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Sucrose metabolism in lactobacilli and bifidobacteria

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ABBREVIATIONS

aa	amino acid(s)
FFF	field-flow-fractionation
B.	*Bifidobacterium*
Blast	basic local alignment search tool
bp	base pairs
CAC	chemically acidified control
DY	dough yield (flour-water content)
DP	degree of polymerisation
DSC	differential scanning calorimetry
E.	*Escherichia*
EPS	exopolysaccharide(s)
FOS	fructooligosaccharide(s)
FPLC	fast protein liquid chromatography
G-1-P	α-glucose-1-phosphate
G-6-P	glucose-6-phosphate
GaOS	galcatooligosaccharide(s)
GlgP	glucan (glycogen) phosphorylase(s)
Gly-P	glycerol-3-phosphate(s)
GOS	glucooligosaccharide(s)
GPC	gel permeation chromatography
G-Ps	collective term for G-1-P, G-6-P and gly-3-P
Gtf	glucansucrase(s)
HePS	heteropolysaccharide(s)
HoPS	homopolysaccharide(s)
HPAEC-IPAD	high-performance anion-exchange chromatography and integrated pulsed amperometric detection
HPLC	high-performance liquid chromatography
L.	*Lactobacillus*
LAB	lactic acid bacteria
Lact.	*Lactococcus*
LB	Luria-Bertani medium
Lc.	*Leuconostoc*
LTH	Lebensmitteltechnologie Hohenheim
mMRS	modified de Man, Rogosa, Sharp medium
Mp	molecular weight of the highest peak
Δlev	levansucrase deletion mutant of *L. sanfranciscensis* TMW 1.392
NAD	nicotinamide adenine dinucleotide
ORF	open reading frame
OS	oligosaccharides
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
P_i	inorganic phosphate
PTS	phosphotransferase system
RAPD	randomly amplified polymorphic DNA
RI	refractive index
S.	*Strepctococcus*
SDS	sodium dodecyl sulfate
SucP	sucrose phosphorylase

TLC	thin layer chromatography
TMW	Technische Mikrobiologie Weihenstephan
TPA	texture profile analysis
WPS	water soluble polysaccharides
WT	wildtype

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1 INTRODUCTION

1.1 Sucrose utilization in lactobacilli and bifidobacteria

Sucrose, commonly known as table sugar, is a glycosidic dimer of glucose and fructose (O-β-D-Fru*f*-(2-1)-α-D-Glc*p*). It is the soluble carbohydrate reserve and transport form in plants, which synthesize it during photosynthesis and therefore it is the most abundant disaccharide in the environment. It is an important nutrient for bacteria and yeasts. The hydrolysis product glucose plays a central role in the bacterial metabolism. Glycoside hydrolases from different families can degrade sucrose: e.g. invertases (β-fructofuranosidase) (EC 3.2.1.26, GH family 32), sucrose phosphorylases (EC 2.4.1.7, GH family 13), glucansucrases (EC 2.4.5.1, GH family 70) and levansucrases (EC 2.4.1.10, GH family 68).

The genus *Lactobacillus* is a diverse group that belongs to the lactic acid bacteria (LAB), which are characterized through the production of mainly lactic acid in their carbohydrate metabolism. Lactobacilli are omnipresent in the environment; some species are autochthonous in animals (Tannock 2004). Many species are used in food production and preservation since centuries and nowadays play an important role because some strains of these species are considered probiotic. Bifidobacteria were grouped within the lactobacilli till 1974 but exhibiting a G+C content of more than 50 Mol% in their DNA, in contrast to lactobacilli, whose G+C content varies between 38–50 Mol% they were recognized as a separate genus (reviewed by Ballongue et al. 1998). Bifidobacteria play a key role in the intestinal ecosystem of mammalians. They dominate the microflora of infants fed with breast milk, and still in adults bifidobacteria species are considered to be key commensals in human-microbe interactions (Ballongue 1998; reviewed by Ventura et al. 2004). They have been associated with beneficial health effects what has led to their use in foods as probiotic. Probiotics are defined by the Food and Agricultural Organization of the United Nations and the World Health Organization (FAO/WHO) (2001) as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. To achieve a probiotic status, microorganisms must fulfil a number of criteria, ranging from resistance to bile and acid to survive the passage through the intestine to specific effects on the host.

L. reuteri strains belong to the natural flora of animals e.g. pigs and ducks (Kurzak et al. 1998; Tieking et al. 2005a) and are also dominant in type II (long-term industrial) sourdough fermentations (Vogel et al. 1999). *L. sanfranciscensis* is considered to be a key lactic acid bacterium in sourdough (Gobetti and Corsetti 1997) and is dominant in type I (traditional back slopping procedures) sourdough produced from wheat and rye flour (Hammes et al.

1996). *B. lactis* Bb-12, more precisely *B. animalis* subsp. *lactis* Bb-12 (*B. lactis* TMW 2.530) is a probiotic strain of animal origin adapted to milk with a high colonization capacity to human mucosa (Ouwehand et al. 1999). It is used as a probiotic culture in diary products, especially yoghurt (CHR Hansen, Hoersholm, Denmark). Although the numerous health claims made for probiotics are discussed controversially some evidence for a positive effect upon treatment of acute diarrhoea with *L. reuteri* and *B. lactis* Bb-12 or in the prevention of diarrhoea are available (Simmering and Blaut 2001; Reid and Hammond 2005; Weizman et al. 2005).

1.1.1 Sucrose catabolism in lactobacilli and bifidobacteria

For sucrose catabolism, the disaccharide is transported into the bacterial cell. The major mechanism of sucrose uptake is mediated by phosphotransferase system (PTS) coupled to a phosphoenolpyruvate (PEP) dependent phosphorylation leading to sucrose-6-phosphate (S-6-P). S-6-P is cleaved by sucrose-6-phosphate-hydrolase into glucose-6-P (G-6-P) and fructose. If the fructose moiety is metabolised it is generally phosphorylated by fructokinase (fig. 1A). After formation of G-6-P catabolism in homofermentative lactobacilli mainly occurs via the Emden-Meyerhof pathway to pyruvate. The end product of sucrose fermentation of most homofermentative lactobacilli are two moles lactate per mole glucose from sucrose and theoretically also per mole fructose from sucrose. Alternatively, sucrose gets into the cell via a solute-cation symport system by sucrose permease of the major facilitator superfamily. Heterofermentative lactobacilli were thought not to have an active PTS (Romano 1986). Further they likely use the phosphoketolase pathway because of the lack of fructose-1,6-diphosphate aldolase. However, in the heterofermentative *L. brevis*, at least for fructose, the synthesis of a PTS and glycolytic enzymes was observed under anaerobic conditions (Saier et al. 1996). Heterofermentative lactobacilli form equimolar amounts of lactate, ethanol and CO_2 from the glucose moiety of sucrose and only a part of the fructose moiety is transformed to F-6-P and converted to G-6-P via fructokinase and phosphoglucomutase. The other part of the fructose available from sucrose is used as alternative organic electron acceptor to gain additional ATP by forming acetate instead of ethanol. Fructose is thereby reduced to mannitol to regenerate NAD^+ from NADH, which is formed in the glycolytic pathway (fig. 1A).

Bifidobacteria have a unique glucose metabolism called bifidus pathway or D-fructose-6-phosphate shunt including a partial Embden-Meyerhof pathway. The key enzyme is D-xylose-5-phosphate/D-fructose-6-phosphate phosphoketolase. G-6-P is converted by G-6-P-isomerase for utilization in the F-6-P-shunt. Bifidobacteria theoretically produce three moles

acetate and two moles lactate from two moles glucose but also higher ratios are obtained because of the existence of a pathway that enables bifidobacteria strains to produce ethanol and formate (Bezkorovainy 1989). When fermented with sucrose the fructose moiety can be phosphorylated by a fructokinase (Caescu et al. 2004) to F-6-P and so be incorporated into the bifidus-shunt.

Several glycosyl hydrolases and sucrose gene clusters have been described from LAB and bifidobacteria. Their regulation is usually mediated by transcriptional regulators of the LacI-GalR family and by catabolite repression, though sucrose phosphorylases have not been studied as intensively as hydrolases yet. The LacI-like regulators of bifidobacteria are closely related to the ones of LAB indicating that horizontal gene transfer took place although they are found clustered with sucrose phosphorylase in bifidobacteria. The sucrose phosphorylases, however, seem to have evolved separately in LAB and bifidobacteria (reviewed by Reid and Abratt 2005). In LAB, only the sucrose phosphorylases of *Leuconostoc mesenteroides* (Koga et al. 1991; Kitao and Sekine 1992; Kawasaki et al. 1996; Schwarz and Nidetzky 2006) and *Streptococcus mutans* (Russel et al. 1988 and 1992) are well characterized yet. For *L. reuteri* CRL 1100 an invertase is characterized (Cuezzo de Ginés et al. 2000). In *L. plantarum* lately a sucrose PTS coupled to a β-fructofuranosidase was found that could probably play a role in the metabolism of short chain fructooligosaccharides (Saulnier et al. 2007).

In the genera *Lactobacillus* and *Streptococcus* further glycosyltransferases are widespread which, however, act extracellulary and will be discussed later on as they are not implicitly involved in sucrose catabolism in direct sense.

Bifidobacteria are known to utilize a great variety of carbohydrates and to be able to degrade complex carbohydrates that are not digestible for animals and humans and therefore reach the intestine. In the human intestinal tract sucrose is hydrolyzed and the glucose moiety is transported actively and acts strongly on the level of blood sugar and the secretion of insulin. Intestinal strains therefore do not find pure sucrose as substrate in the large intestine. Raffinose (O-α-D-Gal-(1-6)-O-α-D-Glc*p*-(1-2)-β-D-Fru*f*) and stachyose (O-α-D-Gal-(1-6)-O-α-D-Gal-(1-6)-O-α-D-Glc*p*-(1-2)-β-D-Fru*f*) that are both widespread in plants, however, are not (completely) digested by humans and reach the colon where sucrose is liberated by microbial enzymes. Also from 6-kestose (O-α-D-Glc*p*-(1-2)-β-D-Fru*f*-(6-2)-β-D-Fru*f*) and 1-kestose (isokestose) (O-α-D-Glc*p*-(1-2)-β-D-Fru*f*-(1-2)-β-D-Fru*f*) sucrose can be released. Accordingly to their intestinal habitat, in the genome of *B. longum* NCC2705 many oligosaccharide transporters were identified but only one PTS-type sugar transporter,

probably as a response to competition in the GIT (Schell et al. 2002). Sucrose phosphorylases have been described in *B. longum*, *B. lacits* and *B. adolescentis* (reviewed by Reid and Abratt 2005). In *B. lactis* its expression, transcription and activity were reported to be induced by sucrose and raffinose and to be repressed by glucose. But no direct activity of sucrose phosphorylase on raffinose was found (Trindade et al. 2003).

1.1.2 Production of polymers with higher degree of polymerisation from sucrose

Another group of sucrose hydrolysing enzymes are the above mentioned extracellular glycosyltransferases. Glycosyltransferases are subdivided in glucosyl- and fructosyl-transferases (Gtf and Ftf), which are also termed glucan- and fructansucrases, respectively. Both follow a conserved domain structure with: (i) N-terminal signal peptide, (ii) variable region, (iii) catalytic domain, (iv) C-terminal domain that in glucansucrases is also called glucan binding domain and in fructansucrases possesses a cell-wall anchor motif. An overview on these enzymes is given by van Hijum et al. (2006) and Korakli and Vogel (2006). These extracellular or cell wall bound enzymes use the energy resulting from the cleavage of the glycosidic bond in the sucrose molecule to transfer the glucose or fructose moiety, respectively, to an acceptor molecule and release the other moiety into the extracellular environment from where they can be transported into the cell and further metabolized. Sucrose is the only substrate for glucansucrases, while fructansucrases also can use raffinose (van Geel-Schutten et al. 1999). Depending on the acceptor molecule used, three activities of glycosyltransferases are distinguished: If the acceptor is water, the sucrose molecule is just hydrolyzed (hydrolysis activity). The sugar monomer can also be transferred to another sugar molecule to form oligosaccharides (acceptor reaction) or to a subsequently growing polysaccharide chain leading to exopolysaccharides (EPS) (transferase activity) (fig. 1B). The predominance of one of these activities can be influenced by culture conditions such as sucrose concentration, temperature, pH and availability of acceptor molecules (Tieking et al. 2005a, 2005b; Korakli et al. 2003; Kralj et al. 2004b).

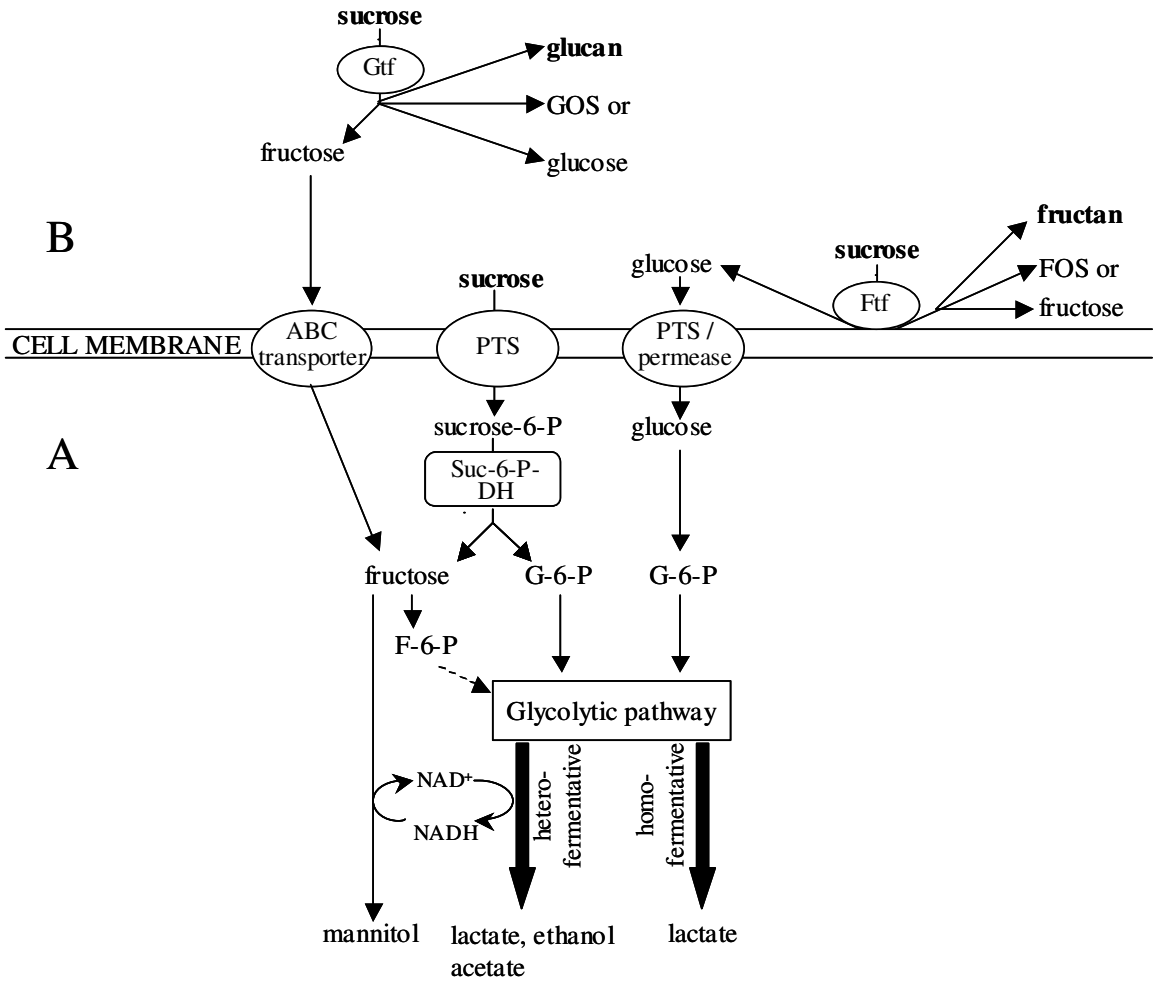

Figure 1 Described metabolic pathways of sucrose metabolism in lactobacilli. **A** Intracellular sucrose catabolism, **B** extracellular action of glycosyltransferases. Gtf, glucosyltransferase; GOS, glucooligosaccharides; Ftf, fructosyltransferase; FOS, fructooligosaccharides. Dashed arrows indicate that intermediates were left out.

Production of exopolysaccharides. EPS made from sucrose through glucan- or fructansucrases are composed of only one type of monomer (glucose or fructose) and are therefore called homoexopolysaccharides (HoPS) (glucans and fructans). They are further characterized through their main type of linkages into dextran (α-(1-6)-glucan), mutan (α-(1-3)-glucan), reuteran (α-(1-4)-glucan), levan (β-(2-1)-fructan) and inulin (β-(2-6)-fructan). Heteropolysaccharides (HePS), in contrast, consist of repeating precursor units made of multiple sugar types (de Vuyst and Degeest 1999). In the formation of HePS various enzymes are involved for the intracellular synthesis of sugar nucleotides and repeating units and the extracellular polymerisation. The term EPS therefore in this work always stands for HoPS.

The production of HoPS is wide spread among lactobacilli isolated from cereal fermentations and intestinal environments (Tieking et al. 2003a and 2005a) but no data are available for

HoPS production in bifidobacteria and only little is known about HePS production in this genus (Abbad-Andaloussi et al. 1995; Roberts et al. 1995). Research is rather focused on the health promoting properties of cell-wall associated HePS (part of the glycocalyx) of bifidobacteria (e.g. Novik et al. 2002; Hosono et al. 1997). Genes related with EPS production in *B. longum* NCC2705 are supposed to be obtained by horizontal gene transfer (Schell et al. 2002). The biological function of HoPS production in lactobacilli is not fully understood. Most of the EPS-forming bacteria can not use their EPS as a nutrient reserve because of the lack of degrading enzymes (Cerning 1990). But EPS were found to protect cells from environmental stresses as e.g. nisin, starvation, membrane stress and low pH (Looijesteijn et al. 2001; Kim et al. 2000; Schwab and Gänzle 2005). EPS is further involved in biofilm formation and cell-cell adherence and therefore may support EPS producing strains, e.g. *L. reuteri*, to survive in intestinal passages or persist in the intestinal tract (Schwab 2006). The expression of some of the glycosyltransferases is increased in the presence of sucrose (Quirasco et al. 1999; Schwab 2006) and it was claimed that EPS production may result in osmotic and energetic advantages (Korakli and Vogel 2006).

HoPS of LAB exhibit a large variety of different linkage types, molecular weights (up to 10^7), degrees of polymerization and branching and therefore have diverse functional properties relating to solubility, conformation or interactions with other bio-molecules. Emerging information about structure-function relationships of glycosyltransferases now allows modification of the linkage types in glucans through directed amino acid substitution in the glucosyltransferases (Kralj et al. 2005 and 2006, for review see van Hijum et al. 2006). Moreover, the polymer properties can be affected by environmental conditions. Sucrose concentration as well as temperature affected the degree of branching and molecular size of dextran from *Lc. mesenteroides* B 512FMCM (Kim, D. et al. 2003).

Strains of *L. reuteri* are known to synthesize the glucans reuteran, dextran, and mutan, as well as the fructans levan and inulin (reviewed by van Hijum et al. 2006). Many strains harbour more than one glycosyltransferase gene (van Geel-Schutten et al. 1999; van Hijum et al. 2001 and 2002; Tieking et al. 2003a; Kralj et al. 2004a). *L. reuteri* TMW 1.106 produces a glucan from sucrose in mMRS medium as well as during sourdough fermentation (Tieking et al. 2003a). Beside a glucosyltransferase it also harbours an inulosucrase (AM293550) (Schwab and Gänzle 2005). *L. sanfranciscensis* TMW 1.392 (isogenic to LTH 2590 and DSM 14272) produces a fructan of the levan-type. Tieking et al. (2005b) demonstrated that the levansucrase deletion mutant of *L. sanfranciscensis*, TMW 1.392Δlev, lost the ability to

metabolise sucrose and to synthesize levan, indicating that the levansucrase is the only enzyme in *L. sanfranciscensis* TMW 1.392 able to hydrolyze sucrose and to produce levan.

Production of oligosaccharides (OS). Oligosaccharide production from sucrose by lactobacilli is described through the acceptor reaction of glycosyltransferases which is influenced by the kind of acceptor present. Maltose or isomaltose act as so called strong acceptors and lead to a decrease in EPS and an increase in OS production, whereas weak acceptors as e.g. fructose evoke only low synthesis of OS (Robty and Walseth 1978). The kind of acceptor also defines if only one or a series of OS with increasing degree of polymerisation (DP) are synthesized (see Korakli and Vogel 2006). Beside sugars also salicyl alcohols and aromatic compounds can act as acceptors (reviewed by van Hijum et al. 2006). Sucrose can only act as acceptor for fructansucrases (van Hijum et al. 2006) leading to 1-kestose and nystose as described for e.g. *L. sanfranciscensis* (Tieking et al. 2005c). The inulosucrase of *L. reuteri* TMW 1.106 produces no EPS and only low levels of 1-kestose and FOS under reference conditions. Under stress conditions, e.g. elevated temperature, its *inu* gene is inducible, whereas the expression of the *gtfA* gene is not regulated by environmental stress (Schwab and Gänzle 2005).

Production of OS in bifidobacteria is mostly described through transgalactosidase activity of β-galactosidases resulting in the formation of galactooligosaccharides from lactose (Dumortier et al. 1990, Møller et al. 2001; Hsu et al. 2007). OS formation from sucrose by bifidobacteria results from the transglucosylation activity of sucrose phosphorylase which implies a different mechanism as the one of galactosidases that work without phosphate. Transglucosylation activity has been described for recombinant sucrose phosphorylases of *B. longum* and *B. adolescentis* (Kim, M. et al. 2003; van den Broek et al. 2004). While in *B. longum* sucrose also acts as acceptor, the enzyme of *B. adolescentis* shows activity with several acceptors but not with sucrose as acceptor.

1.2 Sucrose metabolism used in food fermentations

1.2.1 Sucrose catabolism in food fermentations

Sucrose, mainly obtained from sugar cane and sugar beet, is used as sweetener in food. Because of its fermentation to lactic acid and its conversion to glucan and fructan by oral streptococci it plays an important role in the development of dental caries (Johnson et al. 1977; Rozen et al. 2001). However, in fermented foods the products of sucrose metabolism by bacteria play an important role. Sucrose is naturally present abundantly in plant material and its originally spontaneous fermentation through LAB has a tradition in food making of

thousands of years (e.g. sourdough or sauerkraut) because of the preservative function of the souring and its positive effects on flavour and texture of the fermented foods. In wine or beer, in contrast, LAB fermentations are spoilage agents. For industrial application and controlled fermentations selected strains are used as starter cultures to obtain reproducible fermented foods. An overview of fermented foods and the corresponding LAB and bifidobacteria starter cultures is given by Leroy and de Vuyst (2004). LAB and bifidobacteria fermentations further play an important and traditional role in the dairy industry, though with lactose as the typical carbohydrate source in milk.

1.2.2 Poly- and oligosaccharides produced from sucrose used in food production

EPS act as hydrocolloids and can therefore replace hydrocolloids/gums from plant or other microbial origin as e.g. inulin, guar gum, alginate or xanthan that are used (in some countries) to improve texture and shelf life of diverse foods as e.g. baked products, bakery fillings, ice cream, sausages or salad dressings (Dziezak 1991). The properties of the hydrocolloids vary depending on their chemical structure, molecular mass, shape and rigidity and the great variety of EPS produced from LAB thus provides a huge pool that could be exploited to achieve particular effects. Microbial EPS, e.g. the linear dextran of *Lc. mesenteroides,* is used to form the typical crumb of panettone (Decock and Cappelle 2005).

The biological role of oligosaccharide formation for the producer cells is not completely understood. Recently it was shown that added FOS have protective effects on *L. reuteri* TMW 1.106 during freezing, freeze-drying and storage (Schwab et al. 2007b). On the other hand, oligosaccharides gained interest as they can be used as low cariogenic sugar substitutes (Crittiden and Playne 1996). Nondigestible oligosaccharides can furthermore act as prebiotics. Besides inulin, FOS of the inulin type and *trans*-galactooligosaccharides (GaOS) are classified as prebiotics (Roberfroid 2007) because they reach the intestine in significant amounts and stimulate there the growth and/or activity of health promoting bacteria. For *B. lactis* Bb-12 a FOS hydrolyzing β-fructofuranosidase gene was described by Ehrmann et al. (2003). Prebiotics are used widely in the production of functional food/feed to positively influence the human and animal gut microflora. Generally, FOS for this purpose are obtained from chicory roots or by enzymatic synthesis (reviewed by Sangeetha et al. 2005) as it is also the case for GaOS (Crittiden and Playne 1996). Many bifidobacteria produce GaOS from lactose, some of them were shown to do so during manufacture of yoghurt (Lamoureux et al. 2002). The synthesis of prebiotic GaOS was also reported for β-galactosidases from *L. reuteri* (Splechtna et al. 2006). Production of prebiotics through probiotics can probably lead to

symbiotics which Gibson and Roberfroid (1995) already considered as an interesting and promising functional food and which were subject of various experiments (reviewed by Rastall and Maitin 2002).

Following the consumer's demand for natural and functional foods as well as the necessity of cost reduction, the *in situ* production of EPS and oligosaccharides from sucrose during food production using Generally Recognized As Safe (GRAS)-organisms like LAB as functional starters may provide an advantage and replace the need of additives.

1.2.3 Lactobacilli and bifidobacteria in cereal fermentations

In the bread making industry the use of LAB in sourdough plays an important role. For rye dough, acidification is a prerequisite for the applicability to bread making due to the lack of a gluten network (Hammes and Gänzle 1998). The positive effects LAB have on wheat bread quality are also beginning to receive an increasing amount of attention (Clarke and Arendt 2005). LAB positively affect wheat bread volume, retard staling of the bread (Corsetti et al. 1998 and 2000) and also exert some positive nutritional aspects (Liljeberg 1995). With the production of acetic acid, they contribute to the shelf life and flavour of bread. The latter is also positively influenced by strain dependent amino acid metabolism (Thiele et al. 2002b; Gänzle et al. 2007). Acidification also affects the activity of cereal and bacterial α-amylases, proteases, and the solubility of flour constituents, which indirectly affects flavour, texture and staling of the bread (Clarke and Arendt 2005). Though sucrose is the main easily accessible water soluble carbohydrate source of wheat and rye grains and is also enclosed in 1-kestose and nystose that can be cleaved by bacterial invertases (β-fructofuranosidases) (Brandt 2006), the main production of lactic acid occurs with the glucose liberated throughout the fermentation period from maltose and starch by flour or microbial amylases or added ones (Gänzle 2006). With the addition of sucrose to sourdough made with EPS producing lactobacilli, however, the synthesis of EPS *in situ* can be achieved (Tieking et al. 2003a). This observation is worth of being taken into account as much research is going on in the bakery industry to retard bread staling (reviewed by Gray and Bemiller 2003). Plant or microbial hydrocolloids as well as chemical modifications of these are subject of diverse studies on this phenomenon (Collar 2003; Guarda et al. 2004; Davidou et al. 1996; Christianson et al. 1981) and are also examined for their ability to improve machinability of the dough, and volume and texture of the bread (Rosell et al. 2001; Collar et al. 1999; Armero and Collar 1997). Several studies found out, that hydrocolloids had positive effects on these properties in levels of only 1 % flour weight or lower (Guarda et al. 2004; Brümmer 1977). As additives in Europe (and

Norway and Island) underlie declaration, *in situ* production of EPS through sourdough starters can therefore, under optimized conditions, be an alternative to the use of additives.

L. reuteri strains are among the dominant species in type II sourdoughs (Vogel et al. 1999), which are mostly used in industrialized processes to produce bakery pre-products for dough acidification, and. *L. sanfranciscensis* is the key organism of type I sourdoughs (Gobetti and Corsetti 1997), which are used for acidification and aroma formation in rye and wheat doughs. *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392 (isogenic to LTH2590) were shown to produce EPS during sourdough fermentations (Korakli et al. 2001; Tieking et al. 2003a, 2003b and 2005b). *L. reuteri* LTH 5448 was further reported to produce FOS (Schwab and Gänzle 2006) and *L. sanfranciscensis* TMW 1.392 to synthesize 1-kestose from sucrose in fermentation broth as well as in wheat dough (Korakli et al. 2003; Tieking et al. 2005c). The levan of *L. sanfranciscensis* TMW 1.392 was even shown to be metabolised by bifidobacteria (Korakli et al. 2002) and to selectively stimulate their growth in human faecal flora (Dal Bello et al. 2001).

The use of bifidobacteria in cereal fermentations has also received increasing attention during the last years as a source of probiotics (Kabeir et al. 2005) and because of their phytase activity leading to the reduction of antinutritional properties of phytic acid in cereal fibers (Haros et al. 2005). Also the application of *Bifidobacterium* strains as starters for breadmaking has been explored and human strains were suggested as suitable for replacing *Lactobacillus* strains for this purpose (Palacios et al. 2006).

1.3 Motivation and objectives

The rising interest of the consumers in “natural” foods has led to attempts to produce food without additives. The dairy industry has benefited from the ability of lactic acid bacteria to produce polysaccharides, HePS in this case, for a long time. The high potential of sourdough related and therefore “safe” lactobacilli strains to produce EPS (HoPS) (Tieking et al. 2003a) has induced research concerning the use of EPS produced by lactobacilli or the *in situ* production of EPS in sourdough with the aim to replace the hydrocolloids normally used in the bakery industry for improvement of dough and bread quality (Lacaze et al. 2007; Arendt et al. 2007).

Further, the increasing application of prebiotics makes it interesting to search for unknown oligosaccharides and to test their stimulating effect on probiotic bacteria. Especially the production of prebiotics through probiotics and the creation of new symbiotic is an increasing field in the food research (Lamoureux et al. 2002; Rastall and Maitin 2002).

Thirdly, the effective *ex situ* and *in situ* production of EPS, i.e. synthesis of a maximum of EPS with the required properties accompanied by minimal amounts of byproducts, needs the control of fermentation conditions and hence the knowledge of the biosynthesis and the optimization of the process parameters.

Therefore, the objective of this work was to examine the effect of production conditions on the amount and structure of the glucan formed by *L. reuteri* TMW 1.106 isolated from a type II sourdough. Other pathways of sucrose metabolism should be elucidated, and a possible protective function of the EPS on its producer strain should be tested. The influence of various dough fermentation parameters on the *in situ* production of EPS through *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392 isolated from type I sourdough should be investigated. Furthermore, production of EPS and oligosaccharides from sucrose by the probiotic Bb-12 (*B. lactis* TMW 2.530) should be examined and enzymes involved in its sucrose metabolism should be characterized. Insight should be obtained on the technological effect of EPS on doughs and breads. This should be achieved by the use of a levansucrase deletion mutant in wheat dough fermentations. The comparison of the metabolism and the performance of *L. sanfranciscensis* TMW 1.392 as compared to its levansucrase deletion mutant and the effects on dough and bread characteristics should enable to find benchmarks for *in situ* EPS production.

2. MATERIALS AND METHODS

2.1. Strains, media and growth conditions

Lactobacilli were cultivated anaerobically in modified MRS (mMRS) medium (Stolz et al. 1995) containing 10 g l^{-1} maltose, 5 g l^{-1} glucose and 5 g l^{-1} fructose. Where appropriate 15 g l^{-1} agar was added for solid media. *L. reuteri* strain TMW 1.106 (isolated from sourdough) was grown at 37°C. Dextransucrase and inulosucrase deletion mutants of *L. reuteri* TMW 1.106, provided by Jens Walter, University of Otago, New Zealand (Walter et al. 2005) were grown in mMRS with 5 ppm erythromycin. *L. sanfranciscensis* TMW 1.392 (isogenic to strain LTH 2590 and DSM 14272) was cultivated at 30°C. The levansucrase deletion mutant of *L. sanfranciscensis* TMW 1.392Δlev (Δlev) (Tieking et al. 2005b) was grown with erythromycin to maintain the insertion of the knock out plasmid (5 ppm in liquid medium, 10 ppm in agar plates). For the purpose of comparing the organic acids production of EPS-forming lactobacilli, a selection of 29 strains listed in table 8 were cultivated at 37 or 30°C, respectively, for 24 hours.

Bifidobacteria were cultivated anaerobically at 37°C in bifido-medium containing 10 g l^{-1} glucose, 10 g l^{-1} casein peptone, 5 g l^{-1} meat extract, 5 g l^{-1} yeast extract, 3 g l^{-1} K_2HPO_4*3 H_2O, 1 g l^{-1} Tween 80. 10 g l^{-1} sodium ascorbate, 0.5 g l^{-1} cystein-HCl, 1 ml of a vitamin mix (biotin, cobalamin, folic acid, nicotinic acid, panthothenic acid, pyridoxal, riboflavin, and thiamin, each 0.2 g l^{-1}) and 2 ml of a Mg/Mn/Fe-solution (5 g l^{-1} $MgSO_4$*7 H_2O, 1.875 g l^{-1} $MnSO_4$*H_2O and 1.250 g l^{-1} $FeSO_4$·*7 H_2O) were added sterile filtered after autoclaving. Beside Bb-12 (CHR Hansen, Hoersholm, Denmark) (*Bifidobacterium lactis* TMW 2.530) also *B. adolescentis* TMW 2.454 ^{T} (DSM 20083), *B. breve* TMW 2.447 ^{T} (DSM 20213), *B. infantis* TMW 2.448 ^{T} (DSM 20088), *B. lactis* TMW 2.462 ^{T} (DSM 10140), *B. longum* TMW 2.614^T (DSM 20219) and *B. longum* NCC2705 TMW 2.649 (Nestlé, Vevey, Switzerland) were used in this study.

Escherichia coli strains DH5α, BL 21 (Stratagene, Amsterdam, Netherlands) and Top10 (Invitrogen, Karlsruhe, Germany), were used for cloning procedures. *E. coli* was cultivated aerobically in LB medium at 37°C unless otherwise stated. Where appropriate, 100 µg ml^{-1} ampicillin for maintaining plasmids, and additionally 10 µg ml^{-1} chloramphenicol for cultivating BL 21 respectively, were added. Induction of gene expression was performed with isopropyl-β-D-thiogalactopyranosid (IPTG) or arabinose.

Strains were subcultured at least two times just prior to experimental use. The absence of contaminations was verified by the observation of an uniform colony morphology on agar plates and microscopy.

2.2 General molecular techniques

Cloning, DNA manipulations and agarose gel electrophoresis were performed as described by Sambrook et al. (1989). DNA of the lactobacilli and bifidobacteria was prepared according to Lewington et al. (1987). To check the quality of the DNA, a RAPD-PCR with M13V universal primers (table 1) according to Müller et al. (2001) was carried out. *E. coli* plasmid DNA was isolated with the Plasmid Mini kit from Qiagen (Hilden, Germany). DNA was amplified on a Thermo Cycler (PRIMUS 96 plus, MWG-Biotech AG, Ebersberg, Germany) using Taq-Polymerase and dNTPs from Qbiogene (Qbiogene; MP Biomedicals, Heidelberg, Germany), and primers (table 1) purchased from MWG Biotech (Ebersberg, Germany). In general, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM primer and 1.5 U Taq were used per reaction unless stated otherwise. PCR products were stained with ethidium bromide after electrophoretic separation on agarose gels and visualized with UV transillumination. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) or isolated from agarose gels using an E.Z.N.A. gel extraction kit (Peqlab Biotechnologie GmbH, Erlangen, Germany) following the instructions of the supplier. DNA was sequenced by SequiServe (Vaterstetten, Germany). Restriction enzyme digestions and ligations with T4-DNA ligase were performed following the recommendations of the suppliers (MBI Fermentas GmbH, St. Leon-Rot, Germany). Nucleotide and amino acid sequence analysis was carried out using the DNAsis for Windows software (Hitachi Software Engineetring Co, Yokohama, Japan). Electrocompetent *E. coli* cells were prepared according to Tieking et al. (2005b). *E. coli* transformations were performed by electroporation in 2 mm cuvettes at 2.5 kV, 25 µF and 200 Ω in a BioRad gene pulser (BioRad Laboratories, Hercules, USA). Electroporated *E. coli* cells were incubated for 1h at 37°C and intense shaking (220 rotations min^{-1}). Reagents and enzymes were used according to the suppliers recommendations unless otherwise stated.

Plasmids pGEX-4T-1 (Amersham Biosciences Europe GmbH, Freiburg, Germany) and pBAD/Myc-His B (Invitrogen) were used for cloning and expression of the *gtf106* genes and pGEX-4T-1 and pUC18 (Fermentas) were used for cloning and expression of the *sucP106* gene of *L. reuteri* TMW 1.106 in *E. coli*.

Table 1 Primers used for genetic manipulation.

Primer	Sequence (5`to 3`)	Use
M13V	GTTTTCCCAGTCACGAC	RAPD
616V	AGAGTTTGATYMTGGCTCAG	species identification
609R	ACTACYVGGGTATCTAAKCC	
DexreuV	GTGAAGGTAACTATGTTG	*gtf*-screening[1]
DexreuR	ATCCGCATTAAAGAATGG	
Dexreu_V1	CAAACAATGGGTAACAGC	PCR *gtf*106A
Dexreu_R1	CATCTGAATTATAACTAGCC	
RP_2R	ACGGCAAAGTAGAAACTG	inverse PCR[2] *gtf*106A
RP_4V	GATTCTTATGGTGAAAAAGG	
B_R2	CCTTGGTAGTGAATAAATC	PCR *gtf*106B
B_V3	TTCCTGATGGTAATTCTG	
ORF2_r	CTTGACTTGTGCCATAAG	
GTFAbloV	CAACAATATTATATTGACCC	amplifying probe A
GTFAbloR	TCACTATCCTTATTCCAC	
ORF2_V	TCATGAGAAACTTGGTTG	amplifying probe B
ORF2bloR	GATAATTCAGATGATGCAG	
dNklo3_V	GATGCAT**CCCGGG**TATTAACGGTCAACAATATTATATTGAC	cloning *gtf*106A
Klo3_R	ATATATGG**GCGGCCGC**TAGTTTTTTCTGATCAGCCAAATTAC	
Bklon_Vk	GGTGCAT**CCATGG**ATAATATCAAGAATGCTAATGTTCAATTA	cloning *gtf*106B
Bklon_R2	ATATCGAT**AGATCTC**ATCGTTGAAAATTGGTGCAATTTCACT	
eryV	GACTCAAAACTTTATTACTTC	verifying *lev*-knock out
Lev12V	ATTCGCCTTGTTTATATAG	
sydefd_V	GCTACGACGAGTTCGATC	PCR *glgP_Bb12*
spezGP_R	CGGTCGGAGCTGAAGTAG	
invGP_V	GAAGATGTACGAGCAGGG	inverse PCR[2] *glgP_Bb12*
invGP_R	GTATTCGTCGATCATGATGC	
GP_L_V	TTYTC5ATTGARTTTTTRCC	*glgP*-screening[3]
GP_L_R	TRTTWGCRCCATCAAGTG	
GP_L_V2	AAYGGNGG5YT5GG5AG	*glgP*-screening[3]
GP_L_R2	GT5CC5SW5GCYTCYTT	

Primer	Sequence (5`to 3`)	Use
SucPB_V	CTGCGCACCCGCTTCGAC	PCR
SucPB_2R	CCGGCAGGAAGAACTGCAC	*sucP_Bb12*
SucPL_V	GTTAATTACTTACTCTGAC	*sucP106*-
SucPL_R	CGRTTRATRTT5CG5CCTTC	screening[3]
SucPL_v	TAAAATTATTGGAGGATATC	PCR
SucPL_n	GGTAGTTTATTGAGAGAG	*sucP106*
SPkloU_V	GATGCAT**GAGCTC**GCCAATCAAAAACGAAGC	cloning
SPkloU_R	ATATATGG**GGATCC**TCA(ATG)$_6$TTTTTGTTCCATCACTTTTTC	*sucP106*
SPkloX_V	GATGCAT**GTCGAC**CAATCAAAAACGAAGC	cloning
SPkloX_R	ATATATGG**GCGGCCGC**TTTTTGTTCCATCACTTTTTC	*sucP106*
Uhp_V	TTYGGNATHGG5TAYGG	*uhp*-screening[3]
Uhp_R2	AA5GCNCC5GCRTTRTG	

with 5 for Inosin; Restriction sites are in bold; [1]Kralj et al. (2002), [2] with 3mM $MgCl_2$, 0.2-0.4 mM dNTP´s, 0.1-1 µM primers; [3] master mix modified as described in [2] and 5 preamplification cycles at low annealing temperature (38-42°C).

2.3 2D Gelelectrophoresis

Cells were harvested at an optical density (OD_{590nm}) of 0.4 through centrifugation (2,500*g for 5 min). Proteins in the supernatant were precipitated by addition of 20 % (v/v) trichloroacetic acid (30 % w/v) and incubation on ice for one hour. After centrifugation at 4°C (10,000*g for 10min) the pellet was resuspended in SDS buffer (0.9 % SDS, 100 mmol l^{-1} Trisbase, pH 8.6) with proteinase inhibitor (Pefablock^R; Merck, Darmstadt, Germany) and CHAPS. For extraction of whole cell protein, cells were disrupted by sonification in SDS buffer. The suspension was heated to 95°C for 5 min and cooled (0-4°C) again. The proteins were extracted by shaking 20 min in a dilution with threefold of thiourea lysis buffer (6.10 mol l^{-1} urea, 1.79 mol l^{-1} thiourea, 65.06 mmol l^{-1} CHAPS, 1 % DTT, 0.5 % Pharmalyte 3-10). The samples were centrifuged at 14,000*g for 30 min and the supernatants were stored at –80 °C. Two dimensional electrophoresis and protein quantification was performed according to Behr et al. (2007). Colloidal coomassie (Roti-Blue, Carl Roth GmbH & Co, Karlsruhe, Germany) stained proteins were excised from the gel and sent to the Zentrallabor für Proteinanalytik (Ludwig-Maximilians-Universität München, Germany) for a MALDI-MS analysis.

2.4 High Performance Liquid Chromatography (HPLC)–analysis of fermentation products

Supernatants of liquid fermentations were used directly for HPLC-analysis after centrifugation (14,000*g, 15 min). Analysis of fermentation products from dough fermentations was made from watery extracts of the dough samples. For this purpose, dough samples were diluted in ddH_2O (1:1) and centrifuged (8,000*g, 30 min). Perchloric acid (70 % wt/vol) was used (5 % vol/vol) to remove proteins.

Analysis and detection of carbohydrates and sugar alcohols was done using a Polyspher CHPB column (Merck) coupled to a refractive index (RI) detector (Gynkotek, Germering, Germany) and ddH_2O as mobile phase at a flow rate of 0.4 ml min^{-1}. The injection volume was 20 µl and the column temperature 70°C.

Detection and quantification of organic acids and ethanol was done with a Polyspher OAKC column (Merck, Darmstadt, Germany) and RI detection. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.4 ml min^{-1}, the injection volume was 20 µl and the column temperature was 70°C.

EPS in supernatants were detected and quantified using a Superdex 200 gel permeation chromatography (GPC) column (Amersham Pharmacia Biotech, Uppsala, Sweden) coupled to a RI detector as previously described (Tieking et al. 2003a). For EPS quantification isolated and purified EPS was used for calibration of the GPC column.

Monomer composition and content of EPS was determined after hydrolysis of isolated pure EPS. Pure EPS from fermentations in liquid media was obtained by ethanol-precipitation, dialyzation against ddH_2O (molecular weight cut-off 12-14 kDa) and lyophilisation as described before (Tieking et al. 2005a). Hydrolysis took place by incubation two hours at 80°C with H_2SO_4 added at a final concentration of 4 mol l^{-1} for glucan and 5 % $HClO_4$ in the case of fructan. After neutralization with 4 M KOH samples were analyzed by HPLC for detection and quantification of sugar monomers using a Polyspher OAKC column (Merck) for glucans and an Asahipak NH2P-50 E4 column (SHOWA DENKO Europe GmbH, München, Germany) able to separate fructose from mannitol, arabinose and xylose for fructans and dough samples. The Asahipak NH2P-50 E4 column was used with CH_3CN/H_2O 75/25 as mobile phase at a flow rate of 1 ml min^{-1} at room temperature. Trichloroacetic acid solution (20 %) was used to remove proteins according to the recommendations of the supplier. For the declaration of the estimated amount of EPS (glucan or fructan) in g l^{-1}, the conversion

factor 162 g mol^{-1} was used for glucose or fructose, respectively, and the used HPLC column was calibrated with hydrolyzed standard solutions of purified EPS of the respective strain.

EPS from dough fermentations were precipitated from watery dough extracts together with water soluble polysaccharides (WPS) originating from the flour and were calculated on the basis of the monomer (glucose or fructose) content of WPS extracted from chemically acidified control doughs as described previously (Korakli et al. 2001).

Detection and determination of oligosaccharides was done by high-performance anion-exchange chromatography and integrated pulsed amperometric detection (HPAEC-IPAD) with an AminoPac PA10 column and an ED40 electrochemical detector (both Dionex GmbH, Idstein, Germany) as described by Thiele et al. (2002a). A ternary gradient as described by Tieking et al. (2005c) with the solvents A: water (18 mOhm), B: sodium acetate (1 M) and C: NaOH (0.25 M) was applied. If necessary, samples (especially watery extracts of dough samples) were filtered (Whatman ready-to-use filter units; 0.2 µm; Schleicher & Schuell, Dassel, Germany) and diluted with ddH_2O (1/250) before injection of 20 µl.

Detection and separation of α-glucose-1-phosphate (G-1-P) and glucose-6-phosphate (G-6-P) was done with the Asahipak NH2P-50 E4 column (SHOWA DENKO Europe GmbH, München, Germany) with 10 mM sodium phosphate buffer pH 4.4 as eluent, a flow rate of 1 ml min^{-1} and a column temperature of 40°C. G-1-P (disodium salt, Boehringer, Mannheim, Germany) and G-6-P (disodium salt, Fluka) were used as external standards in the concentration of 20 mg ml^{-1}.

2.5 Characterization of the EPS of *L. reuteri* TMW 1.106

Pure EPS of *L. reuteri* TMW 1.106 was obtained and its monomer composition was determined as described by Tieking et al. (2005a).

Determination of molecular weight and size distribution and hydrodynamic diameter and its size distribution was done by A. Stocker (GSF-National Research Center for Environment and Health, Institute of Ecological Chemistry, Neuherberg, Germany) in coopoeration with Postnova (Postnova, Landsberg, Germany) by asymmetrical flow field-flow-fractionation (FFF) (Focus 1000; Postnova, Landsberg, Germany) coupled to multi-angle light scattering (MALS) (680 nm, PD 2020/PD 2000+; Precision Detectors, Bellingham, MA) and a RI detector (PN3140; Postnova, Landsberg, Germany). The channel dimensions were 335*60*40 mm (Postnova). The spacer had a trapezoidal geometry (thickness 250 µm). The accumulation wall was coated with a regenerated cellulose membrane RC 10 (Postnova) having a Mw cut off of 10 kDa. A precolumn filter unit was placed between channel and detectors containing a

polyetherketone frit with 2 µm pore-size and a teflon microfilter (0.1 µm pore-size) of regenerated cellulose filter paper (all Postnova). The carrier solvents were prepared from ultrapure water (TKA, Niederelbert, Germany). EPS was dissolved in the FFF carrier solvent (0.1 mol l^{-1} $NaNO_3$ containing 0.02 % sodium azide; both p.a. grade purity; Merck, Darmstadt, Germany) to a concentration of 5 mg ml^{-1} and centrifuged for 5 min at 5,000 *g at room temperature. Volumes of 10 µl were directly injected into a manual injection assembly (PN 5100, Postnova) containing a Rheodyne valve and a 10 µl sample loop. All measurements were performed at room temperature. The sample was introduced by a syringe pump at 0.2 ml min^{-1} for 1 min. During injection a focusing flow of 1.8 ml min^{-1} was applied. After injection, a relaxation/focusing flow of 1.8 ml min^{-1} was maintained for 5 min before elution was started with an outlet flow of 1 ml min^{-1}. During the first step of elution the cross-flow rate of 1 ml min^{-1} decreased linearly from 1 to 0 ml ml min^{-1} during 15 min. In a second step, the cross-flow rate was maintained at 0 ml min^{-1} for 10 min to ensure that the entire sample had been eluted. The outlet flow rate was kept constant at 1.0 ml min^{-1}.

The molecular weight was measured by static light scattering. The obtained light scattering data were processed by the Discovery32 software (Precision Detectors, Bellingham, MA). Molecular weight distributions were processed from laser scattering signals (15° and 90°LS) and RI signals. A $\delta n/\delta c$ value of 0.147 from literature was used for light scattering calculations of EPS. Polystyrolsulphonate standards (Postnova, Landsberg, Germany) and bovine serum albumin (BSA) (Sigma-Aldrich, Taufkirchen, Germany) were used for calibration of detectors. The hydrodynamic diameter was determined by dynamic light scattering that does not require any data for $\delta n/\delta c$ or calibration with standards. Shown are reproducible results of triplicate determinations.

One-dimensional ^{1}H-NMR spectra were recorded by P. Kaden (Technische Universität München, Department Chemie, Organische Chemie und Biochemie II, Garching, Germany) on a 600 MHz and a 900 MHz spectrometer (Bruker DMX600 and AVANCE 900, respectively). All spectra were recorded at 298K with a conventional probehead. EPS samples were dissolved in 99.97 % D_2O (Euriso-top). Proton spectra (at least 16 k data points, spectral width up to 12,000 Hz) were apodised by applying a square-sine function prior to Fourier transformation, a fifth-order polynomial baseline correction was performed when necessary.

2.6 Sucrose metabolism by *Lactobacillus reuteri* and *Bifidobacterium lactis* under different fermentation conditions

2.6.1 pH static fermentation experiments with *L. reuteri* TMW 1.106 and TMW 1.974

pH static fermentations were carried out in 350-ml jar fermenters (DCU, B. Braun Biotec International, Melsungen, Germany) each containing 300ml mMRS-sucrose medium (80-120 g l^{-1}) and were made at least in triplicates. Fermenters were inoculated at a 1 % level with cells that were washed once with fermentation medium. The incubation temperature was 40 °C. Medium was adjusted before inoculation to pH 4.0; 4.7; 5.4 and 6.2. 4 mol l^{-1} H_2SO_4 and 4 mol l^{-1} NaOH were added automatically to maintain the pH. A stirring rate of 100 rpm was used. Samples were taken aseptically at regular time intervals until 48 h after inoculation. Bacterial growth was monitored by measuring the optical density at 590 nm (OD_{590nm}) or cell counts. EPS and organic acids were quantified with HPLC as described in **2.4**. EPS produced under different pH values was characterized regarding monomer composition, molecular weight and structure as described above. Fermentation supernatants were further analyzed for the production of oligosaccharides with HPAEC-IPAD as described in **2.4**.

2.6.2 Fermentation with bifidobacteria under stress conditions

Overnight cultures of different *Bifidobacterium* strains were washed twice with fresh bifido-medium with sucrose (100 g l^{-1}) and used for inoculation to an OD_{590nm} of about 0.15. Fermentations with *B. lactis* TMW 2.530 were carried out at different temperatures using water baths at 30, 37, 40, 45 and 50°C and with increasing bile concentrations up to 67g l^{-1} ox gall (dried, unfractionated; Sigma). Growth was monitored by measuring OD_{590nm} at 30-min intervals in Spectrofluor (Tecan). In a second section various *Bifidobacterium* strains (**2.1**) were fermented at 37 and 45°C with and without bile (50 g l^{-1}). Samples were taken aseptically at regular time intervals until 48 h after inoculation. Growth was monitored by measurement of OD_{590nm} using a photometer (Pharmacia Biotech). The pH value of the cultures was determined and fermentation supernatants were analysed by HPLC as described in **2.4** for the production of EPS, organic acids and oligosaccharides.

2.6.3 Cell death kinetics with *L. reuteri* in medium with extreme initial pH values and purified EPS

To investigate a putative protective property of the EPS of *L. reuteri* TMW 1.106 against extreme pH, the microorganisms from an overnight culture in mMRS were washed twice with medium and resuspended in mMRS adjusted to pH 3.0 and pH 8.3 with and without isolated

and purified EPS (25 g l^{-1}) of *L. reuteri* TMW 1.106, respectively. The pH values of the autoclaved media were adjusted with 2 mol l^{-1} HCl and 2 mol l^{-1} NaOH and the media were then sterile filtered. The four cultures were incubated at 37°C until reaching the detection limit of the method used for cell counts. Cell counts were determined by the plate (mMRS) dilution method. Several dilutions in sterile tap water of each sample were plated (Eddy Jet plating machine, IUL Instruments, Germany) in duplicate and plates were incubated for 48 h at 37°C under controlled atmosphere (80 % N_2, 20 % CO_2). Results were expressed as cfu ml^{-1}.

2.6.4 Purification and characterisation of unknown peaks detected after fermentation of *B. lactis* TMW 2.530 and *L. reuteri* TMW 1.106 with sucrose under certain conditions

In preparative HPLC runs with supernatant of *B. lactis* TMW 2.530 fermented at 45°C the peak eluting after the system peak on the Polyspher OAKC column (at about 8.5 minutes) was collected manually behind the RI detector after injection of up to 80 µl and analysed with HPAEC-IPAD and the AminoPac column. Part of the collected fraction was hydrolyzed with HCl (5 M end concentration, 1 h at 80°C). HCl was removed by drying the sample at 70°C with a constant nitrogen flow. The residue was resuspended in ddH_2O to the same concentration as before hydrolysis. Hydrolysate and collected fraction were analyzed by OAKC and AminoPac (dilution rate 1:10). The resulting chromatograms were compared to those of external standards of HPLC-grade glucose and fructose (both Merck). Treatments with α-amylase (from *Bacillus*, Sigma) or α-glucosidase (from *Saccharomyces cerevisiae*, Sigma) were carried out by incubation of the collected fraction with the enzymes (450 Units α-amylase or 1mg ml^{-1} α-glucosidase) at 40°C for 3 h and subsequent analysis by HPLC (OAKC and AminoPac) to check whether the peak consisted of oligosaccharides. Relevant chromatograms were also compared with those of G-1-P and G-6-P as external standards on OAKC, AminoPac and NH2P-50 (**2.4** last paragraph) to investigate whether they result from the activity of a putative sucrose phosphorylase.

2.7 Detection and characterization of two glucansucrases from *L. reuteri* TMW 1.106

2.7.1 Nucleotide sequence analysis of two *gtf* genes from *L. reuteri* TMW 1.106

The sequence of *gtf106A* was obtained by primer walking starting from the sequence obtained with the primer pairs DexReu_V1/R and DexReu_V/R1 (table 1) constructed on the basis of the sequence of *gtfA* of *L. reuteri* LB 121 (Kralj et al. 2002). Primers RP_2R and RP_4V were

designed for inverse PCR (table 1). Chromosomal DNA of *L. reuteri* TMW 1.106 was digested with EcoRV and SacI respectively and ligated with T4 DNA-ligase (all MBI Fermentas GmbH, St. Leon-Rot, Germany), yielding circular DNA as template for inverse PCR. The partial sequence of *gtf106B* was obtained by primer walking starting with primers GTFB (Kralj et al. 2004a) and B_R2, constructed on the sequence of the second ORF obtained with primer RP_R2, and with primer pair B_V3/ORF2_r constructed on the basis of the sequence of *gtfB* from *L. reuteri* LB 121 taking care that it will not bind to the sequence of *gtf106A*.

2.7.2 Cloning, heterologous expression and purification of the glucosyltransferases of *L. reuteri* TMW 1.106

Primers ΔNklo3_V and klo3_R, containing the recognition sites for Cfr9I and NotI, respectively, were used to amplify an N-terminal deletion mutant of *gtf*106A (*ΔN-gtf*106A). Primers Bklon_V and Bklon_R2 containing the recognition sites for NotI and BglII, respectively, were used to amplify an N-terminal deletion mutant of *gtf*106B (*ΔN-gtf*106B) (for list of primers, see table 1). PCR products were digested with the appropriate restriction enzymes (NotI and BglII) and ligated into the corresponding site of the expression vector pGEX-4T-1 for *gtf106A* and pBAD/Myc-His B for *gtf106B*. The resulting constructs were used to transform *E. coli* DH5α as described in **2.2**. Clones were tested for insertion of the desired genes by PCR with primers targeting the catalytic cores of Gtf106A and Gtf106B. Plasmid from positive clones were isolated using E.Z.N.A. plasmid Miniprep KitI (Peqlab Biotechnology GmbH, Erlangen, Germany) and sequenced with primers pGEX-5´and pBAD-5´, respectively to ensure correct ligation and used to transform *E. coli* BL 21 and TOP 10, respectively. Cells of *E. coli* carrying the *ΔN-gtf*106A expression vectors were used to inoculate 200 ml of LB with 0.6 M sorbitol and grown at 37°C to an OD_{600nm} of 0.5. IPTG was added to a final concentration of 0.75µM and cells were further incubated at 30°C for 4 h. Cells of *E. coli* carrying the *ΔN-gtf*106B expression vectors were used to inoculate 200 ml of LB with 0.4M sorbitol and grown at 37°C to an OD_{600nm} of 0.6. Arabinose was added to a final concentration of 10 mM and cells were further incubated at 37°C for 4 h. Samples taken before induction and at the end of the induced growth period were tested for overexpression by SDS-PAGE (12 %) (Laemmli 1970).

For purification of glucansucrases, cells were harvested, washed with binding buffer A (see below) and broken by ultrasonification (5*30 sec with 30 % cycles and 70 % power). For purification of ΔN Gtf106A, the crude extract was loaded onto a GSTrap FF column

(Amersham Pharmacia Biotech) at a flow rate of 1 ml min^{-1}. The column was washed with 10 ml of binding buffer A (1*PBS; 10*PBS: 1.4 M NaCl, 27 mmol l^{-1} KCl, 100 mmol l^{-1} Na_2HPO_4, 18 mmol l^{-1} KH_2PO_4, pH 7.3) at a flow rate of 2 ml min^{-1}. The Glutathion S-transferase (GST) fusion proteins were eluted with a linear gradient to 100 % buffer B (50 mmol l^{-1} Tris, 10 mmol l^{-1} gluthation red., pH 8.0) in 2 ml. GST was removed from the ΔN-Gtf106A protein before elution by on-column cleavage overnight at room temperature with 50 Units mg^{-1} thrombin (Amersham Pharmacia Biotech). For purification of ΔN-Gtf106B, crude extract was loaded on a HisTrap column (1ml) using binding buffer A (0.5 mol l^{-1} NaCl, 50 mmol l^{-1} NaH_2PO_4, 500 mmol l^{-1} Imidazol) and eluted with elution buffer B (0.5 mol l^{-1} NaCl, 50 mmol l^{-1} NaH_2PO_4, 50 mmol l^{-1} Imidazol) using the same gradient as mentioned above. Fractions were collected and assayed for activity (see below). Active fractions were tested for purity by SDS-PAGE, pooled and applied to an Amicon Ultra centrifugal filter device with a nominal molecular weight limit of 50,000 (Millipore, Cork, Ireland) for desalting and removal of thrombin (from the ΔN-Gtf106A fractions). The protein concentration was determined with the Bradford method, using the BioRad Protein Assay (BioRad Laboratories, Hercules, USA) and bovine serum albumin as external standard.

2.7.3 Characterisation of the glucansucrases of *L. reuteri* TMW 1.106

Glucansucrase activity was determined at 45°C unless otherwise stated in reaction buffer containing 300 mmol l^{-1} sucrose and 1 mmol l^{-1} $CaCl_2$. Different pH values of the reaction buffer were prepared by mixing buffer A (100 mmol l^{-1} glacial acetic acid) and buffer B (100 mmol l^{-1} Na_2HPO_4). About 2.5 µg enzyme were incubated in 500 µl reaction buffer. Samples were taken at 60 s time intervals and stopped by adding 1/5 volume of 2 mol l^{-1} NaOH. The amounts of released fructose and glucose by the glucansucrase were determined enzymatically (Roche, Mannheim, Germany) in a microtiter plate assay using Spectraflour (Tecan, Salzburg, Austria). Enzyme activities were calculated as follows: total activity = [fructose released] (µmol)*(mg protein*min)$^{-1}$; hydrolysis activity = [glucose released] (µmol)*(mg protein*min)$^{-1}$; transferase activity = total activity - hydrolysis activity. The EPS produced through ΔN-Gtf106A was quantified after precipitation and characterised as described in **2.4** and **2.5**, respectively.

2.8 Identification and partial characterisation of *B. lactis* and *L. reuteri* sucrose phosphorylases

2.8.1 Preparation of crude extract from *B. lactis*, purification and identification of two enzymes

Crude extract preparation and enzyme purification was done as described essentially by Korakli and Vogel (2003). In the following section are only mentioned modifications of the procedure described there. For preparation of crude extract, 2 l of bifido-medium containing 20 g l^{-1} sucrose were incubated with *B. lactis* TMW 2.530 overnight at 42°C. Cells were harvested, washed, resuspended and subsequently disrupted by ultrasonification at 4°C as described. The cell debris were removed by centrifugation and the supernatant was used as crude extract. It was brought to 40 % saturation with ammonium sulphate, centrifuged at 14,000 g for 15 minutes. The pellet was resuspended in buffer A of Phenyl Sepharose column (50 mM sodium dihydrogenphosphate and 1 M ammonium sulphate, pH 6.5) and pellet as well as supernatant were screened for synthesis of the striking peaks on OAKC and AminoPac column by adding sucrose (final concentration 300 mM) and incubation at 42°C for 3 h and HPLC-analysis. Incubation of crude extract was used as positive control. The active supernatant was loaded on a phenyl Sepharose hydrophobic column (HiLoad™ 16/10, Pharmacia Biotech, Uppsala, Sweden) and a gradient from buffer A to buffer B (50 mM sodium dihydrogenphosphate, pH 6.5) was applied. Fractions were collected and screened for synthesis of the unknown two peaks later identified as hexose-phosphates by HPLC analysis with the OAKC and AminoPac column. Subsequently active fractions were pooled, dialyzed against the loading buffer of the next used column and purified with following columns in the order of nomination: hydroxyapatite column (Econo-Pac CHTII, BioRad, Munich, Germany), anion exchange column (UNO™ Q1R, BioRad) and gelpermeation chromatography (Superdex 200, Amersham Pharmacia Biotech). For the GPC the eluent was 50 mM sodium phosphate with pH 6.9 at a flow rate of 1 ml min^{-1}. After each purification step samples of the active fractions were applied for SDS-PAGE (12 %) (Laemmli 1970). Gels were stained with Coomassie (Roti-Blue, Carl Roth GmbH & Co) and additional by silver staining (Blum et al. 1987) if indicated. Digestion with trypsin and N-terminal sequencing by Edman degradation was done by Toplab GmbH (Martinsried, Germany). MALDI-MS was done by Lars Israel (Adolf-Butenandt-Institut (ZFP), Ludwig Maximilian Universität, München, Germany).

2.8.2 Enzyme assays with purified sucrose phosphorylase of *B. lactis* TMW 2.530 and partial characterization

Partial characterization of the purified enzyme. The purified enzyme (eluent 50 mM sodium phosphate, pH 6.9) was examined for the release of fructose from sucrose, with an enzymatic test and photometrical detection as described in principle in **2.7.3** (Roche, Mannheim, Germany). Assuming the peak to be a glucooligosaccharide, the release of fructose and not of glucose was expected and therefore an increase of extinction at 340 nm was not expected after addition of suspension 1 containing hexokinase and G-6-P-dehydrogenase but only after addition of suspension 2 containing phosphoglucomutase. The enzyme present in 100 µl of the fraction after GPC was mixed with 100 µl of 50 mM sodium phosphate (pH 6.9) and sucrose in concentrations ranging from 0 to 400 mM resulting in end concentrations of 0 to 200 mM. Incubation took place at 50°C. Samples were taken every 30 minutes and the reaction was stopped in a microtiter plate by adding 1/5 volume 2 mol l^{-1} NaOH. For the termination of the temperature and pH optimum sucrose concentration was 60 mM. For the termination of the pH optimum 50 mM sodium phosphate buffer of different pH values was prepared as described in **2.7.3**. Phosphorolysis activity was calculated from released fructose [(µmol)*(mg protein*min)$^{-1}$]. Measurement of released glucose was done as control to exclude contaminant invertase activity.

Verification of the production of G-1-P. The activity of the enzyme identified as sucrose phosphorylase was verified by HPLC with the Asahipak NH2P-50 column as described in **2.4** and thin layer chromatography (TLC). TLC was done as described by Weinhäuslel et al. (1997). In brief, 10 µl of standards (1-2 mg ml^{-1}) and 20 µl of samples were applied to silica plates (Kieselgel 60, Merck). The solvent was butanol/propanol/ethanol/water (2:3:3:3, by vol.). Detection was done with thymol reagent (0.5 g thymol, 90 ml ethanol, 5 ml H_2SO_4) at 150°C.

Action of sucrose phosphorylase of *B. lactis* on other sugars and transglycosylation activity. Purified sucrose phosphorylase of *B. lactis* TMW 2.530 was incubated overnight at 50°C in phosphate buffer (50 mM, pH 6.9) with raffinose (O-α-D-Gal*p*-(1-6)-O-α-D-Glc*p*-(1-2)-β-D-Fru*f*; Fluka), leucrose (O-α-D-Glc*p*-(1-5)-D-Fru*p*; Fluka), lactose (O-β-D-Gal*p*-(1-4)-D-Glc*p*; Merck), maltose (O-α-D-Glcl*p*-(1-4)-D-Glc*p*; Sigma), maltotriose (O-α-D-Glcl*p*-(1-4)-O-α-D-Glc*p*-(1-4)-D-Glc*p*; Sigma) or dextrins (maltodextrin from maize starch, Fluka) as sole carbon sources (final concentrations 100-200 mM) and by incubation with mentioned sugars (final concentration 50 mM) additionally to sucrose (final concentration 100 mM).

Analysis was done by HPLC (OAKC column). To test the possibility of the enzyme to transfer the glucose moiety of G-1-P without the presence of free inorganic phosphate on sucrose (transglycosylation activity), 0.5 ml of purified sucrose phosphorylase was dialyzed 5 h against 3*150 ml citrate buffer (50 mM, pH 6.9) and was then incubated with sucrose and G-1-P dissolved in water. Analysis was done by HPAEC-IPAD and TLC as described above.

2.8.3 Partial nucleotide sequence analysis of genes encoding GlgP and SucP

***B. lactis* TMW 2.530.** Partial sequences of *glgP* and s*ucP* of *B. lactis* TMW 2.530 were obtained by inverse PCR starting from the sequence obtained with the primer pair sydefd_V/spezGP_R for GP_B and SucPB_V/SucPB_2R for SucPB (table 1). The sequence of the primer sydefd_V was deduced from the aa sequence SYDEFD of the peptide obtained by Edman degradation (Toplab GmbH) (**2.8.1**). Primer spezGP_R was constructed on the basis of the sequence of conserved region LLQLRP in *glgP* of *B. longum* NCC2705 (NC 004307.2, AAN24421), *B. longum* DJO10A (http://genome.jgi-psf.org/mic_home.html, ZP_00121604) and *B. adolescentis* ATCC 15703 (NC 008618, AE014295.3). Primers sucPB_V/2R were constructed on the basis of the sequence of conserved regions LRTRFD/VQFFLP in *sucP* of *B. longum* NCC2705 (AE014295), *B. longum* DJO10A (jgi1514_blonDJO10A), *B. adolescentis* ATCC 15703 (AP009256) and *B. animalis* subsp. *lactis* (AAN01604). Primers invGP_V and invGP_ were designed for inverse PCR (table 1). Chromosomal DNA of *B. lactis* TMW 2.530 was digested with EcoRI, NdeI and BglII, respectively and ligated with T4 DNA-ligase (all MBI Fermentas GmbH), for inverse PCR.

***L. reuteri* TMW 1.106.** Primers SucP_LV and SucP__R (table1) were constructed on the basis of the conserved aa sequences LITYSD and EGRNINR of sucrose phosphorylase genes from *Streptococcus mutans* UA159 (AAN58596), *S. suis* 89/1591 (NZ AAFA02000035.1), *Leuconostoc mesenteroides* (D90314) and the sequences of *L. reuteri* JCM 1112 (NZ AAOV01000014.1), *L. reuteri* 1000-23 (NZ AAPZ00000000) and *L. reuteri* ATCC 55730 (DQ466578), upon their publication. To complete the sequence of the *sucP* gene of *L. reuteri* TMW 1.106 primers SucPL_v and SucPL_n (table1) were constructed targeting conserved regions before and after *sucP* in *L. reuteri* JCM 1112 and *L. reuteri* 100-23. For the identification of a putative *glgP* gene in the DNA of *L. reuteri* TMW 1.106 primers GP_L_V/R and GP_L_V2/R2 were constructed on the basis of conserved regions F(L)SIEFLP/TLDGAEI and NGGLGR/KEASGT in *glgP* genes of *L. casei* (ABJ70791), *L. salivarius* (ABE00097), *L. lactis* (AE006303), *Streptococcus pneumoniae* (NP_269418),

Geobacillus stearothermophilus (BAA19591), *B. adolescentis* (BAF38811), *B. longum* (AAN24421) and *E. coli* (NP_417884).

Prediction of signal peptides or transmembrane domains was done with the features SignalP (http://www.cbs.dtu.dk/services/SignalP/) for signal peptides and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) for transmembrane domains.

2.8.4 Cloning and heterologous expression of sucrose phosphorylase of *L. reuteri* in *E. coli* and preliminary characterization of the purified enzyme

Primer pairs SPkloU_V/R and SPkloX_V/R (table 1), containing the recognition sites for SacI/BamHI and SalI/NotI, respectively, were used to amplify the *sucP106* gene from *L. reuteri* TMW 1.106. Further procedure was done as described essentially in **2.7.2**. The vectors pUC18 and pGEX-4T-1 with *E. coli* TOP 10 and BL 21, respectively, were used for cloning. Clones were tested for insertion of the desired gene by PCR with primers sucPL_V and the corresponding SPklo-reverse primer. Plasmids from positive clones were sequenced with primers M13 uni (-43) and pGEX-5`, respectively to ensure correct ligation. Overexpression was induced with 100 to 1000 µM IPTG in LB-medium with or without sorbitol (0.4 M). Further incubation took place at 28 or 30°C and overexpression levels were tested by SDS-PAGE (12 %) (Laemmli 1970). For purification of heterologously expressed *sucP106* a HisTrap column (for pUC18) or a GSTrap FF column (for pGEX-4T-1) were used. Active fractions were tested for purity by SDS-PAGE, pooled and applied to an Amicon Ultra centrifugal filter device with nominal molecular weight limit of 50,000 (Millipore, Cork, Ireland) for desalting and buffer exchange (phosphate buffer, 50mM, pH 6.9). The protein concentration was determined with the Bradford method.

Activity of SucP106 from the pUC18/*E. coli* TOP 10 system was determined spectrophotometrically through the reduction of $NADP^+$ (Sigma) modifying the method described by Koga et al. (1991). The modification consisted in decoupling the activity of SucP106 and the enzyme reactions of phosphoglucomutase (PGM) (from rabbit muscle, Sigma) and G-6-P-dehydrogenase (G-6-P-Dh) (from bakers yeast, Sigma). Fractions with SucP106 were incubated with the equal volume phosphate buffer (50 mM, pH 6.9) and sucrose (final concentration 300 mM) overnight at 42°C. Samples of 50 µl were inactivated by the addition of 1/5 volume of 2 mol l^{-1} NaOH before adding the equal volume of phosphate buffer (50 mM, pH 6.9) with $MgCl_2$ and EDTA (final concentrations 15 mM and 1 mM, respectively). Further 1 µl of $NADP^+$ (30 mg ml^{-1}), G-6-P-Dh and PGM (both 1.6 Units $µl^{-1}$) were added. Incubation and measurement took place at room temperature in a Spectraflour

(Tecan). Phosphorolysis activity was calculated from the reduction of $NADP^+$ to NADPH [(µmol)*(mg protein*min)$^{-1}$].

2.8.5 Construction of primers targeting hexose- and glycerol-3-phosphate transporters

Primers Uhp_V/R2 were constructed on the basis of the conserved regions FGIGYG and HNAGAF in the hexose-phosphate transporter UhpT (CAA50595) of *Lact. lactis* NCDO 763 (ML3) and the glycerol-3-phosphatase transporters of *Lact. lactis* subsp. *cremoris* SK11 (ABJ72147) and *L. brevis* ATCC 367 (ABJ63273) and used for a PCR screening of *Bifidobacterium* and *Lactobacillus* strains.

2.9 Effect of EPS on dough and bread quality through *in situ* production with *L. sanfranciscensis*

The work described in this section was done in cooperation with the partners of the AiF project (AiF-FV 14037 N) Michael Seitter, Steffi Vogelmann and Christian Hertel (Lebensmittelmikrobiologie, Universität Hohenheim, Stuttgart, Germany).

2.9.1 Predough fermentations

Predoughs were prepared with a dough yield (DY = mass of dough/mass of flour*100) of 200. For inoculation of doughs, 10^9 cells from an overnight culture were washed, resuspended in 10 ml of saline buffer and added to the dough to obtain about 10^7 cells per g dough. Control doughs were made and designated as shown in table 2. Yeast invertase (Fluka Riedel-de Haën Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) was applied to Δlev Inv predoughs in a concentration resulting in comparable sucrose hydrolysis as observed in WT predoughs. The data for the chemically acidified and the direct control doughs were kindly provided by M. Seitter and Vogelmann, S (University of Hohenheim, Germany). All doughs were incubated for 22 h at 30°C.

2.9.2 Determination of pH and cell counts in sourdough

At the beginning and end of fermentation, the pH values of the doughs were determined with a glass electrode, and cell counts determined by plating two mMRS-agar plates of three appropriate dilutions. To verify that Δlev mutations were not re-mutated during dough fermentation without erythromycin through the loss of the integrated plasmid, cell counts for Δlev were carried out on mMRS-agar plates with and without erythromycin (10 ppm). To verify that *L. sanfranciscensis* TMW 1.392 and the mutant were the dominant strains in the dough fermentations, colonies of *L. sanfranciscensis* TMW 1.392 wildtype and the Δlev

mutant were randomly checked through RAPD-PCR with M13V universal primers (table 1) and partial sequencing of the 16S rDNA (table 1). The Δlev mutant was controlled for carrying the knock out plasmid disrupting the levansucrase gene through PCR as described by Tieking et al. (2005b) using primers eryV and Lev12V. To ensure the absence of yeasts in the predoughs, YGC (5 g l^{-1} yeast extract, 20 g l^{-1} glucose, 0.1 g l^{-1} chloramphenicol) plates were made and incubated for at least 48 hours at 30°C. All predough fermentations were carried out at least two times.

Table 2 Composition of predoughs (all amounts are in % on flour base) and resulting bread doughs

Predough	wheat flour blend [a]	Water	strain	sucrose	additives	bread dough designation
WT	**100**	**100**	**wildtype**	**10**	**-**	**WT**
Δlev	**100**	**100**	**mutant**	**10**	**-**	**Δlev**
Δlev Inv	**100**	**100**	**mutant**	**10**	**yeast invertase (0.2)**	**Δlev Inv; Δlev Inv EPS**
WT 0%	**100**	**100**	**wildtype**	**-**		**WT 0%; WT 0% EPS**
CAC[b]	**100**	**100**	**-**	**-**	**lacitc and acetic acid (1:1) to pH 3.6; antibiotics**	**CAC**

[a] flour characteristics: 10.3 % protein (Nx5.7), 27.5 % wet gluten content, 11.9 % moister, 0.55 % ash content, >5 mg kg^{-1} ascorbic acid; [b] chemically acidified control

2.9.3 Analysis of fermentation products from sucrose

Analysis of fermentation products was made from watery extracts of freeze-dried dough samples which were dissolved in ddH_2O (1:3). Levan, organic acids and carbohydrates in the extracts were quantified by HPLC as described in **2.4**. 1-kestose was quantified with HPAEC-IPAD according to Thiele et al. (2002a) as described in **2.4**. 1-kestose as external standard was purchased from Fluka (Fluka Riedel-de Haën Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany).

2.9.4 Preparation of bread dough and bread making

Bread doughs were prepared with 10 % predough, 1.8 % (flour base) salt, 2.0 % dry yeast (Uniferm GmbH & Co. KG, Werne, Germany), 3.0 % sodium propionate (Fluka Riedel-de Haën Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) and water up to an optimal dough consistency of 500 Brabender Units (BU) to permit a high water content and comparability. For mixing a spiral kneader (Oase, Osnabrück Germany) was used. Additional breads from the control predoughs Δlev Inv and W T0% were made by adding 0.3 % (flour base) purified levan to the bread dough. Finally six different bread doughs and breads were

compared (table 2). Dough maturing took place at room temperature for 30 min. Pieces of 600 g were then subsequently rounded, moulded and panned in sealed pan sets. Proofing occurred at 30°C with 80 % relative humidity for 1 h. Breads were baked in a multi-deck oven (Wachtel, Hilden, Germany) at 230°C for 1 h. After 2 h of cool down at room temperature, breads were shrink-wrapped in vapour proof PE film tube (0.2 mm, VWR, Darmstadt, Germany) and stored at 25°C.

2.9.5 Evaluation of dough rheology

For the evaluation of the dough rheology and comparison of the performance of WT and Δlev in this regard, the following instruments were used: rheofermentometer (Chopin, Villeneuve La Garenne, France), Brabender Farinograph (Brabender GmbH & Co. KG, Duisburg, Germany), texture analyser (TA-XT2, Stable Microsystems, Surrey, UK) and rapid viscoanalyser (RVA-4, Newport Scientific, Warriewood, Australia). Bread dough stickiness was determined according to Huang and Hoseney (1999). Measurements of extensibility and resistance to extension were done by the micro-extension procedure (Kieffer et al. 1981) and were replicated at least three times for each bread dough. To ensure careful sample preparation for the micro-extension measurements, plastic stripes belonging to the Kieffer mould were used to take out the dough stripes. A hook displacement speed of 2 mm min^{-1} was applied and eight to ten stripes of every dough were measured and averaged. The RVA experiments were done according to ICC method 162 with mixing for 10 s at 960 rpm and than 160 rpm. For testing different EPS, 3.418 g flour (11.9 % moisture content) and 10.5 mg EPS (0.3 % flour base) were mixed with 25 ml water, for testing different predoughs, 10 % of the flour were replaced with freeze-dried dough samples.

2.9.6 Measurement of bread characteristics

The loaf volume was measured by applying the rapeseed displacement method. For each charge, three breads were measured three times each. Texture profile analysis (TPA) was performed using the XT2 Texture Analyzer (Stable Micro Systems, Surrey, UK). The parameter hardness was chosen to describe the crumb firmness and was determined according to the AACC method 74-09 (AACC 1999) from two equal loaves. The retrogradation of starch was measured by differential scanning calorimetry (DSC) according to Leon et al. (1997) with the PerkinElmer DSC 6 (PerkinElmer, Waltham, Massachusetts, USA) and corresponding sealed stainless steel capsules. Hardness and retrogradation enthalpy were fitted to the Avrami equation, which describes the equilibrium crystallization of high polymer

melts. The data was analyzed by means of a restricted model with *n* set to 1 (Rojas et al., 2001) with f=y0+a*(1-exp(-b*x^1)) for TPA and f=a*(1-exp(-b*x^c)) for DSC.

2.9.7 Production of levan

Purified levan of *L. sanfranciscensis* TMW 1.392 was needed for control experiments. *L. sanfranciscensis* TMW 1.392 was incubated in Homohiocchii medium with 100 g l^{-1} sucrose (autoclaved) at 30°C for 48 h. The pH was adjusted to 5.4 (Tieking et al., 2005b) with 2 N NaOH several times during fermentation. Levan isolation and purification was carried out according to Korakli et al. (2003) by precipitation with ethanol for 48h and dialysis for 48h.

2.10 Quantification of lactic and acetic acid produced by EPS forming lactobacilli

29 EPS-forming lactobacilli were examined for their acid production from sucrose. Formation of EPS had been previously assumed by the production of slime and a shiny colony morphology on agar plates containing 80 g l^{-1} sucrose (F. Waldherr, Technische Mirkobiologie, Technische Universität München, Germany, personal communication) or by cultivation in liquid sucrose-medium with 100g l^{-1} sucrose as the sole carbohydrate source followed by GPC analysis as described in **2.4**. For selected strains also the monomer composition of precipitated and hydrolozed EPS had been determined as described previously (Tieking et al. 2003, 2005a). Precultures of EPS forming lactobacilli were washed with sterile tap water and used to inoculate 1 ml mMRS-sucrose medium with 100 g l^{-1} sucrose (autoclaved, pH 5.6) as the sole carbohydrate source in the ratio 2:100. After anaerobical incubation at 30 or 37°C, respectively, for 24 h, final pH was measured and cell counts were made as described in **2.6.3**. Amounts of lactate and acetate produced were determined by HPLC as described in **2.4** and the fermentation quotient (FQ) was calculated (ratio of lactic to acetic acid).

2.11 Influence of dough yield, pH, sucrose concentration and different fermentation substrates on *in situ* EPS production during sourdough fermentation through *L. reuteri* and *L. sanfranciscensis*

2.11.1 Preparation of doughs for the purpose of EPS production

Doughs were prepared as described in **2.9.1** with different types of flours, sterile tap water, 10 % sucrose (flour base) and fermented with *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392 for 48 h at 37 or 30°C, respectively. Control doughs to separate the effects of organic acids and incubation on the amount of water soluble polysaccharides (WPS) extracted

from the flour, were chemically acidified with lactic acid (80 %; Riedel-de Haen Fluka Riedel-de Haën Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) and glacial acetic acid (100 %; Merck, Darmstadt, Germany) (2:1) and adjusted to pH 3.5, unless indicated otherwise. To evaluate the influence of dough yield, pH, sucrose fed-batch and fermentation substrate on *in situ* EPS production, doughs with different formulas were prepared. For doughs with different DY (160, 220, 500, 800) the same wheat flour blend as for the experiments described in **2.9** was used. For the other experiments doughs were prepared with a strong wheat flour (WS), mentioned wheat flour blend (WB; with 60 % WS), a rye-wheat mixture (RW) (50:50 with WS) and with rye bran (RB) and different DY were chosen for good manageability. DY was 200 for WS and WB, 250 for RW and 367 for RB. For the experiments with pH control, the pH was adjusted to pH 4.0 and 4.7 for *L. reuteri* TMW 1.106 and 5.4 and 4.7 for *L. sanfranciscensis* TMW 1.392 according to optimal EPS production through the strains/enzymes (this work and Tieking et al. 2005b). During fermentations the pH was adjusted several times with 2 N NaOH. For the fed-batch fermentations additional 2 g sucrose were supplied after 8, 24, 32 and 48 hours, depending on the time of fermentation. Samples were taken at the start of fermentation, at 8, 24, 48 and 72 hours. Fermentations at the different conditions were carried out at least two times with *L. reuteri* TMW 1.106 and one time with *L. sanfranciscensis* TMW 1.392 if trends were clearly the same as with *L. reuteri* TMW 1.106.

2.11.2 Analysis of the dough samples

Determination of pH values and cell counts were carried out as described in **2.6.3**. Quantification of EPS, organic acids, ethanol and residual sucrose in watery extracts of dough samples was done as described in **2.4**. Oligosaccharides in watery dough extracts from sourdough and chemically acidified control doughs were detected with HPAEC-IPAD and identified partially by comparison with external standards and acceptor reaction products of ΔN-Gtf106A as described below.

2.11.3 Enzymatic synthesis of oligosaccharides and analysis of oligosaccharides produced during dough fermentations

Oligosaccharides were synthesized by ΔN-Gtf106A from *L. reuteri* TMW 1.106 in reaction buffer containing 150 mM sucrose and 100 mM of a probable acceptor. As acceptors substances were chosen: maltose, arabinose, xylose and raffinose that are typically present in flours as well as isomaltose, galactose, lactose, melibiose, mannose and rhamnose. The reaction buffer (pH 5.4) was prepared by mixing buffer A (100 mM glacial acetic acid, 1 mM

$CaCl_2$) and buffer B (100 mM Na_2HPO_4, 1 mM $CaCl_2$); incubation took place at 45°C for 62 h. Products of acceptor reaction were determined by HPAEC-IPAD. 1-kestose (Fluka), isomaltose (Sigma), linear maltooligosaccharides (G_4–G_{10}, Sigma) and erlose (1^F-β-fructofuranosylmaltose) (Fluka) were used as external standards.

2.11.4 Stability of the EPS during storage of the dough at 4-8°C

To evaluate the stability of the EPS produced *in situ*, doughs were stored after fermentation in vapour proof PE bags (0.2 mm) at 4 to 8 °C. After 3 (alternatively 1.5) months, the doughs were again analysed as described in **2.11.2**.

3 RESULTS

3.1 Sucrose metabolism of *Lactobacillus reuteri* and *Bifidobacterium lactis* under different fermentation conditions

3.1.1 Effect of pH on the production of EPS and oligosaccharides by *L. reuteri* TMW 1.106 in relation to its growth

L. reuteri TMW 1.106 was grown at constant pH in mMRS-sucrose (100 g l^{-1}) medium (40°C) at pH values of 4.0, 4.7, 5.4 and 6.2 in order to define the conditions for maximal EPS production. The average yields of EPS after pH static fermentations are shown in figure 2A. Maximal EPS production was observed between pH 4.7 and 5.4. Yields of up to 22 g l^{-1} which correspond to 73 mmol l^{-1} glucose in glucan could be obtained after 48 h. The amount of EPS formed decreased with further increase of the pH value. The average specific yields of EPS after pH static fermentations are shown in figure 2B. Although growth was also highest at pH 4.7 and 5.4 (up to $2.5*10^9$ cfu ml^{-1}) and the total yield of glucan will certainly depend on final density as every cell is supposed to possess a glucansucrase, the EPS yields per OD unit showed no consistent relationship of EPS production and cell density when the pH for growth varied. This observation indicates that the pH influences expression or activity of the glucansucrase. The assumption is further supported by the fact that the synthesis of EPS proceeded after reaching the stationary growth phase as depicted for one representative fermentation in figure 2C. Precipitation, dialysis and hydrolysis of the EPS confirmed that the polymer formed was a HoPS, which consisted predominantly of glucose and about 5 % fructose. At pH 4.0, little EPS was produced and cells grew to $1.9*10^8$ cfu ml^{-1} only. Hydrolysis of sucrose and consumption of glucose and fructose at this pH value was low compared to fermentations at other pH values, consequently, the amounts of the fermentation products lactate and acetate were lower (fig. 2D). At the other pH values, sucrose was almost totally consumed during fermentation. However, in independent experiments variations in lactate, acetate and ethanol production were observed.

As HoPS are synthesized by glycosyltransferases which further can produce oligosaccharides, culture supernatants from pH static fermentations were analyzed with HPAEC-IPAD for the formation of oligosaccharides. *L. reuteri* TMW 1.106 produced small amounts of 1-kestose (β-D-furanosyl-(1-2)-β-D-fructofuranosyl-α-D-glucopyranoside), leucrose (D-glucosyl-α-(1-5)-D-fructopyranose) and smaller quantities of other substances detected with HPEAD-IPAD that were not further characterized. The amount of leucrose varied slightly with the pH value

of the medium and followed the trend of the glucan to increase at higher pH values (data not shown).

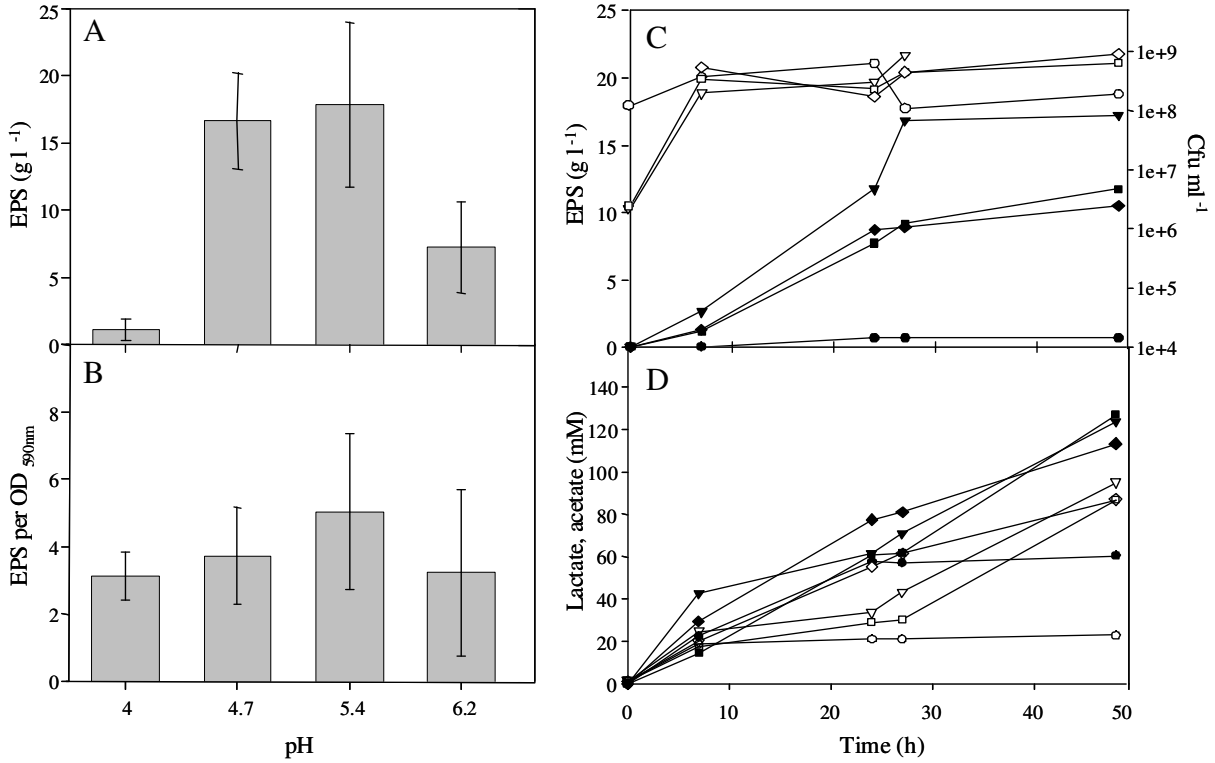

Figure 2 A EPS yields (g l^{-1}) determined with GPC after 48 h of pH static fermentations with *L. reuteri* TMW 1.106 at different pH values and 100 g l^{-1} sucorse. **B** Specific EPS yields per growth (OD_{590nm}). Data are means and standard deviations of four independent fermentations. **C** Fermentation kinetics of growth (cfu ml^{-1}, white symbols) and formation of EPS (black symbols) and **D** lactate (black symbols) and acetate (white symbols) at pH 4.0 (circles), 4.7 (triangles), 5.4 (squares) and 6.2 (diamonds).

3.1.2 EPS synthesis by and growth of *B. lactis* TMW 2.530 at elevated temperature

Parallel studies were focused on the sucrose metabolism of *B. lactis* TMW 2.530 (Bb-12 from Chr. Hansen). *B. lactis* TMW 2.530 was incubated at 30, 37, 40, 45 and 50°C. Optimal growth took place at 40°C, where OD_{590nm} of about 4.5 and a pH of 3.7±0 were reached after 48 hours of fermentation. Growth at 37°C and 30°C was comparable (OD_{590nm} 4.0; pH 3.8±0 at 37°C). At 45°C the strain only grew to OD_{590nm} 3.2 (pH 4.2±0.2) and the lag-phase increased distinctly from 4 h at 37 and 40°C to 24 h. At 50°C only very little growth was seen (OD_{590nm} 0.2) and likewise the production of lactate and acetate was low, leading to a final pH value of 5.4±0.4. The ratio acetate/lactate reached the theoretical value of 1.5 best after 48 hours of fermentation at 40°C (1.6), at 37 and 45°C the ratio was 1.7, at 50°C 2.7 (not determined at 30°C).

No formation of high molecular weight EPS through *B. lactis* TMW 2.530 from sucrose could be detected under reference conditions (37°C). After 48 h of fermentation at 45°C and 50°C little amounts of EPS, in the range of mg ml^{-1}, could be detected in the supernatants with GPC (data not shown). The EPS of *B. lactis* TMW 2.530 eluted at the void volume of the GPC column comparable with EPS from *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392, indicating a high molecular weight EPS with an estimated molecular weight above 10^6. Based on the peak areas of GPC, most EPS was formed at 45°C and less than a half at 50°C. Hydrolysis of the precipitated and dialyzed EPS from a fermentation at 50°C and comparison of the amount of glucose and fructose in bifido-medium with sucrose that had been treated the same way, lead to the suggestion, that *B. lactis* TMW 2.530 synthesized a heteropolysaccharide (HePS) from sucrose that contained fructose and glucose in a ratio about 1.8:1 (fig. 3).

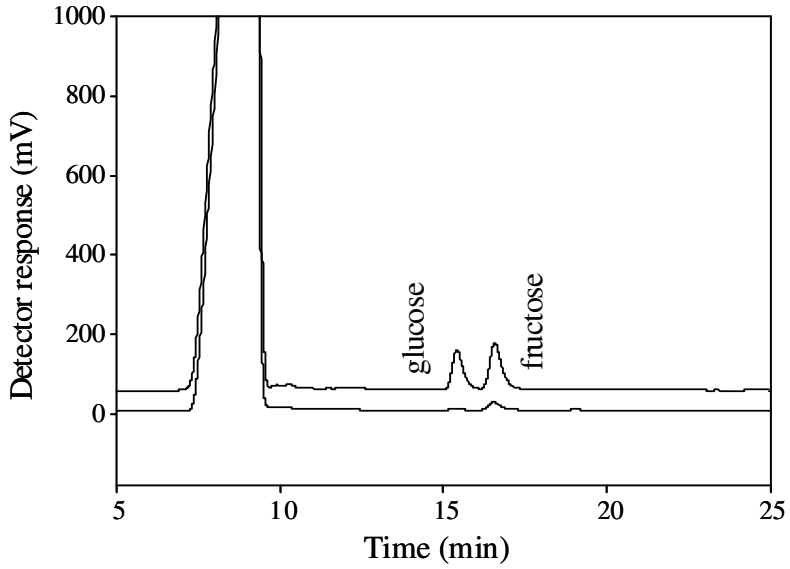

Figure 3 OAKC separation of precipitated, dialyzed and hydrolyzed material from unfermented medium with sucrose (lower line) and supernatant of *B. lactis* TMW 2.530 after fermentation at 50°C in medium with sucrose (upper line, offset 50 mV). Peaks were assigned by the use of external standards.

3.1.3 Effect of EPS in medium with extreme initial pH value on cell death of *L. reuteri*

To examine a possible protective function of the EPS against pH stress, *L. reuteri* TMW 1.106 was incubated in mMRS adjusted to extreme pH values in the presence or absence of EPS isolated from *L. reuteri* TMW1.106 culture supernatants. The addition of EPS to mMRS improved the long-term viability of *L. reuteri* TMW1.106 in media with initial pH values of 3.0 and 8.3 (fig. 4). The pH value of the latter one decreased to 4.4 and 4.6 with and without EPS, respectively, due to lactic acid production of 71 and 69 mmol l^{-1}. In contrast only 11 and

19 mmol l^{-1} lactic acid was detected in the experiment with an initial pH of 3.0 with and without EPS, respectively. The delay of cell death through added EPS was higher (twice the time) in the experiment with an initial pH of 8.3 than in the one with an initial pH of 3.0. Acetic acid was produced in the range from 11 to 18 mmol l^{-1} with and without EPS, respectively.

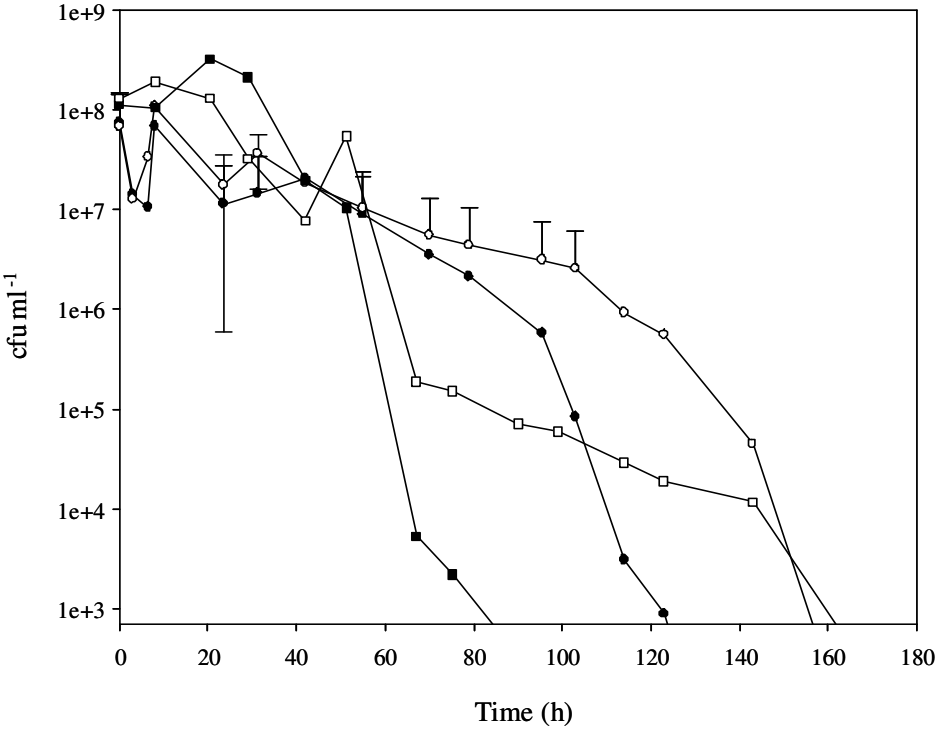

Figure 4 Effect of EPS on survival of *L. reuteri* TMW 1.106 in mMRS medium with an initial pH of 3.0 (circles) or 8.3 (squares) with (white symbols) and without (black symbols) addition of EPS (25 g l^{-1}) of *L. reuteri* TMW 1.106. Data are means and standard deviations of two independent experiments.

3.1.4 Detection of unknown substances produced from sucrose by *L. reuteri* and *B. lactis*

Analysis of the culture supernatants of *L. reuteri* TMW 1.106 after pH static fermentations with HPAEC-IPAD led to the detection of two unknown peaks eluting after about 36 and 39 min (fig. 5, upper line) that were not detected after fermentation without sucrose but increased with augmented sucrose supply (unpublished data Körber/Gänzle, Technische Mikrobiologie, Technische Universität München, Germany) and where therefore thought to be sucrose derivatives. Detectable amounts of these substances were soonest observed after 12 h of fermentation and showed an opposite amount distribution (quantified as peak areas) as the EPS built from the strain at the respective pH values (fig. 6A). The production/detection of these unknown substances, equal to the one of the EPS, seemed not to be growth related (fig.

6B). This trend was proven through various batches of pH static fermentations with *L. reuteri* TMW 1.106 and *L. reuteri* TMW 1.974 (data not shown).

When analysing the supernatants of *B. lactis* TMW 2.530 after fermentation at different temperatures with HPAEC-IPAD for the formation of oligosaccharides, the most obvious detected peaks had the same retention time as the two unknown peaks seen in fermentation supernatants of *L. reuteri* TMW 1.106 after fermentation with sucrose (fig. 5, lower line). The peak areas increased with increasing sucrose concentrations from 2 to 20 g l^{-1} in the fermentation medium (data not shown). The higher the chosen fermentation temperature, the more of the two conspicuous substances was detected in supernatants of *B. lactis* TMW 2.530 (see chapter **3.1.7**).

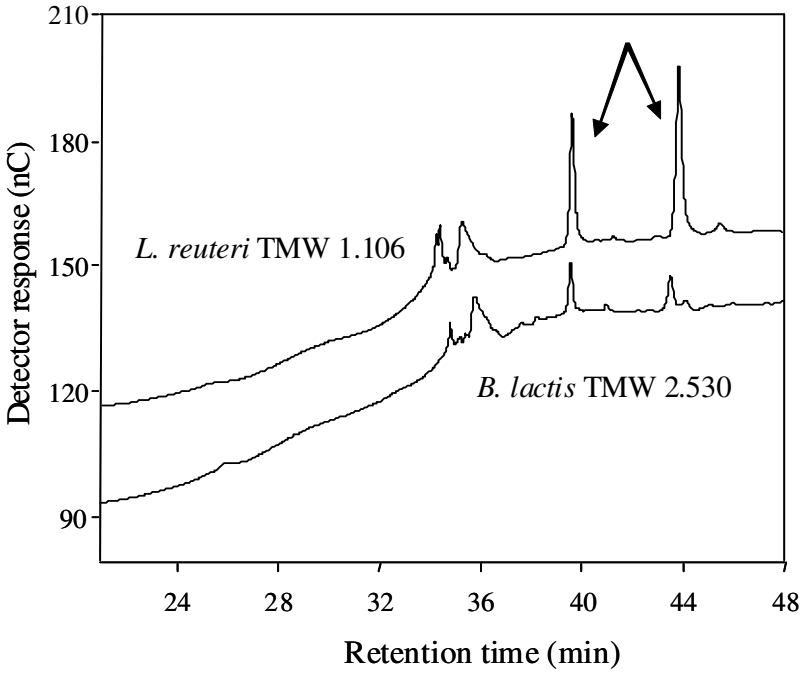

Figure 5 Separation of fermentation supernatants of *L. reuteri* TMW 1.106 after pH statical fermetentation at pH 6.2 (upper trace) and *B. lactis* TMW 2.530 (lower trace) grown on mMRS-S medium by HPAEC-IPAD. Under the prevailing conditions of the HPLC run, the peaks corresponding to the two unknown substances, later identified as G-1-P and G-6-P, eluted at about 39.5 and 44 minutes.

However, for both species, the amounts and the predominance of the first or the second peak varied greatly at repeated fermentations at the same conditions, indicting that there could be a relation between the two substances and that they were convertible in each other or stayed in some dependency. For *L. reuteri* detection of the first peak was more frequent, whereas if both peaks were detected (at higher pH values) the second peak was greater than the first one. For *B. lactis* (and all other examined bifidobacteria strains) occurrence of the second peak was generally more frequent than of the first one.

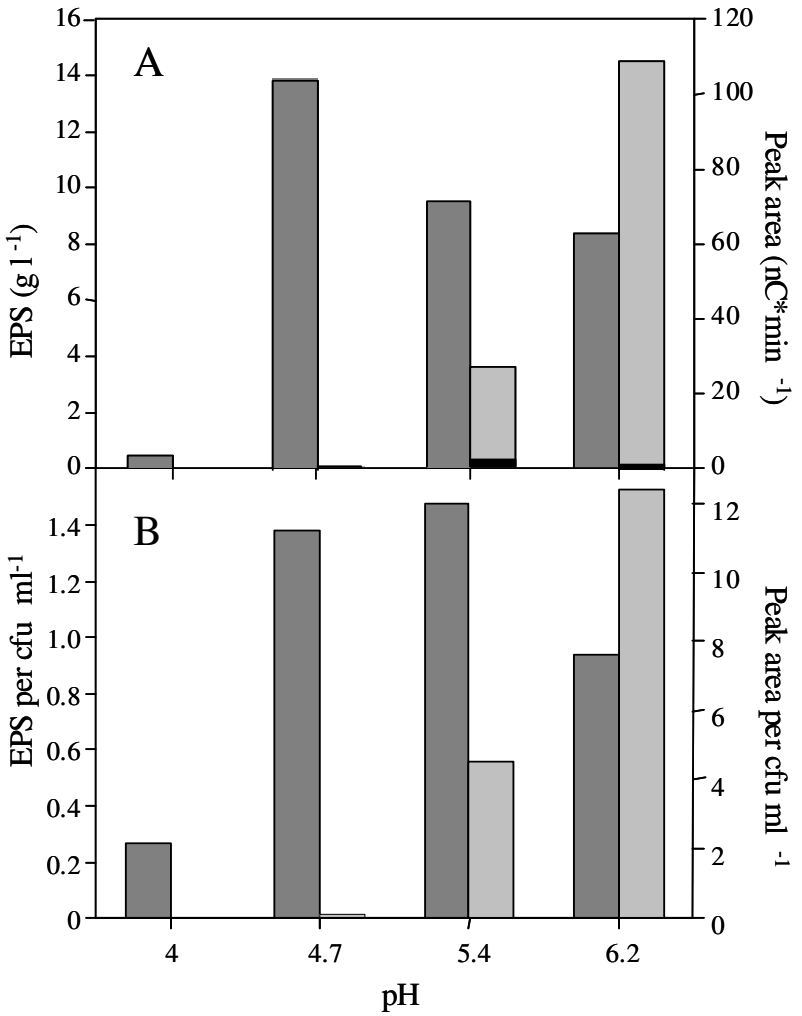

Figure 6 A EPS yields (g l^{-1}) (dark grey bars) and peak areas (nC*min) of the two unknown peaks detected with HPAEC-IPAD at about 36 min (black bars) and 39 min (grey bars) after 48 h of a representative pH static fermentation at different pH values. **D** Specific yields of the not identified substances (sum of peak areas) per growth (cfu ml^{-1}). The depicted trends were confirmed qualitatively through various independent experiments.

3.1.5 Characterization of the unknown substances produced by *L. reuteri* and *B. lactis*

Closer examination of OAKC chromatograms from supernatants of *B. lactis* and *L. reuteri* led to the conclusion, that the unknown substances were also detectable with this method, in fact as one single peak at about 8.5 min close to the peak resulting from the perchloric acid treatment of the supernatant (about 8 min). This peak could only be detected after fermentation under the same conditions, e.g. elevated temperatures for *B. lactis,* when also the two peaks on AminoPac chromatograms were detected (fig. 7A). To prove this, the mentioned peak from supernatant of *B. lactis* after fermentation under elevated temperature was collected after separation on OAKC (without perchloric acid treatment of the supernatant) (fig. 7B lower line) behind the RI-detector and after verification of its purity with the same column

(fig. 7B upper line) the collected peak was analyzed with HPAEC-IPAD giving the two peaks known from AminoPac chromatograms (fig. 7C). Using the OAKC column had the advantage of being less time consuming, easier to handle and allowing the collection of the eluting substances in higher concentrations for further characterization (with the restriction that the two peaks were not separated).

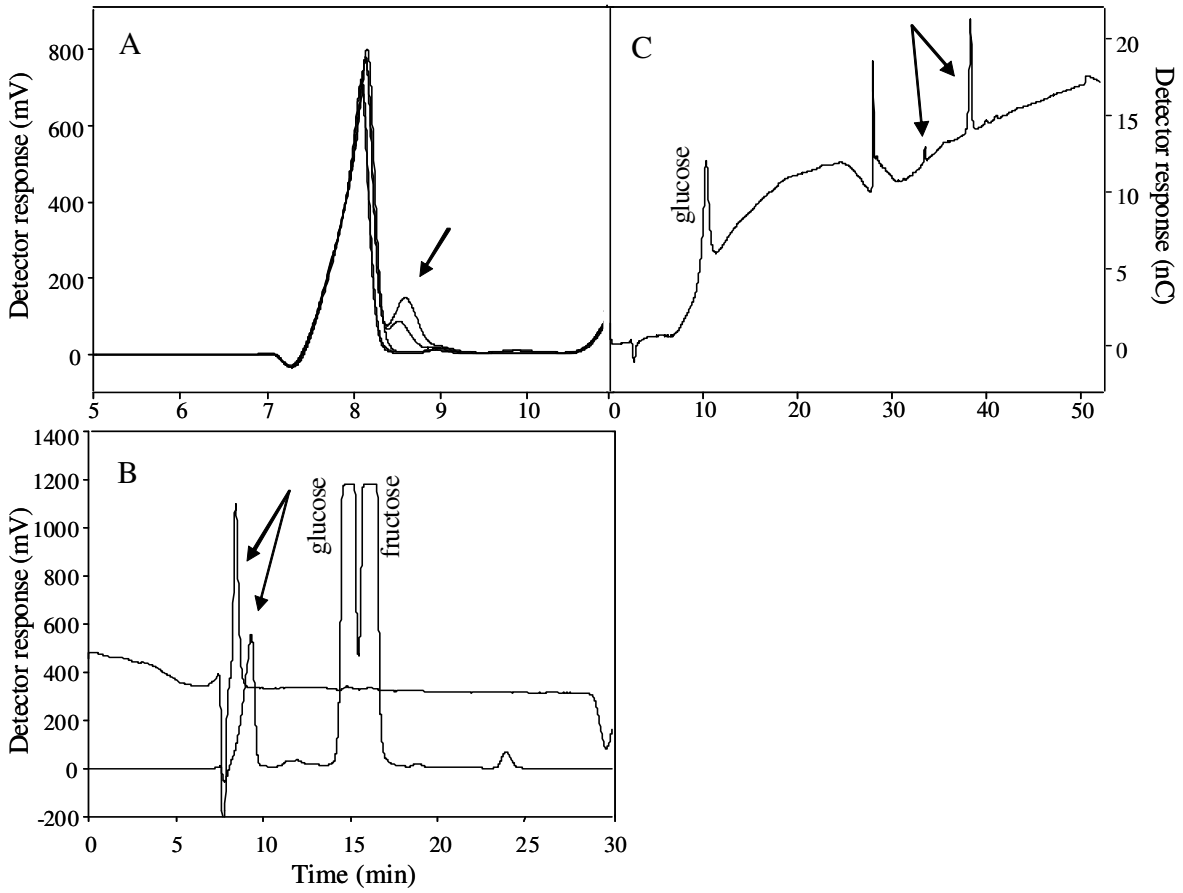

Figure 7 A Separation of fermentation supernatants of *B. lactis* TMW 2.530 grown on bifido-medium with sucrose at different temperatures with the OAKC column. Shown are the first minutes of the runs from supernatants after 48 h of fermentation at 37, 40, 45 and 50°C, and at t0. After fermentation at 45°C (middle trace) and 50°C (upper trace) a peak was detected at about 8.5 minutes after the peak evoked by the perchloric acid treatment. **B** Separation of supernatant of *B. lactis* TMW 2.530 with OAKC column before (lower trace) and after (upper trace) collecting of the unknown peak (marked with arrows) from untreated supernatant. **C** Separation of the collected fraction by AminoPac.

Acid hydrolysis of the collected fraction after preparative runs with the supernatant of *B. lactis* on the OAKC column followed by analysis with OAKC/RI and HPAEC-IPAD demonstrated that the main but not only component of these substances was glucose. Other smaller peaks could not be determined. Treatment with the exoenzyme α-glucosidase led to a decrease of the two unknown peaks in the collected fraction on AminoPac chromatograms (data not shown), confirming that glucose is a component of the unknown substances.

Treatment with the endoenzyme α-amylase, however, did not lead to a decrease of the mentioned peaks in the collected fraction, arguing against the identity as a GOS with higher DP.

As parallel experiments (see chapter **3.3.1**) suggested that a sucrose phosphorylase releasing fructose and α-glucose-1-phosphate (G-1-P) from sucrose is involved in the synthesis of the unknown substances by *B. lactis* TMW 2.530, G-1-P was analysed by HPLC. Comparison of HPLC chromatograms of G-1-P with the ones of supernatants of *B. lactis* TMW 2.530 and *L. reuteri* TMW 1.106 revealed, that the peak eluting earlier from AminoPac has the same retention time as G-1-P. As the two detected unknown substances seemed to be convertible in each other, G-6-P was also analyzed and was shown to have the same retention time as the second peak detected on AminoPac chromatograms of supernatants from *L. reuteri* TMW 1.106 and *B. lactis* TMW 2.530. Accordingly to the observations made after analysis of fermentation supernatants with the OAKC column, G-1-P and G-6-P eluted at the same time as the observed unknown peak from OAKC (about 8.5 minutes) and were not separated by this column (data not shown). The presence of G-1-P and G-6-P in the supernatants of *B. lactis* TMW 2.530 and *L. reuteri* TMW 1.106 was later further sustained by comparing respective fractions of supernatants with external standards on the Asahipak NH2P-50 column separating G-1-P and G-6-P with phosphate buffer pH 4.4 as eluent (fig. 8). Therefore the two substances leading to the two peaks on AminoPac chromatograms with retention times of about 36 and 39 min (depending on not influenceable run conditions) are further referred to as G-1-P and G-6-P but quantified as peak area (after separation with the OAKC column) because the identity of the substances was not known yet at the time when the experiments were done and separation of the two substances with OAKC was not possible.

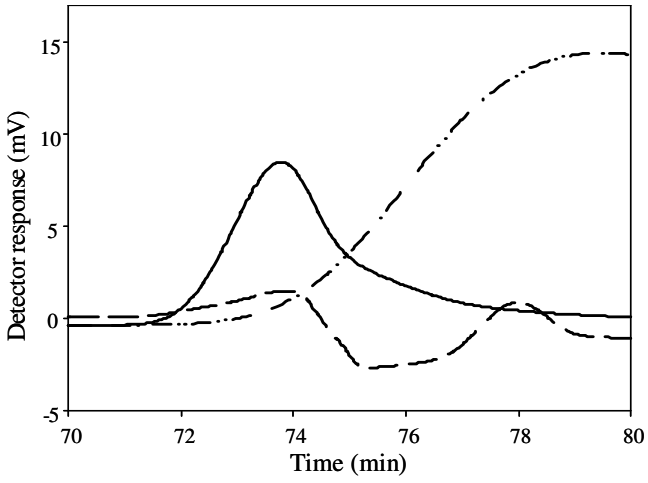

Figure 8 Separation of a respective fraction of fermentation supernatant of *L. reuteri* TMW 1.106 fermented at pH 6.2 (dashed trace) and external standards G-1-P (solid trace) and G-6-P (dashed dotted trace) on the Asahipak NH2P-50 column with phosphate buffer as eluent.

3.1.6 Influence of stress conditions on growth and the production of G-1-P and G-6-P from sucrose through different *Bifidobacterium* strains

After fermentation with *B. lactis* at reference conditions (37°C) detection of G-1-P and G-6-P was not significant and reproducible. In the supernatants of *L. reuteri* TMW 1.106 the corresponding substances were detected after pH static fermentations at pH values 5.4 and 6.2 that probably evoked stress reactions because of increased acid production compared with not pH static fermentation (see table 8). The stress factors elevated temperature and transisohumulon are known to induce the production of EPS and 1-kestose from sucrose in *L. reuteri* strains (Schwab 2006). Therefore *B. lactis* TMW 2.530 as well was cultivated under stress conditions as elevated growth temperature and/or bile (oxgall) and analyzed for the production of the hexose-phosphates.

Growth with bile was tested from 0.7 to 6.7 % (w/v) corresponding to about 0.35 and 3.5*MIC (Margolles et al. 2003) at 37°C. With 6.7 % bile *B. lactis* TMW 2.530 reached only half of the OD_{590nm} as without bile (data not shown). When incubated with 2 % bile (which corresponds to the MIC according to Margolles et al. 2003) *B. lactis* grew to OD_{590nm} 1.9 at 37°C and 0.8 at 45°C in comparison to 4.3 and 3.1 without bile, respectively. Comparing the growth of all examined bifidobacteria (*B. adolescentis* TMW 2.454 ^{T}, *B. breve* TMW 2.447 ^{T}, *B. infantis* TMW 2.448 ^{T}, *B. lactis* TMW 2.462 ^{T}, *B. longum* TMW 2.614^{T} and *B. longum* NCC2705 TMW 2.649), strain specific differences could be observed (fig. 9A). While *B. longum* and the two tested *B. lactis* strains grew well at 45°C and reached at least 50 % of the OD_{590nm} at reference conditions, *B. adolescentis*, *B. infantis* and *B. breve* were impaired drastically. With 2 % bile all strains except *B. adolescentis* reached optical densities above 1 after 48 hours of fermentation. *B. lactis* TMW 2.530 got on best with both stress factors. The amounts of acetate built during 48 h decreased for all strains when subjected to stress, but more clearly with bile than with elevated temperature (fig. 9C). The lactate levels decreased in all strains when incubated at 45°C. Incubation with bile, however, led to an increase of lactate production in both examined *B. lactis* strains, in *B. breve* and one of the two *B. longum* strains (fig. 9D). The theoretical value of 1.5 for acetate/lactate was reached in approximation through all examined strains under reference conditions (37°C without bile), decreased in all strains when fermented with bile (at 37 and 45°C) and (though high standard deviations within two independent experiments) seemed to increase when incubated at 45°C (fig. 9B).

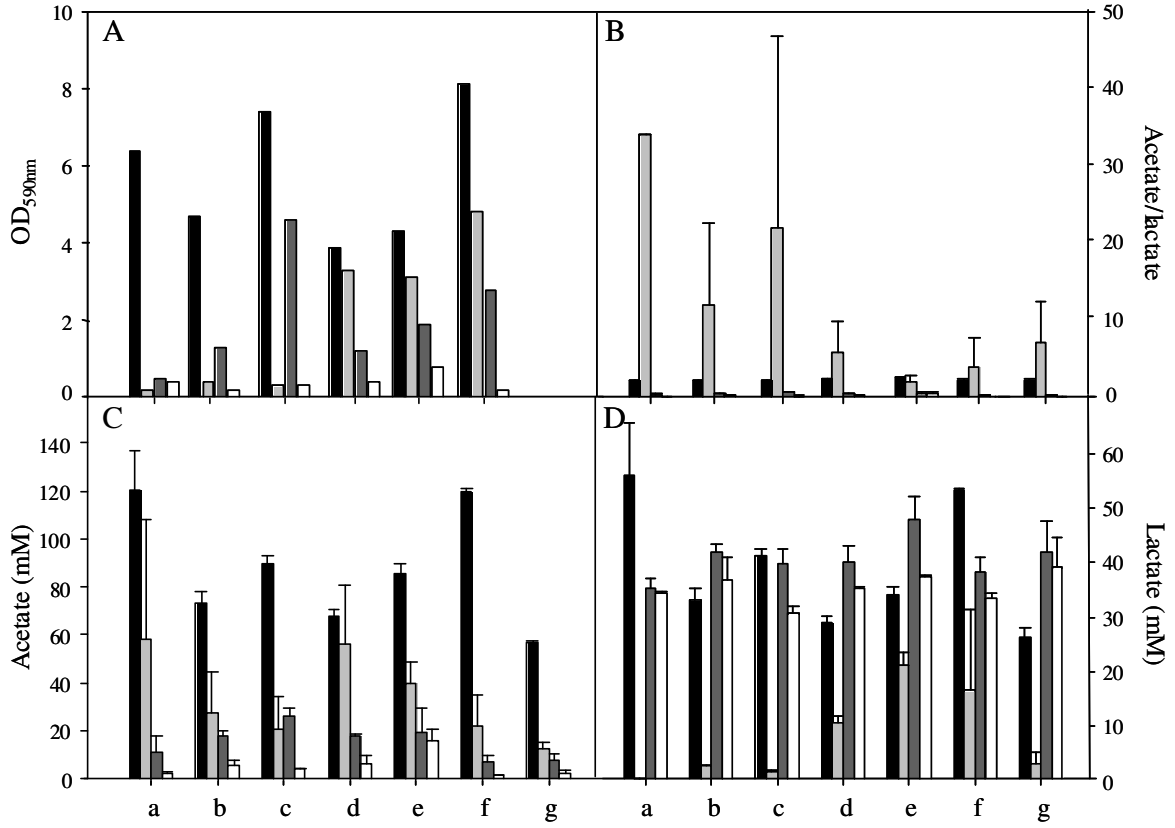

Figure 9 A Optical density of different bifidobacteria strains after 48 h of fermentation under reference conditions (37°C) (black) and stress conditions: 45°C (grey), 37°C with bile (50 g l^{-1}) (dark grey), 45°C with bile (white): a, *B. adolescentis*; b, *B. breve*; c, *B. infantis*; d, *B. lactis* TMW 2.462; e, *B. lactis* Bb-12 and f, *B. longum* TMW 2.614. **B** ratio acetate/lactate and production of **C** acetate, **D** lactate at 37°C (black), 45°C (grey), 37°C with bile (50 g l^{-1}) (dark grey) and 45°C with bile (50 g l^{-1}) (white) through: a, *B. adolescentis*; b, *B. breve*; c, *B. infantis*; d, *B. lactis* TMW 2.462; e, *B. lactis* Bb-12; f, *B. longum* TMW 2.614 and g, *B. longum* NCC2705 TMW 2.649.

Similar to the observation made with increasing temperatures, increasing bile concentrations led to increasing amounts of hexose-phosphates in the supernatant of *B. lactis* TMW 2.530, while the ΔOD_{590nm} decreased from 0.6 to about 0.3. In figure 10 this amounts (in peak area (nC*min)) are depicted in relation to the growth at different temperatures (fig. 10A) and under different bile concentrations (fig. 10B). A screening in bifido-medium with sucrose of various *Bifidobacterium* species for these hexose-phosphates proved, that similar to *B. lactis* TMW 2.530, particularly G-6-P could be detected in the supernatants of all investigated strains. However, the yields differed depending on strains and conditions (fig. 11A). *B. adolescentis*, *B. breve*, *B. infantis* and *B. longum* TMW 2.614 showed significantly increased peaks at 45°C (42.2, 20.3, 12.4, and 14.4 mV*min) while both *B. lactis* strains showed even higher rates at 37°C with bile (50 g l^{-1}), reaching up to 41.9 mV*min under these conditions. The ratio of peak area to OD_{590nm} as visible in figure 11B showed the same

trend for the other tested B*ifidobacterium* strains as for *B. lactis*. Elevated temperature or/and addition of bile led to an increase of this ratio.

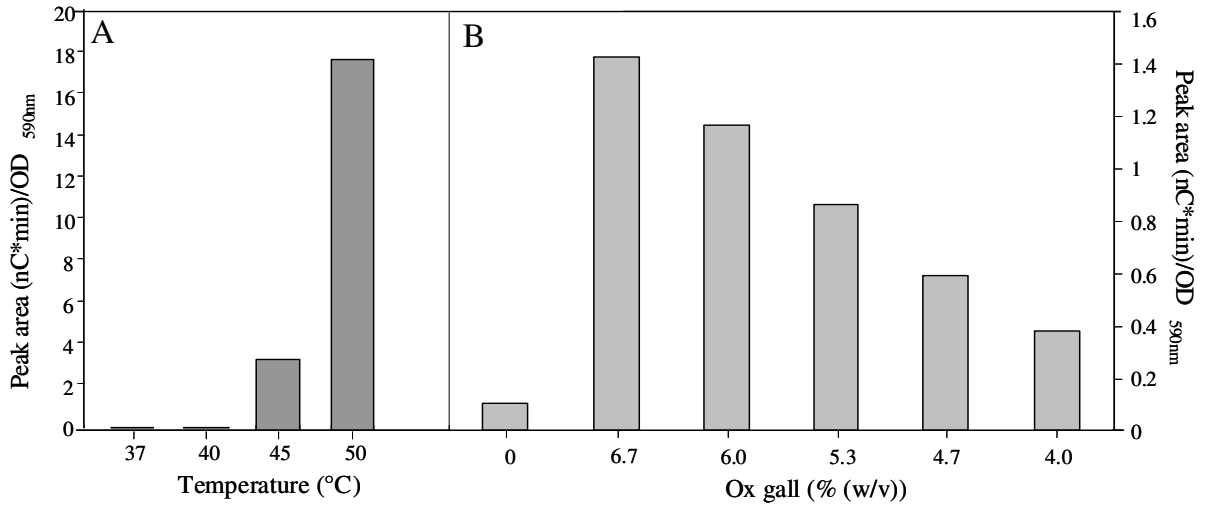

Figure 10 Effect of **A** temperature and **B** bile on the amount of an undefined substance detected on AminoPac (as peak area in nC*min) in relation to the growth (OD_{590nm}) after 48 h of fermentation of *B. lactis* TMW 2.530 at different temperatures

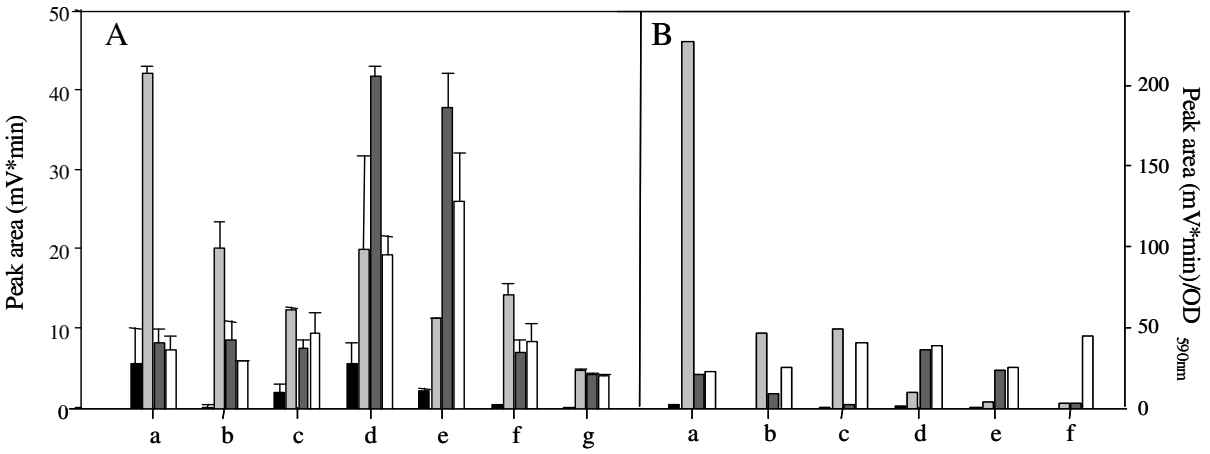

Figure 11 A Effect of temperature and bile on the peak area of the undefined peak and **B** on the relation of the unknown substance to the optical density after 48 h of fermentation of various bifidobacteria strains in sucrose media at 37°C (black), 45°C (grey), 37°C with bile (50 g l^{-1}) (dark grey) and 45°C with bile (50 g l^{-1}) (white): a, *B. adolescentis*; b, *B. breve*; c, *B. infantis*; d, *B. lactis* TMW 2.462; e, *B. lactis* Bb-12; f, *B. longum* TMW 2.614 and g, *B. longum* NCC2705 TMW 2.649.

To evaluate whether the appearance of the hexose-phosphates had to do with the metabolism of the bifidobacteria, the peak area (after separation on OAKC column) was also compared with the glycolysis products lactate and acetate. The higher temperature led to a reduction of the sum of lactate and acetate to maximal 68 % and the addition of bile (50 g l^{-1}) to maximal 59 % compared to reference conditions (37°C without bile) (fig. 12A). The ratio of peak area

to the sum of lactate and acetate was slightly increased through the addition of bile for the two investigated *B. lactis* strains (d and e), while for all other strains, it was clearly increased through elevated temperature without bile (fig. 12B). The mentioned divergence between the investigated strains coincided with the distinct influence of bile and elevated temperature on the occurrence of the unknown peak and the relation between amount of peak and optical density (fig. 11) except the combination elevated temperature with bile because of the very low growth at this condition but an almost similar lactate production.

The hexose-phosphates could not be detected after fermentations with fructose, galactose, glucose, lactose or maltose as sole carbon source or with sucrose at 30°C (only tested for TMW 2.530).

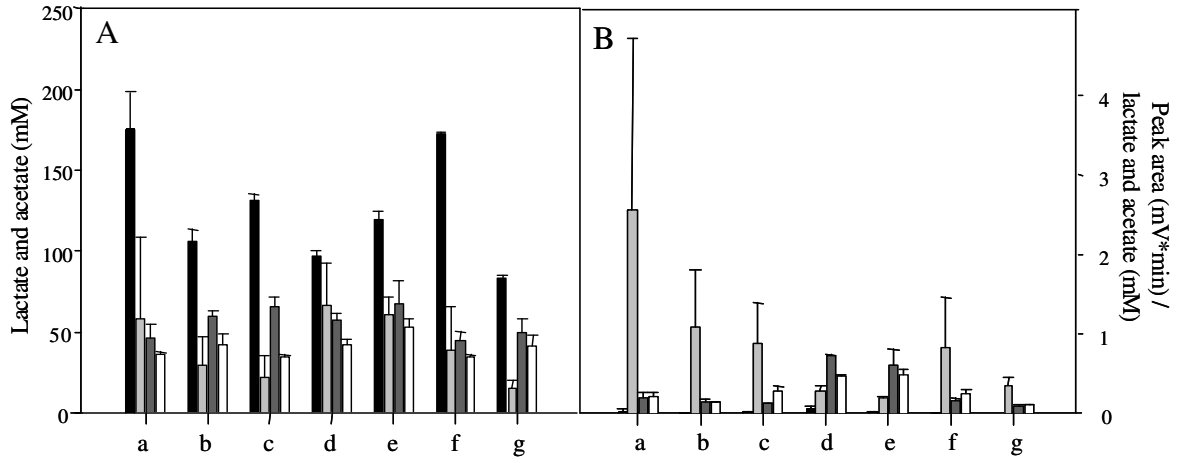

Figure 12 A Effect of temperature and bile on the sum of the metabolites lactate and acetate and **B** the relation of lactate and acetate to the amount of hexose-phosphates detected after separation on OAKC after 48 h of fermentation in sucrose media at 37°C (black), 45°C (grey), 37°C with bile (50 g l^{-1}) (dark grey) and 45°C with bile (50 g l^{-1}) (white): a, *B. adolescentis*; b, *B. breve*; c, *B. infantis*; d, *B. lactis* TMW 2.462; e, *B. lactis* Bb-12; f, *B. longum* TMW 2.614 and g, *B. longum* NCC2705 TMW 2.649.

3.1.7 Investigation on a probable function of extracellular hexose-phosphates

Investigations were made to find out if the hexose-phosphates in the fermentation supernatants of bifidobacteria cells grown under stress conditions have a positive effect or a protective function. As the bifidobacteria strains revealed to grow very slow on agar plates when taken from broth fermentations, no death kinetics could be carried out to investigate a protective function of these substances. Further collection and purification of large amounts of the substances was also not possible. Therefore sucrose media fermented with *B. lactis* TMW 2.530 and a known amount (in mV*min) of hexose-phosphates was inoculated a second time with the same strain and incubated for another 96 hours to see, if the strain was

able to metabolize its own products as a kind of reserve carbohydrate. If residual sucrose was still present in the medium, the peak area of the hexose-phosphates increased, indicating that *B. lactis* did not metabolize them (data not shown). If no residual sucrose was left at the time of the second inoculation, 26 and 59 % of the hexose-phosphates disappeared during 96 hours of incubation at 37 and 45°C, respectively and the addition of bile even led to a higher decrease of 85 and 97 % at 37 and 45°C, respectively (fig. 13A). Concentrations of lactate and acetate increased after reinoculation in the presence of bile. For lactate the increase was about 50 %, for acetate about 17 % when incubated with bile at 37°C and 15 % when incubated with bile at 45°C (fig. 13B). The acetate concentration also increased when incubated at reference conditions (5 %) and at 45°C without bile (9 %). However, no clear relation between the disappearance of the hexose-phosphates and the increase of lactate and acetate could be determined.

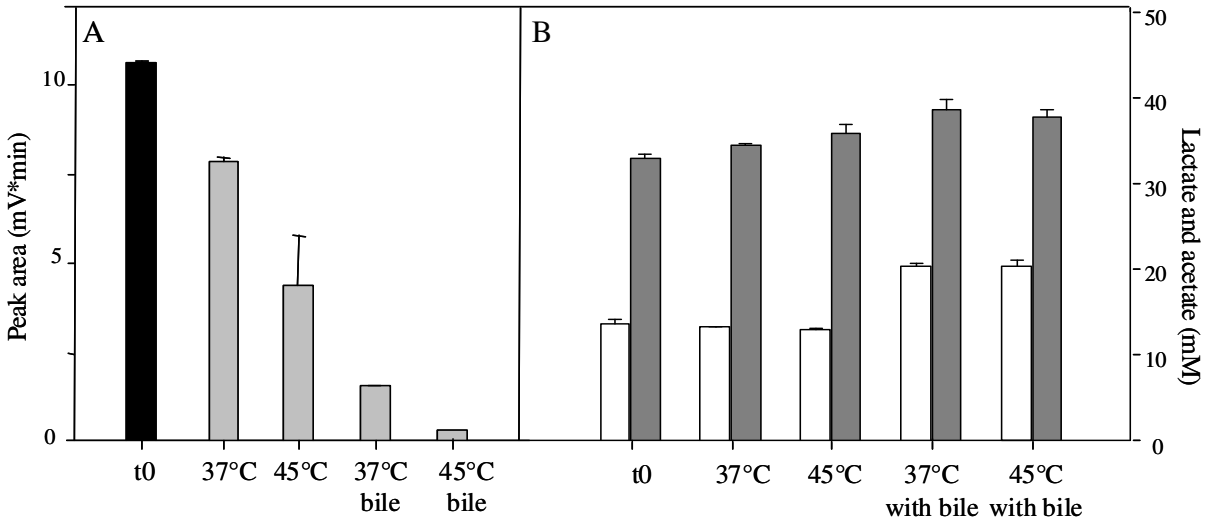

Figure 13 Effect of temperature and bile on the amount of **A** the hexose-phosphates (grey) and **B** of lactate (white) and acetate (dark grey) present in sucrose-free media at t0 (black) and after 48 h of incubation with *B. lactis* Bb-12.

3.2 Detection and characterization of two glucansucrases of *L. reuteri* analysis of their reaction products

3.2.1 Detection, isolation and nucleotide sequence analysis of two glucansucrase genes of *L. reuteri* TMW 1.106

The substances responsible for the two initially unknown peaks detected with HPAEC-IPAD were initially suggested to be glucooligosaccharides (GOS) (Tieking et al. 2005a) because of detected glucose as a hydrolysis product and of an incomplete glucose recovery that are probably produced by the same glucosyltransferase as the EPS. With the aim to find the gene(s) responsible for the synthesized EPS and these putative GOS and to find optimal conditions for the production of EPS, two glucansucrases were detected and sequenced in *L. reuteri* TMW 1.106.

Starting from a known sequence of about 500 bp (Tieking et al. 2005a) with high homology to *gtfA* of *L. reuteri* 121 (Kralj et al. 2002), a sequence of 6135 bp was obtained after several rounds of PCR by primer walking. In this sequence are located one complete open reading frame encoding a putative *gtf* gene (*gtf106A*) with 1782 amino acids (aa) which showed highest similarity to GtfA of *L. reuteri* 121 (92 % identity, 95 % similarity in 1783 aa) (Blastx NCBI; http://www.ncbi.nlm.nih.gov/BLAST/), and two partial open reading frames with homology to GtfB (184 bp upstream of *gtf106A*) and to a putative transposase of *L. reuteri* 121 (Kralj et al. 2004a) (downstream of *gtf106A*) (AY697435). The putative Gtf106A (EF189716) of *L. reuteri* TMW 1.106 shared the common domain structure of glucansucrases from lactic acid bacteria with four distinct domains shown in figure 14A. Compared to GtfA of *L. reuteri* 121, Gtf106A of *L. reuteri* TMW 1.106 has three amino acid replacements in highly conserved regions of the catalytic domain (P1025V, I1029V, N1134S, numbering of GtfA from *L. reuteri* 121, fig. 15).

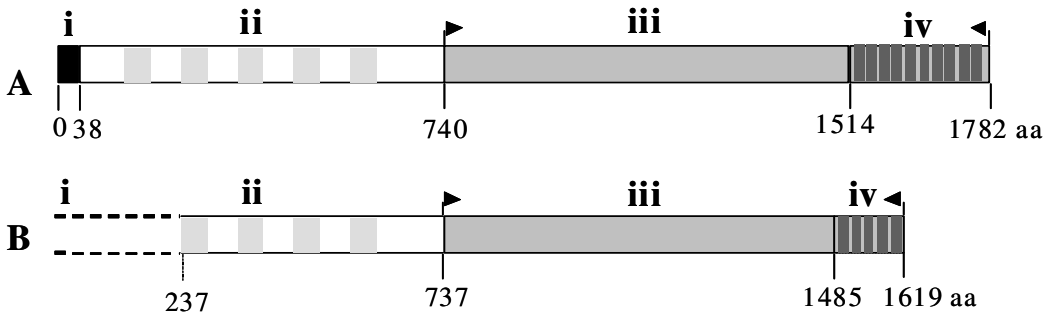

Figure 14 Glucansucrases **A** Gtf106A and **B** Gtf106B from *L. reuteri* TMW 1.106. (i) N-terminal signal peptide, (ii) variable region with RDV repeats (shaded grey), (iii) catalytic domain, (iv) C-terminal (putative) glucan binding domain with conserved YG repeats (shaded grey). The heterologously expressed proteins ΔN-Gtf106A and ΔN-Gtf106B are indicated by small arrows.

Enzyme	Main Linkages		Region II		Region III		Region IV	
Gtf106A	1-6	1017	ANFDSIRVDA**V**DN**V**DADLLNI	1057	HLNILEDWSHADPEY	1127	YTFIRAHD**S**NAQDQINNV
GtfA	1-4/1-6	1016	ANFDSVRVDAPDNIDADLMNI	1056	HINILEDWNHADPEY	1126	YSFVRAHDNNSQDQIQNA
Gtf180	1-6	1017	ANFDGIRVDAVDNVDVDLLSI	1058	HINILEDWGWDDPAY	1129	YNFVRAHDSNAQDQIRQA
GtfML1	1-3	1017	ANFDSIRVDAVDNVDADLLDI	1058	HINILEDWGGQDPYY	1125	YSFIRAHDNGSQDDIKRA
GtfO	1-4	1016	ANFDSVRVDAPDNIDADLMNI	1056	HINILEDWNSSDPNY	1126	YSFIRAHDNNSQDQIQNA
			****.:**** **:*.**:.*		*:******. ** *		*.*:****..:**:*...

Figure 15 Amino acid sequence alignment of conserved regions II, III and IV in the catalytic domains of dextran-, mutan- and reuteransucrases in *L. reuteri*. Gtf106A, *L. reuteri* TMW 1.106 (this work); GtfA, *L. reuteri* 121 (Kralj et al. 2002); Gtf180, *L. reuteri* 180; GtfML, *L. reuteri* ML1 and GtfO, *L. reuteri* ATCC 55730 (all Kralj et al. 2004a). Residues fully conserved in all GTF are underlined; catalytic residues are highlighted grey. Amino acid replacements in Gtf106A compared to GtfA are shown in bold type.

Starting from the second ORF (203 bp) a sequence of another 4154 bp was obtained by PCR and primer walking which showed highest homology (92 % identity, 95 % similarity in 1383 aa) to the inactive glucansucrase GtfB from *L. reuteri* 121 (Kralj et al. 2004a) and was therefore termed Gtf106B (EF189716). GtfB of *L. reuteri* 121 and Gtf106B of *L. reuteri* TMW1.106 exhibit 94 % identity and 97 % similarity on aa level when only the catalytic and the C-terminal domains are considered (Kralj et al. 2004a). The three conserved amino acids already identified as essential for the activity Asp1024, Glu1061 and Asp1133 (numbering of GtfA from *L. reuteri* 121) (Devulapalle et al. 1997) could be identified. The C-terminal domain was very short (fig. 14B) and the highly conserved motif “INGQYY” at the start of the catalytic domain was missing.

3.2.2 Gene expression and Gtf106A and Gtf106B levels in crude cellular extracts at different pH values

To investigate whether the distinct EPS production at various pH values is attributable to pH effects on gene expression or enzyme activity, the expression of Gtf106A and Gtf106B at different pH values was quantified by protein separation on 2D-PAGE. Neither enzyme was detected in the supernatant of sucrose grown cells. In the extract of whole cell protein, Gtf106A and Gtf106B could be identified at all tested pH values by the predicted pI (4.9 and 5.1, respectively) and Mw (198 and 179 kDa, respectively) and appeared as spot rows (fig. 16A). MALDI-MS analysis of the excised protein spots confirmed that all proteins in the spot row at Mw 198 kDa are gene products of *gtf106A*. Two spots from the spot row at Mw 179 kDa were identified as Gtf106B. This phenomenon leads to the suggestion, that both enzymes undergo posttranslational modifications which influence the pI. The quantification of the expression level of *gtf106A* during growth at different pH values was performed according to Mahon and Dupree (2001). Quantification of the whole rows revealed no significant

difference in Gtf106A expression at different pH values. However, by assigning and quantifying the single spots of Gtf106A separately, significant quantitative differences were detected. Namely, the spot with the highest pI (4.83) was not detected at pH 4.0 (fig. 16B).

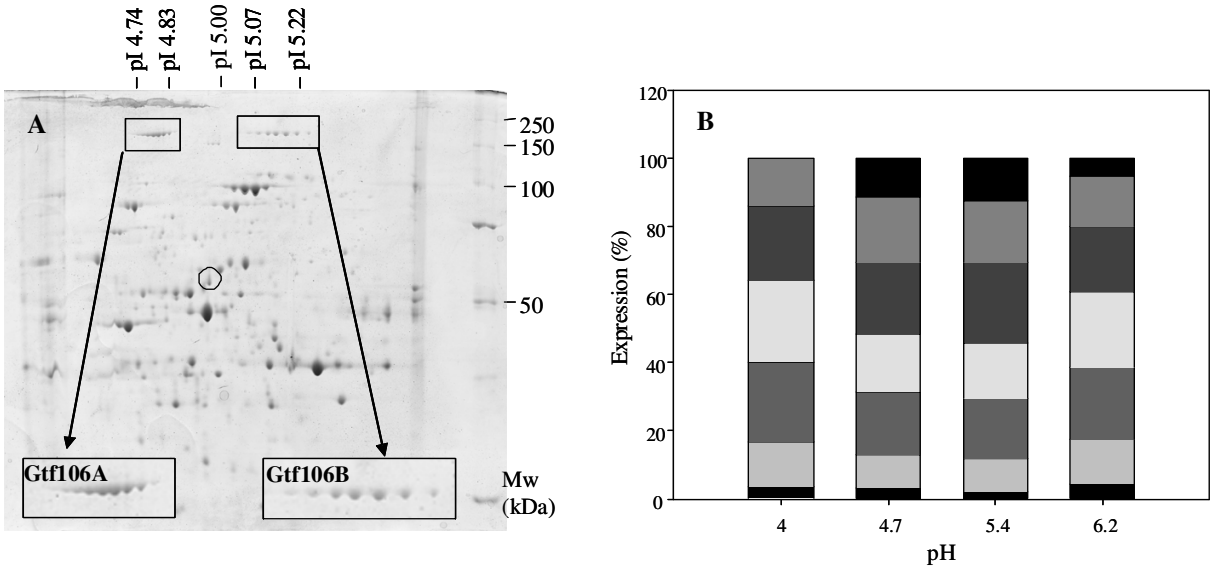

Figure 16 A 2D-gelelectrophoresis of whole cell proteins of *L. reuteri* TMW 1.106. Shown is the gel with proteins formed in pH static fermentation at pH 5.4. For better visualization, blow ups are included of the areas with spots identified as Gft106A and Gtf106B. Gft106A and Gtf106B could be identified at all tested pH values by the predicted pI (4.9 and 5.1 respectively), Mw (198 and 179 respectively) and MALDI-MS analysis. A spot probably belonging to SucP106 with predicted pI and Mw values of 5 and 56 kDa is indicated by an circle (see **3.3.3**). **B** Expression levels of individual Gtf106A spots (pI from bottom to top: 4.74, 4.76, 4.77, 4.79, 4.80, 4.82 and 4.83) at different pH values (bars). Data are

3.2.3 Characterization of the glucansucrases of *L. reuteri* and the effect of pH on their activity by heterologous expression in *E. coli* and analysis of the product spectrum from sucrose and other sugars

Gtf106A and Gtf106B were expressed and purified without their N-terminal domains as the N-terminal domain of glucosyltransferases does not affect their catalytic properties or the EPS structure (Monchois et al. 1999; Kralj et al. 2004b). Both recombinant proteins were purified to homogeneity as judged by SDS-PAGE through affinity chromatography (fig. 17). His-tagged ΔN-Gtf106B exhibited hydrolase but no transferase activity as measured by equal release of glucose and fructose from sucrose. The hydrolysis activity of ΔN-Gtf106B was less than 10 % of the maximal hydrolysis activity of ΔN-Gtf106A at all conditions and was detectable only after 27 h of incubation at 37°C.

ΔN-Gtf106A showed maximal total activity at about pH 4.2 (fig. 18). Maximum of the transferase activity was also in the range of 3.7 to 4.2 (100 %) with still about 50 % relative

activity at pH 5.3. Optimum of the hydrolysis activity was in the range of 4.5 to 5.0. The maximal measured hydrolysis activity was only 23 % of the maximal measured transferase activity. The optimal temperature for total activity of ΔN-Gtf106A was 45°C. The amounts of EPS produced by ΔN-Gtf106A during 48 h at 45°C in Na_2HPO_4/acetate buffer with 300 mmol l^{-1} sucrose were determined with GPC and are shown as bars in figure 18. A total of 26.7 g l^{-1} glucan could be obtained at pH 4.3. ΔN-Gtf106A also produced low amounts of leucrose when incubated with sucrose as sole carbohydrate source (data not shown). When arabinose, xylose, lactose, raffinose or melibiose were present in the reaction buffer additionally to sucrose, the formation of monoaducts was detected (shown for arabinose in fig. 19A). No such acceptor reaction could be seen for galactose, mannose and rhamnose. When maltose or isomaltose were supplied additionally to sucrose, series of peaks were detected (fig. 19B). Comparison with standards of maltooligosaccharides and dextrins with different degree of polymerisation (DP) revealed that malto- and isomaltooligosaccharides were produced through ΔN-Gtf106A from sucrose with maltose and isomaltose as acceptor (data not shown).

Detection of the two unknown peaks after incubation of ΔN-Gtf106A and ΔN-Gtf106B with sucrose was not possible under all conditions tested. This result indicated the existence of other enzymes responsible for their synthesis/detection and supported their later identification as G-1-P and G-6-P instead of GOS.

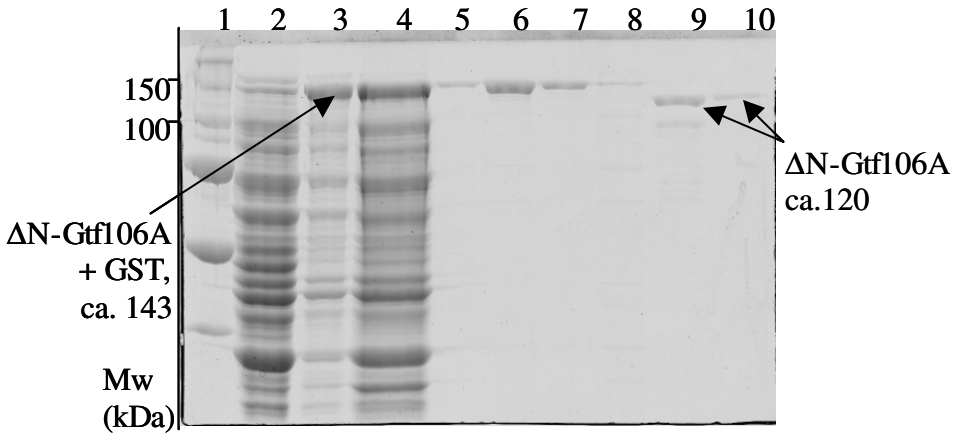

Figure 17 SDS PAGE of crude extract of heterologously expressed ΔN-Gtf106A in *E. coli* BL21. Precision protein standard (line1), not induced crude extract of *E. coli* (line 2), supernatant of induced crude extract (line 3), induced crude extract (line 4), FPLC fractions 14-16 (elution) (lines 5-7), fraction 1 of the washing step (line 8), fraction 1 and 2 after on column cleavage (line 9 and 10). GST, Glutathion S-transferase.

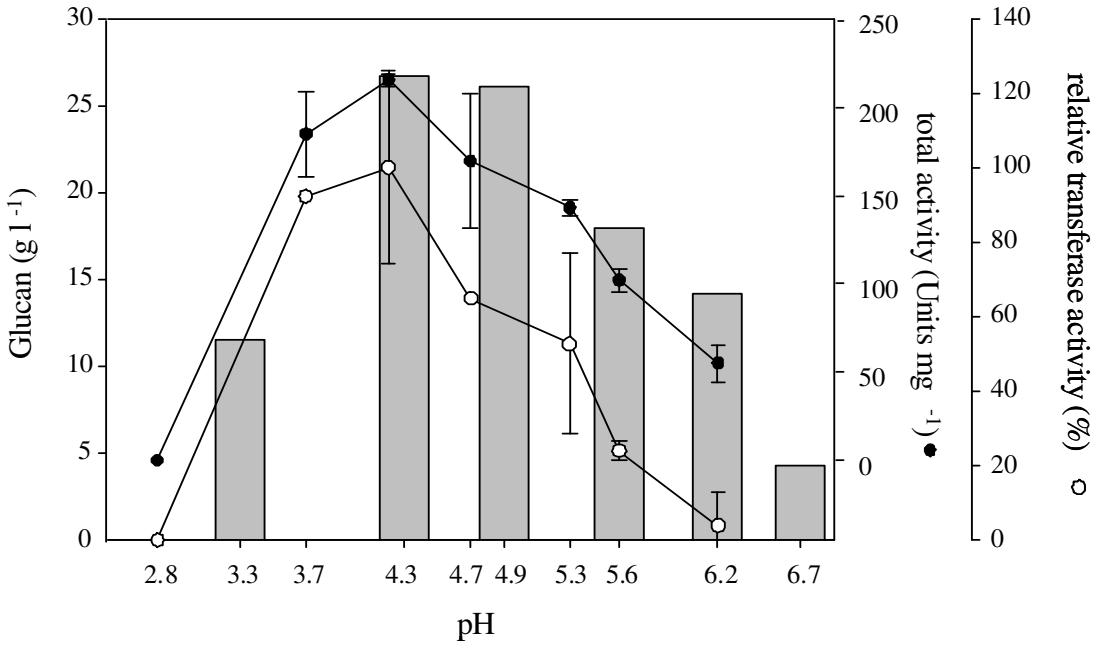

Figure 18 Effect of pH on purified ΔN-Gtf106A total activity (black circles; units mg^{-1} of protein) and relative transferase activity (white circles; % of maximal activity), and the amount of glucan produced (bars). Data shown are means and standard deviations of two experiments. One unit was defined as the release of 1 µmol of monosaccharide per minute.

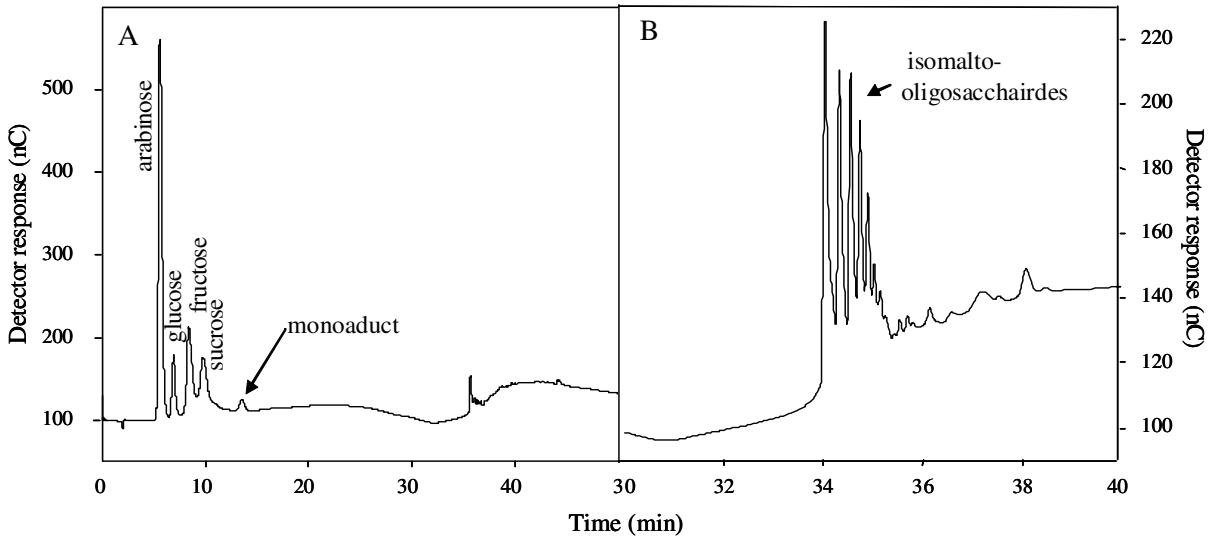

Figure 19 A Monoaduct and **B** isomaltooligosaccharides formed by ΔN-Gtf106A in the presence of sucrose and **A** arabinose or **B** isomaltose, respectively. Sugars were separated by HPAEC-IPAD.

3.2.4 Fermentations with glycosyltransferase deletion mutants of *L. reuteri* TMW 1.106

Fermentations with a dextransucrase and an inulosucrase deletion mutant of *L. reuteri* TMW 1.106 (kindly provided by J. Walter, University of Otago, New Zealand; Walter et al. 2005) were carried out to examine their ability to produce anymore EPS and other sucrose derivatives. This way the presence of another glucansucrase and an altered activity of the N-terminal truncated enzymes ΔN-Gtf106A and ΔN-Gtf106B should be excluded. After adapting to sucrose (in presence of 5 ppm erythromycin) the deletion mutants were shown by HPAEC-IPAD to still produce the unknown substances that are responsible for the two peaks on AminoPac mentioned in **3.1.4** (data not shown). No production of EPS could be detected with GPC-analysis of fermentation supernatant of the dextransucrase deletion mutant verifying that Gtf106A is the only active EPS producing enzyme of the strain. However it was possible to precipitate material with ethanol that amounted to less than 2 % of the EPS produced from the wildtype and was shown after hydrolysis to contain glucose and fructose and other smaller undefined peaks that had also be seen after hydrolysis of the collected fraction containing G-1-P (**3.1.5**) (data not shown).

3.2.5 Analysis of the glucans produced at different pH values

Molecular weight, hydrodynamic diameter and its size distribution of the glucans produced at different pH values were determined using asymmetrical flow-FFF coupled to multi-angle light scattering and RI detection as this is a suitable method for separation and characterisation of large biomacromolecules (Wittgren and Wahlund 2000 and 2002; Lee et al. 2003; Simon et al. 2003; Andersson et al. 2004). Clear differences in molecular masses (Mw and Mp) of the precipitated and purified EPS produced during fermentations with *L. reuteri* TMW 1.106 could be observed (table 3 and figure 20). The molecular mass of the EPS was highest at pH values where the strain also produced most EPS and was lowest at pH 4.0, when the least EPS was produced. For EPS produced at pH values above 4.0, the RI and the 90° light scattering signal of the fractograms indicated two different, not completely resolved size populations (shown for EPS produced fermentatively at pH 6.2 in figure 20A) as compared to the fractogram of EPS produced at pH 4.0 (fig. 20B). This was consistent with the 15° light scattering signals (data not shown) and even more obvious in the figures displaying the size distribution of the hydrodynamic diameter (fig. 20C). As smaller substances elute earlier in the fractogram, the first population belongs to a substance with a lower molecular mass. Most of the smaller size population eluting within a time interval from 5-9 min was detected after fermentations at pH 6.2, accompanied by a lower Mp and by a low

Rhp. The molar mass ranges (peaks from the 90° light scattering signal) and the hydrodynamic diameter of the two populations can be estimated from the Mw and the Rh distributions of the fractograms shown in figure 20A and C. For the smaller population the weight-average molecular weight was about 2-3*10^6 and about 20-30*10^6 for the larger population and the hydrodynamic diameter was about 35 nm and 60 nm, respectively. The polydispersity index (Mw/Mn) was highest for the EPS produced at pH 4.7, what indicates a higher variability of the EPS produced at this pH value.

For the glucans produced with purified ΔN-Gtf106A the pH dependency of the molecular weight was less pronounced. The polydispersity indexes Mw/Mn and Rhw/Rhn (data not shown) of ΔN-Gtf106A-glucans showed the monodispersity of these samples and accordingly the differences between the diverse parameters (Mw, Mn, etc.) were smaller than the ones of EPS from fermentations. The molecular weights and the hydrodynamic diameters of the EPS produced with purified ΔN-Gtf106A were lower as the ones produced during fermentation at the same pH values. Pure mMRS-S medium as a blank sample was extracted as described for EPS and measured using asymmetrical flow-FFF. The absence of any RI response confirmed that EPS derived completely from bacterial fermentations (data not shown).

Table 3. Structural properties of EPS produced under different pH values during fermentation with *L. reuteri* TMW 1.106 (strain) and with purified ΔN-Gtf106A

	pH 4.0		**pH 4.7**		**pH 5.4**		**pH 6.2**	
	Glucan produced by							
	Strain	**ΔN-Gtf106A**	**Strain**	**ΔN-Gtf106A**	**Strain**	**ΔN-Gtf106A**	**Strain**	**ΔN-Gtf106A**
Mw (10^6 Da)	5.4	5.2	12.2	6.2	11.9	7.6	9.1	7.6
Mn (10^6 Da)	2.1	4.7	2.3	5.4	3.0	6.6	2.9	5.8
Mw/Mn	2.5	1.1	5.2	1.2	3.9	1.1	3.1	1.3
Mp (10^6 Da)	2.4	5.6	21	5.6	13.8	6.0	2.4	5.3
Rhw (10^1 nm)	6.9	n.a.	7.4	n.a.	7.4	n.a.	6.3	3.6
Rhp (10^1 nm)	4.9	n.a.	6.8	n.a.	6.3	n.a.	3.5	2.6
linkage types								
α-(1-6) (%)[a]	82.6	83.1	84.8	83.7	84.7	84.7	85.3	n.a.
α-(1-4) (%)	17.4	16.9	15.2	16.3	15.3	15.3	14.8	n.a.

Mw, weight average molecular weight of the probe; Mn, number average molecular weight of the probe; Mp, molecular weight of the highest peak in the probe; Rhw, average hydrodynamic diameter of the probe; Rhp, hydrodynamic diameter of the highest peak in the probe; n.a., not available/comparable

[a] α-(1-6) linkages were detected at ~ 4.90 ppm, α-(1-4) linkages were detected at ~ 5.15 ppm

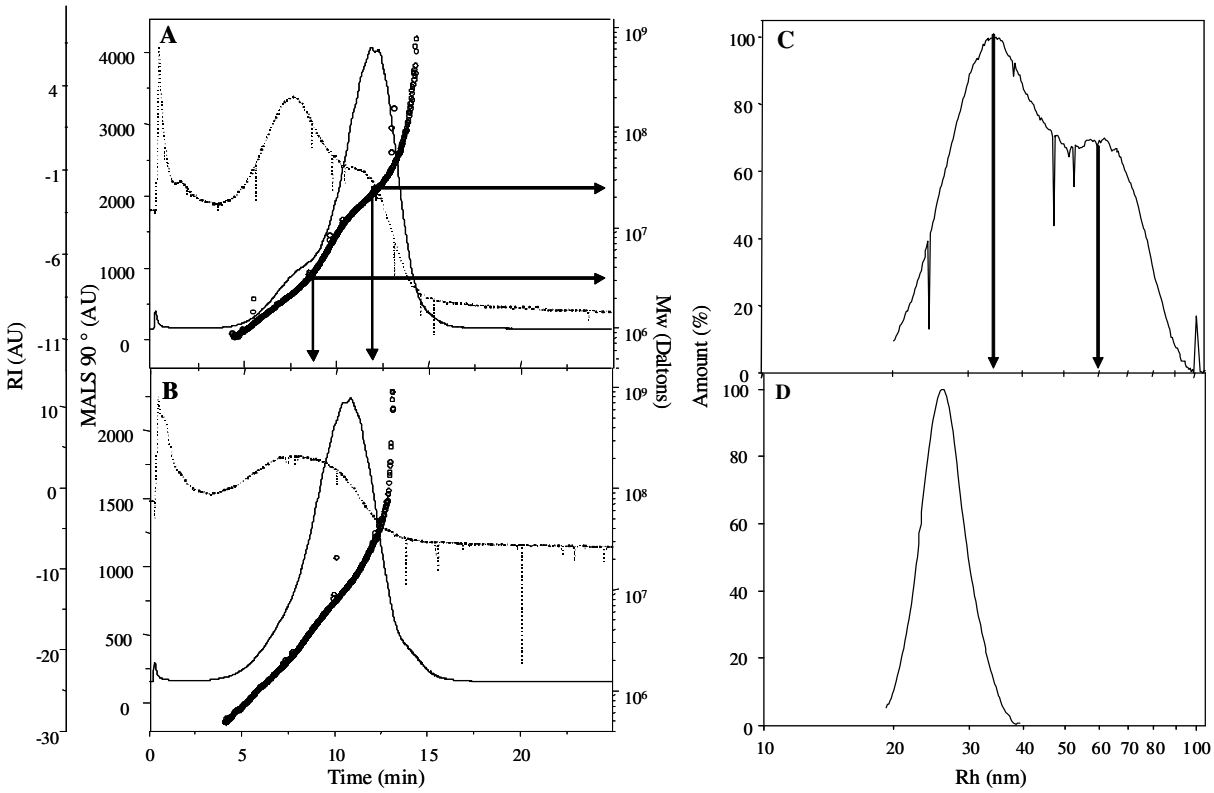

Figure 20 Asymmetrical flow-FFF analysis of the molecular mass of the EPS formed upon pH static fermentation of *L. reuteri* TMW 1.106 at **A** pH 6.2 and **B** pH 4.0. Shown are the light scattering signal at 90° angle (solid line) and RI (dotted line) signals together with the molecular weight distribution of the EPS (circles). Arrows indicate the approximate molecular masses of the two detected peaks based on their retention times. Distribution of the hydrodynamic diameter of the EPS formed at pH 6.2 **C** fermentatively and **D** enzymatically with ΔN-Gtf106A. AU, arbitrary units.

The ^{1}H NMR spectra of the glucans produced through fermentation and with ΔN-Gtf106A were virtually identical (data not shown) but revealed small distinct differences depending on the pH value at which the EPS were produced. Comparison of the anomeric signals with that from potato starch (Gidley 1985) showed that in both glucans, produced fermentatively and with ΔN-Gtf106A, the glucopyranosyl units were linked mainly α-(1-6) but also contained α-(1-4) linkages. According to the general definition of dextrans (a glucan in which α-(1-6) linkages predominate (Leathers. 2002)), these results demonstrate that Gtf106A of *L. reuteri* TMW 1.106 produces a branched dextran. The higher the pH value the less α-(1-4) linkages were observed in the glucan produced by ΔN-Gtf106A in buffer from pH 4.0 to 5.4. The correlation between higher pH and a lower percentage of α-(1-4) linkages could also be seen in the EPS isolated from fermentations. The samples derived from EPS production at pH 6.2 were not comparable to the other results because of a lack of peak separation in the proton spectra.

3.3 Identification of *B. lactis* and *L. reuteri* sucrose phosphorylases and their products

3.3.1 Purification of putative sucrose and glucan phosphorylase enzymes from *B. lactis* TMW 2.530

In order to identify the enzymes responsible for the detection of the two peaks in the supernatant of *B. lactis* TMW 2.530 later identified as hexose-phosphates, enzymes from crude extract of whole cell protein of *B. lactis* TMW 2.530 grown on sucrose were separated stepwise by fast protein liquid chromatography (FPLC). After each step fractions were tested for the appearance of the hexose-phosphate peak(s) by incubation in phosphate buffer with sucrose and HPLC analysis with the OAKC and AminoPac column. Active fractions were pooled and subjected to the next purification step. After five purification steps (precipitation with 40 % ammonium sulphate, phenyl sepharose, hydroxyapatite, anion exchange and GPC), two major protein-bands were obtained on SDS-PAGE. The band with the higher molecular weight (85-100 kDa, depending on protein marker used) could be purified completely (fig. 21A) which was also confirmed through silver staining (fig. 21B). The protein was digested with trypsin and two peptides were N-terminal sequenced by Edman degradation. The obtained sequences HLMDSWMK and SYDEFD(L,Q)LL(F)T (minor sequences in brackets) showed homology to a glucan phosphorylase (ZP_00121604) of *Bifidobacterium longum* DJO0A (NCBI Microbial Genomes Annotation Project 2004) and a glycogen phosphorylase (AAN24421) of *Bifidobacterium longum* NCC2705 (Shell et al. 2002). The other protein with about 50-60 kDa on SDS-PAGE (depending on protein marker used) was not obtainable in a pure form (fig. 21B). Therefore, both proteins (85-100 and 50-60 kDa) were cut out from the gel and analyzed by MALDI-MS. Mass spectrometric data were interpreted by the Matrix Sciences Mascot software and blasted against the NCBI database. The data confirmed the protein with the higher molecular weight to be a α-glucan phosphorylase (EC 2.4.1.1) and led to the identification of the protein with the lower molecular weight as a sucrose phosphorylase (EC 2.4.1.7) with highest similarity to the sucrose phosphorylase (AAN01605) of *B. animalis* (Trinidade et al. 2003). On a 2D-gel made with whole cell protein extract of *B. lactis* TMW 2.530 spots/spot rows could be identified that corresponded to the predicted pI and Mw of the glucan- (pI about 5 and 91-95 kDa) and the sucrose phosphorylases (pI about 4.8 and 56 kDa) from the NCBI data base (fig. 22).

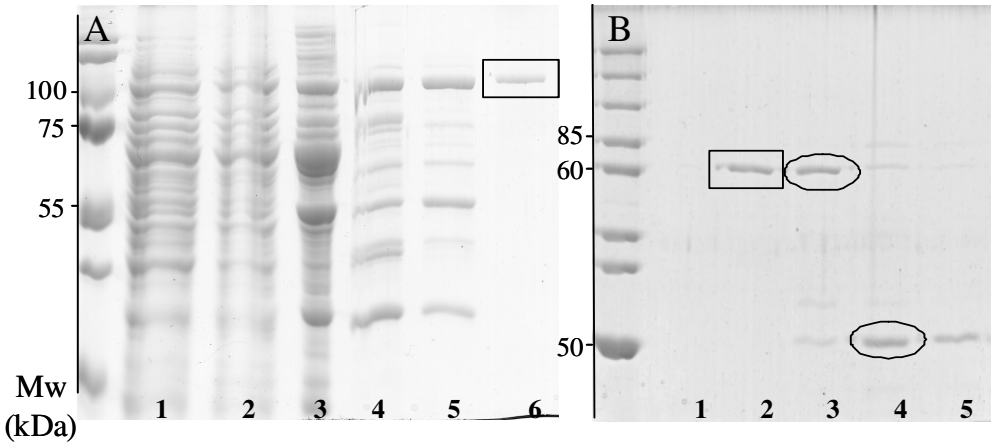

Figure 21 Cell protein profiles of *B. lactis* TMW 2.530 separated on 12 % SDS-PAGE. In **A** are shown after Commassie-staining: crude extract of whole cell protein (lane 1), pellet after precipitation with 40 % ammonium sulphate (lane 2), active fractions (fr. 38-42) after phenyl sepharose (lane 3), active fraction (18) after hydroxyapatite (lane 4), active fraction (14) after anion exchange (lane 5) and active fraction (13) after GPC separation (lane 6). The band marked with a box was identified as α-glucan phosphorylase through N-terminal sequencing by Edman degradation. In **B** are shown after silver staining: fractions 12–16 (lane 1–5) after separation with GPC. The bands marked with ellipses were cut out and analyzed by MALDI-MS identifying the smaller protein that could only be purified partially by the mentioned purification steps as sucrose phosphorylase, and confirming the greater protein to be α-glucan phosphorylase.

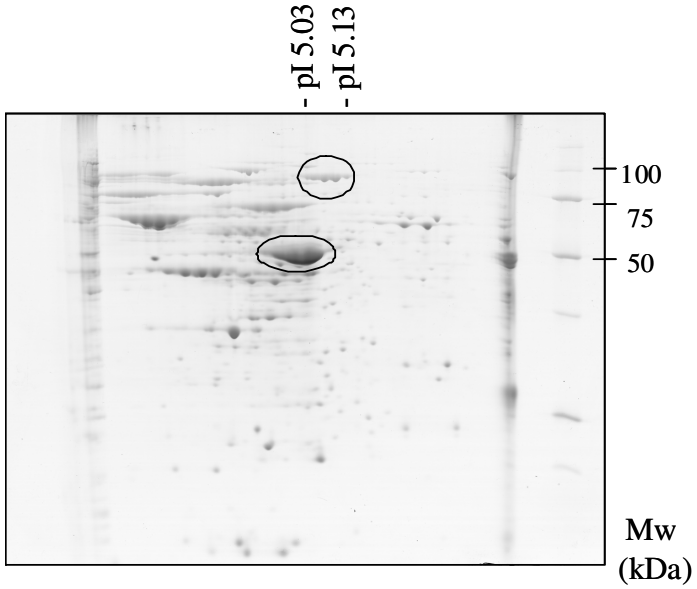

Figure 22 2D-gelelectrophoresis of whole cell proteins of *B. lactis* TMW 2.530 grown in medium with sucrose. Spot rows with the predicted Mw and pI of α-glucan phosphorylase (pI about 5 and 91-95 kDa) and sucrose phosphorylase (pI about 4.8 and 56 kDa) could be identified and are marked with a circle (GlgP) and an ellipses (SucP), respectively.

3.3.2 Partial characterization of the purified sucrose phosphorylase of *B. lactis* TMW 2.530

Parallel to the purification and identification of the sucrose phosphorylase, fractions from the last purification step containing this protein in phosphate buffer were examined spectrophotometrically for the release of fructose from sucrose as this was currently the known educt, while the glucose moiety was converted to sugar-phosphates. The detection of free fructose was significant, whereas the amounts of free glucose were negligible as they did not exceed the tenth of the amount of free fructose (data not shown). These results indicated the absence of a (contaminating) invertase activity but supported the initial idea of GOS formation. The K_m value for the release of fructose from sucrose through the enzyme was 8.7 mM sucrose and the V_{max} was 1.9 µmol $(min*l)^{-1}$ (at 50°C) (fig. 23A). The activity showed Michaelis-Menten characteristics by approximation. Maximum activity (143 Units $µg^{-1}$) with 60 mM sucrose was observed at 55°C (fig. 23B) with about 50 % and 25 % activity at 30°C and 21°C, respectively. No significant pH optimum could be defined. Activity was stable in the range of 180 to 200 Units $µg^{-1}$ from pH 4.0 to 6.4. For higher pH values, no clear results were obtainable due to high variability in four replications.

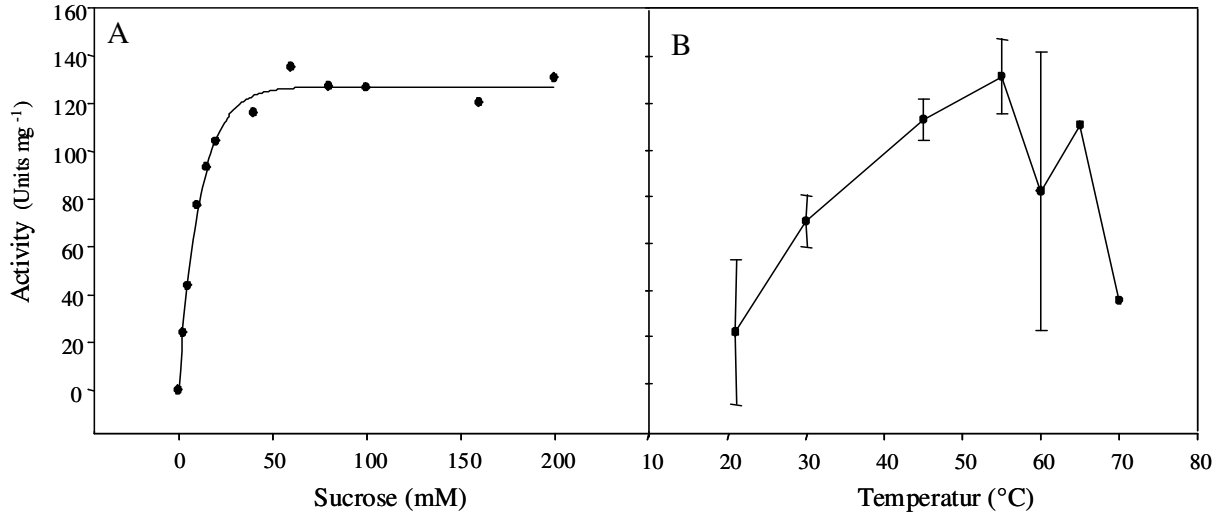

Figure 23 A K_m and V_{max} for the release of fructose through the action of SucP_Bb12. **B** Effect of temperature on the fructose releasing activity of SucP_Bb12. One unit was defined as the release of 1 µmol of monosaccharide per minute. Values are means and standard deviations of two independent experiments.

To prove the identity and activity of the putative sucrose phosphorylase of *B. lactis* TMW 2.530, termed SucP_Bb12, the synthesis of G-1-P through the purified enzyme was verified through HPLC and thin layer chromatography (TLC). Bacterial phosphorylases, except disaccharide phosphorylases, need the 5´-phosphate group of pyridoxal phosphate as a

cofactor (Schinzel and Nidetzky 1999). Therefore, SucP_Bb12 could be characterized using phosphate buffer without pyridoxal phosphate although it was not completely separated from GlgP_Bb12 by the last purification step. GlgP_Bb12, furthermore, was not expected to act on sucrose. Accordingly, the production of G-1-P after incubation with sodium phosphate buffer and sucrose could be observed for the fraction (fr.) containing both enzymes (fr. 14 GPC) but not for the fraction containing only GlgP_Bb12 (fr. 13 GPC), as shown by the chromatograms of HPLC analysis depicted in figure 24A. Confirmation of the activity of the putative SucP_Bb12 in fr.14 and the production of G-1-P was also given through HPLC analysis with the Asahipak NH2P-50 column (fig. 24B) and TLC (fig. 25). As expected, fr. 14 did not show activity any more after dialyzation against citrate buffer and incubation with sucrose because of the lack of phosphate. Further this fraction containing the putative SucP_Bb12 was able to act in the reverse direction and synthesized sucrose from fructose and G-1-P as proven by HPLC.

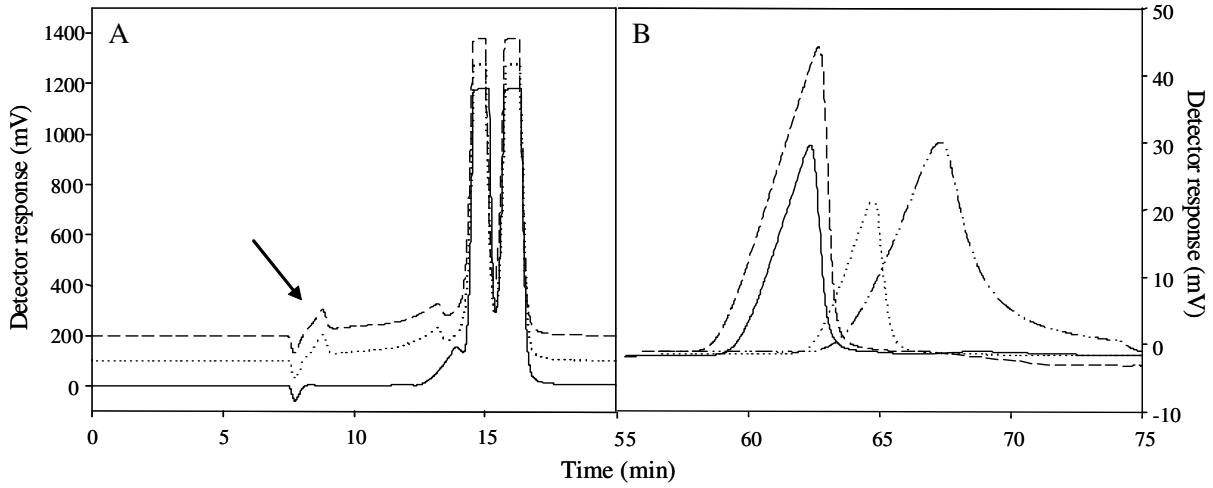

Figure 24 A HPLC separation of the reaction product of GPC fractions 13-15 with sucrose in phosphate buffer. **A** OAKC chromatograms of products of fraction 13, containing GlgP_Bb12 (solid trace), 14 (dotted trace) and 15 (dashed trace) both containing SucP_Bb12 and GlgP_Bb12 (offset 100 mV). **B** NH2P-50 chromatograms of the reaction product of purified SucP_Bb12 (fraction 14) (solid trace) and external standards F-6-P (dotted trace), G-1-P (dashed trace) and G-6-P (dash-dot-dot trace).

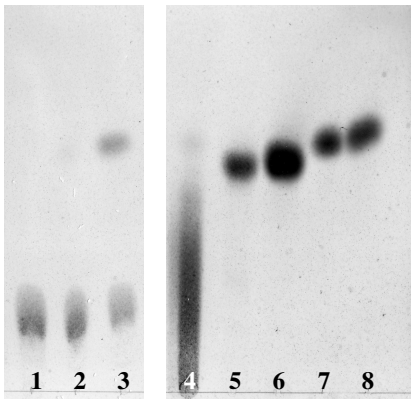

Figure 25 Thin layer chromatography of the reaction product of purified SucP_Bb12 with sucrose and standards. Shown are G-1-P (lane 1), reaction product separated from fructose by GPC with Superdex prepgrade 75 (lane 2), reaction product not completely separated from fructose (line 3), maltooligosaccharides (line 4), maltose (line 5), sucrose (line 6), glucose (line 7) and fructose (line 8).

It was further investigated, whether SucP_Bb12 also acts on other sugars as sole carbon source and whether it has transglucosylation activity as described for the recombinant sucrose phosphorylases of *B. adolescentis* and *B. longum* (van den Broek et al. 2004; Kim, M. et al. 2003). No reaction products could be detected with the OAKC and the AminoPac columns when the partially purified SucP_Bb12 was incubated with maltose, raffinose (O-α-D-Gal*p*-(1-6)-O-α-D-Glc*p*-(1-2)-β-D-Fru*f*), leucrose (O-α-D-Glc*p*-(1-5)-D-Fru*p*) or lactose (O-β-D-Gal*p*-(1-4)-D-Glc*)* as sole carbon sources (100-200 mM in phosphate buffer 50 mM, pH 6.9). After incubation with maltotriose (O-α-D-Glc*p*-(1-4)-O-α-D-Glc*p*-(1-4)-D-Glc*p*) an increase of a peak with the retention time of maltotetraose was detected by OAKC (fig. 26A, upper solid line) and AminoPac (data not shown). This result points at a transglucosylation activity making maltotetraose from maltotriose. When the enzyme was incubated with dextrins (maltodextrin from maize starch) the maltotetraose peak of the dextrins rose likewise (fig. 26B, upper dashed line). In all experiments with other sugars as sucrose as sole carbon sources no G-1-P could be detected. If these sugars were applied in a concentration of 50 mM together with sucrose (100 mM), the peaks with the retention time of G-1-P and G-6-P could be detected and in the case of maltotriose additionally the peak with the retention time of maltotetraose rose (data not shown). However, detection of the peak with the retention time of G-6-P must have been due to a contamination in the partially purified SucP_Bb12 preparation with PGM or another enzyme. Another indication for a probable transglucosylation activity was observed when SucP_Bb12 was incubated with sucrose and G-1-P and had before been dialyzed against citrate buffer, so that the only phosphate available was the one bound to G-1-P. With HPLC analysis through OAKC/RI a new unknown peak was detected (fig. 27) and the sucrose concentration decreased. Though, by HPAEC-IPAD no new peak but only the increase of the peak with the retention time of G-6-P could be detected (data not shown). However, if the purified enzyme was incubated with its collected reaction product from sucrose (assumedly G-1-P) and residual sucrose, likewise on TLC an unknown reaction product with properties of an oligosaccharide (greater than sucrose) could be detected (fig. 28), whereas with OAKC/RI only liberated phosphate was detected (data not shown).

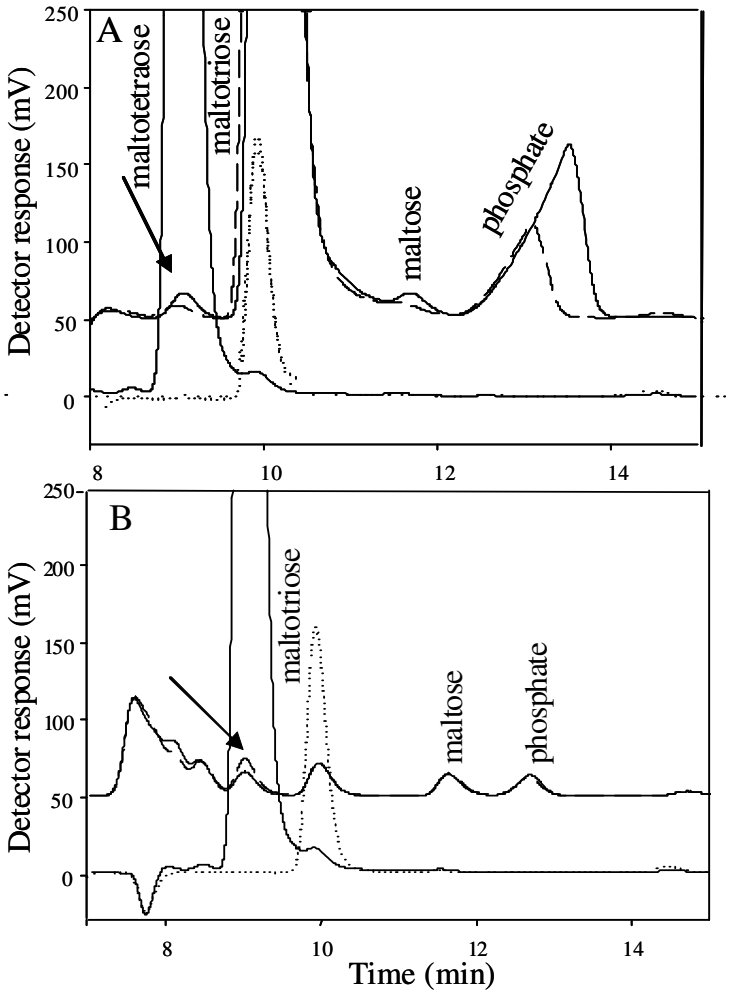

Figure 26 OAKC separation of reaction products of purified SucP_Bb12 (upper traces, offset 50 mV) and external standards (lower traces) maltotriose (dotted trace) and maltotetraose (solid trace) after incubation with **A** maltotriose (before incubation: dashed trace; after incubation: solide trace) and **B** dextrins (before incubation: solid trace; after incubation: dashed trace).

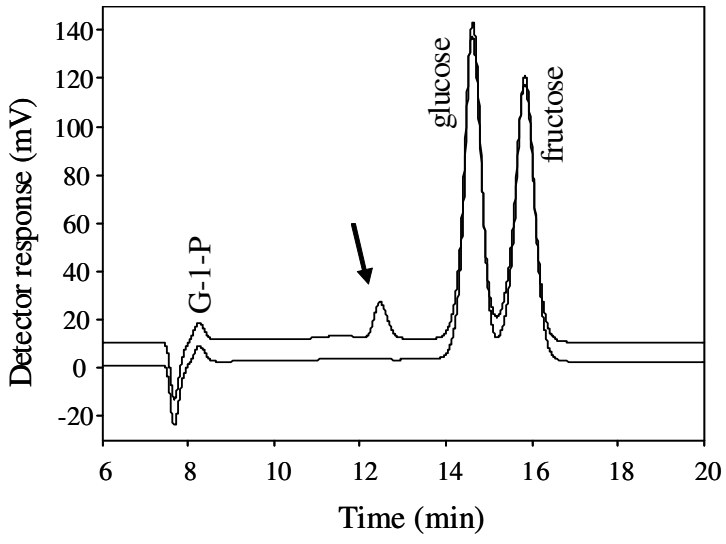

Figure 27 OAKC separation of reaction products after incubation of SucP_Bb12 (dialyzed against citrate buffer) with sucrose and G-1-P (upper trace, offset 10mV) and the negative control (lower trace). Sucrose is hydrolysed by the eluent used with the OAKC column. The resulting and undefined peak is marked with an arrow.

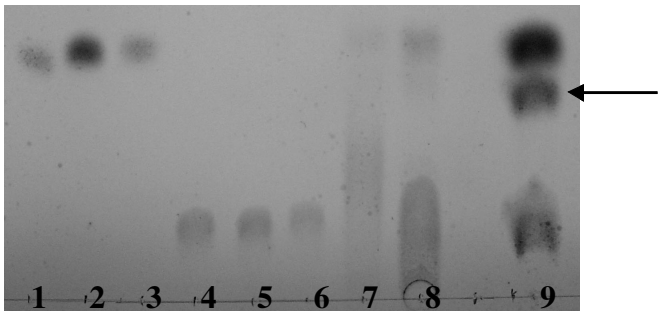

Figure 28 Thin layer chromatography of the reaction product of purified SucP_Bb12 after reincubation with sucrose and its reaction product from sucrose (collected peak from OAKC, assumed to be G-1-P). Shown are maltose (lane 1), sucrose (lane 2), glucose (line 3), G-6-P (line 4), G-1-P (line 5), F-6-P (line 6), maltooligosaccharides (line 7), collected peak (G-1-P) from OAKC with residual sucrose (line 8) and reaction product of concentrated collected fraction and purified SucP_Bb12 (line 9). The resulting and undefined substance is marked with an arrow.

3.3.3 Nucleotide sequence analysis of genes encoding GlgP and SucP in *B. lactis* and *L. reuteri*

In *B. lactis* TMW 2.530 a *glgP* and a *sucP* gene were identified. With primers sydefd_V/spezGP_R obtained from the peptide SYDEFD (**3.3.1**) from *B. lactis* TMW 2.530 and deduced from conserved regions in GlgP from other bifidobacteria a sequence of 3904 bp (appendix) was obtained after several rounds of PCR. This sequence encompassed one complete ORF of 2457 bp that showed highest homology to the glycogen phosphorylase from *Bifidobacterium longum* NCC2705 (AAN24421) (aligned in figure 29) and the glucan phosphorylase from *Bifidobacterium longum* DJO10A (ZP_00121604) (79 % identity, 89 % similarity in 818 and 817 aa, respectively) (Blastx NCBI) and therefore was termed glucan phosphorylase *glgP_Bb12*. The predicted Mw and pI of the protein was 92.7 kDa and pI 5 (www.expasy.org, compute pI/Mw tool). Using SignalP (http://www.cbs.dtu.dk/services/SignalP/) for signal peptide prediction and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) for the prediction of transmembrane domains revealed, that GlgP_Bb12 has no signal peptide but seems to have a strongly significant transmembrane domain (score 1746) from aa 128 to 146 with orientation form inside to outside (marked with a solid box, fig. 29). The nucleotide sequences up- and downstream (each about 700 bp) showed only low homology to some hypothetical proteins of bifidobacteria.

```
B. lactis                                    MTELTAPKSPLTANEFADEIRQALK 25
B. longum              MLTFVRRYLSRGIINTGTYPGRKVRMTEITAPKSPVTAEQFADEIREQLK 50
                                            ***:******:**::******: **

B. lactis              YTQGVTPEQAKTADVYVATATVVRRHLMDSWMKTQQDMINGDTKAVGYLS 75
B. longum              YTQNVTTEQATPADVYVAASKAVRNHLADSWFKTQADTVNGNTKAVGYLS 100
                       ***.**.***..******::..**.** ***:*** * :**:********

B. lactis              AEFLMGKQLRNALLNAGLTPQFEEAVRGLGFDPQAVVDAEYEPGLGNGGL 125
B. longum              AEFLMGKQLRNALLNAGLTEQFDKAVEALGFKVQDVVDAEYEPGLGNGGL 150
                           ****************** **::**..***. * ***************

B. lactis              GRLAACFIDSLASLGVPAFGYGIQYKYGIFRQEFDDEGRQIERPDYWLSN 175
B. longum              GRLAACFIDSLASLGVPAFGYGIQYKYGIFKQEFDKDGKQVETPDYWLAN 200
                       ********************************:****.:*:*:* *****:*

B. lactis              EDPWGHIDYERDQRVNFGGKVVE-ENGKRVWKPSWAVRAIPVDYMVPGYA 224
B. longum              EEPWGHIDYNRDQKVSFGGKVVENADGTKTWQPAWSVRAVPVDYLVPGYK 250
                       *:********:***:*.*******  :*.:.*:*:*:***:****:****

B. lactis              SGRVNTLRLWQARSYDEFDLLTFNKSEYLDAVKPQVKAEDISKVLYPEDS 274
B. longum              SGRVNTLRLWSAKSYDEFDLLAFNRSEYMEAVTPQVKAENISKILYPEDS 300
                       **********.*:*********:**:***::**.******:***:******

B. lactis              TEVGKELRLEQQYFFASASIHDAIRVFYPDTDKPDLTTFADKITFQLNDT 324
B. longum              TKVGKELRLEQQYFFVSASLHDAIRVFYPGQDKPDLTTFPNKIVFQLNDT 350
                       *:**************.***:*********. ********.:**.******

B. lactis              HPVIGIPELMRIMIDEYGYDWDTAWEVTHKTFNYTCHTLLPEALEVWPAS 374
B. longum              HPVIGIPELMRILIDEYGYDWDTAWSITTKTFNYTCHTLLPEALEVWPAS 400
                       ************:************.:* **********************

B. lactis              LIGKLLPRHLEIIERINKQFVSELEAKGASEDQIKRMLILTDDEHPVVRM 424
B. longum              LIGELLPRHLEIIEKINAQFEDELKSKGVDKNTIKDMAIYTGDA---VRM 447
                       ***:**********:** ** .**::**..:: ** * * *.*    ***

B. lactis              AYLATYAGSNVNGVAELHSQLLKDVTLRDFSDVYPAKFKNVTNGVTPRRF 474
B. longum              AYLATYGGSHVNGVAELHSQLLKDVTLKNFSDVYPDKFTNVTNGVTPRRF 497
                       ******.**:******************::****** **.**********

B. lactis              IKLANPRLSELITEGLGTDKWLEDLDLLEGLAPLAQDDEFVKKFAAVKHE 524
B. longum              VKLANPRLSDLITEGLGTDKWLSDLEMLKGLEPLAKDDEFVKKFAAVKKA 547
                       :********:************.**::*:** ***:************:

B. lactis              NKVAFADFSKQRYGAELDANTMFDTMIKRLHEYKRQALKILEIIATYSDI 574
B. longum              NKVDFAAYAKREYGFELDPNTMFNTMVKRLHEYKRQSLKILSVISTYADI 597
                       *** ** ::*:.** ***.****:**:*********:****.:*:**:**

B. lactis              KSGKVNVQDITPRTYIFGAKAAPGYYLAKMTIQLINNVAKVIDNDPDVNG 624
B. longum              KSGKVKAEDVTPRTVFFGAKAAPGYYLAKMTIQLINNVSRVVNNDPDVKG 647
                       *****:.:*:**** :**********************::*::******:*

B. lactis              KIKIFFPWNYNIEVAQMLIPATELDEQISQAGKEASGTSNMKFALNGALT 674
B. longum              KLAVYFPWNYNVRLAQHLIPATDLDEQISQAGKEASGTGNMKFALNGAMT 697
                       *: ::******:.:** *****:***************.*********:*

B. lactis              VGTLDGANVEIRERVGADNFFLFGMTVDEVEKMYEQGYDPSKYYEADPRL 724
B. longum              VGTLDGANVEIRERVGAENFFLFGMTVDEVEKKYAEGYNPASYYEADPRL 747
                       *****************:************** * :**:*:.********
```

```
B. lactis           KAAIDMVADGTFSNGDRNTYAPLVSDWLTKDWFMTLADFSAYHDIQADID 774
B. longum           KHAIDMVADGTFSNGDRNAYSPLVADWLTKDWFMTLADFSAYMDIQSEIE 797
                    * ***************:*:***:***************** ***::*:

B. lactis           ALYADQLEWNRKAILNVANSGYFSSDRSIKDYLDRIWHTRSLDK 818
B. longum           ALYADELEWNRKALLNVANSGYFSSDRSMEDYLERIWHTAPLAD 841
                    *****:*******:**************::***:***** .* .
```

Figure 29 Alignment of a putative glucan phosphorylase of *B. lactis* TMW 2.530 with the most homologous sequence, the glycogen phosphorylase of *B. longum* NCC2705. (AAN2442). The strongly significant transmembrane domain TM with a score of 1746 and predicted orientation form inside to outside is boxed. Another possible TM with a score of only 298 is market by a dashed box. Predicted catalytic residues are shaded grey. The catalytic residues Lys552, Arg553, Lys558 and Thr662 predicted for glycogen phosphorylase from rabbit muscle are highlighted grey.

With primers sucPB_V/2R targeting conserved regions of sucrose phosphorylases of other bifidobacteria, a sequence of 840 bp (appendix) was obtained that showed 100 % homology to the nucleotide sequence of the sucrose phosphorylase from *B. lactis* (AAN01605).

In *L. reuteri* TMW 1.106 no *glgP* gene could be detected with several degenerated primer pairs obtained from conserved regions in *glgP* of *L. casei*, *L. salivarius*, *L. lactis*, *Streptococcus pneumoniae*, *Geobacillus stearothermophilus*, *B. adolescentis*, *B. longum* and *E. coli*. However, a gene coding for a sucrose phosphorylase could be identified in *L. reuteri* TMW 1.106. The first 1150 bp of the sequence of *SucP106* of *L. reuteri* TMW 1.106 were obtained with primers based on conserved regions of *sucP* genes in *L. reuteri*, *S. mutans*, *S. suis* and *Lc. mesenteroides*. Because of the high homology, the lacking bp could be obtained with primers targeting conserved sequences before and after the meanwhile available sequences of *sucP* genes in *L. reuteri* JCM 1112 and *L. reuteri* 100-23. A total of 1539 bp (appendix) was obtained which encompassed one ORF of 1458 bp that was termed *sucP106*. The translated amino acid sequence showed highest homology (99 % identity, 100 % similarity in 457 aa) to SucP of *L. reuteri* JCM 1112 (EAR58730). The predicted Mw and pI of the protein was 56 kDa and pI 5 (www.expasy.org, compute pI/Mw tool). On the 2D-gel made with whole cell protein extract of *L. reuteri* TMW 1.106 after fermentation at pH 6.2 a spot probably belonging to SucP106 could be detected with the predicted pI and Mw from a just newly available sequence of a *sucP* gene of *L. reuteri* JCM 112 (Sequencing of the draft genome and assembly of *L. reuteri* DSM 20016, Joint Genome Institute) (pI 4.9, Mw 50 kDa, fig. 16A small circle). Because of the appearance of G-1-P in the supernatant, the sequence was examined for a probable extracellular location. Signal peptide and transmembrane domain prediction revealed no signal peptide but a preferred transmembrane domain from aa 357 to 377 (fig. 43, marked with a box) with orientation from outside to inside (score 930).

The nucleotide sequences up- and downstream (each less than 50 bp) did not show any homology to known sequences (megablast, http://www.ncbi.nlm.nih.gov/BLAST).

3.3.4 Cloning, heterologous expression of SucP106 in *E. coli* and preliminary characterization of the purified enzyme

The *sucP106* gene was successfully his-tagged and cloned into the restrictions sites for SacI and BamHI of the pUC18 vector but expression in *E. coli* Top10 was very low. The best result was obtained with 200 µM IPTG and without sorbitol, however after purification with a HisTrap column (1ml) (fig. 30A) only a very weak band was visible on SDS-PAGE (data not shown). After overnight incubation of 1.3 µg purified SucP106 in phosphate buffer (50 mM, ph 6.7) with sucrose (90 mM) at 42°C only about 22 µg G-1-P could be detected with a photometrical enzyme assay applying G-6-P-dehydrogenase and phosphoglucomutase and measuring the increase of NADPH (modified according to Koga et al. (1991)). The activity however was too low to characterize the enzyme. Further, in all tests despite G-1-P also some G-6-P could be detected indicating that SucP106 had not been purified completely with the HisTrap system. After cloning *sucP106* successfully into the restriction sites of SalI and NotI of the pGEX-4T-1 vector, a good overexpression in *E. coli* BL21 at any of the tested conditions could be reached (fig. 30B).

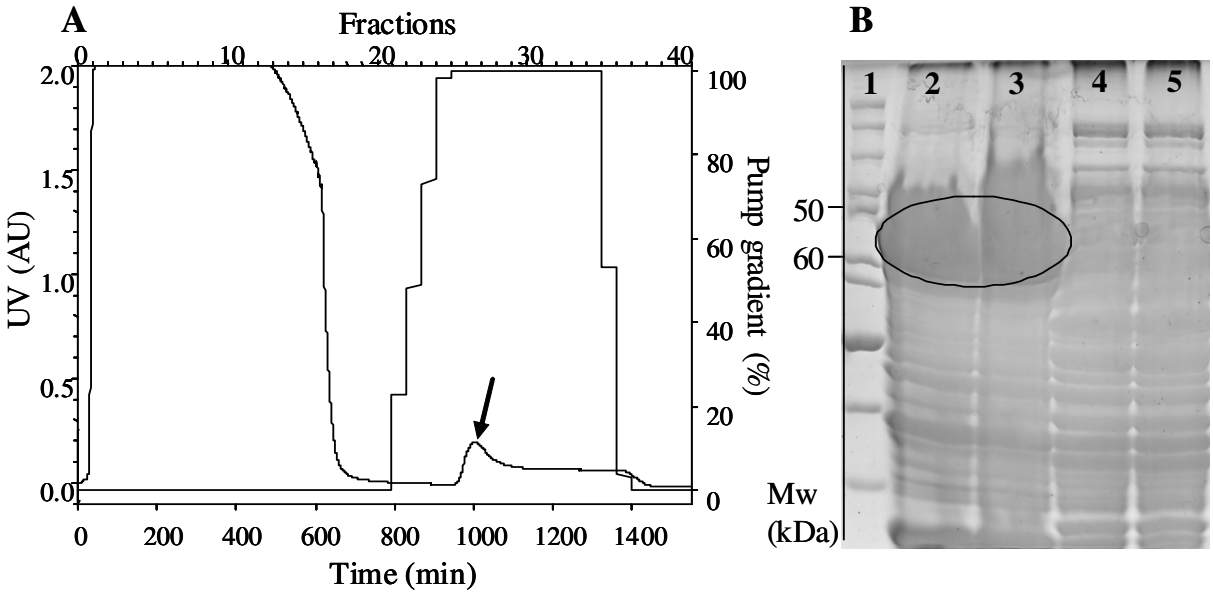

Figure 30A FPLC fractionation of crude extract of heterologous expression of *sucP106* in the pUC18/*E. coli* Top10 system. AU, arbitrary units. **B** SDS-PAGE of heterologously expressed SucP106 in *E. coli*: Precision protein standard (line 1), supernatant of crude extract of the pGEX-4T-1/*E. coli* BL21 system after induction with 100 (line 2) and 500 µM IPTG (line 3), supernatant of crude extract of the pUC18/*E. coli* Top10 system after induction with 100 (line 4) and 500 µM IPTG (line 5). The band of overexpressed SucP106 is marked with an ellipses.

3.3.5 Searching for P_i antiporters in *L. reuteri* TMW 1.106 and *B. lactis* TMW 2.530

The existence of a gene encoding sucrose phosphorylase, which is usually located intracellulary, did not explain the occurrence of G-1-P and G-6-P in the supernatants of *L. reuteri* TMW 1.106 and *B. lactis* TMW 2.530. Therefore, degenerated primers were constructed in order to find P_i-antiporters like GlpT and UhpT described in e.g. *E. coli* that could probably export sugar-phosphates under conditions of excessive sugar supply. Primers Uhp_V/R2 were constructed on the basis of conserved regions in the hexose-phosphate transporter UhpT of *Lactococcus lactis* (CAA50595) and the glycerol-3-phosphatase transporters of *Lact. lactis* (ABJ72147) and *L. brevis* (ABJ63273). With DNA of *L. reuteri* TMW 1.106 amongst others two PCR amplicons with the expected size of about 300 bp (appendix) were obtained by a PCR profile with five preamplification cycles at low temperature (38°C). With DNA of *B. lactis* TMW 2.530 and *B. adolescentis* TMW 2.454^T a 300 and a 600 bp product were obtained (data not shown). The sequences of the two PCR-products obtained with DNA of *B. lactis* TMW 2.530 revealed no homology to any known P_i antiporters. Sequencing of the 300 and 400 bp PCR-products with DNA of *L. reuteri* with Uhp_R2 gave two identical sequences. Their deduced aa sequences had highest homology (81 % identity, 89 % similarity within 77 aa; fig. 31) to the glycerol-3-phosphatase transporter UhpC (ABJ63273) of the major facilitator superfamily from *L. brevis* ATCC 367.

```
L. brevis  MFSFLKPAPDAKVKVPTEKVVGTYRLRQTGVLLSICIGYIGYYIIRLIFTTEQNDIMKAY 60
L. reuteri ------------------------------------------------------------

L. brevis  GFTTADIGLVMSCFGIGYGISKLFMGALSDKSNPKWYLATGLFISAILNFGLGSTRNLYV 120
L. reuteri -------------FGIGYGTSKLFMGALSDKSNTNRYLATGLVISALLNFGLGSTHSLYM 47
                       ****** *************.: ******.***:********:.**:

L. brevis  MMLLMLVMSVAQGMGAAACQRTVQLWWGKKWRGTVYAVWSSAHNAGAFACV..... 448
L. reuteri MMFLMLIMSIAQGMGAAACQRSVQLWXGKKX---------------------------- 78
           **:***:**:***********:**** ***
```

Figure 31 Alignment of the translated nucleotide sequences of a putative P_i antiporter of *L. reuteri* TMW 1.106 with a partial sequence of the most homologous sequence, the glycerol-3-phosphatase transporter UhpC (ABJ63273) of *L. brevis*.

3.4 Application of bacterial sucrose metabolism in food production: *In situ* EPS production through *L. sanfranciscensis* TMW 1.392 in dough and its effect on dough and bread quality

Because of already existing patents for the use of dextran in bread dough/baked products (patent numbers 6627235 and 2983613) a levan producing strain, *L. sanfranciscensis* TMW 1.392, that had been shown to produce EPS in wheat dough (Tieking et al. 2005b) was chosen in curse of the AiF project (AiF-FV 14037 N) to investigate effects of EPS production on dough and bread quality in comparison to a mutant strain disabled to produce EPS.

3.4.1 Performance of *L. sanfranciscensis* TMW 1.392 and its levansucrase deletion mutant in wheat dough: growth and metabolites formed

L. sanfranciscensis TMW 1.392 wildtype (WT) and its levansucrase deletion mutant TMW 1.392Δlev (Tieking et al. 2005b) (Δlev) were grown in wheat dough with a dough yield of 200 with 10 % sucrose (w/w flour) for 22 hours at 30°C. Two control doughs used to separate the effects of organic acids and levan were made and treated the same way. Yeast invertase was given to the mutant dough (Δlev Inv) to hydrolyze sucrose and to offer the possibility to the mutant to perform a similar metabolism as the WT. Further, WT was fermented without sucrose (WT 0%). Data for chemically acidified doughs had been obtained with the same flour and were kindly provided from the partners of the AiF project (AiF-FV 14037 N) (Seitter, M., Vogelmann, S. and Hertel, C., Lebensmittelmikrobiologie, Universität Hohenheim, Stuttgart, Germany).

Cell counts for the mutant *L. sanfranciscensis* TMW 1.392Δlev on agar plates with and without erythromycin were in the same decimal power. As depicted in figure 32, the average viable cell counts were $2*10^8$ for the wildtype whereas the mutant only reached a maximum of $4*10^7$. The pH of the dough after 22 hours was lowest in dough fermented by the wildtype without sucrose (3.44) and highest in dough fermented by the mutant with sucrose but without invertase to hydrolyze the sucrose (3.59). The spectra of metabolites formed in the different doughs differed significantly and reproducibly (table 4), which provides further evidence for the differential growth of the respective strains. The metabolites formed by the mutant *L. sanfranciscensis* TMW 1.392Δlev are comparable to the amounts of the metabolites formed by the wildtype fermented without added sucrose due to the activity of flour invertase on sucrose and maltose naturally present in flour. However, the wildtype formed the most lactic acid when fermented without sucrose, and resulted in the highest fermentation quotient (FQ).

Compared to the wildtype, the mutant produced remarkably less acids. Even the addition of yeast invertase did not lead to the same pattern of metabolites as observed in the WT doughs but resulted in increased acetic acid level and therefore, the lowest FQ. Levan was only produced in noticeable amounts when the wildtype was incubated in dough with sucrose. Average amounts of estimated 4.5 g kg^{-1} flour were reached.

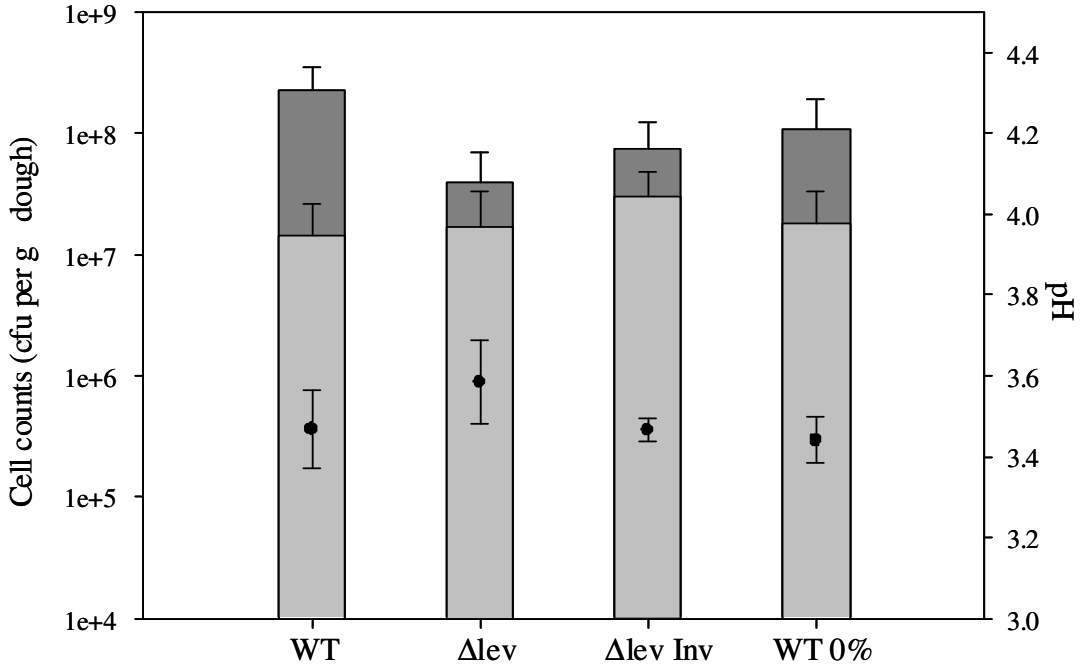

Figure 32 Colony forming units (bars) and pH values (circles) in wheat dough with 10 % sucrose (w/w) after incubation (grey bars) and 22 hours of fermentation (dark grey bars) at 30°C with *L. sanfranciscensis* TMW 1.392 wildtype (WT) and its levansucrase deletion mutant TMW 1.392Δlev (Δlev). Control doughs to separate the effects of organic acids and levan were made with mutant, 10% sucrose and yeast invertase (Δlev Inv) and with wildtype without sucrose (WT 0%). Data are means and standard deviations of at least three independent experiments.

Table 4 Metabolites (mmol kg^{-1}, flour base) in predoughs fermented by wildtype (WT) and Δlev mutant (Δlev) of *L. sanfranciscensis* TMW 1.392 for 22 hours at 30°C. Data are representative for several equal fermentations with a coefficient of variation of maximal 11 % and a standard deviation of two measurements of maximal ±3 %.

	lactate	acetate[b]	FQ[a]	ethanol[b]	mannitol	glucose	fructose	sucrose[c]	levan[d]
WT	142.05	55.34	2.6	36.90	99.46	187.00	18.00	73.61	14.4
Δlev	93.70	23.04	4.1	36.80	13.33	2.00	0.00	286.03	<1
Δlev Inv	109.85	81.24	1.4	0.00	179.92	178.00	62.00	0.00	<1
WT 0%	167.67	20.03	8.4	38.11	14.50	8.00	0.00	0.00	<1

[a] FQ, fermentation quotient; [b] ethanol may be partially lost during lyophilization; [c] sucrose at 0 hours: 384 mmol kg^{-1} (11 mmol kg^{-1} originating from the wheat flour); [d] fructose in levan (mmol kg^{-1}, flour base), estimated 5.2 g kg^{-1} (flour base) levan

Production of oligosaccharides by *L. sanfranciscensis* TMW 1.392 in wheat dough with sucrose is expectable because of the availability of various possible acceptors (Tieking et al. 2005b and 2005c). However, detection of oligosaccharides with the AminoPac column from the freeze dried dough samples was very low. Production of noticeable amounts of 1-kestose and nystose was not stated. Two peaks, one possibly belonging to erlose, were detected in only some of the repeated fermentations and they were also present in doughs fermented by the levansucrases deletion mutant.

3.4.2 Influence on dough rheology

To evaluate the influence from *L. sanfranciscensis* TMW 1.392 wildtype and the Δlev mutant on the dough rheology, pre- and bread doughs were made and analyzed by various methods. No significant effect of levan (added and *in situ* produced) could be seen by rheofermentometer measurements which describe dough stability and gaseous release. However, when the control doughs made to separate the effects of an altered metabolic pathway/performance of the mutant (organic acids) and levan were considered, an effect caused by organic acids was observed. Bread doughs made with predough WT 0%, having the highest lactate content, showed the lowest dough development characteristics, whereas bread doughs made with Δlev Inv which had the highest acetate content, showed the best gas retention coefficient and the lowest percentile in height loss [(Hm-h)/Hm] followed by the chemically acidified control (CAC), which also reached the highest total gas volume (table 5).

Table 5 Development and gaseous release characteristics of doughs from rheofermentometer testing

	Dough development			**Gaseos release**						
	T1	**Hm**	**(Hm-h) *Hm^{-1}**	**H`m**	**T`1**	**Tx**	**total vol.**	**CO_2 lost vol.**	**retention vol.**	**retention coeff.**
	(min)	(mm)	(%)	(mm)	(min)	(min)	(ml)	(ml)	(ml)	(%)
WT	129	43.5	48.5	64.9	99	97	1335	172	1162	87.2
Δlev	130	47.1	49.5	65.5	105	91	1379	183	1195	86.8
Δlev Inv	151	47.8	16.1	58.9	111	109	1189	94	1094	92.1
Δlev Inv EPS	148	47.1	16.7	58.6	111	115	1168	89	1079	92.4
WT 0%	136	36.6	66.4	56.5	105	109	1177	119	1057	89.9
WT 0% EPS	126	34.4	100[a]	56.5	99	103	1187	129	1058	89.2
CAC	123	44.8	28.8	58.8	75	79	1524	-[b]	-[b]	-[b]

[a] probably a measurement error; [b] data not available

Table 6 Farinograph mixing characteristics of doughs. Data are means and standard deviations of two independent experiments.

	absorption[a]	**DDT**[b]	**stability**	**degree of softening 10´´**	**degree of softening 12´´**	**pH predough**
	(%)	(min)	(min)	(BU)	(BU)	
WT	57.7 ± 1.1	1.8 ± 0.3	5.1 ± 0.7	64.3 ± 7.6	73.0 ± 8.5	3.5 ± 0.0
Δlev	57.8 ± 0.4	1.7 ± 0.1	6.0 ± 0.4	50.0 ± 9.6	60.0 ± 11.1	3.6 ± 0.1
Δlev Inv	57.1 ± 0.9	1.6 ± 0.3	5.5 ± 0.7	60.3 ± 9.7	68.3 ± 8.7	3.5 ± 0.0
Δlev Inv EPS	58.3 ± 0.8	1.6 ± 0.1	4.6 ± 0.8	67.7 ± 13.6	78.3 ± 13.8	3.5 ± 0.0
WT 0%	57.4± 0.4	1.9 ± 0.2	5.5 ± 0.8	56.0 ± 9.6	64.7 ± 10.7	3.5 ± 0.0
WT 0% EPS	58.5 ± 0.4	1.6 ± 0.0	4.8 ± 0.2	54.0 ± 4.4	67.3 ± 4.6	3.5 ± 0.0
CAC	60.6 ± 0.2	1.5	7.9	-[c]	-[c]	3.6

[a] at 500 FU; [b] dough development time, [c] data not available

Data from the farinograph showed a positive effect of EPS on the water absorption of the bread doughs, as it increased about 1 % after the addition of levan (table 6). The *in situ* production of levan with the wildtype was not enough to have reproducible positive effects on water absorption. Dough mixing stability, however, decreased slightly with the addition of levan, and was highest when the predough Δlev with the highest pH was used. The dough fermented with WT did not reach better results than the one fermented with Δlev.

For dough rheology measurements all doughs were adjusted to a optimal dough consistency of 500 Brabender Units (BU), which is the practical measure used in bakeries to permit high water content and comparability. The measurement errors of bread dough stickiness and extensograph analysis (extensibility and resistance) were relatively high (5-15 %) and the values varied widely within the independent experiments. No significant effects of levan or different acid contents on stickiness could be seen, probably because of the premise to adjust all doughs to 500 BU. With the extensograph also no significant difference could be measured. However, the extensograph analysis revealed some observable trends. Resistance of the doughs was lower when levan was added or produced *in situ* (fig. 33A), and the higher acetate content produced in the Δlev Inv sample led to an increase of the resistance of the dough while at the same time the extensibility decreased (fig. 33B). Consequently, the ratio between resistance and extensibility (R/E) was highest for the Δlev Inv doughs (12.8±0.5), and was significantly reduced by the addition of levan (8.6±0.6). For the other doughs, no significant differences in R/E could be observed (data not shown). Further, we observed clear differences in the temporal changes of the dough stripes characteristics between stripes form

bread dough with or without EPS. For each bread dough 10 to 20 bread dough stripes were measured by a micro-extension procedure. For calculation of the averaged resistance (and extensibility), the first and last few stripes and measurements were discarded. However, great differences in the rheological behaviour of the bread dough with increasing number of measured stripes and therefore increasing resting time (although one measurement only took 20 seconds) were observed. Changing in the dough characteristics to higher extensibility and lower resistance (indicated by arrows in fig. 34) during the short duration of the measurement procedure was more pronounced when no EPS had been added. Figure 34 shows the extensograph curves from the first and the last stripes of bread dough WT 0% EPS (A) and of WT 0% (B) (both from the same predough WT 0%), which indicates that with EPS, theoretically, measurement of about 20 stripes was possible and lead to only small changes in dough consistence during the time of measurement. Without EPS, however, consistence of the dough changed within only ten stripes. The same trend was observed for stripes of bread dough Δlev Inv EPS and of Δlev Inv (both from the same predough Δlev Inv) (data not shown). These results suggest that dough with EPS is less sensitive to time delays at room temperature.

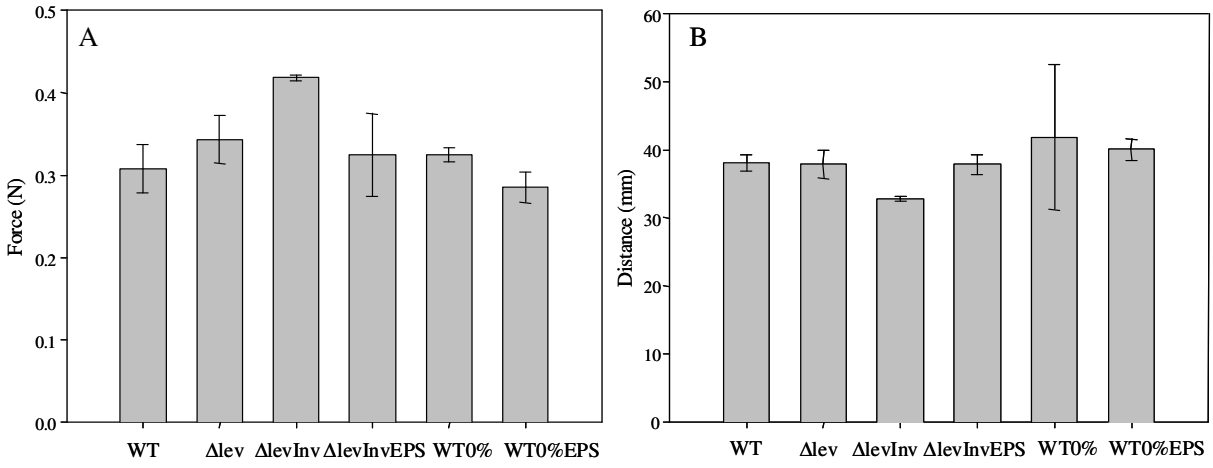

Figure 33 A Resistance to extension, expressed as force (N), and **B** extensibility, expressed as distance (mm), of bread doughs from to independent experiments. Data are means and standard deviations of at least 8 dough stripes per dough and two

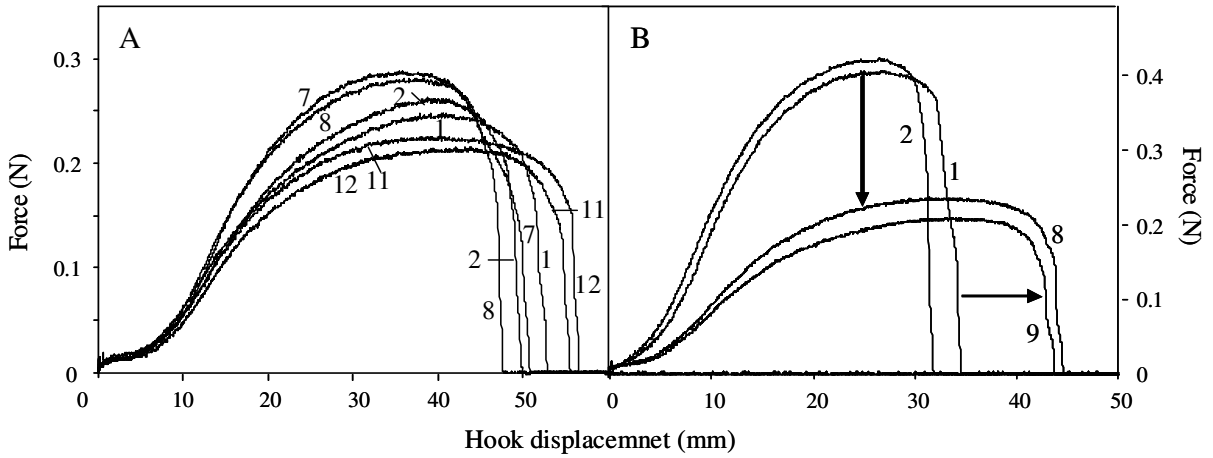

Figure 34 Force as a function of hook displacement (distance). For each bread dough 10 to 20 bread dough stripes were measured by micro-extension procedure. For calculation of the averaged resistance and extensibility, the first and last few stripes and measurements were discarded. However, great differences in the rheological behaviour of the bread dough with increasing number of measured stripes and therefore increasing resting time were observed. Compared are bread doughs **A** WT 0% EPS and **B** WT 0%. Depicted are the first and last measured stripes. Numbers indicate the number of the measured stripe.

Measurements with the rapid visco analyzer (RVA) to elucidate the pasting properties of the different predoughs did not reveal significant and reproducible differences when freeze-dried predoughs were used in an amount to replace 10 % of the flour normally used in the RVA. Generally, the use of commercial guar and xanthan showed substantial higher effects as visible in figure 35A. However, significant differences could be observed when different purified EPS were used without predough as a kind of system test. The fructan of *L. sanfranciscensis* LTH 2590 showed a slightly higher viscosity as the one of *L. sanfranciscensis* TMW 1.392 (fig. 35B). The use of an unbranched dextran in comparison to both levans showed that the dextran had more influence on the pasting properties, especially on the peak viscosity and the viscosity at the end of holding at 50°C (marked with an arrow; fig. 35C).

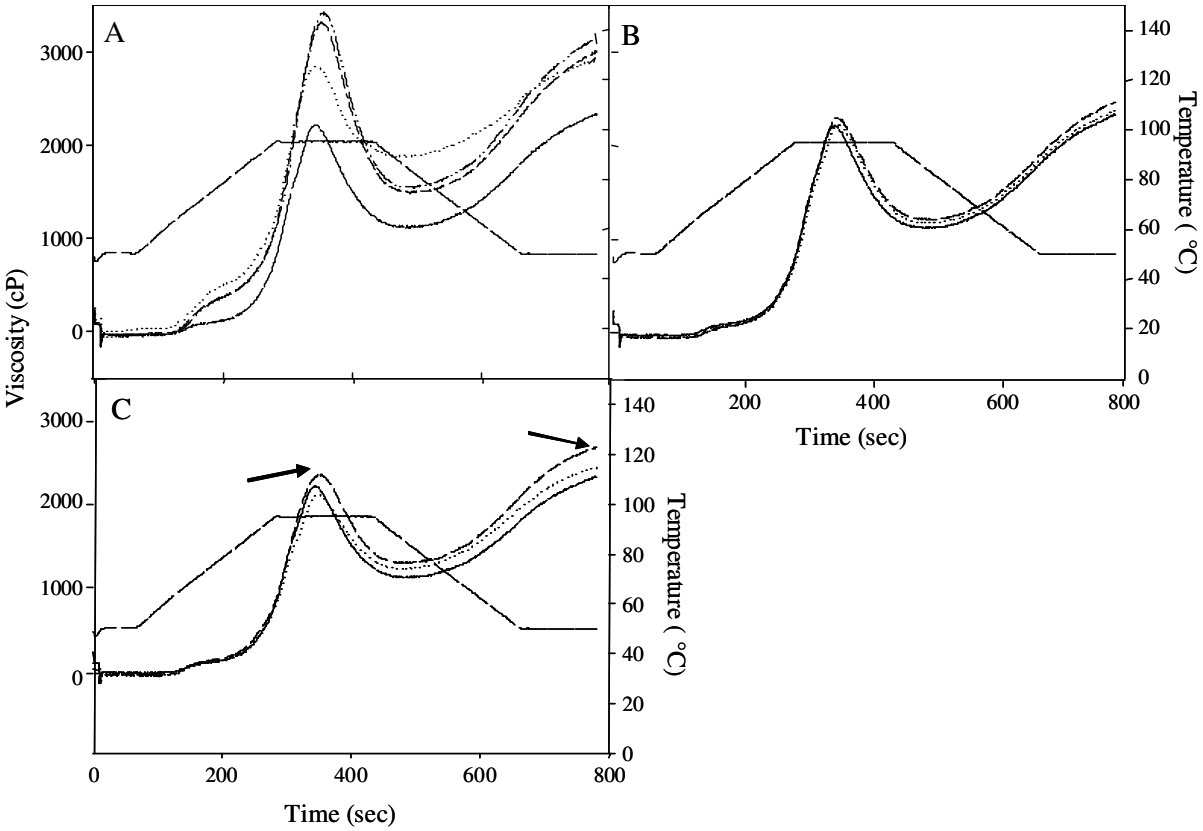

Figure 35 Pasting curves of RVA measurements and applied temperature profile (long dashed line). Shown are pasting curves of flour blend without addition (solid line) and with addition of **A** 2 % xanthan (dotted line), 2 % guar gum (dashed line), 2 % guar gum and Δlev Inv predough (dashed-dotted line); **B** Δlev Inv predough and 2% levan (flour base) from *L. sanfranciscensis* TMW 1.392 (dotted line) or from LTH 2590 (dashed line); **C** 5 % levan (TMW 1.392) (dotted line) or 5 % unbranched dextran (dashed line). cP, Centipoises.

3.4.3 Volume of breads made with predoughs of wildtype and mutant of *L. sanfranciscensis* TMW 1.392

The averaged volumes of the six different breads are given in table 7 (sixth column). The methodical error of triple measurements of the same bread was maximal 3 %; the one of the triple measurement of three equal breads per predough was maximal 2.5 %. Comparison of WT and Δlev breads, however, was not possible because of the poor reproducibility within four similar experiments. However, when 0.3 % levan was added to the bread doughs made with the predough Δlev Inv or WT 0%, the loaf volume increased about 1.0 % and 2.0 %, respectively, compared to the use of the same predough without addition of EPS. The volume of all breads was higher than the volume of the bread made with chemically acidified predough (seventh row). Breads made with the predough Δlev Inv, which had the highest value of acetic acid, produced the lowest volumes.

3.4.4 Staling of bread made with predoughs of WT and Δlev of *L. sanfranciscensis* TMW 1.392

The hardness (F) of the breads was determined by texture profile analysis (TPA), according to AACC method 74-09 (AACC 1999), over a period of 12 days. Retrogradation of starch, namely amylopectin was determined with differential scanning calorimetry (DSC) according to Leon et al. (1997) over a period of eight days. Hardness and retrogradation enthalpy were fitted to the Avrami equation which describes the equilibrium crystallization of high polymer melts. For comparability data were analyzed by means of a restricted model with n set to 1. Maximal hardness F_∞-F_0 (N) (corrected with the initial hardness F_0), maximal enthalpy ΔH_∞ (J/g) (with day 0 = 0 retrogradation) and the Avrami constants k_{TPA} and k_{DSC} were consulted (table 7). Lower k values describe slower firming and staling rates. The averaged Avrami constants k_{TPA} and k_{DSC} were reduced in breads made with WT predough when sucrose was added (0.263 in comparison to 0.369 for TPA and 0.206 to 0.219 for DSC) and hence, levan was produced. This positive effect was even more pronounced, particularly in TPA, when levan was added directly to the bread doughs (0.155). The same effect of added EPS to bread dough was also observed for k_{TPA} of the Δlev Inv dough (0.149 in comparison to 0.226). In the DSC measurements, the k_{DSC} of the Δlev Inv breads did not follow this trend. Breads made with Δlev predough had a slightly higher averaged k_{TPA} value than the ones with WT predough, but surprisingly a lower averaged maximal hardness was observed. However, the averaged maximal hardness was reduced when EPS had been added to bread doughs. Generally, results were better and comparability was easier when the same predough was used, e.g. WT 0% for breads WT 0% and WT 0% EPS, than in the WT and Δlev batch. Figure 36 shows the kinetics of bread firming when EPS was added to bread doughs made with WT 0% (A) and Δlev Inv (B). At day eight of storage WT 0% breads had a crumb hardness of 19.1 N compared to WT 0% EPS breads of only 15.7 N, and Δlev Inv breads had a crumb firmness of 18.7 N compared to Δlev Inv EPS breads of only 17.6 N. A probable effect of different metabolisms, more precisely, different amounts of lactic acid produced and hence different pH in the predough can be assumed. The averaged maximal hardness, F_∞-F_0 (table 7), is highest for the WT 0% breads (with the highest production of lactic acid) and lowest for the Δlev breads (with the lowest lactic acid production). For the maximal enthalpy (ΔH_∞) no such trends could be observed.

Table 7 Avrami parameters (with n set to 1) of TPA and DSC and bread volumes; pH and FQ of the corresponding predoughs. Data are means of all similar experiments.

	TPA[a]		DSC[b]		bread characteristics		predough	
sample	F_∞-F_0 (N)	k_{TPA} (days^{-1})	ΔH_∞ (J/g)	k_{DSC} (days^{-1})	**loaf volume** (ml)	(%)[c]	**pH**	**FQ**
WT	17.11 ± 2.64	0.263	1.14 ± 0.10	0.206	2040 ± 109	+ 12.1	3.46	2.6
Δlev	16.24 ± 1.97	0.272	1.15 ±0.14	0.205	2070 ± 90	+ 13.7	3.62	4.1
Δlev Inv	17.81 ± 0.91	0.226	1.20 ± 0.13	0.279	1978 ± 38	+ 9.2	3.46	1.4
Δlev Inv EPS	17.22 ± 2.55	0.149	1.29 ± 0.01	0.295	1997 ± 32	+ 9.7	3.46	1.4
WT 0%	18.58 ± 1.05	0.369	1.23 ± 0.01	0.219	1994 ± 121	+ 9.6	3.36	8.4
WT 0% EPS	17.49 ± 0.59	0.155	1.21 ± 0.01	0.166	2033 ± 42	+ 11.7	3.36	8.4
CAC	23.33 ± 2.03	0.188	1.31 ± 0.04	0.266	1820 ± 18	-	3.60	-

[a] texture profile analysis; [b] differential scanning calorimetry, [c] in relation to the bread made from the chemically acidified control dough (CAC)

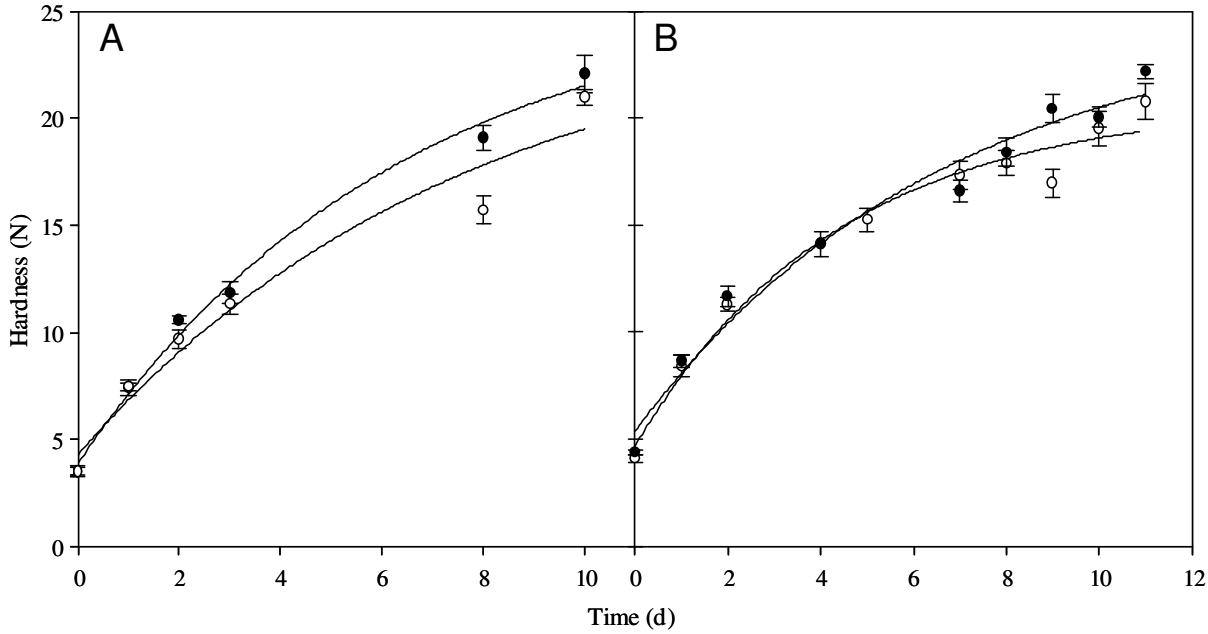

Figure 36 Kinetics of crumb hardness of breads fitted to the Avrami equation with n set to 1. **A** Breads made with WT 0% predough without EPS (black symbols) and with EPS (white symbols); **B** breads made with Δlev Inv predough without (black symbols) and with EPS (white symbols). Data are means and standard deviations of measurements of ten slices of every bread.

3.5 Screening for the amount of lactic and acetic acid produced by EPS forming lactobacilli in mMRS-S

Lactate and acetate were identified to affect the dough rheology and the bread characteristics and possibly counterbalance positive effects of EPS produced additionally/alternatively from sucrose. Therefore, heterofermentative EPS forming LAB were compared concerning the production of lactic and acetic acid from sucrose. For simplification of the screening, strains were grown in mMRS-sucrose. Within one genus, the FQ was quite similar with exception of the genus *L. pontis*, where *L. pontis* TMW 1.1115, had a higher FQ value as the other two examined *L. pontis* strains (table 8). The absolute amounts of lactate and acetate formed during 24 hours varied, partially founded on the different growth but without a comparable growth relatedness within the genera. Two-thirds of the strains showed FQ values between one and 2.5. Only within the genus *L. reuteri*, FQ values smaller than one were observed. *L. collinoides* TMW 1.1166, *L. frumentum* 1.187 and *L. pontis* 1.1115 had FQ values above 3.5. Some strains produced almost equal amounts of lactate and acetate, accompanied by the production of glycerol (not quantified) and a FQ lower than 1.5, in most cases. However not all strains of one genus produced glycerol (2 of 6 *L. sanfranciscensis* and 2 of 3 *L. pontis* strains).

Comparing the production of organic acids in respect of the kind of EPS produced (as far as known), it was clearly visible, that the average FQ of glucan-forming LAB was smaller than the one of the fructan-forming strains (1.0±0.12 and 1.65±0.65, respectively) (with exception of TMW 1.1115). All glucan forming strains synthesized glycerol.

Table 8 Cell counts, pH, and production of lactate and acetate (and glycerol) through EPS-forming lactobacilli after 24 h of fermentation in MRS-S

	TMW#	EPS monomer; descr.[2]		ferm. temp. (°C)	cfu ml^{-1} (*10^8)	pH	lactate (mM)	acetate (mM)	FQ[3]	Glycerol
L. mucosae	1.141	F[1]	.	37	1.1	3.3	92.52	70.46	1.3	+
L. mucosae	1.81	F	1+	30	28.8	3.6	42.19	40.26	1.1	+
L. pontis	1.675	F		37	7.0	3.4	66.81	55.10	1.2	
L. pontis	1.1115	F		37	7.2	4.3	19.35	4.49	4.3	+
L. pontis	1.397	G[1]	+/-	37		3.4	60.49	54.93	1.1	+
L. reuteri	**1.106**	**G**		**37**	**0.6**	**3.7**	**33.43**	**28.95**	**1.2**	+
L. reuteri	1.138	G		37	11.8	3.4	63.13	59.56	1.1	+
L. reuteri	1.272	G		30		3.9	24.58	29.86	0.8	+
L. reuteri	1.974	G		37	1.5	3.7	36.12	39.87	0.9	+
L. reuteri	1.976	G		37	1.3	3.6	39.47	43.55	0.9	+
L. reuteri	1.1274	n.d.		37	1.5	3.5	58.33	49.78	1.2	+
L. sanfranciscensis	1.53	F		30	1.9	4.5	12.28	12.22	1.0	+
L. sanfranciscensis	1.54	F		30	7.6	3.5	51.25	23.21	2.2	
L. sanfranciscensis	1.896	F		30	6.8	4.2	17.13	12.51	1.4	+
L. sanfranciscensis	1.953	F		30	6.6	3.8	34.98	11.56	3.0	
L. sanfranciscensis	1.1149	n.d.		30	5.8	4.1	18.94	12.36	1.5	
L. sanfranciscensis	**1.392**	**F**	**2++**	**30**	**1.1**	**3.6**	**48.24**	**21.17**	**2.3**	
L. buchneri	1.75	n.d.	2+	37	11.6	3.7	51.46	18.59	2.8	
L. brevis	1.57	n.d.	1+	30	1.6	4.7	9.31	11.72	0.8	-
L. collinoides	1.1166	G, F	+/-	26	22.0	4.1	19.35	4.49	4.3	
L. fermentum	1.187	F, (G)	3+	30	37.6	3.6	60.61	16.61	3.7	
L. frumenti	1.665	F		37	0.9	3.5	55.59	35.93	1.6	
L. frumenti	1.666	F	2+	37	7.8	3.4	75.43	50.76	1.5	+
L. hammesii	1.1236	n.d.	1+/-	30	7.6	4.0	24.15	10.29	2.4	+
L. hilgardi	1.45	n.d.	+/-	30	17.6	3.7	44.66	17.42	2.6	
L. kunkeei	1.1258	G	3+	30	28.2	3.7	32.87	31.95	1.0	-
L. lindneri	1.88	n.d.		28-30	26.4	3.5	62.31	28.18	2.2	
L. parabuchneri	1.429	n.d.	3+	30	27.8	3.8	39.73	18.92	2.1	
L. suebicus	1.428	n.d.	+/-	30	21.4	3.5	53.37	26.13	2.0	

[1] F: fructan, G: glucan, ; n.d., not determined; [2] determined by F. Waldherr and co-workers, Technische Mikrobiologie, Technische Universität München, Germany: numbers describe relative shiny colony morphology on agar plates with sucose; +/-, +, ++ describe relative amounts of slime produced after 48 h on agar plates with sucrose.; [3] FQ: fermentation quotient

3.6 *In situ* production of metabolites from sucrose

The *in situ* produced levan from *L. sanfranciscensis* TMW 1.392 did not have the desired effects on dough and bread quality. Instead effects of the levan apparently were counteracted by the formation of acids. The use of a not further characterized glucan, however, showed higher effects in all experiments in which it was used. Therefore, experiments were carried out where a glucan and a fructan producer should be compared concerning the amounts of EPS and organic acids produced during dough fermentations. *L. reuteri* TMW 1.106 was chosen because its EPS production is well characterized and more efficiently and the strain had the highest FQ amongst all tested glucan forming strains described in **3.5**.

3.6.1 Influence of dough yield (DY) on EPS contents in dough

Wheat doughs from commercial wheat flour (type 550) with 10 % sucrose (flour base) and DY values of 160, 220, 500 and 800 were fermented with *L. reuteri* TMW 1.106 or *L. sanfranciscensis* TMW 1.392. Water soluble polysaccharides including the microbial EPS were extracted and hydrolyzed. To ensure practical relevance of the results, these were interpreted along rules applied by bakers aming at maximal EPS content per kg flour obtainable under different conditions. Despite the experimental error which is higher when EPS has to be extracted from a complex food matrix like dough than from media, clear trends could be observed that have more importance than absolute amounts. In figure 37A one of two fermentations of *L. reuteri* in wheat dough is depictured. In the doughs with DY 500, the amount of glucose in WPS increased significantly even in the first 8 hours. After 23 h the maximal EPS content (per kg flour) was detected with DY 500, whereas with DY 220 the amount of glucose in WPS still increased. In both experiments done the amount of glucose in WPS at DY 160 was comparable to the one in the control doughs (taking in to account the experimental error). This observation indicated that in the control dough polysaccharides were released from the flour through the acidic conditions and/or flour enzymes. A DY value of 800 did not lead to a higher glucan content (per kg flour) as compared to DY 500 (data not shown). Cell viability was comparable up to 23 h (with a little but not reproducible delay in DY 160) and maximal cell counts of $2*10^9$ (cfu per kg flour) were observed. In DY 160 and 220 cell viability was stable until 48 h, whereas cell counts of *L. reuteri* TMW 1.106 in dough with DY 500 and 800 decreased drastically. Acetate contents (per kg flour) were similar in all doughs (54.2±1.5 mM after 48 h), lactate contents (per kg flour) were highest in doughs with

the highest DY (142.8 mM after 48 h) and lowest in the ones with lowest one (77.1 mM after 48 h), which was also reflected in the pH values DY (fig. 37A).

With *L. sanfranciscensis* TMW 1.392 dough fermentations at different DY showed clearly the same trends. In doughs fermented with *L. sanfranciscensis* TMW 1.392 the amount of fructose in WPS (per kg flour) was highest at the highest DY (fig. 37B) after 24 h and the amount of extractable fructan then decreased drastically till 48 h. Acetate levels in *L. sanfranciscensis* doughs were about 44.9±4.9 mM in all doughs, the lactate content was highest in the dough with highest DY (500) (207.6 mM after 48 h) and lowest in the one with DY 160 (90.8 mM after 48 h) (all per kg flour). With both strains the FQ decreased with decreasing DY, from 2.7 to 1.5 for *L. reuteri* and 4.4 to 1.9 for *L. sanfranciscensis*. The ratio of monomers from EPS in WPS to lactate, which gives insight into the conversion of monomers either into EPS or lactate was highest in DY 500 at 24 h and in DY 220 at 48 h (data not shown).

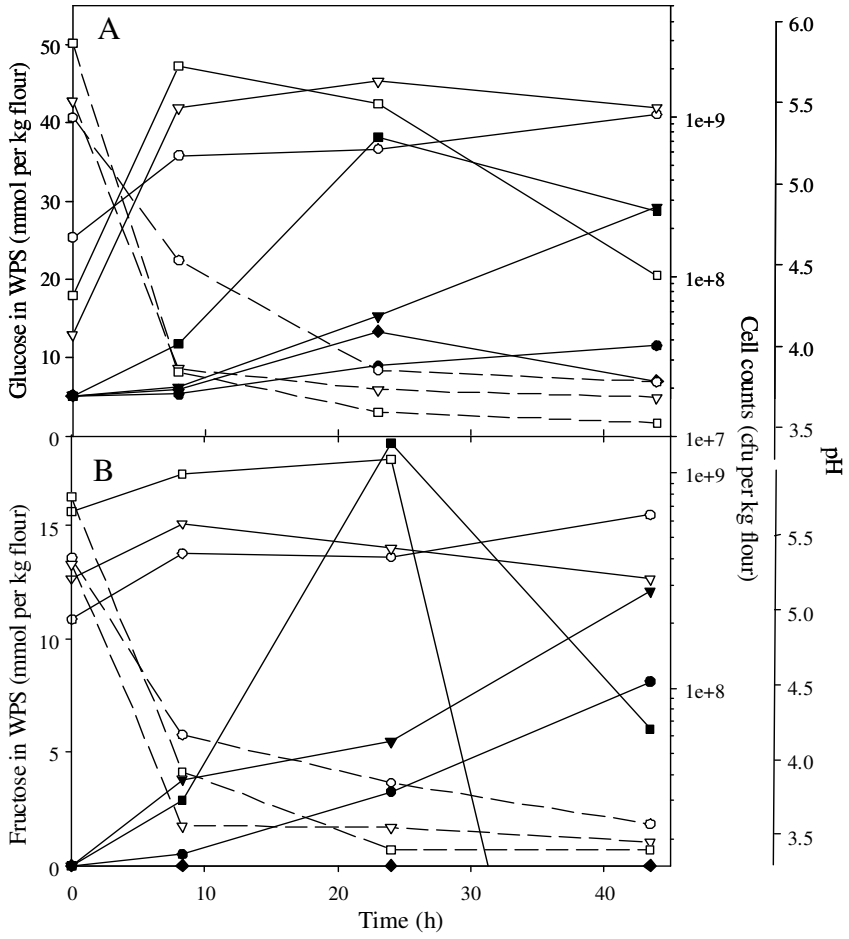

Figure 37 Kinetics of EPS yields (mmol monomer in water soluble polysaccharides (WPS) per kg flour) (balck symbols), growth (cfu) (white symbols) and pH (dotted lines) in dough fermentations with **A** *L. reuteri* TMW 1.106 and **B** *L. sanfranciscensis* TMW 1.392 at different dough yields (DY): DY 160 (circles), DY 220 (triangles), DY 500 (squares) and chemically acidified control dough (diamonds) at DY 220.

3.6.2 Influence of controlled pH and sucrose fed-batch on EPS contents in dough

Dough fermentations were carried out at controlled pH at the pH optima for the transferase activities of both glycosyltransferases, the levansucrase of *L. sanfranciscensis* (Tieking et al. 2005b) and Gtf106A of *L. reuteri* (this study). In dough with pH 4.0 no EPS production by *L. reuteri* TMW 1.106 could be detected in comparison to the release of WPS in the control dough with pH control. In dough with pH 4.7 the EPS production was higher than in dough without pH control (fig. 38A). Absolute amounts of glucose in WPS varied within replicates because of the use of two different wheat flours. Augmentations to 126 % and 155 % in comparison to the dough without pH control could be reached with pH control during two days. When determined, hardly any residual sucrose could be detected after two days of fermentation in dough without pH control (1.5 mmol) in doughs with pH control to 4.7 sucrose was not detectable after two days. With pH regulation at 4.7 in wheat dough fermented with *L. sanfranciscensis* TMW 1.392 an augmentation of fructose in WPS to 113 % could be reached (fig. 38B). At pH 5.4 instead, the amount of WPS was less than in dough with the same DY but no pH regulation.

If additional sucrose was supplied in small amounts after about 8, 24 and 48 h (sucrose fed-batch) to pH controlled wheat dough (pH 4.7) the yield of EPS-monomers in WPS could be further increased (fig. 38, squares). Augmentations to 143±1.4 % for *L. reuteri* and to 122 % for *L. sanfranciscensis* were measured in comparison to the doughs with pH control only. For *L. reuteri* no residual sucrose could be detected at the end of fermentation, whereas in the *L. sanfranciscensis* doughs, sucrose accumulated to 56 % after 2 days of fermentation in the fed-batch dough. In the pH regulated dough sucrose was almost consumed totally (7 mmol left), whereas in the dough without pH control 24.6 mmol per kg flour of sucrose could be detected.

Cell viability of *L. reuteri* and *L. sanfranciscensis* in the different doughs was similar, although a small percentage of cells in the doughs probably died in fermentations with pH control every time the pH was readjusted because of the direct contact to NaOH. The production of organic acids was higher in doughs with pH regulation but correct calculation was not possible because of the adjustment of pH with lactate and acetate.

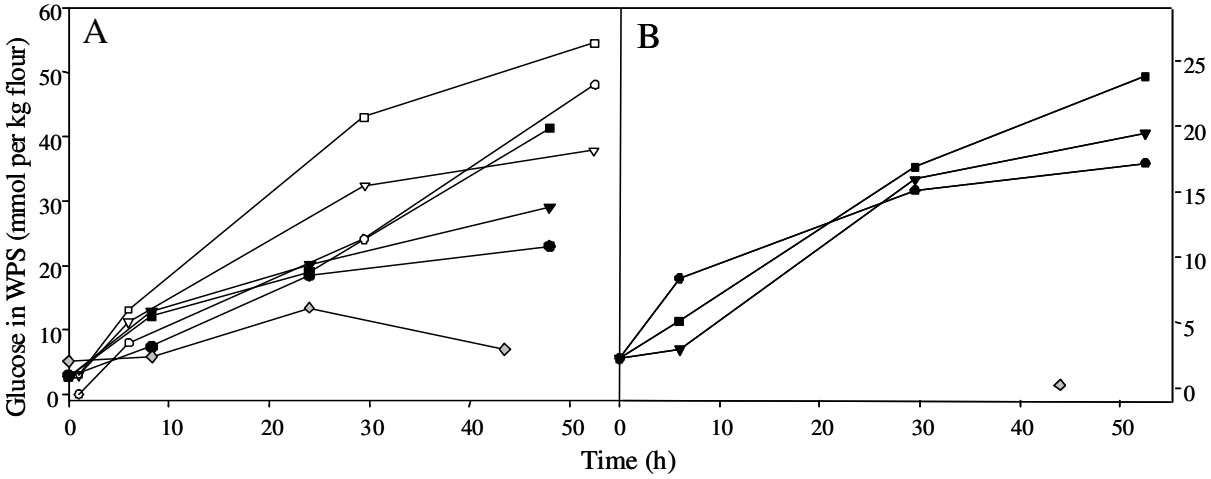

Figure 38 Kinetics of EPS yields (mmol monomer in water soluble polysaccharides (WPS) per kg flour) in wheat dough fermentations with pH control and sucrose fed-batch with **A** *L. reuteri* TMW 1.106 (grey: control, black and white: two independent experiments) and **B** *L. sanfranciscensis* TMW 1.392: no pH control (circles), pH 4.7 (triangles), pH 4.7 and fed-batch (squares) and chemically acidified controle at pH 4.7 (diamonds).

3.6.3 Influence of different fermentation substrates on EPS contents in dough

Doughs were prepared with a wheat flour blend (WB), a strong wheat flour (WS), a rye-wheat mixture (RW) (50:50) and with rye bran (RB), each with 10 % sucrose (flour base). From the parallel fermented control doughs maximal 8.9 mmol glucose and 5.9 mmol fructose could be isolated from WB, 6.9 and 3.1 mmol from WS, 6.5 and 2.3 mmol from RW and 5.7 mmol glucose and 2.8 mmol fructose from RB. In figure 39A the mean amounts of glucose in WPS from two independent experiments with *L. reuteri* are depictured. It was striking, that the amount of glucose in WPS increased much more in rye bran during the first hours, which indicates that the EPS production started earlier in rye bran than in the other substrates. However, the yield of extractable EPS from rye bran decreased after 24 h. The amount of produced and extracted EPS of the other substrates varied too much between similar experiments to see clear trends. Whereas at 24 h still most EPS was extractable from rye bran, at 48 h the extractable amounts did not differ widely. The pH in rye bran decreased to 3.9, while in the other substrates 3.5 was achieved. The amounts of lactate and acetate (per kg flour) were highest in the rye bran ferment (222±52 mM and 141±27 mM after 24 h, 247±19 mM and 163±12 mM after 48 h), and lowest in the wheat doughs (WB) (68±6 mM and 43±1 mM after 24 h, 73±10 mM and 49±13 mM after 48 h) (fig. 39B). Cell viability (per kg flour) at 24 h was best in RB (4.7-9.5*10^9) and RW (3.9-4.3*10^9), but in RW cell counts had decreased drastically till 48 h, reaching the lowest level within all substrates(data not shown).

Cell viability of *L. sanfranciscensis* TMW 1.392 was also best in RB followed by RW. Similar to *L. reuteri*, cell counts of *L. sanfranciscensis* decreased drastically in RW and RB till 48 h. The *in situ* EPS production through *L. sanfranciscensis* was not as reproducible as through *L. reuteri* TWM 1.106. It appeared to be best in RW during the first hours, but at 48 h the extractable amount of fructose in WPS from RW had decreased to 60 % and after 48 h most fructose in WPS was detected in WS doughs. The pH in rye bran sank to 3.7-3.8, while in the other substrates values from 3.3 to 3.5 were achieved. Similar to the *L. reuteri* doughs, in the *L. sanfranciscensis* doughs the amounts of lactate and acetate were highest in the rye bran ferment (279±21 mM and 160±37 mM after 24 h, 255±26 mM and 155±40 mM after 48 h), and lowest in the wheat doughs (WB) (112±60 mM and 46±19 mM after 24 h, 130±14 mM and 56±8 mM after 48 h). However, the FQ in the *L. sanfranciscensis* doughs was always slightly higher than in the *L. reuteri* doughs. The ratio of monomers from EPS in WPS to lactate was higher in wheat doughs for both strains because of the high lactate production in the rye substrates, but more as twice with *L. reuteri* than with *L. sanfranciscensis* (data not shown).

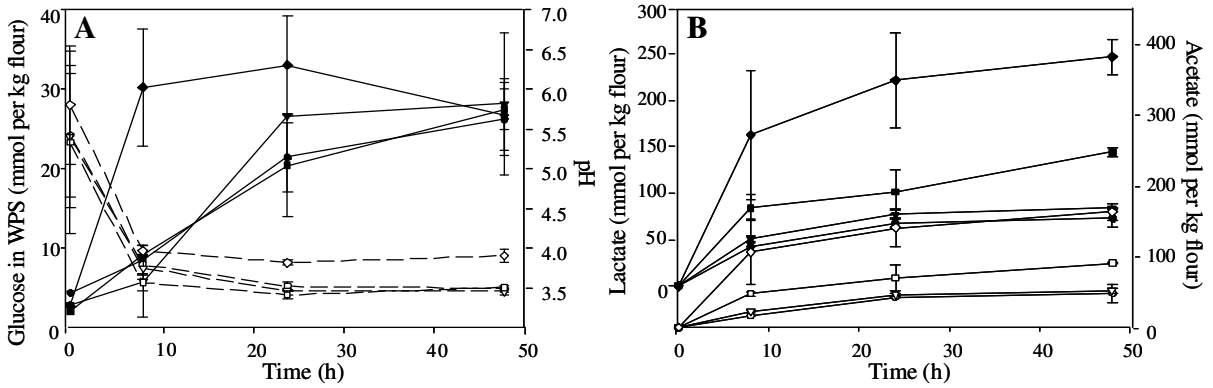

Figure 39 Kinetics of **A** glucose in WPS (black symbols) and pH (white symbols), **B** lactate (black symbols) and acetate (white symbols) in dough fermentations with *L. reuteri* TMW 1.106 with different fermentation substrates: wheat blend (circles), wheat flour strong (triangles), rye flour (squares) and rye bran (diamonds). Data are means with standard deviations from two independent experiments.

3.6.4 Oligosaccharide production through *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392 *in situ* under different conditions

As synthesis of oligosaccharides competes with EPS production, some doughs were analyzed by HPAEC-IPAD for oligosaccharide synthesis in dough. Due to various poly- and oligosaccharides of flour origin, which also can be partially degraded during fermentation, a high variety of metabolites can act as acceptors for oligosaccharide synthesis. This fact makes it difficult to identify oligosaccharides from dough extracts. In wheat dough extracts from

L. reuteri TMW 1.106 a peak series could be detected eluting after the system OH-peak like after incubation of ΔN-Gtf106A with sucrose and maltose or isomaltose (chapter **3.1.5**) (data not shown). Comparison with the chromatogram from the chemically acidified control dough proved, that the peak series did not result from an α-amylase activity on starch/amylose. In wheat doughs with different DY, most peaks of this series appeared at DY 160; pH regulation in combination with sucrose fed-batch led also to a higher oligosaccharide production. No difference was detectable between WB and WS and in RW and RB no such peak series were produced, but two not identified peaks appeared that were not detected in the control doughs and were not further characterized. In the doughs produced with different substrates and *L. sanfranciscensis* TMW 1.392 only very low amounts of 1-kestose were produced. In RW three unknown peaks were detected and at the same time the amount of detectable arabinose decreased, indicating that arabinose could have acted as an acceptor for one of these not identified oligosaccharides (data not shown). Regulation of the pH to 4.7 did not lead to a higher oligosaccharide production but additional fed-batch led to an augmentation of 1-kestose. After 30 h about 11 mM fructose had been used for levan production in the not pH regulated dough without fed-batch, in the fed batch system 14 mM had been used for levan and about 16 mM for 1-kestose synthesis.

3.6.5 Stability of the EPS during storage of the dough at 4-8°C

Practical application of EPS containing pre-ferments requires storage. Therefore, the stability of the EPS upon storage was investigated. After a period of storage in vapour proof PE bags at 4 to 8°C doughs with different fermentation substrates (WS, WB, RW, RB) were analysed again. The amount of glucose in WPS extracted from all *L. reuteri* doughs increased drastically during storage for three months. The average augmentation led to 122 %. Cfu of bacillary bacteria decreased to $1\text{-}2*10^6$ during storage. In RW and WB growth of yeasts was detected. The level of organic acids and ethanol increased in all doughs (max 150 % in doughs without yeasts), however the pH also increased slightly. In a second experiment over three months, the amount of glucose in WPS had even risen to 245 % in pH regulated wheat dough with sucrose fed-batch. Lactate levels had decreased to minimal 89 %, acetate and ethanol levels had increased to maximal 132 %. No yeasts were detected on agar plates.

The storage experiments with doughs from *L. sanfranciscensis* TMW 1.392 did not provide reproducible results or trends. In a first experiment over 3 month maximal 15 % of the produced levan could be extracted from all doughs. In a second experiment over 1.5 months, in contrast, the amount of levan increased in RW.

4. DISCUSSION

Exopolysaccharide and oligosaccharide synthesis from sucrose by lactobacilli or other probiotic bacteria enabling the design of novel cereal based products requires a thorough understanding of their sucrose metabolism these substances are derived from. This work showed that *L. reuteri* TMW 1.106 is able to use sucrose in two pathways: employing a glucansucrase to form a dextran, which was shown to exert a protective function against low pH, and oligosaccharides; or employing the energy saving sucrose phosphorylase to gain energy from sucrose via catabolism. The letter enzyme was also identified to paly a role in the sucrose metabolism of *B. lactis* Bb-12 TMW 2.530 yet known to harbour an invertase (Ehrmann et al. 2003). It was further shown that EPS has positive effects in the dough and bread system, which, however, can be counteracted by the formation of acids if the EPS is produced *in situ* in preferments. As the latter is the only way industrial practice can easily exploit these compounds, selecting the appropriate producer strain and optimization of the conditions of EPS synthesis are the keys for the transfer of the basic knowledge into industrial practice. It was shown, that the specific design of fermentations along the optimum conditions of enzyme activity and bacterial growth can help to exploit EPS for the baking industry.

4.1 Influence of pH on glucansucrases of *Lactobacillus reuteri* TMW 1.106

Many *L. reuteri* strains from different habitats produce EPS (Tieking et al. 2003a, 2005a). Several of them have more than one glycosyltransferase (van Geel-Schutten et al. 1999; van Hijum et al. 2001 and 2002; Tieking et al. 2003; Kralj et al. 2004a). This study has shown, that *L. reuteri* TMW 1.106 harbours two glucansucrases, Gtf106A, a dextransucrase, and Gtf106B, that are highly homologous to the reuteransucrase GtfA and the inactive GtfB of *L. reuteri* 121, respectively (Kralj et al., 2002 and 2004a). The strain further harbours one inulosucrase, that does not produce EPS under normal culture conditions (Schwab and Gänzle 2006). The presence of another active glycosyltransferase was excluded by fermentations with the glucansucrase deletion mutant of the strain (provided by Jens Walter, University of Otago, New Zealand; Walter et al. 2005).

The his-tagged ΔN-Gtf106B, comparable to GtfB (Kralj et al. 2004a), had no transferase activity; however, it exhibited hydrolysis activity. The sequence of Gtf106B has the catalytic residues with crucial roles in formation of the glycosyl-enzyme complex and the transition state stabilisation (Asp1024, Glu1061, Asp1133, His1132 and Gln1514) and therefore probably has some hydrolysis activity (for review see van Hijum et al. 2006). But like the

GtfA mutant H1065S:A1066S from *L. reuteri* 121 that does not have transferase activity, Gtf106B also has Ser at position 1065 (Kralj et al. 2005). This work determined that dextran production of *L. reuteri* TMW1.106 is attributable to Gtf106A and is dependent on the environmental pH. Moreover, glucan had a protective effect for the bacterium against low pH and therefore probably contributes to the pronounced acid tolerance of *L. reuteri,* which is relevant to its occurrence in the gastrointestinal tract of animals and in sourdough.

The synthesis of EPS through *L. reuteri* TMW 1.106 in pH static fermentations was highest in the pH range of 4.7 to 5.4 although it was shown not to be growth related. The latter finding is in accordance with results published recently by Årsköld et al. (2007) for *gtfO* of *L. reuteri* ATCC 55730. However, the specific yields of EPS were higher at pH 5.4 in this study and amounts obtained were far higher than the ones reported by Årsköld et al. (2007). Poor growth and low EPS yields were specifically observed at pH 4.0, challenging an assumed protective function of EPS against low pH. However, because sucrose was the only carbohydrate source in the growth media, poor growth of the strain at pH 4.0 is likely to be attributable to the low hydrolysis activity of ΔN-Gtf106A at this pH value and the probably low activity of SucP106, as sucrose phosphorylases from e.g. *Lc. mesenteroides* strains are reported to work best at high pH values (Silverstein et al. 1967; Kawasaki et al. 1996; Goedl et al. 2007). And as intracellular pH regulation requires ATP (Booth 1985) for proton translocation by an ATPase, *L. reuteri* TMW 1.106 probably favours sucrose utilization for energy supply over forming EPS if the initial pH of the fermentation is already low. However, optimal transferase activity of ΔN-Gtf106A was observed in the range of pH 3.7 to 4.2 which supports again a protective function of the EPS formed against low pH. Also the N-terminal domains of glucansucrases have been reported not to play a significant role in the enzyme activity and the linkage-type and molecular mass of the EPS produced (Abo et al. 1991; Monchois et al. 1999; Kralj et al. 2004b), no data are available of the pH optima of deletion mutants. Protonation of Glu (pK_A= 4.2) or His (pK_A= 6.0) in the catalytic domain may contribute to pH-dependent catalytic properties of Gtf106A. And as protonable groups also appear in the N-terminal domain of Gtf106A, it cannot be excluded, that its deletion may have influenced the pH-dependent activity of the enzyme (Karlson et al.1994).

Glucansucrases can occur in a cell free and a cell bound form (van Geel-Schutten et al. 1999). Because neither Gtf106A nor Gtf106B could be detected in the concentrated supernatant of *L. reuteri* TMW 1.106, the enzymes likely are bound to the cell surface. EPS produced by a cell-wall bound enzyme surrounds the cell at least for the time of chain elongation. Moreover,

the low pH optimum of the transferase activity of ΔN-Gtf106A is in keeping with a protective function of dextran against environmental challenges such as low pH. This was also substantiated by the measurements of survival at low pH in the presence of EPS (fig. 4).

The analysis of protein expression by 2D SDS-PAGE revealed that Gtf106A and Gtf106B were transcribed at all four tested pH values and that the expression was not significantly lower at pH 4.0 than at higher pH values. This was also seen after qualitative analysis of the *gtf106A* and *gtf106B* mRNA levels (data not shown). Accordingly, Wall et al. (2007) also did not find changes in the expression of one of the glucansucrase genes found for *L. reuteri* ATCC 55730 (Båth et al. 2005) in connection with pH changes. Thus, the low dextran yield in pH static fermentations at pH 4.0 is not attributable to a decrease in gene expression as was the case for the dextransucrase gene in *Lc. mesenteroides* (Quirasco et al. 1999) or the *gtfBC* genes in *S. mutans* (Li and Burne 2001) and *gtfO* of *L. reuteri* ATCC 55730 (Årsköld et al. 2007). Remarkably, Gtf106A and Gtf106B were expressed as isomers differing in their pI, indicating posttranslational modifications that influence the pI (Baumann and Meri 2004). Such polymorphisms can be evoked by posttranslational modifications as e.g. phosphorylation (e.g. Ser or Thr), glycosylation (e.g. Thr or Asp) or deamidation (e.g. Asn or Gln). Such modifications often take place parallel to protein folding (Baumann and Meri 2004) and are frequently noticed in proteins with high molecular weights (unpublished observations; Behr, Technische Mirkobiologie, Technische Universität München, Germany). Asn (10.21 Mol%) is the most frequent amino acid in Gtf106A, followed by Thr (8.24 Mol%) and Asp (7.91 Mol%) and about half of the Asn, Thr, Asp, Ser and Gln residues are located in the N-terminal domain that had been deleted for cloning and may therefore explain the altered pH optimum. The Gtf106A isomer with the highest pI was not detected in cell extracts of *L. reuteri* grown at pH 4.0 which indicates that the posttranslational modifications may depend on the environmental pH and may contribute to differences in the amount of glucan produced by native Gtf106A at pH 4.0.

The glucan produced in pH static fermentations and by the purified ΔN-Gtf106A enzyme showed virtually the same NMR-spectra at the different pH conditions which demonstrates that Gtf106A is responsible for EPS production by *L. reuteri* TMW 1.106. This conclusion was supported by fermentations with a glucansucrase deletion mutant of *L. reuteri* TMW1.106 (provided by Jens Walter, University of Otago, New Zealand; Walter et al. 2005). The EPS yield achieved with the glucansucrase deletion mutant from sucrose amounted only to maximal 2 % of the yield obtained with the wildtype strain and the hydrolysate of the

precipitated material contained, as expected, relatively less glucose and more fructose than the one found after fermentation of the inulosucrase deletion mutant of the same strain.

Asymmetrical flow-FFF analysis showed that the molecular mass and the polydispersity of dextran produced through *L. reuteri* TMW 1.106 was dependent on the environmental pH. Benincasa et al. (2002) also observed an influence of culture conditions (incubation time, nutrient and pH) on the molecular mass of pullulan produced through *Aerobasidium pullulans* and postulated that culture conditions have to be strictly controlled to customize the microbial EPS production. Dextran from fermentations of *L. reuteri* at pH 4.0 showed the highest monodispersity concerning the data for molecular mass and hydrodynamic diameter. In keeping with our observations, D. Kim et al. (2003) observed polydisperse dextran produced from a purified dextransucrase and stated that the pH has a higher impact on the molecular weight than on branching. The higher values of Mp and Rhp of the EPS from fermentations at pH 4.7 and 5.4 indicate that at these pH values polymers with high molecular mass and high hydrodynamic diameter are present in the highest concentration. After fermentation at pH 6.2, in contrast, most of a population with lower Mw and Rh was present. It can not be excluded that the inulosucrase of *L. reuteri* TMW 1.106 (Schwab and Gänzle 2006) contributed to polymer production at higher pH values. Though, only the EPS isolated from fermentations at pH 4.7 showed a slightly higher percentage of fructose after hydrolysis (data not shown). The optimum activity of inulosucrase of *L. reuteri* 121 was observed in the pH range of 5.0 - 5.5, however, Inu predominantly produced oligosaccharides and only small amounts of inulin (van Hijum et al. 2003). SucP106 may also contribute to the production of oligosaccharides as for sucrose phosphorylases of *Lc. mesenteroides* strains transglucosylation activity resulting in OS has been described (Kitao and Sekine 1992; Kawasaki et al. 1996) and a possible transglucosylation activity of SucP106 could not be excluded in the course of this work. The optimum of transglucosylation activity of sucrose phosphorylase of *Lc. mesenteroides* is at high pH (about 5-6) (Silverstein et al. 1967). Furthermore, G-1-P is also precipitable with ethanol (Russell et al. 1988) but should have been lost during dialyzation because of the cut-off of 12-14 kDa. However, a smaller and former not identified peak visible on chromatograms of dialyzed and hydrolyzed precipitated material from fermentations with *L. reuteri* TMW 1.106 and its dextransucrase deletion mutant (**3.2.4**) and the one of hydrolized G-1-P (**3.1.5**) had the same retention time as phosphate (about 13.4 min) on OAKC (data not shown) and was arguing for an insufficient removal of G-1-P through dialyzation or probably the presence of phosphorylated glucooligosaccharides through transglucosylation activity of Suc106P.

D. Kim et al. (2003) revealed high temperatures (37-45°C) and relatively low sucrose concentrations (0.1-0.3 M) as preferred conditions for high molecular weight dextran, which coincides with our findings for the optimal temperature for the activity of ΔN-Gtf106A (45°C) and for the maximal sucrose concentration for highest average EPS-yields (max. 100 to 120 g l^{-1}) (unpublished data Körber/Gänzle, Technische Mikrobiologie, Technische Universität München, Germany).

The structure of *L. reuteri* TMW 1.106 dextran, 83-85 % α-(1-6) linkages and 15–17 % α-(1-4) linkages, is similar to the dextran produced by the GtfA mutant N1134S:N1135E:S1136V of *L. reuteri* 121. Comparable to Gtf106A, this GtfA mutant has Ser at position 1134 instead of Asn which confirms that the conserved regions II and IV and specifically the amino acid at position 1134 are the main determinants of the structure of the glucan produced (Kralj et al. 2005 and 2006). Whether the different linkage and size distribution has any effect on the speculated functions of the EPS, for example in foods, remains to be demonstrated. Martin and Hoseney (1991) suggested that for the delay of bread staling the branching of dextrin is not as important as its molecular weight.

If EPS production is desired, oligosaccharides are undesirable side products. Comparable to *L. reuteri* 121, respectively its GtfA, *L. reuteri* TMW 1.106, respectively its Gtf106A and ΔN-Gtf106A, produced small amounts of leucrose and panose in slightly increasing amounts with increasing pH values following the trend observed for the synthesis of glucan. On the other hand, some oligosaccharides have positive effects for example on the survival of microorganisms under stress-conditions (Schwab 2006), may serve probiotic functions, or are used as fat replacers. Oldenhof et al. (2005) reported about positive effects of maltodextrins as a bulking agent on the survival of *L. bulgaricus* during freeze-drying. Isomaltooligosaccharides may have a selectively stimulating effect on gut bacteria (Roberfroid 2007). Dextrins are traditionally used in the food industry for the manufacture of confectionery and as fat replacer (Belitz et al. 2001). ΔN-Gtf106A was shown to produce a series of isomalto- and maltooligosaccharides (dextrins) from sucrose and (iso)maltose as acceptors and maltooligosaccharide synthesis was also shown *in situ* (**3.6.4**).

However, under all conditions tested (especially high pH values), whether ΔN-Gtf106A nor ΔN-Gtf106B produced the substances responsible for the two peaks observed on AminoPac chromatograms from supernatants of fermentations at pH 5.4 and especially 6.2 that were thought to be glucooligosaccharides produced by the same glucosyltransferase and led to the suggestion that these two substances must be built by other enzymes.

4.2 Evidence of another sucrose degrading enzyme of *L. reuteri* TMW 1.106

The observation, that the dextransucrase mutant of *L. reuteri* TMW 1.106 (provided by Jens Walter, University of Otago, New Zealand) was able to grow in MRS with antibiotics and sucrose as sole carbon source after adapting to increasing concentrations of sucrose (and decreasing concentrations of glucose) without producing noteworthy amounts of EPS was also made by Schwab (2006) and led to the suggestion, that this strain must have another sucrose hydrolyzing enzyme. Fermentations with this deletion mutant with sucrose revealed further that the mutant also synthesized the two substances leading to the initially unidentified peaks on AminoPac chromatograms. These results finally confirmed that GtfA106 was only responsible for the pH dependent production of the dextran and that no altered activity of the N-terminal truncated enzymes ΔN-Gtf106A and ΔN-Gtf106B led to the loss of ability to synthesize the unknown substances but that *L. reuteri* TMW 1.106 must have another enzyme responsible for their production and pH dependent occurrence in the supernatant. The two unknown peaks resulting from supernatants of *L. reuteri* TMW 1.106 were later identified as G-1-P and G-6-P based on consistent results of HPLC, TLC and an enzymatic test according to Koga et al. (1991). In a previous paper (Tieking et al. 2005a) we postulated the substances to be GOS with a DP of over 4 because of results of gel permeation chromatography. Probably, as also suggested by Russell et al. (1988) this mis-interpretation could have resulted from a charge interaction of the glucose-phosphates and the column matrix. The assumption of the presence of a sucrose phosphorylase gene in *L. reuteri* TMW 1.106 was brought through observation of the same peaks on AminoPac chromatograms of supernatants of *B. lactis* TMW 2.530 and the identification of SucP in *B. lactis* TMW 2.530 followed by enzymatic test with the purified enzyme and with the purified substances. By PCR a *sucP* gene could be also identified in *L. reuteri* TMW 1.106 and its activity was proven after heterologous expression in *E. coli*. This result was also confirmed by the release of draft genome sequences of *L. reuteri* 100-23 (NZ AAPZ00000000) and JCM 1112 (Sequencing of the draft genome and assembly of *L: reuteri* DSM 20016, Joint Genome Institute). Recently *L. reuteri* ATCC 55730 was also described to harbour an active sucrose phosphorylase (Båth et al. 2005; Årsköld et al. 2007) and expression data of SucP106 were reported by Schwab et al. (2007a) who found sucrose induced expression of the sucrose phosphorylase of *L. reuteri* TMW 1.106 in contrast to its constitutive expression of Gtf106A. The catalytic residues common in members of the glycoside hydrolase family 13 Asp192 and Glu232 that had been described in *sucP* of *B. adolescentis* (AF543301; *B. adolescentis* numbering) through Sprogøe

et al. (2004) and, in the case of Asp192, had been confirmed in *sucP* of *Lc. mesenteroides* (Schwarz and Nidetzky, 2006) could also be found in the *sucP* gene of *L. reuteri* TMW 1.106 (fig. 43, highlighted grey). Although models of transmembrane domains (TMpred on www.ch.embnet.org) are purely speculative and a score of 930 for the preferred TM-model for SucP106 with outside to inside orientation is not significant (likewise predicted (score) for sucP of *B. longum* (597), *B. adolescentis* (559) and *B. animalis* (580) and SucP of *L. reuteri* JCM 1112 (930)) such a localisation would place mentioned catalytic residues extracellulary and therefore make the detection of G-1-P in the supernatant possible. However, in this case, the organism would need a transport system to transport G-1-P into the cell to avoid cross-feeding of competitors as e.g. *E. coli* that was reported to be able to use G-1-P as carbon and phosphate source when grown in minimal medium with G-1-P as only carbon or phosphate source, repectively (Pradel and Boquet 1991). Båth et al. (2005), however, did not describe the sucrose phosphorylase of *L. reuteri* ATCC 55730 to have a TM domain and to be located extracellular.

The model of sucrose metabolism in *L. reuteri* TMW 1.106 may therefore be extended as depicted in figure 40A. Beside the utilization of sucrose by the constitutively expressed GtfA106, sucrose presumably gets into the cell via a sucrose permease and is subsequently phosphorylated by sucrose phosphorylase overexpressed in the presence of sucrose (Schwab et al. 2007a) leading to G-1-P and fructose. Phosphoglucomutase then converts G-1-P to G-6-P which enters the glycolytic pathway. In contrast to sucrose transport and hydrolysis by a sucrose PTS-system and sucrose-6-P-hydrolase this way is energy saving. The additional presence of an invertase in this strain was excluded by Schwab et al. (2007a).

In all tested *L. reuteri* strains (TMW 1.138, 1.272, 1.968, 1.969, 1.973, 1.975, 1.974, 1.976 and 1.979) that were shown in prior studies (Tieking et al., 2005a and unpublished data) to accumulate G-1-P in fermentation supernatants after 48 hours of fermentation (no pH control; pH of mMRS-S at t_0: 6.2), a probable sucP gene could be detected with primers SucPL_V/R (some data shown in fig. 41). Positive results were also obtained with DNA of EPS-negative but G-1-P-positive (Tieking, unpublished data) *L. reuteri* strains (TMW 1.973, 1.975 and 1.968) and with DNA of *L. animalis* TMW 1.971. In supernatants of *L. animalis*, however, no G-1-P-peak had been detected under reference conditions, probably because the gene is not expressed or active in that strain or G-1-P is not released in the supernatant.

No data are available about characterized sucrose phosphorylases of lactobacilli. The pH optimum for the phosphorolysis reaction of characterized sucrose phosphorylases from *Lc. mesenteroides* strains is reported to be at high pH (7-7.5) (Silverstein et al. 1967;

Kawasaki et al. 1996). The optimal temperature of SucP of *B. adolescentis* is described to be high (48°C; van den Broek et al. 2004). These results are in accordance with the detection of G-1-P only after fermentations at pH 5.4 and especially at pH 6.2 and no detection of it after fermentation of *L. reuteri* TMW 1.106 at 30°C. In accordance with data from *B. lactis* (Trindade et al. 2003) and *B. longum* (Kullin et al. 2006), expression of SucP106 is induced by sucrose and also by raffinose and repressed by glucose (Schwab et al. 2007a). However, no activity of SucP106 with raffinose could be detected spectrophotometrically and by HPLC and these findings are again in accordance with published data (e.g. Trindade et al. 2003). In *S. mutans* the sucrose phosphorylase gene is organized in one operon with a transport system for raffinose and co-transcribed with α-galactosidase that releases sucrose from raffinose (Russell et al. 1992). *L. reuteri* TMW 1.106 was able to grow on raffinose as sole carbon source and G-1-P was detected after fermentation (data not shown), probably also through action of a galactosidase on raffinose followed by the action sucrose phosphorylase on the released sucrose (fig. 40B).

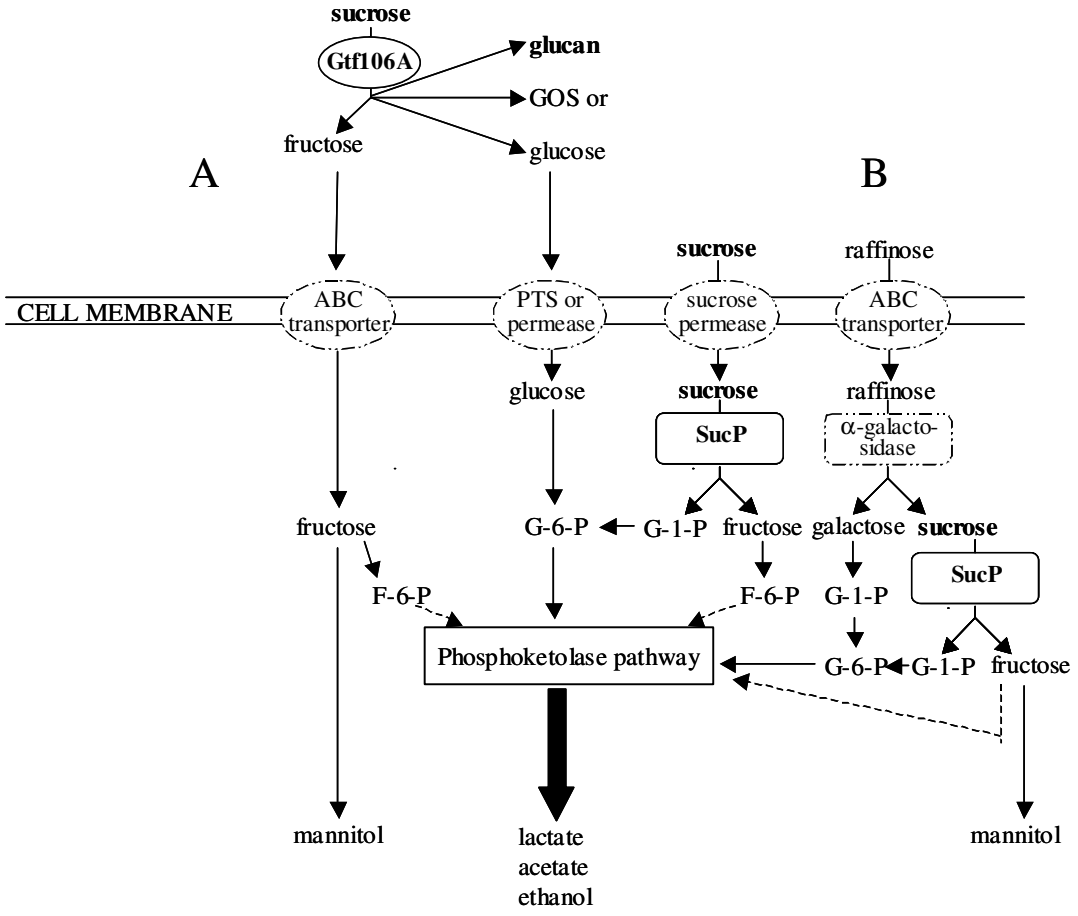

Figure 40 Suggested metabolic pathways of **A** sucrose and **B** raffinose metabolism of *L. reuteri* TMW 1.106. Gtf, glucosyltransferas; GOS, glucooligosaccharides; SucP, sucrose phosphorylase. Dashed arrows indicate that intermediates were left out; the existence of proteins in dash-dot-dotted lines is not proven.

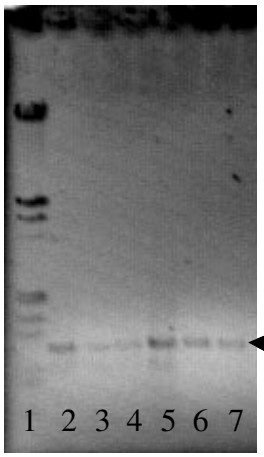

Figure 41 Agarosegel with PCR-products obtained with primers SucPL_V/R and DNA of *L. reuteri* strains TMW 1.106 (line 2), 1.974 (line 3), 1.976 (line 4), 1.979 (line 5), 1.272 (line 6) and 1.138 (line 7) and molecular weight standard (line 1).

4.3 Sucrose utilization of *B. lactis* TMW 2.530 through sucrose phosphorylase

B. longum NCIBM 702259^T was described to harbour two genes for sucrose utilization. One β-fructofuranosidase (CscA) clustered with a sucrose permease and a sucrose phosphorylase (ScrP) clustered with a putative membrane protein with homology to a sucrose symporter (Kullin et al. 2006). Transcriptional regulatory studies led to the suggestion that the sucrose phosphorylase of this strain cleaves the sucrose that results from α-galactosidase activity on raffinose and the β-fructofuranosidase cleaves glucose from fructose-oligosaccharides. Raffinose and FOS are supposed to be transported into the cell by *B. longum* NCC2705 with an ATP-binding cassette transporter. Recently it was reported about a putative sucrose permease in *B. longum* that is more similar to systems in LAB than in other actinomycetes (Parche et al. 2007). Sucrose phosphorylases of bifidobacteria, however, had been described to form a cluster that is distinct from the one of LAB (Kullin et al. 2006; Reid and Abratt 2005). *B. lactis* TMW 2.530 was shown in this study to grow on sucrose and raffinose (data not shown) and to have an active sucrose phosphorylase. In previous studies this strain was also shown to have a β-fructofuranosidase (Ehrmann et al. 2003) identical to the one of the type strain *B. lactis* DSM10140 that, heterologously expressed and purified, showed 100 % activity against sucrose. Sucrose metabolism in *B. lactis* TMW 2.530 therefore is supposed to follow similar pathways as described for *B. longum* though no investigations had been made about the way of sucrose uptake or the metabolism of FOS (fig. 42).

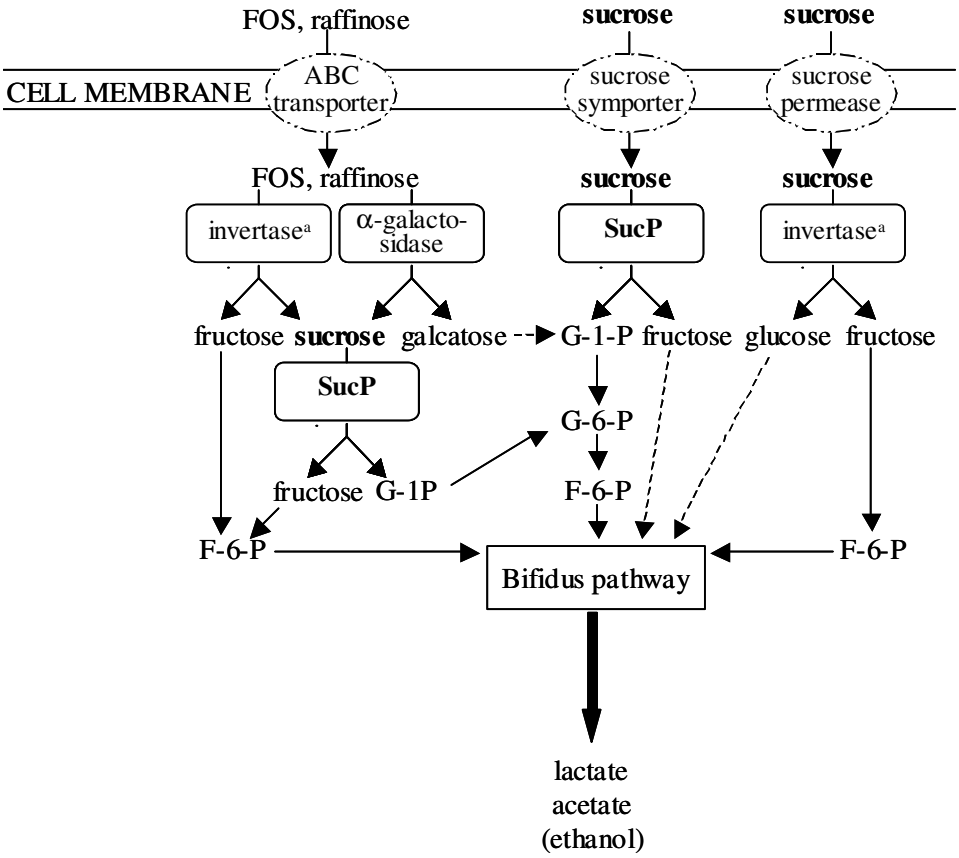

Figure 42 Suggested metabolic pathways of sucrose, raffinose and FOS metabolism of *B. lactis* Bb-12. FOS, fructooligosaccharides; SucP, sucrose phosphorylase. Dashed arrows indicate that intermediates were left out. [a] Ehrmann et al. 2003.

As well for all other bifidobacteria strains examined in this study a suitable PCR product with primers SucPB_V/2R was obtained (data not shown) which indicates the presence of a *sucP* gene. In figure 43 the alignment of the translated nucleotide sequences of *sucP* from *B. lactis* TMW 2.530 (partial sequence) and *L. reuteri* TMW 1.106 is depicted. The mentioned catalytic residues 13 Asp192 and Glu232 described in *sucP* of *B. adolescentis* (AF543301; *B. adolescentis* numbering) and *Lc. mesenteroides* (Schwarz and Nidetzky, 2006) and also found in *sucP106* of *L. reuteri* TMW 1.106 were also conserved in *sucP* of *B. lactis* TMW 2.530 (fig. 43, highlighted grey). The G+C content of the nucleotide sequences, however, was typically for lactobacilli (37.2 % for *sucP106*) and bifidobacteria (58.6 % for the partial *sucP_Bb12*) and sustains the current opinion that *sucP* genes have evolved separately in these two genera.

```
L. reuteri  MPIKNEAMLITYSDSMGKNIKETHEVLKNYIGDAIGGVHLLPFFPSTG--DRGFAPYRYD 58
B. lactis   ------------------------------------CILPFFTPFDGADAGFDPIDHT 22
                                                 :****.. .  * ** *  :

L. reuteri VVDSAFGNWDDVEALGEDYYLMFDFMINHISKKSEMYQDFKKKHDDSKYNDFFIRWEKFW 118
B. lactis  KVDARLGDWDDIAELAKTHDIMVDAIVNHMSWQSKQFQDVLANGEDSEYYPMFLTMSSVF 82
            **: :*:***:  *.: : :*.* ::**:* :*: :**.  : :**:*  :*:  ...:

L. reuteri EKAGKNRPTQEDVDLIYKRKDKAPKQEITFDDGTTENLWNTFGEEQIDINVKSKVANEFF 178
B. lactis  PDG----ATEEELAGIYRPRPGLPFTHYSFAG-KTRLVWVTFTPQQVDIDTDSAKGWEYL 137
            ..    .*:*::  **: :   *  . :* . .*. :* **  :*:**:..*  . *::

L. reuteri KETLIDMVKHGADMIRLDAFAYAIKKVGTNDFFVEPEIWDLLNEVQDILAPYKAIILPEI 238
B. lactis  MSIFDQMSKSHVKYIRLDAVGYGAKEAGT-SCFMTPKTFELISRLREEGAKRGLEILIEV 196
            . : :* *  .. *****..*. *:.** . *: *: ::*:..:::  *     ** *:

L. reuteri HEHYTIPQKISQHDFFIYDFTLPMTTLYTLYSGKTNRLAKWLKMSPMKQFTTLDTHDGIG 298
B. lactis  HSYYKKQVEIAAKVDRVYDFALPPLLLHSLFTGKVDALAHWTEIRPNNAVTVLDTHDGIG 256
           *.:*.   :*: :   :***:**   *::*::**.: **:* :: * : .*.********

L. reuteri VVDAKDILTDDEIEYASNELYKVGANVKRKYSSAEYNNLDIYQINSTYYSALGDDDKAYL 358
B. lactis  VIDIG----------------------------------------------------SDQ 264
           *:*                                                      :

L. reuteri LSRAFQVFAPGIPMVYYVGLLAGSNDLELLEKTKEGRNINRHYYTKEEVAQEVQRPVVKN 418
B. lactis  LDRSLKGLVPDADVDT---------------------------------------------- 274
           *.*::: :.*

L. reuteri LLDLLAWRNKFAAFDLDGSIDVETPTETTIKITRKDKDGKNVAVLDADAANKTFTITANG 478
B. lactis  ------------------------------------------------------------ 280


L. reuteri EKVMEQK 485
B. lactis  -------
```

Figure 43 Alignment of the translated nucleotide sequences of *sucP* from *L. reuteri* TMW 1.106 and *B. lactis* TMW 2.530 (partial sequence). The catalytic Asp192 (Schwarz and Nidetzky 2006) is in bold, other conserved aa described in different members of family GH-13 (Sprogøe et al. 2004) are shaded grey.

Only little data are available about sucrose phosphorylases of bifidobacteria. The determined K_m value of the purified SucP_Bb12 from *B. lactis* TMW 2.530 by fractionating is similar to that reported for SucP of *Lc. mesenteroides* (Goedl et al. 2007). The optimal temperature of recombinant SucP of *B. adolescentis* was reported to be 48°C (van den Broek et al. 2004), for the fractionated SucP_Bb12 it was 55°C in accordance with detection oft G-1-P only after fermentations at elevated temperatures but not at 30°C (data not shown).

For several glycosidases transglycosylation activity with a great variety of acceptors (alcoholic or phenolic OH groups of various substances) has been described, as e.g. for α-galactosidase of *B. adolescentis* (van Laere et al. 1999), β-glycosidase of *Thermus caldophilus* (Choi et al. 2003), amylosucrase of *Neisseria polysaccharea* (Albenne et al. 2002) and several sucrose phosphorylases in, for instance, *Lc. mesenteroides* (Kitao and

Sekine 1992; Kawasaki et al. 1996), *B. longum* (Kim, M. et al. 2003) and *B. adolescentis* (van den Broek et al. 2004). New products of transglycosylation activity may be interesting subjects for investigations on new prebiotics or bifidogen substrates (van den Broek et al. 2004), non cariogenic sweeteners (Kitao and Sekine 1992), antioxidative prodrugs (Kitao et al. 2000) or just continuous and efficient production of G-1-P (Goedl et al. 2007). The optimal pH values for transglycosylation activity in mentioned enzymes were mostly described to be at about pH 6, for SucP of *Lc. mesenteroides* strains it was lower than the one for the phosphorolysis action (pH 5-6 versus pH 7-7.5) (Silverstein et al. 1967). Transglucosylation activity for SucP with sucrose as acceptor has been described for *Lc. mesenteroides* ATCC 12291 (Kitao and Sekine 1992) and for *B. longum* SJ32 (Kim, M. et al. 2003). In contrast to our results, M. Kim et al. (2003) could see a series of reaction products on TLC plates, whereas after incubation of fractionated SucP_Bb12 with G-1-P and sucrose only one unknown substance larger than sucrose was seen on TLC (fig. 28) and an unknown peak could be detected with the OAKC column (fig. 27). This educt of SucP activity with sucrose could not be identified yet. It was shown, that the peak did not belong to melezitose (O-α-D-Glc*p*-(1-3)-O-β-D-Fru*f*-(2-1)- α–D-Glc*p*), resulting from the transfer of glucose from G-1-P to the fructose moiety of sucrose making a 1-3 linkage. Transfer of glucose from G-1-P to the glucose moiety of sucrose making a 1-6 linkage would lead to gentianose (O-β-D-Glc*p*-(1-6)- α–D-Glc*p* (1-2)-β-D-Fru*f*) that occurs in rhizomes of gentian plants. A maltooligosaccharide disproportion reaction suggested for the SucP of *B. lactis* TMW 2.530 with maltotriose and dextrins has also been described for amylosucrase from *Neisseria polysaccharea* (Albenne et al. 2002). On the other hand, maltotriose had been examined not to be an acceptor for other bacterial sucrose phosphorylases (Kitao and Sekine 1992; Kawasaki et al. 1996; van den Broek 2004).

4.4 Evidence of a phosphate-transporter in *L. reuteri*

One reason for the detection of glucose-phosphates in the supernatants of *L. reuteri* TMW 1.106 and *B. lactis* TMW 2.530 could be cell lysis, also suggested by Fraenkel (1968). The hexose-phosphates were detected only after at least 12 h of fermentations and cell lysis could have happened more frequently at higher pH values because of a higher production of organic acids. Furthermore, Zárate et al. (2000) and de Valdez et al. (1997) described that the permeability of the cell membrane of propionibacteria and *L. reuteri* increased after they had been treated with bile. Pradel and Boquet (1991) supposed leakage of G-1-P through the cell

membrane at high cytoplasmic concentrations and even thought of leakage of PGM to the periplasm of *E. coli*. On the other hand, there exist several phosphate transporters in e.g. *E. coli*, *Lact. lactis* (former *S. lactis*) and *Staphylococcus aureus*, that exchange G-6-P or *sn*-Gly-3-P for inorganic phosphate (P_i), which is required for energy supply, DNA, RNA and phospholipid synthesis (reviewed by Maloney 1990; van Veen 1997). *E. coli* is reported to have four phosphate transport systems: Pst, requiring ATP; Pit, regulated by the proton motive force and though inhibited by low internal pH, and the antiporters GlpT and UhpT, using *sn*-Gly-3-P or G-6-P, respectively (van Veen 1997). Though later ones usually release P_i under physiological conditions and accumulate sugar-phosphates intracellulary (van Veen 1997) under the excessive sucrose supply in this study (about 300 mM in mMRS-S) they probably release *sn*-Gly-3-P or G-6-P and take up P_i (less than 50 mM in mMRS-S). External G-6-P was detected during pH static fermentations in increasing amounts from pH 5.4 to 6.2, values that do not support the transport of inorganic phosphate via a Pit system, if existing. Further, UhpT of *Lact. lactis* is described to have maximal activity at pH 7 (Maloney et al. 1984) and to act electroneutral and in a pH-dependent stoichiometry exchanging P_i and G-6-P in the ratio 1:1 at pH 5.2 and 2:1 at pH 7.0 (reviewed by Maloney 1990). As the fermentations with *L. reuteri* TMW 1.106 were done with abundant sucrose G-6-P should be available through action of SucP106 and PGM. Further, Kadner et al. (1992) and Fraenkel (1968) reported toxic effects of elevated sugar-phosphate levels in cells of *E. coli* and Fraenkel (1968) also reported the detection of extracellular G-6-P.

For *L. reuteri* TMW 1.106 the existence of a gene similar to the gly-3-P transporter of *L. brevis* could be identified with primers constructed on conserved regions of G-6-P and gly-3-P transporters in *Lact. lactis* and *L. brevis*. Gly-3-P is generated in phospholipid degradation for glyconeogenesis or can proceed from glyceraldehyde-3-P from glycolysis (or alternatively from G-6-P over F-1,6-diP) for phospholipid synthesis (especially lipoteichoic acids for the cell wall of LAB). Accordingly, analysis of glycerol-phosphates through HPLC with OAKC and AminoPac columns revealed, that α-glycerol-3-phosphate eluted more or less at the same time as G-1-P on both columns (fig. 44A and B) and therefore probably was also present in fermentation supernatants of *L. reuteri* TMW 1.106 and not distinguished with the methods used. However, for *E. coli* it has been described, that UhpT has a broad specificity also transporting gly-3-P and glyceraldehyde-3-P (Guth et al. 1980) justifying the suggestion that the putative gly-3-P transporter of *L. reuteri* TMW 1.106 also accepts G-6-P. Furthermore, Blastp (Blastp NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) of the hexose-phosphate transporter UhpT from *Lact. lactis* NCDO 763 (ML3) revealed 100 % identity (and

100 % similarity) to the glycerol-3-phosphatease transporter of *Lact. lactis* subsp. *cremoris* MG1363, numerous other hits for gly-3-P transporters but no other hit for a G-6-P transporter in gram negative bacteria (within 80 hits) which indicates that no such gene is characterised in LAB. Although for *L. reuteri* no UhpT or GlpT data are available so far but only phosphate transporters requiring ATP, the data described for *Lact. lactis* and *E. coli* would be in accordance with findings in this study. The stoichiometric properties detected for UhpT from *Lact. lactis* could also explain the higher detection of extracellular G-6-P at pH 6.2 than at pH 5.2 through a positive feedback reaction: higher pH could have led to higher Suc106P activity and to more G-6-P after action of PGM, but, in turn, to the need of more internal P_i.

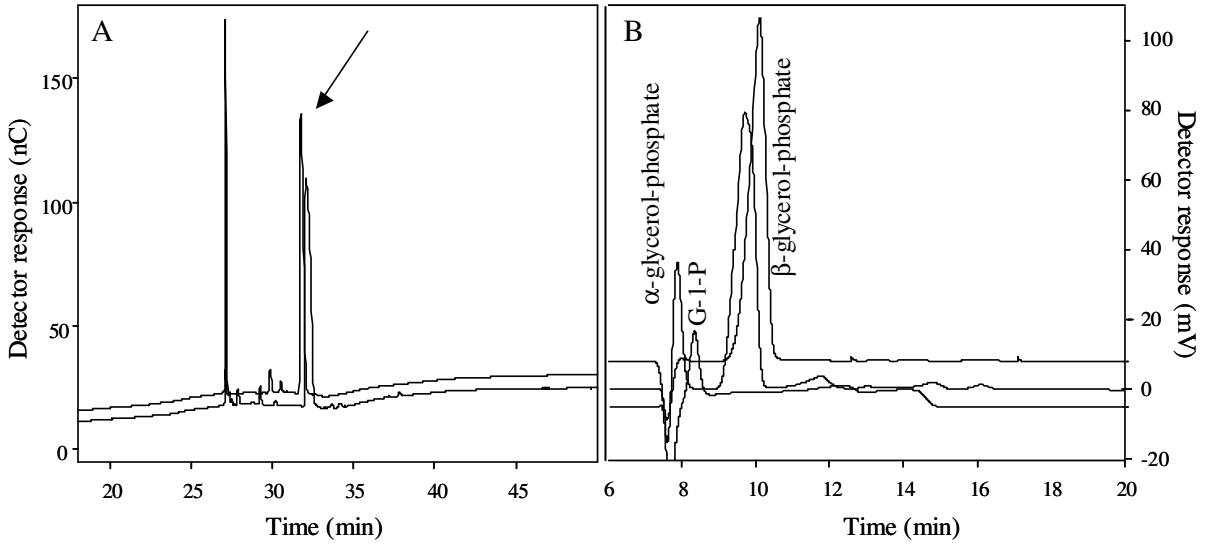

Figure 44 Chromatograms of glycerol-phosphates (50% α and 50% β; Sigma) **A** on the AminoPac column (lower trace) together with G-1-P (upper trace, offset 5 nC) and **B** on the OAKC column (middle trace) together with natrium-β-glycerolphosphate (upper trace, offset 5 mV) and G-1-P (lower trace, offset -5mV).

G-1-P is described not to be a substrate for UhpT (Maloney et al. 1984), but the relevant peak was also detected in supernatants of *L. reuteri* TMW 1.106 fermented at pH 5.4 and 6.2 and its identity was proven through enzymatic tests according to Koga et al. (1991). Pradel and Boquet (1991) described a phosphate-starvation induced G-1-P transport through UhpT in *E. coli* mutants. Therefore G-1-P transport in *L. reuteri* TMW 1.106 might have occurred because of abundant sucrose and the activity of SucP106.

A positive PCR-result could be obtained for *L. reuteri* TMW 1.974, 1.975, 1.976 and TMW 1.979 (data not shown) but not for all *L. reuteri* strains accumulating G-Ps (collective term for G-1-P, G-6-P and gly-3-P) in the supernatant. With *L. animalis* no amplicon was generated in PCR with primers for Uhp_V/R2, which was in accordance with the absence of G-Ps in fermentation supernatants of this strain.

In known genome sequences of *Bifidobacterium* strains various phosphate transport systems are listed (http://genome.jgi-psf.org: *B. longum* DJO10A: jgi1931, jgi2524, jgi2620-2, jgi2624; *B. longum* NCC2705: Schell et al. 2002), including ABC transporters and proteins, which are partially homologous to inorganic phosphate transporters form *Staph. aureus* and *E. coli*. Probably *B. lactis* TMW 2.530 also has a system analogue to UhpT or GlpT of *E. coli* (reviewed by van Veen 1997) that could explain the higher levels of especially G-6-P detected under heat and bile stress. In the experiment reported in **3.1.7** *B. lactis* TMW 2.530 showed the ability to metabolise it again when no sucrose was available and to use it as a carbohydrate source as indicated through increasing amounts of lactate and acetate. This increase in metabolic products was distinctly more pronounced in the presence of bile than heat, thus under conditions when *B. lactis* TMW 2.530 also released most G-Ps. These results argue for the presence of a transport system for G-Ps like GlgP or UhpT of *E. coli* (van Veen 1997) in *B. lactis* TMW 2.530 that act in the direction inside to outside if enough carbohydrate is present and in the direction outside to inside when the carbohydrate source is exhausted and energy is needed for e.g. detoxification of bile salts. Though, this theory would lead to cross-feeding of concurrent bacteria like *E. coli* in the natural habitat.

4.5 GlgP in *B. lactis* and *L. reuteri*

For *B. lactis* TMW 2.530 the presence of a α-glucan phosphorylase highly similar to the glycogen phosphorylase of *B. longum* NCC2705 was proven. Glycogen or α-glucan phosphorylases (EC 2.4.1.1) catalyse the reversible release of α-D-G-1-P from glycogen and α-1,4-D-glucan with the cofactor pyridoxal phosphate (reviewed by Schinzel and Nidetzky 1999). Consistent with findings of Vernazza et al. (2006) *B. lactis* TMW 2.530 was able to grow on medium (in the presence of pyridoxal 5´-phosphate) with starch or dextrins as sole carbon source (tested without stress conditions). HPLC analysis after fermentation with dextrins showed that *B. lactis* TMW 2.530 could degrade them completely, independent of the DP, whereas *L. reuteri* TMW 1.106 only was able to degrade dextrins with higher DP (data not shown). Because of the lack of a signal peptide GlgP_Bb12 from *B. lactis* TMW 2.530 is not an extracellular enzyme, but the model of a transmembrane domain (TMD) from aa 128 to 146 indicates, that it is bound to the membrane. The catalytic residues Lys552, Arg553, Lys558 and Thr662 predicted for glycogen phosphorylase from rabbit muscle by Catalytic Site Atlas Version 2.2.5 (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/CSA/) were also found in GlgP_Bb12 (fig. 29; highlighted grey) and, under the permission of the TMD

from inside to outside, would be located extracellulary and therefore be able to act on high molecular weight exopolysaccharides. However, no peak corresponding to G-1-P could be detected in supernatants of *B. lactis* TMW 2.530 after fermentation with starch or dextrins without stress conditions: Probably GlgP_Bb12 was not active under these conditions and *B. lactis* TMW 2.530 has other hydrolases for degrading dextrins and starch, or G-1-P was transported into the cell. Beside the strongly preferred model of a TMD from inside to outside, there is also a not significant second model from outside to inside indicated as dashed box. The latter one, however, would place the catalytic residues to the cytoplasm and would require a transport system for glucan or, in case of the existence of another extracellular glucan cleaving enzyme, for glucan derived oligosaccharides into the cell. For *B. longum* NCC 490, an endogalactanase has been described by Hinz et al. (2005), which is located extracellulary anchored to the cell membrane and would require a galactooligosaccharide transporter. The genome sequence of *B. longum* NCC2705 showed some putative transporters for oligosaccharides and a glycogen phosphorylase (Schell et al. 2002) but no glucose-phosphate transporter has been described yet. Though, the genes up- and downstream of glycogen/glucan phosphorylase of *B. longum* NCC2705 and DJO10A are not well characterized so far. In *S. salivarius* and *E. coli* the glucan phosphorylase genes are located in the glycogen synthesis operon and are co-induced with glycogen synthase and ADP-glucose synthase in the stationary growth phase (reviewed by Schinzel and Nidetzky 1999). While GlgP of *E. coli* is reported to prefer glycogen over dextrins, glycogen phosphorylase of *S. salivarius* is more active on maltodextrins. The authors supposed a role of GlgP in stress situations (for energy supply) and an adaptation to the needs of each individual bacteria strain harbouring such a gene. It was also speculated, that *B. longum* as a GIT inhabitant can break down glycans produced by the epithelial cells in the GIT (Schell et al. 2002). Also other intestinal strains or bacteria associated with epithelia have a *glgP* gene as e.g. *B. adolescentis* (AP009256), *E. coli* (AAG58534), *Salmonella enterica* (AE014613) and *S. pyogenes* (YP060347).

No evidence for the presence of a glucan phosphorylase gene in *L. reuteri* TMW 1.106 could be provided with any of the primers constructed and tested. This result indicates, that the sometimes observed decrease of detectable EPS during pH static fermentations longer that 24 hours (data not shown) is not caused by the activity of known phosphorylases. However, *L. reuteri* TMW 1.106, as well as *B. lactis* TMW 2.530 having a glucan phosphorylase, were shown to grow well in medium with dextrins or starch as only carbon source, and wildtype as well as dextransucrase mutant were able to grow in wheat dough without sucrose (Schwab

2006). By HPLC analysis the degradation of some peaks belonging to dextrins with higher DP and at the same time appearance of some new peaks (with the retention time of maltotetraose) but not the ones with retention times of G-1-P or G-6-P could be detected (data not shown) which indicates that there must be other enzymes degrading dextrin or starch. Though, in the unfinished genome sequence of *L. reuteri* 100-23, no maltodextrin phosphorylase or amylomaltase is listed that would be necessary to break down dextrins or starch. *L. reuteri* JCM 1112 only possesses a maltose phosphorylase gene (http://genome.jgi-psf.org).

4.6 Influence of stress factors on sucrose metabolism of different *Bifidobacterium* strains

It is described in the literature that ox gall induces the expression of general stress response proteins in *B. longum* NCIMB 8809 (Sánchez et al. 2005). Further are described some relations between the resistance to bile salts, survival at low pH and the expression or activity of glycolytic enzymes in bifidobacteria (e.g. Noriega et al. 2004) or cross-protective stress responses between heat, low pH and bile in bifidobacteria and lactobacilli (Saarela et al. 2004). Therefore it was investigated in this study, if exposure to stress conditions influenced the sucrose metabolism of *B. lactis* TMW 2.530 and if a relation could be found to the detection of hexose-phosphates in the supernatant. Rezzonico et al. (2007) found significant induction of the expression of genes relevant for carbohydrate transport and metabolism under heat stress in *B. longum.* Sánchez et al. demonstrated, that adaptation of some bifidobacteria to high bile salt concentrations was associated with increased activity of xylose-5-P/F-6-P phosphoketolase (F6PPK) (EC 4.1.2.22) (2004), which is the key enzyme in the carbohydrate metabolism of bifidobacteria, and described overexpression of xylose-5-P/F-6-P phosphoketolase, glyceraldehyde-3-phosphate dehydrogenase C and the following enzymes towards the formation of lactate in *B. longum* under bile stress (2005). As an effect lower acetate/lactate ratios under bile stress (Sánchez et al. 2005) and, in the absence of bile, higher acetate/lactate ratios in bile resistant strains compared to their not resistant original strains (Sánchez et al. 2004) were reported. In our experiments the amount of acetate decreased in all strains and under all stress conditions, but more with bile than under elevated temperature (45°C). The lactate levels decreased for all strains when incubated at 45°C in this study. Incubation with bile, however, led to different responses among the strains. These findings indicate that there exist differences in the response to elevated temperature and presence of bile and, that divergences even occur within one species. Nonetheless, the ratio acetate/lactate

decreased for all strains when incubated with bile as also reported for *B. longum* through Sánchez et al. (2005).

An adaptive response to stress-conditions has probably also been observed in this study as an increased ratio (lactate +acetate)/OD after the examined strains had been exposed to heat or bile (5 % (w/v) oxgall). We considered this observation as low growth but high metabolism because of stress. However, Vernazza et al. (2006) found out, that for some strains of bifidobacteria, which were also used in this study, the optical density did not reflect very well the real level of bacteria and the number of cfu ml^{-1}. The authors reported *B. lactis* Bb12 (TMW 2.530) to be highly tolerant against low pH (pH 2) and bile (0.5 % (w/v) oxgall at pH 8) but its OD_{630nm} to remain low in contrast to the one of *B. longum* 20219 (TMW 2.641) and *B. adolescentis* 20083 (TMW 2.454). In our study, the optical density of all strains decreased with bile (5 % (w/v) oxgall) but the decrease was lowest for *B. infantis* TMW 2.448 ^{T} (DSM 20088) and *B. lactis* TMW 2.530 (Bb12) and TMW 2.462 ^{T} (DSM 10140). These findings are in accordance with the ones of Vernazza et al. (2006) that *B. lactis* Bb12 and *B. infantis* were more resistant to bile as the other strains tested. Under elevated temperature (45°C), in accordance with data from Lamoureux et al. (2002), the two *B. lactis* strains with a probable animal origin (Ventura et al. 2004) grew better than the other strains that are all of human origin.

Furthermore, after exposure to one or both stress factors, for all tested bifidobacteria strains enhanced amounts of G-1-P and G-6-P were detected by HPLC. The expression of *glgP* of *B. longum* was shown to be induced by bile (Sánchez et al. 2005). However, extracellular activity of GlgP on sucrose as abundant carbohydrate in our fermentations is not described and not probable. As concomitant growth of the *Bifidobacterium* strains was reduced under stress (measured by optical densitometry), a positive correlation between growth and the detection of G-1-P and G-6-P remained unlikely. This suggests that their occurrence in the supernatant might in deed be caused by cell lysis. However, the hypothesis of a stress response would be supported by results of Noriega et al. (2004) and Sánchez et al. (2004, 2005) together with findings of Grill et al. (1995), that the activity of F6PPK, which is overexpressed and activated by stress, is regulated by the ratio of ATP/ADP and inorganic phosphate: at low pH conditions a PMF driven transport system for inorganic phosphate would not work but higher hydrolytic activity (as stress response) should provide more ATP, more G-6-P and more gly-3-P for an energy dependent phosphate-transport or an antiport system. Increase of gly-3-P in *B. longum* NCIMB 8809 under stress conditions was indirectly

shown through the more than 4-fold induction of glyceraldehyde-3-phosphate dehydrogenase C and phosphoglycerate kinase expression (Sánchez et al. 2005).

4.7 EPS production from sucrose under elevated temperature

There are only few data in the literature about EPS production by bifidobacteria. Most of the available data are concerning cell-wall polysaccharides (e.g. Novik et al. 2002). Rezzonico et al. (2007) suggested an effect of heat treatment on carbohydrate metabolism and biosynthesis of cell wall/membrane by doing transcriptom analysis. Roberts et al. (1995) suggested that an EPS coating might be beneficial for bifidobacteria to survive acids and bile during the passage through the human stomach and to adhere to the intestinal epithelium.

Bifidobacterium genes coding for enzymes responsible for EPS production were found to exhibit deviations in the G+C content that normally is high in this genus and therefore indicate a horizontal gene transfer (reviewed by Ventura et al. 2004). HePS production by bifidobacteria is described especially from lactose (Roberts et al. 1995; Abbad-Andaloussi et al. 1995). The examined EPS in these studies consisted more or less of equimolecular ratios of galactose and glucose (and some acidic compounds). In this study, however, EPS production of *B. lactis* TMW 2.530 from lactose was not evaluated, but small amounts of HePS were detected to be produced from sucrose. Roberts et al. (1995) also determined HePS production through *B. longum* from sucrose but to a lower extent than from lactose. Manca de Nadra et al. (1985), however, reported the production of a HePS consisting of glucose and fructose from different carbohydrate sources through *L. bulgaricus*. At elevated temperatures (45 and 50°C) *B. lactis* TMW 2.530 was shown to produce HePS from sucrose. Elevated temperature is also known to induce the production of EPS and 1-kestose in *L. reuteri* strains (Schwab 2006). Thus, 40°C turned out not to be a stress factor for *B. lactis* TMW 2.530, probably because of its animal origin (Lamoureux et al. 2002). In contrast to observations made by Abbad-Andaloussi et al. (1995), *B. lactis* TMW 2.530 did not produce most HePS under the conditions for best growth, but under stress conditions. No investigations have been made on the enzymes responsible for the HePS production of *B. lactis* TMW 2.530. G-1-P is a general precursor in the formation of sugar nucleotides that are parts of the repeating units in heteropolysaccharides (de Vuyst et al. 2001). However, whether UDP-glucose pyrophosphorylase nor dTDP-glucose pyrophosphorylase, the enzymes reacting on G-1-P in the pathway of HePS production are known so far in the genomes of B. longum NCC 2705 and B. *adolescentis* ATCC 15703.

While stress induced EPS formation may help the bacterium in survival it can also be exploited in the design of functional foods. An increased production of HePS through the probiotic culture Bb-12 (*B. lactis* TMW 2.530) at elevated temperature like they are used for the production of yogurt therefore could be beneficial for the food's texture. Additional production of oligosaccharides through a probable transglucosylation activity of the strain's sucrose phosphorylase could be interesting for the production of prebiotic food. The examination of latter activity remains to be undertaken.

4.8 Influence of EPS and acid production through *L. sanfranciscensis* TMW 1.392 on wheat dough and bread quality

4.8.1 Different performance of wildtype and mutant strains in wheat dough fermentations

Inactivation of the levansucrase in *L. sanfranciscensis* TMW 1.392 (Tieking et al. 2005b) led to different overall performance of the knock out mutant *L. sanfranciscensis* TMW 1.392Δlev (Δlev), visible in lower cell counts, a different metabolite pattern and decreased acidification of the Δlev doughs. At the end of predough fermentation, the wildtype (WT) reached cell counts ranging from 1 to $3*10^8$, whereas those of the Δlev mutant ranged from 1 to $9*10^7$on agar plates with erythromycin. However, the competitiveness of the mutant in the doughs was given due to the high inoculum cell number, which, on the other hand, was accompanied by a low multiplication factor (max. $2*10^2$). Some pre-experiments in medium demonstrated that when grown alone in medium, *L. sanfranciscensis* TMW 1.392Δlev reached about 80 to 90 % of the maximal optical density of WT both with and without sucrose (data not shown). These findings indicate that expression of a levansucrase is advantageous for *L. sanfranciscensis* TMW 1.392 in his natural habitat sourdough. Similar observations were made by Schwab (2006) for *L. reuteri* strains, in which deletion of fructosyltransferases caused impaired growth in wheat sourdough. This may be explained by the lack of the protective function of EPS against low pH suggested by Kim et al. (2000) and in this work, or the reduced energy supply for the mutant, as Lev is the only enzyme in *L. sanfranciscensis* TMW 1.392, which hydrolyzes sucrose (Tieking et al. 2005b) and delivers fermentable sugar in addition to forming EPS.

The knock out mutant produced low amounts of lactate and acetate in the same range as described by Tieking et al. (2005b), since some sucrose had been hydrolyzed in the Δlev dough due to flour invertase. However, FQ resulting from the Δlev doughs were higher than

in WT doughs because of the low availability of fructose as electron acceptor and so the energy recovery for Δlev was low too. If sucrose was hydrolyzed completely by added yeast invertase, the Δlev mutant produced the highest amounts of acetate and mannitol, which resulted in the lowest FQ. Dough fermentations with the wildtype without added sucrose resulted in the highest lactic acid production and in the highest FQ, which was also reported by Tieking et al. (2005b).

The different metabolite pattern complicated the direct comparison of the dough and bread characteristics from WT and Δlev and partly counterbalanced the observation of the effect of the levan produced *in situ* by *L. sanfranciscensis* TMW 1.392 WT. However, the control doughs made with the wildtype without sucrose (WT 0%) and the mutant with sucrose and yeast invertase (Δlev Inv) with the addition of purified levan (WT 0% EPS and Δlev Inv EPS) gave good insights into the effect of both, levan and an altered metabolism resulting in different organic acids yields on dough and bread characteristics. For these controls, a better comparability was ensured due to the use of one predough for making bread doughs with and without added levan.

4.8.2 Effect of EPS production on dough rheology and bread quality

The positive effects of hydrocolloids in bread making could be confirmed by adding levan to the bread doughs (0.3 % = 3 g/kg flour), as it increased the water absorption and the bread volume, yet retarded the firming of the crumb. However, the effects observed with levan of *L. sanfranciscensis* TMW 1.392 were smaller than the ones presented in literature for e.g. the hydrocolloids xanthan and HPMC (Collar et al. 1999; Rosell et al. 2001;). This can mostly be attributed to the lacking production of acids in these studies and that hydrocolloids had been applied in higher amounts. Furthermore, hydrocolloids have different effects on dough and bread properties because of their different chemical structure, shape, rigidity and molecular size, which affect their proposed associations with starch (Christianson et al. 1981), interactions with wheat proteins (Huebner and Wall 1979), and interactions of gluten network and starch (Martin and Hoseney 1991; Davidou et al. 1996). Comparability with data from literature is additionally difficult because of different dough making procedures, which affect either constant dough consistence or constant water percentage. Most data available on the rheological influence of hydrocolloids are produced with charged, substituted or modified hydrocolloids, which probably exert effects on the properties of the hydrocolloid itself and have even stronger effects on dough and bread characteristics. However, the results of Brandt

et al. (2003), made with *L. sanfranciscensis* LTH 1729, that *in situ* produced levan was more effective than added one, could not be confirmed.

In our study, extensibility of the doughs increased (better reproducibility with Δlev Inv) with the addition of EPS and R/E decreased (significant for Δlev Inv). This result is in agreement with the findings of Rosell et al. (2001) but not with the ones of Brandt et al. (2003), who found no influence of levan on extensibility. Ribotta et al. (2005) also described an increase in extensibility with both guar gum and λ-carrageenan, whereas xanthan had no influence. Our extensograph results suggest further, that dough with levan is less sensitive to time delays (longer resting times) at room temperature causing decrease of resistance to extension and so enables flexible processing times.

Sourdoughs with a long fermentation period (12-20 h) are usually incorporated with 10-20 % to the bread dough (Seiffert 2006). With the incorporation of 10 % predough into the bread dough in this study, EPS amounts of maximal 0.52 g kg^{-1} (average 0.45 g per kg flour; 0.045 %) could be reached. This is only about 15 % of the amount of levan effectively added to the control doughs in this study and much less than other commonly used hydrocolloids (Brümmer et al. 1977; Armero and Collar 1997; Rosell at al. 2001; Guarda et al. 2004). Still, the predoughs WT showed better results than the predoughs WT 0% concerning the water absorption and constants k_{TPA} and k_{DSC}. Since the acid contents in the doughs were different, the better results with WT may have additional metabolic reasons.

Generally, for all measurements that were also done with purified unbranched dextran, other effects could be observed for the dextran than for the branched levan of *L. sanfranciscensis* TMW 1.392. The dextran reached a higher gas retention coefficient (2 %) than the levan (only made once). In the RVA experiments the glucan clearly showed highest effects and the levan of LTH 2590 had more effect on the peak viscosity than the one of TMW 1.392, which indicates higher interactions between these EPS and starch (Christianson et al. 1981). As the pasting profiles from the RVA correlate highly with bread staling characteristics and can serve as predictors (Collar 2003), this observation might explain the better results with predough of LTH 2590 for TPA and DSC compared to TMW 1.392 (personal communication M. Seitter, Universität Hohenheim, Germany) although the strains are thought to be isogenic (oligosaccharide production in wheat dough was also more pronounced through LTH 2590). These findings are further in accordance with some rheometer measurements with EPS in watery suspension carried out with the levan of *L. sanfranciscensis* TMW 1.392, the branched dextran of *L. reuteri* TMW 1.106 and the unbranched dextran of *L. curvatus* TMW 1.624

which revealed, that the unbranched dextran of *L. curvatus* had the highest viscosity and was the only EPS showing visco-elastic behaviour. Lacaze et al. (2007) made similar observations with the long linear dextran of *Lc. mesenteroides* and proposed a good line up of this EPS and facilitated hydrogen interactions. The levan of *L. sanfranciscensis* TMW 1.392, however, showed the lowest viscosity and therefore the lowest effect (unpublished results, H. Grepka, Diplomarbeit, Technische Mikrobiologie, Technische Universität München, Germany).

4.8.3 Effect of organic acid production on dough rheology and bread quality

Acidification of sourdough is important for flavour, rope inhibition, quality and shelf life of bread, and it influences structure-forming components and enzymatic activities (reviewed by Clarke and Arendt 2005; Arendt et al. 2007). The different degrees of acidification of the predoughs and bread doughs showed altered effects on dough and bread characteristics. In agreement with Armero and Collar (1996) and Clarke et al. (2002), the water absorption and dough stability decreased with the use of predoughs. The highest stability was seen in doughs of predough with the highest pH. The adjustment of all bread doughs at 500 BU followed the practical approaches used in bakeries, which test their systems along their capacity for water incorporation. As expected, it did produce significant differences in dough rheology. The trend that doughs made with the predough Δlev Inv with the highest acetic acid level reached the highest resistance to extension and showed the lowest extensibility is in agreement with Tanaka et al. (1967), who discovered that a drop of pH to 4.2 with acetic acid in the presence of salt (and at 500 BU) led to an increase in resistance. But in general, the micro-extension experiment with the Kieffer rig and the texture analyser led to large variations and a low reproducibility, probably caused by variations in deformation history of the dough stripes as discovered by Dunnewind et al. (2004). The measurements with the rheofermentometer showed remarkable differences in dough development and gaseous release probably caused by different lactic and acetic acid levels, which influenced yeast metabolic activity. The highest acetic acid level (Δlev Inv) evoked the lowest high loss ((Hm-h)/Hm) and the best gas retention coefficient. The loaf volumes of the breads corresponded to observations made with the rheofermentometer (total gaseous volume), as the Δlev breads had the highest averaged volume and the Δlev Inv and the WT 0% breads were in the same range and showed lower loaf volumes. The Δlev Inv breads were even smaller than a direct control treated the same way (personal communication M. Seitter, Universität Hohenheim, Germany), which contradicts the general opinion that the use of sourdough increases bread volume. But compared with the chemically acidified control, all sourdough breads had a higher volume.

These findings are in agreement with those made by Barber, S. et al. (1991) and Barber, B. et al. (1992), who found the highest volumes in breads made from wheat sourdough with the highest FQ (about 4-5) and negative effects after the additional incorporation of lactic or acetic acid. N. Vermeulen (Technische Mikrobiologie, Technische Universität München, Germany) found out, that bread doughs made with 10 % predough of pH 3.6 using different acids had a lower pH value when acetic acid was added and that these breads also had the lowest volumes due to the inhibition of yeasts by acetic acid (personal communication, unpublished data).

Consideration of the relationship between bread crumb hardness and acidity levels reveals a striking observation that the ones with highest acid content (WT 0% most lactic acid, Δlev Inv most acetic acid) and lowest pH (WT 0%) showed the highest maximal hardness (table 7, 2nd column). The findings are in accordance with the results obtained with chemically acidified doughs adjusted to different pH values (personal communication M. Seitter; Universität Hohenheim, Germany). Barber et al. (1992) also found higher crumb hardness changes in breads with added acetic and especially lactic acid. However, in our tests, bread was made in closed tins and thus had equal volumes. The DSC results were not significantly different because of high standard deviations. Starch retrogradation is described to be influenced by starch acid hydrolysis and α-amylase activity (Martin and Hoseney 1991). The optimum of wheat α-amylase is pH 4.5-4.6 and 54-56°C (Lee and Urnau 1969), so the activity might have been low in all doughs but was masked by the acid hydrolysis of starch (Barber et al. 1992).

4.8.4 Applicability of *L. sanfranciscensis* TMW 1.392 for *in situ* EPS production

All measurements showed that there must be additional reasons beyond EPS production and acidification for the different rheological properties of sourdoughs and qualities of sourdough breads as already stated by e.g. Clarke et al. (2002) and Corsetti et al. (2000). Protein degradation during fermentation with cereal proteases (pH 3.0-4.0) and/or bacterial ones might be one reason (Clarke et al. 2004). In the first instance, this work showed that the use of a knock out mutant is helpful to judge overall performance of a strain, while the interpretation of specific effects, such as levan production, must consider all changes in its metabolism. The amount of *in situ* produced EPS by *L. sanfranciscensis* TMW 1.392 seemed to not be sufficient to exert positive effects on bread quality with the use of 10 % predough. However, Crowley et al. (2002) and Collar et al. (1994) argued for a maximal use of 20 and 10 % sourdough for wheat bread, respectively. The negative effects caused by an excess of acids (Δlev Inv FQ 1.2±0.2 or Wt 0% FQ 8.1±0.7) on bread volume and crumb hardness were

indeed presented in this work. Therefore, we considered *L. sanfranciscensis* TMW 1.392 problematic for *in situ* EPS production approaches. Strains and/or conditions must be found to maximise *in situ* EPS production and at the same time to optimise acid production to a certain quotient which allows acceptable volume, crumb structure and flavour of breads. Thus, when EPS producing strains are screened for dough applications, their metabolite pattern, final fermentation pH and fermentation quotient should be considered.

4.9 Screening for the amount of lactic and acetic acid produced by EPS forming lactobacilli

As high amounts of organic acids possibly counterbalance positive effects of *in situ* produced EPS on bread volume and crumb hardness, 29 EPS forming lactobacilli were examined for their acid production from sucrose consulting the FQ. For bakers, the FQ is traditionally characteristic for bread quality. It can, theoretically, be influenced through variation of aeration, DY or temperature and addition of sucrose or fructose and citrate (Röcken 1999), whereas Röcken et al. (1992) found out that the addition of electron acceptors (e.g. fructose) is more efficient as the variation of DY. In the literature, different FQ are described to be optimal, depending on the desired effect and the flour used. A FQ of about 4 in wheat dough was postulated to be optimal for bread quality (Barber, S. et al. 1991; Barber, B. et al. 1992). For rye acidification it is a prerequisite for baking. Concerning the importance of acetate for microbial stability and flavour of bread, FQ values between 2 and 2.7 (Hammes and Gänzle 1998) or even lower (Spicher 1983) were described to be optimal.

The screening took place in medium (mMRS-S), so that the results will not be fully comparable with data from dough fermentations because of the higher buffer capacity of dough. Compared with fermentations in dough, the average FQ of *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392 were slightly lower in the fermentations in medium (1.2 and 2.3) than in wheat dough (averaged 1.7 and 2.6 after 24 h). However, the majority of the examined strains had a FQ in medium far below the proposed optimal value for bread volume of about 4 (Barber et al. 1991). The strain reaching a FQ value closest to 4 in the medium was *L. fermentum* TMW 1.187 (FQ 3.78) which, however, produced less levan as *L. sanfranciscensis* TMW 1.392. Though, to finally find out promising candidates for an *in situ* production of EPS in dough, the amounts of EPS produced effectively remains to be determined for all strains. As suspected, most fructan producing strains (especially *L. sanfranciscensis* strains) produced less acetate than glucan forming strains (especially *L. reuteri* strains). Apparently they used the fructosyl unit from sucrose for EPS synthesis and

to a lower extent as an alternative electron acceptor, which would lead to the production of acetate instead of lactate (Stolz et al. 1995). This result sustained the assumption, that the synthesis of EPS (fructan in this case) has some energetic advantage for the bacteria and led to the postulation, that the use of a fructan forming strain is more promising in respect of an optimal FQ although the use of an undefined glucan showed more positive effects on dough and bread quality (**4.8.2**). Nevertheless, different strains will produce different glucans that will have distinct effects and the amount of EPS produced *in situ* under the supplied conditions has to be considered as well (not checked *in vitro* in this study). Furthermore, also the ability to ferment pentosans available in flour that will increase the acetic acid level (Gobbetti et al. 1999) is to be taken in to account when selecting a starter strain.

4.10 Investigation of *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392 in different dough systems with sucrose supplementation

The experiments discussed in **4.8.2** made with RVA and rheofermentometer led to the suggestion, that glucan may be more efficient in influencing dough and bread quality and examination of EPS producing lactobacilli for their FQ showed, that there is some difference between glucan and fructan forming strains. Therefore, the glucan forming strain *L. reuteri* TMW 1.106 and the fructan forming strain *L. sanfranciscensis* TMW 1.392 were compared in dough fermentations for EPS and acid production under different conditions.

Because wheat and rye flour naturally contain polysaccharides that undergo changes in water solubility through flour or microbial enzymes and acidification, it is difficult to calculate the exact amounts of EPS produced in dough. Moreover it can not be excluded, that the extractability of EPS from the dough matrix changes during the fermentation. However, reproducible trends of EPS production under different conditions could be observed in this work.

4.10.1 Influence of dough yield on the EPS contents produced *in situ* by *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392

With increasing DY for both strains higher EPS but also higher lactate contents (per kg flour) had been detected. The latter finding confirms the description of Decock and Cappelle (2005). A parallel increase of acetate with increasing DQ as described by various authors (reviewed by Neysens and de Vuyst 2005) could not be observed. For HePS production positive as well as negative effects of reduced a_w values have been reported (Liu et al. 1998; Looijesteijn and Hugenholtz 1999). With the prolongation of the fermentation the EPS yields in DY 220 could

be further increased, which led to a higher ratio of monomers of EPS in WPS to lactate. As the DY value will influence the flavour profile of a sourdough and determines its pumpability, the value will probably be selected depending on the production facilities and the demanded product qualities. For dried sourdoughs the higher acetic acid content with higher DY can be partially decreased depending on the process used (Brandt 2007), but lactic acid will be concentrated.

4.10.2 Influence of pH on the *in situ* EPS production

The positive effects of pH regulation for the production of glucan through *L. reuteri* in medium could also be seen in dough. At pH 4.7 the amount of glucan increased, whereas at pH 4.0 almost no EPS was formed. Maximal estimated amounts of 9.6 and 12.1 g glucan per kg flour could be reached with pH control after 23 and 44 h, respectively, in comparison to 3.0 and 7.2 g in the corresponding dough without pH control. For dough fermentations with *L. sanfranciscensis* TMW 1.392, similar to the ones with *L. reuteri* TMW 1.106, a pH regulation at pH 4.7 led to an improved EPS formation.

For both strains, the optimal pH for *in situ* production of EPS was different from the one for the purified heterologously expressed enzyme in buffer (**3.2.3**; Tieking et al. 2005b). For *L. reuteri* TMW 1.106 this observation had already been made for pH static fermentations in mMRS-S medium and indicated that results from enzyme characterizations are not implicitly transferable to microbial fermentations.

As expected, the maintenance of the pH at a moderate level led to higher amounts of organic acids in doughs as well as in liquid medium for both strains (compared to data for pH statically fermentations of *L. sanfranciscensis* TMW 1.392 in medium of Korakli et al. 2003). This effect has to be taken into account regarding the possible negative effects of an excess of acids. Further, in all fermentations done, the synthesis of organic acids was drastically enhanced through prolongation of the fermentation time (48 h), whereas in most cases the EPS production did not increase proportionally.

4.10.3 Influence of fed-batch on the *in situ* EPS production

In fermentations of *L. reuteri* TMW 1.106 in medium with sucrose we had seen in prior experiments that higher EPS yields could be obtained by increasing the sucrose concentration up to 350 mM. At 470 mM sucrose the EPS yield decreased again, probably due to enzyme inhibition (unpublished data, Körber/Gänzle, Technische Mikrobiologie, Technische Universität München, Germany). Starting with 10 % sucrose in doughs (about 300 mM) should be gradable and the augmentation had expected effects. Sucrose fed-batch additional to

pH control led to the highest EPS yields reached after one or two days with both strains. With prolongation of the sucrose fed-batch fermentation to 76 h, glucan yields of *L. reuteri* still increased appreciable (54 %) whereas lactate and acetate concentrations did not increase in the same way (about 20 and 25 %, respectively). The EPS formation through *L. sanfranciscensis* TMW 1.392 with fed-batch, in contrast, seemed to reach a maximum at about 48 h and sucrose accumulated later on.

4.10.4 Influence of different fermentation substrates on the *in situ* EPS production

EPS production was most efficient and very fast through *L. reuteri* in RB. The high amount of glucan produced in RB during the first hours could be interesting, as rye bran on the one hand is a co-product that accumulates during the production of rye flour and on the other hand could be used to produce high-fibre health-contributing products with nutritional benefits that nowadays have a high consumer acceptance. Sourdough fermentation was found to be an effective method to solubilise dietary fibre (arabinoxylans) of rye (Hansen et al. 2002), to have positive effects on the applicability of (wheat) bran for bread production (Salmenkallio-Marttila et al. 2001) and to decrease the phytic acid content accompanied by an increase of the bioavailability of magnesium (Lopez et al. 2001). Latter effect is caused by phytases that recently were described to have more activity in some bacterial strains, e.g. bifidobacteria and *L. reuteri*, than have cereal derived phytases (Palacios et al. 2007). No experiments combining rye bran and sucrose-fed-batch had been made, but would probably be worth to undertake. However, rye bran ferment also had the highest acid contents because of the highest buffering capacity and this is in accordance with results from Hansen and Hansen (1994) that reported more (lactic) acid production in wholemeal flour. Using RB as fermentation substrate for *L. sanfranciscensis* TMW 1.392 led also to best growth but not to best EPS formation.

4.10.5 Alternative production of oligosaccharides from sucrose

If the formation of EPS should be optimized, also the alternative production of oligosaccharides from sucrose has to be considered. For *L. reuteri* TMW 1.106 peak series on HPAEC-IPAD chromatograms from wheat doughs were detected that were similar to maltooligosaccharide series produced by the purified enzyme ΔN-Gtf106A (**3.1.5**). As maltose is present naturally in flour and is a strong acceptor (Dols et al. 1998), we supposed, that *L. reuteri* TMW 1.106 produced maltooligosaccharides (dextrins) in wheat dough. Apart from being an alternative product to glucan, these oligosaccharides may have positive effects on bread-staling as dextrins are the products of α-amylase acting on starch. They were

proposed to be responsible for the anti-staling effect of α -amylases (Martin und Hoseney 1991; Rojas et al. 2001) which are traditionally used as baking additives. Only small amounts of the peak corresponding to G-1-P were detected in doughs fermented with *L. reuteri* TMW 1.106. These findings would fit with a correlation of their detection and the need of phosphate (**4.4**), as phosphate that is present in sourdoughs mainly bound in phytate and will be released in the course of fermentation and so get available (Lopez et al. 2001).

For *L. sanfranciscensis* TMW 1.392 the production of 1-kestose (augmentation to 260 %) during fed-batch fermentations exceeded the formation of levan (augmentation to 110 %). These results are in accordance with data for the purified levansucrase of the strain (Tieking et al. 2005b) and findings from fermentations in medium, where 1-kestose production increased exponential when the sucrose concentration exceeded 300 mM (Korakli et al. 2003). Therefore, sucrose fed-batch seemed not to be the suited tool to augment the levan production in *L. sanfranciscensis* TMW 1.392.

4.10.6 Stability of *in situ* produced EPS during storage of the predough

During storage of the sourdoughs at 4-8°C, the dextran of *L. reuteri* TMW 1.106 seemed to be stable. No decrease in glucan in WPS was detected; in contrast, the amount of glucose in extracted WPS increased to about 200 % in fed batch doughs. Some belated test experiments (incubation in mMRS-S for 48 hours at 37°C and afterwards 10 days at 4-8°C without or with washing the cells before) showed that *L. reuteri* TMW 1.106 still produced some glucan (additional 20 %) from sucrose medium during storage at 4-8°C and that washed cultures produced the same amount of glucan in the same time at the same conditions. Lactate, acetate and ethanol levels did not increase, which indicated that the organism was not active under this conditions, but Gtf106A, supposed to be cell-bound (**3.2.2**), was still active. Therefore augmentation in glucan during storage of sourdough with *L. reuteri* TMW 1.106 could be possible if sucrose is left or fed before storage.

The levan of *L. sanfranciscensis* TMW 1.392 appeared to be stable for maximal 1.5 month. Kawakami et al. (2005) described a fructan degrading enzyme from wheat that gets induced through cold stress and degrades β-2,1 and β-2,6 linkages. However, related experiments with *L. sanfranciscensis* as described above for *L. reuteri* gave no coherent information as *L. sanfranciscensis* TMW 1.392 was still active and produced acids as well as levan at 4-8°C. The levansucrase which is supposed to be cell bound (Tieking et al. 2005b), however, was not active in washed cultures.

However, it had been observed earlier in fermentations in liquid medium, that the dextran of *L. reuteri* TMW 1.106 was partially degraded upon prolonged fermentations (24-48 h) especially at pH 4.7 and 5.4. This phenomenon was also observed in fermentations with RB and with wheat at DY 500, thus it could always be observed at conditions that led to high EPS production in the first 24 hours of fermentation. Various EPS degrading enzymes had been described in the literature (Colby and Russell, 1997; Pham et al. 2000; Degeest et al. 2002) but were not investigated in *L. reuteri* and *L. sanfranciscensis*.

4.10.7 Final comparison of *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392

The *in situ* produced amounts of EPS by both strains reported in **3.4** and former studies (Korakli et al. 2001; Tieking et al. 2003a) could be augmented by optimizing the fermentation conditions. However, the amounts that would have positive effects on dough and bread quality as described in literature (Rosell et al. 2001) and suggested in **4.8**, would only be reached with incorporation of 20 % predough in bread doughs. This would introduce a considerable amount of acids and proteolytically degraded gluten. Generally, the *in situ* formation of EPS was better for *L. reuteri* TMW 1.106 under all tested fermentation conditions. The highest measured conversion from sucrose to EPS was 11.9 % (after one day in RB) for *L. reuteri*, whereas it was only 5.6 % for *L. sanfranciscensis* (after two days in WB at pH 4.7). The average FQ levels were very low for *L. reuteri* TMW 1.106 in all experiments and it remains to be evaluated if the dextran of the strain is more powerful as the levan of *L. sanfranciscensis* TMW 1.392 and can compensate negative effects of a very low FQ.

5 SUMMARY

Sucrose is the most abundant disaccharide in the environment and an important carbohydrate source for bacteria. The main end products from sucrose metabolism for the acquisition of energy in lactobacilli and bifidobacteria are lactate and acetate, or ethanol. Many lactobacilli also synthesize extracellular polysaccharides and oligosaccharides from sucrose. The benefit they draw from that is not yet fully understood. However, HePS produced by LAB from lactose have been used in the diary industry for decades, whereas the knowledge about the production of HoPS by lactobacilli and the idea of using them in food production are quite new.

L. reuteri TMW 1.106 and *L. sanfranciscensis* TMW 1.392 are typical sourdough lactic acid bacteria strains producing EPS, which is not exploited intentionally for the use in baked goods. In addition to the use of the purified EPS compounds, the *in situ* production of EPS by these strains under optimized conditions is tempting, as it could replace the addition of hydrocolloids to baked goods, and even be promising for the generation of healthier foods. The nutritional value of cereal derived goods could further be enhanced by the incorporation of bifidobacteria, which already have an established function in dairy products. In this work, the sucrose metabolism of these bacteria was investigated, as it significantly influences the performance of these bacteria in food systems and delivers EPS and oligosaccharides with food structural and nutritional impacts.

This work showed that *L. reuteri* TMW 1.106 and *B. lactis* TMW 2.530 have two ways of sucrose utilization, formation of extracellular polymers and intracellular catabolism through sucrose phosphorylases, whereas the existence of the latter one was not seen in *L. sanfranciscensis* TMW 1.392.

Aiming at maximal EPS production and the understanding of a protective function of EPS, part of this work focused on the influence of pH on the sucrose metabolism of *L. reuteri* TMW 1.106. Results indicated that the strain harboured two glucansucrases, Gtf106A and Gtf106B, whereas Gtf106B only exhibited hydrolysis but not transferase activity. The pH value had most influence on the amount and molecular weight, and to a less extent on the structure of the EPS which was shown to be a branched dextran and to have protective effects for the strain against low pH. Up to 22 g l^{-1} EPS could be obtained at the optimal pH range (4.7–5.4). The pH influence on the amounts of detected EPS was shown to be attributable to a pH dependent transferase activity of Gtf106A and not to a pH dependent expression. At low pH (4.0), a deviant polymorphism of the glucansucrase on 2D-PAGE compared to the ones

from higher pH values was seen, and a relationship was attributed to the observed divergence between the transferase activity of the recombinant ΔN-Gtf106A and the *in vitro* EPS production at this pH. ΔN-Gtf106A was further shown to produce oligosaccharides with maltose and isomaltose as acceptor molecules. Two other substances derived from sucrose and thus affecting the EPS yields indirectly were identified in this work as G-1-P and G-6-P and a putative hexose-phosphate/P_i antiporter could also be discovered in the genome of *L. reuteri* that is possibly responsible for their extracellular occurrence. Increased activity of the detected SucP106 at neutral pH values as known for sucrose phosphorylases of other bacteria was suggested indirectly by increased detection of G-1-P from pH 4.7 to 6.2.

For *B. lactis* TMW 2.530 the formation of a HePS from sucrose and the extracellular occurrence of hexose-phosphates was detected under elevated fermentation temperatures (above 45°C). A sucrose phosphorylase and a glucan phosphorylase protein, both releasing G-1-P, were identified in this strain. The optimal temperature for the partially purified SucP_Bb12 was 55°C. With maltotriose and sucrose as acceptor the enzyme showed transglucosylation activity that can probably be exploited for the production of new oligosaccharides. Further, results were obtained indicating that after depletion of sucrose *B. lactis* uses the hexose-phosphates as a substrate, especially under the same stressing factors that had evoked the release of hexose-phosphates before.

Another part of this work concerned sucrose metabolism in sourdough fermentations, the performance of a levansucrase deletion mutant in dough and the influence of EPS and acidification on dough and bread quality. It was shown that EPS has positive effects in the dough and bread system, which, however, can be counteracted by the formation of acids if the EPS is produced *in situ* in preferments. The levansucrase deletion mutant of *L. sanfranciscensis* TMW 1.392 was impaired in growth in dough and produced wheat sourdoughs with low acid content, a FQ of 4.1 and the highest mixing stability. The breads made with this dough had the highest volume compared with ones made with doughs with higher lactic or higher acetic acid content, despite the addition of EPS. The high lactic acid level produced by the *L. sanfranciscensis* wildtype in dough without supplemented sucrose negatively influenced crumb hardness, whereas a high amount of acetic acid showed negative effects on the bread volume. Application of purified levan to the fermented doughs had positive effects on the water absorption, bread volume and firming of the crumb. *In sit*u production of levan with *L. sanfranciscensis* in wheat dough, however, was too low under the conditions used to show significant results. The use of a glucan resulted in higher effects concerning the dough and bread quality.

For further optimization of the *in situ* production of EPS in dough a glucan and a fructan producing strain, *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392, were compared. With *L. reuteri*, higher amounts of EPS were obtainable in dough, but similar to *L. sanfranciscensis*, EPS yields were lower than in liquid medium. The synthesis of EPS could be augmented for both strains with increased DY and by pH regulation and maintenance at 4.7. However, under these conditions the acid production also increased significantly. For *L. reuteri,* a batch to which sucrose was added again during wheat dough fermentation (fed-batch) turned out to be promising, whereas with *L. sanfranciscensis* thereby the synthesis of 1-kestose exceeded the levan production. *L. reuteri* synthesized moderate amounts of maltooligosaccharides in wheat doughs, which probably have anti-staling effects. Further, both strains grew very good in rye bran substrate. *L. reuteri* therein produced high glucan yields during the first hours of fermentation leading to the highest measured conversion from sucrose to EPS (11.9 %) after only one day of fermentation, whereas it was only 5.6 % for *L. sanfranciscensis* (after two days in wheat blend pH 4.7). However, the average FQ levels were lower for *L. reuteri* TMW 1.106 in all experiments, especially in rye bran, and it remains to be evaluated if the dextran of the strain is more powerful as the levan of *L. sanfranciscensis* TMW 1.392 and can compensate negative effects of a low FQ. Nevertheless, the high EPS production in rye bran is promising because it might positively influence the applicability of rye bran doughs for baking processes and for the production of high-fibre bakery products.

6 ZUSAMMENFASSUNG

Saccharose ist das in der Umwelt am häufigsten vorkommende Disaccharid und eine wichtige Kohlenhydratquelle für Bakterien. Die Hauptendprodukte des Energiestoffwechsels aus Saccharose durch Laktobazillen und Bifidobakterien sind Milchsäure und Essigsäure oder Ethanol. Viele Milchsäurebakterien bilden aus Saccharose auch extrazelluläre Poly- und Oligosaccharide (OS). Der Nutzen den sie daraus ziehen wird bis heute nicht vollständig verstanden. Dennoch werden die durch Milchsäurebakterien von Laktose gebildete Heteropolysaccharide seit Jahrzehnten in der Milchindustrie genutzt, während das Wissen über die Bildung von Homopolysacchariden durch Laktobazillen und die Idee, diese in der Lebensmittelproduktion einzusetzen, relativ neu sind.

Lactobacillus reuteri TMW 1.106 und *Lactobacillus sanfranciscensis* TMW 1.392 sind typische Milchsäurebakterien im Sauerteig und bilden gleichzeitig Exopolysaccharide (EPS), die in der Herstellung von Backwaren nicht bewusst genutzt werden. Zusätzlich zu der Verwendung von EPS-Präparaten ist die *in situ* Synthese von EPS durch diese Stämme unter optimierten Bedingungen interessant, da sie den Zusatz von Hydrokolloiden bei der Produktion von Backwaren ersetzen und darüber hinaus aussichtsreich für die Erzeugung nutritiv wertvoller Nahrungsmittel eingesetzt werden könnten. Der Nährwert getreidebasierter Lebensmittel könnte ferner durch die Zugabe von Bifidobakterien, welche bei der Produktion fermentierter Milchprodukte bereits eine bewährte Funktion haben, gesteigert werden. In dieser Arbeit wurde der Saccharosemetabolismus dieser Bakterien untersucht, da er das Verhalten der Bakterien in Lebensmittelsystemen entscheidend beeinflusst und EPS und OS mit Bedeutung für die Struktur und den nutritiven Wert des Lebensmittels liefert.

Die Arbeit zeigte, dass *L. reuteri* TMW 1.106 und *Bifidobacterium lacits* TMW 2.530 zwei Wege für die Saccharoseverwertung besitzen: die Bildung extrazellulärer Polymere und die intrazelluläre Verstoffwechselung durch Saccharosephosphorylase, wobei für *L. sanfranciscensis* TMW 1.392 die Existenz des letzteren nicht festgestellt wurde.

Ein Teil dieser Arbeit konzentrierte sich auf den Einfluss des pH-Werts auf die Glukanproduktion durch *L. reuteri* TMW 1.106 mit dem Ziel, die EPS-Produktion zu optimieren und eine mögliche schützende Funktion des EPS zu verstehen. Es stellte sich heraus, dass der Stamm zwei Glukansucrasen hat, Gtf106A und Gtf106B, wobei Gtf106B nur Hydrolyse- aber keine Transferaseaktivität aufwies. Den größten Einfluss hatte der pH-Wert auf die Menge und auch auf das Molekulargewicht, jedoch nur in einem geringeren Maße auf die Struktur des EPS. Es handelt sich um ein verzweigtes Dextran, welches einen schützenden

Effekt gegenüber niedrigem pH für den Stamm aufweist. Im optimalen pH-Bereich (4,7-5,4) konnten bis zu 22 g l^{-1} EPS gewonnen werden. Es zeigte sich, dass die pH-Abhängigkeit der detektierbaren Menge an EPS der pH-abhängigen Transferaseaktivität und nicht einer pH-abhängigen Expression der Gtf106A zuzuschreiben ist. Bei niedrigem pH-Wert (4,0) wurde auf 2D-PAGE ein von den bei höheren pH-Werten abweichender Polymorphismus der Glukansucrase beobachtet und ein Zusammenhang mit der bei diesem pH festgestellten Divergenz zwischen der Transferaseaktivität der rekombinanten ΔN-Gtf106A und der EPS-Produktion *in vitro* vermutet. Für ΔN-Gtf106A wurde ferner die Bildung von OS mit Maltose und Isomaltose als Akzeptormolekül festgestellt. Zwei andere aus Saccharose gebildete Substanzen, die indirekt die EPS-Ausbeute beeinflussen, wurden in dieser Arbeit als Glukose-1-Phosphat (G-1-P) und Glukose-6-Phosphat identifiziert. Auch ein putativer Transporter für Hexosephosphat im Austausch gegen inorganisches Phosphat wurde im Genom von *L. reuteri* entdeckt, welcher möglicherweise für deren extrazelluläres Auftreten verantwortlich ist. Eine gesteigerte Aktivität der Saccharosephosphorylase von *L. reuteri* bei neutralem pH, wie sie für Saccharosephosphorylasen anderer Bakterien bekannt ist, wurde indirekt durch gesteigerte Detektion von G-1-P bei pH-Werten von 4,7 hin zu 6,2 bestätigt.

Für *B. lactis* TMW 2.530 wurde die Bildung von Heteroexopolysacchariden aus Saccharose und extrazelluläres Auftreten von Hexosephosphaten bei erhöhten Fermentationstemperaturen (über 45°C) detektiert. In diesem Stamm wurden eine Saccharosephosphorylase und eine Glukanphosphorylase, welche beide G-1-P freisetzen, identifiziert. Die optimale Aktivität der partiell aufgereinigten Saccharosephosphorylase lag bei 55°C. Das Enzym wies Transglukosylierungsaktivität mit Maltotriose und Saccharose als Akzeptor auf, welche möglicherweise für die Herstellung neuer OS genutzt werden kann. Ferner gab es Hinweise darauf, dass *B. lactis* die Hexosephosphate nach Aufbrauchen der Saccharose als Substrate verwendet. Dies geschieht vor allem unter den gleichen Stressbedingungen, die zuvor deren Freisetzung hervorgerufen hatten.

Ein weiterer Teil dieser Arbeit beschäftigte sich mit dem Saccharosemetabolismus von Laktobazillen in Sauerteigfermentationen, dem Verhalten einer Levansucrase-Deletionsmutante im Teig und dem Einfluss von EPS und Säuerung auf die Teig- und Brotqualität. Es wurde gezeigt, dass EPS positive Effekte auf das Teig- und Brotsystem ausübt, denen die Bildung von Säuren während der *in situ* Produktion des EPS in den Vorteigen entgegenwirken kann. Das Wachstum der Levansucrase-Deletionsmutante von *L. sanfranciscensis* TMW 1.392 war im Teig beeinträchtigt. Die resultierenden Weizensauerteige wiesen einen geringen Säuregehalt, einen Fermentationsquotienten von 4,1

und die höchste Stabilität gegenüber Kneten auf. Die Brote aus diesem Teig hatten das größte Volumen im Vergleich zu solchen, die, trotz Zugabe von EPS, mit Teigen mit mehr Milch- oder Essigsäure hergestellt worden waren. Der hohe Milchsäuregehalt, der durch den Wildtyp *L. sanfranciscensis* im Teig ohne zugesetzte Saccharose erreicht wurde, übte einen negativen Einfluss auf die Krumenfestigkeit aus, wogegen ein hoher Gehalt an Essigsäure negative Effekte auf das Brotvolumen aufwies. Der Zusatz von gereinigtem Levan zu fermentierten Teigen hatte positive Effekte auf die Wasserabsorption, das Brotvolumen und das Hartwerden der Krume. Die *in situ* Produktion von Levan durch *L. sanfranciscensis* im Weizenteig war jedoch unter den verwendeten Bedingungen zu niedrig, um signifikante Ergebnisse zu erzeugen. Zugesetztes Glukan führte zu einer deutlicheren Verbesserung der Teig und Brotqualität.

Für die weitere Optimierung der *in situ* Produktion von EPS wurden ein Glukan- und ein Fruktanbildner, *L. reuteri* TMW 1.106 und *L. sanfranciscensis* TMW 1.392, verwendet. Mit *L. reuteri* konnten höhere Mengen an EPS im Teig erreicht werden, aber ähnlich wie bei *L. sanfranciscensis* war die EPS-Ausbeute im Teig niedriger als im Flüssigmedium. Mit beiden Stämmen konnte die EPS-Synthese mit erhöhter Teigausbeute und pH-Regulation auf den konstanten Wert von 4,7 gesteigert werden. Die Säureproduktion stieg jedoch in beiden Fällen ebenfalls signifikant an. Für *L. reuteri* erwies sich eine Saccharosenachdosierung während der Fermentation von Weizenteigen als vielversprechend, während mit *L. sanfranciscensis* hierbei die Synthese von 1-Kestose die Levanproduktion überstieg. *L. reuteri* bildete moderate Mengen an Maltooligosacchariden im Weizenteig, welche möglicherweise Anti-Staling-Effekte haben. Ferner wuchsen beide Stämme sehr gut in Roggenkleie. *L. reuteri* produzierte darin während der ersten Stunden der Fermentation große Mengen an Glukan, was zu der höchsten Umwandlung von Saccharose in EPS (11,9 %) nach nur einem Tag Fermentation führte, wogegen mit *L. sanfranciscensis* nur 5,6 % erreicht werden konnten (nach zwei Tagen Fermentation in schwachem Weizenmehl bei pH 4,7). Der durchschnittliche Fermentationsquotient (FQ) jedoch war mit *L. reuteri* in allen Experimenten, vor allem mit Roggenkleie niedriger. Es bleibt deswegen offen, ob dessen Dextran um so viel wirkungsvoller ist als das Levan von *L. sanfranciscensis,* so dass die negativen Effekte eines niedrigen FQ kompensiert werden. Dennoch ist die gute EPS-Bildung in Roggenkleie vielversprechend, da sie die Backfähigkeit solcher Teige und so die Anwendbarkeit von Roggenkleie zur Herstellung von ballaststoffreichen Backerzeugnissen positiv beeinflussen kann.

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8 APPENDIX

> Partial sequence of the sucrose phosphorylase of In *B. lactis* TMW 2.530.

TGCATTCTGCCGTTCTTCACCCCGTTCGATGGCGCGGACGCAGGCTTCGACCCGATCGACCA
CACCAAGGTCGACGCCCGCCTCGGTGACTGGGATGACATCGCCGAGCTCGCCAAGACGCATG
ACATCATGGTCGATGCCATCGTCAACCACATGAGCTGGCAGTCCAAGCAGTTCCAGGATGTG
CTCGCGAACGGCGAGGATTCCGAGTACTACCCGATGTTCCTCACGATGAGCTCCGTGTTCCC
CGACGGCGCGACCGAGGAGGAGCTCGCCGGCATCTACCGCCCGCGCCCGGGCCTGCCGTTCA
CCCACTACAGCTTCGCCGGCAAGACCCGCTTGGTGTGGGTCACGTTCACGCCGCAGCAGGTC
GACATTGACACCGACTCCGCCAAGGGCTGGGAATACCTGATGTCGATCTTCGACCAGATGAG
CAAGTCGCACGTCAAGTACATTCGCCTCGACGCCGTCGGCTACGGCGCCAAGGAGGCCGGCA
CCAGCTGCTTCATGACCCCCAAGACCTTCGAACTCATCTCGCGACTGCGCGAGGAGGGTGCC
AAGCGCGGTCTGGAGATTCTCATCGAGGTGCACTCGTACTACAAGAAGCAGGTGGAGATCGC
CGCCAAGGTGGATCGCGTCTATGACTTCGCGCTGCCGCCATTGCTGCTGCACTCGCTGTTCA
CCGGCAAGGTGGACGCGCTCGCGCACTGGACCGAGATTCGCCCGAACAATGCGGTCACCGTG
CTCGACACGCACGACGGCATCGGCGTCATCGACATCGGCTCCGACCAGCTCGACCGTTCGCT
CAAGGGTCTGGTGCCCGACGCGGACGTCGACACA

> Sequence of *B. lactis* TMW 2.530 containing one complete ORF of 2457 bp that showed highest homology to the glycogen phosphorylase from *Bifidobacterium longum* NCC2705 (AAN24421) (aligned in figure 29). Start and the stop codon of the putative glucan phosphorylase of *B. lactis* TMW 2.530 are in bold.

NNTNAAGTGAGTAGCGCACGNCCANNGCCATGGTGGNNCGNNCGAGCCGGGGTGCGATGTCG
AGGGCCGGCGTGGTNTCGGCTGCTGCGCGCGCCGCCGTGCACCAATCGTCGTATGNNNGCAT
GCCGCGCATCATATCTTGCTTACGAATTGCTGCCATACTGTGATTCTGCATCGAACGCTCGA
TAACAATGGGCTGTTTACCCATGGGGCATTAATCCTTGTGAAGACGCTATGGTGATTGCGAA
AAACTCGTTTTCTCCGCGTAAAAAGAGACGCCTGGCGGCTGTATTCGTCTCTTTTTACGTCA
CGGTGAAAGTCATAGGGGAGGAGAAGGGGAAAAGGGGAGAGAGAAGAAGTGTGGAGGTCTGT
TGTTCTGCGTAAAAAGTGACATTTGGTAGTCGTATTTGTCTCTTTTTACGCCAAGGGAGGCG
TCAAAGAGGGATGCGTCAAAGGGTGTTTTGCGTCAGGGGCTGTTACACCAAAAGGAGGGGAG
GGGTAAGCCATGAGATGCAAGGGTGGTCGCGGTAACGAGAGAAGAGTAGTGTGCGGTATATG
GATACCAACGTTTGCAATGGTCGCCGCAGTAAACAGTGCGCGAAACGAATGCGGAAAACAGA
GCGTTTTTCGTGAAAGGAGCAAGCGGTGGTATATTTTATGAGAACGTATGCAAAAACGGATG
CAGCAATGCCCGCCGACGCGCACGTTCGATGAGGAACATATAAAGGGAGTCACGT**ATG**ACCG
AACTAACGGCACCTAAGTCCCCGCTGACCGCCAACGAATTCGCTGACGAAATTCGACAGGCG
CTCAAGTACACACAGGGGGTCACCCCGGAACAGGCCAAGACGGCCGATGTCTATGTTGCCAC
TGCAACCGTGGTCCGCCGTCATCTCATGGATTCCTGGATGAAGACCCAGCAGGACATGATCA
ACGGCGACACGAAGGCTGTGGGCTACCTGTCCGCGGAATTCCTGATGGGCAAGCAGCTGCGC
AACGCATTGCTCAACGCGGGCCTCACCCCGCAGTTCGAAGAGGCCGTGCGCGGCCTCGGCTT
CGACCCGCAGGCAGTGGTCGACGCGGAATACGAACCGGGCCTGGGCAACGGCGGTCTCGGCC
GTCTCGCAGCCTGCTTCATCGATTCGCTCGCCTCGCTCGGCGTTCCCGCCTTCGGCTACGGC
ATCCAGTACAAGTACGGCATCTTCCGCCAGGAATTCGATGACGAGGGTCGCCAGATCGAGCG
TCCGGACTACTGGCTGTCCAATGAGGACCCGTGGGGGCACATCGACTACGAGCGCGATCAGC
GCGTCAACTTCGGCGGCAAGGTCGTCGAGGAGAACGGCAAGCGTGTGTGGAAGCCGAGCTGG
GCCGTGCGCGCGATCCCGGTGGACTACATGGTGCCCGGCTACGCCTCGGGTCGCGTGAACAC
GCTGCGTCTGTGGCAGGCCCGCTCCTACGACGAGTTCGACCTGCTCACGTTCAACAAGTCCG
AGTACCTCGACGCGGTGAAGCCGCAGGTCAAGGCCGAGGATATCTCCAAGGTGCTCTACCCG

GAGGACTCCACCGAGGTGGGCAAGGAACTGCGTCTCGAACAGCAGTACTTCTTCGCCAGCGC
CTCCATTCACGATGCGATTCGCGTGTTCTACCCGGACACCGACAAGCCGGATCTGACCACCT
TCGCCGACAAGATCACGTTCCAGCTCAACGACACCCACCCGGTGATCGGCATTCCGGAGCTC
ATGCGCATCATGATCGACGAATACGGCTACGACTGGGACACCGCCTGGGAAGTCACCCACAA
GACCTTCAACTATACCTGCCACACGCTGCTTCCGGAAGCACTGGAAGTGTGGCCGGCATCGC
TCATTGGCAAGCTGCTGCCGCGCCACCTCGAGATCATCGAGCGCATCAACAAGCAGTTCGTC
TCCGAGCTCGAGGCCAAGGGTGCCAGCGAAGACCAGATCAAGCGCATGCTGATCCTCACCGA
CGACGAGCACCCGGTGGTGCGCATGGCCTACCTCGCCACCTACGCCGGCTCCAACGTGAACG
GCGTCGCCGAGCTGCACTCCCAGCTGCTCAAGGATGTCACGCTGCGTGACTTCTCCGACGTC
TACCCGGCGAAGTTCAAGAACGTGACCAACGGCGTGACCCCACGCCGCTTCATCAAGCTGGC
CAATCCGCGCCTCTCCGAGCTCATCACCGAGGGTCTCGGCACCGACAAGTGGCTTGAGGACC
TCGATCTGCTCGAAGGCCTCGCGCCGCTCGCCCAGGACGACGAGTTCGTGAAGAAGTTCGCC
GCCGTGAAGCACGAGAACAAGGTCGCATTCGCCGACTTCTCCAAGCAGCGCTACGGCGCCGA
ACTCGATGCCAACACGATGTTCGACACGATGATCAAGCGTCTGCACGAATACAAGCGCCAGG
CACTGAAGATCCTCGAGATCATCGCCACGTACTCCGACATCAAGAGCGGCAAGGTCAACGTC
CAGGACATCACGCCGCGCACCTACATCTTCGGTGCGAAGGCCGCCCCGGGCTACTACCTCGC
CAAGATGACGATCCAGCTCATCAACAACGTGGCCAAGGTGATCGACAACGATCCGGATGTCA
ACGGCAAGATCAAGATCTTCTTCCCGTGGAACTACAACATCGAGGTCGCGCAGATGCTCATC
CCGGCCACCGAGCTCGACGAGCAGATCTCGCAGGCCGGCAAGGAGGCCTCGGGCACCTCGAA
CATGAAGTTCGCCCTCAACGGCGCGCTCACCGTGGGCACGCTCGACGGCGCGAACGTGGAGA
TTCGCGAGCGCGTGGGCGCCGACAACTTCTTCCTCTTCGGCATGACCGTCGACGAGGTGGAG
AAGATGTACGAGCAGGGCTACGACCCGAGCAAGTACTATGAGGCCGACCCGCGTCTGAAGGC
CGCGATCGACATGGTTGCCGACGGCACGTTCTCGAACGGCGACAGGAACACCTACGCCCCGC
TGGTCTCCGACTGGCTGACGAAGGACTGGTTCATGACGCTGGCCGACTTCTCCGCCTACCAT
GACATTCAGGCCGATATCGACGCACTGTATGCCGACCAGCTCGAATGGAACCGTAAGGCGAT
TCTCAACGTCGCCAACTCCGGTTACTTCAGCTCCGACCGTTCGATCAAGGACTACCTCGACC
GCATCTGGCACACCCGTTCGCTCGATAAG**TGA**TGAACGGTAATCTGTTCGCAGATTCCTGAT
GGGACGCTTTCGGCCTCCGCTCGCATATGCAGGCGGAGGCCGAAAGCATATTGCGCGAGCGT
ATCTATCTGTTGTGCATTTGCGTAAATAGAGACGCATAGCGCTCGCGGGTGTCCGAAATTAC
GCAAGATGGACGGGGCGGTCTGCGGATATGCAAAGGCGCCGGGTACTCGAACACGGTACCCG
GCGCCCTGCCGTACATGTGTGAACTCAGGCGGCGTCCTTTGCGGCGTCCGCAGCCGGCTGGT
CAAGATCGTCCTCGGTCTTGTCCTGTGCCAGGGTCAGGTCGACCGGCGACGAGCTGTTCTCC
CACGGCTCCTCCCACGGATCGTAATCCGGGTTCTGCTGCTTGTAGATGTACAGACCAACCAC
AGCCGCAAGCAGACCACCGAACAGCAGTGCAAAGAACTTCCAACCGTTTGAAGACTTCTTCT
CCATGACGGCTCCTTTCAGTATTCTGGTCGTCCGGTTCGGCCCGGTCGACTCTGCCTTCCAT
CATAAGCCCCACATCGCAAGATGACGCATATTGTGCTCATGGCAAGCGGATGACGCGTGATG
TCTTAAGTCCCCAATGATACTCCAGTTCCATGGCGATTCGGTAGTCGGTGCCACGGAGTGTG
CCTGGCTGTGCGAATTCGATGACGAGGGTCGCCAGATCGAGCGTCCGGACTACTGGCTGTCC

> Sequence of L. reuteri TMW 1.106 containing one complete ORF of 1458 bp that showed highest homology (99 % identity, 100 % similarity in 457 aa) to sucrose phosphorylase of *L. reuteri* JCM 1112 (EAR58730). Start and the stop codon of the sucrose phosphorylase of *L. reuteri* TMW 1.106 are in bold.

GTAGCCTCTTTAAAATTATTGGAGGATATCATT**ATG**CCAATCAAAAACGAAGCAATGTTAAT
TACTTACTCTGACTCAATGGGTAAAAATATTAAAGAAACTCATGAAGTATTAAAGAACTATA
TCGGTGATGCAATCGGTGGTGTTCACTTACTTCCATTCTTCCCATCAACCGGTGACCGTGGT
TTCGCACCATACCGTTACGATGTTGTTGATTCTGCTTTTGGTAACTGGGACGATGTTGAAGC
ATTGGGTGAAGACTACTACTTAATGTTTGACTTCATGATTAACCATATTTCCAAGAAGTCTG

AAATGTACCAAGACTTCAAGAAGAAGCACGATGATTCTAAGTACAACGACTTCTTTATCCGT
TGGGAAAAGTTCTGGGAAAAAGCTGGTAAGAACCGTCCAACTCAAGAAGATGTTGATTTGAT
TTACAAGCGGAAAGATAAGGCTCCTAAGCAAGAAATTACCTTTGATGATGGTACTACTGAAA
ACTTATGGAACACTTTCGGTGAAGAACAAATTGATATTAATGTTAAGAGTAAGGTAGCTAAC
GAATTCTTCAAGGAAACATTAATTGACATGGTTAAGCACGGTGCTGATATGATTCGTCTTGA
TGCCTTTGCTTACGCTATCAAGAAGGTTGGTACTAATGATTTCTTTGTTGAACCTGAAATCT
GGGATCTTTTAAATGAAGTTCAAGATATTTTGGCTCCATACAAGGCCATCATCCTTCCTGAA
ATTCACGAACACTACACCATTCCACAAAAGATTTCACAACATGACTTCTTCATCTATGACTT
TACCTTACCAATGACTACTCTTTATACCCTTTACTCTGGTAAGACTAACCGCCTTGCTAAGT
GGTTAAAGATGTCACCAATGAAACAATTTACTACTCTTGATACTCATGATGGTATTGGGGTT
GTTGATGCTAAGGATATCTTAACTGATGATGAAATCGAATATGCATCCAACGAATTATACAA
GGTTGGTGCTAACGTTAAGCGGAAATACTCAAGTGCTGAATACAACAACTTGGATATTTACC
AAATTAACTCTACTTACTACTCTGCATTAGGTGACGATGACAAAGCTTACTTGCTTTCTCGT
GCATTCCAAGTATTTGCACCTGGTATTCCAATGGTTTATTATGTTGGATTACTTGCTGGTTC
AAATGACCTTGAATTGCTTGAAAAGACTAAGGAAGGTCGTAACATTAACCGTCACTACTACA
CTAAAGAAGAAGTTGCACAAGAAGTTCAACGTCCAGTAGTTAAGAACCTCTTAGACTTACTT
GCATGGCGGAACAAGTTTGCAGCCTTTGATCTTGATGGTTCAATTGATGTGGAAACACCAAC
TGAAACAACCATTAAGATTACACGGAAGGATAAGGATGGCAAGAATGTTGCTGTTCTTGACG
CTGATGCTGCTAACAAGACTTTCACTATTACAGCTAATGGCGAAAAAGTGATGGAACAAAAA
TAGACATTAAATTAATTAGTAATCTCTCTCGAAATAAAATAGGTAGTATCC

> Sequence of L. reuteri TMW 1.106 obtained with primers Uhp_V/R2. The deduced aa sequences had highest homology (81 % identity, 89 % similarity within 77 aa; fig. 30) to the glycerol-3-phosphatase transporter UhpC (ABJ63273) of the major facilitator superfamily from *L. brevis* ATCC 367.

GGNGTTTCTTTCCCCNCCATAATTGAACAGAACGTTGACATGCCGCTGCTCCCATCCCTTGA
GCAATCGACATAATTAACATTAGAAACATCATCATATATAGGCTATGAGTAGAGCCTAAGCC
GAAGTTAAGCAGTGCAGAAATAACCAATCCGGTTGCTAAATAACGGTTAGTGTTACTTTTAT
CACTTAATGCTCCCATAAATAGTTTTGAAGTTCCATACCCAATCCCAAAA

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