Lehrstuhl für Technische Mikrobiologie

Production of prebiotic exopolysaccharides by lactobacilli

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Doctoral thesis

Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

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Abbreviations frequently used in this thesis:

aa	amino acid
AFLP	Amplified Fragment Length Polymorphism
bp	base pairs
cfu	colony forming units
dNTP	desoxynucleoside triphosphate
dTDP	desoxythymidine diphosphate
DP	degree of polymerization
EMBL	European Molecular Biology Laboratory
EPS	exopolysaccharide
FOS	fructooligosaccharide
GOS	glucooligosaccharide
HMW	high molecular weight
HoPS	homopolysaccharide
HePS	heteropolysaccharide
HeOS	heterooligosaccharide
HPAEC-IPAD	High Performance Anion Exchange Chromatography with Integrated Pulsed Amperometric Detection
HPLC	High Performance Liquid Chromatography
LAB	lactic acid bacteria
LMW	low molecular weight
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
GlcNAc	N-acetyl-glucosamine
GalNAc	N-acetyl-galactosamine
GPC	gel permeation chromatography

RAPD	Randomly Amplified Polymorphic DNA
RI	refractive index
RT-PCR	reverse transcrictase PCR
SDS	sodium dodecyl sulfate
UDP	undecaprenylphosphate
WS-PS	water soluble polysaccharides

Contents

1 Introduction

1.1 Lactic acid bacteria in cereal fermentations

Following a long tradition, sourdough is used as leavening agent in bread production. Sourdough fermentations as well as baking agents based on sourdoughs retained their importance in contemporary baking technology because of the improved aroma, texture, and shelf life of sourdough breads (14, 86, 100). The production of a wide variety of traditionally prepared baked goods continues to rely exclusively on the use of sourdough as leavening agent. In most industrial applications, sourdough fermentation or dried sourdough preparations are added to the bread doughs additionally containing bakers yeast as leavening agent (34, 35, 109). The knowledge of the metabolic activities and corresponding genes of sourdough lactic acid bacteria responsible for their positive influence on bread quality is a prerequisite for the deliberate choice of starter cultures for specific applications.

The predominant microorganisms isolated from traditional sourdoughs (type I doughs) sustained by continuous propagation are yeasts and lactic acid bacteria, mainly *Lactobacillus sanfranciscensis* and *Lactobacillus pontis*. In industrial sourdoughs prepared by using elevated temperatures and / or longer fermentation times (type-II-sourdough) as well as in cereal fermentations in tropical climates, thermophilic, acid tolerant lactobacilli such as *L. pontis*, *Lactobacillus panis*, *Lactobacillus reuteri, Lactobacillus amylovorus*, and *Lactobacillus frumenti* are predominant (3, 66, 109). Microbiotae of type II sourdoughs are phylogenetically closely related or even identical to those species of lactobacilli most frequently found in the digestive tract of humans and animals, for example *L. reuteri* and *Lactobacillus acidophilus*.

1.2 Exopolysaccharides from lactic acid bacteria

Bacterial polysaccharides that are secreted into the environment are termed exopolysaccharides (EPS). The description of EPS formation by wine-spoiling lactic acid

bacteria dates back to Pasteur (Pasteur 1861, as cited by Leathers) (56). Orla-Jensen (1943) described EPS formation from sucrose by *Leuconostoc* spp., mesophilic lactobacilli and pediococci and indicated the role of EPS formation in the spoilage of apple cider and beer (74).

Two classes of EPS from lactic acid bacteria can be distinguished, homopolysaccharides (HoPS), which are extracellulary synthesized from sucrose and heteropolysaccharides (HePS) with (ir)regular repeating units that are synthesized from intracellular sugar nucleotide precursors. Several recent reviews outline the current state of the scientific knowledge on biosynthesis, genetics, diversity, and application of HePS formation by lactic acid bacteria (6, 12, 20, 21, 40, 41, 43, 55, 108).

Studies on the application of EPS-forming starter cultures have primarily focused on HePS from lactobacilli in dairy fermentations. HePS are composed of regular repeating units consisting of three to eight carbohydrate units. In addition to glucose and galactose, HePS often contain rhamnose, N-acetyl-glucosamine (GlcNAc), N-acetyl-galactosamine (GalNAc), and phosphates (21, 55). Glucose-1-phosphate and fructose-6-phosphate are key intermediates linking HePS biosynthesis to the general energy metabolism (5, 6, 19, 82). Glucose-1-P is converted to the sugar nucleotides dTDPrhamnose, UDP-galactose, or UDP-glucose, Fructose-6-P serves as precursor for UDP-GalNac and GDP-fucose (6, 21). The repeating unit is assembled from the sugar nucleotides by sequential activity of dedicated glycosyltransferases and is attached to the membrane carrier undecaprenylphosphate during assembly. This lipid II carrier is also involved in assembly and export of murein repeating units. Export of the repeating units is thought to occur through a „flippase“, followed by extracellular polymerisation. The enzymes involved in export and polymerisation of HePS from LAB are homologous to proteins involved in biosynthesis of the *O*-antigens of Gram-negative bacteria (6, 40)

The HePS gene clusters of lactic acid bacteria contain genes coding for regulation, polymerisation, export, and chain length determination, and (phospho-) glycosyltransferases, the synthesis of these sugar nucleotides is carried out by housekeeping enzymes (6, 40). HePS application is currently limited to „ropy“ dairy starter cultures employed to improve the texture of yoghurt and other fermented milk products (20, 43, 55). Both the amount and the size of HePS produced may be influenced by appropriate choice of growth conditions. The polysaccharide concentration in laboratory media and fermented milk is typically in the range of 50 – 800 mg L^{-1} and does not exceed 1.5 g L^{-1} (18, 19, 20, 21). It was shown that these amounts effectively improve fermented milk texture when produced *in situ*, but are much less effective when externally added prior to fermentation by a EPS-negative strain.

HoPS are composed of only one type of monosaccharide and it can be distinguished between extracellular glucans and fructans. They are synthesized by extracellular glucosyl- and fructosyltransferases (glycosyltransferases) using sucrose as the glycosyl donor. Glycosyltransferases are transglycosylases which are able to use the energy of the osidic bond of sucrose to catalyze the transfer of a glucose or fructose moiety to a glycosyl acceptor molecule (64). The characterization of EPS biosynthetic enzymes from LAB was carried out mainly with strains of the genera *Streptococcus* and *Leuconostoc* (81, 83). Several glycosyltransferases have been characterized from these organisms that produce a large variety of polymers including fructans of the levan- and inulin-type, the α-glucans dextran, mutan and alternan, and β-glucans. More recently, the potential of lactobacilli to produce HoPS was evaluated. The production of extracellular glucan and fructan by *L. reuteri* LB 121 was described by van Geel Schutten et al., who performed a screening of lactobacilli from different origins. Furthermore, glucansucrases from *L. reuteri*, *Lactobacillus parabuchneri*, *Lactobacillus sake* and *Lactobacillus fermentum* were described by Kralj et al. (52). Korakli et al. observed the production of fructan from sucrose by *L. sanfranciscensis* TMW 1.392,

which was later characterized as levan, concomitant with the production of kestose (47, 48, 92)

Figure 1.

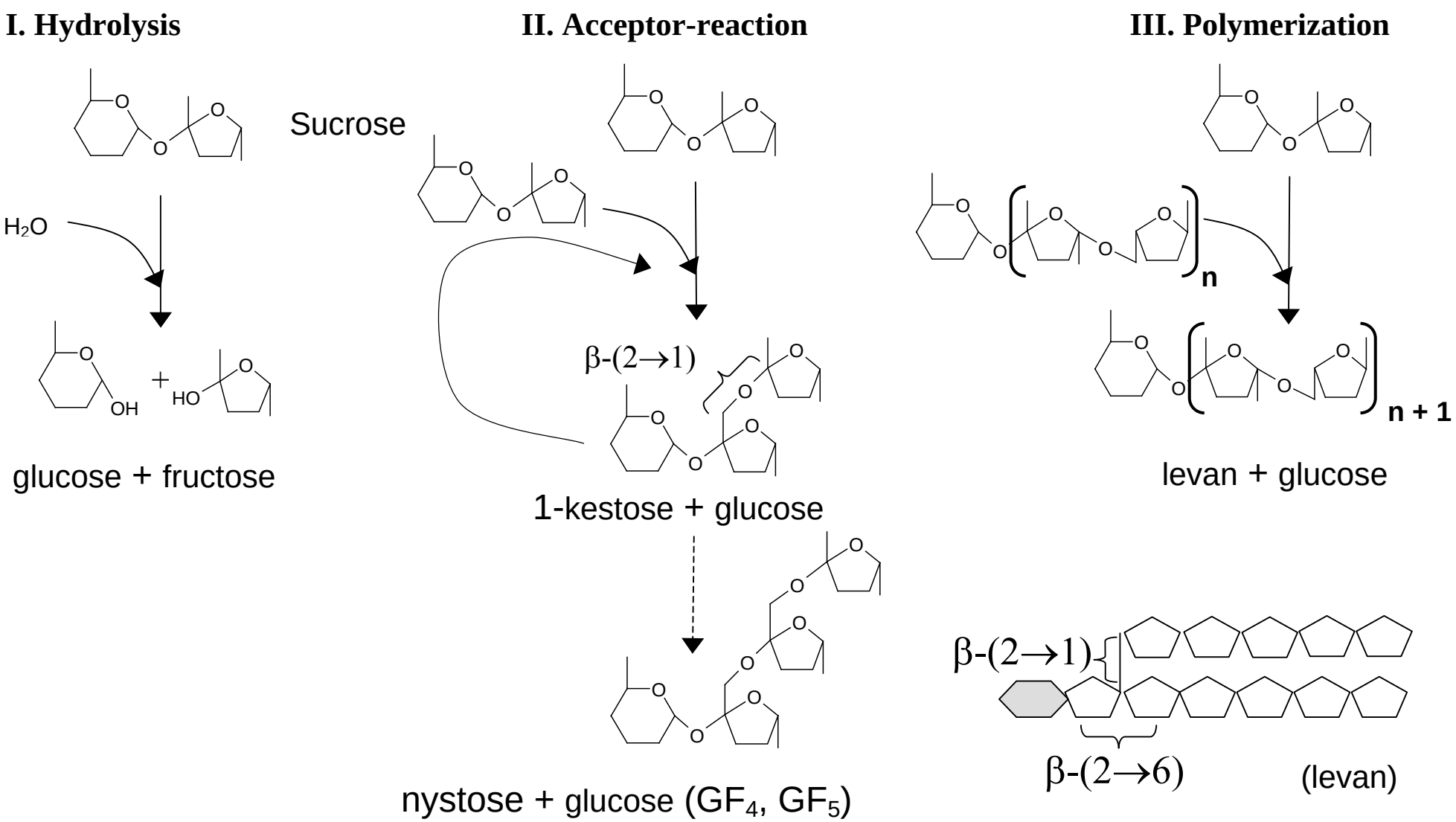
I. Hydrolysis
II. Acceptor-reaction
III. Polymerization
Sucrose
H2O
OH
HO
glucose + fructose
β-(2→1)
1-kestose + glucose
nystose + glucose (GF4, GF5)
n
n + 1
levan + glucose
β-(2→1)
β-(2→6)
(levan)

Figure 1. Schematic overview of hydrolysis-, acceptor-, and polymerization reactions catalyzed by bacterial glycosyltransferases using the example of levansucrases. The carbohydrate moieties are drawn to indicate the carbon atoms, and the oxygen atoms of interest. The linkage types of interest are indicated. In addition to sucrose, fructosyltransferases of lactobacilli use raffinose as substrate. Kestose and nystose are a product of the acceptor-reaction, and act themselves as fructosylacceptors when present in high concentrations. Carbohydrates other than sucrose, kestose, and nystose may be used as fructosylacceptors but corresponding products have not yet been described from fructosyltransferases of lactobacilli.

1.3 Glucan and fructan biosynthetic enzymes

Generally, three reactions are catalyzed by glycosyltransferases, the hydrolysis of sucrose, the formation of oligosaccharides with a degree of polymerization ranging from 2 – 6, and the formation of polysaccharides with a relative molecular mass (M_R) of more than 10^6 (64, 84). As an example, Figure 1 shows the reactions catalyzed by levansucrases. The enzyme catalyzes a hydrolysis reaction (I) using water as an acceptor molecule. In an acceptor reaction (II), sucrose or kestose act as acceptors, yielding in the ß-(2→1) linked oligosaccharides kestose and nystose, respectively. In addition to sucrose and kestose, other carbohydrates may act as acceptors when present in high concentrations (26, 38, 39). The polymerisation (III) of fructose to a levan chain yields a high-molecular-mass polymer with ß-(2→6) linkages in the main chain that may be branched with ß-(2→1) linkages. Fructosyltransferases use not only sucrose, but also raffinose as substrate (84). Thus, by a single enzyme several types of linkages between the fructosyl moieties are formed, and a variety of acceptors may participate in the reaction to yield a broad range of products, namely various oligosaccharides.

Using a high-resolution 3D structure of the *B. subtilis* levansucrase, the identification of the catalytic nucleophile, general acid/base catalyst and transition state stabilizer were enabled

(62). Altering the corresponding amino acid residues Asp249, Asp404 and Glu503 in the levansucrase from *L. reuteri* LB121 resulted in a clear loss of activity (76).

Fructan- and glucansucrase enzymes from LAB are extracellular or cell wall bound enzymes. Their consensus domain organisation is shown in Figure 2. Glucansucrases have an average M_R of 160k and are composed of four domains: domain A, a N-terminal export signal; domain B, a variable region with no apparent role in catalysis; domain C, the highly conserved catalytic core with about 900 amino acid residues; and domain D, a C-terminal domain covering 300 to 400 residues, which are responsible for glucan binding (83). As an exception to this common domain structure of glucansucrases, an enzyme from *Leuconostoc mesenteroides* was recently characterized that contains two catalytic domains in the order A-B-C1-D-C2 (9). The catalytic domains of glucansucrases share homologies to glucan-hydrolyzing enzymes such as amylases and insight into the amino acid residues involved in catalysis now allow to alter the polymer structure by site-directed mutation in the glucansucrase enzymes (64). For example, mutations in the asp-569 of glucosyltransferase I from *Streptococcus downei* yielded enzyme variants producing a α-(1→6) linked glucan in addition to the α- (1→3) linked glucan formed by the wild type enzyme (65).

Glucansucrases

Fructosyltransferases

Figure 2. Domain organisation of glucosyltransferases and fructosyltransferases from LAB. A: N-terminal signal sequence, glucansucrases and levansucrases are extracellular. B: variable region. C: highly conserved catalytic domain. D: glucan binding domain consisting of a series of repeating units. E: cell wall anchor domain.

Fructosyltransferases from LAB generally consist of an export signal (domain A), a N-terminal variable domain (domain B), the catalytic domain with conserved residues (domain C), but in contrast to glucansucrases they are usually lacking a polymer-binding domain. C-terminal cell wall anchor motifs (domain E) are common in levansucrases from LAB (81, 105)

1.4 Application of EPS from lactobacilli: overview

Microbial polysaccharides have rheological properties that match the industrial demands and can be produced in large amounts and high purity. Since the 1940ies, dextran and levan have found numerous pharmaceutical and food applications. Fructose-oligosaccharides (FOS) have interesting properties for food applications as they have a low sweetness compared to sucrose, are essentially calorie-free, and noncariogenic (116). Food applications of inulin and FOS are based mainly on their prebiotic properties (84). Remarkably, the levan from *L. sanfranciscensis* LTH 2590 (isogenic to *L. sanfranciscensis* TMW 1.392) also exhibits prebiotic effects as demonstrated *in vitro* by two different experimental approaches (16, 46). Fructans play a role in the cellular stress tolerance of plants and the mechanisms of protection involve the stabilization of membranes (73). Dextran from *Lc. mesenteroides* protects the producer strain during starvation survival at alkaline and acidic conditions (42). The property of FOS and EPS to protect bacteria against various stresses will enable their application to improve the survival and activity of LAB during the preparation and storage of starter cultures.

The addition of plant polysaccharides is a common practice in the production of bread or frozen dough to improve textural properties and shelf life of bread. Several patents claim that fructan or FOS improve rheological properties of wheat doughs and bread quality (96, 115). Dextran from *Lc. mesenteroides* finds commercial application in baking improvers (17). A study performed by Brandt et al. (10) provided evidence that EPS effectively improve dough rheological parameters and bread quality. Remarkably, EPS produced *in situ* was more effective when compared to externally added levan and addition of 1 % (flour base) sucrose to wheat doughs sufficed to induce polymer formation by lactobacilli to effective concentrations (10). Formation *in situ* of EPS from sucrose results in further metabolites such as mannitol, glucose, and acetate, that may contribute to the improved bread quality (49). Polymers produced from lactobacilli thus may be expected to beneficially affect one or more of the following technological properties of dough and bread: (i) water absorption of the dough, (ii) dough rheology and machinability, (iii) dough stability during frozen storage, (iv) loaf volume and (v) bread staling.

1.5 Motivation and objectives

Following the identification and classification of lactic acid bacteria from cereal fermentations in the past three decades (110), basic and applied sciences now face the challenge to identify functional traits within these bacteria to fully exploit their metabolic potential for the production of baked goods. The deliberate exploitation of desirable metabolic activities is enabled by the demonstration of the contribution of individual metabolic traits on bread quality and will ultimately enable the selection of the most suitable starter cultures for baking applications "*in silico*" (24). The suitability of this approach was demonstrated using the example of arginine metabolism of *L. pontis* and its impact on bread flavor (24, 31, 100). In the past years it has initially been reported that cereal associated LAB of the species *L. sanfranciscensis* and *L. reuteri* are capable to produce large amounts of polysaccharides and

oligosaccharides, which may beneficially affect bread flavor, texture, and shelf life (10, 102, 108). In this work, the general potential of cereal associated lactobacilli to produce HoPS *in vitro* and during sourdough fermentation should be evaluated based on these initial reports on fructan and glucan production by lactobacilli. Furthermore, the aim was to gain knowledge on the genetic determinants of EPS formation by *L. sanfranciscensis* and the biochemical properties of the biosynthetic enzymes. The biodiversity of glycosyltransferases in lactobacilli for use in sourdough fermentation was examined.

2. Materials and Methods

2.1 Strains, media and growth conditions

Lactobacilli were cultivated anaerobically in MRS medium modified according to Stolz et al. (95), containing 10 g x L^{-1} maltose, 5 g x L^{-1} glucose and 5 g x L^{-1} fructose (mMRS-maltose) , or 35 g x L^{-1} maltose and 50 g x L^{-1} sucrose (mMRS-malsuc). In general, all strains of *L. sanfranciscensis* were incubated at 30 °C, all intestinal strains as well as sourdough isolates of *L. reuteri*, *L. frumenti*, *L. pontis* and *L. panis* at 37 °C.

For the purpose of screening for EPS-production, mMRS was used containing 100 g x L^{-1} sucrose as sole carbon source (mMRS-suc). The medium pH was adjusted to 6.2 before autoclaving (121 °C, 20 min), and sugars were autoclaved separately from the other medium components. Where appropriate, 10 μg x L^{-1} erythromycin was added. Prior to each experiment, strains were inoculated on plates and the absence of contaminants was verified by the observation of an uniform colony morphology of the inoculum used for the experiments.

E. coli strains DH5 α, JM109 DE3, and XL1 were used for cloning procedures. *E. coli* was cultivated aerobically in Luria-Bertani (LB) medium at 37 °C unless stated otherwise. Where appropriate, 100 μg ampicillin x L^{-1} for maintaining plasmids or 40 μg x L^{-1} isopropyl-*β*-D-thiogalactopyranosid (IPTG) for induction of gene expression were added. Solid media contained 15 g x L^{-1} agar-agar.

2.2 EPS-screening

2.2.1 Screening of cereal associated lactobacilli for EPS synthesis

107 strains of the genera *Lactobacillus* and *Weissella* that were previously isolated from sourdough fermentations, sour wort fermentation, and spoiled beer were screened for EPS formation. This selection comprised strains of *Lactobacillus alimentarius* (2 strains), *Lactobacillus amylolyticus* (1) *L. amylovorus* (2), *Lactobacillus brevis* (5), *Lactobacillus*

buchneri (1), *Lactobacillus farciminis* (3), *Lactobacillus fructivorans* (1), *L. frumenti* (7), *Lactobacillus fermentum* (2), *Lactobacillus mindensis* (3), *L. panis* (2), *Lactobacillus pentosus* (3), *Lactobacillus plantarum* (6), *L. pontis* (9), *Lactobacillus rhamnosus* (1), *L. reuteri* (5), *Lactobacillus sakei* (1), *Lactobacillus sanfranciscensis* (30), *Lactobacillus suebicus* (2), *Lactobacillus viridescens* (1), *W. confusa* (3), and 21 strains of *Lactobacillus* that were not classified to species level.

In a first screening, all strains were incubated for 60 h at 30 °C in 200 µL mMRS-suc. After incubation, the cells were removed by centrifugation (1500 x g, 10 min) and 100 µL of the supernatants were analyzed by gel permeation chromatography (GPC) coupled to a refractive index (RI) detector to detect high molecular weight (HMW) EPS. 20 µL culture supernatant were injected on a Superdex 200 GPC column (Amersham Pharmacia Biotech, Uppsala, Sweden), the samples were eluted with 50 mM sodium phosphate, pH 6.9 at a flow rate of 0.6 mL min^{-1}. Gel filtration high molecular weight and low molecular weight calibration kits (Amersham Pharmacia, Uppsala, Sweden) were used for calibration of the GPC column. The GPC column employed separates polymers with a relative molecular weight (M_r) ranging from 10^4 and 5×10^6.

In a second step, EPS-positive strains were inoculated in 600 µL mMRS-suc and after fermentation the supernatants were precipitated by adding 2 volumes of ethanol and storage at –20 °C for at least 1 hour. After centrifugation (10 min, 1200 x g) the precipitates were dried under vacuum, redissolved in 300 µL demineralized water and analyzed by GPC as described above. The first screening and the second screening were carried out each in two representative experiments (total of four replicates for EPS-positive strains) with consistent results.

2.2.2 Screening of lactobacilli isolated from ducks for EPS synthesis

A total of 23 lactobacilli, isolated from the duck`s crop or intestinum, were screened for HoPS-production and oligosaccharide-synthesis. These organisms had been previously isolated from 12 ducks of varying ages and identified to species level by Kurzak et al (53). The selection of bacteria comprised strains of *Lactobacillus salivarius* ssp. *salicimus* (5 strains), *Lactobacillus agilis* (1), *Lactobacillus delbrueckii* ssp. *lactis* (1), *L. reuteri* (10), *Lactobacillus animalis* (2) and *Lactobacillus acidophilus* (4). To avoid selection of isogenic organisms, strains of the same species were chosen from different Randomly Amplified Polymorphic DNA (RAPD) clusters (53). All intestinal strains were grown anaerobically at 37 °C. Screening for EPS-production was performed in culture supernatants as described.

2.2.3 Isolation of lactobacilli from pig feces and screening for EPS production

Isolation of lactobacilli from pig feces was performed by Dr. Maher Korakli from the Lehrstuhl für Technische Mikrobiologie, Freising, Germany.

For the determination of lactobacilli in the intestinal microflora of pigs, pig feces was diluted and plated onto mMRS. Colonies with different morphologies were subcultured twice by dilution streaks on mMRS plates to obtain pure cultures. As a subsequent step, DNA was isolated from the different isolates and AFLP was performed as described below. At least one representative strain of each AFLP cluster was chosen and 16S rDNA was partially amplified using the primer combination 616V and 609R (Table 1). Identification to species level was achieved by sequencing the 16S rDNA amplicons. Furthermore, a total of 19 lactobacilli that were isolated from 20 pigs were screened for EPS-production as described. *L. sanfranciscensis* TMW 1.392 and *L. reuteri* TMW 1.106 were used as reference strains for fructan and glucan production, respectively.

2.2.4 Monosaccharide composition of EPS

To analyse the monosaccharide composition of the EPS, positive strains were inoculated on 10 mL mMRS-suc medium. Strains of *L. sanfranciscensis* were incubated at 30 °C, all intestinal strains and sourdough isolates of *L. reuteri*, *L. frumenti*, and *L. pontis* were incubated at 37 °C. After 60 hours the culture supernatants were precipitated with ethanol and redissolved in demineralised water as described above. A preparative GPC run was performed with 200µl injection volume at a flow-rate of 0.6 mL min^{-1}, and the polymer-peak-fractions were collected in an elution volume ranging from 7 to 10 mL corresponding to a M_r above 10^6. The polymer-fractions containing high molecular weight EPS were dried in vacuum and dissolved in 800 µL demineralised water. To hydrolyse the EPS, 15 % (v/v) perchloric acid (70 %) was added and the samples were heated to 80 °C for 1 hour. To precipitate perchlorate, 250 µL 5M KOH were added, precipitated potassium perchlorate was removed by centrifugation (4 °C, 12000 x g, 5 min), and the supernatant was used for the analysis of monosaccharides in the hydrolysate. The monosaccharide compositions were analysed by high-performance liquid-chromatography (HPLC) using a Polyspher CH PB column (Merck, Darmstadt, Germany) and RI detection as described in **2.3**. For peak identification, an external standard was used containing arabinose, fructose, glucose and xylose. The determination of the monosaccharide composition was done in two independent experiments with consistent results.

2.2.5 Preparation of doughs for the purpose of EPS screening

Sourdough fermentations for the purpose of EPS screening were carried out by Dr. Maher Korakli from the Lehrstuhl für Technische Mikrobiologie, Freising, Germany, essentially as described (49), who kindly provided experimental data. For the fermentation of doughs, 5 strains forming fructan (*L. sanfranciscensis* TMW 1.392, *L. frumenti* TMW1.103, TMW1.660, TMW1.669 and *L. pontis* TMW1.675), one strain forming glucan (*L. reuteri*

TMW1.106), and one EPS-negative control, *L. sanfranciscensis* LTH 2581, were used. Doughs were prepared with 100 g wheat flour (ash content = 510-630 mg / 100 g), 12 g sucrose, and 100 g sterilized tap water. For inoculation of doughs, cells from 10 mL of overnight cultures of each strain in mMRS-suc were harvested by centrifugation, resuspended in 5 mL of physiological salt solution, and added to the doughs. Aseptically fermented control doughs were prepared without inoculum and 20 ppm chloramphenicol and 10 ppm erythromycin were added to inhibit microbial growth. Control dough and doughs with *L. sanfranciscensis* were incubated for 24 h at 30 °C, all other doughs were incubated at 37 °C. Determination of pH and cell counts in sourdoughs. Cell counts were determined on mMRS-maltose agar. Appropriate dilutions were plated using a spiral plater (IUL, Königswinter, Germany), and plates were incubated at 30 °C for 48 h under anaerobic conditions. The pH-values of the dough were determined with a glass electrode.

2.3 High Performance Liquid Chromatography (HPLC)-analytics

Analysis and quantification of organic acids was performed using a polyspher OAKC column (Merck, Darmstadt, Germany) and refractive index (RI) detection. The injection volume was 20 µL, 5 mM H_2SO_4 at a flow rate of 0.4 mL min^{-1} was used as mobile phase and the column temperature was maintained at 70 °C.

Analysis of carbohydrates was done using a polyspher CH PB column (Merck, Darmstadt, Germany) and RI detection. The injection volume was 20 µL, demineralised H_2O at a flow rate of 0.4 mL min^{-1} was used as mobile phase and the column temperature was maintained at 70 °C.

Detection and determination of oligosaccharides was performed by high-performance anion-exchange chromatography and integrated pulsed amperometric detection (HPAEC-IPAD) as described by Thiele et al. (101). Therefore, a ternary gradient was employed using water with

a resistance of 18 mOhm (solvent A), 1 M sodium acetate (solvent B) and 0.25 M NaOH (solvent C): -27 min, 0 % B and 0 % C; -21min, 0 % B and 12 % C; 0 min, 0 % B and 12 % C; 2 min, 0 % B and 10 %C; 11 min, 0% B and 20 % C; 18 min, 0% B and 30 % C; 28 min, 32 % B and 24 % C; 39 min, 40 % B and 24 % C; 52 min, 50 % B and 50 % C; with injection at t = 0 min. The column was washed with 50 % B and 50 % C for 20 min after each sample. External standards containing the respective substance were used for quantification of products.

2.4 Molecular techniques.

2.4.1 General molecular techniques

General techniques regarding cloning, DNA manipulations and agarose gel electrophoresis were done as described by Sambrook et al. (89). Chromosomal DNA of lactobacilli was isolated according to the method of Lewington et al. (58) from cultures that were grown to stationary growth phases in 10 mL mMRS-maltose. This procedure was modified for *L. animalis* TMW1.971, because of problems with DNA digestions by restriction enzymes due to insufficient DNA-quality. DNA therefore was purified from the 25-fold amount of culture and the DNA-isolation procedure was modified with respect to the following steps: Cell lyses was enhanced by freezing and thawing the cell pellets and by an enlarged lysis period of 16 hours at 4 °C. Prior to the first step of precipitating, 3 subsequent steps of purifying were included by shaking 1 volume of the sample with 2 volumes of (i) phenol, (ii) a 24:1 mixture of chloroform-isoamylalcohol mixed with phenol 1:1 and (iii) chloroform-isoamylalcohol (24:1); each step of shaking was performed for 10 min and followed by centrifugation for 60 min at 8000g x min^{-1} and 4 °C.

To check the yield and quality of the DNA preparations, a RAPD-PCR was carried out with M13 universal primers (Table 1), as described by Müller et al (68)**.**

E. coli plasmid DNA was isolated with the Plasmid Mini Kit from Quiagen (Hilden, Germany) according to the instructions of the manufacturer.

Restriction endonuclease digestions and ligations with T4-DNA ligase were performed following the recommendations of the suppliers (MBI Fermentas, St. Leon-Rot, Germany).

Table 1. Primers used for genetic manipulations

Primer	Sequence (5` to 3`)	Use
LevV	GAY GTI TGG GAY WSI TGG C	PCR
LevR	TCI TYY TCR TCI SWI RMC AT	PCR
Lev5R	TGT AAT TAG ACA CAT AAC	Inverse PCR
Lev6V	TTC ACC ATT AGT ATC CAC	Inverse PCR
Lev7R	AT TAA AAT TGA AGC TGA TAC	Inverse PCR
Lev8V	GGT GAT AAT GTT GCT ATG ATC G	Inverse PCR
Lev10V	GGT TGA CTG GAG TTT GAT TAA C	Inverse PCR
Lev11R	TGA CAT TCT TGC CGA CGT AG	PCR
Lev12V	AT TCG CCT TGT TTA TAT AG	PCR
LevH	GTT GAT GAG GCT TTA TGC	PCR
P1_HisV	ATA TAC ATA TGG CTG TTG AGA ACA ATA	PCR and cloning
P2_HisV	ATA TAC ATA TGG ATA CTA AAA CTG CTG	PCR and cloning
HisR	TAT AGG ATC CCC GTT GGT CCA CAA AAT	PCR and cloning
KnockoutV	ATA TAG ATA TCG ATA ATA GTC AAT TAA	PCR and cloning
KnockkoutR	ATA TAG ATA TCA CTA ATG GTG AAT AAA	PCR and cloning
PtaV	ATT AGT TTT TCC TGA AGG GG	PCR
PtaR	ATC AAC AAT TTC AAT GCC ATC	PCR
T7	GTA ATA CGA CTC ACT ATA GGG C	PCR
eryV	GAC TCA AAA CTT TAT TAC TTC	PCR
Dex3R	TTG GCC AAC TTT GTA GGC	Inverse PCR
Dex4V	TAA GAC AAC AAC AGA TGG	Inverse PCR
Dex5R	AAA ATC AGT GAT CAG ATC	Inverse PCR
Dex6V	ATA AGT ATG GTT CTT ACC	Inverse PCR
Dex7V	GGT ATT ATT TCG ACA ACG	Inverse PCR
Dex9R	CCC TGT CAA TAA AGC ACC	Inverse PCR
Dex12R	CGT TGC ATA GCT CCG TTC	Inverse PCR

Primer	Sequence (5` to 3`)	Use
Dex13R	CTG ATT GAA CTA CGT TAG	Inverse PCR
Dex14R	CTT ATC GTC ACC GTA AGC	Inverse PCR
Dex15R	GCC ATA AAT CAT CCA GCC	PCR
Dex16V	TGA CAC CTT TAA TGG TGC	Inverse PCR
Dex17V	GGT AAA CAT TGG GTA TTT GC	PCR
M13V	GTT TTC CCA GTC ACG AC	species identification
dexwobR	YTGRAARTTISWRAAICC	screening
dexwobV	GAYGCIGTIGAYAAYGTI	screening
DexreuV	GTGAAGGTAACTATGTTG	screening
DexreuR	ATCCGCATTAAAGAATGG	screening
levV	GAYGTITGGGAYWSITGGC	screening
levR	TCITYYTCRTCISWIRMCAT	screening
Dexani_exV	GCTGCCTACAAAGTTGGC	RT-PCR
Dexani_exR	ACCCCATACCTTGAGTCC	RT-PCR
616V	AGAGTTTGATYMTGGCTCAG	species identification
609R	ACTACYVGGGTATCTAAKCC	species identification
Pre-M	GATGAGTCCTGAGTAAC	AFLP
Pre-E	GACTGCGTACCAATTCA	AFLP
E*-A[b]	GACTGCGTACCAATTCA	AFLP
M-CT	GATGAGTCCTGAGTAACT	AFLP

Primers used for PCR and cloning procedures with I for Inosin; Y for C or T; S for G or C; W for A or T; R for A or G; M for A or C according to International Union of Biochemistry group codes. *Nde*I, *BamH*I and *EcoR*V restriction sites are underlined.

PCR was carried out in thermocyclers (Eppendorf, Hamburg, Germany) by using taq polymerase from Promega (Mannheim, Germany) and dNTPs from Diagonal (Waldeck, Germany). In general, 3 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 µM primer and 1.5 U taq were used per reaction unless stated otherwise. Amplifications products were separated on 1 % agarose gels, stained with ethidium bromide, and visualized with UV transillumination. PCR-products were purified using the QIAquick PCR Purification Kit (Quiagen, Hilden, Germany) according to the instructions of the supplier, and sequenced by SequiServe (Vaterstetten,

Germany). Nucleotide and amino acid sequence analysis was carried out using the DNASis for Windows software (Hitachi Software Engineering Co, Yokohama, Japan). *E. coli* transformations were performed with a Bio-Rad gene pulser apparatus (Bio-Rad Laboratories, Hercules, Calif.) in 0.2 mm cuvettes at 2.5 kV, 25 µF and 200 Ω.

2.4.2 Construction of primers targeting fructosyl- and glucosyltransferases

Primers to amplify fructansucrase genes were constructed to target conserved amino acid sequences of levansucrases of lactic acid bacteria. The amino acid sequences DVWDSWP and DEV(I,L)ER are conserved in levansucrases from *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus amyloliquefaciens*, *Streptococcus mutans*, *Streptococcus salivarius* (European Molecular Biology Laboratory (EMBL), Heidelberg, Germany, www.embl.heidelberg.de, accession numbers P05655, P94468, P21130, P11701, and Q55242, respectively) and in fructosyltransferases of *L. reuteri* strain 121 (104, 106). The sequences of the degenerated primers are given in Table 1.

For the detection of glucosyltransferase genes in the screened organisms, primers were constructed that were targeting conserved regions in the catalytic domains of known bacterial glucansucrases. According to Monchois et al. (64), sequence alignments of catalytic domains from known bacterial glucansucrases were used to construct degenerated primers from the amino acid sequences DAVDNV and EGFSNF. (Table 1).

2.4.3 Isolation of mRNA and RT-RCR

Total RNA was isolated from cultures of *L. sanfranciscensis* TMW1.392, *L. sanfranciscensis* TMW 1.53, *L. reuteri* TMW 1.106, *L. curvatus* TMW 1.624 and *L. animalis* 1.971 grown to the exponential growth phase (OD 578 nm = 0.2) in mMRS-suc and mMRS-maltose. From the cultures were taken 0.6 mL and resuspended in 1.2 mL stop buffer (RNA protect bacteria reagent, Quiagen, Hilden, Germany). The isolation of RNA was performed using the RNeasy

Plant Mini Kit (Quiagen, Hilden, Germany). In the RNA preparations, DNA was digested by incubation with RQ1 RNAsefree DNAse (Promega, Mannheim, Germany). RT-PCR was performed using 100 U M-MLV reverse transcriptase and primed with 20 μg mL^{-1} of hexameric random primers (rRNase H minus and random primers from Promega, Mannheim, Germany). From the cDNA library, an internal fragment of the levansucrase was amplified with primers Lev3V and LevshortR matching internal sequences of the 800 bp LevV - LevR levansucrase fragment of *L. sanfranciscensis* (Table 1). Primer pairs dexreuV/R were used for the gtfA gene of *L. reuteri* TMW1.106, and dexani_exV/R for the gtf gene of *L. animalis* 1.971 (Table 1). PCR was also carried out with DNAse digested RNA preparations to ensure complete hydrolysis of chromosomal DNA in the RNA preparations.
PCR with the mentioned primers was also carried out with DNAse digested RNA preparations to ensure complete hydrolysis of chromosomal DNA in RNA preparations.

2.4.4 Amplified Fragment Length Polymorphism (AFLP)

AFLP typing was performed by Rossica Valcheva from the Laboratoire de Microbiologie Alimentaire et Industrielle QM2A, ENITIAA, Nantes Cedex, France, essentially as described by Vos et al. (111) as modified by Schmidt et al (91). In brief, AFLP procedure consisted of the subsequent steps (i) restriction of genomic DNA, (ii) ligation with adapters, (iii) preliminary PCR (pre-PCR), (iv) selective amplification with labelled primers and (v) automated laser fluorescence analysis (ALFA) of the amplification products with an ALF-sequencer (Amersham, Freiburg Germany) and data processing using the applied maths software from BioNumerics (Sint-Martens-Latem, Belgium).

2.4.5 Plasmids

The plasmid pLP3537 (79), which is replicating in lactobacilli and carries erythromycin and ampicillin resistance genes, was used for optimising the transformation efficiency of *L. sanfranciscensis* TMW1.392. A *Lactobacillus* suicide vector was constructed by ligating the 1.4-kb erythromycin resistance cassette of pLP3537 into the *Hind*III and *Xba*I sites of pSP72 (Promega, Mannheim, Germany). The resulting plasmid pME-1 was transformed in *E.coli* XL1 and used for the construction of the integration vector pME-1knock as described below. The vector pET-3a (Novagen, Callbiochem-Novabiochem, Schwalbach, Germany), modified by the addition of a 6x-histidin tag, was used for cloning and heterologous expression of the levansucrase-gene.

2.4.6 Nucleotide sequence analysis of the levansucrase gene sequence from *L. sanfranciscensis* TMW 1.392

Based on the 800 bp fragment of the levansucrase gene from *L. sanfranciscensis* identified with the degenerated primers LevV / R and subsequent sequencing, primers Lev5R and Lev6V were designed for inverse PCR (Table 1). Chromosomal DNA of *L. sanfranciscensis* TMW 1.392 was digested with *Pst*I, religated, and used as template for inverse PCR to yield a product with the size of approximately 2800 bp. Sequencing of this PCR product using primers shown in Figure 1 and Table 1 yielded a sequence with an overall size of 3623 bp, comprising two open reading frames (Figure 1).

2.4.7 Nucleotide sequence analysis of the glucansucrase gene sequence from *L. animalis* TMW 1.971

Primers Dex3R and Dex4V were designed for inverse PCR (Table 1), based on the 1200 bp fragment of the glucansucrase gene from *L. animalis* identified with the degenerated primers

DexwobV/R and subsequent sequencing. Chromosomal DNA of *L. animalis* TMW 1.971 was digested with *Hind*III, religated, and used as template for inverse PCR to yield a product with the size of approximately 2200 bp. Sequencing of this PCR product using the primer-combinations dex5R/6V, dex 9R/4V, dex9R/6V, dex12R/6V and dex 13R/7V (Figure xy and Table 1) yielded a sequence with an overall size of 3800 bp. In a subsequent step, *Nde*I digested and religated DNA templates were used to obtain a total sequence of 5141 bp with the primer combination Dex 14R/16V. Primers dex15R, dex17V, dexklonV and dexklonR were used in combinations to close gaps in the sequence.

2.4.8 Cloning, heterologous expression and purification of the levansucrase

Primers P1_HisV/P_HisR and P2_HisV/P_HisR carrying *Nde*I and *BamH*I restriction sites (Table1) were used to amplify ORFs corresponding to a complete levansucrase sequence lacking the N-terminal leader peptide (*levΔhis*) and a truncated levansucrase sequence missing the N-terminal repeating units (*levCDΔhis*), respectively. Both ORFs were truncated at the C-terminal LPXTG motif (Figure 2). The PCR-products obtained with *L. sanfranciscensis* genomic DNA and the appropriate primers were digested with *Nde*I and *BamH*I restriction enzymes and ligated into the expression vector pET3a, downstream of the inducible lacZ promotor and in frame upstream of a 6 x histidin-tag to obtain C-terminally his-tagged proteins. The resulting constructs were used to transform *E. coli* DH5 α. Clones were screened for correct inserts by colony-PCR. Positive colonies were inoculated on 50 ml LB with ampicillin for 16 hours and used for plasmid isolation as described above. Plasmids were sequenced with T7 plasmid primer to ensure a correct in frame ligation and used to transform *E. coli* JM109 DE3.

Cells of *E. coli* JM109 DE3 carrying the *levΔhis* or the *levCDΔhis* expression vectors were inoculated on 200 ml LB and grown at 37 °C to an optical density (590 nm) of 0.6. IPTG was added and cells were grown at 30 °C for 12 hours to an optical density of 1.0. Cells were

harvested, washed with 50 ml of binding buffer A (50 mM NaH_2PO_4, 10 mM imidazole, 300 mM NaCl) and broken by ultrasonification (5 x 30 sec with 50 % cycle, 90 % power). Cell debris was removed by centrifugation (10000 x g, 10 min, 4 °C) and the supernatants were applied to a HiTrap chelating nickel-column (Amersham, Freiburg, Germany) with a flow of 1.5 mL x min^{-1}. The column was washed with 5.0 ml of 20 % buffer B (50 mM NaH_2PO_4, 300 mM imidazole and 300 mM NaCl) and the his-tagged proteins were eluted with a linear gradient from 20 % buffer B to 100 % buffer B. Fractions were collected and assayed for levansucrase activity. Active fractions were dialyzed overnight against 25 mM sodium acetate pH 5.4 and their protein composition was determined with SDS-PAGE (54). The protein concentration of fractions with the purified levansucrase enzymes was determined with the Bradford method, using the BioRad Protein Assay (BioRad, Laboratories, Hercules, USA) and bovine serum albumine as external standard.

2.4.9 Insertional inactivation of the levansucrase gene by single crossover integration

A 1079-bp fragment of the levansucrase-gene was obtained with PCR using primers KnockoutV and KnockoutR (Table 1), carrying *Eco*RV restriction sites. Digestion and ligation into the *Eco*RV restriction site of plasmid pME1 resulted in the nonreplicating integration vector pME1_knock, which was cloned in *E. coli* DH5α and isolated with the Plasmid Midi Kit from Quiagen as recommended by the supplier.

Electrocompetent cells of *L. sanfranciscensis* were obtained with a method that was based on a protocol of Berthier et al. (1). The strain was grown on 1 L mMRS-maltose medium supplemented with 1 % (w/v) glycine to an optical density (590 nm) of 0.7. The cells were harvested by centrifugation at 4 °C (4000 x g, 15 min) and washed four times with 50 ml of 10 mM $MgCl_2$ solution, once with glycerine (10 % v/v) and once with glycerine-sucrose solution (10 % v/v, 0.5 M). Cells were resuspended in 8 ml of the glycerine-sucrose solution and stored at – 80 °C in 100 µl aliquots. All washing and storage solutions were cooled on ice

to 0 °C. Electroporation of 100 µl cell suspension was performed in 0.2 mm cuvettes in a Bio-Rad gene pulser apparatus (Bio-Rad Laboratories, Hercules, Calif.). The transformation efficiency of *L. sanfranciscensis* TMW1.392 was optimised using the replicating plasmid pLP 3537 by varying different combinations of voltage and resistance. The highest transformation efficiency was observed with 1000Ω/1.2 kV at 25 µF. After electroporation, 1 mL mMRS4 was added followed by 3 hours of incubation at 30 °C before plating on mMRS-maltose agar with 10 ppm erythromycin.

Integration frequencies were estimated as ratios of the clones obtained with pME1_knock to the number of pLP3537 transformants.

Electrocompetent cells of *L. sanfranciscensis* TMW1.392 were transformed with the nonreplicating plasmid pME-1_knock and screened for erythromycin-resistant colonies. Cells from these colonies were subcultured and harvested for DNA isolation. PCR with primers targeting the regions upstream and downstream of the levansucrase gene (Lev12V and Lev11R, respectively) and the plasmidborn regions from pME-1_knock (eryV and T7, Table1) were used to verify insertion of plasmid pME-1_knock into the levansucrase gene. PCR-products with expected sizes of 2100 bp (T7/Lev11R) and 3000 bp (Lev12V/eryV) were obtained with a deletion mutant strain named 1.392Δlev. To ensure a single crossover integration of pME-1_knock into the levansucrase gene of *L. sanfranciscensis* 1392Δlev, the PCR products obtained with primers T7/Lev11R and Lev12V/eryV were sequenced.

L. sanfranciscensis TMW1.392 wild-type and the 1392Δlev mutant strain were inoculated on mMRS-suc and grown for 48 hours. Cell-free supernatants were analysed for EPS formation with GPC, sucrose consumption as well as fructose and kestose formation were analysed by HPLC as described in **2.3**.

2.4.10 Isolation of RNA from dough fermented with *L. sanfranciscensis* TMW 1.392 and TMW 1.392Δlev, RT-RCR and amplification of a levansucrase and phosphotransacetylase internal fragment from cDNA

Sourdoughs with *L. sanfranciscensis* TMW 1.392 and TMW 1.392Δlev, that were prepared as described in **2.5** were fermented to a pH of 4.5 (exponential growth phase). For the purpose of total RNA isolation, cells were harvested from 50 gram sourdough that was diluted in 100 mL Tris-HCl buffer (50 mM, pH 8.0) and centrifuged at 1500 x g and 4 °C for 5 min. The supernatant was transferred to a 50 mL tube and centrifuged again for 15 min at 4500 x g and 4 °C. After redissolving of the cell pellet in 2 mL of Tris-HCl buffer, one volume of RNA-protect solution (Quiagen, Hilden, Germany) was added.

The isolation of RNA was performed using the RNeasy Plant Mini Kit (Quiagen, Hilden, Germany) following the instructions of the manufacturer. In the RNA preparations, DNA was digested by incubation with RQ1 RNAsefree DNAse (Promega, Mannheim, Germany). RT-PCR was performed using 100 U M-MLV reverse transcriptase and primed with 20 µg mL^{-1} of hexameric random primers (Promega, Mannheim, Germany). From the cDNA library, a fragment of the levansucrase was amplified using taq-polymerase and primers Lev8V and LevH, matching a region which was located downstream of the single crossover integration of pME1_knock into the *L. sanfranciscensis* genome. Furthermore, a PCR was carried out with primers Pta V/R (Table 1) targeting the phosphotransacetylase gene of *L. sanfranciscensis* (45). PCR with Lev8V and LevH and Pta V/R primers was also carried out with DNAse digested RNA preparations to ensure complete hydrolysis of chromosomal DNA in RNA preparations.

2.5 Characterization of the sucrose metabolism of *L. sanfranciscensis* TMW 1.392 and TMW 1.392Δlev in medium and doughs

L. sanfranciscensis TMW 1.392 and TMW 1.392Δlev were grown in mMRS-malsuc to stationary growth phases (optical density 1.4 at 590 nm, pH 3.5). Cells were removed by centrifugation at 17000 x g for 15 min and the fermentation products in the supernatants were analyzed with HPLC. Analysis and quantification of organic acids and carbohydrates was performed using a polyspher OAKC column and a polyspher CH PB column, respectively, (both Merck, Darmstadt, Germany) and RI-detection as described in **2.3**, levan was quantified by GPC as described in **2.2.1**. Determination of kestose levels in culture supernatants was performed by HPAEC-IPAD as described in **2.3**. External standards containing fructose, glucose, ethanol, mannitol, 1-kestose, nystose, lactic and acetic acid were used for quantification of products.

For the analysis of fermentation patterns, wheat doughs were prepared with and without sucrose and fermented with *L. sanfranciscensis* TMW 1.392 or TMW 1.392Δlev. For inoculation of doughs, about 10^9 cells from an overnight culture in mMRS were washed with sterile water and resuspended in 10 mL of tap water. Doughs were prepared with 100 g wheat flour type 550 (ash content = 510-630 mg / 100 g), 90 g sterile tap water, 10 mL cells of strain TMW 1.392 or TMW 1.392Δlev, and 20 g sucrose where indicated. The pH-values of the doughs were determined with a glass electrode. For determination of cell counts, appropriate dilutions of the doughs were plated on mMRS-maltose agar and plates were incubated at 30 °C under anaerobic conditions. Analysis of EPS-contents were performed as described in **2.2.5**. For analysis with HPLC, dough samples were weighed, diluted with demineralized water and centrifuged at 17000 x g for 30 min to remove solids. Analysis and quantification of organic acids and carbohydrates in the supernatants was performed by HPLC. Data concerning dough analysis presented in **3.2** are representative for quadruplicate independent fermentations with consistent results.

2.6 Characterization of the levansucrase from *L. sanfranciscensis*

Levansucrase activity was determined at 28 °C in reaction buffer (25 mM sodium acetate buffer, pH 5.4, containing 1mM $CaCl_2$ and 90 mM sucrose as final concentrations) unless stated otherwise. The final concentration of levansucrase enzymes ranged between 0.5 and 1 µg x mL^{-1}. For the determination of the K_m -values, the sucrose concentration was varied in the range of 0 to 500 mM. For the determination of the temperature optimum, reactions were carried out in reaction buffer at temperatures ranging from 0 to 60 °C. For the determination of the pH optimum were prepared buffer A (100 mM sodium acetate, pH 2.0, 1mM $CaCl_2$ and 90 mM sucrose) and buffer B (100 mM Na_2PO_4, pH 9.0, 1mM $CaCl_2$ and 90 mM sucrose) and these buffers were mixed to obtain final pH values ranging from 3.5 to 7.9. To perform enzyme kinetics, the reaction was started by adding 10 µl enzyme-solution to 90 µl of reaction buffer. Samples were taken at 60 sec time intervals and stopped with 1/10 volume 2 M sodium hydroxide. One volume of 100 mM sodium acetate, pH 5.4, was added to the samples and the glucose and fructose concentrations were determined enzymatically (Test kit glucose and fructose, Roche Biochemicals, Mannheim, Germany). The overall activity of the enzymes as well as the contribution of hydrolase and transferase activities was calculated as follows:

overall activity = [glucose released] (µmol) x (mg protein x min)$^{-1}$

hydrolase activity = [fructose released] (µmol) x (mg protein x min)$^{-1}$

transferase activity = overall activity – hydrolase activity

To determine the influence of pH, temperature, and sucrose concentration on the composition of products formed from sucrose, the enzymes were incubated overnight under the following conditions: (i) 40 °C, pH 4.5, and 50, 62.5, 125, 250, or 500 mM sucrose. (ii) 40 °C, 500 mM sucrose, and pH 3.5, 4.5 or 5.5. (iii) pH 4.5, 500 mM sucrose, and 20, 40, or 50 °C. In these samples were quantified sucrose and the products glucose, fructose, and 1-kestose by high-performance liquid-chromatography (HPLC) using a Polyspher CH PB column (Merck,

Darmstadt, Germany) and RI detection as described. The concentration of levan was determined as described in **2.2,** using levan that was purified from culture supernatants of *L. sanfranciscensis* TMW1.392 was as external standard.

2.7 Partial purification and characterization of oligosaccharides from enzymatic reactions and sourdoughs

The levansucrase of *L. sanfranciscensis* TMW 1.392 was purified to homogeneity after heterologous expression in *E. coli* JM109 as described in **2.4.8**. Enzymatic reactions were carried out at 37 °C in 10 mM Na-acetate buffer pH 5.4 with an enzyme concentration of 0.5 $\mu g \times mL^{-1}$, containing 1 mM L^{-1} $CaCl_2$, 0.4 Mol L^{-1} sucrose or 0.4 Mol L^{-1} raffinose, and where indicated 0.4 Mol L^{-1} of the major low molecular weight carbohydrates present in wheat doughs, maltose, maltotriose, xylose, and arabinose. The reaction mixture was incubated for 20h and stored frozen for further analysis.

Enzymatic reactions were carried out in 1 mL volume with levansucrase using sucrose and the major low molecular weight carbohydrates present in wheat doughs, maltose, maltotriose, xylose, and arabinose as described above. After overnight incubation, the reaction mixture was separated on GPC column (Amersham Pharmacia Biotech, Freiburg, Germany) eluted with demineralised water a a flow of 0.4 mL min^{-1} and carbohydrates were detected with an RI detector as described in **2.3**. In order to estimate the degree of polymerisation (DP) of the oligosaccharides based on their molecular weight, the column was calibrated using glucose, sucrose, raffinose, and stachyose as molecular weight standards. Peaks corresponding to tri, tetra- or pentasaccharides were collected, pooled, and dried under vacuum. When maltotriose or raffinose was used as acceptor carbohydrate, peaks corresponding to tetra- and pentasaccharides were collected. The dried oligosaccharides were redissolved in 0.5 mL demineralised water and subjected to a second chromatographic separation on the same GPC column. Peaks corresponding to oligosaccharides with a DP of (3 or 4) and (4 or 5) were

pooled, dried under vacuum, and redissolved in 0.2 mL demineralised water. These fractions were analyzed for the oligosaccaride composition by HPAEC-IPAD as described below and it was verified that no contaminating mono- di- or trisaccharides were present (data not shown). Furthermore, oligosaccharides in these fractions were hydrolysed by incubation for 1h with 5 % perchloric acid at 90 °C. The hydrolysates were neutralised by addition of 4M KOH. Monosaccharides in the hydrolysates were quantified by HPLC using the CHPB® column (Merck, Darmstadt, Germany) eluted with deionised water at a flow of 0.4 mL min^{-1} and RI detection as described.

Wheat doughs were fermented with 1.392 and 1.392Δlev as described in **2.5.** For oligosaccharide analysis, dough samples were weighed, diluted with demineralised water and centrifuged at 17000 x g to remove solids. These supernatants were diluted 1:100 and used for oligosaccharide analysis as described below.

Invertase treatment of enzymatic reactions and aqueous dough extracts were carried out by incubation with yeast invertase with a concentration of 100 μg x L^{-1} for 3 h at 37 °C.

2.8 Chromatographic separation of oligosaccharides

The separation of oligosaccharides was achieved by HPAEC-IPAD as described in **2.3**. For improved separation of oligosaccharides, the gradient was modified as follows: 0 min, 0 % B and 12 % C; 2 min, 10 % B and 12 % C; 11 min, 20 % B and 12 % C; 18 min, 0 % B and 30 %C; 36 min, 36 % B and 24 % C; 45 min, 40 % B and 24 % C. The column was washed with 50 % B and 50 % C for 20 min after each sample. Maltose, maltotriose, raffinose, melibiose, and mixtures of inulin-type FOS (Actilight, degree of polymerisation, DP, 2 to 8, Gewürzmüller, Stuttgart, Germany) or maltose-oligosaccharides (DP4 – 10, Sigma, Deisenhofen, Germany) were used as external standards.

3 Results

3.1 In situ production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria

Based on initial reports on HoPS-formation by lactobacilli (102, 103), a screening was performed among cereal associated lactobacilli to investigate the potential of these organisms to produce glucans and fructans *in situ* and *in vitro*. The monosaccharide composition of the EPS was determined and a molecular screening was performed using primers targeting conserved domains of bacterial levansucrases.

3.1.1 Screening of sourdough lactobacilli for EPS production

A total of 107 strains of the genera *Lactobacillus* and *Weissella* were cultivated on medium containing excessive amounts of sucrose and screened by GPC for EPS-production. This selection comprised mainly strains isolated from sourdough fermentations, sour wort fermentations, and spoiled beer. Examples for chromatograms from culture supernatants of EPS-positive and EPS-negative strains are shown in Figure 3. A peak corresponding to high molecular weight EPS is observed at 7.2 mL elution volume, corresponding to a M_r above 10^6. EPS with a molecular weight of 10^5 to 10^6 was not observed in any of the strains and low molecular weight (LMW) EPS is not detectable with this method, because the medium used contains polysaccharides or other compounds eluting in the M_r range of 10^4 to 10^5.

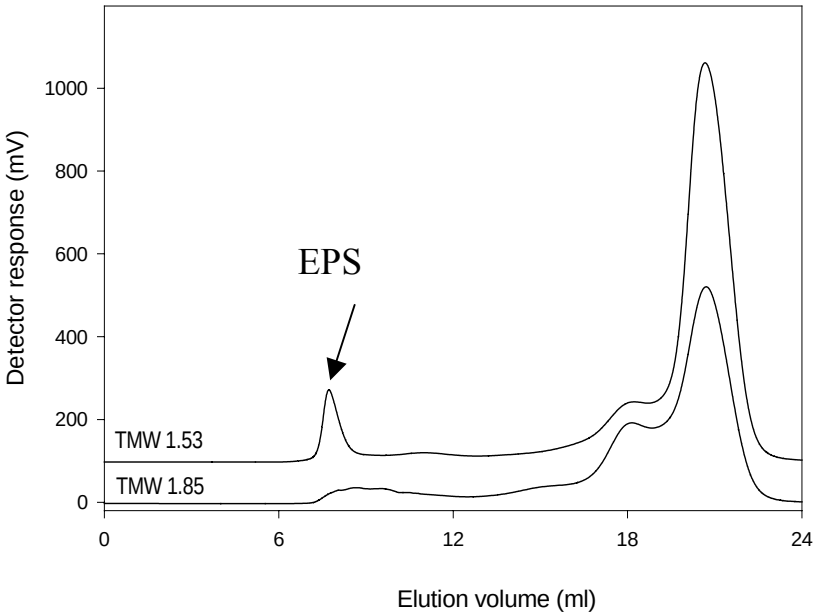

Figure 3. GPC-chromatograms from culture supernatants of *L. sanfranciscensis* TMW 1.53 (EPS-positive) and *L. pontis* TMW1.85 (EPS-negative). A peak corresponding to high molecular weight EPS is observed at 7.2 ml elution volume. The medium used is essentially free of compounds with an M_r above 10^5 but LMW-EPS is not detectable, because it is covered by a LMW peak from the medium.

Twenty-two of the 107 strains were found to produce high molecular weight EPS. In table 1 are listed the EPS-forming strains, their origin, and the type of EPS formed. The EPS was termed fructan or glucan if fructose or glucose accounted for greater 95 % of sugars liberated by hydrolysis, respectively. Three strains were found to produce small amounts of polysaccharides with unknown composition. Because of the low amounts of polysaccharides formed by these strains and / or incomplete hydrolysis of the polysaccharides, the analysis of the respective hydrolysates as performed here did not allow an unambiguous identification of the monosaccharides. Production of fructan and glucan was detected in 15 and 4 strains, respectively. Fructan-forming species were *L. sanfranciscensis*, *L. frumenti*, *L. pontis*, *L. reuteri*, *L. panis* and *W. confusa*. Glucan-forming species were *L. reuteri*, *W. confusa* and *Lactobacillus* spp. TMW1.624. Based on the correlation of peak areas of EPS in

Table 2. EPS-forming species and their origin.

organism and TMW number	**EPS-formation[a)]**	**origin**	**PCR-product with Lev-primer**
L. sanfranciscensis 1.392[c)]	fructan	Strain D1 (=LTH2590), sourdough (1)	800 bp
L. sanfranciscensis 1.53	fructan	DSM 20451, sourdough (44)	800 bp and 700 bp
L. sanfranciscensis 1.54[c)]	fructan	LTH1729, sourdough (4)	no
L. sanfranciscensis 1.896	fructan	sourdough	800 bp
L. sanfranciscensis 1.953	fructan	sourdough	800 bp
L. sanfranciscensis 1.1149	unknown[d)]	sourdough	no
L. frumenti 1.103	fructan	sourdough	800 bp
L. frumenti 1.660	fructan	sourdough (68)	no
L. frumenti 1.665	fructan	sourdough (68)	no
L. frumenti 1.666	fructan	DSM 13145^T, sourdough (65, 66, 68)	no
L. frumenti 1.669	fructan	sourdough (68)	800 bp
L. pontis 1.1115	fructan	sourdough	no
L. pontis 1.675	fructan	sourdough (68)	no
L. pontis 1.682	unknown [d)]	sourdough (68)	no
L. panis 1.649	fructan	DSMZ 6036, sourdough (113)	no
L. reuteri 1.693	fructan[b)]	DSMZ 20016, intestinal isolate	no
L. reuteri 1.977	glucan	duck colon (1)	no
L. reuteri 1.106	glucan	sourdough	no
L. reuteri 1.109	unknown [d)]	sourdough	no
L. curvatus 1.624	glucan	sourdough	no
W. confusa 1.617	glucan	sourdough	no
W. confusa 1.934	fructan	sourdough	no

Table 2: [a)] EPS formation was determined in four independent experiments, and the monomer composition of EPSs were determined in two independent experiments. [b)] The amount of fructan produced by *L. reuteri* TMW 1.693 was significantly lower compared to the other fructan-forming strains.[c)] Fructan formation by strains TMW 1.392 (=LTH2590) and TMW1.54 (=LTH1729) was previously described (16, 47).
[d)] Unknown composition of EPS because of the low amounts of polysaccharides formed and / or incomplete hydrolysis of the polysaccharides.

GPC chromatograms as well as that of monosaccharides in HPLC chromatograms to the EPS concentration of culture supernatants, it can be estimated that the amount of glucan and fructan formed by these strains during growth in mMRS-sucrose ranges from 2 to 20 g L^{-1} as previously described for *L. sanfranciscensis* TMW 1.392 (49). The strain *Lactobacillus* spp. TMW 1.624, was identified as *L. curvatus* by partial sequencing of the 16 S rDNA, using the primers 616V/616R (Table 1).

3.1.2 PCR-screening for a levansucrase gene and sequence of the amplification product

To determine the presence of levansucrase genes, DNA was isolated from the 15 fructan-forming strains, 4 glucan-forming strains, and 16 EPS-negative organisms.

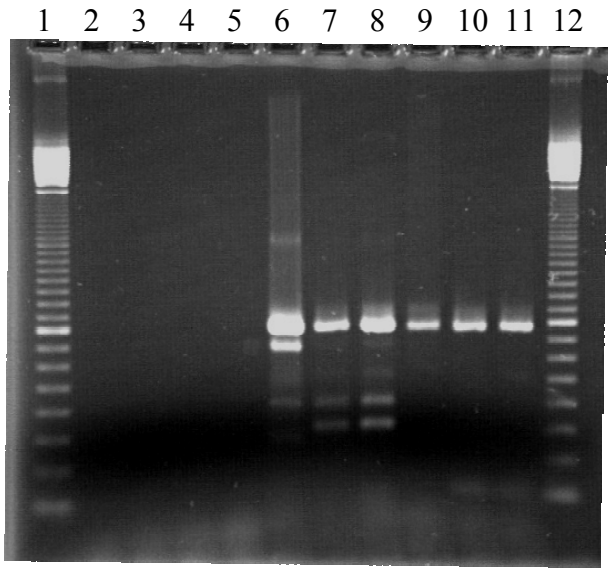

Figure 4. Agarosegel with PCR-products obtained with LevV-LevR primers and DNA from EPS-positive and EPS-negative lactobacilli as template. Lane 1 and 12: 100 bp standard. lanes 2 to 5: PCR with non-fructan-forming strains. Lanes 6 to 11: fructan-forming lactobacilli; lane 6, 7, 8, and 9: *L. sanfranciscensis* TMW1.53, TMW1.953, TMW 1.896, and TMW1.392; lanes 10 and 11: *L. frumenti* TMW1.669 and TMW1.103.

Primers were constructed targeting conservative regions of known bacterial levansucrases and a PCR was carried out.

In Figure 4 the amplification products are shown which could detected from 6 of the 15 fructan-forming strains. One major PCR product was observed in 6 of the 15 fructan-forming strains. The size of 800 bp corresponds to length of the amplicon calculated from known sequences of bacterial levansucrases, 700 to 900 bp. No PCR product was detected if DNA from fructan-negative strains was used as template (Figure 4 and data not shown).

3.1.3 Comparison of the sequence of levV-levR PCR-products with other bacterial levansucrases

The amplification products from *L. frumenti* TMW 1.103 and TMW 1.669 and from *L. sanfranciscensis* TMW 1.53, TMW 1.392, TMW 1.896 and TMW 1.953 were purified and sequenced. Based on searches using the EMBL protein- and nucleotide databases, the amplicons were identified as partial sequences of levansucrase genes. The deduced amino acid sequences of genes amplified from *L. sanfranciscensis* TMW 1.53 and *L. frumenti* TMW 1.103 were compared to known sequences of *L. reuteri*, *S. mutans* and *S. salivarius* (Figure 5). The partial sequences of *L. sanfranciscensis* TMW 1.392, TMW 1.896 and TMW 1.953 were identical to that one of *L. sanfranciscensis* TMW 1.53, and the partial sequences of *L. frumenti* TMW1.669 was identical to that one of *L. frumenti* TMW 1.103 (data not shown).

A

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                          10         20         30         40         50
INULO.AMI          1 ------PVQD VRTGQVANWN GYQLVIAMMG IPNQNDN--H IYLLYNKYGD
LEVREU.AMI         1 ------PVQD PVTGYVSNYM GYQLVIAMMG IPNSPTGDNH IYLLYNKYGD
SEQ103.AMI         1 ---------- ---------- --------MG IPNTPYGDNH IYLLYNKYGD
SEQ53.AMI          1 ---------- -VTGYVSNYK GYQLVIAMMG IPNTPNGDNH IYLLYNKYGD
SSALIV.AMI         1 DVWDSWPVQD AKSGVVSNWN GYQLVISMAG APNK--NSNH IYLLYRKYGD
SMUTANS.AMI        1 ---------- ---------- ---------- --NTNDN--H IYLLYNKYGD
                          60         70         80         90        100
INULO.AMI         51 NELSHWKNVG PIFGYNSTAV SQEWSGSAVL NSDNSIQLFY TRVDTSDNNT
LEVREU.AMI        51 NDFSHWRNAG SIFGTKETNV FQEWSGSAIV NDDGTIQLFF TSNDTSDYKL
SEQ103.AMI        51 NDFSHWRNAG SIFGNKENNV YQEWSGSAIV NNDGSIQLFY TSNDTSDFKL
SEQ53.AMI         51 NDFSHWRNAG SIFGTNENNV YQEWSGSAIV NDNGTIQLFY TSNDTSDYKL
SSALIV.AMI        51 NDFTHWKNAG PIFGYNALED DQQWSGSATV NSDGSIQLYY TKNDTSGGKL
SMUTANS.AMI       51 NNFDHWKNAG SIFGYNETPL TQEWSGSATV NEDGSLQLFY TKVDTSDKNS
                         110        120        130        140        150
INULO.AMI        101 NHQKIASATL YLTDNNGNVS LAQVANDHIV FEGD--GYYY QTYDQWKATN
LEVREU.AMI       101 NDQRLATATL NLNVDDNGVS IKSVDNYQVL FEGD--GFHY QTYEQFA---
SEQ103.AMI       101 NDQKLATATL HLNVDDNGVS IASVDNNHVI FEGD--GYHY QTYQQFA---
SEQ53.AMI        101 NDQRLATATL NLDVDDNGVA IKSVDNYHIL FEGD--GFHY QTYDQFA---
SSALIV.AMI       101 NWQQLASATL NLAVENDEVV IKSVENDHIL FGGD--NYHY QSYPKFMSTF
SMUTANS.AMI      101 NNQRLATATV NLGFDDQDVR ILSVENDKVL TPEGVMAYHY QSYQQWRSTF
                         160        170        180        190        200
INULO.AMI        151 KG-------- --ADNIAMRD AHVIEDDNGD RYLVFEASTG LENYQGEDQI
LEVREU.AMI       151 ------NGKD RENDDYCLRD PHVVQLENGD RYLVFEANTG TEDYQSDDQI
SEQ103.AMI       151 ------EGKD RKDDDYCLRD PHVVQLTNGD RYLVFEANTG KEDYQSDEQI
SEQ53.AMI        151 ------NGKD RKNDDYCLRD PHVVQSENGD RYLVFEANTG MEDYQSDDQI
SSALIV.AMI       151 DDDHNHDGNP DRTDNYCLRD PHIIEDN-GS RYLIFESNTG DENYQGEKQI
SMUTANS.AMI      151 TG-------- --ADNIAMRD PHVIED NGD RYLVFEASTG TENYQGEDQI
                         210        220        230        240        250
INULO.AMI        201 YNWLNYGGDD AFNIKSLFRI LSNDDIKSRA TWANAAIGIL KLNKDEKNPK
LEVREU.AMI       201 YNWANYGGDD AFNIKSSFKL LNNKKDRELA GLANGALGIL KLTNNQSKPK
SEQ103.AMI       201 YNWANYGGDD AFNIKSFFKL LNNKKDRELA GLANGSLGIL KLNNNQDNPE
SEQ53.AMI        201 YNWANYGGDD AFNIKSFFKL LNNKNDRELA SLANGAIGIL KLNNDQTNPK
SSALIV.AMI       201 YKWSNYGGDD AFNLKSFLNI VNNKHLYNLA SWANGSIGIL KLDDNEKNPS
SMUTANS.AMI      201 YNFTNYGGSS AYNVKSLFRF LDDQDMYNRA SWANAAIGIL KLKGDKKTPE
                         260        270        280        290        300
INULO.AMI        251 VAELYSPLIS APMVSDEI-- .......... .......... ..........
LEVREU.AMI       251 VEEVYSPLVS TLMACDEV-- .......... .......... ..........
SEQ103.AMI       251 VDEVYSPLVS TLMA------ .......... .......... ..........
SEQ53.AMI        251 VEEVYSPLVS TLM------- .......... .......... ..........
SSALIV.AMI       251 VAELYTPLVT SHMVTDEVER .......... .......... ..........
SMUTANS.AMI      251 VDQFYTPLLS STMVS----- .......... .......... ..........
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B

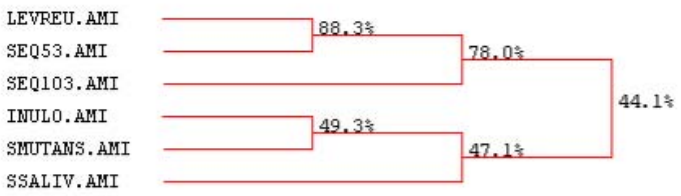


Figure 5. Partial levansucrase-sequences of *L. sanfranciscensis* TMW 1.53 (Seq 53) and *L. frumenti* TMW1.103 (Seq 103) (Panel A). Multiple alignment with the partial levansucrase-sequences from *L. reuteri, S. mutans* and *S. salivarius* and two fructansucrases from *L. reuteri* (LevReu, 103, Inulo, 106) leads to the genealogical tree shown in panel B. The partial sequences of *L. sanfranciscensis* LTH2590, TMW 1.896 and TMW 1.953 were identical to that of *L. sanfranciscensis* TMW 1.53, and the partial sequences of *L. frumenti* TMW1.669 was identical to that of *L. frumenti* TMW 1.103 (data not shown).

By comparison of the amino acid sequences of the levansucrase-fragments, *L. sanfranciscensis* levansucrase revealed an identity of 88.3 % with the gene fragment of *L. reuteri*, 78 % with that from *L. frumenti* and 44.1 % with fragments of *S. salivarius* and *S. mutans*. The corresponding partial sequences of levansucrases from *Bacillus amyloliquefaciens* and *B. stearothermophilus* are only 15.8 % identical to those from *Lactobacillus* and *Streptococcus* (data not shown).

Using DNA from *L. sanfranciscensis* TMW1.53 as template for the lev-PCR, additionally a minor band with lower molecular weight was detected. Sequencing of this PCR-product and comparison with the EMBL nucleotide database did not reveal significant homologies to levansucrases or any other documented DNA sequences.

3.1.4 Expression of the levansucrase gene in *L. sanfranciscensis*

To determine whether the putative levansucrase genes encompassing the 800 bp LevV – LevR PCR product are expressed in *L. sanfranciscensis*, PCR targeting an internal sequence of the LevV – LevR PCR product was carried out using cDNA of *L. sanfranciscensis* TMW 1.392 and TMW 1.53. RNA was isolated from cultures growing in the presence or absence of sucrose. In Figure 6 are shown the amplification products obtained from chromosomal DNA from either strains, and from cDNA libraries of either strain growing on mMRS-maltose or

mMRS-sucrose. The PCR products had the expected size of 120 bp. Expression of the putative levansucrase gene was observed in either strain growing on maltose or sucrose as carbon source.

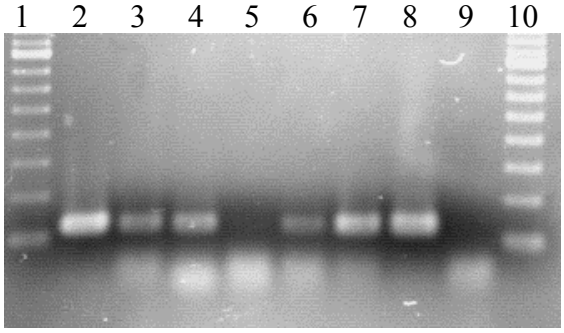


Figure 6. Agarosegel with PCR-products obtained with Lev3V – LevshortR primers using chromosomal DNA or cDNA libraries from EPS-positive *L. sanfranciscensis* strains as template. It was ensured by Lev3V-LevshortR PCR that RNA preparations used as template for the RT-PCR were free of DNA. Strains were grown on mMRS-maltose or mMRS-sucrose. Lanes 1 and 10: molecular weight standard, lanes 5 and 9: negative control (no template). Lanes 2 – 4: TMW 1.392, chromosomal DNA (lane 2), cDNA from maltose grown cells (lane 3)or sucrose grown cells (lane 4). Lanes 6 – 8: TMW 1.53. chromosomal DNA (lane 6), cDNA from maltose grown cells (lane 7)or sucrose grown cells (lane 8).

3.1.5 *In situ* EPS-formation in sourdough

Experiments concerning EPS screening in sourdough fermentation were kindly performed by Dr. Maher Korakli from the Lehrstuhl für Technische Mikrobiologie, Freising, Germany. Sourdough fermentations were carried out using 5 strains producing fructan and one strain producing glucan in order to compare the EPS-formation in sourdough with the results obtained in vitro. Sourdough fermentation and analysis of the dough on the presence of EPS was carried out as described. Dough fermented with the EPS-negative strain *L.*

*sanfranciscensi*s LTH2581, and dough in which microbial growth and metabolism was inhibited by antibiotics served as negative controls. Table 2 shows the monosaccharide composition of water soluble polysaccharides extracted from the sourdoughs. Low amounts of fructose were found in water-soluble polysaccharides (WS-PS) from control doughs fermented with EPS-negative *L. sanfranciscensis* or aseptic fermented doughs. More than tenfold higher fructose levels in WS-PS were found in all doughs fermented with fructan-producing strains, indicating fructan formation by these strains during sourdough fermentations. Dough fermented with *L. sanfranciscensis* LTH 2581 or fructan-forming strains contained less than 1 mMol kg^{-1} glucose in WS-PS. In the control dough with antibiotics a higher content of glucose in WS-PS was observed because in this dough amylases were not inhibited by acidification, resulting in generation of water-soluble polysaccharides from insoluble starch during fermentation. A significantly higher content of glucose was observed in dough fermented with the glucan-forming strain *L. reuteri* TMW1.106, indicating glucan formation from sucrose by this strain. The amounts of high-molecular weight fructan and / or glucan can be estimated to range between 0.5 g / kg flour (*L. frumenti* TMW 1.103) and 2 g / kg flour (*L. sanfranciscensis* TMW 1.392). An increase of arabinose and xylose levels in water soluble polysaccharides indicated a solubilization of arabinoxylans during fermentation in all doughs. The content of arabinoxylans in WS-PS was not appreciably affected by fermentation of EPS-positive or EPS-negative lactobacilli when compared to the aseptic control dough.

Table 3. Characterization of wheat sourdoughs fermented with EPS-positive and EPS-negative lactobacilli, and monosaccharide composition of water-soluble polysaccharides extracted from these doughs. Experimental data were kindly provided by Dr. Maher Korakli from the Lehrstuhl für Technische Mikrobiologie, Freising, Germany

Strain used for sourdough fermentation		**pH**	**cell count log(cfu g^{-1})**	**glucose**	**xylose**	**arabinose**	**fructose**
				[mmol kg^{-1}]			
All doughs	t=0h	6.07±0.03	6.80±0.80[a)]	0.70±0.04	4.85±0.05	4.78±0.10	0.10±0.01
Control dough (with antibiotics)	t=24h	6.05±0.16	3.98±0.28	2.87±0.33	15.97±0.72	12.90±1.03	0.13±0.02
***L. sanfranciscensis* LTH2581**	t=24h	3.49±0.23	9.11±0.21	0.37±0.20	15.80±0.55	11.05±0.67	0.18±0.09
***L. sanfranciscensis* LTH2590**	t=24h	3.79±0.07	8.86±0.18	0.44±0.23	12.37±0.05	10.04±0.21	6.08±1.00[b)]
***L. frumenti* TMW 1.103**	t=24h	3.42±0.14	9.57±0.09	0.81±0.30	12.70±0.08	12.05±1.24	1.11±0.30[b)]
***L. frumenti* TMW 1.660**	t=24h	3.47±0.04	8.63±0.37	0.76±0.51	14.49±1.11	13.60±0.46	3.28±0.00[b)]
***L. pontis* TMW 1.675**	t=24h	3.40±0.07	9.10±0.16	0.64±0.33	13.04±0.23	12.06±1.19	1.39±0.09[b)]
***L. frumenti* TMW 1.669**	t=24h	3.44±0.04	9.36±0.13	1.49±0.50	13.87±1.18	12.84±0.30	2.33±0.11[b)]
***L. reuteri* TMW 1.106**	t=24h	3.52±0.06	9.38±0.23	3.58[b)]±0.89	13.12±1.05	12.95±0.74	0.57±0.03

Data are means ± standard deviations of two independent experiments. The aseptic control dough and sourdoughs inoculated with *L. sanfranciscensis* LTH2581 and LTH2590 were incubated at 30 °C, other doughs at 37 °C.

[a)] The cell counts in control dough at t = 0h was 2.50 ± 0.50.

[b)] The amount of high molecular weight fructan and / or glucan can be estimated by comparison of fructose or glucose levels in water soluble polysaccharided from control doughs (EPS negative strain and aseptic control) compared to levels of fructose or glucose levels in water soluble polysaccharides from doughs fermented with EPS-positive strains: LTH 2590: 2 g fructan / kg flour; TMW 1.103: 0.3 g fructan / kg flour; TMW 1.660: 1.0 g fructan / kg flour; TMW 1.675: 0.4 g fructan / kg flour; 0.7 g fructan / kg flour; 1.0 g / kg flour. [c)]

3.2 Molecular and functional characterization of a levansucrase from *Lactobacillus sanfranciscensis*

The screening of cereal associated lactobacilli revealed that approximately 20 % of the screened organisms are capable to produce extracellular HoPS. In other bacteria, e.g. of the species Bacillus, Streptococcus and Leuconostoc, it is known that glucans and fructans are formed from sucrose by the activity of a single enzyme, i.e. glucan- or fructansucrases. A 800 bp sequence could be amplified in this work from fructan-forming lactobacilli by PCR targeting conserved domains of levansucrases of lactic acid bacteria. Database searches revealed a high similarity with known levansucrase genes. These levansucrase genes were detectable in 6 of the 15 fructan-forming strains and expression of these gene could be verified in *L. sanfranciscensis*. Taken together, these results suggest that a levansucrase is responsible for fructan-formation in *L. sanfranciscensis* strains. *L. sanfranciscensis* belongs to the predominant organisms in sourdough fermentations, and so the genetic determinants of the levan production of a selected *L. sanfranciscensis* strain was investigated in more detail.

3.2.1 Nucleotide sequence analysis of the *L. sanfranciscensis* TMW1.392 levansucrase gene

A total sequence of 3623 bp was obtained after several rounds of inverse PCR based on the partial sequence of the *L. sanfranciscensis* levansucrase described above and sequencing of the PCR products (Figure 1). One complete open reading frame (ORF1) encoding a putative levansucrase gene (2637 bp, starting at position 705) and a partial ORF with homology to an IS 153-element of *L. sanfranciscensis* (23, EMBL AJ239042) were located on this DNA segment (ORF2, Figure 1). Two putative start codons were identified in ORF1: (i) an ATG codon located at position 705 and (ii) an ATG codon located at position 726. An imperfect Shine-Dalgarno sequence (AAGGAA) 9 bp upstream from the ATG codon at position 705 was identified. Corresponding putative –10 and –35 sequences could be found at positions

664 and 641 (TATAAT and ATGTTA), revealing 100 % and 50 % identity with the –10 TATAAT and –35 TTGACA consensus sequences proposed for lactobacilli (61, 80). Furthermore, the resulting N-terminal amino acid (aa)-sequence has high homology to the N-terminal aa-residues of a levansucrase and an inulosucrase from *L. reuteri* LB121 (EMBL accession numbers AF465251 and AF459437) and matches the criteria of an export-signal (see Figure 2 and explanation downwards). Therefore, the ATG codon at the nucleotide-position 705 was considered as the start codon for the levansucrase gene in *L. sanfranciscensis*. A stop codon occurs at position 3342, followed by a putative termination signal at position 3394 with a pallindromic sequence (CTG AAG AAT ATT ATT ATT CTT CAG) indicating that the levansucrase gene is expressed as monocistronic mRNA.

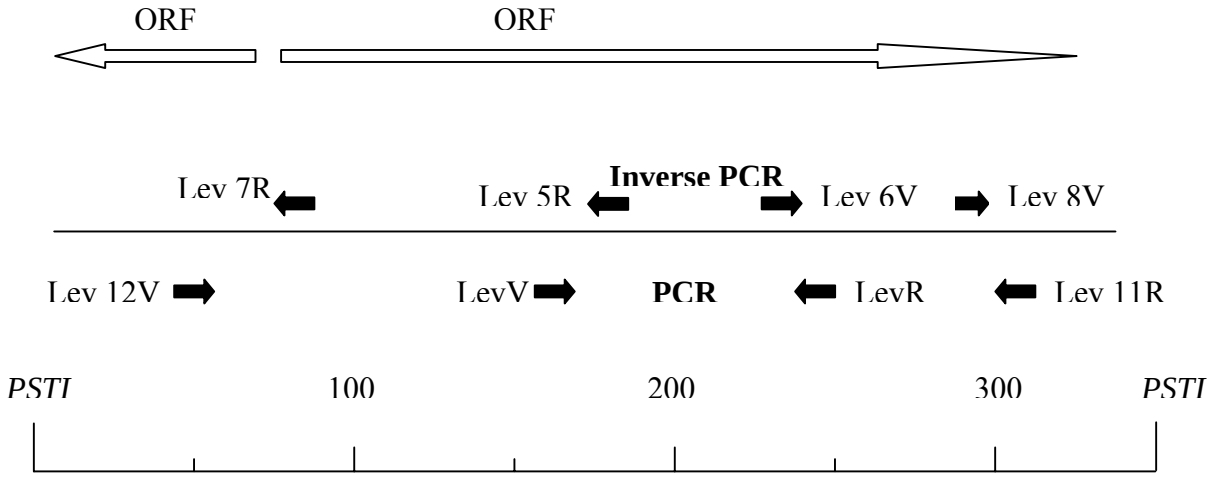


Figure 7. Strategy for the sequencing of the levansucrase-gene from *L. sanfranciscensis* TMW 1.392 by using inverse PCR.

3.2.2 Analysis of the amino acid (aa)sequence and alignments with bacterial levansucrases

Starting at nucleotide-position 705, ORF2 revealed a 879 aa protein with a predicted relative molecular weight M_R of 94930 and a predicted pI of 4.69. Blast searches showed highest similarities with a levansucrase from *L. reuteri* (76 % identity and 82 % similarity in 773

amino acids), an inulosucrase from *L. reuteri* (53 % identity and 68 % similarity in 757 amino acids) and a fructosyltransferase from *Streptococcus salivarius* (EMBL L08445; 46 % identities and 61 % similarity in 789 amino acids).

Four regions can be designated in the amino acid sequence. (**i**) a N-terminal leader peptide up to aa residue 37; (**ii**) a domain containing 7 x 16 direct repeats between aa residues 99 and 211; (**iii**) a highly conserved region of 541 aa residues containing the catalytic domain and (**iv**) a C-terminal cell wall anchor (Figure 8). The N-terminal sequence contains 37 amino acids that match all criteria of N-terminal signal peptides from secreted proteins as known from Gram-positive bacteria, consisting of a charged N-region from aa residue 1 to approximately 13, a hydrophobic H-region from residue 14 to approximately 32, and a C-region between position 33 and 37 with a putative cleavage site (Figure 8, 36). The SignalP-HMM prediction method according to Nielsen and Krogh (71) identified this N-terminal region as signal peptide with a probability of 98,6 % and predicted a cleavage site between positions 35 and 36.

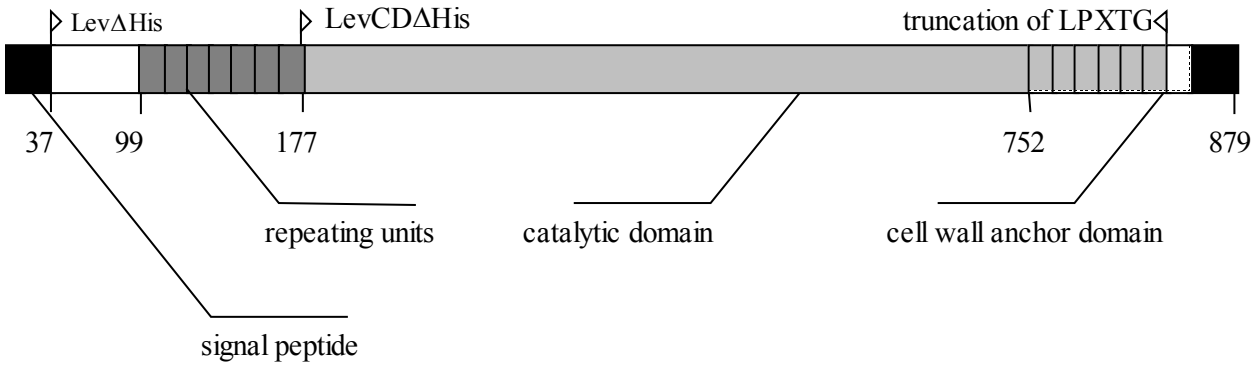


Figure 8. Schematic structure of the levansucrase from *L. sanfranciscensis* LTH2590. Four characteristic protein domains identified by BLAST searches are indicated: **i,** signal peptide for export, **ii,** repeating units (7 x 16 aa), **iii**, catalytic domain, and **iv**, cell wall anchor domain. The heterologous expressed proteins LevΔHis and LevCDΔHis with the truncation of the C-terminal cell wall anchor are indicated by small arrows in the drawing.

The signal peptide is followed by a domain containing 7 x 16 direct repeats (DNATSGSTKQESSIAN) that have no homology with any known fructosyltransferase. Blast searches revealed similarities but low homologies with repeats from other surface-located or secreted proteins from a wide range of organisms, for instance from *Anaplasma marginale* (EMBL AY010244) or from *Enterococcus hirae* (EMBL AF167576).

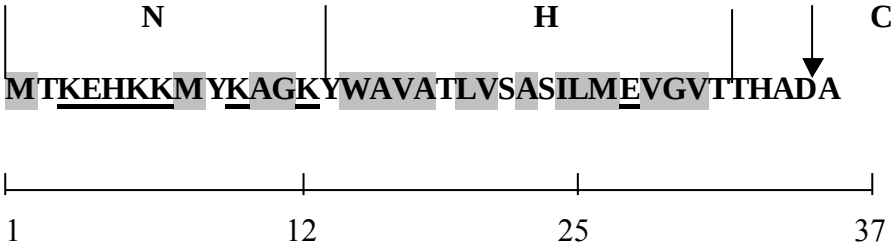
Domain (**iii**) belongs to the glycoside hydrolase family 68 of the levansucrases and invertases, containing aa residues that have been described as highly conserved in bacterial levansucrases (http//:pfam.wustl.edu). The RDP-motif at position 464 has been reported to play a role in catalysis (94), furthermore, a 20 aa-residue long "sucrose-box" from position 381 to 400 is highly conserved in bacterial fructosyltransferases as well as in invertase enzymes (70, 78).

Blast searches (http//:pfam.wustl.edu) revealed that region (**iv**) harbours a cell wall anchor with a stretch of 6 x 12 direct repeats (PVNPSQPTTPAT) containing the motif PXX and a LPXTG motif followed by a range of hydrophobic amino acids (Figure 9). Repeats containing the PXX motif are common to cell wall anchor domains of cell wall bound proteins in Gram-positive bacteria. The LPXTG motif is conserved within the sorting signal of all known cell-wall anchored proteins of Gram-positive bacteria (69). The threonyl residue of this motif is covalently linked to the murein interpeptide after proteolytical cleavage between the glycine and threonine residues.

3.2.3 Insertional inactivation of the levansucrase gene and sucrose metabolism by the levansucrase deficient strain

L. sanfranciscensis TMW 1.392 was transformed with the non-replicating plasmid pME-1_knock. Sequencing of the disrupted levansucrase gene ensured that a single crossover integration of pME-1_knock into the chromosomal levansucrase gene of TMW 1.392Δlev has taken place (Figure 10). The strain TMW 1.392Δlev grew on mMRS-sucrose plates with erythromycin and a smooth colony morphology was observed, whereas *L. sanfranciscensis*

A



B

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Lb.sanf_Levsuc    PHQPVNPSQPTTPATPVNPSQPTTPATPVNPSQPTTPATPVNPSATTTPATPVNPSATTT 809
Lb.reut_Inulsuc   ---------PHDPATPNDPETPTTPETPETPNTPKTPKTPENPGTPQTPNTPN------T 743
Lb.reut_Levsuc    PHQPVQPKIDQPDQQPSG--QNTKNVTPGNGDKPAGKATPDNTNIDPSAQPSGQNTNIDP 745
                                  *  .    *.  ** . . *    ** *..   :. ..       .

Lb.sanf_Levsuc    PAKPVNPSQPTTPAKPVQAGQATATNFVDQRLPQTGENNSQSQTMSFIGILLAMFGSLLG 869
Lb.reut_Inulsuc   PEIPLTPETPKQPE--------TQTN---NRLPQTGNNANKAMIGLGMGTLLSMFG---- 788
Lb.reut_Levsuc    SAQPSGQNTKNVTP----GNEKQGKNTDAKQLPQTGNKS--GLAGLYAGSLLALFG---- 795
                  .  *   .  . .          .*   ::*****::   .     * **::**

Lb.sanf_Levsuc    FLGIKKRRND 879
Lb.reut_Inulsuc   LAEINKRRFN 798
Lb.reut_Levsuc    LAAIEKRHA- 804
                  :  *:**:
```

Figure 9. Amino acid sequence of the signal peptide (panel A) and the cell wall anchor domain (panel B) of the levansucrase of *L. sanfranciscensis*. Panel A: The N-region is rich in positively charged amino acids, the hydrophobic H-region and the C-region with the putative cleavage site were assgined based on the SignalP-HMM output as described by Nielsen and Krogh (1998). Positively charged residues are underlined, hydrophobic amino acids are shaded grey, and the predicted cleavage site of the leader peptide is indicated by an arrow. Panel B: Alignment of the amino acid sequence of the C-terminal cell wall-anchor domain of the *L. sanfranciscensis* levansucrase with the corresponding sequences from the levansucrase of *L. reuteri*, and an inulosucrase from *L. reuteri* (EMBL accession numbers AF465251 and AF459437). Fully conserved residues are indicated by an asterisk, fully conserved stronger groups are indicated by an colon, and fully conserved weaker groups by a period. The proline residues in the cell wall-anchor region are printed in bold letters and the hydrophobic stretch is shaded grey. An arrow indicates the putative cleavage site in the LPXTG-motif conserved in cell-wall anchor domains of Gram-positive bacteria (underlined).

TMW 1.392 showed a slimy colony-form on mMRS-sucrose due to levan-formation (Figure 11). The ability of the wild type and mutant strain to metabolise sucrose was determined by HPLC analysis of cell-free supernatants from cultures grown on mMRS-sucrose to stationary growth phase (pH 3.5, optical density 1.4 at 590 nm).

The wild type strain, *L. sanfranciscensis* TMW 1.392, metabolised sucrose with formation of acetate, mannitol, glucose, kestose and levan (Table 2). In contrast, *L. sanfranciscensis* TMW

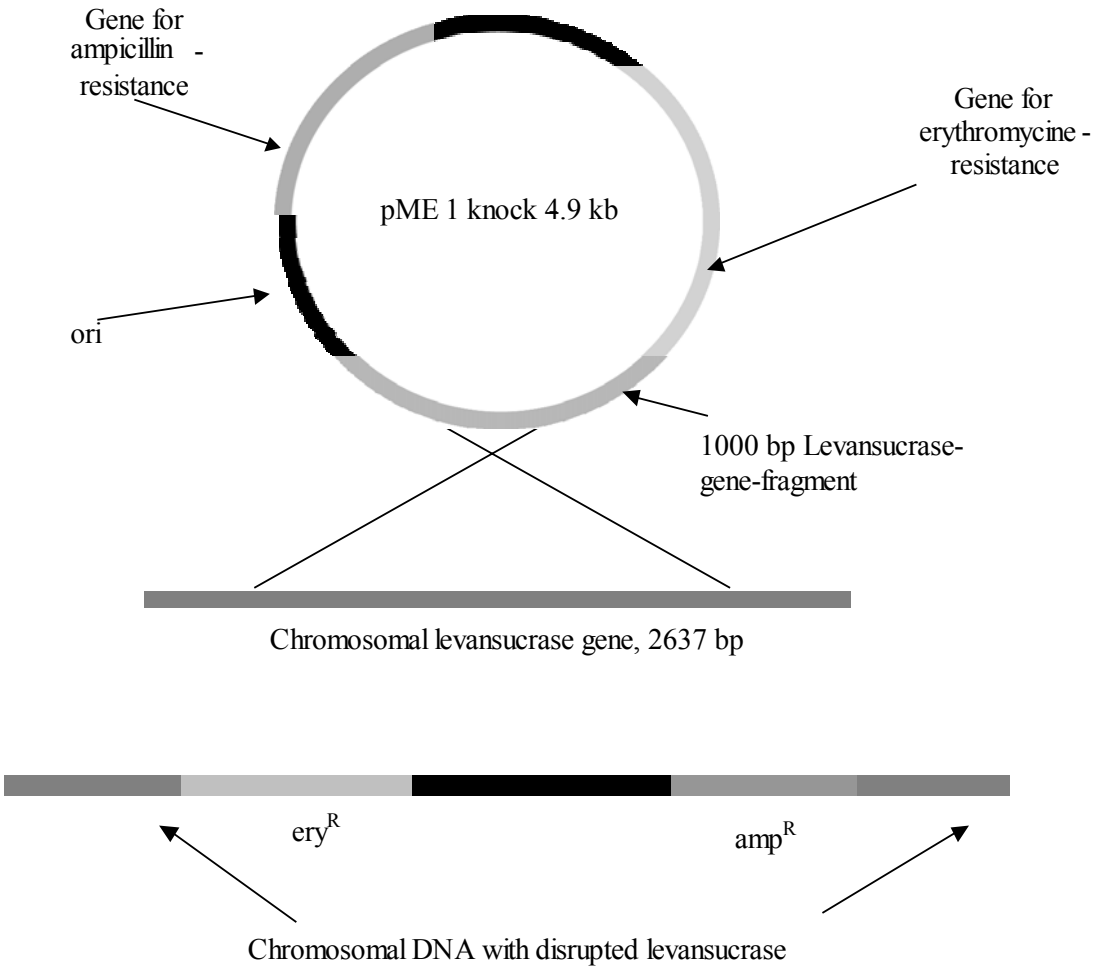

Figure 10. Schematic illustration of the levansucrase gene-disruption by integration of the non-replicating plasmid pME1_knock with homologous recombination

1.392Δlev did not metabolise sucrose. Accordingly, glucose was not liberated from sucrose, and no mannitol, acetate, kestose and levan was observed in the supernatants. These results strongly indicate that levansucrase is the only enzyme capable of sucrose-hydrolysis in this strain of *L. sanfranciscensis*.

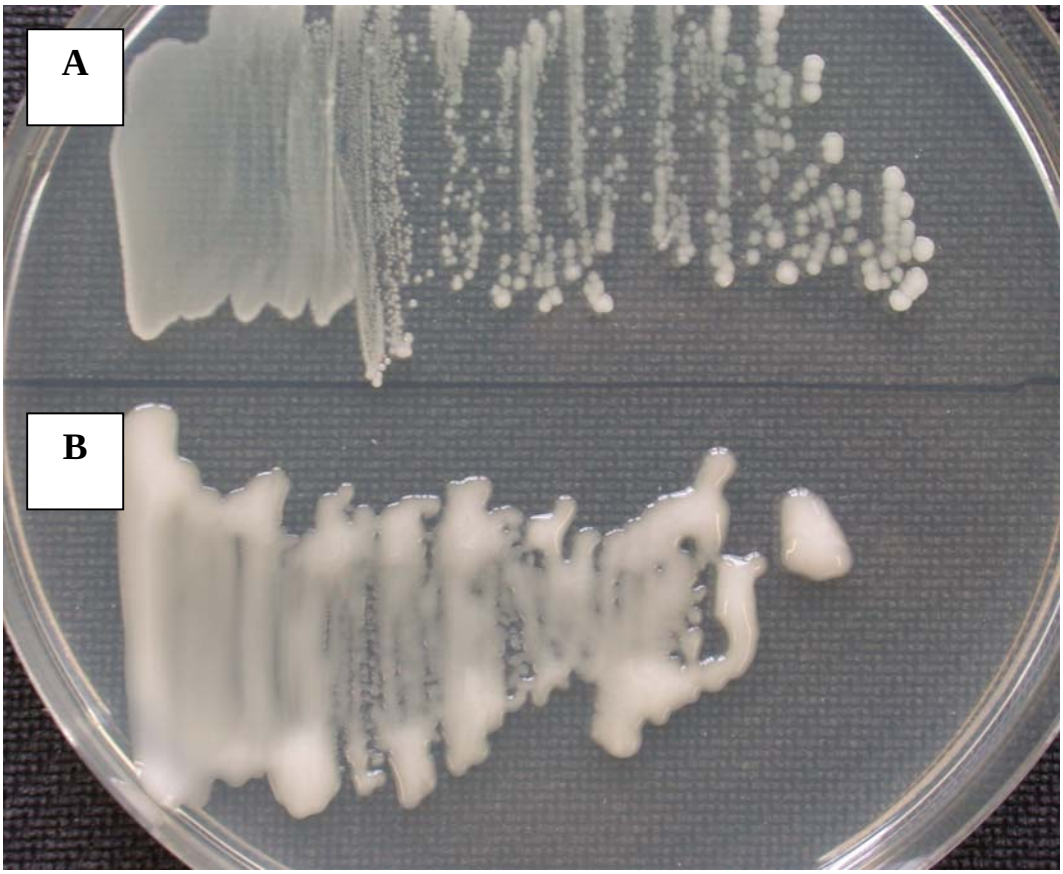

Figure 11. Colony morphology of *L. sanfranciscensis* TMW 1.392Δlev (panel A) and TMW 1.392 (panel B) on mMRS-suc. The wild-type strain showed a slimy colony form due to levan-formation. In contrast, the mutant strain exhibited no EPS formation.

3.2.4 Dough fermentations with the wild type strain and the levansucrase deficient strain.

Both strains were grown in wheat doughs with and without 9 % (w/w) sucrose for 24 hours. The resulting pH values were 3.5 in all doughs and the obtained cell counts were in a range between 4×10^7 and 3×10^8 cfu. Figure 12 demonstrates that of both strains the growth rates in sourdough were comparable. To demonstrate the correlation between levansucrase-

expression and fermentation patterns in sucrose containing doughs, mRNA was isolated from dough fermentations with the mutant and the wild type strain at a pH of 4.5 (exponential growth phase). After RT-PCR, PCR was performed using primers that were targeting the levansucrase gene and the house-keeping gene phosphotransacetylase (43). The levansucrase gene is expressed in dough by the wild type strain but not by the deletion mutant TMW1.392Δlev (Figure 13).

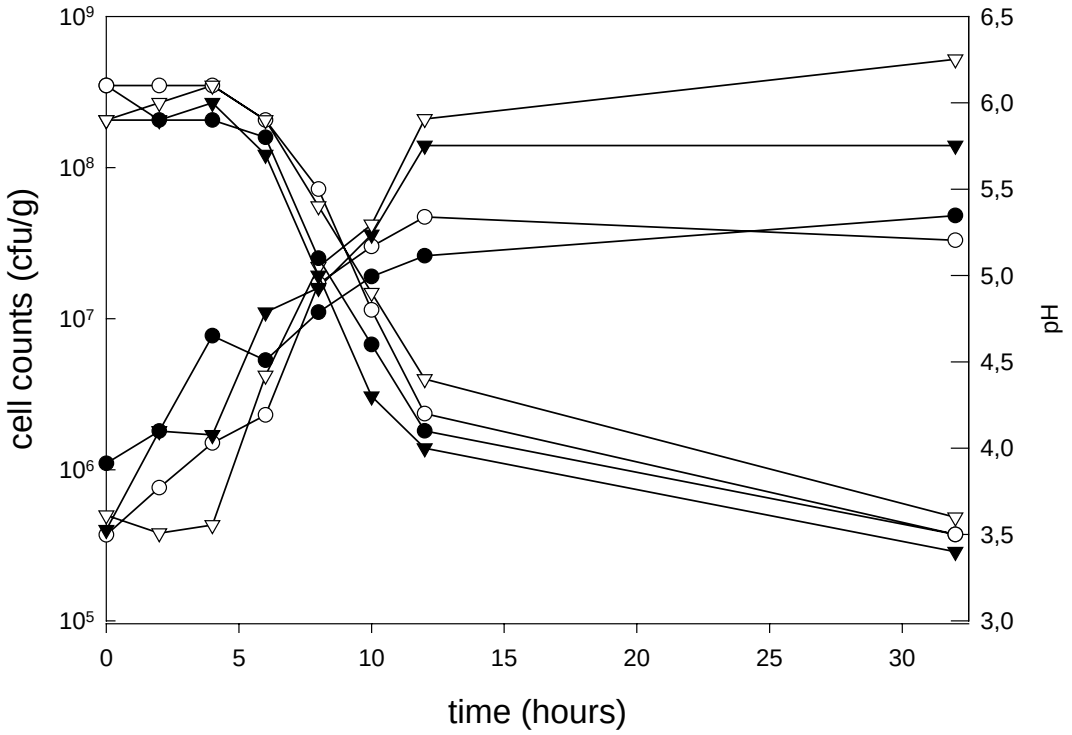

Figure 12. Growth rates and pH values of the mutant strain *L. sanfranciscensis* TMW 1.392Δlev (black symbols) and the wild strain TMW 1.392 (open symbols) in doughs with (▼) and without sucrose (●).

The level of metabolites in dough without added sucrose did not differ appreciably between strains TMW 1.392 and TMW 1.392 Δlev. This indicates that the flour invertase activity suffices to hydrolyse the sucrose levels naturally present in dough (0.3-0.4 %). In doughs where sucrose was added, TMW1.392 formed less lactate, and the levels of acetate and

mannitol were strongly increased compared to doughs without sucrose. Additionally, 21.8 mMol x kg^{-1} dough kestose and 2.0 g x kg^{-1} dough fructan were formed, corresponding to12.6 mMol x kg^{-1} fructose moieties in the polymer, and glucose accumulated to large concentrations. In doughs fermented with the mutant strain, acetate and mannitol levels were only slightly enhanced as a result of flour invertase activity, kestose and EPS were not formed, and only minor levels of glucose accumulated based on flour enzymatic activities. (Table 4)

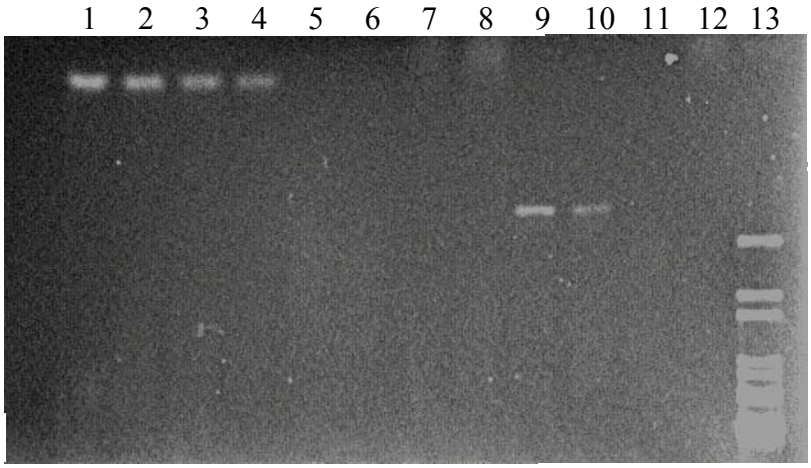

Figure 13. Agarosegel with PCR-products obtained with Lev8V/LevH primers and Pta V/R primers using cDNA libraries from *L. sanfranciscensis* TMW 1.392 and TMW 1.392Δlev strains as template. Primers Lev8V/ LevH were targeting the levansucrase gene, whereas PCR with primers Pta V/R was targeting the phosphotransacetylase gene of *L. sanfranciscensis* and provided a positive control for successful isolation and transcription of RNA. Amplification products had the expected sizes of 450 bp for the lev-primer and of 150 bp for the Pta primer. Strains were grown on doughs with 9% sucrose to a pH of 4.5. It was ensured by PCR that RNA preparations used as template for the RT-PCR were free of DNA. Lane 1 to 6: PCR with primers Pta V/R. Lane 1 and 2: cDNA from TMW 1.392Δlev, lanes 3 and 4: cDNA from TMW1.392, lanes 5 and 6: PCR with RNA of both strains as template (negative control). Lanes 7 – 12: PCR with primers Lev8V/LevH primers. Lanes 7 and 8: cDNA from TMW1.392Δlev, lanes 9 and 10: cDNA from TMW1.392, lanes 11 and 12: PCR with RNA of both strains as template (negative control). Lane 13: molecular weight standard.

Table 4.

	Wild type strain in mMRS-malsuc [mmol x L^{-1}]	Mutant strain in mMRS-malsuc [mmol x L^{-1}]	Wild type strain in dough with 9 % sucrose [mmol x kg^{-1}]	Wild type in dough [mmol x kg^{-1}]	Mutant strain in dough with 9 % sucrose [mmol x kg^{-1}]	Mutant strain in dough [mmol x kg^{-1}]
lactate	97.0	70.0	75.1 ± 0.3	102.0 ± 1.8	91.0 ± 2.9	95.3 ± 9.0
acetate	9.3	0.0	42.4 ± 1.5	8.2 ± 1.4	13.5 ± 0.1	7.1 ± 1.4
ethanol	83.0	63.0	25.2 ± 2.0	84.2 ± 1.8	69.6 ±0.8	80.0 ± 6.0
kestose	2.0	0.0	20.8 ± 0.3	0.0	0.0	0.0
glucose	18.8	4.4	175.6 ± 2.0	34.0 ± 2.7	27.7 ± 1.2	23.1 ± 2.6
fructose	0.0	0.0	26.9 ± 2.0	0.0	0.0	0.0
mannitol	18.1	0.0	74.6 ± 3.4	10.8 ± 0.0	21.1 ±1.4	9.1 ± 1.5
fructan[a]	3.8	0.0	12.6 ± 0.8	< 1	< 1	< 1

Data concerning dough analytics are means ± standard deviations of two measurements and are representing one of four fermentations with consistent results.
a: fructose moieties in fructan

3.2.5 Heterologous expression and biochemical characterization of the recombinant enzymes.

Two versions of the levansucrase of *L. sanfranciscensis* TMW 1.392 were heterologously expressed in *E. coli*: (i) LevΔHis, starting at aa-position 36 with a truncation of the N-terminal leader peptide at the predicted cleavage-site. (ii) LevCDΔHis starting at aa-position 177 with a truncation of the region encoding the 7 x 16 aa repeats. The direct repeats were truncated to investigate whether they affect enzyme activity or the pattern of products formed. A truncation downwards of the C-terminal cell-wall-anchoring LPXTG-motif was made in both enzymes in accordance to the predicted cleavage site of the native enzyme in *L. sanfranciscensis*. Both recombinant proteins could be purified to homogeneity by affinity chromatography. The active fractions contained a single band after separation of proteins by SDS PAGE (Figure 12). The protein LevΔhis had an apparent M_R of 105 k, which is about 20 % percent larger than the predicted M_R of 87 k. Sequencing of the plasmid construct revealed a correct size of the insert.

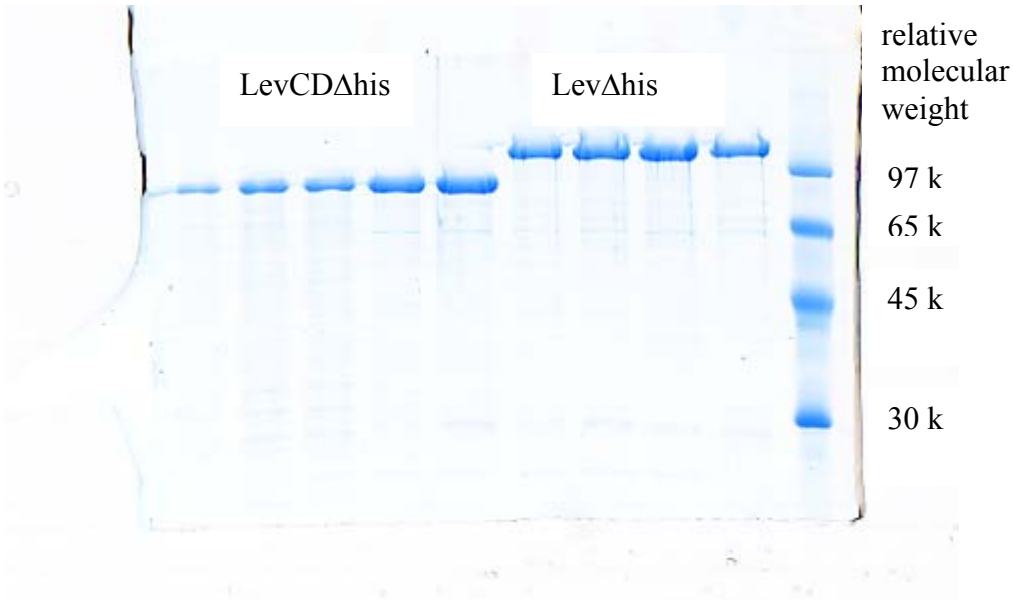

Figure 14. SDS-page with active fractions of the purified, heterologously expressed enzymes LevCDΔhis and LevΔhis. It is apparent from the molecular weight standard that LevΔhis is exceeding the expected size of 87 k

Large differences between the apparent M_R as judged by SDS-PAGE and the M_R derived from mass spectrometry analysis and / or DNA-sequence data was also observed for the native and his-tagged fructosyltransferases from *L. reuteri* (103, 107), and a fructosyltransferase from *S. salivarius* (94). The M_R of LevCDΔhis derived from SDS-PAGE analysis was 75 k, which is consistent with the expected size of 73 k, and indicates that the unusual electrophoretic mobility is caused by the N-terminal domain of the enzyme. The recombinant enzymes both exhibited hydrolase activities as well as transferase activities as outlined below.

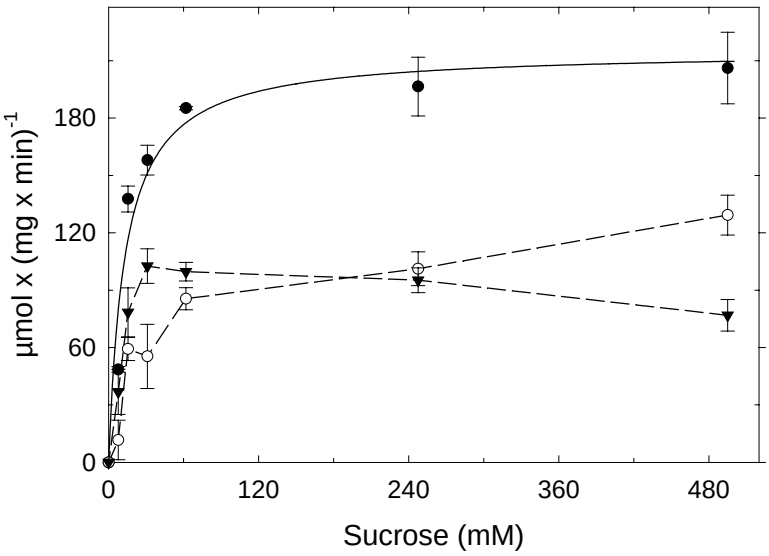

Figure 15. Michaelis-Menten plot of the levansucrase of *L. sanfranciscensis* which was purified after heterologuous expression in *E. coli* (levΔHis). Shown are the overall activity (●), the transferase activity (❍), and the hydrolase activity (▼). Results were calculated on the basis of the overall activity (release of glucose) and the hydrolase activity (release of fructose). Shown are means ± standard deviation of two independent experiments performed in duplicate. The data for the overall-activity were fitted to the Michaelis Menten equation (solid line).

The overall activity of the enzyme LevΔhis exhibits Michaelis-Menten-characteristics by approximation (Figure 15) and the K_m - and V_{max}-values at 28 °C were determined as 13.1 ± 3.4 mM sucrose and 206 ± 19 μmol x mg^{-1} x min^{-1}, respectively. The transferase and hydrolase activities do not follow Michaelis-Menten-kinetics. Whereas the transferase-activity is not saturated by increasing sucrose concentrations, the hydrolase-activity is inhibited by sucrose concentrations exceeding 90 mM. Accordingly, at sucrose concentrations below 200 mM, the hydrolase activity was predominant, whereas transferase activity exceeded the hydrolase activity at sucrose concentrations above 200 mM. The pH- and temperature-optima of LevΔhis are shown in Figure 16. Enzyme-activity was optimal at a pH of 5.4 and was above 50 % of the optimal activity between pH-values 4.0 and 6.2, which reflects the pH range permitting growth of *L. sanfranciscensis*. A pH above 7 led to a complete inhibition of the

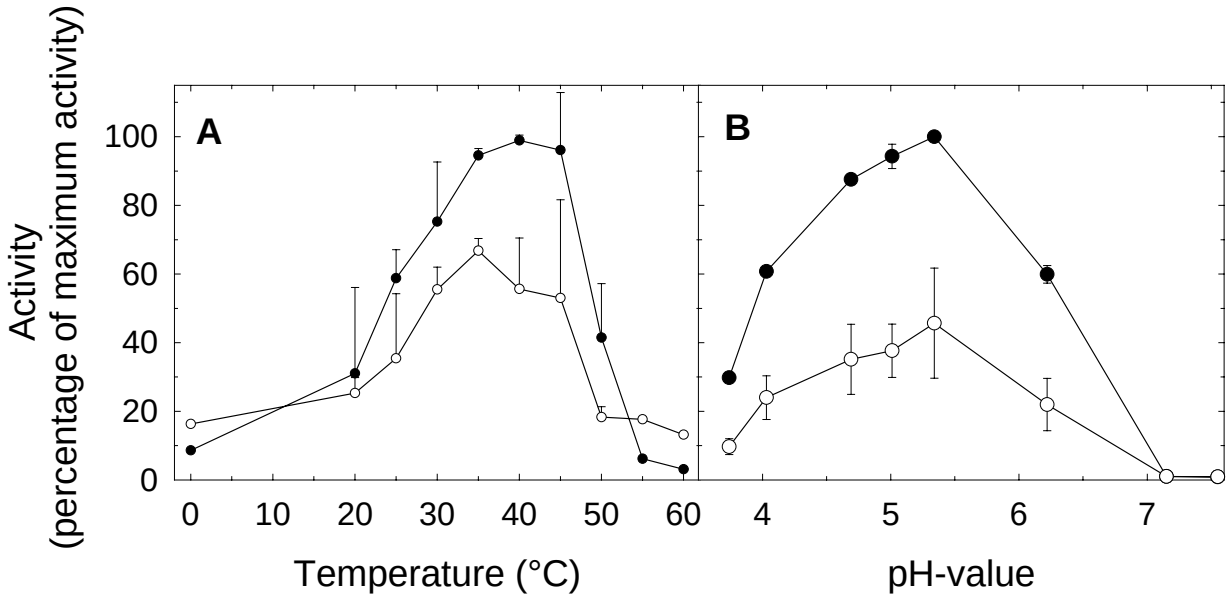

Figure 16. Temperature- and pH-optimum of the *L. sanfranciscensis* levansucrase purified after heterologuous expression in *E. coli* (levΔHis). The enzyme activity relative to the activity at 40° C and pH 5.4 is shown. Symbols indicate the overall activity (release of glucose, ●), and transferase activity (❍, release of fructose). Datum points are means ± standard deviation of two (temperature) or three (pH) independent experiments.

levansucrase (Figure 16). Optimum activity was observed at temperatures between 35 and 45 °C. The pH or the temperature exerted minor effects only on the ratio of transferase activity to the overall activity.

K_m - and V_{max} values were furthermore determined for the truncated enzyme LevCDΔHis and for the overall activity at 28 °C was observed a K_m-value and a V_{max} of 14.5 ± 4.2 and 201 ± 17 μmol x mg^{-1} x min^{-1}, respectively. Thus, the activity of LevCDΔHis showed no significant difference to the activity of LevΔHis. Furthermore, the relative contributions of hydrolase and transferase activities to the overall activity of LevCDΔHis were virtually identical to those values obtained with the enzyme LevΔHis (data not shown).

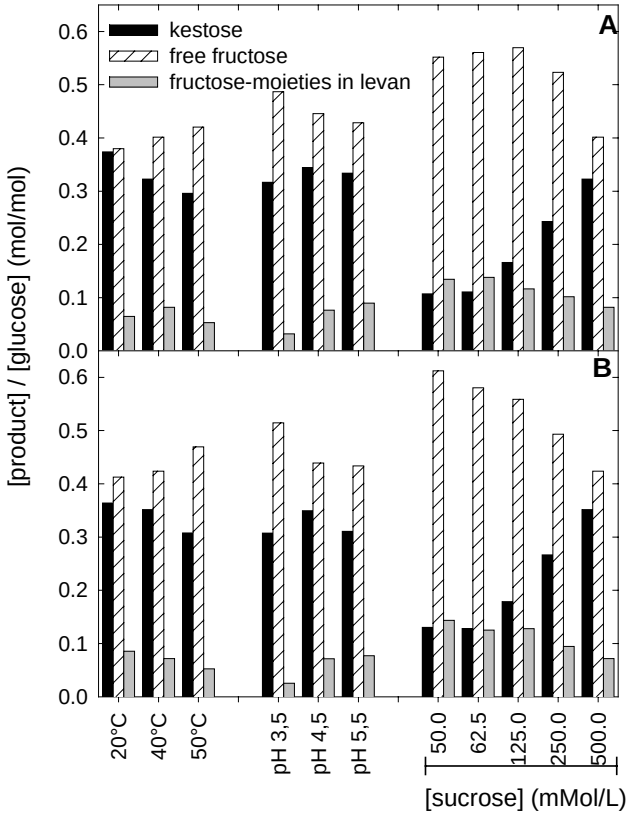

Figure 17. Products of the *L. sanfranciscensis* levansucrase purified after heterologuous expression in *E. coli* (LevΔHis) and the enzyme LevCDΔHis lacking the N-terminal repeating units. Enzymes were incubated for 16 h at various temperatures, pH-values, and sucrose concentrations. Reference conditions were 40 °C, pH 4.5 and 500 mM sucrose. The products of the fructosyltransferase activity and the hydrolase activity were calculated

in relation to the glucose released. The sum of kestose, fructose, and fructose from levan relative to the glucose released was 0.9 ± 0.1 (theoretical value 1). Panel A: levΔHis enzyme, Panel B: levCDΔHis enzyme. Results are representative for four (kestose, fructose) and two (fructose from levan) independent experiments.

To determine whether transferase activity resulted in formation of 1- kestose or levan due to varying conditions, the enzyme was incubated for 16 hours and the products of the enzymatic reaction were quantified. The pattern of products formed by the LevΔHis and LevCDΔHis enzymes at various levels of sucrose-concentrations, temperature and pH are depicted in Figure 17. Levan was quantified on a GPC column, and levan formed under any condition had a relative molecular weight of $\geq 5 \times 10^6$. In addition to the products fructose, kestose, and levan, the formation of nystose and higher FOS was observed (Figure 18). However, FOS other than kestose accounted for less than 10 % of the products under any conditions and, accordingly, the sum of kestose, fructose, and fructose from levan relative to the glucose released was 0.9 ± 0.1. No significant differences between the two enzymes LevΔHis and LevCDΔHis concerning the composition of products were observed. A temperature shift from 20 to 50 °C resulted in slightly higher proportions of free fructose and lower amounts of 1-kestose and levan. The pH only slightly affected the ratio of hydrolase activity to transferase activity. At pH 5.5, less fructose was formed compared to pH 3.5. However, the proportion of levan was increased more than two-fold upon a shift of the pH from 3.5 to 5.5.

In agreement with kinetic data from Figure 4, increasing sucrose concentrations favoured the transferase activity compared to the hydrolase activity. Moreover, the transferase activity resulted in formation of roughly equal amounts of kestose and levan at low sucrose concentrations, whereas kestose levels by far exceeded the levan levels when the reaction was carried out at 500 mM sucrose.

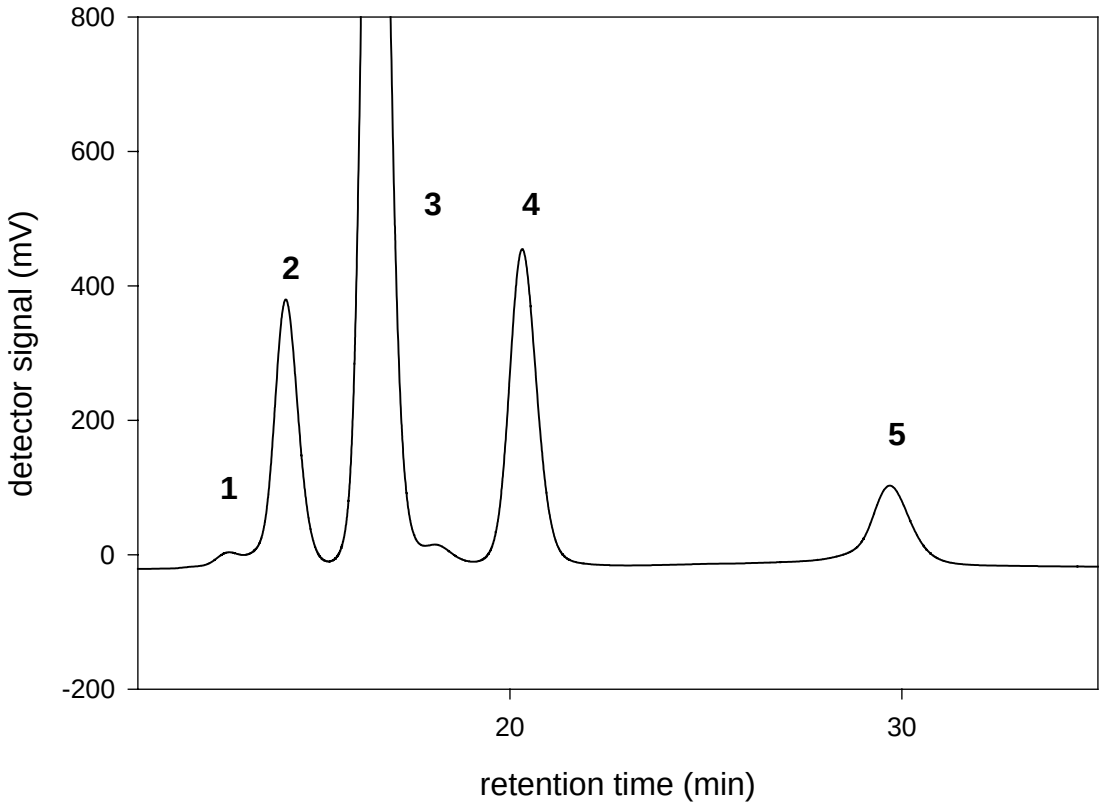

Figure 18. Chromatogram demonstrating the enzymatic production of the oligosaccharides (1) nystose and (2) 1-kestose after incubation of the heterologously expressed and purified *L. sanfranciscensis* levansucrase for 16 h at 40 °C with 500 mM sucrose at pH 4.5. Furthermore the enzymatic products (4) glucose and (5) fructose as well as the substrate (3) sucrose are visible.

3.3 Evidence for formation of heterooligosaccharides (HeOS) by *Lactobacillus sanfranciscensis* during growth in wheat sourdough

Literature provides knowledge on formation of HeOS by levansucrases in presence of sucrose and adequate acceptor carbohydrates (26, 38, 39). Therefore, experiments were carried out to determine if HeOS`s are generated by the levansucrase positive strain *L. sanfranciscensis* TMW 1.392 *in situ* while sourdough fermentation, using the purified levansucrase and the levansucrase deficient strain *L. sanfranciscensis* TMW 1.392Δlev as tools for an analytical setup.

3.3.1 Enzymatic synthesis of fructooligosaccharides (FOS) and HeOS

In order to determine the retention times of HeOS on the HPAEC-IPAD system, HeOS were synthezised in enzymatic reactions with levansucrase using sucrose as fructosyl-donor and the corresponding carbohydrates as fructosyl-acceptors. Although the elution order of heterogeneous sugar oligomers on HPAEC-IPAD can not be predicted, oligomers of a homologous series differing only in their DP generally elute in the order of their molecular weight. Although our analytical setup allowed the separation of maltose-oligosaccharides and inulin-type FOS up to a DP of 8 (data not shown), however, sufficient separation of HeOS with a DP of 4 or more was not achieved in those cases where oligomers of two or more homologous series were present. Therefore, peaks were assigned to HeOS based on the following considerations. (i) comparison of the retention times to FOS external standards. (ii) assignment of additional peaks in reactions with fructosyl-acceptors to HeOS known to result from the (repetitive) acceptor reaction with maltotriose, maltose, xylose, and arabinose, which allowed identification of oligomers with a DP of 3 or less. (iii) preparation of tetra-, penta- and hexasaccharides in two consecutive GPC runs, followed by HPAEC-IPAD analysis of the oligosaccharides, and the determination of the monomer composition of oligosaccharides.

3.3.2 FOS and HeOS from sucrose or raffinose as fructosyl-donor and -acceptor

Levansucrase activity with sucrose as the only substrate is known to yield inulin-type FOS with a DP of up to 5. The reaction of the *L. sanfranciscensis* levansucrase with 0.4 M sucrose as fructosyldonor and –acceptor yielded 1-kestose (GF2), nystose (GF3) and FOS with a DP of 5 or greater (GF4, GF5, and GF5), which were identified using FOS as external standards (Figure 19 A).

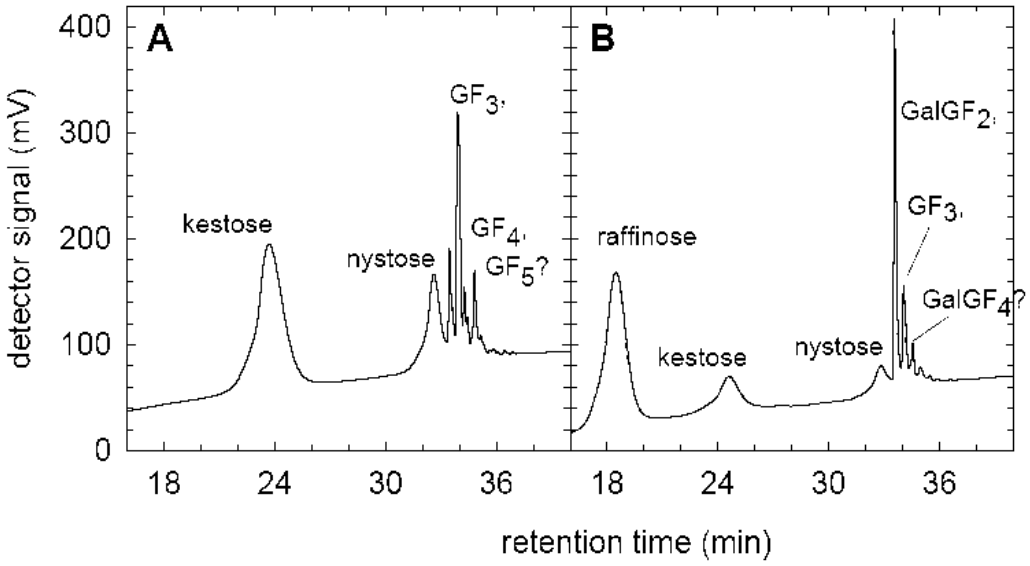

Figure 19. Separation of oligosaccharides by HPAEC-IPAD resulting from the levansucrase reaction with 0.4 Mol L^{-1} sucrose (PanelA) or raffinose (PanelB). Peaks were assigned based on external standards or literature data on products of levansucrase activity as described in the text. Glucose, fructose, sucrose, and melibiose eluted prior to the retention time cut-off at 16 min (not shown).

In reactions of levansucrase with raffinose as fructosyl-donor and -acceptor, melibiose and 1F-β-fructosylraffinose (GalGF2) is formed (38). In the reaction of the *L. sanfranciscensis* levansucrase with 0.4 M raffinose as fructosyl-donor, peaks corresponding to tetra-, penta-, and hexasaccharides were present in addition to melibiose, raffinose, kestose, and nystose. The major oligosaccharide peak at 34 min was assigned to GalGF2. HeOS from this reaction

were prepared by two consecutive runs on a GPC colums and collection of peaks corresponding to tetra- and pentasaccharides. Analysis of this oligosaccharide preparation by HPAEC-IPAD demonstrated that the preparation was free of oligomers with a DP of 3 or less, nystose was not present and GalGF2 was depleted (Figure 20 A). The molar monomer ratio in this oligosaccharide preparation upon hydrolysis was Gal:Glu:Fru 1:2:5, which demonstrates that raffinose acts as fructosyl-acceptor to yield oligosaccharides with a DP of 4 - 5.

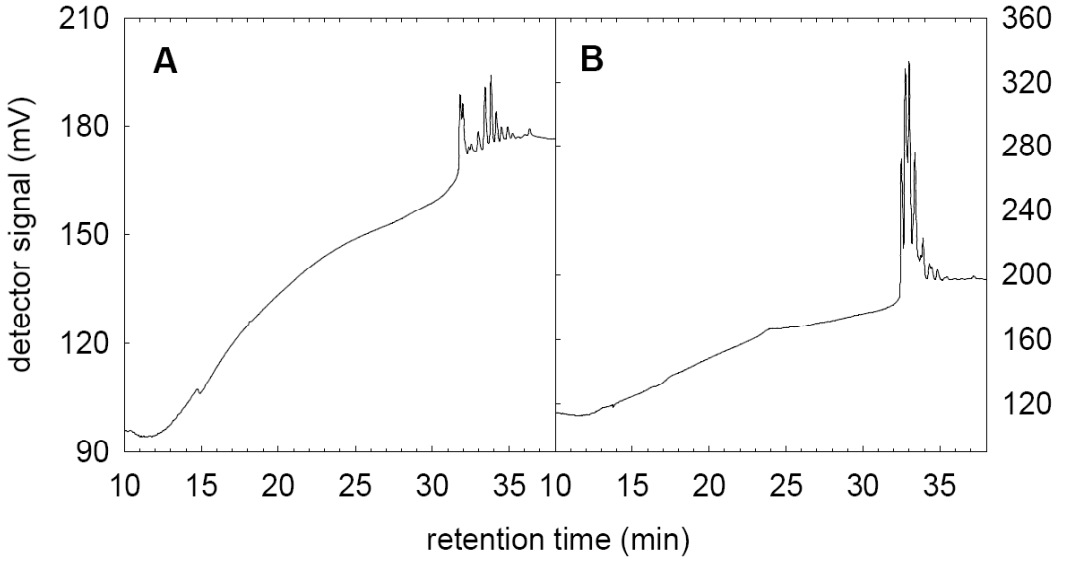

Figure 20. Separation of oligosaccharides resulting from the levansucrase reaction in the presence of raffinose (A) or sucrose and arabinose (B), followed by two consecutive GPC runs to remove mono-, di-, and trisaccharides. Likewise, oligosaccharides form the maltotriose, maltose and xylose acceptor reactions were prepared (data not shown).

3.3.3 HeOS from maltose and maltotriose as fructosyl-acceptors

The levansucrase reaction with sucrose as fructosyl-donor and maltotriose or maltose as acceptor yields 1F-β- fructofuranosylmaltotriose (G3F) or 1F-β-fructofuranosylmaltose (G2F) in addition to the FOS-series (39). In the reaction of the *L. sanfranciscensis* levansucrase with 0.4 M sucrose and 0.4 M maltotriose or maltose were present the FOS kestose and nystose (Figure 21A and 21B). The major HeOS in reactions with maltose eluting at 35 min was

attributable to G2F, oligomers with a DP or 4 or greater that were hydrolysed by invertase were additionally present (Figure 21B). The unambiguous assignment of HeOS from the maltotriose-acceptor reaction was not possible because three homologous series of oligosaccharides were present, maltooligosaccharides, FOS resulting from the acceptor-reaction with sucrose, and additionally the oligosaccharides G3F, G3F2 and G3F3 resulting from the acceptor-reaction with maltotriose (Figure 21A). This complex mixture of oligomers was not sufficiently separated with our analytical setup. However, all oligomers from the maltotriose-acceptor reaction eluting after 35 min were hydrolysed by invertase treatment (Figure 21A) and can not be assigned to FOS (compare to Fig 19A), indicating the presence of G3Fx HeOS. Accordingly, in the monomers from pentasaccharides prepared by GPC (data not shown), a glucose to fructose ratio of 2.5:1 was determined. Because the maltotriose preparation used in our experiments contained trace amounts of maltose and maltotetraose (Figure 3A, lower trace and data not shown), additional oligosaccharides from maltose and maltotetraose may be present in the reaction products.

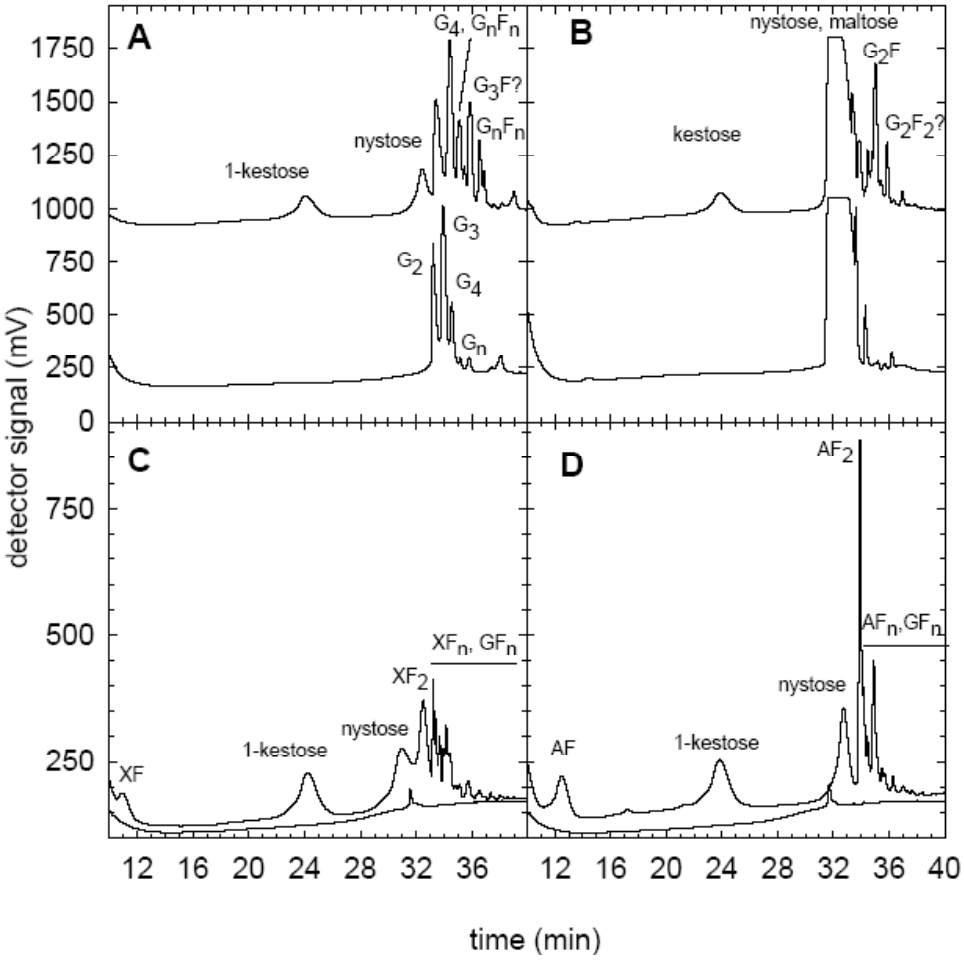

Figure 21. Separation of oligosaccharides by HPAEC-IPAD resulting from the levansucrase reaction with 0.4 Mol L^{-1} sucrose as fructosyl-donor and 0.4 Mol L^{-1} maltotriose (A), maltose (B), xylose (C), or arabinose (D) as fructosyl-acceptors (upper traces). The same reaction products were separated after hydrolysis of FOS and HeOS by yeast invertase (lower traces) Peaks were assigned based on external standards or literature data on products of levansucrase activity as described in the text. Glucose, fructose, sucrose, xylose and arabinose eluted prior to the retention time cut-off at 16 min (not shown).

3.3.4 HeOS from xylose or arabinose as fructosyl-acceptors

The levansucrase reaction in the presence of xylose and arabinose, the products xylsucrose (xylosyl-β-fructofuranoside, XF), 1F-β-fructosyl-xylsucrose (XF2), and di- and trifructosyl-xylsucrose are obtained, from arabinose was characterized arabsucrose (arabinosyl-β-

fructofuranoside, AF) (26, 38, 75, 97). In reactions of the *L. sanfranciscensis* levansucrase with xylose and arabinose, the disaccharides xylsucrose and arabinosucrose were present in substantial amounts (Figures 21C and 21D), and the major peak at 32 min is attributable to XF2 and AF2, respectively. The presence of FOS resulting from the acceptor-reaction with xylose and arabinose was substantiated by preparation of oligosaccarides with a DP of 3 or more via GPS (Figure 20B and data not shown) and analysis of their monomer composition. The ratio of glucose : pentose : fructose in tri, tetra- and pentasaccharides from reactions with xylose and arabinose were 3:1:5.5 and 2:1:4, respectively, demonstrating the presence of oligosaccharides with a DP of 3 or greater in enzymatic reactions with xylose and arabinose as fructosyl-acceptors.

3.3.5 Oligosaccharides formed during sourdough fermentations with *L. sanfranciscensis*

To determine whether HeOS are formed during growth of *L. sanfranciscensis* TMW 1.392, fermentations in wheat dough were carried out in the presence of 10 % sucrose. Doughs fermented with *L. sanfranciscensis* TMW 1.392 were compared to doughs fermented with an isogenic levansucrase-negative derivative of this strain, *L. sanfranciscensis* TMW1.392Δlev. Moreover, aqueous extracts of wheat doughs were compared to aqueous extracts treated with yeast invertase to eliminate fructose-oligosaccharides originating either from the levansucrase reaction, or from flour. The chromatograms of dough extracts and invertase treated dough extracts are shown in Figure 22. Major peaks that were present in the doughs fermented with *L. sanfranciscensis* TMW 1.392 but not in doughs fermented with *L. sanfranciscensis* TMW 1.392Δlev eluted at 12.5, 23.5, 34.0, 34.75 and 35.25 min (Fig 22A). Chromatograms of extracts from doughs fermented with TMW 1.392 and TMW 1.392Δlev were virtually identical after treatment with invertase, indicating that all these peaks are indeed attributable to FOS or HeOS (Figure 22B). 1-Kestose and AF can be assigned based on external standards and the retention times of the respective compounds derived from the enzymatic reactions,

respectively (Figure 21D). The peak eluting at 35 min may corresponds to G2F, AFn or G3F originating from the acceptor-reaction with maltose, arabinose, or maltotriose (Figures 21A, 21B, 21C). Because maltose is by far the most abundant carbohydrate in sourdough, the formation of G2F is more likely than that of other HeOS. Low amounts of 1-kestose originating from flour were also present in doughs fermented with *L. sanfranciscensis* TMW 1.392Δlev, nystose coelutes with maltose and can therefore not be identified unambiguously. FOS or HeOS eluting between 33.5 and 34.75 min could not be assigned to individual compounds because several FOS and HeOS with DP > 3 eluting in that range were generated in enzymatic reactions. Invertase treatments of extracts from doughs fermented with strains TMW 1.392Δlev or TMW 1.392 generated an oligosaccharide eluting at 12 min. This peak was absent in TMW 1.392Δlev fermented doughs but was also observed in invertase treated dough extracts from chemically acidified doughs (data not shown).

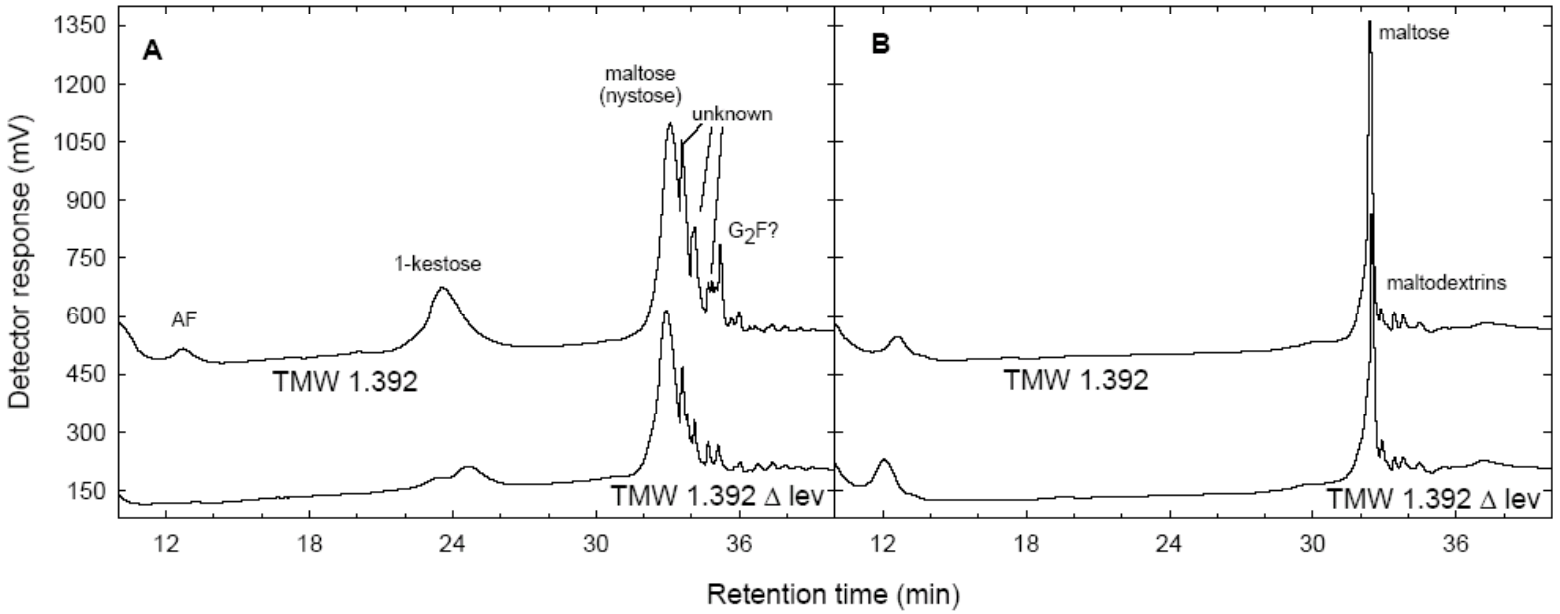

Figure 22. Separation of oligosaccharides extracted from wheat doughs by HPAEC-IPAD. (A) analysis of sourdough aqueous extracts fermented with *L. sanfranciscensis* TMW 1.392 (upper trace) or *L. sanfranciscensis* TMW 1.392Δlev (lower trace). (B) analysis of invertase treated sourdough aqueous extracts fermented with *L. sanfranciscensis* TMW 1.392 (upper trace) or *L. sanfranciscensis* TMW 1.392Δlev (lower trace). Peaks were assigned based on the comparison of the retention times with external standards, and standards derived from enzymatic reactions (Figure 3). Mannitol, glucose, fructose, sucrose, and melibiose eluted prior to the retention time cut-off at 10 min (not shown)

3.4 Extracellular homopolysaccharides and oligosaccharides from intestinal lactobacilli

Screening of cereal lactobacilli described in Chapter **3.1** has shown that species of lactobacilli that also occur in intestinal tract. Therefore, lactobacilli isolated from two different intestinal habitats were screened for the production of extracellular HoPS and oligosaccharides, and genes corresponding to gtfs and ftfs were identified with PCR methods.

3.4.1 Screening of lactobacilli isolated from ducks for HoPS synthesis

Lactobacilli of duck origin were inoculated in liquid mMRS-sucrose medium and screened for HoPS production, using GPC and RI-detection as analytical tools. Eight HoPS-positive strains could be identified among 23 screened lactobacilli. The monomer composition of the EPS was determined by hydrolysis of the purified polymers and HPLC-analysis of the monosaccharides. Three strains of *L. reuteri* and one strain of *L. animalis* were identified as glucan producers, whereas 4 strains of *L. acidophilus* produced fructan (table 5).

3.4.2 Production of oligosaccharides by HoPS-producing strains

To evaluate whether oligosaccharides are formed by HoPS-positive strains, culture supernatants of the fructan producing strains *L. acidophilus* TMW1.986 and 1.989, and of the glucan producing strains *L. reuteri* TMW1.974 and 1.976 were analysed by HPAEC-IPAD. Strains TMW1.986 and 1.989 produced kestose on mMRS-sucrose as identified by external standards of kestose and nystose (Figure 23 and data not shown). The glucan-positive strains TMW1.974 and 1.976 also exhibited oligosaccharide formation, which is obvious from two oligosaccharide peaks in the chromatograms (Figure 23 and data not shown). The retention times allow the conclusion that the major peak has a degree of polymerization greater than three, whereas the minor peak is likely to be associated with a disaccharide.

Table 5. HoPS producing lactobacilli isolated from ducks or pigs

Organism	**EPS formation[a]**	**glycosyltransferase genes detected with PCR**
Lactobacilli of duck origin		
***L. reuteri* TMW 1.974**	glucan[a)]	fructosyltransferase and glucansucrase
L. reuteri TMW 1.976	glucan[a)]	fructosyltransferase and glucansucrase
L. reuteri TMW 1.979	glucan[a)]	fructosyltransferase and glucansucrase
L. animalis TMW 1.971	glucan[a)]	glucansucrase
L. acidophilus/johnsonii TMW 1.986	fructan[a)]	fructosyltransferase
L. acidophilus/johnsonii TMW 1.987	fructan[a)]	fructosyltransferase
L. acidophilus/johnsonii TMW 1.989	fructan[a)]	fructosyltransferase
L. acidophilus/johnsonii TMW 1.991	fructan[a)]	fructosyltransferase
Lactobacilli of porcine origin		
***L. reuteri* TMW 1.272**	glucan[a)]	none
L. reuteri TMW 1.138	glucan[a)]	none
L. crispatus TMW 1.144	fructan[b)]	fructosyltransferase
L. acidophilus TMW 1.142	fructan[b)]	fructosyltransferase
L. mucosae TMW 1.141	fructan[b)]	fructosyltransferase

[a)] Amounts of EPS were in the range between 0.5 g L^{-1} and 2.0 g L^{-1}
b) Amounts of EPS was less than than 0.1 g L^{-1}

Oligosaccharides were identified as GOS based on the following observations: (i) fructose from sucrose was quantitatively recovered as mannitol in fermentations with TMW1.974 and 1.976 in mMRS-sucrose, but glucose recoveries in lactate and HMW glucans were incomplete, indicating GOS formation (data not shown). (ii) Oligosaccharides were resistant to enzymatic and chemical treatments which hydrolyse β-(1,2) and β-(2,6) fructans but do not hydrolyse glucans.

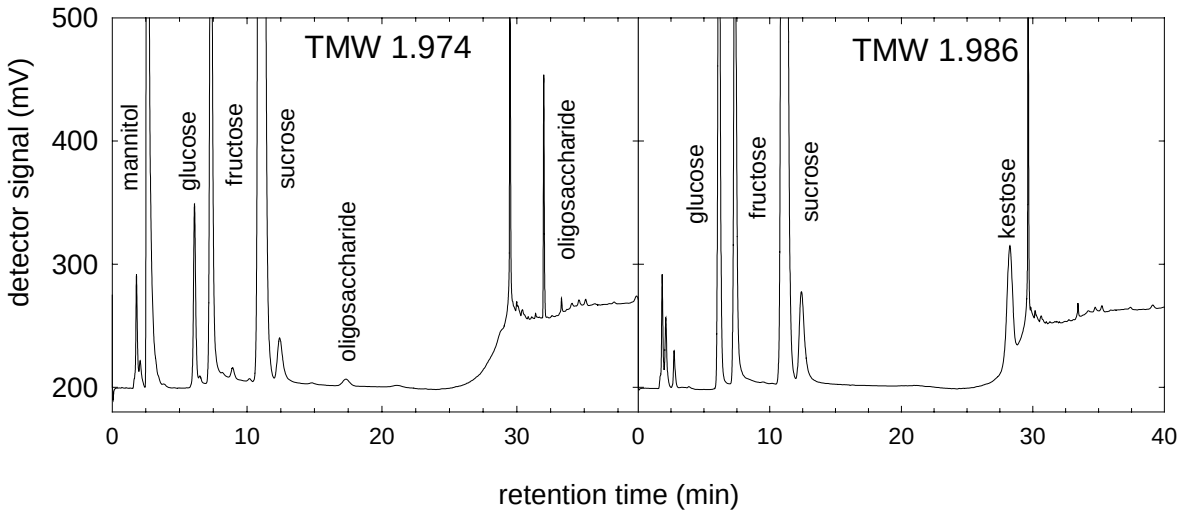

Figure 23. HPLC analysis of culture supernatants from *L. acidophilus* TMW1.986 and *L. reuteri* TMW 1.974 grown on mMRS-sucrose for 48 hours. Peaks were assigned based on external standards. Comparable results were obtained with *L. acidophilus* TNMW 1.989 and *L. reuteri* TMW 1.976 (data not shown).

3.4.3 Molecular screening for genes encoding glucosyl- and fructosyltransferases

A screening for ftf and gtf genes in the HoPS producing strains of duck origin was performed with degenerated primers, which were obtained from conserved sequences of known bacterial enzymes. Chromosomal DNA of the fructan- or glucanforming lactobacilli was used as template. Using the specific primers dexreuV/dexreuR, which were deduced from the gtfA nucleotide sequence of *L. reuteri* LB 121, PCR-products with the expected size of 600 bp were obtained from all glucan-forming *L. reuteri* strains of duck origin (table 5). Sequencing revealed four identical sequences with 90 % identity to the internal aa fragment of the GtfA recently identified by Kralj et al. (50).

```
                                                ⇓□□□
LeumesDEX         LLQIAADYFKLAYGVDQNDATANQHLSILEDWSHNDPLYVTDQGSNQLTMDDYVHTQLIW 60
LmesDsrb742       LLQIAADYFKAAYGVDKNDATANQHLSILEDWSHNDPEYVKDLGNNQLTMDDYMHTQLIW 60
LeuconDsrT        LLQIAADYFKAAYGVDKSDAISNQHVSILEDWSDNDAEYVKDNGDNQLSMDNKLRLSLKY 60
Lanimalis         ALSIAGDYLKAAYKVGQNDATANKHISILEDWNDKDPEYVNSIGNPQLTMDDYIVQQLKF 60
Lbreuteri         -MNIAQDYFNAAYGMD-SDAVSNKHINILEDWNHADPEYFNKIGNPQLTMDDTIKNSLNH 58
                   :.** **:: ** :. .** :*:*:.*****.. *. *... *. **:**: :  .*
                                                          ♦⇓
LeumesDEX         SLTK-----SSDIRGTMQRFVDYYMVDRSNDSTENEAIPNYSFVRAHDSEVQTVIAQIVS 115
LmesDsrb742       SLTK-----DMRMRGTMQRFMDYYLVNRNHDSTENTAIPNYSFVRAHDSEVQTVIAQIIS 115
LeuconDsrT        SLTMPAVDQYGNKRSGLEPFLTNSLVDRTNDSTDNTAQPNYSFVRAHDSEVQTVIAEIIK 120
Lanimalis         SLGQ-----APDKVDRMQRFKEWYLVDRSKDNTENTAIPNYSFVRAHDASVQEDILQLIQ 115
Lbreuteri         GLSD------ATNRWGLDAIVHQSLADRENNSTENVVIPNYSFVRAHDNNSQDQIQNAIR 112
                  .*             :: :    :.:* ::.*:* . ********** . *  * : :

LeumesDEX         DLYPDVENSLAPTTEQLAAAFKVYNEDEKLADKKYTQYNMASAYAMLLTNKDTVPRVYYG 175
LmesDsrb742       ELHPDVKNSLAPTADQLAEAFKIYNNDEKQADKKYTQYNMPSAYAMLLTNKDTVPRVYYG 175
LeuconDsrT        QRIDPDSDGLSPTMDQLTEAFKIYNADQLKTDKEFTQYNIPSTYATILTNKDTVPRVYYG 180
Lanimalis         DTTGKPWG--VYTNEELQQGLKDYMADQKLTNKKYNRYNIPSSYAILLTNKDTIPRVYYG 173
Lbreuteri         DVTGKDYH----TFEDEQKGIDAYIQDQNSTVKKYNLYNIPASYAILLTNKDTIPRVYYG 168
                  :          * ::   .:. *  *:  : *::. **:.::** :******:******

LeumesDEX         DLYTDDGQYMATKSPYYDAINTLLKARVQYVAGGQ-----------SMSVDSND-VLTSV 223
LmesDsrb742       DLYTDDGQYMANKSPYFDAINGLLKSRIKYVAGGQ-----------SMAVDQND-ILTNV 223
LeuconDsrT        DMYTDDGQYMATKSLYYDAIDTLLKSRIKYVSGGQTMSMKYMQGDSSMAADSYRGILTSV 240
Lanimalis         DLYSDAGKYMAEKSIYFDAIDNLLKTRTKYIAGGQ-----------TLDVDGHD-VLTSV 221
Lbreuteri         DLYTDGGQYMEHQTRYYDTLTNLLKSRVKYVAGGQS--------MQTMSVGGNNNILTSV 220
                  *:*:* *:**  :: *:*::  ***:* :*::***           :: ..    :**.*

LeumesDEX         RYGKDAMTASDTGTSETRTEGIGVIVSNNAELQLEDGHTVTLHMGAAHKNQAYRALLSTT 283
LmesDsrb742       RYGKGAMSVTDSGNADTRTQGIGVIVSNKENLALKSGDTVTLHMGAAHKNQAFRLLLGTT 283
LeuconDsrT        RYGNGAMTATDAGTNETRTQGIAVIESNNPDLKLSSTDQVVVDMGIAHKNQAYRPALLTT 300
Lanimalis         RFGKGALNVTDKGTSETRTQGMGLIISNNNSLKLNDGEKVVLHMGAAHKNQAYRAVMLSS 281
Lbreuteri         RYGKGAMTATDTGTDETRTQGIGVVVSNTPNLKLGVNDKVVLHMGAAHKNQQYRAAVLTT 280
                  *:*:.*:..:* *. :***:*:.:: **. .* *   . *.:.** ***** :*  : ::
```

Figure 24. Alignment of the internal Gtf fragment of *L. animalis* 1.971 with the four most similar sequences from *Leuconostoc mesenteroides* NRRL B-512F (LeuconDsrT, AB020020), *Lc. mesenteroides* B-742CB (LmesDsrb742, AF294469), *Lc. mesenteroides* NRRL B-512F (LeumesDEX, U81374) and *L. reuteri* LB 121 (Lbreuteri, AX306822). Regions with strong homologies among bacterial Gtfs (64) are shaded grey. ⇓, putative catalytical residue (64); □, putative residue which may play a role in the binding with acceptor molecules and in the transfer of the glucosyl residue (64); ♦, putative residue stabilizing the transition state (64). Fully conserved residues are indicated by an asterisk, fully conserved stronger groups are indicated by an colon, and fully conserved weaker groups by a period.

Using the primer combination DexwobV/DexwobR, a PCR product was obtained with the DNA from *L. animalis* TMW1.971. Sequencing of the 1200 bp-product and analysis of the deduced aa-sequence revealed high homology to bacterial gtfs; the enzyme belongs to the family 70 of glycosyl hydrolases (http//:pfam.wustl.edu). Blast searches revealed the highest degree of identical amino acids (57 %) with a glucansucrase from *Lc. mesenteroides* (accession number U81374). Alignments of the internal Gtf fragment of *L. animalis* TMW1.971 and the four most similar Gtf sequences available in public databases are given in figure 24. The fragment comprises the catalytical core of the enzyme and harbours aa residues that are putatively involved in catalysis, binding of acceptor molecules and stabilising the transition state, respectively (64). The expression of the gtf gene of *L. animalis* TMW 1.971 was shown by using PCR targeting an internal sequence using cDNA as template (data not shown).

Using the primer combination LevV/R, PCR products of 800 bp length were obtained with the fructanforming organisms *L. acidophilus* TMW 1.986, 1.987, 1.989, and 1.991. Sequencing of the amplification products showed 100 % identity of the four gene fragments (data not shown). Analyses of the deduced aa sequence revealed high homology to bacterial ftfs; the enzymes belong to the family 68 of the glycosyl hydrolases. The highest degree of

identical amino acids (68 %) was observed with a levansucrase from *L. reuteri* (AF465251). An alignment with the five most similar sequences is given in figure 24.

3.4.4 Identification of lactobacilli from the pig feces and their ability for HoPS formation

To evaluate the potential of lactobacilli from a different intestinal habitat to produce HoPS, lactobacilli were isolated from pig feces. 19 pig isolates and 9 reference strain were clustered based on their AFLP patterns using the BioNumerics software package (data not shown). At least one representative strain of each cluster was chosen and identified to species level on the basis of partial 16S rDNA sequences. With the exception of the two strain of *L. mucosae*, all strains exhibiting a similarity level of 55 % or higher could be alloted to the same species. The total *Lactobacillus* cell count was $5 \times 10^8 - 2 \times 10^9$ cfu g^{-1} and the species *L. mucosae* (2 strains), *L. reuteri* (6), *L. crispatus* (7), *L. acidophilus* (1), *L. mucosae*(1) and *L. johnsonii*(1) were identified. A physiological and molecular screening was performed with these strains to identify HoPS producers and gtf or ftf genes, respectively. Two *L. reuteri* strains produced a glucan, but no gtf-PCR product was obtained with the chromosomal DNA of the glucan positive *L. reuteri* strains, neither with dexwobV/R, nor with primers targeting the gtfA gene described by Kralj et al. (50) (table 5).

Ftf-amplicons could be achieved with the DNA of three *L. acidophilus* strains and one strain each of *L. mucosae* and *L. crispatus*, concomitant with fructan production in mMRS-sucrose (table 5).

```
Inuloreu        VQDVRTGQVANWNGYQLVIAMMGIPN--QNDNHIYLLYNKYGDNELSHWKNVGPIFGYNS 58
Ljohnsoni       VQDAKTGYVSNWNGYQLVIGMMGVPN--VNDNHIYLLYNKYGDNDFNHWKNAGPIFGLG- 57
Strepsali       VQDAKSGVVSNWNGYQLVISMAGAPN--KNSNHIYLLYRKYGDNDFTHWKNAGPIFGYNA 58
Lsanfranc       VQDP-TGYVSNYKGYQLVIAMMGIPNTPNGDNHIYLLYNKYGDNDFSHWRNAGSIFGTNE 59
Lbreuteri       VQDPVTGYVSNYMGYQLVIAMMGIPNSPTGDNHIYLLYNKYGDNDFSHWRNAGSIFGTKE 60
Lacidophi       VQDPITGYVSNYKGYQLVIAMMGMPK--KNDNHIYLLYNKYNDNNFSHWRNAGSIFGYEE 58
                ***  :* *:*: ******.* * *:   ..*******.**.**::.**:*.*.***
                                 ⇓
Inuloreu        TAVSQEWSGSAVLNSDNSIQLFYTRVDTSDNNTNHQKIASATLYL--TDNNGNVSLAQVA 116
Ljohnsoni       TPVIQQWSGSATLNKDGSIQLYYTKVDTSDNNTNHQKLASATVYLNLEKDQDKISIAHVD 117
Strepsali       LEDDQQWSGSATVNSDGSIQLYYTKNDTSGGKLNWQQLASATLNL--AVENDEVVIKSVE 116
Lsanfranc       NNVYQEWSGSAIVNDNGTIQLFYTSNDTSDYKLNDQRLATATLNL--DVDDNGVAIKSVD 117
Lbreuteri       TNVFQEWSGSAIVNDDGTIQLFFTSNDTSDYKLNDQRLATATLNL--NVDDNGVSIKSVD 118
Lacidophi       TPDLQEWSGSAIVNKDDSIQLFYTRNDTSNGKINDQQLATANLKL--RVDNNGVSIVSVD 116
                    *:***** :*.:.:***::*  ***. : * *::*:*.: *    ::. : :  *
                                                        ⇓
Inuloreu        NDHIVFEGDGYYYQTYDQWKATNKG----------ADNIAMRDAHVIEDDNGDRYLVFEA 166
Ljohnsoni       NDHIVFEGDGYHYQTYDQWKETNKG----------ADNIAMRDAHVIDDDNGNRYLVFEA 167
Strepsali       NDHILFGGDNYHYQSYPKFMSTFDDDHNHDGNPDRTDNYCLRDPHIIED-NGSRYLIFES 175
Lsanfranc       NYHILFEGDGFHYQTYDQFANGKDRK---------NDDYCLRDPHVVQSENGDRYLVFEA 168
Lbreuteri       NYQVLFEGDGFHYQTYEQFANGKDRE---------NDDYCLRDPHVVQLENGDRYLVFEA 169
Lacidophi       NDHVIFIGDSKKYQTYDQFANGINRN---------KDNYTLRDPHVVEEENGDRYLVFEA 167
                * :::* **.  **:* ::    . .          *:  :**.*::: .**.***:**:

Inuloreu        STGLENYQGEDQIYNWLNYGGDDAFNIKSLFRILSNDDIKSRATWANAAIGILKLNKDEK 226
Ljohnsoni       STGTENYQGDDQIYQWLNYGGTNKDNLGDFFQILSNSDIKDRAKWSNAAIGIIKLNDDVK 227
Strepsali       NTGDENYQGEKQIYKWSNYGGDDAFNLKSFLNIVNNKHLYNLASWANGSIGILKLDDNEK 235
Lsanfranc       NTGMEDYQSDDQIYNWANYGGDDAFNIKSFFKLLNNKNDRELASLANGAIGILKLNNDQT 228
Lbreuteri       NTGTEDYQSDDQIYNWANYGGDDAFNIKSSFKLLNNKKDRELAGLANGALGILKLTNNQS 229
Lacidophi       NTGSDNYQGDNQVYNWTNYGGNDKFNVRNFLDYFDNDNDKALASAANGALGILKLSGEQN 227
                .** ::**.:.*:*:* **** :  *: . :  ..*..    *  :*.::**:**  : .

Inuloreu        NPKV--AELYSPLISAPMV 243
Ljohnsoni       NPSV--AKVYSPLISAPM- 243
Strepsali       NPSV--AELYTPLVTSHM- 251
Lsanfranc       NPKV--EEVYSPLVSTLMA 245
Lbreuteri       KPKV--EEVYSPLVSTLMA 246
Lacidophi       NPIVEPENVYSPLVTSLMA 246
                :* *.. ::*:**::: *
```

Figure 25. Alignment of the internal Ftf fragment of *L. acidophilus* with the inulosucrase of *L. reuteri* LB 121 (Inuloreu, AF45943) and the Ftfs of *L. reuteri* LB 121 (Lbreuteri, AF465251), *L. johnsonii* (Ljohnsoni, AE017202), *Streptococcus salivarius* (Strepsali, L08445). Regions with strong homologies among Ftfs (70, 78) are shaded grey; ⇓, aa residues which may play a role in catalysis (94, 1). Fully conserved residues are indicated by an asterisk, fully conserved stronger groups are indicated by an colon, and fully conserved weaker groups by a period.

3.5 Isolation and nucleotide sequence analysis of the *L. animalis* strain 1.971 glucansucrase gene

Screening of intestinal lactobacilli with molecular tools revealed hitherto undescribed genes of Ftf and Gtf enzymes among strains of the species *L. acidophilus* and *L. animalis.* Based on these results, the sequence of a glucansucrase from *L. animalis* TMW 1.971 was characterized.

3.5.1 Nucleotide sequence analysis

Based on the partial sequence of the *L. animalis* glucansucrase (Chapter 3.4), a total sequence of 5141 bp was obtained after several rounds of inverse PCR and subsequent sequencing of the PCR-products (Figure 26). One complete open reading frame (ORF1), encoding a putative glucansucrase gene (4752 bp, starting at position 52), was located on this DNA segment. A start codon (ATG) could be located at position 52, and an imperfect Shine-Dalgarno sequence at position –19 (AGGAGT). A stop codon occurs at position 4804, but no clear termination signal could be identified downstream of ORF1. Moreover, at position 4970, a start codon of an further ORF could be identified, harboring another secretion signal with high homology to that of the levansucrase from *L. sanfranciscensis* TMW 1.392.

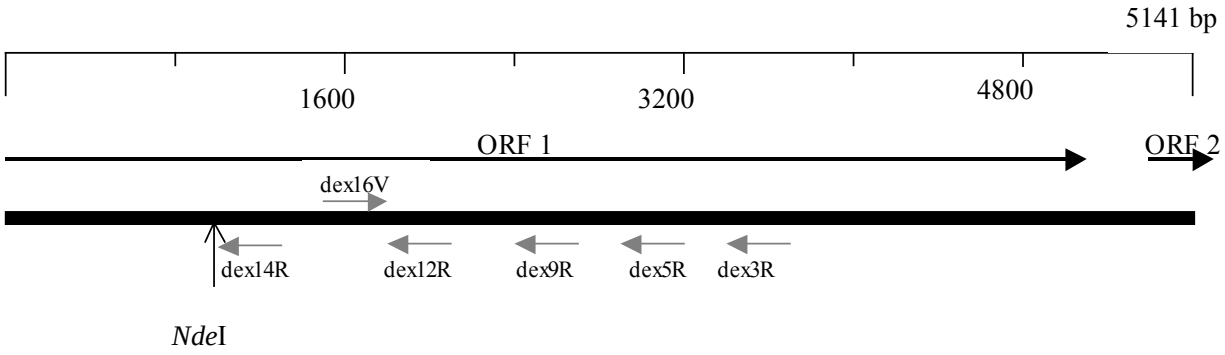

Figure 26. PCR-strategy for the isolation of the dextransucrase gene from *L. animalis* TMW 1.971

3.5.2 Amino acid sequence analysis

Translation of ORF1 and blast searches with the deduced aa sequence revealed high similarities to dextransucrases from *Leuconostoc mesenteroides* strains with 50 % identity and 65 % similarity (EMBL accession numbers AJ250127, AY017384, U81374 and AY 142210). Very similar to the levansucrase of *L. sanfranciscensis* TMW 1.392, the first 40 amino acids were predicted to harbor a secretion signal with a probability of 100 %, using the SignalP-HMM prediction method according to Nielsen et al. (71). A cleavage site between the amino acids at position 39 and 40 was predicted with a probability of 97,6 % (Figure 27).

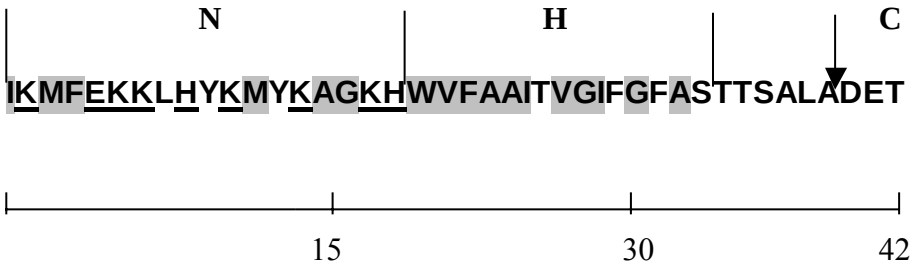

Figure 27. Signal peptide of the glucansucrase of *L. animalis*. The N-region is rich in positively charged amino acids, the hydrophobic H-region and the C-region with the putative cleavage site were assgined based on the SignalP-HMM output as described by Nielsen and Krogh (67). Positively charged residues are underlined, hydrophobic amino acids are shaded grey, and the predicted cleavage site of the leader peptide is indicated by an arrow.

The signal peptide is followed by a variable domain with irregular repeating units, that is described in glucansucrases from other species and appears to have no apparent function for catalysis or substrate binding (64). Seven repeats could be identified and blast searches revealed 28 % identity and 44 % similarity of this region with a glucosyltransferase from *Streptococcus gordonii*. No hits could be achieved when searching matches with protein families (http:\\pfam.wustl.edu).

The putative glucan binding domain (GBD) consists of four repeats with an average length of 23 amino acids, that are weakly conserved in other glucansucrases (Figure 28)

```
YG1             -YYNSEGEIMKNAFVKDG-KNNW-- 21
YG2             YYFDNDGNMVTNTALTIDSDAQVAD 25
YG3             YYFLSNGISLRDGFVQLA-NGDVY- 23
YG4             -YYDINGRKLKNGKVTVN-NVEYT- 22
                 *:  :*  : :  :    . :
```

Figure 28. Alignment of four repeats (YG1-YG4) that could be identified in the glucanbinding domain of the dextransucrase from *L. animalis* TMW 1.971.

4 Discussion

4.1 Screening of cereal lactobacilli for EPS synthesis and genes coding for EPS synthesizing genes

The microflora of traditionally prepared sourdoughs typically consists of two to five strains in a single dough, rarely exceeding these numbers. As approximately 20 % of the sourdough-strains screened in this work were found to produce EPS from sucrose, it is likely that any given sourdough contains EPS-producing lactobacilli. The frequency of fructan- or glucan-positive strains was highest in the phylogenetically closely related species *L. reuteri*, *L. frumenti*, *L. panis*, and *L. pontis* originating from type II sourdoughs or the intestinal tract. Van Geel-Schutten et al. (102) characterized 2 strains of *L. reuteri* among 182 lactobacilli of various origins as potent producers of EPS. Outside of the genus *Lactobacillus*, glucan and fructan formation has been found in *Leuconostoc* spp. and oral streptococci. Strains of species dominating type II sourdough fermentations are frequently found in the intestinal tract of mammals and birds (53, 59, 98, 113). In particular, virtually all species of lactobacilli detected in pig intestines are also recognized as organisms dominating type II sourdough fermentations (3, 93, 112), although evidence for the occurrence of *L. pontis* and *L. panis* in pig intestines is based on culture independent techniques only. Remarkably, the frequency of EPS-forming strains of lactobacilli is highest in intestinal isolates and species typical for intestinal microbiotae. It is tempting to speculate whether glucan- and fructan-formation are physiological properties with relevance for the survival of lactobacilli during gastro-intestinal transit.

Glucans and fructans are formed from sucrose by the activity of a single enzyme, i.e. glucan- or fructansucrases. Two fructosyltransferases from *L. reuteri* strain 121 were recently characterized (104, 106). Generally, bacterial levansucrases exhibit fructosyltransferase activities resulting in the formation of the inulin-type fructooligosaccharides kestose and

nystose (98, 25). Accordingly, kestose and nystose production were observed in *L. reuteri* strain 121 where levansucrase is the only fructosyltransferase which is transcribed and active (103, 106). The second fructosyltransferase gene in *L. reuteri* 121 exhibits high homology to bacterial levancsucrases, however it is not expressed under the conditions used. The gene product exhibited fructosyltransferase activity upon heterologous expression and was termed "inulosucrase" based on the formation of inulin, kestose and nystose (106). A 800 bp sequence could be amplified in this work from fructan-forming lactobacilli by PCR targeting levansucrases of lactic acid bacteria and database searches revealed a high similarity with known levansucrase genes. These levansucrase genes were present only in 6 of the 15 fructan-forming strains and in none of the fructan-forming *L. reuteri* strains. The fructan of the Lev-PCR positive strain *L. sanfranciscensis* LTH2590 (=TMW 1.392) was previously characterized by enzymatic digestion and reported to be of the levan type (16). An internal fragment of the putative levansucrase gene was amplified from cDNA libraries of *L. sanfranciscensis* TMW 1.392 and TMW 1.53, indicating levansucrase expression in these strains (this study), leading to the conclusion that fructan formation by *L. sanfranciscensis* is due to a levansucrase enzyme, which was confirmed by the experiments discussed in Chapter **4.2**.

L. reuteri 121 contains at least genes for two fructansucrases and one glucansucrase, exhibits glucansucrase and levansucrase activities, and produces two types of EPS, glucan and / or levan, depending on environmental conditions (50, 103, 104, 105). *L. sanfranciscensis* LTH1729 was reported to produce levan (16) but was Lev-PCR negative (**4.1**). Furthermore, 8 strains were additionally described here that produce fructan(s) not characterized on structural level but are Lev-PCR negative. Therefore, it can be anticipated that a further characterization of genes responsible for glucan- and fructan-formation in lactic acid bacteria may provide new types of glycosyltransferases.

Korakli et al. (49) have shown by the use of ^{13}C labeled sucrose the formation of up to 3.6 g high molecular weight fructan per kg flour by *L. sanfranciscensis* LTH2590 in wheat and rye sourdoughs. Fructan formation in dough was detected by a higher ^{13}C content of water soluble polysaccharides in dough, and a 10fold higher content of fructose in WS-PS upon fermentation compared to control doughs. By use of the same methodology it could be demonstrated that those strains producing EPS in mMRS-sucrose also formed EPS during sourdough fermentation in the presence of sucrose. The amounts of high molecular weight fructan and / or glucan can be estimated to range between 0.3 and 2 g EPS / kg flour based on the glucose and fructose levels in water soluble polysaccharides. These amounts are comparable to the fructan levels previously reported (49) and are higher than levels of hetero-expolysaccharides produced by dairy lactic acid bacteria during growth in milk, 10 – 200 mg per liter milk (55). Hydrocolloids such as xanthan or modified cellulose significantly affect dough rheology and bread texture at levels of 0.1 %-1 % of flour base (13, 87) and levan formed by *L. sanfranciscensis* LTH2590 was shown to affect rheological properties of wheat doughs at a level of 0.1 % flour base (11) EPS formed during sourdough fermentation can be assumed to be technologically relevant.

Bacterial levansucrases characterized usually have invertase activity in addition to the levan-sucrase activity (37, Chapter **3.2**). Because fructose is used as electron acceptor by hetero-fermentative lactobacilli from sourdough, resulting in concomitant production of acetate instead of ethanol, sourdough fermentation by fructan-forming strains in the presence of sucrose results in higher acetate contents of the dough (49). The formed acetate affects sensorial qualities and improves the shelf life of the bread. During fermentation of wheat doughs with 12 % sucrose with fructan-forming *L. sanfrancisensis*, more than 50 % of the sucrose were metabolised during fermentation (49, see also **3.2**). Because sourdough preferments are generally included in wheat bread formulas at levels below 30 %, 12 %

sucrose addition at the preferment stage results in sucrose levels at or below 2 % flour base in the bread dough. The addition of 2 % sucrose is commonly used in wheat bread formulas.

EPS from LAB may influence the intestinal flora, because oligofructose and fructans of the levan- and inulin-type are known to preferentially stimulate the growth of bifidobacteria (8, 16, 60). Possible health benefits achieved through stimulation of growth and metabolism by dietary oligofructose or fructans have been proposed (28, 57, 63, 85, 99, 114). In **3.1**, it was shown that two thirds of EPS-producing strains form fructan. The levan produced by *L. sanfranciscensis* LTH2590 is metabolised by bifidobacteria (46), and selectively stimulated growth of bifidobacteria during cultivation of human fecal microflorae *in vitro* (16). However, wheat and rye flours contain about 6.6 and 8.5 % arabinoxylans, 1.4 and 2 % β-glucans, and 1 and 4 % fructans, respectively. Therefore, it remains to be established whether or not fructans produced during sourdough fermentation exert an additional effect on the composition and activity of the intestinal microflora, and human health.

In conclusion, it was shown that the production of EPS from sucrose is a metabolic activity wide spread in sourdough lactic acid bacteria. Lev-PCR produced false negative, but not false positive results and thus allows a rapid screening of isolates on fructan formation. EPS-positive strains formed technologically relevant amounts of EPS during sourdough fermentation. These results will allow the deliberate use of EPS-forming lactobacilli in bread production to achieve the substitution of additives currently used in bread production by glucans or fructans formed *in situ*. The links between type II sourdough and intestinal microflorae on the level of species composition and EPS production may prove to be helpful for the further development of pre- and probiotic concepts.

4.2 Isolation and characterization of a levansucrase from *L. sanfranciscensis* TMW 1.392

In Chapter **3.2**, the molecular and functional characterization of the levansucrase of *L. sanfranciscensis* TMW 1.392 is described.

A 2300 bp ORF was identified from the genomic DNA of *L. sanfranciscensis* TMW 1.392 coding for a protein with a predicted relative molecular weight (M_R) of 90 k with high homology to known bacterial levansucrases. This levansucrase is expected to be a cell wall bound enzyme because of the presence of an export signal peptide (71) at the N-terminus and a cell wall anchor (69) at the C-terminal end of the protein, respectively.

Van Hijum et al. (105, 107) recently characterized two fructosyltransferase genes from *L. reuteri* and the sequence and domain organisation of these two enzymes is highly homologuous to the *L. sanfranciscensis* levansucrase. All three enzymes harbour a proline-rich region (PXX) as cell wall anchor domain, a cell wall-anchoring LPXTG motif followed by a hydrophobic stretch of aa residues which is terminated by three positively charged amino acids. In contrast to the enzymes from *L. reuteri*, the sequence of *L. sanfranciscensis* contains direct repeats of 6 x 12 aa residues within the proline rich cell wall anchor domain. Moreover, the *L. sanfranciscensis* levansucrase contains an unusual sevenfold repetition of the 16 aa residues DNATSGSTKQESSI(V)AN close to the N-terminus. These repeating units exhibit no homology to any known fructosyltransferase and cover about 13 % of the total protein-length. With few exceptions, fructosyltransferases from other bacteria described in literature do not contain such N-terminal variable domains. *L. reuteri* Ftf`s also possess repeats subsequent to the N-terminal leader peptide, but of a smaller number, shorter in overall-length and different in sequence. A recently characterized inulosucrase from *Leuconostoc citreum*, also exhibits N-terminal repeating units (72). A combination of N-terminal leader peptide and a variable region of repeating units comparable to the organisation of the levansucrase of *L. sanfranciscensis* described here frequently occurs in glucosyltransferases from lactic acid bacteria. Investigations with glucosyltransferases from lactic acid bacteria lacking the N-

terminal repeating units demonstrated that these do not contribute to any aspect of the catalytic activity of these enzymes. Their function remains unknown (64). As shown in **3.2**, a truncated version of the *L. sanfranciscensis* levansucrase lacking the N-terminal repeats was expressed and its activity was compared to the wild type enzyme. No effect of the N-terminal repeating units on the kinetic properties of the levansucrase or the spectrum products produced from sucrose was observeable, arguing against a possible role of the N-terminal repeating units in catalysis.

Many Gram-positive bacteria use cell wall anchored proteins to adhere to extracellular matrixes, e.g. biofilms or host tissue, and these cell wall bound proteins frequently contain N-terminal repeating units that are involved in the adherence properties (69). Lactobacilli of intestinal origin frequently express cell wall bound glycosyltransferases or fructosyltransferases and all glycansucrases from lactobacilli characterized to date contain to a various extent N-terminal repeating units with no apparent function for the catalytic activity of the enzymes. It is tempting to speculate that these cell-surface located N-terminal repeats in glycansucrases contribute to the interaction of these organisms to external surfaces.

The ability to use sucrose as carbon source is a metabolic trait that is not widespread among the species *L. sanfranciscensis*. Korakli et al. investigated the sucrose metabolism of *L. sanfranciscensis* TMW 1.392 in pH-static fermentations. Based on their observations of kestose and EPS-formation together with fructose liberation from sucrose, they postulated that sucrose metabolism in this strain is due to a levansucrase enzyme (48). The comparison of strains TMW 1.392 and TMW 1.392Δlev demonstrated that formation of 1-kestose and levan as well as sucrose metabolism is indeed both catalysed by a single levansucrase enzyme (Figure 29). In medium with maltose and sucrose as carbon sources acetate and mannitol were not formed by the levansucrase negative mutant; accordingly, in fermentations with sucrose-containing doughs the formation of acetate, ethanol, free glucose, mannitol and free fructose were striking different. Furthermore, kestose and EPS were not formed by the mutant. Taken

together, it is fair to assume that the activity of glycansucrases have the most influence on fermentation patterns and therefore on rheological and sensorical qualities in foods with sucrose-based formulas, when fermented with *L. sanfranciscensis*.

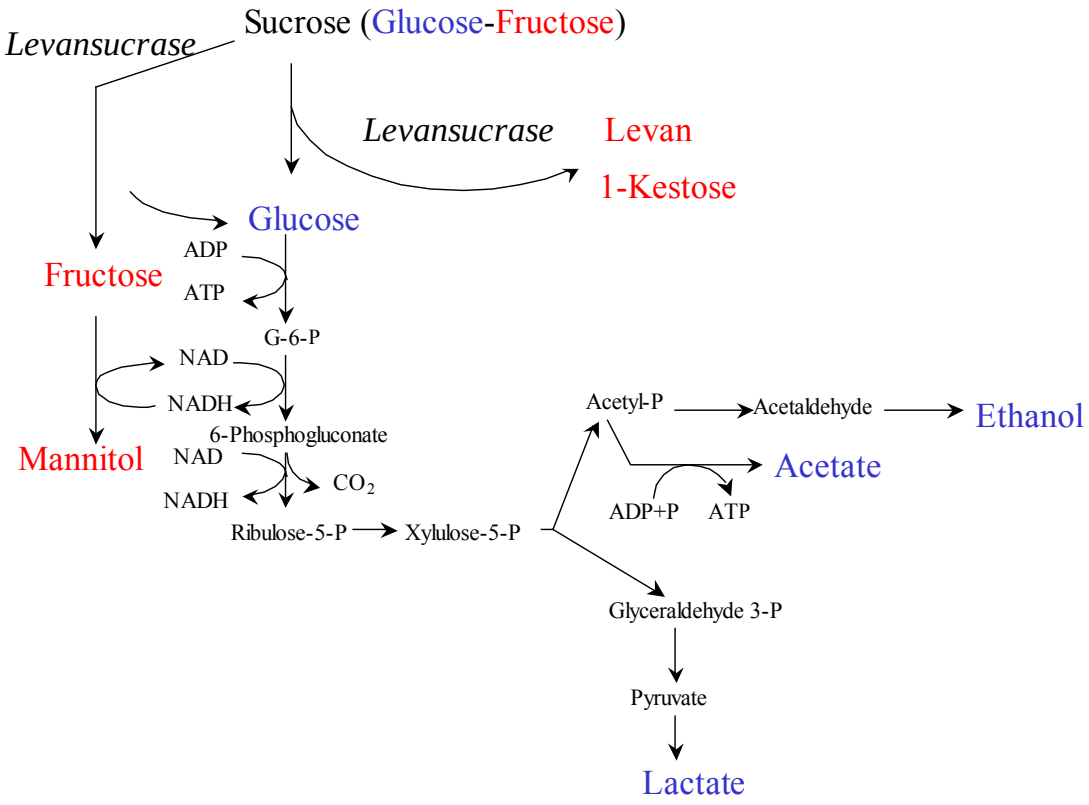

Figure 29. Sucrose metabolism by *L. sanfranciscensis* TMW 1.392.

Levan and 1-kestose were major products from sucrose metabolism of *L. sanfranciscensis* in dough and in medium. Remarkably, the strain *L. sanfranciscensis* TMW 1.392 produced in wheat dough 20 mMol x L^{-1} or 11 g x L^{-1} of the prebiotic FOS 1-kestose. This is the first report that lactic acid bacteria produce prebiotic FOS *in situ* during food fermentations. The high levels of maltose and maltose-oligosaccharides in dough do furthermore support the

formation of further oligosaccharides by the transferase reaction, which was investigated in **3.3** and is discussed downwards.

The product spectrum obtained from bacterial levansucrases is modulated by the substrate concentration. Using levansucrases from *Bacillus subtilis* and the inulosucrase from *L. reuteri*, an increase of the transferase activity compared to the hydrolase activity was observed when the sucrose concentration was increased (25, 105). Moreover, increasing concentrations of sucrose increased the yield of oligosaccharides from sucrose in conversions with a levansucrase from *Zymomonas mobilis* (15). In **3.2** was analysed the ratio of fructose, kestose, and levan produced by the *L. sanfranciscensis* levansucrase from sucrose at various levels of pH, temperature and substrate concentration. Shifts in the composition of products were observed upon variation of the temperature or the pH. The formation of levan was favored over sucrose hydrolysis or kestose-formation at pH 3.5 or 20 °C. A major effect of the substrate-concentration on the ratio of fructose to kestose or levan was apparent and an increase of the sucrose concentration from 50 to 500 mMol L^{-1} resulted in a 3-fold higher yield of kestose, a concomitant reduction of fructose, and slightly reduced levan levels. The pattern of products observed after the enzymatic reaction of the purified levansucrase is in general agreement with the pattern of products observed upon growth of *L. sanfranciscensis* in wheat doughs or mMRS-medium. Here, fructose, which was further reduced to mannitol, was the most important product of levansucrase activity, followed by kestose and levan, the latter accounting for less than 20 % of the overall enzymatic turnover. In agreement with the effect of sucrose on levansucrase activity, Korakli et al. (48), reported increased formation of kestose with increasing sucrose concentrations in pH-static fermentations at pH 5.6 with *L. sanfranciscensis* LTH 2590 (= TMW 1.392). Moreover, the hydrolase activity of the levansucrase was inhibited by fructose added to the growth medium.

4.3 Heterooligosaccharides in sourdough fermentations

In chapter **3.3** evidence is provided for the formation of hetero-oligosaccharides (HeOS) by *L. sanfranciscensis* during growth in sourdough. To identify the various HeOS based on HPAEC-IPAD analysis, HeOS standards were synthesised by enzymatic reactions with *L. sanfranciscensis* levansucrase in the presence of the corresponding acceptor carbohydrates. Preliminary evidence was provided that hitherto undescribed tri-, tetra- and pentasaccharides are formed by levansucrase in the acceptor reaction with maltotriose, maltose, arabinose, and xylose. Because external standards for the various HeOS are unavailable, HeOS previously reported as products from transfructosylation were identified by comparison of the products obtained from the levansucrase reaction in the presence and absence of fructosyl-acceptors. The HeOS 1^F-β-fructosylraffinose, 1^F-β-fructofuranosylmaltotriose, 1^F-β-fructofuranosylmaltose, xylsucrose, 1^F-β-fructosyl-xylsucrose, and arabsucrose were characterized from the levansucrase reactions with the corresponding acceptor carbohydrates (26, 38, 39, 75, 97). These investigators found identical HeOS using levansucrase enzymes from the phylogenetically diverse bacteria *Aerobacter levanicum* (member of *Enterobacteriaceae*, current taxonomic position unknown), *Bacillus subtilis*, and *Rahnella aquatilis (*previously: *Erwinia herbicola*)*.* Bacterial levansucrases share a substantial degree of homology in their primary sequences and the catalytic residues and substrate binding domains are well conserved (62, 105, 107,). The catalytic properties of the *L. sanfranciscensis* levansucrase with respect to substrate specificity, polymer- and oligomer formation are well in agreement with kinetic data from other bacterial levansucrases. Therefore, there is little doubt that the major HeOS from the acceptor reactions with *L. sanfranciscensis* levansucrase are identical to those described previously using levansucrases from other bacterial species. To demonstrate the presence of hitherto undescribed, heterologuous tetra- and pentasaccharides, compounds with the appropriate molecular weight were separated from mono-, di- and trisaccharides by two consecutive GPC runs. The determination of the

monomer composition of this oligomers demonstrated for xylose and arabinose as acceptor molecules that HeOS with a DP of 3 or more were present in the HeOS mixture prepared by GPC. In the case of raffinose and maltotriose, HeOS with a DP of more than four were formed. The characterization of these oligomers, however, requires improved analytical methods to achieve the separation of the complex mixture of oligosaccharides generated by the levansucrase enzyme.

The ability of *L. sanfrancsicensis* to form HeOS in wheat sourdough was determined by comparing the oligosaccharide patterns in dough fermented with the isogenic, levansucrase-positive and levansucrase-negative strains TMW 1.392 and TMW 1.392Δlev, respectively. *L. sanfranciscensis* TMW 1.392Δlev is unable to metabolise sucrose because the levansucrase is the only enzyme in the parent strain acting on sucrose. The development of cell counts, lactate levels, and pH values during fermentation with these two strains was virtually identical. Therefore, any effect of flour enzymes on the formation or removal of endogenous oligosaccharides was identical in the two doughs, and differences in the levels of oligosaccharides result from levansucrase activity. Indeed, the oligosaccharide levels in these two doughs were identical after removal of FOS and HeOS with yeast invertase. The unidentified carbohydrate generated by treatment of dough extracts with yeast invertase may result from the hydrolysis of FOS originating from flour, or from the transferase activity of invertase (26, 27). Based on the comparison of oligosaccharides present in extracts from doughs fermented with *L. sanfranciscensis* TMW 1.392 and TMW 1.392Δlev, at least five FOS or HeOS could be identified which result from levansucrase activity. Maltose is the major carbohydrate in either wheat or rye doughs after sourdough fermentation and is therefore readily available as fructosyl-acceptor. Substantial levels of arabinose, xylose, and maltodextrins are additionally present (7, 88). Their levels are enhanced by the use of pentosanases and amylases in bread applications (22). The formation of 1-kestose was reported in 3.2. Arabsucrose and 1^F-β-fructofuranosyl-maltose were identified here by use of

the standards synthesised in enzymatic reactions. Leavening of bread doughs may be achieved through the metabolic activity of heterofermentative lactic acid bacteria in the absence of yeasts (29). In industrial practice, bakers yeast is the leavening agent most commonly used. Because FOS and HeOS produced in dough by the *L. sanfranciscensis* levansucrase are hydrolysed by yeast invertase, these compounds are not likely to be present in bread. Nevertheless, improved knowledge on the formation of FOS and HeOS during growth of levansucrase-positive lactobacilli may enable the directed generation of cereal-based, functional ingredients for food and feed use and the formation of HeOS by *L. sanfranciscensis* needs to be taken onto account when effects of sucrose metabolism of this strain on bread and dough quality are studied. To date, some studies give evidence for an influence of HoPS on bread and dough quality (10, 32). Grepka has shown changes of rheological parameters, when purified levan of *L. sanfranciscensis* was added in concentrations of 0.5 % (w/w) and 1.0 % (w/w) (32). Furthermore, glucan of *L. curvatus* TMW 1.624, which was identified as dextran by nuclear-magnetic-resonance (NMR)-spectroscopic measurements, showed a clear improvement of water resorptions and caused also an increase of bread volumes of up to 15 % (32). Facing the variety of changes in the fermentation pattern of sucrose-containing sourdoughs due to the activity of levansucrase, it is fair to assume that influences on taste, rheological parameters and volume of bread are numerous and are not only attributable to the formation of EPS. In accordance, Brandt et al. observed a more effective improvement of the bread quality by *in situ* formed levan in sourdoughs, than by the addition of purified levan to control sourdoughs (10).

The characterization of the levansucrase described in 3.3 will enable the optimization of dough fermentations with the aim to improve bread quality through increased levels of levan and / or kestose in dough. Furthermore, the comparison of the performance of the levansucrase deletion mutant strain and the wild type strain in baking applications will result

in an improved understanding of the effect of levan and kestose on dough rheology, bread texture, and bread staling.

4.4 Screening of lactobacilli isolated from the intestinum of ducks and pigs

The genus *Lactobacillus* has been proposed to have a significant potential to produce various HoPS (102, 51). As described in **3.1**, a total of 107 *Lactobacillus* strains from sourdough was screened for EPS production and a high proportion of HoPS-producing strains was found especially among those species which can also be found frequently in gastrointestinal ecosystems, e.g. *L. reuteri* and *L. pontis*. In this work, *Lactobacillus* strains from defined intestinal habitats, ducks and pigs, were screened for the ability to produce HoPS as well as oligosaccharides, and genes coding for fructosyl- and glucosyltransferases in HoPS-positive strains were identified. Remarkably, a high proportion of the isolates, 30 % and 26 % of lactobacilli of duck and pig origin, respectively, exhibited glucan or fructan production from sucrose.

It is remarkable that cereal and intestinal lactic microflorae share common properties not only with respect to their composition, but also with respect to metabolic properties. Moreover, it has repeatedly been reported that cereal lactobacilli representative for type II cereal ecosystems exhibit certain probiotic trains *in vitro* and *in vivo* (109, 33, 30, 77). It remains unknown whether EPS formation provides an ecological advantage to lactobacilli in the intestinal tract. On the one hand, EPS formation was shown to improve the starvation survival of *Leuconostoc mesenteroides* at extremes of pH (42). In analogy, EPS may enhance the survival of lactobacilli during gastro-intestinal transit. On the other hand, a facilitation of the adhesion to animal crop and mucosa cells, similar to the adhesion of oral streptococci to the tooth enamel using biofilm formation, is not likely because sucrose is not present in the gut.

A PCR based screening method was recently proposed as an efficient tool to detect HoPS-producing lactobacilli (52)**.** Ftf and gtf genes could be detected by PCR in eight out of 10

intestinal HoPS producing strains as described in **3.4**. Two glucan forming strains were PCR-negative. The screening among sourdough isolates as described in **3.1** revealed positive PCR reactions with 6 of 15 fructan –forming strains. Thus, a screening exclusively based on PCR techniques may lead to false negative results, either because of an inadequate primer design or because target sequences are less conserved than the corresponding sequences in known bacterial glycosyltransferases. Furthermore, silent genes encoding Ftfs may result in false positive results (105). Two novel glycosyltransferase genes could be detected in our work based on partial amino acid sequences of their catalytic domains. In *L. animalis*, it was verified that the gtf gene is expressed and therefore contributes to the glucan formation observed in this organisms. This finding further substantiates previous reports that a large variety of GTF and FTF enzymes is found in the genus *Lactobacillus* that may be used for the production of polymers with a wide range of different glycosidic bonds and various branching patterns (52).

In addition to the hydrolysis of sucrose and polymer synthesis, Ftfs and Gtfs from streptococci and *Leuconostoc* spp. catalyse the synthesis of oligosaccharides from sucrose (64). As shown in **4.4**, all tested HoPS-positive lactobacilli were also able to produce oligosaccharides. *L. acidophilus* strains produced the prebiotic trisaccharide kestose, and *L. reuteri* strains produced GOS which remain to be characterized on structural level. This is the first description of oligosaccharide-synthesis by glucanforming lactobacilli. The large number of HoPS positive strains among LAB of intestinal origin makes these organisms an excellent source of enzymes for industrial production of poly- and oligosaccharides.

HoPS of the levan- and inulintype, as well as kestose and nystose have been described as prebiotic (8, 16, 46). Regarding the widespread use of *L. animalis*, *L. reuteri* and *L. acidophilus* as starter cultures for food fermentation, and as probiotics, the ability of these organisms to produce extracellular oligosaccharides and polymers with prebiotic features creates interesting possibilities to produce fermented food of food additives that possess

synbiotic properties, and opens new perspectives in the application about pro-, pre- and synbiotics.

4.5 Molecular characterization of a glucansucrase from *L. animalis*

The glucansucrase from *L. animalis* was characterized on a molecular level. The amino acid sequence reflected a structure that was similar to those from glucansucrases that have recently been described (64, 51). Furthermore, the catalytic domain appeared to be well conserved, which allows the conclusion that mode of catalytic activity and substrate specifity is in accordance with those from other bacterial glucosyltransferases. Remarkably, the levansucrase from *L. sanfranciscensis* and the glucosyltransferase from *L. animalis* both exhibit a N-terminal leader peptide which could be identified as leader peptide, as well as an extensive variable region with no apparent functionality, followed by the catalytic domain. The catalytical residues are followed by a region that could be identified as glucan binding domain. Different from the levansucrase of *L. sanfranciscensis*, no C-terminal cell wall anchor domain could be identified, which is also in accordance with the structure known from other bacterial glucosyltransferases (64). Thus, it can be estimated that the glucosyltransferase from *L. animalis* TMW1.971 is an exoenzyme that is secreted into the environment.

As a noticeable fact, the gene sequence was not terminated by a corresponding signal, but was followed by a second open reading frame. ORF 2 had a N-terminal peptide sequence with striking similarities to the leader peptide from *L. sanfranciscensis* levansucrase, so it is tempting to speculate whether ORF 2 is also coding for an enzyme involved in HoPS production and ORF 1 and ORF 2 are members of a “glycansucrase isle” within the genome of *L. animalis* TMW 1.971.

L. animalis is a bacterium which has been described as probiotic organism (53). Investigations concerning the metabolic abilities to produce HoPS with prebiotic properties and the molecular background is of high interest.

5 Summary and concluding remarks

The potential of lactobacilli to produce homopolysaccharides of the fructan and glucan type remained unknown up to the late 1990`s. A first report on the ability of lactobacilli to produce fructans and glucans was given by van Geel-Schutten et al. in 1998, who performed a screening among 140 lactobacilli in medium with high sucrose content, revealing several strains that produced HoPS from sucrose in amounts of more than 1000 mg x L^{-1} (102). Recently, fructan formation was described for the sourdough isolate *L. sanfranciscensis* TMW 1.392 (47).

To evaluate the distribution and diversity of EPS formation among sourdough lactic acid bacteria, a screening was performed among 107 *Lactobacillus* strains with cereal origin to identify EPS producers as well as the monosaccharide composition of these polysaccharides and the existence of genes coding for levansucrases.

Twenty-two of the 107 strains were found to produce EPS with a molecular weight of more than 10^6, comprising 15 fructan producers, 4 glucan producers and 3 strains producing heteropolysaccharides of unidentified composition. Fermentations of sucrose-containing doughs were performed with a selection of EPS-positive strains, revealing *in situ* EPS formation in all cases.

In 6 of the 15 fructan-forming strains fragments of levansucrase genes with the size of 800 bp were identified by PCR with degenerated primers, targeting conserved domains of known bacterial enzymes. Analyses of the gene fragments revealed similarities of the deduced amino acid sequences with enzymes of *S. mutans* and *S. salivarius*. Furthermore, the expression of the levansucrase gene in *L. sanfranciscensis* TMW 1.392 was shown by RT-PCR, targeting the levansucrase gene.

Based on these findings, the complete levansucrase gene sequence of *L. sanfranciscensis* TMW 1.392 was characterized with an inverse PCR approach, which revealed an ORF of 2637 bp, encoding an enzyme with high homologies to a levansucrase and an inulosucrase

from *L. reuteri*. The corresponding aa sequence showed a N-terminal leader peptide, harboring a secretion signal and a proteolytical cleavage site, followed by a domain of repeating units with no homologies to other known proteins.

The well conserved catalytical domain showed a clear membership of the glycoside hydrolase family of the levansucrases and invertases, followed by a C-terminal cell wall anchor domain. The enzyme was heterologously expressed in *E. coli* with and without truncation of the N-terminal repeating units and characterized biochemically. The *L. sanfranciscensis* levansucrase clearly showed invertase and levan-forming activity as well as kestose-forming activity, the latter exceeded the levan formation at sucrose concentrations above 50 mM. The range of products was influenced by the sucrose concentrations, but was only slightly affected by changing of temperatures and pH. No differences could be observed between the native and the truncated enzyme regarding K_m-values, V_{max} and the range of products.

To investigate the influence on sucrose metabolism in dough and medium, a levansucrase negative mutant was constructed by integration of the nonreplicating plasmid PME-1_knock, harboring a fragment of the *L. sanfranciscensis* levanucrase, into the genomic DNA of *L. sanfranciscensis* TMW 1.392. The levansucrase negative strain *L. sanfranciscensis* 1.392Δlev showed neither kestose and levan formation, nor sucrose utilization in sucrose containing medium, providing evidence that levansucrase is the only enzyme that is responsible for sucrose utilization in this strain. Dough experiments revealed comparable growth rates of both strains, but completely different fermentation patterns in sucrose containing doughs, regarding the concentrations of mannitol, free glucose, ethanol and acetic acid. In doughs with sucrose, levan and kestose were produced by the wild strain, but not by the mutant. This is the first report of kestose formation in a food fermentation.

Bacterial levansucrases have been described in literature to produce not only levan and fructooligosaccharides when acting on sucrose as substrate, but also to form heterooligosaccharides in presence of other sugars that may act as acceptor molecules (38, 39,

26). Incubation of the purified levansucrase enzyme from *L. sanfranciscensis* with sucrose in combination with arabinose, xylose, maltose, maltotriose and raffinose yielded in the HeOS`s arabsucrose, xylsucrose, fructofuranosylmaltose, fructofuranosylmaltotriose and fructosylraffinose, which could be assigned by an analytical approach combining HPAEC-IPAD and GPC.

Using these enzymatically designed sugars as external standards and TMW 1.392Δlev as negative control, it could be demonstrated that fructofuranosylmaltose was present in sucrose containing doughs fermented with *L. sanfranciscensis* TMW 1.392, together with four other oligosaccharides that could not be identified unambiguously.

The screening showed an accumulation of EPS forming organisms among those lactobacilli that can also be found in the intestinum and those that are phylogenetically closely related to intestinal species. Therefore, lactobacilli that have been isolated from the duck's and pig's intestinum were screened for EPS-formation and oligosaccharide synthesis. 30 % and 26 % of lactobacilli of duck and pig origin, that have been identified by molecular techniques, exhibited glucan and fructan formation from sucrose. The fructanforming organisms, namely strains of the species *L. acidophilus*, showed furthermore formation of fructose and kestose and oligosaccharides could also be detected in supernatants of glucanforming *L. reuteri* strains, which showed retention times on HPAEC-IPAD different from those of kestose and nystose. This is the first description of oligosaccharide synthesis by glucanforming lactobacilli.

Ftf- and gtf- genes could be identified in eight of ten intestinal EPS producing strains. The gtf gene fragment from *L. animalis* TMW 1.971 was investigated in more detail, revealing an ORF of 4725 bp, encoding a putative glucansucrase gene with homologies to gtfs from *Lc. mesenteroides*, exhibiting a N-terminal signal peptide, a variable domain, a catalytic domain as well as a glucan binding domain located at the C-terminus. Because of the missing cell-wall anchor region, it is very likely that *L. animalis* 1.971 gtf is secreted into the environment.

Finally, it can be stated that among cereal and intestinal lactobacilli exists a huge potential for the *in situ* formation of homopolysaccharides as well as fructooligosaccharides, glucooligosaccharides and heterooligosaccharides in technological relevant amounts in food fermentations. These carbohydrates may face the demands for sugar replacers to be not cariogenic, having a low glycemic response and low caloric values and additionally may possess prebiotic properties. Moreover, polysaccharides of the levan- and dextran-type are known to influence the rheological behaviour of food. In the case of the levansucrase from *L. sanfranciscensis*, it has been shown that the spectrum of sugar-products from sucrose can be influenced by the sucrose concentration as well as by the presence of acceptor-carbohydrates such as maltose and arabinose. This creates possibilities for the design of functional cereal foods containing mixtures of diverse *in situ* formed sugars with prebiotic properties as well as texturising features and rheological properties, that face the growing consumers demand for "natural" and "clean label" foodstuff. The occurence of EPS- and oligosaccharide forming in intestinal organisms that are known to be probiotic opens new perspectives for the applications of synbiotic lactobacilli in food fermentations, and rises the question of a possible role of EPS in the microbial community of the intestinum.

6 Zusammenfassung und Ausblick

Die Befähigung von Lactobazillen zur Bildung von Homopolysacchariden war bis in die späten 1990er weitgehend unbekannt. Erste Untersuchungen zur Bildung von Fructanen und Glucanen durch diese Organismengruppe wurden von van Geel Schutten et al. durchgeführt, welche 140 Lactobazillen auf Medium mit hohem Saccharosegehalt zogen und bei einigen Stämmen die Bildung von EPS in Mengen von mehr als 1 g x L^{-1} detektierten (102). Etwa gleichzeitig wurde von Korakli et al. über die Bildung von hochmolekularem Fructan durch das Sauerteigisolat *L. sanfranciscensis* TMW 1.392 berichtet (47).

Um die Verbreitung der EPS-Bildung unter Sauerteigbakterien und die Zusammensetzung der gebildeten Polysaccharide zu charakterisieren, wurden 107 Stämme der Gattung Lactobacillus auf saccharosehaltigem Medium gezüchtet und auf die Bildung von Homopolysacchariden hin untersucht. Weiterhin wurde die Monomerzusammensetzung der detektierten Polysaccharide analysiert und nach Genen gesucht, die für Levansucrasen codieren. Unter den 107 Stämmen konnten 22 identifiziert werden, die EPS mit einem Molekulargewicht von mehr als 10^6 bildeten. 15 bildeten Fructan, vier Glucan und drei Stämme bildeten Heteropolysaccharide deren Zusammensetzung nicht weiter analysiert wurde. Mit einer Auswahl dieser Stämme wurden Fermentationen in saccharosehaltigen Teigen durchgeführt. In allen Teigen konnte eine EPS-Bildung während der Fermentation nachgewiesen werden. In sechs der 15 Fructanbildner konnten mittels PCR und degenerierten Primern, die konservierte Bereiche von bekannten bakteriellen Levansucrasen als Zielsequenz hatten, 800 bp große Gen-Fragmente detektiert werden, die durch Homologievergleiche als Levansucrasen identifiziert wurden. Ähnlichkeiten bestand mit Enzymen von *S. mutans* und *S. salivarius*. Die Expression dieses Gens in *L. sanfranciscensis* TMW 1.392 wurde mit einer RT-PCR bestätigt.

Ausgehend von diesen Beobachtungen wurde das Levansucrase Gen von *L. sanfranciscensis* TMW 1.392 vollständig charakterisiert. Mittels inverser PCR-Techniken wurde ein offener

Leserahmen in der Größe von 2637 bp gefunden, welcher für ein Enzym mit großen Ähnlichkeiten zu einer Levansucrase und einer Inulosucrase von *L. reuteri* codierte. Die Analyse der Aminosäurensequenz ergaben ein N-terminales Sekretionssignal mit einer proteolytischen Schnittstelle, gefolgt von einer Domäne mit sich wiederholenden Bereichen, die keinerlei Homologie zu bekannten Proteinen aufwies. Weiterhin zeigte sich eine gut konservierte katalytische Domäne und ein Zellwandanker am C-Terminus des Enzyms.
Das Enzym wurde einmal vollständig und einmal ohne die N-terminale Domäne in *E. coli* kloniert und heterolog exprimiert. Die gereinigte Levansucrase wurde biochemisch charakterisiert, wobei sich sowohl Invertase-Aktivität, als auch fructanbildende und kestosebildende Aktivität nachweisen liesen. Letztere war bei Saccharose-Konzentrationen größer 50 mM gegenüber der Levanbildung dominierend. Das Spektrum an gebildeten Produkten konnte durch die Saccharosekonzentration maßgeblich beeinflusst werden, durch eine Änderung von pH und Temperatur aber nur unwesentlich. Zwischen der trunkierten und der nativen Enzymvariante konnte kein Unterschied beobachtet werden hinsichtlich der K_m-Werte, der maximalen Umsatzrate und des Spektrums an gebildeten Produkten.
Um den Einfluss der Levansucrase auf den Saccharosestoffwechsel zu studieren, wurde eine Levansucrase-negative Mutante von *L. sanfranciscensis* TMW 1.392 konstruiert. Hierfür wurde ein nicht-replizierendes Plasmid, in welches ein Fragment der Levansucrase kloniert worden war, mittels homologer Rekombination in das Genom von *L. sanfranciscensis* TMW 1.392 integriert und das Levansucrase-Gen somit zerstört. Die Mutante *L. sanfranciscensis* TMW 1.392Δlev zeigte in saccharosehaltigem Medium weder Levan- noch Kestosebildung und war zur Verstoffwechslung von Saccharose nicht befähigt. Levansucrase ist somit das einzige Enzym in *L. sanfranciscensis* TMW 1.392, welches die Verstoffwechslung von Saccharose ermöglicht. Teigexperimente zeigten unterschiedliche Fermentationsmuster von *L. sanfranciscensis* TMW 1.392 und TMW 1.392Δlev in saccharosehaltigen Teigen bei vergleichbaren Wachstumsraten. Die Konzentrationen an Mannit, freier Glucose, Ethanol und

Essigsäure waren in Teigen mit Saccharose stark verschieden, jedoch in Teigen ohne Zusatz von Saccharose vergleichbar; in Teigfermentationen mit dem Wildstamm erfolgte die Bildung von Kestose und EPS, in Fermentationen mit der Mutante nicht. Dies ist der erste Nachweis von Kestosebildung in einer Lebensmittelfermentation.

Wie durch Literaturdaten belegt wird, bilden bakterielle Levansucrasen neben Levan und Fructooligosacchariden auch Heterooligosaccharide in Gegenwart von geeigneten Zuckern als Akzeptoren für die freigesetzten Fructosemoleküle (26, 38, 39). Bei Inkubation der gereinigten Levansucrase mit Saccharose in Kombination mit Arabinose, Xylose, Maltose, Maltotriose und Raffinose entstanden die Heterooligosaccharide Arabinosucrose, Xylsucrose, Fructofuranosylmaltose, Fructofuranosylmaltotriose und Fructosylraffinose, was analytisch mittels HPAEC-IPAD und Gelpermeationschromatographie gezeigt werden konnte. Mit diesen enzymatisch generierten Standards und TMW1.392Δlev als Negativkontrolle konnte nachgewiesen werden, daß Fructofuranosylmaltose zusammen mit vier anderen unidentifizierten Heterooligosacchariden in Fermentationen von saccharosehaltigen Teigen durch *L. sanfranciscensis* TMW 1.392 gebildet wird.

Wie die Untersuchungen an getreideassoziierten Lactobazillen ergeben hatte, ist die Fähigkeit EPS zu bilden unter den Arten gehäuft, die auch in intestinalen Systemen vorkommen bzw. mit diesen phylogenetisch eng verwandt sind. Daher wurden in einem weiteren Ansatz Lactobazillen, die aus dem Faeces von Schweinen und Enten isoliert und mit molekularbiologischen Methoden identifiziert worden waren, auf EPS- und Oligosaccharidbildung untersucht. 30 % und 26 % der untersuchten Stämme aus Ente und Schwein zeigten Glucan- und Fructanbildung; weiterhin zeigten alle untersuchten EPS-Bildner Oligosaccharidbildung.

Ausgehend von dem Genfragment der Dextransucrase von *L. animalis* wurde die Gesamtsequenz analysiert. Mit inverser PCR wurde ein Leserahmen in der Größe von 4752 bp identifiziert; Sequenzanalysen zeigten ein N-terminales Sekretionssignal, gefolgt von einer

variablen Domäne, einer katalytischen Region und einer glucanbindenden Domäne am C-Terminus. Da in der Sequenz kein Zellwandanker identifiziert wurde, handelt es sich mit großer Wahrscheinlichkeit um ein Protein, das in den Extrazellulärraum sekretiert wird.
Abschließend kann das Resümee gezogen werden, daß es innerhalb der Gattung Lacobacillus ein großes Potential für die *in situ* Bildung von Homopolysacchariden, Fructooligosacchariden, Glucooligosacchariden und Heterooligosacchariden gibt, die in Lebensmittelfermentationen in technologisch relevanten Mengen gebildet werden können. Diese Zucker könnten ganz oder teilweise die Anforderungen an Saccharoseaustauschstoffe erfüllen, welche nicht kariogen, einen niedrigen glykemischen Effekt sowie einen geringen Brennwert haben sollten; zudem werden den Fructooligos und Fructanen prebiotische Eigenschaften zugeschrieben. Es ist weiterhin bekannt, daß Polysaccharide vom Levan- und Dextrantyp das rheologische Verhalten von Lebensmitteln beeinflussen.
Im Fall der Levansucrase von *L. sanfranciscensis* konnte gezeigt werden, daß das Spektrum an gebildeten Produkten durch die Konzentration an Saccharose beeinflußt werden kann, ebenso wie durch das Vorhandensein von geeigneten Zuckern als Fructosylakzeptoren, wie zum Beispiel Maltose und Arabinose. Basierend auf diesen Erkenntnissen ist die Entwicklung funktioneller fermentierter Lebensmittel auf Getreidebasis ermöglicht, die eine Mischung aus alternativen Zuckerstoffen enthalten und zudem die wachsende Forderung der Verbraucher nach „natürlicher“ und „zusatzstofffreier“ Nahrung erfüllen. Durch den Einsatz Levansucrase-positiver Sauerteigbakterien in saccharosehaltigen Teigen läßt sich zudem der Gehalt an Acetat deutlich erhöhen, was Geschmack und Haltbarkeit des Brotes positiv beeinflußt.
Die Befähigung probiotischer, intestinaler Lactobazillen zur Bildung prebiotischer EPSe und Oligosaccharide eröffnet neue Perspektiven in der Entwicklung synbiotischer fermentierter Lebensmittel und wirft die Frage auf nach einer möglichen Rolle dieser Substanzen in der mikrobiologischen Lebensgemeinschaft des Darms.

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8 List of publications that resulted from this dissertation

Original papers

M. Tieking, M. A. Ehrmann, R. F. Vogel, and M. G. Gänzle. 2004. Molecular and functional characterization of a levansucrase from the sourdough isolate *Lactobacillus sanfranciscensis* TMW 1.392. Appl. Microbiol. Biotechnol. **66:**655-663

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Tieking, M., M. Korakli, M. A. Ehrmann, M. G. Gänzle, and R. F. Vogel. 2003. In situ production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. Appl. Environ. Microbiol. **69:**945-52.

(Dough analytics in this work were performed by Dr. Maher Korakli, from the "Lehrstuhl für Technische Mikrobiologie", Freising, Germany.)

Tieking, M., M. Korakli, S. Kaditzky, R. Valcheva, M. G. Gänzle, and R. F. Vogel. Exopolysaccharides and glucoseoligosaccharides of intestinal lactobacilli. J. Appl. Microbiol., accepted for publication.

(Isolation and characterization of lactobacilli with pig origin in this work were performed by Dr. Maher Korakli from the "Lehrstuhl für Technische Mikrobiologie", Freising, Germany and Rossica Valcheva from the Laboratoire de Microbiologie Alimentaire et Industrielle QM2A, ENITIAA, Nantes Cedex, France.)

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