Functional characterization of sucrose phosphorylase and scrR, a regulator of sucrose metabolism in Lactobacillus reuteri

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Abstract

Lactobacillus reuteri harbors alternative enzymes for sucrose metabolism, sucrose phosphorylase, fructansucrases, and glucansucrases. Sucrose phosphorylase and fructansucrases additionally contribute to raffinose metabolism. Glucansucrases and fructansucrases produce exopolysaccharides as alternative to sucrose hydrolysis. L. reuteri LTH5448 expresses a levansucrase (ftfA) and sucrose phosphorylase (scrP), both are inducible by sucrose. This study determined the contribution of scrP to sucrose and raffinose metabolism in L. reuteri LTH5448, and elucidated the role of scrR in regulation sucrose metabolism. Disruption of scrP and scrR was achieved by double crossover mutagenesis. L. reuteri LTH5448, LTH5448ΔscrP and LTH5448ΔscrR were characterized with respect to growth and metabolite formation with glucose, sucrose, or raffinose as sole carbon source. Inactivation of scrR led to constitutive transcription of scrP and ftfA, demonstrating that scrR is negative regulator. L. reuteri LTH5448 and the LTH5448ΔscrP or LTH5448ΔscrR mutant strains did not differ with respect to glucose, sucrose or raffinose utilization. However, L. reuteri LTH5448ΔscrP produced more levan, indicating that the lack of sucrose phosphorylase is compensated by an increased metabolic flux through levansucrase. In conclusion, the presence of alternate pathways for sucrose and raffinose metabolism and their regulation indicate that these substrates, which are abundant in plants, are preferred carbohydrate sources for L. reuteri.
Key words: sucrose, sucrose phosphorylase, lactobacillus, *Lactobacillus reuteri*, sourdough

**List of abbreviations**

LAB, lactic acid bacteria

GI, gastrointestinal

PTS, phosphotransferase system

ScrP, sucrose phosphorylase

ScrR, sucrose regulator

LevS, levansucrase

FtfA, fructosyltransferase A

ScrP, sucrose phosphorylase

MRS, deMan-Rogosa-Sharpe medium

LB, Luria-Bertani

HPLC, high performance liquid chromatography

HPAEC-PAD, high performance anion exchange chromatography with integrated pulsed amperometric detection

qPCR, quantitative real time polymerase chain reaction

FOS, fructo-oligosaccharides
1. Introduction

*Lactobacillus reuteri* is a stable member of sourdough microbiota (Gänzle and Vogel, 2002; De Vuyst and Neysens, 2005) that occurs predominantly in industrial sourdough fermentations prepared for the production of baking improvers (Brandt, 2007) and in cereal fermentation in tropical climates (Vogel et al., 1999). Sucrose is the most abundant carbon source in ungerminated cereal grains. Wheat and rye grains contain more than 0.6% and 1.2% sucrose, respectively, and sucrose is commonly used in bread formulas. Raffinose is additionally present in concentrations of 0.1 – 0.4% (Belitz et al., 2004). The ability of *L. reuteri* to metabolize sucrose and raffinose as sole carbon sources contributes to its ecological fitness in food fermentations, and impacts the quality of fermented cereal products (Figure 1, Tieking et al., 2005; Schwab et al., 2007; Teixeira et al., 2012).

Lactobacilli harbour a multitude of enzymes and transport systems involved in sucrose metabolism (Kaplan and Hutkins, 2003; Barrangou et al., 2006; van Hijum et al., 2006; Goh et al., 2006; Saulnier et al., 2007). Sucrose metabolism is mediated by β-fructofuranosidases (SacA or BfrA) catalyzing hydrolysis of sucrose or sucrose-phosphate (Saulnier et al., 2007; Nakai et al., 2012; for review, see Gänzle and Follador, 2012). The intracellular sucrose phosphorylase (ScrP) or extracellular glucansucrases and fructansucrases complement or substitute β-fructofuranosidases in few *Lactobacillus* spp. (van Hijum et al., 2006; Gänzle and Follador, 2012). Fructansucrases but not glucansucrases also use raffinose, stachyose, and verbascose as substrates (van Hijum et al., 2006; Teixeira et al., 2012).

The expression of sucrose PTS systems is regulated by operon-specific (local) transcriptional regulators, in conjunction with the catabolite control protein A, CcpA, a
transcriptional regulator of the LacI/GalR family (Reid and Abratt, 2005, Andersson et al., 2005 Monedero et al., 2007, Francke et al., 2008). In L. plantarum, L. paracasei and L. acidophilus, sucrose metabolic genes are induced by sucrose or short chain fructo-oligosaccharides. MrmR / SacR were identified as putative local regulators of the sucrose catabolic operons on the basis of sequence similarities to other regulatory proteins (Goh et al., 2006; Saulnier et al., 2007). Taken together, these mechanisms mediate repression of carbon catabolite operons in the presence of more favourable carbon sources, and expression in the presence of the substrates.

Genome sequence data of more than 10 strains of L. reuteri in combination with functional analysis of sucrose metabolic enzymes demonstrate that sucrose utilization in this species is mediated by extracellular glucansucrases or fructansucrases and sucrose phosphorylase. Sucrose PTS systems or intracellular β-fructofuranosidases are absent (Schwab et al., 2007; Frese et al., 2011; Teixeira et al, 2012, Gänzle and Follador, 2012). The expression of sucrose phosphorylase and levansucrase in L. reuteri is induced by sucrose but the expression of glucansucrases is not influenced by the carbon source (Schwab et al., 2006; Årsköld et al., 2007). The regulation of sucrose catabolism in L. reuteri thus differs fundamentally from L. plantarum and L. acidophilus, the two species of lactobacilli for which experimental data is available (for review, see Gänzle and Follador, 2012). It was the aim of this study to characterize the regulation of sucrose metabolism in L. reuteri. The study employed L. reuteri LTH5448, a sourdough isolate harbouring a sucrose phosphorylase (scrP) and a levansucrase (ftfA) (Schwab et al., 2007; Teixeira et al., 2012). The role of the putative sucrose regulator scrR was elucidated by
quantification of the expression of sucrose metabolic genes in a \textit{scrR} mutant and the wild type strain.

\section*{2. Materials and methods}

\subsection*{2.1 Strains, media and growth conditions.}

Bacterial strains and plasmids used in this study are shown in Table 1. \textit{Escherichia coli} JM109 (Promega, Nepean, Canada) was cultured in Luria-Bertani (LB) broth at 37°C. \textit{L. reuteri} strains were anaerobically cultivated at 37 °C in modified deMan-Rogosa-Sharpe medium (mMRS) (Stolz et al., 1995) or MRS containing 20 g L\textsuperscript{-1} sucrose (sucMRS); 20 g L\textsuperscript{-1} raffinose (rafMRS); 20 g L\textsuperscript{-1} fructo-oligosaccharides (fosMRS); and 20 g L\textsuperscript{-1} each of glucose and sucrose (glusucMRS) as carbon sources. Fructo-oligosaccharides were obtained from Orafti (Tienen, Belgium) with a degree of polymerization of 3 – 8. HPLC analysis verified that glucose, fructose and sucrose were essentially absent (data not shown). Other carbohydrates were purchased with 99% purity from Sigma (Oakville, Canada).

The production of oligosaccharides and exopolysaccharides was also monitored in MRS containing 100 g L\textsuperscript{-1} sucrose (suc100MRS). Ampicillin (100 mg L\textsuperscript{-1}) or erythromycin (500 mg L\textsuperscript{-1}) was added to LB for selecting antibiotic-resistant \textit{E. coli}. Erythromycin (10 mg L\textsuperscript{-1}) was added to mMRS medium to select erythromycin-resistant \textit{L. reuteri}.

\subsection*{2.2 General molecular methods.}

Genomic DNA was isolated from overnight cultures grown in mMRS using DNeasy Blood & Tissue kit (Qiagen, Mississauga, Canada). DNA was amplified by polymerase chain reaction (PCR) using Taq DNA polymerase and dNTPs from Invitrogen (Burlington, Canada). Primer design for sequencing \textit{scrR}, \textit{scrP}, and the flanking regions
of the genes was based on the genome sequence of *L. reuteri* 100-23. Primers were purchased from Integrated DNA Technologies (Coralville, USA) and are listed in Table 2. PCR products were visualized after electrophoretic separation on agarose gels. DNA sequencing was performed by Macrogen (MacrogenUSA, Rockville, USA).

2.3 Phylogenetic analysis of *scrR*.
Sucrose regulators and CcpA were aligned using CLUSTALW implemented in BioEdit. Phylogenetic analysis of ScrR sequences was performed using MEGA (Tamura et al., 2011) and the maximum likelihood method applying the Jones-Taylor-Thornton (JTT) substitution model. Bootstrap support was calculated for 100 replicates.

2.4 Generation and verification of *L. reuteri* LTH5448Δ*scrP* and LTH5448Δ*scrR* mutants.
Sequencing of *scrP* and *scrR* genes in *L. reuteri* LTH548 was achieved with primers specific for the corresponding loci in the genome sequenced strain *L. reuteri* 100-23 (Gene bank Accession number AAPZ00000000.2, primers listed in Table 2). In-frame truncation of *scrP* and *scrR* genes was achieved with the temperature-sensitive shuttle vector pJRS233 (Su et al., 2011). In brief, flanking fragments of the target genes were amplified from genomic DNA of *L. reuteri* LTH5448 by PCR (primers are listed in Table 2). The amplicons were ligated into pGEMTeasy vector (Promega) to generate pScrP-A, pScrP-B, pScrR-A, and pScrR-B (Table 1). The flanking fragments of *scrP* and *scrR* from these plasmids were digested with the respective restriction enzymes (New England Biolabs, Pickering, Canada), purified, and ligated into pGEMTeasy to create pScrP-AB and pScrR-AB. The co-ligated DNA fragments in pScrP-AB and pScrR-AB were cut with the respective restriction enzymes, and ligated into pJRS233 using T4 DNA ligase.
(Epicentre, Markham, Canada). The resulting plasmids pKO-\(scrP\)-AB and pKO-\(scrR\)-AB were electrotransformed into competent \(L.\ reuteri\) LTH5448 cells suspended in water with 30% (v/v) polyethylene glycol (MW 3350; J.T. Baker Chemical, Phillipsburg, NJ). Transformants were grown in mMRS broth containing erythromycin at 42°C for 80 generations to select for single crossover mutants. \(L.\ reuteri\) with pKO-\(scrP\)-AB and pKO-\(scrR\)-AB integrated into chromosome were cured by culturing in mMRS broth at 37°C for 100 generations. Erythromycin-sensitive double crossover mutants were identified by replica plating on mMRS and mMRS-erythromycin agar. The in-frame deletion of \(scrP\) and \(scrR\) in \(L.\ reuteri\) LTH5448\(\Delta scrP\) and \(L.\ reuteri\) LTH5448\(\Delta scrR\), respectively, was confirmed by PCR. DNA sequencing was conducted to verify the deletion region using sequencing primers (Table 2).

2.5 Isolation of mRNA and synthesis of cDNA libraries.

RNA was isolated from exponentially growing cells (OD\(_{600}\) of 0.5) of \(L.\ reuteri\) LTH5448 and its mutant LTH5448\(\Delta scrR\) in mMRS, gluMRS, sucMRS, rafMRS or glusucMRS. Two volumes of RNAprotect (Qiagen) were added, and cells were harvested by centrifugation at 5000 \(\times\) g. Cells were resuspended in 1 mL TRIzol (Invitrogen), and disrupted using a bead beater (Biospec products, Bartlesville, USA) and 0.1 mm zirconia-silica beads. The supernatant was transferred to 1.5 mL Eppendorf tubes for RNA isolation according to TRIzol manufacturer’s manual. Reagents and enzymes for DNase I treatment and reverse transcription were purchased from Promega (Madison, USA) and used according to the manufacturer’s instructions. Transcription of \(scrP\), \(scrR\), and \(ftfA\) in wild type \(L.\ reuteri\) LTH5448 grown in mMRS and sucMRS was analysed by PCR using the primers shown in Table 2.
2.6 Quantification of transcriptional levels of the scrP, scrR and ftfA genes in response to glucose, sucrose, and raffinose.

Quantitative analysis of the expression of scrP, scrR, and ftfA in L. reuteri LRH5448 and LTH5448ΔscrR was carried out using cDNA as a template in quantitative PCR (qPCR) reactions. DNase-I treated mRNA preparations were used as negative control; chromosomal DNA was used as positive control. Amplifications were carried out in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with primers listed in Table 2. Melting curve analysis and determination of amplicon size by agarose gel electrophoresis verified the specific amplification of the appropriate cDNA. Expression levels of scrP, scrR and ftfA in cultures grown in mMRS, gluMRS, rafMRS or glusucMRS were calculated by the ΔΔC_T method according to Pfaffl (2001). The phosphoketolase gene (pho) was used as housekeeping gene. Maltose and / or sucrose rather than glucose are preferred carbon sources for L. reuteri (Stolz et al., 1993; Schwab et al., 2007); therefore, cultures grown in sucMRS were used as reference conditions. Gene expression was quantified in duplicate from cDNA libraries obtained from three (scrP and ftfA) or two (scrR) independent experiments.

2.7 Determination of growth kinetics and metabolite formation.

Growth of L. reuteri LTH5448, LTH5448ΔscrP, and LTH5448ΔscrR was assessed in mMRS, sucMRS, rafMRS or fosMRS. Media were inoculated with 1% of washed overnight cultures, incubated at 37°C, and growth was monitored by changes in the optical density (OD) at 600 nm over 24 hours. Samples were taken for metabolite analysis by HPLC as described (Teixeira et al., 2012). Data shown are mean values for
duplicate (\textit{L. reuteri} LTH5448 and LTH5448\textit{ΔscrP}) or triplicate (LTH5448\textit{ΔscrR}) independent experiments.

Oligosaccharide and levan formation by \textit{L. reuteri} LTH5448 and LTH5448\textit{ΔscrP} was assessed in succ100MRS. Media were inoculated with overnight cultures and incubated at 37°C. Samples were taken for analysis by HPAEC-PAD and size exclusion chromatography as described (Galle et al., 2010). The amount of high molecular weight levan (relative molecular weight of more than $10^5$) in succ100MRS was calculated using purified levan from \textit{L. reuteri} LTH5448 purified by ethanol precipitation, dialysis, and phenol-chloroform extraction (Wang et al., 2010) as standards. Levan with intermediate molecular weight ($10^4$ – $10^5$) was not quantified in culture supernatants due to interference of media components. Data shown are mean values for duplicate (FOS) or triplicate (levan) independent experiments.

Polysaccharides from culture and supernatant were hydrolyzed with 2M H$_2$SO$_4$ at 80°C for 2 hour in order to differentiate bound levan from levan released into the culture medium. The monosaccharides obtained from acid hydrolysis were quantified with an Aminex 87H column as described (Schwab et al., 2007). To assess levan hydrolysis by \textit{L. reuteri} LTH5448 and LTH5448\textit{ΔftfA}, cells from overnight cultures were washed with 50 mmol L$^{-1}$ phosphate buffer (pH 6.5) and incubated with 5 g L$^{-1}$ levan for 24h. Samples were taken periodically and levan was quantified by size exclusion chromatography as described above. The incubation in phosphate buffer avoids interference with media components, and thus allows quantification of levan with intermediate molecular weight (molecular weight range $10^4$ -> $10^6$).

2.8 Nucleotide Accession numbers.
The nucleotide sequences of scrP and scrR in L. reuteri LTH5448, and the sequences of truncated scrP and scrR in L. reuteri LTH5448ΔscrP and L. reuteri LTH5448ΔscrR are deposited in GenBank (accession number KC539460, KC539461, KC539462, and KC539463, respectively.

3. Results

3.1 Identification of genes coding for sucrose phosphorylase and a putative sucrose dependent regulator in L. reuteri LTH5448.

A partial sequence of scrP coding for sucrose phosphorylase was previously identified in L. reuteri LTH5448 (Schwab et al., 2007). Sequencing of this gene and its flanking regions confirmed the presence of a sucrose phosphorylase in L. reuteri LTH5448. The gene sequence and the genetic organization (Figure 2A) are homologous to L. reuteri 100-23.

Bioinformatic analysis of the genome of L. reuteri 100-23 identified the putative sucrose regulator scrR. The scrR gene was flanked by divergently oriented muramidase and a hypothetical protein (Figure 2B) and located distant from scrP or ftfA. An scrR gene (length 942 base pairs) was amplified by PCR in L. reuteri LTH5448. In L. reuteri LTH5448, the coding region of scrR and scrP and their flanking sequences were highly homologous to L. reuteri 100-23. The prediction of putative regulator ScrR encodes 314 amino acid residues. The gene is present in all sequenced genomes of L. reuteri strains with 96-98% homology, and is highly homologous to sucrose regulators of Streptococcus mutans (ScrR, U 46902) (48% identity in 307 aa) and L. acidophilus NCFM (MsmR, 68% identity in 141 aa). ScrR of L. reuteri clustered with sucrose regulators of other lactic acid bacteria and bifidobacteria in a cluster distant from the global regulator CcpA.
The putative sucrose regulator ScrR possesses the N-terminal helix-turn helix (HTH) DNA binding domain of the LacI-GalR-type transcriptional regulators, and the core unit with a motive indicative of periplasmic binding proteins and the sugar binding domain of the LacI family. Qualitative analysis of cDNA libraries of *L. reuteri* LTH54448 indicated that *scrR* is expressed in *L. reuteri* LTH5448 (data not shown).

### 3.2 Generation of *scrR* and *scrP* deletion mutants of *L. reuteri* LTH5448

To identify the regulatory role of *scrR* in transcription of *scrP* and *ftfA*, and the impact of *scrP* on sucrose utilization, double crossover mutants of *L. reuteri* LTH5448 were generated. The truncation in *scrP* of *L. reuteri* LTH5448Δ*scrP* was confirmed by PCR with the primers *pho*-KO1b-F-*XhoI* and *pho*-KO4b-R-*PstI* (Table 2). PCR amplified the expected amplicons of 3.2 kb and 1.8 kb with DNA from *L. reuteri* LTH5448 and LTH5448Δ*scrP*, respectively, as template. A deletion in *scrR* of *L. reuteri* LTH5448Δ*scrR* was verified by PCR with the primers *reg*-KO1a-F-*XhoI* and *reg*-KO4b-R-*HindIII* (Table 2), yielding amplicons of 3.3 kb and 2.6 kb for *L. reuteri* LTH5448 and LTH5448Δ*scrR*, respectively. DNA sequencing verified the truncation of *scrP* and *scrR* in *L. reuteri* LTH5448Δ*scrP* and LTH5448Δ*scrR*, respectively.

### 3.3 Expression of *scrP* and *scrR* in *L. reuteri* LTH5448 and LTH5448Δ*scrR*.

The transcription of *scrP*, *scrR*, and *ftfA* in wild type *L. reuteri* and its LTH5448Δ*scrR* mutant in response to glucose, sucrose, and raffinose was quantified relatively to its expression in sucMRS (Table 3 and data not shown). The wild type strain showed comparable expression of *scrR* when grown with or without sucrose (data not shown). *ScrP* was expressed in presence of sucrose and raffinose, but was not repressed by glucose. In contrast to the wild type strain, the LTH5448Δ*scrR* mutant strain showed
expression of \textit{scrP} when grown without sucrose (Table 3). The levanuscrase FtfA was also highly expressed in the presence of sucrose but expression levels were much lower when sucrose was absent. Comparable to the expression of \textit{scrP}, expression of \textit{ftfA} in \textit{L. reuteri} LTH5448 was not repressed by glucose and deletion of \textit{scrR} resulted in constitutive expression of the enzyme (Table 3). These data demonstrate that \textit{scrR} codes for a transcriptional regulator of the LacI/GalR-family that mediates repression of sucrose metabolic genes in the absence of sucrose.

\textbf{3.4 Growth kinetics and carbohydrate metabolism in mMRS}

In order to determine the contribution of \textit{scrP} and \textit{scrR} to sucrose metabolism, growth and carbohydrate metabolism of \textit{L. reuteri} LTH5448 and its mutants were compared in media with different carbon sources. There were no differences in the growth of \textit{L. reuteri} LTH5448, LTH5448\textDelta{scrP} and LTH5448\textDelta{scrR} during growth in mMRS, sucMRS or rafMRS (Figure 4). Growth and metabolism of \textit{L. reuteri} LTH5448 and LTH5448\textDelta{scrR} were also compared in gluMRS. The results confirmed that disruption of \textit{scrR} does not result in a phenotype that is detectable by metabolite analysis (data not shown). Samples obtained at 24 hours of fermentation showed that all strains completely metabolized the sugars in all three media (data not shown), formed comparable amounts of lactate, acetate, and ethanol, and reduced comparable amounts of fructose to mannitol (Table 4). These similarities can be explained by the fact that the expression of sucrose metabolic genes is not different between \textit{L. reuteri} LTH5448 and LTH5448\textDelta{scrR} when the substrate is present. In the absence of the substrate, the expression of sucrose metabolic genes in \textit{L. reuteri} LTH5448\textDelta{scrR} does not influence sucrose metabolism. In \textit{L. reuteri} LTH5448\textDelta{scrP}, FtfA activity compensates for the absence of ScrP.
fosMRS, all strains grew poorly and did not produce considerable amounts of organic acids (data not shown).

### 3.5 Synthesis of levan and fructo-oligosaccharides

*L. reuteri* LTH5448 and LTH5448ΔscrP produced FOS and levan during growth in suc100MRS. Wild type and LTH5448ΔscrP produced four different FOSs (FOS1-FOS4) (Figure 5). Maximum levan levels were 4.6 g L⁻¹ in *L. reuteri* LTH5448 and 6.7 g L⁻¹ in LTH5448ΔscrP after 6 and 8 hours of fermentation, respectively (Figure 5B). Levan concentration increased during the exponential phase of growth followed by an apparent decrease during subsequent incubation. The quantification of total fructans after hydrolysis of levan and FOS with H₂SO₄ indicated that this decrease is attributable to partial hydrolysis of levan to polysaccharides with intermediate molecular weight that were not accounted by the analytical tools employed. Indeed, inubation of *L. reuteri* LTH5448 or LTH5448ΔftfA with purified levan in phosphate buffer resulted in a reduced molecular weight of levan but not in hydrolysis to fructose or FOS, excluding that FtfA was responsible for the decrease in levan concentration during stationary growth phase (data not shown).

### 4. Discussion

A multitude of enzymes, transport systems, and regulatory elements mediate sucrose metabolism in lactobacilli (van Hijum et al., 2006; Goh et al., 2006; Saulnier et al., 2007). Metabolic pathways for the metabolism of sucrose and fructo-oligosaccharides partially overlap (Kaplan and Hutkins, 2003; Goh et al., 2006; Saulnier et al., 2007; Gänzle and Follador, 2012). Transcriptome analysis in *L. acidophilus* suggested that metabolism of sucrose but not metabolism of FOS in *L. acidophilus* is likely mediated by a sucrose PTS.
system (Barrangou et al. 2006). Functional analysis of sucrose metabolism in *L. plantarum* and *L. paracasei* demonstrated that transport and hydrolysis of sucrose as well as short chain FOS are mediated by phosphotransferase systems and associated (phospho)-β-fructofuranosidases (Saulnier et al., 2007; Goh et al., 2006).

In *L. reuteri* LTH5448, sucrose metabolism occurs via two alternative enzymes, the extracellular levansucrase FtfA and the intracellular sucrose phosphorylase ScrP. Sucrose phosphorylase is inducible by sucrose and raffinose in *L. reuteri*, *L. acidophilus* and *Bifidobacterium animalis* (Trindade et al., 2003; Barrangou et al., 2006, Schwab et al., 2007; Teixeira et al., 2012) but does not recognize FOS or raffinose as substrate (Kawasaki et al., 1996; van der Broek et al., 2004). The induction of ScrP by raffinose is thus likely mediated by the intracellular release of sucrose from raffinose by intracellular α-galactosidase activity (Yoon and Hwang, 2008; Teixeira et al., 2012).

Sucrose and raffinose utilization by *L. reuteri* LTH5448 was not impaired by deletion of sucrose phosphorylase as ScrP and FtfA are equally suited to support rapid turnover of sucrose (this study, Schwab et al., 2007). Remarkably, *L. reuteri* LTH5448ΔscrP produced about 25% more levan, confirming that the lack of sucrose phosphorylase is compensated by increased turnover by levansucrase as sole sucrose converting enzyme. Formation of 3.3 g levan / kg by *L. reuteri* during growth in sourdough improved bread volume, and delayed staling (Galle et al., 2012); the use of sucrose phosphorylase-negative strains may thus be beneficial in applications aiming at a high yield of polymeric fructan.

This study identified a putative sucrose regulator ScrR of *L. reuteri* with homology to regulatory proteins of the GalR-LacI family including MsmR and SacR. Structurally
related ScrR regulate the (phospho)-β-fructofuranosidases ScrA and enzymeII\textsubscript{Suc} of sucrose PTS systems in \textit{S. mutans}, \textit{P. pentosaceus}, \textit{L. lactis} (Figure 3) and sucrose phosphorylases in \textit{Bifidobacterium lactis} (Gering and Brückner, 1996; Luesink et al., 1999; Hiratsuka et al., 1998; Trinidad et al., 2003). In contrast to \textit{L. plantarum} and \textit{L. acidophilus}, genes coding for sucrose metabolic enzymes and their regulation in \textit{L. reuteri} are not encoded on a single operon but are on three distinct genetic loci. Moreover, sucrose metabolic enzymes in \textit{L. reuteri} do not support catabolism fructo-oligosaccharides other than sucrose but contribute to metabolism of raffinose-family oligosaccharides (Teixeira et al., 2012; this study).

Inactivation of \textit{scrR} in \textit{L. reuteri} LTH5448 resulted in constitutive transcription of \textit{ftfA} and \textit{scrP} when the strain was grown in the presence of media containing glucose, indicating that \textit{scrR} is a transcriptional repressor similar to \textit{scrR} of \textit{L. lactis}, \textit{S. mutans}, and \textit{Staphylococcus xylosus} (Gering and Brückner, 1996; Luesink et al., 1999; Hiratsuka et al., 1998). Regulation of gene expression by \textit{scrR} in \textit{L. reuteri} is thus substrate controlled, comparable to the function of \textit{scrR} of \textit{B. animals} (Trindade et al., 2003) and \textit{S. mutans}, \textit{msmR} and \textit{scrR} of \textit{L. acidophilus} and \textit{L. plantarum}, respectively. Moreover, \textit{scrR} in \textit{L. reuteri} regulated the expression of \textit{ftfA}. Regulation of fructansucrases by \textit{scrR} was not observed in past studies on transcriptional regulators of sucrose metabolism in lactic acid bacteria. In \textit{L. sanfranciscensis}, levansucrase is the only enzyme capable of sucrose hydrolysis and is constitutively expressed (Tieking et al., 2005). In addition to its contribution to sucrose and raffinose metabolism in \textit{L. reuteri}, levansucrase also provides protection against environmental stressors and its expression is induced by heat stress and sublethal concentrations of proton-ionophores (Schwab et al., 2006). The finding that \textit{ftfA}
is regulated by scrR adds to our understanding of the multiple roles of levansucrases in
the ecology of L. reuteri (Gänzle and Schwab, 2009).

In conclusion, L. reuteri takes a very different approach to utilize sucrose and to regulate
sucrose metabolic genes when compared to its closely related organisms. Lactobacilli
have adapted to nutrient rich habitats by reduction of the genome size (Makarova and
Koonin, 2007). Among lactobacilli, L. plantarum has retained a relatively large genome
size and the ability to metabolise a large spectrum of carbohydrates, and is found in
diverse habitats (Makarova and Koonin, 2007; Siezen and van Hylckama Vlieg, 2011).
Glucose is metabolized preferentially over maltose or sucrose, reflecting carbon
catabolite repression (Andersson et al., 2005; Saulnier et al., 2007). L. reuteri underwent
a more extensive reductive evolution to adapt to specific vertebrate hosts (Walter, 2008;
Frese et al., 2011). In these narrow ecological niches, particularly the upper intestine of
(grain-feeding) animals, sucrose and raffinose are major carbohydrate sources. This study
demonstrates that sucrose metabolism in L. reuteri is not subject to repression by glucose.
L. reuteri apparently has abandoned regulatory circuits to repress sucrose metabolic
enzymes in presence of glucose. This preferential metabolism of sucrose contributes to
the ecological fitness of L. reuteri (Sims et al., 2011; Walter et al., 2008). Differences in
the regulation of sucrose metabolism between L. reuteri and other lactobacilli thus reflect
the ecological adaptation of L. reuteri.

Sourdough isolates of L. reuteri do not represent a separate, extra-intestinal evolutionary
lineage of L. reuteri but are of intestinal origin (Su et al. 2012). The concurrent
expression of sucrose (and maltose) metabolic enzymes matches the carbohydrate
availability in cereal fermentations, and accounts for the simultaneous utilization of
carbohydrates during growth in sourdough (this study, Gänzle et al., 2007). The simultaneous utilization of maltose and sucrose also determines the formation of the exopolysaccharides reuteran and levan as well as acetate, and thus has a substantial impact on bread quality (Tieking et al., 2003; Kaditzky and Vogel, 2008; Galle et al., 2012).

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References


Figure legends.

**Figure 1.** Sucrose and raffinose utilization in *L. reuteri* LTH5448 (modified from Teixeira et al., 2012). FtfA, levansucrase, α-Gal, α-galactosidase, SucP, sucrose phosphorylase. The scheme does not indicate the transport proteins involved in metabolism because transport proteins with specificity for sucrose, melibiose, or raffinose are not annotated in *L. reuteri* genomes, and have not been characterized on the biochemical level.

**Figure 2.** Gene loci encoding sucrose phosphorylase (*scrP*) (**Panel A**) and its putative regulator (*scrR*) (**Panel B**) in *L. reuteri* 100-23 (Gene bank Accession number AAPZ00000000.2). The lines below represent the homologous sequences in *L. reuteri* LTH5448. PCR primers used for the generation of LTH5448Δ*scrR* and LTH5448Δ*scrP* mutants are indicated by arrows.

**Figure 3.** Phylogenetic analysis of sucrose specific regulator ScrR and related transcriptional factors in the LacI/GalR family. The distance tree was constructed using the maximum likelihood method. Bootstrap values were calculated with 100 replicates.

**Figure 4.** Growth of *L. reuteri* LTH5448 (●), *L. reuteri* LTH5448Δ*scrP* (○) and *L. reuteri* LTH5448Δ*scrR* (▼) with maltose, glucose and fructose (mMRS); sucrose (sucMRS); or raffinose (rafMRS) as substrate. Data shown are mean values for duplicate (*L. reuteri* LTH5448 and LTH5448Δ*scrP*) and triplicate (LTH5448Δ*scrR*) independent experiments.

**Figure 5.** Formation of fructooligosaccharides and levan during growth of *L. reuteri* LTH5448 and *L. reuteri* LTH5448Δ*scrP* in presence of 100 g L⁻¹ sucrose. **Panel A.** FOS formation was quantified after 24 hours of incubation. **Panel B.** Levan concentration in
the culture supernatant of *L. reuteri* LTH5448 (●) and *L. reuteri* LTH5448ΔscrP (○).

Data are shown as means ± standard deviation of three independent replicates. Significant differences (P<0.001) between the levan concentrations in *L. reuteri* LTH5448 and LTH5448ΔscrP were determined by Student’s *t*-test and are indicated by an asterisk.
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> JM109</td>
<td>Cloning host for pGEMTeasy- and pJRS233-derivative plasmids</td>
<td>Promega</td>
</tr>
<tr>
<td><em>L. reuteri</em> LTH5448</td>
<td>Sourdough isolate</td>
<td>Schwab et al. 2007</td>
</tr>
<tr>
<td><em>L. reuteri</em> LTH5448Δ<em>scrP</em></td>
<td>Wild type strain derivative with a deletion in <em>scrP</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>L. reuteri</em> LTH5448Δ<em>scrR</em></td>
<td>Wild type strain derivative with a deletion in <em>scrR</em></td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEMTeasy</td>
<td>Cloning vector used in <em>E. coli</em>; 3.0 kb; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pScrP-A</td>
<td>pGEMTeasy containing a 0.9 kb sequence upstream of <em>scrP</em>; 3.9 kb; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pScrP-B</td>
<td>pGEMTeasy containing a 0.9 kb sequence downstream of <em>scrP</em>; 3.9 kb; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pScrP-AB</td>
<td>pGEMTeasy containing the upstream and downstream sequences of <em>scrP</em>; 4.8 kb; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pScrR-A</td>
<td>pGEMTeasy containing a 1.2 kb sequence upstream of <em>scrR</em>; 4.2 kb; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pScrR-B</td>
<td>pGEMTeasy containing a 1.2 kb sequence downstream of <em>scrR</em>; 4.2 kb; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pScrR-AB</td>
<td>pGEMTeasy containing the upstream and downstream sequences of <em>scrR</em>; 5.4 kb; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJRS233</td>
<td>Shuttle vector used in the hosts <em>E. coli</em> and <em>L. reuteri</em>; 6.0 kb; Erm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Perez-Casal et al. 1993</td>
</tr>
<tr>
<td>pKO-<em>scrP</em>-AB</td>
<td>pJRS233 containing 1.8 kb of the flanking sequences of <em>scrP</em>; 7.8 kb; Erm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pKO-<em>scrR</em>-AB</td>
<td>pJRS233 containing 2.4 kb of the flanking sequences of <em>scrR</em>; 8.4 kb; Erm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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</table>

Amp<sup>r</sup>, Erm<sup>r</sup>, ampicillin and erythromycin resistance, respectively
### Table 2. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’ – 3’)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td><code>scrP</code></td>
<td><code>pho-KO1b-F-XhoI</code></td>
<td>CCGCTGGAGGTATGAAGGTACAACC</td>
<td>Insert primer</td>
</tr>
<tr>
<td></td>
<td><code>pho-KO2-R-XbaI</code></td>
<td>GCTCTAGATTTAGTCAGAGTAAGTAATTAAC</td>
<td>Insert primer</td>
</tr>
<tr>
<td></td>
<td><code>pho-KO3-F-XbaI</code></td>
<td>GCTCTAGATTTCACTATTACAGCTAACCAGCGCAA</td>
<td>Insert primer</td>
</tr>
<tr>
<td></td>
<td><code>pho-KO4a-R-PstI</code></td>
<td>AACGTCGAGGCCTGGAATTTCTACC</td>
<td>Insert primer</td>
</tr>
<tr>
<td></td>
<td><code>pho-1b-F</code></td>
<td>GTATGAAGGTACAACC</td>
<td>Sequencing</td>
</tr>
<tr>
<td></td>
<td><code>pho-4a-R</code></td>
<td>GCTTTGAATTTCTACC</td>
<td>Sequencing</td>
</tr>
<tr>
<td></td>
<td><code>SucPho F</code></td>
<td>CTGGTAAAGCGTGCAACb)</td>
<td>qPCR</td>
</tr>
<tr>
<td></td>
<td><code>SucPho R</code></td>
<td>CAGTTAAGATATCTTACATb)</td>
<td>qPCR</td>
</tr>
<tr>
<td><code>scrR</code></td>
<td><code>reg-KO1a-F-XhoI</code></td>
<td>CCGCTGGAGTAATTTCAAATAGGTATG</td>
<td>Insert primer</td>
</tr>
<tr>
<td></td>
<td><code>reg-KO2-R-BamHI</code></td>
<td>CGGGATTCCTCATTGAGCAACATCTTTAAC</td>
<td>Insert primer</td>
</tr>
<tr>
<td></td>
<td><code>reg-KO3-F-BamHI</code></td>
<td>CGGGATTCCTAAACTTGTTCCCGTTGA</td>
<td>Insert primer</td>
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<tr>
<td></td>
<td><code>reg-KO4b-R-HindIII</code></td>
<td>CCCAAACGTTCTTCAATGGTCAAAAT</td>
<td>Insert primer</td>
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<tr>
<td></td>
<td><code>reg-5-F</code></td>
<td>CCGTGGATTGTTCAATATG</td>
<td>Sequencing</td>
</tr>
<tr>
<td></td>
<td><code>reg-6-R</code></td>
<td>CTGTTGAACGAACTTCAG</td>
<td>Sequencing</td>
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<tr>
<td></td>
<td><code>reg-9-F</code></td>
<td>TGGGGATAGAGGAATATC</td>
<td>qPCR</td>
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<tr>
<td></td>
<td><code>reg-10-R</code></td>
<td>CGATTGATGGAATGGAAC</td>
<td>qPCR</td>
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<tr>
<td><code>pho</code></td>
<td><code>Phoket V</code></td>
<td>GTAACCTTCAAGGAATCCb)</td>
<td>qPCR</td>
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<td><code>Phoket R</code></td>
<td>CGTCTTTACGCTTCTCTG</td>
<td>qPCR</td>
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<td><code>ftfA</code></td>
<td><code>Leu1001 V</code></td>
<td>GAATGGCTATCAACCTTG</td>
<td>qPCR</td>
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<tr>
<td></td>
<td><code>Leu1001 R</code></td>
<td>CTTCTACTTGCCTGTTCb)</td>
<td>qPCR</td>
</tr>
</tbody>
</table>

<sup>a</sup> Restriction enzyme sites are indicated in italic form

<sup>b)</sup> Primers from Schwab et al., 2007; other primers were designed in this study
Table 3. Sucrose phosphorylase and levansucrase expression in *L. reuteri* LTH5448 and LTH5448Δ*scrR*. Data are shown as means ± standard deviation of triplicate independent experiments.

<table>
<thead>
<tr>
<th><em>L. reuteri</em> strain</th>
<th>Target gene</th>
<th>Sugar composition of media</th>
<th>Log₁₀ [relative gene expression]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose, Maltose, Fructose</td>
<td>Glucose</td>
</tr>
<tr>
<td>LTH5448</td>
<td><em>scrP</em></td>
<td>-2.17 ± 0.45</td>
<td>-2.76 ± 0.29</td>
</tr>
<tr>
<td>LTH5448Δ<em>scrR</em></td>
<td></td>
<td>0.02 ± 0.23</td>
<td>0.58 ± 0.21</td>
</tr>
<tr>
<td>LTH5448</td>
<td><em>ftfA</em></td>
<td>-1.87 ± 0.27</td>
<td>-1.61 ± 1.12</td>
</tr>
<tr>
<td>LTH5448Δ<em>scrR</em></td>
<td></td>
<td>-0.67 ± 0.53</td>
<td>-0.20 ± 0.77</td>
</tr>
</tbody>
</table>

Gene expression was calculated by the ΔΔCt method using wild type cells grown in presence of sucrose as reference conditions; the phosphoketolase gene *pho* was used as endogenous control.
Table 4. Formation of organic acids of *L. reuteri* LTH5448 and LTH5448ΔscrP mutant during growth in mMRS with maltose, glucose and fructose; sucrose (sucMRS); or raffinose (rafMRS) as substrate. Data are shown as means ± standard deviation of duplicate independent experiments.

<table>
<thead>
<tr>
<th>Medium</th>
<th><em>L. reuteri</em> strain</th>
<th>Mannitol [mmol L⁻¹]</th>
<th>Lactate [mmol L⁻¹]</th>
<th>Acetate [mmol L⁻¹]</th>
<th>Ethanol [mmol L⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>mMRS</td>
<td>LTH5448</td>
<td>18 ± 0</td>
<td>77 ± 4</td>
<td>10 ± 1</td>
<td>58 ± 6</td>
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<tr>
<td></td>
<td>ΔscrP</td>
<td>19 ± 1</td>
<td>76 ± 4</td>
<td>10 ± 1</td>
<td>53 ± 7</td>
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<tr>
<td>sucMRS</td>
<td>LTH5448</td>
<td>14 ± 0</td>
<td>26 ± 0</td>
<td>11 ± 0</td>
<td>10 ± 2</td>
</tr>
<tr>
<td></td>
<td>ΔscrP</td>
<td>14 ± 1</td>
<td>25 ± 2</td>
<td>10 ± 2</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>rafMRS</td>
<td>LTH5448</td>
<td>15 ± 0</td>
<td>61 ± 3</td>
<td>10 ± 2</td>
<td>49 ± 1</td>
</tr>
<tr>
<td></td>
<td>ΔscrP</td>
<td>18 ± 2</td>
<td>62 ± 1</td>
<td>10 ± 1</td>
<td>46 ± 4</td>
</tr>
</tbody>
</table>
Figure 1. Teixeira et al.
Figure 2. Teixeira et al.

A

Permease \[\rightarrow\] \(\text{scrP}\) \[\rightarrow\] Hypothetical protein

\(\text{pho-KO1b-F}\) \(\rightarrow\) \(\text{pho-KO2-R}\) \(\rightarrow\) \(\text{pho-KO3-F}\) \(\rightarrow\) \(\text{pho-KO4a-R}\)

B

Muramidase \[\rightarrow\] \(\text{scrR}\) \[\rightarrow\] Permease

\(\text{reg-KO1a-F}\) \(\rightarrow\) \(\text{reg-KO2-R}\) \(\rightarrow\) \(\text{reg-KO3-F}\) \(\rightarrow\) \(\text{reg-KO4b-R}\)
Figure 3. Teixeira et al.
Figure 4. Teixeira et al.