD2**	11	452	Q8XEV7	51.5	1, 2	FHS	**LR3_02825**	HF82_05735	HQ33_08370	HN00_00840	Lreu23DRAFT_3462	cds1089	2506440742
L-arabinose	AraE1	12	387	P31122	29.8	3	DHA1	**LR3_05370**	HF82_07440	HQ33_00710	HN00_07160	Lreu23DRAFT_4690	cds545	2506439633
	AraE	12	472	C4B4V9	72.7	4	SP	-	HF82_06075	-	**HN00_01255**	Lreu23DRAFT_3536	cds38	2506439466
	AraE2	12	435	P96710	30.6	5, 6	SP	**LR3_04700**	HF82_04025	HQ33_00215	HN00_04590	Lreu23DRAFT_4584	cds334	2506439802
D-xylose	**XylT**	12	466	O52733	67.2	7	SP	**LR3_02915**	-	-	-	Lreu23DRAFT_3480	cds874	-
	XynT	11	500	P96792	35.3	-	GPH	**LR3_02925**	-	-	-	Lreu23DRAFT_3482	cds877	-
D-glucose	**GlcU**	10	288	P40420	38.0	8	GRP	**LR3_07890**	HF82_06485	HQ33_06895	HN00_06430	Lreu23DRAFT_4885	-	2506440027
	GlcU1	10	287	A0A0E0ZTM0	38.6	9	GRP	**LR3_02885**	HF82_05795	HQ33_08220	HN00_00900	Lreu23DRAFT_3474	cds1077	2506440754
	GlcU2	12	392	Q04DP6	26.8	10)	GT	**LR3_07180**	HF82_08080	HQ33_03965	HN00_02370	Lreu23DRAFT_4316	-	(2506440383… 2506440382)
D-galactose	**GalP**	13	651	Q9X761	41.8	11	GPH	**LR3_04320**	HF82_07560	HQ33_00580	n.a.[e]	Lreu23DRAFT_4663	cds570	2506440314
D-maltose	**MalT**	12	450	Q9A612	44.9	12	GPH	**LR3_08075**	HF82_06335	-	HN00_04890	Lreu23DRAFT_4916	cds783	2506440002
MOS[a]	**MalT1**	12	462	Q9A612	27.2	12	GPH	**LR3_09920**	HF82_08930	HQ33_08125	HN00_03255	Lreu23DRAFT_4937	cds1065	2506440913
	MalT2	12	456	Q8EEC4	27.6	13	GPH	**LR3_09935**	HF82_08945	HQ33_08120	HN00_03240	Lreu23DRAFT_4938	cds1067	2506440910
D-sucrose	**ScrT**	12	406	Q04DP6	26.8	10	GT	**LR3_01255**	HF82_04270	HQ33_08175	HN00_01910	Lreu23DRAFT_4471	cds1267	2506440958
D-lactose/ D-raffinose	**LacS**	12	641	P23936	38.2	14	GPH	**LR3_00360**	HF82_02760	HQ33_03895	HN00_02605	Lreu23DRAFT_5165	cds1091	2506439790
D-melibiose	SotB1	12	387	Q9S3J9	29.6	-	DHA1	**LR3_04870**	HF82_10260	HQ33_00040	HN00_03045	Lreu23DRAFT_4547	cds368	2506440515
	SotB2	12	390	Q9S3J9	26.2	-	DHA1	**LR3_04495**	HF82_04330	HQ33_00405	HN00_07465	Lreu23DRAFT_4624	cds663	2506440701
D-gluconate	**GntP**	9	379	P12012	55.1	15, 16	GntP	**LR3_09825**	HF82_00365	HQ33_07405	HN00_05280	Lreu23DRAFT_3217	cds1449	2506440538

[a] MOS: maltooligosaccharides

[b] Gene symbols are printed in in bold if they are located adjacent to functionally related genes; other genes are not located in proximity to functionally related gene (clusters)

[c] FHS: Fucose: H^+ Symporter Family; DHA1: drug: H^+ antiporter-1 Family; SP: Sugar Porter Family; GPH: Glycoside-Pentoside-Hexuronide: Cation Symporter Family; GT: Glucose Transporter Family; GntP: Gluconate:H+ Symporter (GntP) Family

[d] The genes that were used as queries for Blastp anlaysis are printed in bold; "-"indicates absence of genes; genes in different strains with identical symbols share homology of 98% or greater.

[e] GalP is present in LTH5448 but not annotated in genome file uploaded to Genbank.

References: 1, Christensen et al., 2003; 2, Martinez-Jéhanne et al., 2009; 3, Koita and Rao, 2012; 4, Wang et al., 2013; 5, Ferreira and de Sá-Nogueira, 2010; 6, Sá-Nogueira and Ramos, 1997; 7, Chaillou et al., 1998; 8, Fiegler et al., 1999; 9, Aké et al., 2011; 10, Kim et al., 2011; 11, Grossiord et al., 2003; 12, Lohmiller et al., 2008; 13, Rodionov et al., 2010; 14, Poolman et al., 1992; 15, Fujita et al., 1986; 16, Yoshida et al., 1995

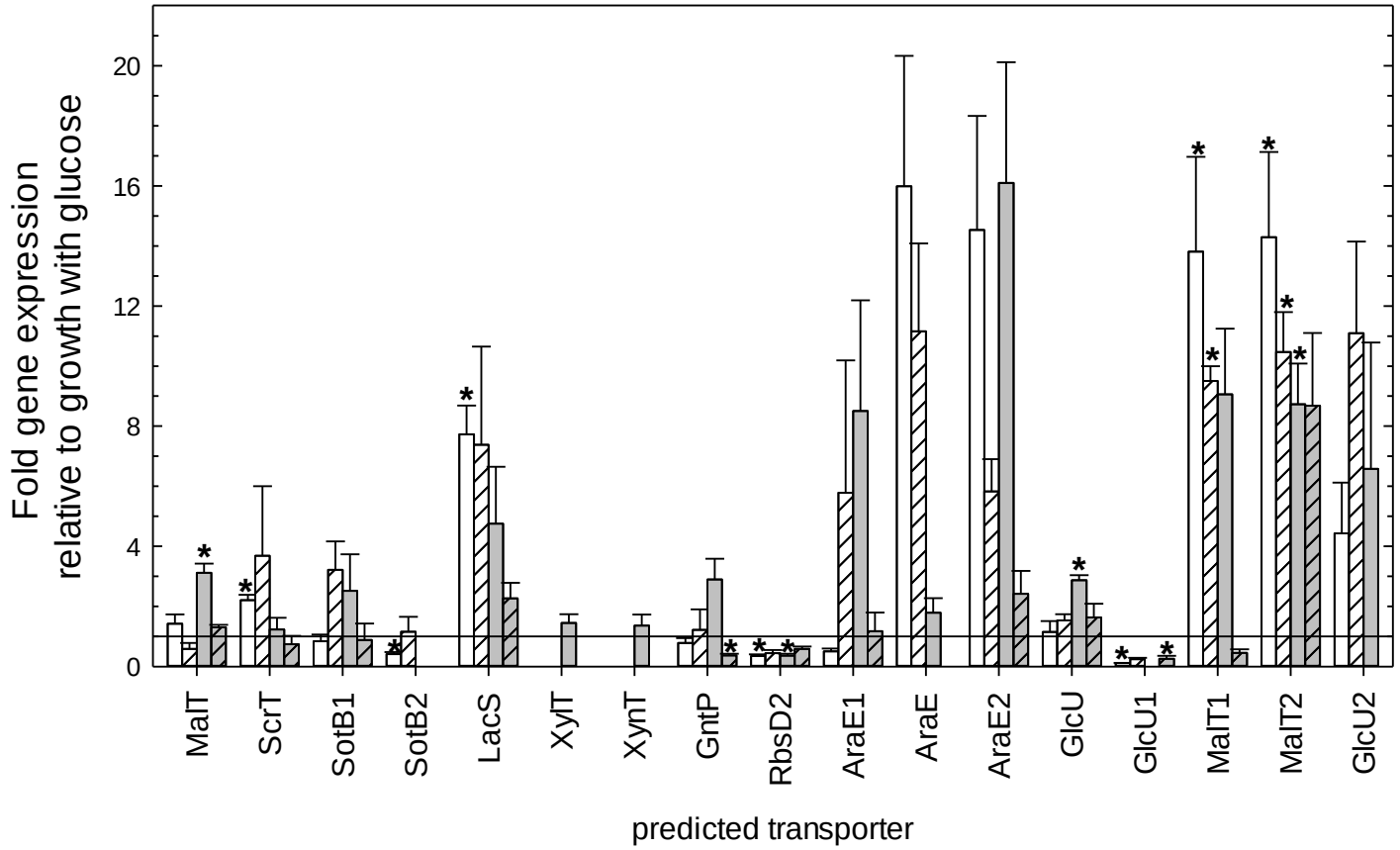


Figure 2. Expression of predicted transporter genes by *L. reuteri* during growth in sourdough. White bars, *L. reuteri* LTH5448, white, hatched bars, *L. reuteri* LTH5448 with baker's yeast, gray bars, *L. reuteri* 100-23, gray, hatched bars, *L. reuteri* TMW1.656. Sourdoughs were fermented at 37°C until the dough pH reached pH 4.5, corresponding to the exponential phase of growth. Gene expression was quantified relative to the expression in chemically defined medium with glucose as sole carbon source; the horizontal line represents unity (gene expression equivalent to gene expression at the reference conditions). Results are shown as means ± standard error of triplicate biological repeats, each sample was analyzed in technical duplicates. Genes that were differentially expressed ($P<0.05$) relative to expression by the same strain at reference conditions are marked with an asterisk. Genes coding for XylT and XynT are present only in L. reuteri 100-23.

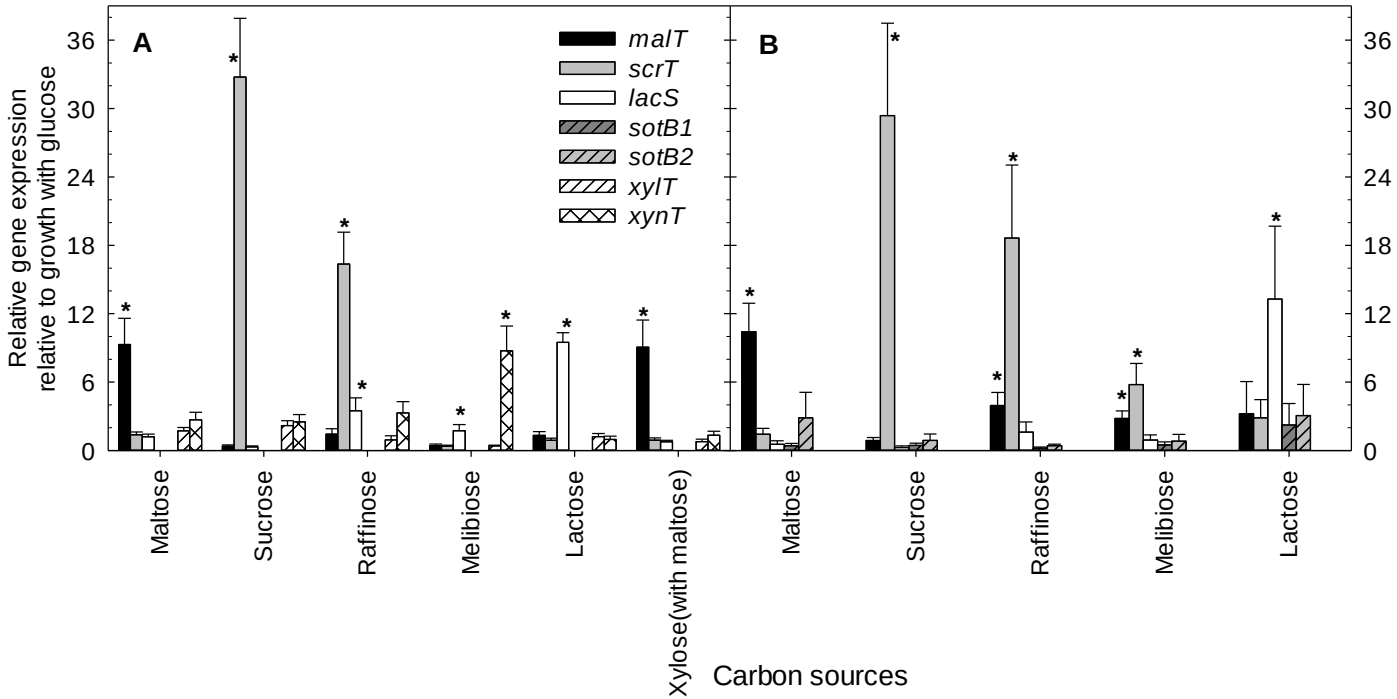
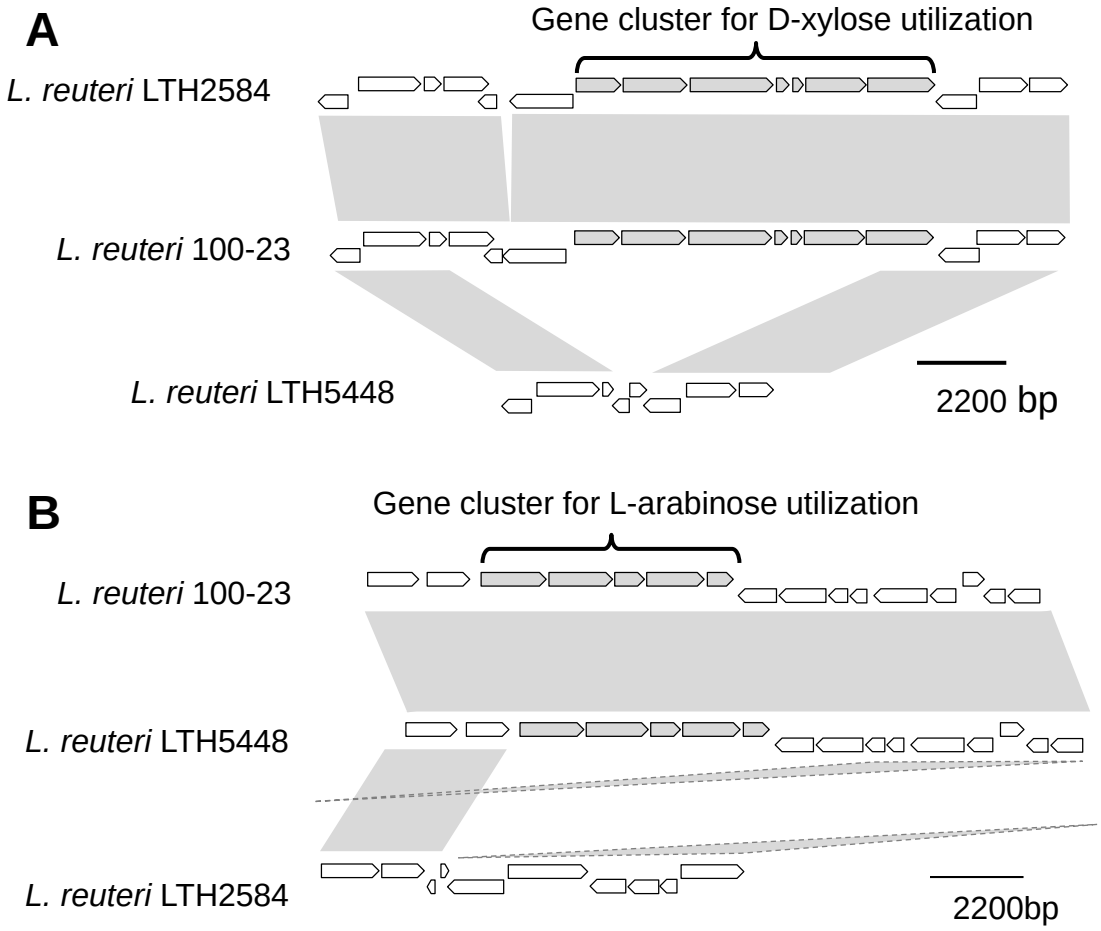
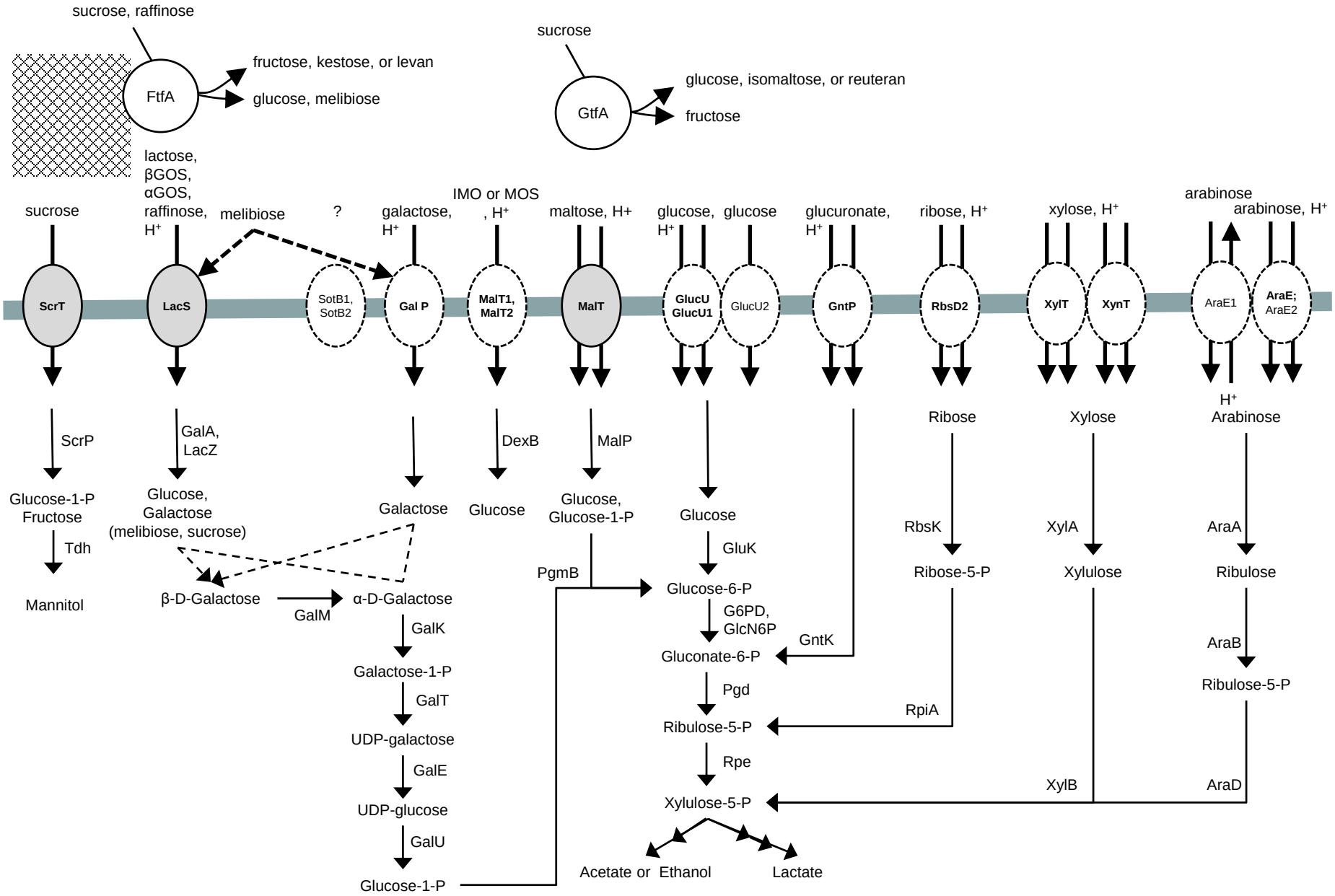


Figure 3. Gene expression of predicted sugar transporters in *L. reuteri* 100-23 (Panel A) and LTH5448 (Panel B). Gene expression was quantified in cultures growing in chemically defined media with different carbon sources relative to expression in medium with glucose. Data represent mean ± standard deviations of 5 replicates. Genes that were differentially expressed ($P<0.05$) relative to expression by the same strain at reference conditions are marked with an asterisk. The mRNA levels of sotB1 or sotB2 in samples of *L. reuteri* 100-23 were below the detection limit**.**



Zhao and Gänzle, **Figure 1.**



Zhao and Gänzle, **Figure 4**