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Novel fructans from acetic acid bacteria

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ABBREVIATIONS

A.	*Acetobacter*
aa	amino acid
AAB	acetic acid bacteria
AF4	asymmetric flow field-flow fractionation
Amp	ampicillin
APS	ammonium persulfate
Asn	asparagine
Asp	aspartic acid
bp	base pair
C	carbon
cfu	colony forming units
cm	centimeter
COSY	^{1}H-^{1}H two-dimensional NMR homonuclear chemical shift correlation
Cys	cysteine
Da	Dalton
dg	degenerate
DNA	deoxyribonucleic acid
D_2O	deuterium oxide
DP	degree of polymerization
DSM	Deutsche Sammlung von Mikroorganismen
DTT	dithiothreitol
E.	*Escherichia*
EDTA	ethylene diamine tetraacetic acid
EPS	exopolysaccharide
Fig.	figure
FOS	fructooligosaccharide

FTF	fructosyltransferase
ftf	fructosyltransferase gene
g (centrifugation)	relative centrifugal force
g (weight)	gram
G.	*Gluconobacter*
Ga.	*Gluconacetobacter*
Gln	glutamine
Glu	glutamic acid
GTF	glycosyltransferase
H	hydrogen
h	hour
HePS	heteropolysaccharide
HMQC	^{13}C-^{1}H two-dimensional NMR; heteronuclear multiple quantum coherence
HoPS	homopolysaccharide
HPLC	high-performance liquid chromatography
HPMC	hydroxypropylmethylcellulose
Hz	Hertz
IS	insertion sequence
K	Kelvin
k	constant
L	liter
L.	*Leuconostoc*
LAB	lactic acid bacteria
LB	lysogeny broth
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
Leu	leucine
M	molar
m	meter

MALS	multi-angle light scattering
M_i	molar mass
mi	modified inverse
min	minute
mMRS	modified deMan, Rogosa, Sharp medium
M_N	number average molar mass
msf	modified site-finding
msp	modified single primer
M_W	weight average molar mass
MWCO	molecular weight cut off
M_Wi	molecular weights
N	Newton
N.	*Neoasaia*
Na	natrium/sodium
NaG	natrium/sodium-gluconate medium
NBRC	biological resource center
NCBI	national center for biotechnology information
n_i	number of molecules
NMR	nuclear magnetic resonance
nt	nucleotide
OD	optical density
o/n	overnight
ORF	open reading frame
p	probability
PAGE	polyacrylamide gel electrophoresis
PAPE	phage associated promoter element
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PDB	protein data bank
PDI	polydispersity index
ppm	parts per million
$R_G i$	radiuses of gyration
RI	refractive index
rms	root mean square
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
RT	retention time
s	second
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
si	standard inverse
sp.	*species*
TCA	tricarboxylic acid
TEMED	tetramethylethylendiamine
T_m	melting temperature
TMW	Technische Mikrobiologie Weihenstephan
U	unit
UDP	uridin diphosphate
V	volt
v_G	hydrodynamic coefficient
v/v	volume/volume
vs.	versus
w/v	weight/volume
w/w	weight/weight
Z.	*Zymomonas*

CONTENTS

1. INTRODUCTION

1.1 Phylogeny and ecology of acetic acid bacteria

Acetic acid bacteria (AAB) are Gram-negative, obligate aerobic, mesophilic rods, which are usually isolated from acidic, alcoholic and sugary environments, such as vinegar, spoiled beverages, fruits, nectars or flowers, due to their unique ability to incompletely oxidize a wide range of alcohols and sugars to their corresponding acids (Cleenwerck & De Vos, 2008). Based on comparative analysis of 16S rRNA gene sequences, they belong to the class of α-proteobacteria and, therein, to the family of *Acetobacteraceae*. On generic level, they are sub-divided into the genera *Acetobacter, Gluconobacter*, *Gluconacetobacter, Granulibacter*, *Asaia, Swaminathania*, *Kozakia*, *Neoasaia, Acidomonas*, *Saccharibacter*. While the number of species descriptions among AAB is permanently increasing, new isolates are hardly determinable to the species level by using the 16S rRNA gene sequence as sole phylogenetic marker (Jakob et al., 2012). The main characteristic of AAB is their ability – according to their name – to convert ethanol *via* two successive oxidative steps to acetic acid. Therefore, members of the genus *Frateuria*, which phylogenetically (16S rRNA gene) belong to the γ-proteobacteria, are additionally considered as AAB because of their oxidative metabolism (Yamada & Yukphan, 2008). To date, the three main genera of AAB, i. e. those with the highest number of described species, are *Acetobacter*, *Gluconobacter* and *Gluconacetobacter*. Whereas *Acetobacter* and *Gluconacetobacter* strains prefer alcohols as substrates for incomplete oxidations and, therefore, are commonly detected in alcoholic habitats, *Gluconobacter* strains are specialized on incomplete oxidations of sugars and mainly occur in sugary environments (Deppenmeier & Ehrenreich, 2009). *Acetobacter* and *Gluconobacter* strains exhibit physiological differences among each other regarding respiratory ubiquinone types (9 and 10, respectively) and over-oxidation of produced acetate to CO_2 and H_2O (characteristic for *Acetobacter*). *Gluconacetobacter* strains again are physiological "intermediates" of these two genera due to their ability for over-oxidation of acetate and their common ubiquinone type 10 (Yamada & Yukphan, 2008). In addition to their occurrence in sugary/alcoholic habitats, some AAB are symbiotic or pathogenic for insects (Crotti et al., 2010; Roh et al., 2008). Some studies report about clinical AAB isolates, which were abundant in patients with underlying chronic diseases and/or indwelling devices (Alauzet et al., 2010). Several members of the genera *Acetobacter*, *Gluconacetobacter* and *Swaminathania* are associated with plants, thereby being able to fix atmospheric nitrogen and to act as plant growth promoting bacteria (Pedraza, 2008). Traditional symbiotic associations of AAB with other microorganisms, such as lactic acid bacteria (LAB) and yeasts, are found in kombucha or kefir (Dufresne & Farnworth, 2000; Gulitz et al., 2011).

1.2 Physiology of AAB

The physiology/metabolism of AAB was barely understood for several years and was highlighted by genome-sequencing of *G. oxydans* 621H (Prust et al., 2005). This strain lacks complete glycolytic and

tricarboxylic acid (TCA) pathways and, therefore, uses diverse membrane-bound dehydrogenases for rapid incomplete oxidations of primary/secondary alcohols as well as different sugars. The resulting products (gluconic/acetic acid(s), aldehydes, ketones) are almost completely secreted into the extracellular space, thereby lowering the extracellular pH, which again inhibits the growth of competing microorganisms (Deppenmeier & Ehrenreich, 2009).

Incomplete oxidations are predominantly performed by membrane-bound dehydrogenases, which are anchored in the cytoplasmic membrane and whose active centers are oriented into the periplasmic space. Therefore, substrates must not be transported into the cytoplasm, and reaction products do not accumulate in the cytoplasm but can subsequently be secreted into the extracellular milieu. In this way, membrane-bound dehydrogenases can rapidly transfer released electrons into the respiratory chain for reduction of oxygen and energy conservation. The detailed mechanisms for oxygen consumption and energy generation are not clearly understood because *Gluconobacter* and its close relatives lack functional complexes such as cytochrome c oxidase (Matsushita et al., 1994). Moreover, theoretical reconstruction of the respiratory chain (derived from the genome sequence) of *G. oxydans* 621 H revealed a generally low potential for translocation of protons across its cytoplasmic membrane due to the presence of few oxidases, which can pump protons in the course of redox reactions/oxygen reduction (Deppenmeier & Ehrenreich, 2009). Alternatively, *G. oxydans* 621 H has the ability to transport sugars and polyols into the cytoplasm and to use functional pentose phosphate/Entner Doudoroff pathways as well as a set of soluble dehydrogenases for “intermediary” metabolism/energy generation.

Peters et al. (2013a) demonstrated, that acetate can alternatively be produced by *G. oxydans* 621H *via* decarboxylation of pyruvate and subsequent oxidation to acetate. The existence of this secondary pathway for acetate formation was also reported for *Acetobacter pasteurianus* (Chandra Raj et al., 2001). Over-oxidation of acetate to CO_2 and H_2O seems to be typical for members of the genera *Acetobacter*, *Gluconacetobacter*, *Acidomonas*, *Asaia* and *Kozakia* in contrast to *Gluconobacter* strains (Deppenmeier & Ehrenreich, 2009). However, there are different activity levels (no, weak or strong oxidizers) regarding incomplete oxidation of ethanol to acetate and over-oxidation of acetate to CO_2 and H_2O within strains of the same species (Yamada et al., 2000; Lisdiyanti et al., 2002). Therefore, strains should be individually checked with respect to these features. Moreover, AAB have developed some further, strain-specific mechanisms for (acetic) acid resistance, such as e. g. intracellular detoxification of acetic acid by citrate synthase or active export of acetic acid to the extracellular space *via* efflux pumps (Raspor & Goranovic, 2008). The unique metabolism of AAB enables them to rapidly consume excessive substrates in sugary/alcoholic/acidic environments. However, the lack of complete pathways for highly effective aerobic respiration/energy generation typically leads to low biomass yields (Deppenmeier & Ehrenreich, 2009).

1.3 Biotechnological applications of AAB and their use in foods

As AAB are able to incompletely oxidize diverse sugars and alcohols by membrane-bound dehydrogenases, the concomitant accumulation of the respective reaction products in fermentation broths is exploited in several (food) biotechnological applications (Raspor & Goranovic, 2008). Although *Acetobacter* and *Gluconacetobacter* strains can usually over-oxidize acetate to CO_2 and H_2O (1.2), they are used for the production of vinegar, thereby efficiently and rapidly converting ethanol-containing solutions to acetic acid by membrane-bound alcohol-/aldehyd-dehydrogenases. On the contrary, *Gluconobacter* strains, which cannot over-oxidize and, therefore, prinicipally accumulate acetate (1.2), are commonly not suited/used for vinegar production due to their principal specialization on sugars and polyols (1.1) resulting in relatively low conversion yields of ethanol to acetate (Macauley et al., 2001). *Gluconobacter* strains are rather used as starters during manufacture (malt fermentation) of "Bionade", thereby efficiently oxidizing glucose to gluconic acids, which yield the typical taste of these beverages, by membrane-bound glucose/gluconate dehydrogenases (Kowalsky et al., 2011). Furthermore, strains of the genus *Gluconobacter* are actually applied for stereo- and regioselective bioconversions of special substrates, whose corresponding oxidative products gain much interest in the chemical/pharmaceutical industry. They are involved in the (enzymatic) production of vitamin C, (keto)gluconic acids, dihydroxyacetone, L-ribulose, D-tagatose, miglitol as well as further chiral aldehydes and acids (De Muynck et al., 2007).

Apart from these classical biotechnological applications, which mainly use individual, specialized strains for incomplete oxidation of certain substrates, the metabolic activity of AAB, coexisting with metabolic active LAB/yeasts, is traditionally used in some naturally fermented foods. In kombucha, a refreshing tea beverage, the microbial consortium of a so called tea fungus, consisting of several AAB and yeast species, ferments the drink. The tea fungus is structurally built up by different AAB strains (mainly *Gluconacetobacter xylinus*) capable of producing a cellulose network, in which the symbiotic microorganisms are located (Dufresne & Farnworth, 2000). Furthermore, AAB oxidize ethanol to acetic acid during curing of cocoa beans, which, in this way, can be further processed to cocoa or chocolate (Raspor & Goranovic, 2008). Moreover, AAB are commonly detected in fermented kefir beverages, actually without known functions (Gulitz et al., 2011).

1.4 Exopolysaccharide production by AAB

AAB are well known to produce diverse extracellular polysaccharides (EPSs) for biofilm formation (Deppenmeier & Ehrenreich, 2009). The most prominent EPS derived from AAB is water-insoluble cellulose (β-(1→4)-linked glucan), being the main component of “mother of vinegar” and “tea fungus”, which are commonly used as starter consortia to produce vinegar or kombucha, respectively. Such cellulose pellicles are formed by *Acetobacter* and *Gluconacetobacter* strains in static cultures, thereby naturally self-organizing/floating at surfaces of fermentation vessels for sufficient oxygen

supply. In this way, AAB keep up a controlled metabolic activity and stabilize themselves and other symbiotic microorganisms such as yeasts (kombucha). Due to their advantageous properties in comparison to plant derived celluloses (higher purity, higher molecular weight, higher crystallinity indices, higher tensile strengths and water holding capacities), microbial cellulose can be used in textile, paper, food and pharmaceutical industry as well as in waste treatment, broadcasting, mining and refinery (Chawla et al., 2009). Microbial cellulose is mainly recovered from strains of *Gluconacetobacter xylinus*, which polymerize UDP-glucose with the membrane-anchored enzyme cellulose synthase. Ribbon-like cellulose bundles consisting of individual cellulose chains are subsequently secreted to the outside of the cell, where they finally self-assemble into cellulose fibrils (Chawla et al., 2009). Moreover, different AAB strains produce strain-specific heteropolysaccharides (HePS), which can consist of monomers such as glucose, rhamnose, galactose, mannose and glucuronic acid (Kornmann et al., 2003; Serrato et al., 2013; Ali et al., 2011). HePS such as xanthan usually exhibit complex (branched) structures, which are more often desired in food formulations due to their thickening, gelling and stabilizing properties at low concentrations (De Vuyst & Degeest, 1999). While biosynthetic pathways of HePS in AAB are barely investigated, a set of intracellular isomerases and sugar-activating enzymes was supposed to mediate gluconacetan biosynthesis in *Gluconacetobacter xylinus* I-2281 (Kornmann et al., 2003). An operon coding for pol genes (polABCDE) is responsible for pellicle formation (HePS: glucose, galactose, rhamnose) in *Acetobacter tropicalis* SKU1100. Disruption of polB yielded pellicle negative strains with higher sensitivity against acetic acid (Deeraksa et al., 2005). Furthermore, unique dextran dextrinases have been identified in different *Gluconobacter* strains, which catalyze the formation of high molecular weight dextrans (α-(1→6)-linked glucans) from maltodextrins (Naessens et al., 2005a). These dextrans were reported to exhibit unexpected and so far not exploited properties in contrast to commercially available dextrans (Mao et al., 2012). The ability of some AAB strains to produce fructans from sucrose is explicitly depicted in section 1.8.

1.5 Occurrence of fructans

Many plant, fungal and bacterial species are capable of producing fructans (polymers consisting of *β*-D-fructose units), which can be basically distinguished by their type of linkages. So far, two main types of fructans have been identified: inulins (*β*-(2→1)-linked *β*-(D)-fructofuranosyl polymers) and levans (*β*-(2→6)-linked *β*-(D)-fructofuranosyl polymers). While inulins are widely distributed in plants (e. g. barley, wheat, oat, chicory, sunchoke, artichocke, asparagus, salsify, onion) and are produced solely by a few LAB and fungal strains, levans are synthesized by many gram-positive and gram-negative bacteria and are solely found in some grasses (Vijn & Smeekens, 1999; Velazquez-Hernandez et al., 2009). Plant and microbial fructans especially differ in their respective degrees of polymerization (DP); plant fructans are generally reported to have a DP < 100, whereas microbial fructans can reach a DP of up to ~ 10000 fructose monomers (Velazquez-Hernandez et al., 2009).

Therefore, plants usually accumulate high amounts of short-chain fructans, which are typically termed "fructooligosaccharides" (FOS) having a DP < 10, while bacteria produce macroscopic observable slimy/mucous substances, which predominantly consist of long-chain/high molecular weight fructan. These principal differences in molecular weight imply the respective biological roles of fructans; whereas plant fructans are stored intracellularly in vacuoles as "sugar-like" reserve carbohydrates and cold resistance regulators, microbial fructans are produced extracellularly and mainly act as biofilm material during colonization of eukaryotic hosts (Velazquez-Hernandez et al., 2009).

1.6 Bacterial fructan biosynthesis

Bacterial levans or inulins are produced by extracellular levan- or inulosucrases, respectively, both of which belong to the glycoside hydrolase (GH) 68 family of enzymes according to the similarity of their amino acid sequences (Pons et al., 2004). The major substrate for these fructosyltransferases (FTFs) is sucrose. As a first step, the disaccharide is bound to the active centre of the corresponding enzyme and cleaved, which results in the release of glucose. The remaining bound fructose moiety can then be transferred to the growing polymer chain with a terminal sucrose molecule as starting precursor for polysaccharide formation. Alternatively, water can serve as acceptor and reach the catalytic centre of the corresponding FTF after cleavage of donor sucrose, which results in the release of initially bound fructose and the complete hydrolysis of sucrose (Van Hijum et al., 2006). Therefore, FTFs exhibit transferase (transfructosylation) as well as hydrolysis activities, which compete against each other depending on environmental conditions such as pH, temperature, ionic strengths or substrate concentrations (Van Hijum et al., 2006).

GH 68 FTFs exhibit a five-bladed β-propeller fold and a highly conserved sucrose-binding pocket, which comprises three acidic amino acid residues (2x Asp, 1x Glu) for substrate binding (Meng & Fütterer, 2003; Martinez-Fleites et al., 2005; Pijning et al., 2011). These residues function as catalytic nucleophile, transition-state stabilizer and acid-base catalyst in a supposed double-displacement reaction (ping-pong type of mechanism), which involves formation and subsequent hydrolysis of a covalent glycosyl–enzyme intermediate (Ozimek et al., 2006; Pijning et al., 2011). While the exact mechanisms for fructan polymerization are not clearly understood to date, it was demonstrated, that product specifities of levansucrases from *Zymomonas mobilis* depend on the formation of ordered microfibrils, which are composed of multiple (oligomerized) enzymes, that promote synthesis of high molecular weight fructan (Goldman et al., 2008). Furthermore, Pijning et al. (2011) reported about almost identical active sites in inulosucrases and levansucrases and suggested amino acid residues of a non-conserved, variable 1B-1C loop to be responsible for product specifity (inulin *vs*. levan) of GH 68 FTFs. FTFs from gram-positive bacteria possess signal peptides, which are cleaved off during transport to the outside of the cell. Furthermore, they are supposed to be cell-wall anchored/associated due to (partially) existing anchoring motifs (LPXTG) in their C-terminal domains and are calcium-

dependent. On the contrary, FTFs from gram-negative bacteria are completely secreted into the extracellular environment *via* unknown mechanisms (with the exception of *Gluconacetobacter diazotrophicus* levansucrase; 1.8) and exhibit no metal (cofactor) dependence (Van Hijum et al., 2006).

1.7 Applications of fructans

Plant derived inulins (recovered from chicory roots) have gained much interest in food applications over the last decades due to their health promoting properties as non-digestive dietary fibres (Kaur & Gupta, 2002). Inulins exhibit especially prebiotic/bifidogenic effects and increase the body absorption of calcium. Furthermore, inulins are supposed to prevent colon cancer and to activate positive digestive functions. Therefore, they are generally regarded as safe additives in foods (Coussement, 1999) and are used as low-calorie, non-cariogenic sweeteners and representative prebiotics in a multitude of food products (confectionery, fruit preparations, milk desserts, yogurt and fresh cheese, baked goods, chocolate, ice cream and sauces) (Kaur & Gupta, 2002). Apart from its beneficial features, plant inulin has been considered to be responsible for increased flatulences at high uptaken doses (high osmotic pressure) (Coussement, 1999). Due to these predominantly promising properties, several studies demonstrated the fructan (inulin and levan) producing potential of food-grade LAB such as e. g. *Lactobacillus reuteri* or *Lactobacillus sanfranciscensis* (Van Hijum et al., 2006; Korakli & Vogel, 2006), which are natural starter cultures for e. g. sourdough fermentations. In this way, health promoting fructans should be produced *in situ* during natural food fermentations without any need of declaration as additives, thereby enabling the manufacture of clean label products. Although levan of *Lactobacillus sanfranciscensis* extends the shelf-life of breads (Kaditzky, 2008a) and stimulates the growth of intestinal bifidobacteria (Korakli et al., 2002), the potential of microbial fructans for technological improvement of foods is actually not commercially exploited due to lacking systematic studies on the functionality of these sugar polymers (structure-function relationships). Moreover, microbial fructans are still considered as substitutes for plant inulins, i. e. many studies are focused on the optimization of microbial FOS production, while the unknown hydrocolloid character of microbial high molecular weight fructan for improvement of foods has so far not been investigated. Microbial high molecular weight levans rather exhibit antitumour and even antiviral activities (Abdel-Fattah et al., 2012; Calazans et al., 2000; Esawy et al., 2011) or are promising emulsifiers and encapsulating agents in a wide range of products, including biodegradable plastics, cosmetics, glues, textile coatings and detergents (Banguela & Hernandez, 2006).

1.8 Fructan producing AAB

Some AAB strains produce levan (Arrieta et al., 1996; Lisdiyanti et al., 2002; Kato et al., 2007; Kornmann et al., 2003; Paul et al., 2011) or possess genes coding for levansucrases (Sakurai et al., 2011; Prust et al., 2005). However, solely the levansucrase of *Gluconacetobacter diazotrophicus*,

which is known to act as N_2-fixing plant-growth promoter in rhizospheres of plants (e. g. sugarcane), was investigated in detail and actually represents one of the model enzymes for understanding the biochemical and functional properties of GH 68 FTFs (Martinez-Fleites et al., 2005). This protein is unique for AAB and, in general, for known levansucrases of gram-negative bacteria due to its unusual amino acid sequence, which comprises a signal-peptide being cleaved off during transport to the outside of the cell (Arrieta et al., 2004). In this context, a gene cluster coding for a type II secretion machinery is directly associated with the respective levansucrase nucleotide sequences in 14 different *Ga. diazotrophicus* strains. On the contrary, similar genetic organizations for levansucrase secretion have not been detected in genomes of AAB (Prust et al. 2005; Ge et al., 2013). Furthermore, this enzyme is constitutively secreted into supernatants of fermentation broths (Arrieta et al., 1996), contains an intramolecular disulfide-bridge (Betancourt et al., 1999) and exhibits unusual catalytic properties, as it produces relatively high amounts of prebiotic FOS (Tambara et al., 1999). Recently, the importance of *Ga. diazotrophicus* levansucrase for increased tolerance against dessication, osmotic stress and formation of biofilms has been pointed out (Velazquez-Hernandez et al., 2011). However, little overall knowledge exists about functional fructan-biosynthesis in different genera of AAB. Consequently, fructan production by AAB is actually not (commercially) exploited in contrast to cellulose production.

1.9 Aims of this work

The present work should give new insights into

(1) the structure and functionality of fructans from AAB

(2) the genetic and enzymatic background of fructan biosynthesis in/by selected AAB.

For these purposes, different fructan producing AAB strains should at first be identified/selected, whose synthesized, isolated fructans should be investigated regarding their chemical identity and molecular weight distributions using HPLC, NMR spectroscopy and AF4-MALS-RI. Some of these fructans should exemplary be used as additives for baking experiments to possibly demonstrate their applicability as hydrocolloids and to concomitantly establish comparative structure-function relationships. Moreover, fructan-producing enzymes should be identified using different genetic/enzymatic methods and be compared among each other regarding their principal genetic/enzymatic diversity.

In this way, the (non-exploited) potential of AAB to produce fructans, both of which are widely used in (food) biotechnological applications, should be highlighted. Obtained results should sustainably impress the current general knowledge about fructan biology/biotechnology.

2. MATERIAL AND METHODS

2.1 Strains, media and growth conditions

All components used for the cultivation of microorganisms were either autoclaved or sterilized (by filtration) before usage. Sugars and nitrogen sources were autoclaved separately.

2.1.1 Acetic acid bacteria

A total of 22 strains of the genera *Gluconobacter (G.)*, *Gluconacetobacter (Ga.)*, *Acetobacter (A.)*, *Neoasaia (N.)* and *Kozakia (K.)* were (initially) used in this study. AAB strains were generally cultivated aerobically (180-200 rpm for liquid cultures) at 30 °C in/on sodium-gluconate medium (NaG) (20 g/L sodium gluconate, 3 g/L yeast extract, 2 g/L peptone, 3 g/L glycerol, 10 g/L mannitol, optional 20 g/L agar, pH adjusted to 6.0). For analysis of secreted extracellular proteins of *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400 the nitrogen sources of usual NaG medium (yeast extract and peptone) were replaced by 3 g/L casein acid hydrolysate (Sigma-Aldrich, Germany). This modified NaG medium was additionally supplemented with sucrose (1 and 10 g/L; 2.6.1), cysteine (20 mg/L), tryptophan (20 mg/L) and 1 mL/L of a vitamin mix consisting of 200 mg/mL $MgSO_4$ x $7H_2O$, 38 mg/mL $MnSO_4$ x H_2O, 0.2 mg/mL thiamine, 0.2 mg/mL niacine, 0.2 mg/mL folic acid, 0.2 mg/mL pyridoxal, 0.2 mg/mL pantothenic acid and 0.2 mg/ml cobalamine.

2.1.2 Lactic acid bacteria

The water-kefir isolate *Leuconostoc (L.) mesenteroides* TMW 2.1073 was cultivated aerobically (150 rpm for liquid cultures) in/on mMRS medium (5 g/L glucose, 10 g/L maltose, 5 g/L fructose, 10 g/L peptone, 5 g/L yeast extract, 5 g/L meat extract, 4 g/L K_2HPO_4 x $3H_2O$, 2.6 g/L KH_2PO_4, 3 g/L NH_4Cl, 1 g/L Tween 80, 0.5 g/L Cysteine-HCl, optional 20 g/L agar, pH adjusted to 6.2) supplemented with 1 ml/L vitamin mix (2.1.1). For induction of dextransucrase secretion, *L. mesenteroides* TMW 2.1073 was pre-cultivated in vitamin-supplemented mMRS medium, which had been diluted 1:1 with a 0.2 M sucrose solution.

2.1.3 *Escherichia* strains

Escherichia (E.) coli strains DH5α (containing either pBADMycHis (Invitrogen) or pBluescript II SK (Stratagene) vectors), Top 10 (Invitrogen; for heterologous expression) and codon-optimized Rosetta (Novagen; for heterologous expression; selection marker: 68 µg/mL chloramphenicol) generally were cultivated aerobically (180 rpm) at 37 °C in lysogeny broth (LB) medium (10 g/L peptone; 5 g/L yeast extract; 5 g/L NaCl). To select recombinant plasmid harbouring *E. coli* cells LB media were additionally supplemented with 100 µg/mL ampicillin (Amp 100). For induction of polysaccharide synthesis by recombinant *E. coli* cells 60 g/L sucrose and 2 g/L arabinose (induction) were added to solid LB Amp 100 media.

2.2 EPS recovery

2.2.1 Conventional EPS production

For isolation of EPSs from liquid media, a single colony of the EPS producing strain was used to inoculate 50 mL of NaG medium (without sucrose) and incubated for 24 h on a rotary shaker (180 rpm). After washing of the cells in 50 mL NaG medium, 1 L of NaG medium containing 80 g/L sucrose was inoculated with 10 mL of the overnight culture, respectively. For recovery of sufficient EPS amounts for baking experiments, 5 x 1 L of NaG media were inoculated with 10 mL of the same pre-culture of the corresponding strain, respectively. After aerobic cultivation of the main cultures for 48 h at 30 °C on a rotary shaker (180 rpm), cells were removed by centrifugation (5500 rpm, 10 min) and the supernatant was mixed with 2 volumes of cold ethanol and stored for 2 days at 4 °C to precipitate EPSs according to the method described by Tieking et al. (2005). The EPS precipitates must not be centrifuged using NaG medium as they settle down at the bottom of the used precipitation flask as transparent, mucous substances. Consequently, the supernatants were discarded, the precipitates were air-dried and re-dissolved in ddH_2O. The re-dissolved EPSs were furthermore dialyzed (molecular weight cut-off: 14 kDa) against ddH_2O for at least 48 h at 4 °C to remove precipitated low molecular weight substances. Finally, the purified EPSs were lyophilized and quantified by weighing.

2.2.2 Enzymatic fructan production

Gluconobacter species TMW 2.1191 was pre-cultivated aerobically (200 rpm, 30 °C) in a 2 L Erlenmeyer flask supplemented with 200 mL glucose-enriched NaG medium (20 g/L Na-gluconate; 3 g/L yeast extract; 2 g/L peptone from casein; 10 g/L mannitol; 3 g/L glucose; 3 g/L glycerol) to a final OD_{600} = 2.5. Cells were separated by centrifugation (5000 g; 10 min), re-suspended in 200 mL Na-acetate buffer (pH 5.0; 0.1 M) and centrifuged again (5000 g; 10 min). Cells were subsequently re-suspended in 200 mL Na-acetate buffer (pH 5.0; 0.1 M; supplemented with 0.1 M sucrose) and incubated for 3 h (200 rpm; 30 °C) in a 2 L Erlenmeyer flask. Finally, cells were separated by centrifugation (7000 g; 10 min) and discarded. The enzyme-containing supernatant was mixed with 800 mL Na-acetate buffer (pH 5.0; 0.1 M; supplemented with 0.1 M sucrose) and incubated for 24 h without stirring (30 °C). The solution was dialyzed (MWCO: 3 kDa) against ddH_2O for 48 h. The purified solution was freeze-dried. The recovered EPS was subsequently quantified by weighing.

2.2.3 Enzymatic glucan production

L. mesenteroides TMW 2.1073 was pre-cultivated aerobically (150 rpm, 30 °C) in a 100 mL Erlenmeyer flask supplemented with 20 mL liquid mMRS medium (diluted 1:1 with 0.2 M sucrose) to a final OD_{600} = 0.5. Cells were separated by centrifugation (5000 g; 10 min), re-suspended in 20 mL Na-acetate buffer (pH 5.0; 0.1 M) and centrifuged again (5000 g; 10 min). Cells were afterwards re-

suspended in 20 mL Na-acetate buffer (pH 5.0; 0.1 M; supplemented with 0.1 M sucrose and 0.05% $CaCl_2$) and incubated for 2 h (150 rpm; 30 °C) in a 50 mL Sarstedt tube. Finally, cells were separated by centrifugation (7000 g; 10 min) and discarded. The enzyme-containing supernatant was mixed with 160 mL of Na-acetate buffer (pH 5.0; 0.1 M; supplemented with 0.1 M sucrose and 0.05% $CaCl_2$) and incubated for 24 h without stirring (30 °C). The solution was dialyzed (MWCO: 3 kDa) against ddH_2O for 48 h. The purified solution was freeze-dried. The recovered EPS was subsequently quantified by weighing.

2.2.4 Heteropolysaccharide production

K. baliensis DSM 14400 was cultivated aerobically (180 rpm) at 30 °C for 24 h in 1 L NaG-medium without supplemented sucrose. The fermented, cell containing medium was afterwards stored for 3 days at 4 °C without stirring. Cells were afterwards separated from the fermentation broth by centrifugation (5500 rpm). Macromolecular HePS was subsequently precipitated by addition of 2 L ethanol (4 °C, 48 h). The precipitated HePS was collected by centrifugation (4 °C, 7000 g), air-dried, re-dissolved in ddH_2O and dialyzed against ddH_2O (MWCO: 14 kDa) for 48 h. The solution was finally freeze-dried. The recovered EPS was quantified by weighing.

2.3 Baking experiments

2.3.1 Baking of wheat breads

The isolated fructans of *Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533T, *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400 were used to bake 8 breads with 1% and 8 breads with 2% added EPS (w/w flour). As a control 8 breads without added EPS were baked simultaneously in each test series. The basic ingredients for 8 breads without EPS were 1000 g wheat flour, 50 g baker´s yeast, 15 g NaCl and 600 mL water. When EPS was added, the water content of this recipe was reduced corresponding to the used EPS amount (e. g. 10 g EPS + 590 mL water). The ingredients were mixed, dispersed and fermented for 30 min at 30 °C (70% humidity). The dough was then divided into pieces of 205 g and fermented for another 30 min at 30 °C (70% humidity). Finally, the bread dough loaves were baked at 230 °C for 25 min. After cooling down for 1 h at room temperature the wheat breads could be used for the following measurements.

2.3.2 Volume measurements

The specific volume of wheat breads was determined using a laser volumeter (TexVol Instruments, Sweden, BVM-L series). Therefore, each of the breads was positioned on a shaft and analyzed by a laser sensor that moves around the rotating test object. The volume (mL) of all breads was measured on the same day of baking (day 0), after cooling down the baked breads.

2.3.3 Texture profile analysis (TPA)

TPA measurements were done on day 0, day 1, day 4 and day 7 after bread baking to document and compare the staling of the control and EPS breads. On day 0 two control and two EPS breads were cut into equal pieces (2.5 cm broadness, three slices of bread per baked bread) and analyzed (after determination of the specific volume, see 2.3.2). The hardness of bread slices was measured with a texture analyzer (TexVol Instruments, Sweden, TVT-300XP), whose plunger worked with a crosshead speed of 1 mm/s and a penetration depth of 1 cm. The resistance of the crumb to the plunger was detected in Newton (N). For documentation of staling the remaining 6 EPS and 6 control breads of each test series were vacuum packaged in 3 plastic bags (2 EPS- and two control breads per bag) on day 0 and stored at room temperature. On day 1, day 4 and day 7 after bread baking the texture of these breads was then analyzed as described above.

2.4 Structural analyses of EPSs

2.4.1 NMR spectroscopy

In order to determine the linkage types of fructans and glucans, lyophilized EPSs were dissolved in D_2O (10 g/L). Afterwards, nuclear magnetic resonance (NMR) spectra were recorded at Bruker Avance 300 MHz at 293 K. Chemical shifts are reported in ppm (δ units) downfield from 3-(trimethylsilyl)-propionic acid-d4 (D_2O). ^{13}C NMR spectra were accumulated with a 45° pulse, 7.5/µs, 18,000 Hz spectral width, a 4 s delay between pulses and 4096 repetitions. Homonuclear shift-correlated (COSY) spectra were obtained using spectra widths of 2400 Hz and 2400 Hz, with 2 K and 128 data points in the F2 and F1 dimensions, respectively. The delay between pulses was 1.8 s and the number of repetitions 32. Heteronuclear shift-correlated two-dimensional (2D) experiments were carried out using an F2 spectral width of 12500 Hz and an F1 width of 2400 Hz; 64 time points were accumulated with 1 K and 128 data points in F2 and F1, respectively. The delay between pulses was 1.7 s and the number of repetitions 64. Apart from the isolated EPS samples, powdered inulin from chicory roots (Sigma-Aldrich, Germany) was analyzed as described above acting as comparative control substance.

2.4.2 AF4-MALS-RI

Asymmetric flow field-flow fractionation (AF4) (Wyatt Technology, Germany) was used to separate fructan molecules according to the theoretical principles described by Rübsam et al. (2012) and Nilsson (2012). For determination of the corresponding molecular weights (M_Wi) and sizes of the separated molecules (radiuses of gyration, R_Gi), the AF4 system was coupled to multi-angle laser light scattering (MALS) (Dawn, Heleos II, Wyatt Technology, Germany) and a refractive index (RI) as quantitative detector (Agilent Series 1200 G1362A, Agilent Technologies, Germany). For each EPS analysis, three individual 100 µL samples (re-dissolved in ddH_2O, 1 g/L) were injected to the channel.

For the separation process, the inserted spacer had a height of 350 µm and a width of 21.5 mm at the widest position approximately, at which the sample was first focused (3.5 min) in the long channel (240 mm) before it was eluted to the detectors. Measurement was carried out at 25°C. Carrier eluent was composed of 0.05 M $NaNO_3$ and 0.003 M NaN_3 dissolved in Millipore water. Fructan molecules were separated on a 5 kDa membrane (Nadir regenerated cellulose, Wyatt Technology, Germany). Flow conditions were as follows: injection (0.2 mL/min), elution (1 mL/min) and cross flow (3 mL/ min → 0.1 mL/min within 30 min). The cross flow was afterwards kept at 0.1 mL/min for 30 min and finally reduced to 0 mL/min within 18 min. The intensities of scattered light originating from the separated molecules using MALS were simultaneously measured at 18 different scattering angles (in the range of 10° to 160°). Concentrations of polysaccharides in aqueous solutions were determined with the coupled RI detector using a refractive index increment (*dn/dc*) value of 0.146 mL/g. Before analysis of the samples, the accuracy of the calibration of the analytics was verified. Pullulan standards (1 g/L, PSS GmbH, Germany) of known molar masses were measured to prove the calibration. Blank runs were additionally performed as control to be able to subtract the resulting baselines from sample baselines. Weight average (M_W) and number average (M_N) molar masses of the four different isolated fructans were calculated from the relationships

$$M_W = \frac{\sum n_i \cdot M_i^{\,2}}{\sum n_i \cdot M_i} \tag{1}$$

$$M_N = \frac{\sum n_i \cdot M_i}{\sum n_i} \tag{2}$$

where n_i represents the number of molecules with molar masses M_i.

M_W was used to calculate the average degree of polymerization *DP* of the isolated fructans following the equation

$$DP = \frac{M_W}{162.16} \tag{3}$$

where 162.16 Da is the molar mass of *β*-(2→6)-linked fructose in the corresponding fructan.

The quotient of M_W and M_N yields the polydispersity index (*PDI*) of the isolated polymer

$$PDI = \frac{M_W}{M_N} \tag{4}$$

Hydrodynamic coefficients ν_G of the different levan fractions were obtained from the regression lines of the log-log plots of $R_G i$ against $M_W i$ according to the relationship

$$R_G\, i = k_{R_G i} \cdot \left(M_W i\right)^{\nu_G} \tag{5}$$

(Nilsson, 2012), where $k_{R_G i}$ is represented as substance dependent constant.

2.4.3 Sugar monomer analysis of EPSs

The monosaccharide types incorporated in the corresponding sugar polymer(s) were determined *via* high performance liquid chromatography (HPLC) analysis. Therefore, lyophilized EPS was dissolved in ddH_2O (1 g/L) and subsequently treated with 15% (v/v) perchloric acid (70%). The sample was then incubated for at least 3 h at 100 °C to hydrolyze the polymers and after this centrifuged (13000 g) at 4 °C for 10 min to remove precipitated proteins. Finally, the supernatant was analyzed *via* HPLC using a Polysphere OAKC column (Merck, Germany) coupled to a refractive index (RI) detector (Gynkotek, Germany) according to the method of Kaditzky & Vogel (2000b). The type of monosaccharide present in the investigated polymer lastly could be determined according to its retention time using the standards glucose, fructose, rhamnose and galactose.

2.5 Genetics

2.5.1 General molecular techniques

Genomic DNA was isolated following the instructions of the E.Z.N.A. Bacterial DNA Kit (Omega Bio-tek, Norcross, USA). *E. coli* plasmid DNA was purified using the peqGOLD Plasmid Miniprep Kit (peqlab, Germany). Restriction endonuclease digestions, dephosphorylation with shrimp alkaline phosphatase (SAP) and ligations with T4 DNA ligase were performed as recommended by the suppliers (Fermentas, Germany). Unknown DNA sequences were amplified using Taq-DNA-Polymerase (Qbiogene, USA). For amplification of known DNA sequences as well as 16S rRNA genes the proofreading Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland) was used. When specific primer sets were put in the reaction mixture, PCR cycle conditions were routinely performed according to the manufacturers´ instructions. Oligonucleotide primers for PCR reactions were obtained from MWG Biotech AG (Germany). Potentially complex amplification products resulting from degenerate or modified single-primer PCR were sequenced by Sequiserve (Germany). All other PCR products were sequenced by GATC Biotech (Germany). Preparative DNA isolations from agarose gels were performed with the peqGOLD Gel extraction Kit (peqlab, Germany), PCR products were purified using the E.Z.N.A. Cycle-Pure Kit (Omega Bio-tek, Norcross, USA).

2.5.2 Identification of levansucrase encoding genes

Primers used for PCR reactions are listed in table 1. Identification schemes for initially unknown genes, which include used primers, methods and restriction endonucleases, are depicted in Fig. 7 and Fig. 13. Open reading frames (ORFs) were identified as described in 3.3.1 and 3.3.4.

2.5.2.1 Core sequence detection

To identify core sequences of the unknown *ftf*-genes, degenerate (dg) PCR reactions were performed using a gradient cycler (Eppendorf, Germany), which allowed optimization of primer annealing using

different annealing temperatures for multiple samples containing equal reaction mixtures. Typical PCR conditions were as follows: initial denaturation at 94 °C for 3 min; 35 cycles: denaturation at 94 °C for 30 s, annealing for 1 min ± 30 s (temperatures were chosen in 1 °C steps approximately in the range of the melting point T_m (primer) – 10 °C; time was varied if necessary according to the results) and extension at 72 °C (duration adapted to the expected size of the PCR product); final elongation at 72 °C for 10 min. Reaction mixtures generally were prepared according to the suppliers´ instructions. Clear bands of the expected size were cut out of the gels, following preparative DNA extraction and subsequent sequencing as described above.

2.5.2.2 Identification of parts flanking the identified core regions

Based on core sequence information, specific inverse primers (5´ → 3´, table 1) were designed targeting to the direction of the missing part of the corresponding *ftf*-gene. For standard inverse PCR (siPCR) genomic DNA was digested with a restriction endonuclease, which could not cut in the known sequence. DNA subsequently was religated and used as template for siPCR. In a modified inverse PCR (miPCR) approach, digested DNA was cloned into plasmid pBluescript II SK, which was initially treated with the same restriction enzyme. The resulting constructs afterwards were used as template for miPCR using inverse and plasmid primers (T7/T3), which flank the vector´s multiple cloning site. Modified single primer PCR (mspPCR) was performed using solely one inverse primer in a temperature gradient PCR reaction. PCR products of equal size, which showed a constantly increasing intensity towards the primer´s T_m after staining were preparatively purified and sequenced with a second inverse primer, which again binds specifically in the expected amplicon. For modified site-finding PCR (msfPCR) (Tan et al., 2005) random primer SF49+ (containing a 6 x N anchor) and an inverse primer were used for an initial PCR reaction. The resulting product mixture served as template for a second PCR reaction with random primer SF49- (without anchor) and a second inverse primer. Positive PCR amplicons were subsequently sequenced and annotated to the corresponding core sequences.

2.5.3 Cloning and heterologous expression of complete FTFs

Newly identified, putative *ftf*-gene sequences of *Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533T, *Gluconobacter species* TMW 2.1191 and *N. chiangmaiensis* NBRC 101099 (truncated, without signal peptide) and the levansucrase gene of the genome sequenced strain *G. oxydans* 621 H were cloned into expression vectors of the pBADMycHis series (Invitrogen, Germany), which possess a C-terminal 6 x histidine tag for facilitated purification. Primers for amplification of the corresponding *ftf*-genes contained 5´-overhangs with restriction sites (underlined in table 1), allowing in-frame cloning into the respective multiple cloning site. Restriction, SAP treatment and ligation were performed according to the manufacturers´ instructions (2.5.1). The constructs were transformed into competent *E. coli* Top 10 (and additionally *E. coli* Rosetta in the case of *N. chiangmaiensis*), which

were generated by the rubidium chloride method *via* heat shock transformation. Cells harbouring plasmids were selectively cultivated on LB Amp 100 media and subsequently transferred to LB Amp 100 media supplemented with 6% (w/v) sucrose and 0,2% (w/v) arabinose. In this manner, positive *E. coli* clones expressing the corresponding levansucrase as an active enzyme could be selected due to their ability to produce mucous polysaccharide in contrast to the wildtype. The correct *ftf-gene* insertion and sequence of the insert were furthermore proven by control PCR using plasmid primers flanking the multiple cloning site and by subsequent sequencing.

Table 1: Primers used in this study. Restriction sites are underlined and in bold.

Strain	Primer	Sequence (5´→ 3´)	Use
G. sp. TMW 2.767	Av	GCNGAYGCNATGAAR	dgPCR
	Ar	NGTNGGNGCNAGNGTNCC	dgPCR
	Fratseq_F7	CACATCGTTGGACATGACC	miPCR
	Fratseq_R1	CCCTGATGGTGTGTATGG	miPCR
	T7	GTAATACGACTCACTATAGGGC	miPCR
	T3	AATTAACCCTCACTAAAGGG	miPCR
	FtfFratBAD_F	GC**CCATGG**CTAATGCTATTTCCAGCCGAATTC	PCR + cloning
	FtfFratBAD_R	GC**TCTAGA**AGGGCGCGAACGTCATAG	PCR + cloning
G. cerinus	Z1F	ACRACGACVATGCCG	dgPCR
	Z1R	CSGTCTGGTCATTSACGC	dgPCR
	LevCF1	CGCAGCGCACCACTGTCCCA	siPCR
	LevCR1	CTGGCCCGGTGTGTCTCCGG	siPCR
	LevCF2	AAATCTTGGCGTTGGTCTTA	siPCR
	LevCR2	CTTGCACCGACGGTTCGTCT	siPCR
	FtfCerBAD_F	GC**CTCGAG**TAATGCTATTTCCTCCCAGTC	PCR + cloning
	FtfCerBAD_R	GC**TAAGCT**TGGCTCTGACGTCATACGCC	PCR + cloning
G. sp. TMW 2.1191	DgAlbF	GATCCGACGACGACMATG	dgPCR
	DgAlbR	CCGVTCACCRTCSAGAAC	dgPCR
	LevAF1	TGGCATCTGCCAGAATG	mspPCR
	LevAR1	CATGTCGTCTTCCAGGAC	msfPCR
	LevAF2	GTAGGACGGAACAGACTCG	sequencing
	LevAR2	CTGACCTATCTGTTCACGATC	msfPCR
	SF49+	GCACTGCTATGCTTACTGGNNNNNN	msfPCR
	SF49-	GCACTGCTATGCTTACTGG	msfPCR
	FtfAlbBAD_F	GC**CTCGAG**TAACGTGGTTTCCAGCACAC	PCR + cloning
	FtfAlbBAD_R	GC**AAGCTT**GGAACGCTTGTCAAAGGCC	PCR + cloning
G. oxydans 621H	FtfOxBAD_F	GC**CCATGG**CTAACGCTGTTTCCAGCACG	PCR + cloning
	FtfOxBAD_R	GC**TCTAGA**AGGGAACGCTTGTCCCAGG	PCR + cloning
N. chiangmaiensis	DgAAB_F	GARTGRTCNGGNTCN	dgPCR
	DgAAB_R	NCKYTCNGTYTGRTCRTT	dgPCR
	T7	GTAATACGACTCACTATAGGGC	miPCR
	T3	AATTAACCCTCACTAAAGGG	miPCR
	LevNF1	GTAAACCAGACATGCTGGAA	siPCR
	LevNR1	GATGCTGCGGATCTTGGATA	siPCR
	LevNF6	CATCTGGGCTGATGGAAG	miPCR
	FtfNeoBAD_F	GC**CCATGG**CTCAAACAACATCACCTGCGC	PCR + cloning
	FtfNeoBAD_R	GC**TAAGCT**TTTGCAAAAAATTACTGATTTG	PCR + cloning
K. baliensis	DgAAB_F	GARTGRTCNGGNTCN	dgPCR
	DgAAB_R	NCKYTCNGTYTGRTCRTT	dgPCR
	T7	GTAATACGACTCACTATAGGGC	miPCR
	T3	AATTAACCCTCACTAAAGGG	miPCR
	LevKR3	AGGCCAACATCGGGTTAGCT	miPCR
	DgKoz_F	CCCAGYATTCATACGCARCA	dgPCR
	LevKF4	TTGTTCCTATTGAGCAAGCG	dgPCR
	LevKF5	CGACGTTAGGATCGGAATG	miPCR
	LevKF3	AGGCGTAATATTGCCGCCGC	mspPCR
	LevKF6	CCAATATTACTGCCTGAAGG	sequencing
	KLevS_F(1)	ATGAATCTCGGAATTTCCGC	PCR
	KLevS_F(2)	CTTCGCATTGGACGATCGCC	PCR
	KLevS_R(1)	TCAGGAATTCCATTCTTTAT	PCR
	KLevS_R(2)	TGGATTTCCGTCAGGAAGGT	PCR
Each strain	616V	AGAGTTTGATYMTGGCTCAG	16S PCR
	630R	CAKAAAGGAGGTGATCC	16S PCR

2.6 Enzymology

2.6.1 Recovery of proteins from fermented NaG media

Cells of *N. chiangmaiensis* and *K. baliensis* were at first pre-cultivated in 50 mL modified NaG medium (+ 10 g/L sucrose, 2.1.1) for 12 h at 30 °C and 180 rpm. Afterwards, cells were harvested by centrifugation, resuspended in 10 mL of fresh NaG-medium and used for inoculation of 1 L modified NaG medium (+ 1 g/L sucrose, 2.1.1). This medium again was fermented for 12 h at 30 °C and 180 rpm. Cells were finally separated from the broth and discarded. The recovered supernatant was treated step-wise with ammonium sulfate (final saturation: 100%) for out-salting of secreted proteins. Out-salting was conducted under mild stirring (50 rpm) at 4 °C for 1 h. Afterwards, samples were centrifuged at 14000 g and 4 °C for 30 min. Supernatants were discarded, the obtained brownish pellets were re-suspended in (preferably) low amounts (~ 1 mL per 200 mL initial supernatant) of 0.1 M Na-phosphate buffer (pH 7.0). These samples were finally desalted/concentrated using Amicon® Ultra-4 Centrifugal Filter Units (30 kDa MWCO; Merck Chemical GmbH, Germany) as recommended by the manufacturers to remove excessive ammonium sulfate.

2.6.2 Recovery of proteins from fermented Na-acetate buffers

Cells of *Gluconobacter species* TMW 2.1191 and *K. baliensis* DSM 14400 were pre-cultivated aerobically (200 rpm) at 30 °C in 50 mL glucose-enriched (3 g/L) NaG medium to a final OD_{600} of 2.5 and 1.8, respectively. Afterwards, cells were separated from the medium by centrifugation, washed once in 50 mL 0.1 M Na-acetate buffer (pH 5.0), separated again and re-suspended in 50 mL 0.1 M Na-acetate buffer (pH 5.0 + 0.1 M sucrose). After incubation at 30 °C and 200 rpm for 3 h, cells were separated from the solution *via* centrifugation (7000 g) and discarded. The recovered solution was dialyzed (MWCO: 3 kDa) for 48 h against ddH_2O and finally lyophilized.

2.6.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For separation of proteins SDS gels (Laemmli, 1970) were prepared using the materials of a Mini-PROTEAN set (BioRad, Gemany). The recipe for two separating SDS gels (10%) was as follows: 4.2 mL ddH_2O, 3.3 mL acrylamide (30%), 2.5 mL lower Tris (1.5 M Tris-HCl, pH 8.8, 0.4% SDS), 10 µL TEMED (tetramethylethylendiamine), 100 µL APS (ammonium persulfate, 10%). These chemicals were mixed, immediately pipetted into (two) prepared glass sandwiches and covered with 25 µl isopropanol to obtain a straight gel surface. After polymerization of the gel the residual isopropanol was discarded. The separating gel was afterwards overlaid with a stacking gel consisting of 2.6 mL ddH_2O, 1 mL upper Tris (0.5 M Tris-HCl, pH 6.8, 0.4% SDS), 440 µL acrylamide (30%), 6 µL TEMED and 40 µl APS (10%). Proper combs were finally inserted into the non-polymerized stacking gel to generate sample slots. Protein separation was conducted in 1x Tris-glycine-SDS running buffer (3 g/L Tris base, 14.4 g/L glycine, 1 g/L SDS) at 100 V for ~ 1 h.

2.6.3.1 Sample preparation

Before loading on SDS gels, samples were generally mixed with Laemmli sample buffers (2x) consisting of 4% (w/v) SDS, 20% (v/v) glycerol, 120 mM Tris-HCl (pH 6.8), 0.02% (w/v) bromophenol blue and 0.2 M DTT. Desalted samples recovered from NaG fermented supernatants (2.6.1) were typically mixed in equal ratios (15 µl : 15 µl) with 2x sample buffers and shortly heated (95 °C; 10 min) before protein separation. Lyophilized samples recovered from fermented Na-acetate buffers (2.6.2) were at first (partially) re-dissolved in 0.1 M or (in some cases) 1 M DTT solutions (50 mg sample per 500 µl DTT solution) and treated with 160 µl 2 x sample buffers. At this point, samples were jellylike due to the presence of levan. Therefore, samples were heated (95 °C) for at least 1 h and thoroughly vortexed every 10 min. Hot liquid samples (50 µl) were immediately loaded on SDS gels and electrophoretically separated to prevent extensive re-gelatinization of levan.

2.6.3.2 Coomassie staining

Proteins on SDS gels were usually visualized by colloidal Coomassie staining (Roti-Blue, Carl Roth GmbH, Germany). SDS gels were at first incubated in fixing solution (79 mL ddH_2O, 1 mL o-phosphoric acid (85%), 20 mL methanol) for 1 h at room temperature, afterwards transferred in staining solutions (60 mL ddH_2O, 20 mL methanol, 20 mL Roti-Blue) and finally incubated at room temperature o/n. After staining, gels were thoroughly rinsed with ddH_2O and analyzed.

2.6.3.3 Reverse staining and renaturation of proteins

For recovery of partially re-activated/renaturated enzymes, proteins were visualized by reverse staining after SDS-PAGE as described by Hardy et al. (1996) to avoid the formation of protein-dye complexes. For this purpose gels were soaked in 0.2 M imidazole solution (+ 0.1% SDS) for 10 min and afterwards immersed in 0.2 M zinc-sulfate solution for 30-60 s (development). In this way, the respective colorless/transparent protein bands could be localized and excised from the gel. Gel pieces were subsequently washed in repeating steps in phosphate buffered saline (PBS) solutions containing 100 mM EDTA or 0.1% Triton X-100 to complex zinc ions or remove SDS, respectively, as described by Hardy et al. (1996). Finally, proteins were passively eluted from crushed gel pieces by vigorous vortexing in 100 µl 0.1 M Na-acetate buffer (pH 5.0). Residual gel material was removed by centrifugation (10000 g, 10 min). The supernatant was afterwards directly used for activity assays.

2.6.4 Determination of fructosyltransferase activities

Activities of fructosyltransferases were calculated by determination of enzymatically released/consumed sugars *via* HPLC analysis using a Rezex RPM ion-exclusion column (Phenomenex, Germany) coupled to a refractive index (RI) detector (Gynkotek, Germany). Water flow (mobile phase) was kept constant at 0.6 mL/min during each run. Before sample measurement

calibration curves were established using the standards sucrose, glucose and fructose in different concentrations (1-500 mM). Since quantification was possible in the linear region 5-100 mM for each of these sugars, respectively, samples were diluted 1:10 with 30 mM NaOH (to additionally stop the enzymatic reaction; final pH ~ 12.2). A typical assay contained equal volumes of enzymatic solution and 2x stock assay buffer (0.2 M Na-acetate buffer, pH 5.0, 0.46 M sucrose). Enzymatic conversions were performed for 6 h at 30 °C to allow levan polymerization. Overall activities are expressed in Units (U), which are defined as µmol (totally consumed sucrose)/mL (protein sample) * min. Transferase activity was detected as distinctly positive value after subtracting totally released fructose from totally consumed sucrose, which again reflected the approximate amount of totally released glucose.

2.6.5 Protein identification

Coomassie stained proteins of interest were excised from SDS gels and sent to the "Zentrallabor für Proteinanalytik" (ZfP, Ludwig-Maximilians-Universität München). After tryptic digests and modifications of the respective proteins for proper separation and mass spectra generation by LC-MS/MS, the obtained mass spectra were compared with hypothetically tryptically digested proteins of the domain *Eubacteria* (deposited at NCBI) using Matrix Science Mascot software (Perkins et al. 1999). Additionally, the "Mascot generic format (.mgf) formatted" files derived from LC-MS/MS analysis, were processed to peptide sequences with PepNovo (Frank et al., 2005a/b; 2007) and "blasted" against available proteoms of AAB, as also described by Behr et al. (2007), to finally confirm the obtained Mascot search results.

2.7 Computational analysis

Gene sequences were edited and analyzed in terms of ORFs, dyad symmetries and energy calculations for potential hairpin structures using Clone Manager 5 (Sci Ed Central, USA). Alignments were created using CLUSTALW (EMBL-EBI, UK) and graphically optimized with ESPript (Gouet et al., 1999). Phylogenetic trees were calculated with BioNumerics 6.50 (Applied Maths, Belgium). Newly identified 16S rRNA and levansucrase gene sequences of *Gluconobacter* strains were deposited at the European Nucleotide Archive (ENA). Protein structures were predicted using the modelling package "MODELLER" provided on the $(PS)^2$ server (Chen et al., 2006). Thereby, the crystal structure of *Ga. diazotrophicus* (PDB-File: 1W18_A, Martinez-Fleites et al., 2005) served as template for predictive protein assembly. Protein 3D structures (PDB-Files) were graphically visualized with PyMOL software (Schrödinger, USA). Signal peptides were determined with the SignalP 4.0 server (Petersen et al., 2011). NMR spectra were evaluated and graphically optimized with MestReNova software (Mestrelab Research, Spain). AF4-MALS-RI data were processed with ASTRA V 5.3.4.19 software (Wyatt Technology, Germany). HPLC runs were controlled with Chromeleon Software (Dionex, Germany).

2.8 Microscopy

Microorganisms and EPS were microscopically investigated using an Axiostar Plus microscope (Zeiss, Germany). Lyophilized EPS was therefore re-dissolved in ddH_2O (1 g/L), spotted on a glass slide and observed at thousandfold magnification in phase contrast mode. Digital photographs were recorded with a coupled AxioCam digital camera (ICC, Zeiss, Germany).

3. RESULTS

3.1 Selection of effective fructan producers

Since fructans are produced from sucrose by FTFs, a total of 22 AAB strains were screened for their ability to produce slimy, mucous homopolysaccharides (HoPS) on sucrose containing agar plates (table 2). Since GH 70 glycosyltransferases (GTFs) such as dextransucrases also use sucrose as substrate for glucan production, HoPS were isolated in small scale (100 mL) from producing strains and analyzed regarding their monomer compositions *via* HPLC analysis.

Table 2: Screening of AAB strains for their ability to produce HoPS from sucrose

Strain	Origin	EPS producing activity
G. oxydans	DSM 2343 (621 H)	+
G. oxydans	DSM 50049	+
G. oxydans	TMW 2.339 (isolate from palm wine)	+
G. oxydans	DSM 3503	+
G. oxydans	DSM 46615	+
G. oxydans	DSM 2003	+
G. species	DSM 3504	+
G. species	TMW 2.767 (isolate from water kefir)	+++
G. frateurii	DSM 7146	+
G. cerinus	DSM 9533	+++
G. cerinus	TMW 2.155	+
G. species	TMW 2.1191 (isolate from water kefir)	+++
Ga. liquefaciens	DSM 5603	-
Ga. sacchari	DSM 12717	-
Ga. azotocaptans	DSM 13594	-
Ga. hansenii	DSM 5602	-
Ga. johannae	DSM 13595	-
Ga. oboediens	DSM 11826	-
Ga. intermedius	DSM 11804	-
A. pasteurianus	DSM 2324	-
N. chiangmaiensis	NBRC 101099	+++
K. baliensis	DSM 14400	+++

Strains, which were not received from commercial strain collections, were identified by 16S rRNA gene analysis and subsequent blasting (NCBI). EPS producing activity was observed macroscopically as follows: (+) weak, (+++) strong and (-) no production of mucous EPS on sucrose containing agar plates.

Five strains were identified as effective HoPS producers and selected for further studies: *Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533, *Gluconobacter species* TMW 2.1191, *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400. Sucrose-containing agar plates inoculated with these strains were completely covered with mucous polysaccharide after 48 h of incubation. Some strains (all of the genus *Gluconobacter*) showed a weak tendency to form mucous colony morphologies. Fig. 1 illustrates the differential capability of some selected *Gluconobacter* strains to produce mucous polysaccharides. Preliminary EPS isolation studies with all positively identified strains (14) in small scale (100 mL liquid medium) and subsequent determination of the isolated amounts of EPS confirmed these macroscopic observations (data not shown). Each of these isolated EPS types represents a homopolysaccharide consisting of fructose moieties (fructan) as only

fructose could be detected as single peak after hydrolysis of these EPS and subsequent HPLC analysis. On the contrary, the tested strains of the genera *Acetobacter* (1) and *Gluconacetobacter* (7) were not able to produce EPS from sucrose. The phylogenetic positions of *Gluconobacter species* TMW 2.767 and *Gluconobacter species* TMW 2.1191 are presented in section 3.3.2. Since *Gluconobacter species* TMW 2.1191 was isolated in the later course of this work, the isolated fructan of this strain was not used for comparative baking experiments (3.5).

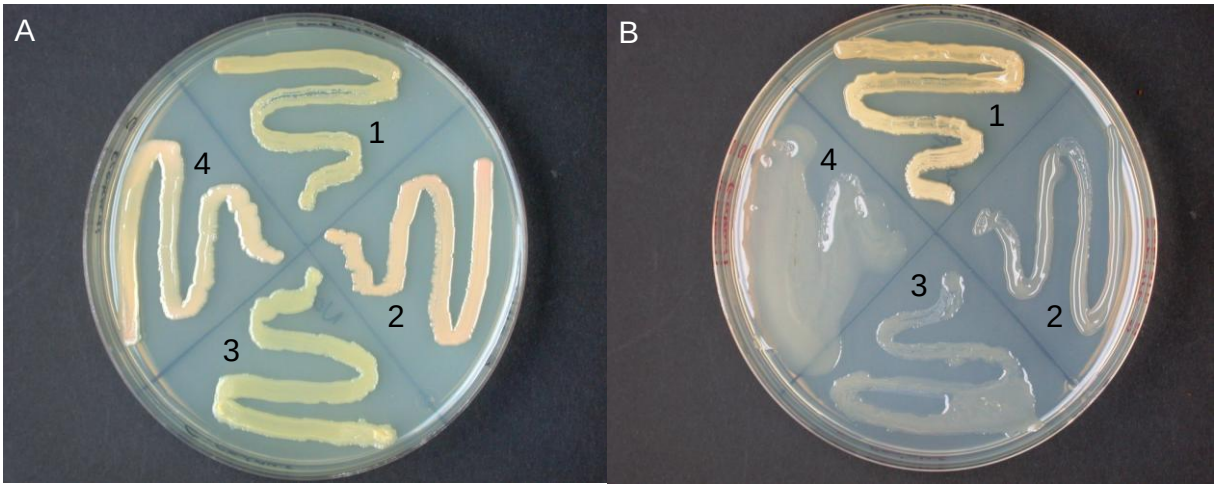

Fig. 1: Morphologies of *Gluconobacter oxydans* 621H (1), *Gluconobacter species* TMW 2.1191 (2), *Gluconobacter cerinus* DSM 9533T (3) and *Gluconobacter species* TMW 2.767 (4) on modified gluconate media without (A) or with supplemented sucrose (B) (8% w/v).

3.2 Structural analyses of EPSs

For later experiments, such as identification of the corresponding fructan producing enzymes (3.3, 3.4) and practical application of fructans (3.5), it was necessary to determine the produced fructan types (inulin *vs*. levan) and to investigate the macromolecular composition of the synthesized polymer fractions. In this way, it should be clarified, if fructans were produced by inulo- or levansucrases and if observed effects in practical applications could be correlated with structural features of the used fructan additives. Therefore, fructans of *Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533T, *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400 were isolated from 5 L of NaG media (+ 80 g/L sucrose), respectively. In this way, total EPS amounts of 59.5 g (*Gluconobacter species* TMW 2.767), 31.5 g (*G. cerinus* DSM 9533), 36.5 g (*N. chiangmaiensis* NBRC 101099) and 39 g (*K. baliensis* DSM 14400) were recovered, which could be used for baking experiments (3.5) and could be structurally analyzed as depicted in sections 3.2.1 and 3.2.2.

3.2.1 Determination of fructan/linkage types

The recorded ^{13}C-NMR spectra of the isolated fructans were compared with (previously) measured chemical shift values for inulin from chicory roots and for levan from *Acetobacter* (*A.*) *xylinum* (actually renamed as *Gluconacetobacter xylinus*) (Tajima et al., 1998) (table 3). According to this, the

isolated fructans of the selected AAB strains exhibited highly similar ^{13}C shift values compared to those reported for levan of *A. xylinum* (Tajima et al., 1998). On the contrary, the ^{13}C values measured for inulin differed in a range of about 0.8 - 1.1 ppm from those measured for fructans from AAB strains (table 3). Furthermore, no additional signals apart from the 6 main carbon signals could be measured in the ^{13}C-spectra of *Gluconobacter species* TMW 2.767, *G. cerinus*, *N. chiangmaiensis* and *K. baliensis* indicating very few, not detectable or no carbon atoms being involved in branching. The ^{1}H NMR spectra as well as the chemical shift correlated *COSY* and *HMQC* spectra of all isolated fructans were identical as well. A small single signal at about 4.95 ppm could be observed in the anomeric regions of each of the four ^{1}H NMR spectra (data not shown), which might be due to glycosidic protons present in the terminal sucrose molecules of the respective polymer chains. Because no main signals could be detected in these anomeric regions, which potentially would result from glycosidic protons in α-/β-linked glucans, the fructan type of these polymers (no linked hydrogens present at *C2* in β-(2→6) or β-(2→1) linked fructans) is confirmed. This furthermore is in agreement with the missing coupling of the corresponding *C2* atoms with hydrogens derived from the recorded *HMQC* spectra.

Table 3: ^{13}C NMR chemical shifts of fructans produced by *Gluconobacter species* TMW 2.767, *G. cerinus*, *N. chiangmaiensis*, *K. baliensis* and inulin from chicory roots.

Fructan	Chemical shifts (ppm) Carbon atom					
	C1	**C2**	**C3**	**C4**	**C5**	**C6**
G. species 2.767	62.32	106.71	78.72	77.66	82.8	65.9
G. cerinus	62.71	107.08	79.11	78.04	83.17	66.26
N. chiangmaiensis	62.57	107.09	79.11	78.04	83.18	66.28
K. baliensis	62.57	106.97	78.98	77.92	83.06	66.16
A. xylinum * Levan	62.6 *	106.9 *	79.0 *	77.9 *	83.0 *	66.0 *
Inulin	63.71	106.1	79.78	77.07	83.91	64.97

(*) data were obtained from Tajima et al. (1998)

Moreover, two dimensional NMR (*COSY*, *HMQC*) yielded the same assignments for carbons and hydrogens present in β-(2→6)-linked fructofuranoses as reported for levan from *A. xylinum* (Tajima et al., 1998). Since these spectra were identical for the isolated fructans of the selected AAB strains, Fig. 3 and Fig. 4 exemplary demonstrate and explain these assignments for the fructan of *G. cerinus*. Consequently, fructans of *Gluconobacter species* TMW 2.767, *G. cerinus*, *N. chiangmaiensis* and *K. baliensis* could be identified as β-(2→6)-linked β-(D)-fructofuranosyl polymers, namely levans (Fig. 2). Moreover, in the later course of this work (3.4.3), the fructan produced by *Gluconobacter species* TMW 2.1191 was investigated by ^{13}C-NMR spectroscopy regarding its linkage types and could be identified as linear levan due to its characteristic chemical shifts, which are representative for linear levans (Appendix A13) (table 3).

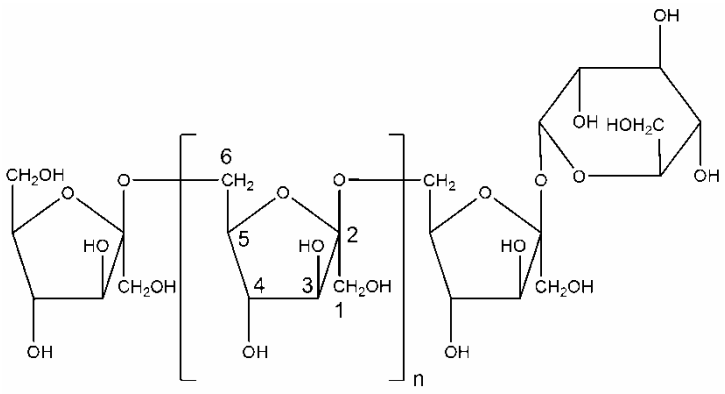

Fig. 2: Proposed structure for levans isolated from *Gluconobacter species* TMW 2.767, *G. cerinus, N. chiangmaiensis, K. baliensis* and *Gluconobacter species* TMW 2.1191.

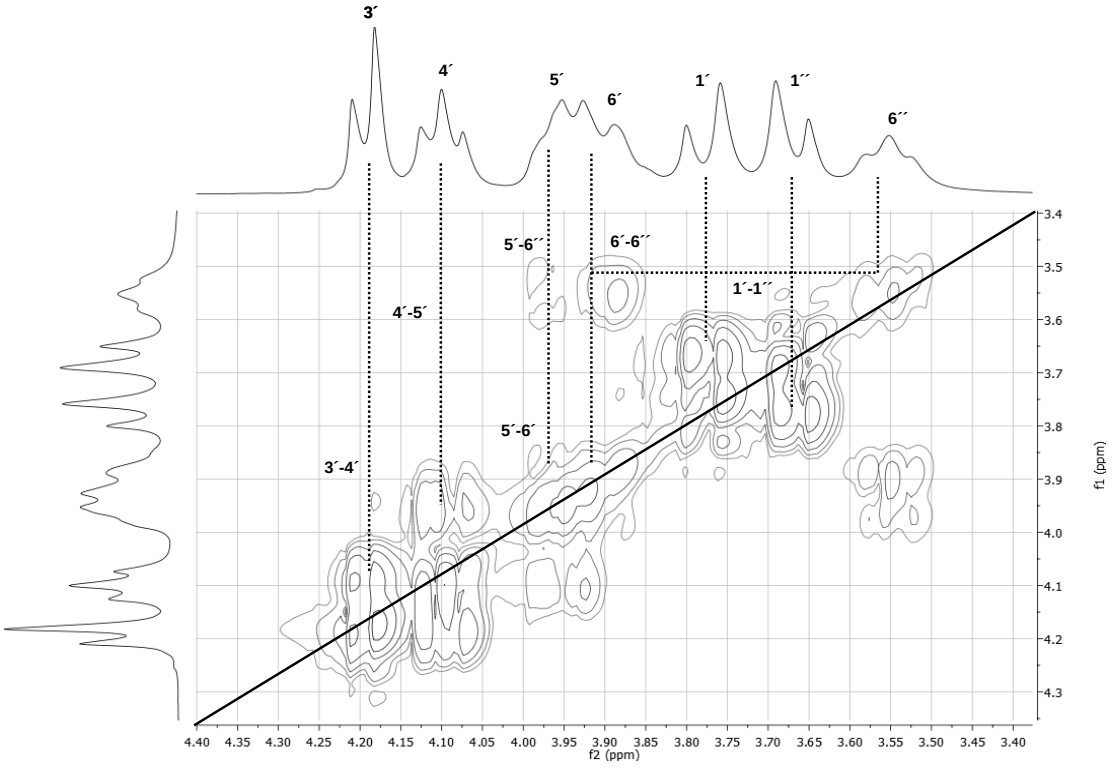

Fig. 3: ^{1}H-^{1}H 2D NMR homonuclear chemical shift correlation (*COSY*) spectrum of fructan isolated from *G. cerinus* DSM 9533T. Vertical dotted lines symbolize the coupling of the respective protons with their corresponding next located protons. Doublet peaks (1´-H, 1´´-H, 3´-H) result from coupling with one next located proton (1´-H ↔ 1´´-H, 3´-H ↔ 4´-H), triplet peaks (4´-H, 6´´-H) from coupling with two next located protons (4´-H ↔ 3´-H/5´-H, 6´´-H ↔ 5´-H/6´-H). The large chemical shift difference between 6´-H and 6´´-H can be explained by the simultaneous shielding effects of oxygen in the fructose residue and by the glycosidic bound oxygen according to Tajima et al. (1998). The shielding effect causes an overlapping of 5´-H and 6´-H signals.

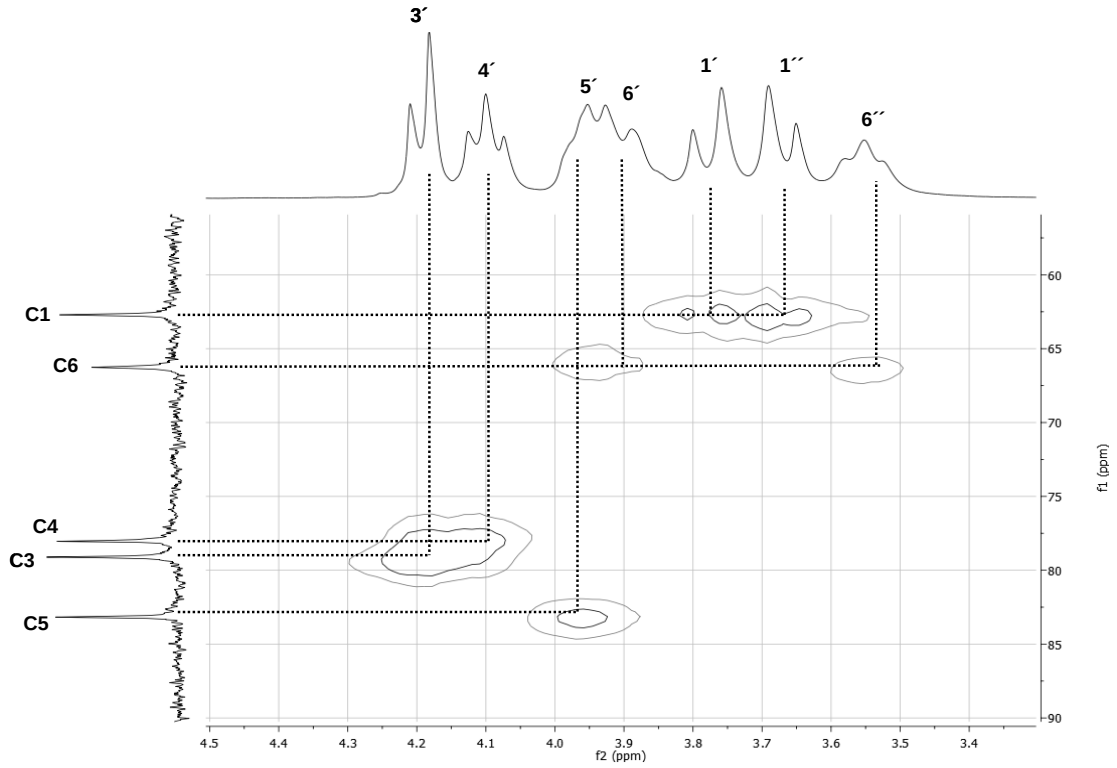

Fig. 4: ^{13}C-^{1}H 2D NMR heteronuclear multiple quantum coherence (*HMQC*) spectrum of fructan isolated from *G. cerinus* DSM 9533T showing attributed carbon atom signals. Dotted lines connect the corresponding carbon and hydrogen signals assigned in Fig. 3. Carbon atoms can be designated as *C1*-*C5* according to their coupling to hydrogens. *C2* (excluded for better resolution of the spectrum), which is involved in the glycosidic linkage of *β*-(2→6)-linked fructans and thus harbours no bound hydrogen, consequently yields no coupling signal to hydrogen (see also Fig. 3).

3.2.2 Determination of molecular weights and particle sizes

Data derived from AF4-MALS-RI were processed to comparative plots, which depict the differing macromolecular compositions of fructans from *Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533T, *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400. Fig. 5 shows the cumulative distributions of obtained molecular weights (M_Wi, Fig. 5A) and molecule sizes (R_Gi, Fig. 5B) for the isolated levan polymer fractions of the four selected AAB strains. In order to investigate the continuity of molecule growth in each fraction and to describe the conformational shapes of these molecules in aqueous solution (obtained from the calculated hydrodynamic coefficients v_G; equation 5, 2.4.2), R_Gi were furthermore logarithmically plotted against their corresponding M_Wi (Fig. 6). Levan polymer fractions were divided into four overlapping M_Wi/R_Gi and three individual M_Wi/R_Gi ranges among each other to establish a relationship between molecular weight/molecule size distributions of the investigated levans and observed functional effects in wheat breads (3.5). Table 4 summarizes M_Wi/R_Gi ranges, the percentage of molecules that were present in these ranges (calculated from data involved in Fig. 5A and 5B, respectively), and the individual hydrodynamic coefficients v_G obtained from the log-log plots of the corresponding R_Gi *vs.* M_Wi.

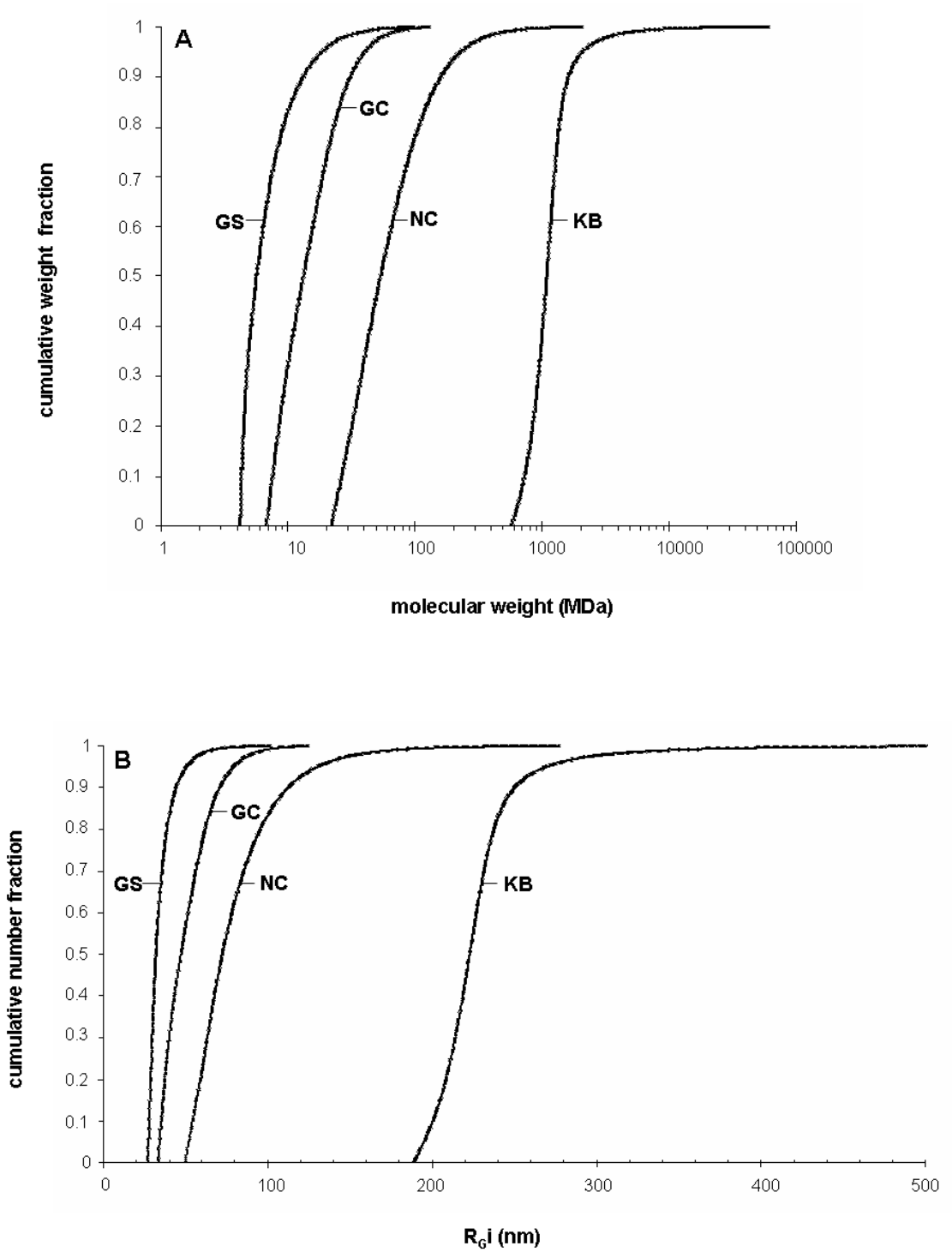

Fig. 5: Cumulative $M_W i$ (A) and $R_G i$ (B) distributions of fructans isolated from *Gluconobacter species* TMW 2.767 (GS), *G. cerinus* (GC), *N. chiangmaiensis* (NC) and *K. baliensis* (KB). Depicted data are mean values of three independent measurements.

In contrast to their basic structure (linearity) and linkage types (3.2.1), levan fractions isolated from the selected AAB strains significantly differed in terms of their molecular weight distributions (Fig. 5A). Whereas *Gluconobacter species* TMW 2.767 and *G. cerinus* synthesized polymer fractions in the range of 4-98 MDa (*Gluconobacter species* TMW 2.767*)* and 6-98 MDa (*G. cerinus*) (100% and 99%

of molecules, respectively), *N. chiangmaiensis* and *K. baliensis* produced portions of comparatively higher molecular weight levan molecules, exhibiting molecular weight ranges of 100-575 MDa (*N. chiangmaiensis*: ~20% of molecules) or even 1000-2000 MDa (*K. baliensis*: ~75% of molecules). Besides, *N. chiangmaiensis* synthesized a large fraction of molecules in the range of 22-98 MDa (77%), which was also detected to a significant lower extent in levans of *Gluconobacter species* TMW 2.767 (3%) and *G. cerinus* (21%). M_Wi/DP of the whole levan fractions of each AAB strain were calculated as 13.3 MDa/8.2x10^4 (*Gluconobacter species* TMW 2.767), 26.3 MDa/1.6x10^5 (*G. cerinus*), 208.9 MDa/1.3x10^6 (*N. chiangmaiensis*) and 2466 MDa/1.5x10^7 (*K. baliensis*). The highest polydispersity index (M_W/M_N) was observed for levan from *N. chiangmaiensis* (2.51) indicating a broad distribution of molecules, which exhibited different molecular weights (see also Fig. 5A and Table 4). More narrow molecular weight distributions were detected for levans of *Gluconobacter species* (M_W/M_N: 1.72), *G. cerinus* (M_W/M_N: 1.53) and *K. baliensis* (M_W/M_N: 1.98).

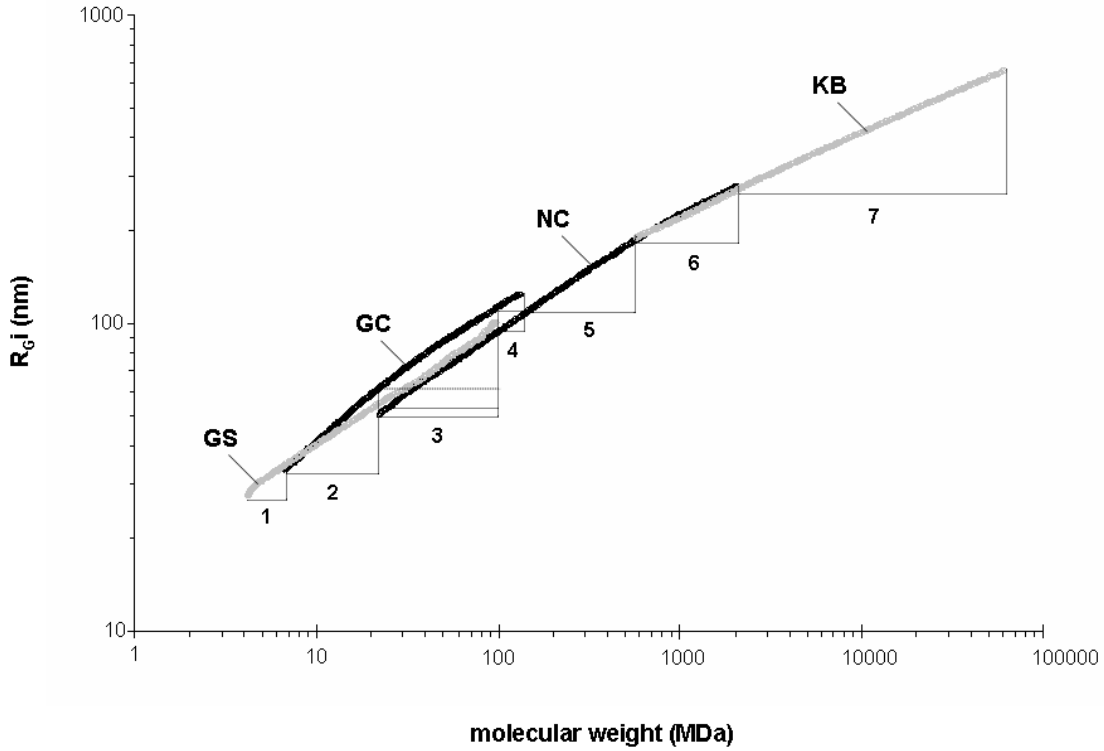

Fig. 6: Conformation plot (*log-log* plot of R_Gi versus M_Wi) of fructans isolated from *Gluconobacter species TMW 2.767* (GS), G. *cerinus* (GC), *N. chiangmaiensis* (NC) and *K. baliensis* (KB). Depicted data are mean values of three independent measurements. Levan fractions were separated into individual (1:GF, 5:NC, 7:KB) and overlapping (2:GF/GC, 3:GF/GC/NC, 4:GC/NC, 6:NC/KB) ranges.

In aqueous solutions the size of levan molecules present in all isolated levans continuously increased with their molecular weight (Fig. 5 and 6). Detected R_Gi value ranges in the overlapping molecular weight fractions 2, 3, 4, 6 slightly differed among the different levans (table 4), but laid within similar R_Gi ranges. The hydrodynamic coefficients v_G of the whole levan fractions (calculated from all data points of each isolated fructan) were 0.44 (*G. cerinus*), 0.39 (*Gluconobacter species* TMW 2.767), 0.38 (*N. chiangmaiensis*) and 0.27 (*K. baliensis*). Noticeably, the highest v_G values were calculated in

the corresponding lowest molecular weight levan fractions 1 (*Gluconobacter species* TMW 2.767), 2 (*G. cerinus*), 3 (*N. chiangmaiensis*) and 6 (*K. baliensis*) of each isolated levan (table 4). Therefore, levan molecules in all isolated levans appeared to adopt a more compact conformation with increasing molecular weight (with the exception of *Gluconobacter species* TMW 2.767 fraction 2→3, possibly due to small detection errors, table 4) in aqueous solution.

Table 4: M_Wi/R_Gi ranges and hydrodynamic coefficients of levan fractions (according to Fig. 6) isolated from *Gluconobacter species* TMW 2.767 (GS), *G. cerinus* (GC), *N. chiangmaiensis* (NC) and *K. baliensis* (KB).

		Levan fraction (Fig. 6)						
	Strain	**1**	**2**	**3**	**4**	**5**	**6**	**7**
M_Wi range (MDa) (percentage of molecules)	GS	4-6 (64%)	6-22 (33%)	22-98 (3%)				
	GC		6-22 (78%)	22-98 (21%)	98-133 (1%)			
	NC			22-98 (77%)	98-133 (9%)	133-575 (13%)	575-2071 (1%)	
	KB						576-2069 (95%)	2069-61637 (5%)
R_Gi range (nm) (percentage of molecules)	GS	27-34 (64%)	34-54 (33%)	54-100 (3%)				
	GC		33-61 (78%)	61-111 (21%)	111-124 (1%)			
	NC			50-92 (77%)	92-104 (9%)	104-186 (13%)	186-277 (1%)	
	KB						189-269 (95%)	269-658 (5%)
Slope of regression line (v_G)	GS	0.46	0.38	0.40				
	GC		0.52	0.40	0.34			
	NC			0.40	0.40	0.39	0.31	
	KB						0.28	0.26

Values for M_Wi/R_Gi ranges and percentages of molecules in these ranges (in parentheses) were calculated from data involved in Fig. 5. Slope values v_G were calculated from the regression lines of the different levan fractions (Fig. 6). Depicted data are mean values of three independent measurements. The coefficients of determination were >0.99 for all calculated slopes.

3.2.3 Identification of a novel heteropolysaccharide from *K. baliensis* DSM 14400

K. baliensis DSM 14400 exhibited slimy colony morphologies on NaG-agar plates, which were not supplemented with sucrose as substrate for EPS formation. This observation was especially made when inoculated agar plates were stored in a fridge at 4 °C for a few days. This potential EPS was subsequently isolated from a NaG liquid culture with a total yield of about 1.5 g/L. A part of the lyophilized material was hydrolyzed and analyzed regarding its sugar monomer composition *via* HPLC. Three peaks were detected after acidic hydrolysis and identified as glucose (RT: 13.8), galactose (RT: 15.5) and fructose (RT: 17.7) in a molar ratio of about 6:2:1 (calculated from standardized peak areas), respectively. Consequently, *K. baliensis* DSM 14400 produces a unique

heteropolysaccharide consisting of glucose, galactose and fructose when grown in NaG medium without supplemented sucrose.

3.3 Genetics and enzymology of levan synthesis

Since fructans of *Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533, *Gluconobacter species* TMW 2.1191 (appendix A13), *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400 could be identified as levans (3.2.1), genomes of related organisms were checked for existing levansucrases, whose nucleotide sequences were used to create degenerate primers (based on conserved regions among these known levanuscrases) for amplification of core sequences of the respective genes. Several PCR methods (2.5.2) were finally applied to detect ORFs coding for levansucrases, which were subsequently cloned into *E. coli* expression strains. In this way, it should be proved, if these genes can be processed to active enzymes. Newly identified levansucrases were phylogenetically and structurally compared among each other. The subdivision of individual sections corresponds to the chronological process of levansucrase identification/characterization.

3.3.1 Identification of levansucrase encoding genes in genomes of *Gluconobacter* strains

In a first step, degenerate primers Av and Ar were constructed from conserved motifs ADAMK and GTLAPT, located in the N- and C-terminal domain of known levansucrases of *G. oxydans*, *Ga. xylinus* and *Zymomonas mobilis*, respectively. In this way, an expected 1000 bp core fragment of *Gluconobacter species* TMW 2.767 levansucrase was obtained. Two miPCR approaches lastly yielded a 1955 bp chromosomal fragment, which included a complete ORF (1335 bp), putatively coding for a levansucrase in *Gluconobacter species* TMW 2.767 (Fig. 7A). Using primers Av and Ar, no positive PCR products could be amplified for *G. cerinus* DSM 9533T. Therefore, an alignment including the levansucrase gene sequences of *Ga. xylinus* (AB034152), *G. oxydans* 621H (NC_006677) and *Gluconobacter species* TMW 2.767 was calculated to identify conserved regions in known levansucrases of AAB. In this way, Z1F and Z1R were created, which amplified the expected core fragment of approximate 750 bp in size. SiPCR finally was performed to detect a complete ORF coding for a levansucrase in *G. cerinus* DSM 9533T (Fig. 7B). Furthermore, dyad symmetries were detected downstream from the levansucrase genes of *Gluconobacter species* TMW 2.767 and *G. cerinus* DSM 9533T (54 nt and 51 nt, respectively), possibly being involved in hairpin formation (17.9 kcal mol^{-1} and 18.6 kcal mol^{-1}, respectively) and thus possibly acting as rho-independent transcription terminators. These potential hairpins are composed of a twelve base pair stem with a loop of 5 unpaired bases followed by 5 uracil moieties (*Gluconobacter species* TMW 2.767) and a thirteen base pair stem with a loop of 8 unpaired bases followed by 7 uracil moieties (*G. cerinus* DSM 9533T). To enhance the possibility to amplify quickly a core-sequence of the unknown *ftf*-gene of *Gluconobacter species* TMW 2.1191, primers DgAlbF and DgAlbR were deduced from an alignment of known levansucrase gene sequences of the *Gluconobacter* strains *G. oxydans* 621H, *Gluconobacter species*

TMW 2.767 and *G. cerinus* DSM 9533T. Using this primer set, solely one PCR approach sufficed to obtain the expected core amplicon (~ 1100 bp) of a putative levansucrase in *Gluconobacter species* TMW 2.1191. Because of rare available suitable restriction sites and endonucleases, mspPCR and msfPCR were performed to finally detect the missing terminal regions of this gene (Fig. 7C). Due to lacking downstream sequence information no potential transcription terminators could be predicted as described for *Gluconobacter species* TMW 2.767 and *G. cerinus* DSM 9533T. Additional sequence information derived from the flanking regions of the predicted levansucrase genes revealed no homologies to proteins of known function using the Blast-Function of GenBank.

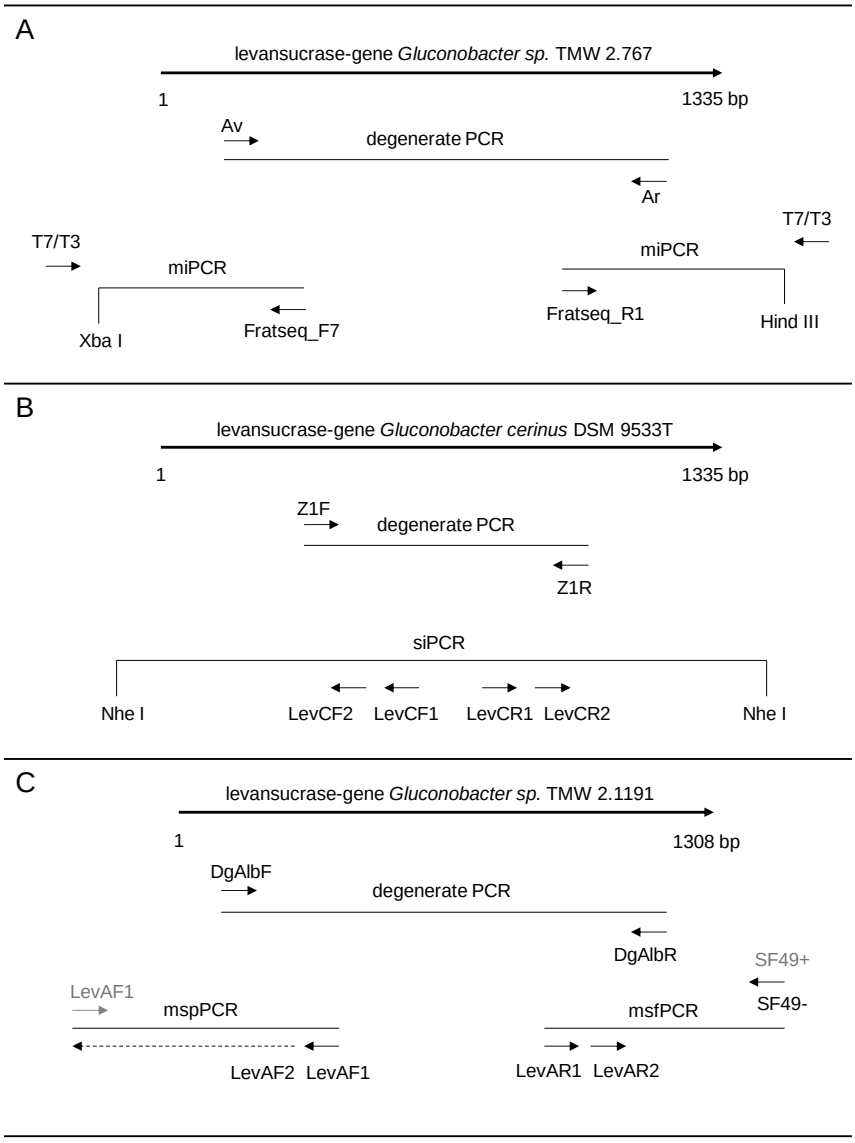

Fig. 7: Schematic representation of the methods used to obtain unknown ORFs coding for levansucrases of (A) *Gluconobacter species* TMW 2.767, (B) *Gluconobacter cerinus* DSM 9533T and (C) *Gluconobacter species* TMW 2.1191. ORFs were identified in the chronological order A→C. An initial alignment including the amino acid sequences of levansucrases of *Zymomonas mobilis* (AAA27696), *Gluconobacter oxydans* 621H (AAW60646) and *Gluconacetobacter xylinus* (BAA93720) was generated to create degenerate primers Av and Ar. Further degenerate primers were constructed involving newly identified sequences to enhance the possibility for positive amplicons. Grey primer names and arrows indicate unspecific binding. Primer LevAF2 was solely used as sequencing primer (dotted arrow). The used restriction endonucleases are named below vertical lines.

3.3.2 Phylogenetic comparison of *Gluconobacter* strains and levansucrases

For species identification, the isolates were subjected to sequencing of their 16S rRNA genes. The phylogenetic position of these strains based on 16S rRNA gene analysis is presented in Fig. 8, which also corroborates the finding of Yamada & Yukphan (2008), that *Gluconobacter* species can phylogenetically be divided into the *G. cerinus* and *G. oxydans* group. Recently, the insect-pathogenic species *G. morbifer* was isolated and described as new species (Roh et al., 2008), which takes a distant position compared to all other *Gluconobacter* species (Fig. 8). *Gluconobacter species* TMW 2.767 shares 99% sequence similarity (based on 1355 bp) with the type strains of *G. frateurii* and *G. japonicus*, which again possess identical 16S rRNA genes. *Gluconobacter species* TMW 2.1191 shares 100% sequence similarity (based on 1358 bp) to the type strain of *G. albidus*, which forms white colonies as included in its species description (Yukphan et al., 2004). However, *Gluconobacter species* TMW 2.1191 forms pink-coloured colonies (Fig. 1A) like the type strain of *G. roseus* (Malimas et al., 2008).

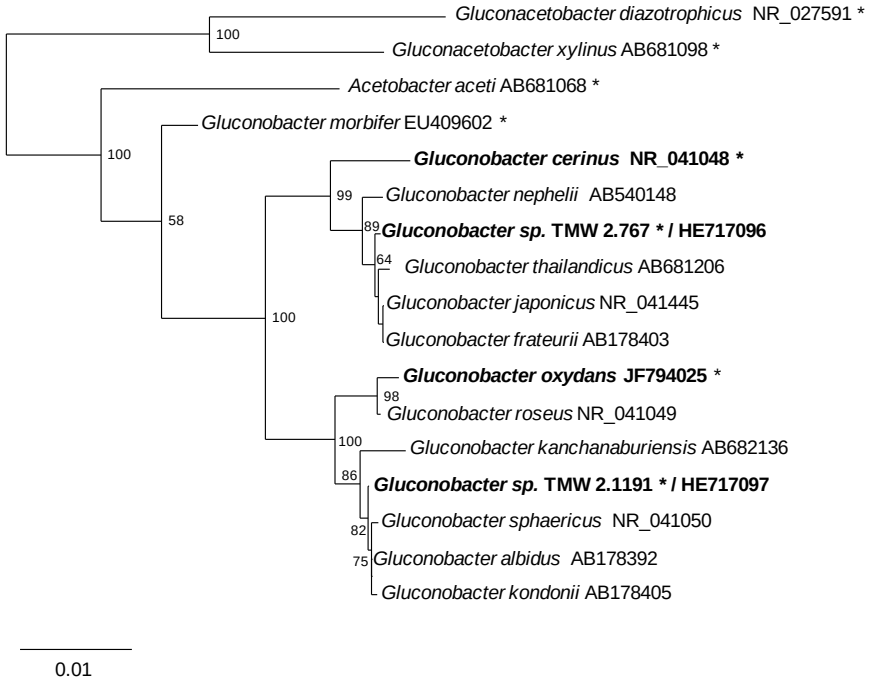

Fig. 8: Phylogenetic tree calculated on the basis of 16S rRNA gene sequences using the neighbour-joining method. The dendrogram includes all type strains of the genus *Gluconobacter* and AAB strains, which possess levansucrase-encoding genes (indicated by asterisks). The scale-bar indicates number of changes per nucleotide. Strains investigated in this study are highlighted in bold. Numbers following species names represent the accession numbers of the corresponding 16S rRNA genes as deposited in GenBank. Bootstrap values indicated at nodes are derived from 1000 replications.

A similar phylogenetic grouping as the one based on 16S rRNA gene analysis is obtained upon comparison of the newly identified FTF proteins depicted in Fig. 9. Levansucrases of *Gluconobacter species* TMW 2.767 and *G. cerinus* DSM 9533T (91% positives in 445 amino acids) as well as levansucrases of *Gluconobacter species* TMW 2.1191 and *G. oxydans* 621H (94% positives in 436

amino acids) (see also Fig. 10) can phylogenetically be grouped in different subclusters (Fig. 9). Thus, the levansucrases investigated in this study show the highest similarity to those of their closest related species. Together with levansucrases of *G. morbifer*, *Ga. xylinus* and *Acetobacter aceti* and those from ethanologenic *Zymomonas (Z.) mobilis species* (α-proteobacteria, *Sphingomonadeceae*), they form an independent cluster as also described by Velazquez-Hernandez et al. (2009). Species similarity is not inevitably associated with levansucrase similarity as shown for the levansucrase of *Ga. diazotrophicus*, which forms an independent cluster with those originating from different *Burkholderia* species (β-proteobacteria) (Fig. 9). In contrast, it´s next related species *Ga. xylinus* (based on 16S rRNA gene analysis, Fig. 8) possesses a levansucrase, which is more similar to those of *Gluconobacter* species (Fig. 9). In conclusion, the newly identified proteins can be classified as FTFs belonging to the GH 68 family based on their phylogenetic grouping and sequence similarity.

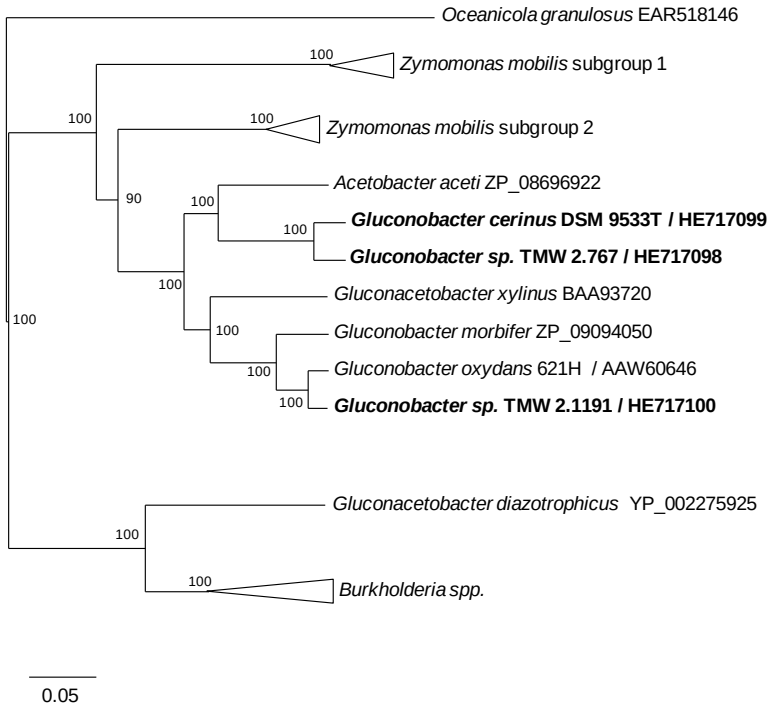

Fig. 9: Phylogenetic tree including all so far identified levansucrases from AAB species and their next related enzymes according to Velazquez-Hernandez et al. (2009). Similarities were calculated using the neighbour-joining method. Numbers following species names represent the accession numbers of the corresponding proteins as deposited in GenBank. Newly identified FTFs are highlighted in bold. The scale bar represents number of changes per amino acid. The levansucrase protein of *Oceanicola granulosus* (EAR51814) was used as an outgroup. Bootstrap values indicated at nodes are derived from 1000 replications. Compressed branches are composed of the following accession numbers: *Zymomonas mobilis* subgroup 1 (YP_004662017, AAC36942, YP_003225996), *Zymomonas mobilis* subgroup 2 (YP_004662018, BAA04475, AAA27695, AAA27702), *Burkholderia spp*. (ZP_02904976, ZP_02379902, ZP_02885256, ACC75109, ZP_02365208, ZP_0250513, ZP_02385398, ZP_02371500, YP_001117258, ZP_02893677, ZP_02906975, ZP_04941833).

3.3.3 Structural features and heterologous expression of *Gluconobacter* levansucrases

The levansucrases of the *Gluconobacter* strains investigated in this study are structurally composed of several domains, which share identical or similar sequences. To demonstrate similarities *versus*

differences their protein sequences are compared in Fig. 10 and depicted as red and yellow blocks, respectively.

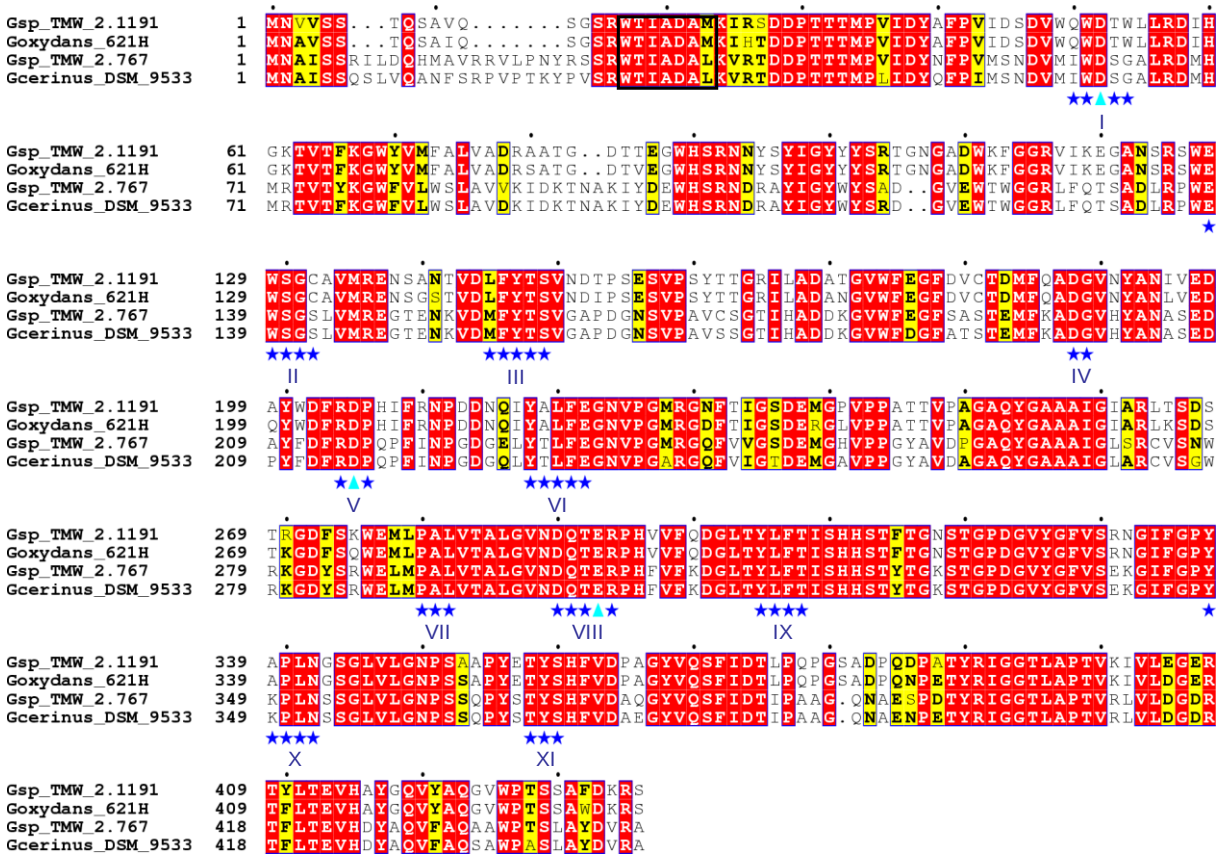

Fig. 10: Alignment of newly identified levansucrases of *Gluconobacter species* TMW 2.1191, *Gluconobacter species* TMW 2.767 and *Gluconobacter cerinus* DSM 9533T including the levansucrase sequence obtained from the genome sequenced strain *Gluconobacter oxydans* 621H (accession number AAW60646). Red coloured groups represent highly conserved, identical sequence patterns, whereas yellow coloured groups indicate well conserved residues within a group, which again are symbolized in bold. Blue asterisks in row indicate conserved motifs in the catalytic domains of fructosyltransferases belonging to the GH 68 family according to Velazquez-Hernandez et al. (2009), Meng & Fütterer (2003), Martinez-Fleites et al. (2005) and van Hijum et al. (2006), which are (if identified) functionally designated as (I) catalytic nucleophile, (II) sucrose box 1, (III) sucrose box 2; being involved in acceptor recognition, (IV) being involved in acceptor recognition, (V) catalytic centre; transition state stabilizing; being involved in sucrose hydrolysis and fructan polymerization (VI) being involved in sucrose hydrolysis + transfructosylation, (VII) being conserved among GH 68 family proteins (VIII) catalytic centre + acid-base catalyst, (IX) being conserved in microbial fructosyltransferases (X) being conserved in the GH 68 family and (XI) orientating the catalytic nucleophile for transfructosylation. Light blue triangles symbolize acidic residues $Asp^{52/62}$, $Asp^{205/215}$ and $Glu^{293/303}$, which are supposed to act as catalytic nucleophile, transition state stabilizer and general acid-base catalyst, respectively, and are strictly conserved among members of the glycoside hydrolase families 32, 43, 62 and 68 (Pons et al., 2004). The bolded box indicates a motif, which is conserved in the N-terminal domain of FTFs from gram-negative bacteria (Velazquez-Hernandez et al., 2009).

Moreover, no predictable signal peptides could be detected, whereas the conserved motif WTIADA(M/L) is located in the N-terminal domain (Fig. 10). Both features have been described as typical features of levansucrases from gram-negative bacteria (Velazquez-Hernandez et al., 2009). The respective predicted molecular weights are 47.8 kDa (*Gluconobacter species* TMW 2.1191), 47.9 kDa (*G. oxydans* 621H), 49.2 (*G. cerinus* DSM 9533T) and 49.5 kDa (*Gluconobacter species* TMW

2.767). These differences in size are mainly due to missing amino acids in the N-terminal domain of FTFs from *Gluconobacter species* TMW 2.1191 and *G. oxydans* 621H. In this domain the highly similar levansucrases of *Gluconobacter species* TMW 2.767 and *G. cerinus* DSM 9533T (91%) exhibit their strongest sequence differences (solely 5 positives in amino acids 6-24, Fig. 10). The 11 motifs shown in Fig. 10 at some points exhibit slight differences in amino acids compared to the consensus sequences described by Velazquez-Hernandez et al. (2009). This may indicate that further amino acids could be involved in the catalysis of levan formation. Key amino acids $Asp^{52/62}$, $Asp^{205/215}$ and $Glu^{293/303}$ involved in substrate binding (Fig. 10) are also highly conserved in these proteins. Fig. 11 demonstrates the capability of recombinant *Escherichia coli* Top 10 cells, which harbour the newly identified levansucrase genes, to form polysaccharide from sucrose.

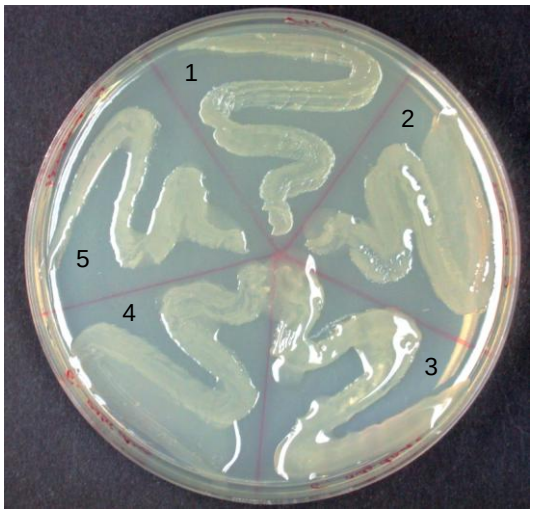

Fig. 11: Recombinant *Escherichia coli* Top 10 cells cultivated on LB media supplemented with 6% (w/v) sucrose and 0.2% (w/v) arabinose. (1) *E. coli* + empty pBAD vector, (2) *E. coli* + FTF of *Gluconobacter species* TMW 2.767, (3) *E. coli* + FTF of *G. cerinus* DSM 9533T, (4) *E. coli* + FTF of *Gluconobacter species* TMW 2.1191, (5) *E. coli* + FTF of *G. oxydans* 621H.

Heterologous expression of the corresponding *ftf*-genes in *E. coli* can lead to recombinant strains being able to produce mucous substances from sucrose. However, insertion of the corresponding gene into the used expression vector and subsequent transformation into *E. coli* cells not necessarily yielded recombinant *E. coli* cells capable of producing mucous polysaccharides. Only about 10% of transformants, which harboured expression vectors containing *ftf*-genes as proven by control PCR, exhibited this macroscopic observable feature. Replica plating of all EPS producing *E. coli* mutants on LB media without sucrose yielded their wildtype-typical colony morphologies, respectively. The nucleotide sequences of these genes contain a few minor codons, which are rarely used by *E. coli*: *Gluconobacter species* TMW 2.767 (1x CGA, 2x CCC); *G. cerinus* DSM 9533T (3x CGA, 3x CCC); *Gluconobacter species* TMW 2.1191 (3x CCC); *G. oxydans* 621H (1x AGG, 3x CCC). In conclusion, it has been demonstrated that these genes can be heterologously expressed as active proteins by *E. coli* cells.

3.3.4 Identification of levansucrase genes in genomes of *N. chiangmaiensis* and *K. baliensis*

Since usage of degenerate primer sets, which were initially designed for identification of core sequences of *Gluconobacter* levansucrases (3.3.1), yielded no positive PCR amplicons using template DNA of these two strains, degenerate primers DgAAB_F and DgAAB_R were constructed from catalytic motifs EWSGS (sucrose box 1) and NDQTER (catalytic centre, acid-base catalyst) (Fig. 10) being conserved among *Gluconobacter sp.* TMW 2.767, *Gluconobacter cerinus* DSM 9533T and *Gluconacetobacter diazotrophicus* PAI5. In this way, expected 550 bp core fragments coding for levansucrases in *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400 were obtained. The residual parts of these genes were identified by chromosomal walking using suitable PCR methods (Fig. 13), respectively.

Whereas the levansucrase-gene of *N. chiangmaiensis* codes for one comprehensible ORF (1776 bp), the levansucrase-gene of *K. baliensis* is disrupted by an IS5 element located in the N-terminal domain of the putatively inactive, original enzyme, which again would exhibit a highly similar sequence compared to the putative one of *N. chiangmaiensis*. This transposable element might have integrated *via* a mechanism, which duplicated the nucleotide sequence GGCCCTACCG at the corresponding insertion ends. The residual sequence of this IS element shares highest similarity (99%) with a plasmid encoded IS5 element from *Acetobacter pasteurianus* IFO 3283 (accession number: AP011171). One incomplete ORF (539 aa) including a levansucrase encoding sequence was found in the whole detected nucleotide sequence (2829 bp) of *K. baliensis*, which starts at the coding end of the inserted IS5 element (TA**ATG)** and comprises all conserved (catalytic) motifs of GH 68 FTFs, but contains a stop codon (TAG) at amino acid position 12 (Appendix A3/A4). Blasting of amino acids 13-18 (TELCNRA) yields hits with highest similarities to glycosyltransferase motifs of two *Burkholderia* species (100%), whereas amino acids 20-536 (PTV…) constitute the expected levansucrase sequence (deduced from alignments of known levansucrases). The putative motif TELCNRAGPTV again is encoded in the insertion/duplicated sequence. The putative levansucrase sequence of *N. chiangmaiensis* and the levansucrase sequence of *K. baliensis* (starting from PTV…) share highest sequence similarity with the levansucrase sequence of the N_2-fixing endophyte *Gluconacetobacter diazotrophicus* and with those of different *Burkholderia* species (Fig. 12, appendix A5) and, therefore, form an independent cluster to other known levansucrases of AAB.

Theoretically, an ORF coding for a levansucrase could be transcribed from the detected nucleotide sequence of *K. baliensis* starting from position 1411. This putative truncated levansucrase (472 amino acids), however, would lack the motif WTIADA(M/L), which is highly conserved among levansucrases of gram-negative bacteria (3.3.3), but would contain all so far known motifs being involved in enzymatic catalysis of FTFs. Both putative (original) N-terminal domains of *N. chiangmaiensis* and *K. baliensis* levansucrases possess(ed) signal peptides comprising 30 amino acids,

being cleaved off during transport to the outside of the cells, respectively. Furthermore, downstream from the (partially) levansucrase coding sequences of both strains, fragments of genes were detected (BfpH and GspE, Fig. 13), which are part of a levansucrase-type II dependent secretion operon in *Gluconacetobacter diazotrophicus* (Arrieta et al., 2004). Moreover, dyad symmetries were found downstream from the (partial) levansucrase genes (stop codons) of *N. chiangmaiensis* and *K. baliensis* (9 nt and 11 nt, respectively), possibly being involved in hairpin formation (18.2 kcal mol^{-1} and 18.8 kcal mol^{-1}, respectively) and thus possibly acting as rho-independent transcription terminators. The detected nucleotide sequences of both strains contain – in contrast to the investigated *Gluconobacter* strains (3.3.3) - a large number of codons, which are rarely used by *E. coli* (appendix A1/A3). Putative levansucrases of *N. chiangmaiensis* without predicted signal peptide were cloned into *E. coli* Top 10 and codon-optimized *E. coli* Rosetta cells, respectively. However, recombinant *E. coli* cells harbouring correct inserts of these iso-forms were not able to produce polysaccharide from sucrose and accumulated large intracellular inclusion bodies as microscopically observed (data not shown). No attempts were made to clone shortened, hypothetical levansucrase ORFs of *K. baliensis* into *E. coli* strains for heterologous expression because of its principal high similarity to *N. chiangmaiensis* levansucrase (appendix A5), which again could not be functionally expressed in different recombinant *E. coli* hosts as described above.

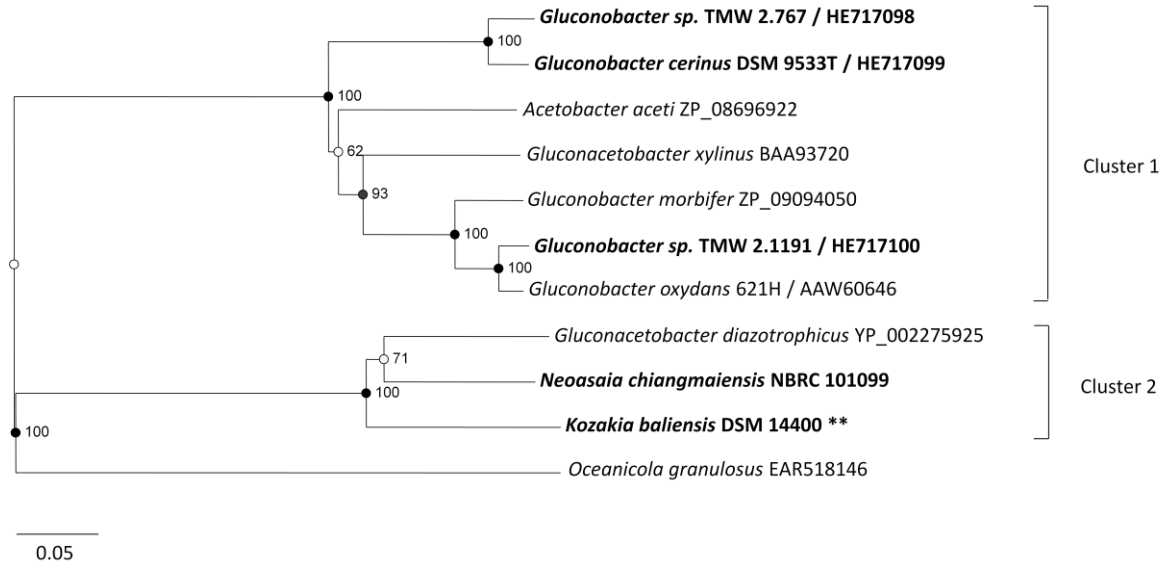

Fig. 12: Phylogenetic tree including all so far identified levansucrases from AAB species. Similarities were calculated using the neighbour-joining method. Numbers following species names represent the accession numbers of the corresponding proteins as deposited in GenBank. Newly identified FTFs are highlighted in bold. The scale bar represents number of changes per amino acid. The levansucrase protein of *Oceanicola granulosus* (EAR51814) was used as an outgroup. Bootstrap values indicated at nodes are derived from 1000 replications. ** For phylogenetic calculations the truncated (putative original) levansucrase sequence (PTV...; 519 amino acids) of *K. baliensis* DSM 14400 was used.

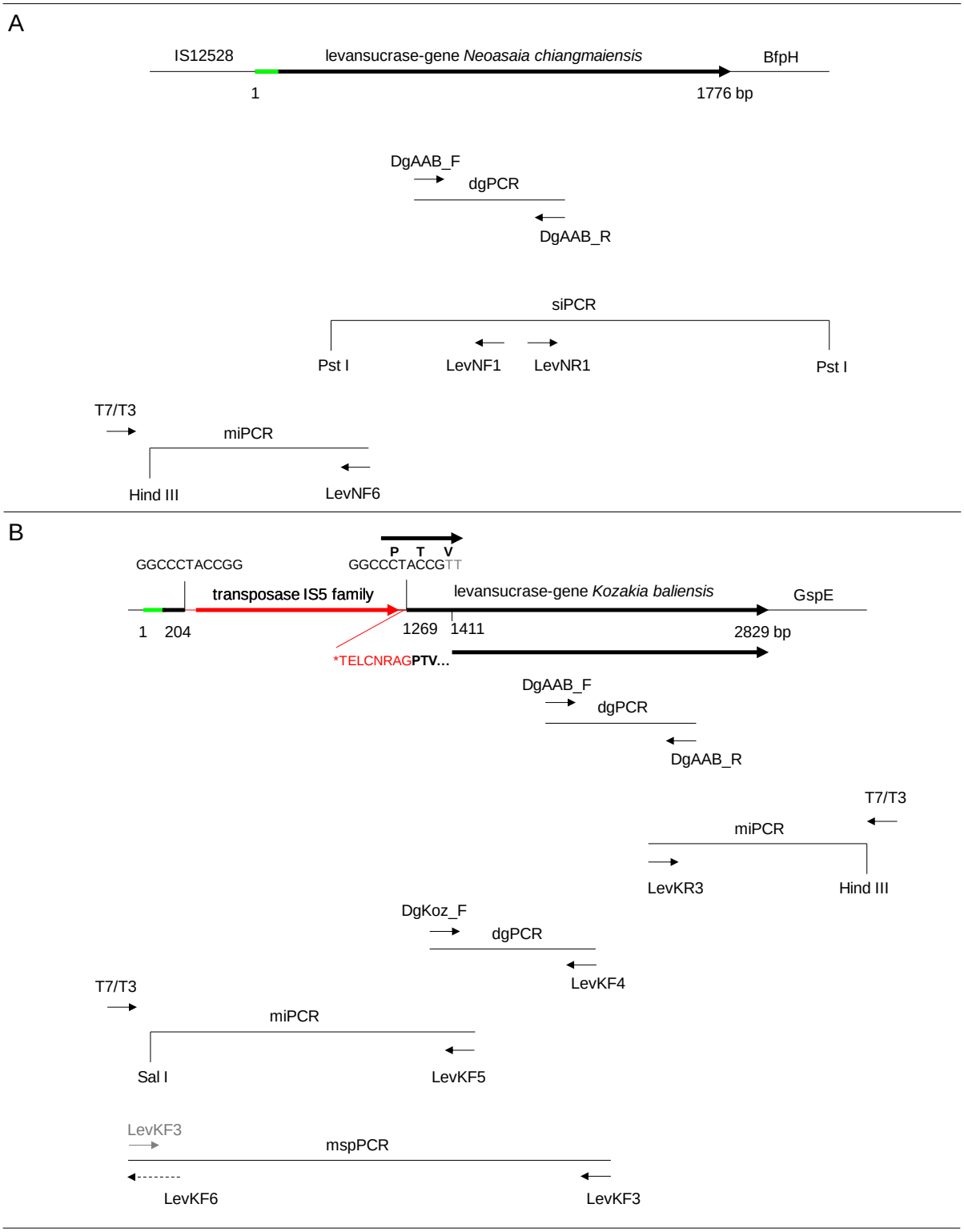

Fig. 13: Schematic representation of the methods used to obtain the genetic organization of unknown ORFs coding for levansucrases of (A) *Neoasaia chiangmaiensis* NBRC 101099 and (B) *Kozakia baliensis* DSM 14400. Grey primer names and arrows indicate unspecific binding. Primer LevKF6 was solely used as sequencing primer (dotted arrow). The used restriction endonucleases are named below vertical lines. Green fragments indicate putative signal peptides as predicted by the SignalP 4.1 server. Insertion element characters in (B) are marked in red. The ORF in (A) is flanked upstream by a gene fragment coding for an insertion element (IS12528) found on plasmid pGOX2 (*G. oxydans* 621H; CP000005.1). See text for further explanations.

3.3.5 Analysis of secreted proteins of *N. chiangmaiensis* and *K. baliensis*

Since the levansucrase gene of *N. chiangmaiensis* could not be heterologously expressed in different *E. coli* hosts and no comprehensible levansucrase ORF could be detected for *K. baliensis* levansucrase (3.3.4), fermented culture supernatants (NaG media, 2.6.1) of these strains were investigated regarding their extracellular protein profiles for possible identification of the corresponding FTFs *via* peptide sequencing. Desalted samples of both strains were loaded on 10% SDS gels to visualize secreted proteins, respectively (Fig. 14).

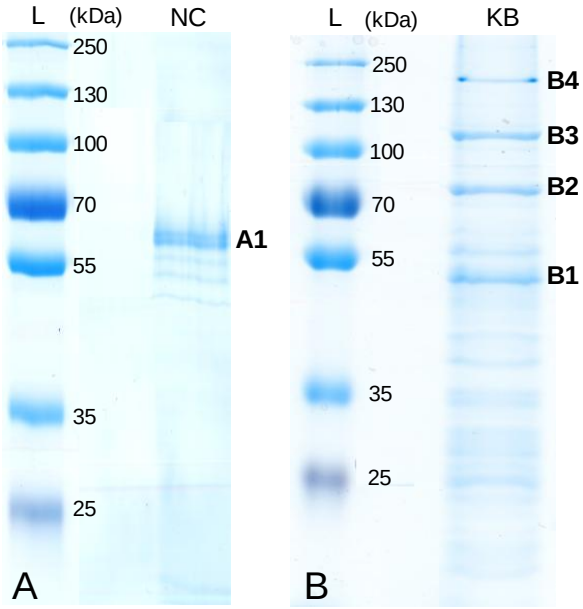

Fig. 14: SDS-PAGE (10%) of secreted proteins recovered from *N. chiangmaiensis* (NC; A) and *K. baliensis* (KB; B). Numbered bands were excised and analyzed *via* peptide sequencing. L: Protein pre-stained ladder (Fermentas, St. Leon-Roth, Germany).

Whereas both desalted protein samples exhibited residual overall levansucrase activities of about 100 U/mL (*N. chiangmaiensis*) and 34 U/mL (*K. baliensis*), the concentrated extracellular protein patterns of both strains differed significantly among each other (Fig. 14). While *K. baliensis* secreted four major and several other protein(s) into the culture supernatant, *N. chiangmaiensis* secreted few, but solely one distinct major protein(s) (Fig. 14; ~ 60 kDa), which coincides with the predicted molecular weight of the putative protein without signal peptide (61.03 kDa; 3.3.4; appendix A2). Protein A1 was additionally visualized by reverse staining and subsequently purified/partially re-activated from the excised gel slice with a residual overall levansucrase activity of about 5 U/mL. However, LC-MS/MS analyses (a total of 3) failed to detect reliable peptide fragments of this putative levansucrase and other potential proteins and, therefore, yielded no positive results. On the contrary, proteins B1 and B2 of *K. baliensis* (Fig. 14B) could be identified *via* LC-MS/MS analysis and subsequent Mascot search as elongation factor Tu and chaperone DnaK, respectively (identical peptide fragments with the respective proteins of *Gluconobacter oxydans* 621H, appendix A8/A9). Proteins B3 and B4 seemed to

be aggregated forms of these two proteins, as peptide fragments of both proteins were detected in both samples, respectively. Re-activation of protein(s) (aggregates) purified from excised gel slices 1-4 of *K. baliensis* yielded no residual overall levansucrase activities.

3.3.6 Structural features of *N. chiangmaiensis* putative levansucrase

Due to its relatively high similarity to the levansucrase of *Ga. diazotrophicus* (466 positives in 553 aa, appendix A5), a 3D model could be calculated for the putative levansucrase of *N. chiangmaiensis* based on the available crystal structure of *Ga. diazotrophicus* levansucrase (Fig. 15A; Martinez-Fleites et al., 2005) (Fig. 15A).

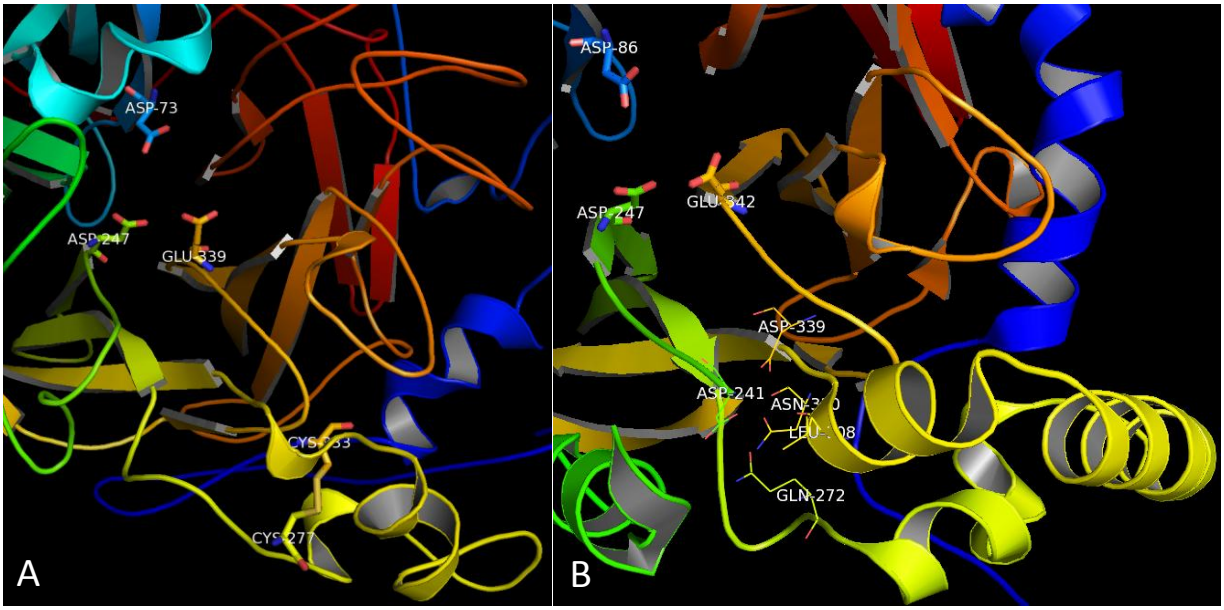

Fig. 15: Ribbon diagrams of *N. chiangmaiensis* putative levansucrase (A) and *Bacillus subtilis* levansucrase (B; Meng & Fütterer, 2003) showing the respective sucrose binding sites (Asp 73/86, Asp 247, Glu 339/342) as well as the corresponding location of an intramolecular disulfide bridge (Cys 277-Cys 333) in *N. chiangmaiensis* putative levansucrase (A) and of the Ca^{2+}-binding site in *Bacillus subtilis* levansucrase (B), which involves amino acids Asp 241, Gln 272, Leu 308, Asn 310 and Asp 339 (Meng & Fütterer, 2003).

The levansucrase model of *N. chiangmaiensis* exhibits a conserved intramolecular disulfide bridge (Cys 277-Cys 333), which was shown to correctly orientate the respective catalytic residues Asp 309 and Glu 401 to their substrate sucrose in *Ga. diazotrophicus* levansucrase (Martinez-Fleites et al., 2005). The truncated form of *K. baliensis* levansucrase (3.3.4), as well as several levansucrases from strains of the genera *Burkholderia*, *Arthrobacter*, *Actinomyces* and *Clavibacter*, which form an independent cluster of levansucrases based on sequence similarity (Velazquez-Hernandez et al., 2009), also possess conserved cysteine residues at the respective positions possibly being involved in disulfide bridge formation (Martinez-Fleites et al., 2005). However, there is no conservation of these cysteine residues among levansucrases of AAB and, generally, members of the GH 68 family of proteins. Moreover, the location of these disulfide bridges in levansucrases of *Ga. diazotrophicus* and

N. chiangmaiensis is found in regions, respectively, being structurally equivalent to the Ca^{2+}-cofactor-binding-site of *B. subtilis* levansucrase as depicted in Fig. 15B (Meng & Fütterer, 2003), which again was reported to analogously function as a fold-stabilizing site for the proper orientation of catalytic residues Asp 247 and Glu 342 to the substrate sucrose.

3.4 Recovery of native levansucrases in buffered solutions

No comprehensible levansucrase ORF could be detected in the genome of *K. baliensis* (3.3.4) and the extracellular protein profile of this strain (while exhibiting overall levansucrase activity) turned out to be rather complex for identification of a secreted levansucrase (3.3.5). Therefore, it was tested, if levan-producing AAB strains could be triggered to secrete levansucrases in a "minimium" system, that lacks excess carbon and nitrogen sources, which again might induce the release of multiple proteins. In this way, the detection of secreted "background" proteins should be reduced, while the amount of levansucrases should be increased, which again should be identified by peptide sequencing. This system was especially effective using cells of *Gluconobacter species* TMW 2.1191 and (to a lower extent) using those of *K. baliensis*. Other levan-producing AAB strains were not investigated in more detail regarding this system during this work.

3.4.1 Preliminary experiments

Preliminary experiments revealed that *Gluconobacter species* TMW 2.1191 could convert a sucrose supplemented, 0.1 M Na-acetate buffer (pH 5.0 + 0.1 M sucrose) into a whitish turbid solution during aerobic incubation for a few hours. The whitish material was subsequently precipitated with ethanol, purified and identified as fructan *via* HPLC analysis. This observation was not constantly reproducible, if cells were used, which were stored for different times on NaG agar plates. Consequently, it could be assumed that the ability of *Gluconobacter species* TMW 2.1191 to produce EPS in a buffer, that lacks essential nutrients like nitrogen-sources, might be growth-dependent. Therefore, cells of *Gluconobacter species* TMW 2.1191 pre-cultivated in liquid NaG medium were harvested at different time points, afterwards incubated aerobically in the equal volume of 0.1 M Na-acetate buffer (pH 5.0) supplemented with 0.1 M sucrose for 3 h and finally discarded after separation (centrifugation) from the turbid solution. The turbid buffered solutions were microscopically observed for remaining cells and randomly plated on NaG plates to exclude a cell dependent turbidity increase. In this way, solely few or no cells (< 50 cfu/mL) remained in the solution after centrifugation. Cell-free solutions were completely whitish, if cells of *Gluconobacter species* TMW 2.1191 originated from the mid to late exponential phase (OD_{600} ~ 2.0-3.0) and were less (slightly) turbid, if initial cells originated from stationary pre-cultures (OD_{600} ~ 3.1-3.3). Cells originating from the lag phase (no observable turbidity of pre-culture) were not able to generate turbid buffered solutions. Moreover, cells harvested from the mid to late exponential phase were not able to secrete EPS forming enzymes in a 0.1 M Na-phosphate buffer (pH 7.0) (with or without supplemented sucrose, 0.1 M) and in a

0.1 M Na-acetate buffer (pH 5.0), which had not been supplemented with sucrose, respectively. Glucose or fructose as well as combinations thereof (each 0.1 M) did not induce levansucrase secretion at pH 5.0 and pH 7.0. Therefore, secretion of enzymes was both dependent on the used pH and the inducer sucrose. However, fermented cell-free supernatants of NaG medium grown to the exponential or stationary growth phases of *Gluconobacter species* TMW 2.1191, could be used to produce fructan when supplemented with sucrose. Consequently, *Gluconobacter species* TMW 2.1191 secretes levansucrases both constitutively and inducibly. Recovery of native enzymes was most effective for *Gluconobacter species* TMW 2.1191 and was also positive (slightly turbid solutions) for *K. baliensis* DSM 14400 originating from the exponential growth phase (0.1 M Na-acetate buffer, pH 5.0 + 0.1 M sucrose, 3 h of aerobic incubation).

3.4.2 Analysis of natively recovered enzymes

Dialyzed, cell-free solutions (50 mL) obtained after 3 h of incubation of *Gluconobacter species* TMW 2.1191 and *K. baliensis* DSM 14400 in 0.1 M Na-acetate buffer (pH 5.0 + 0.1 M sucrose) were lyophilized to concentrate extracellularly secreted enzymes, subsequently re-dissolved in water, treated with different Laemmli sample buffers and finally loaded on 10% SDS gels (Fig. 16).

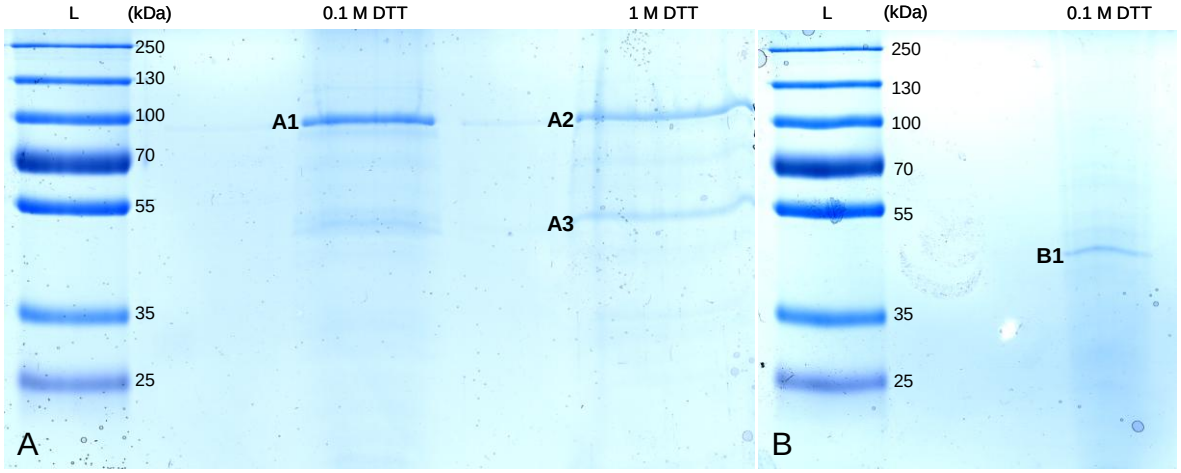

Fig. 16: SDS-PAGE (10%) of secreted proteins recovered from *Gluconobacter species* TMW 2.1191 (A) and *K. baliensis* DSM 14400 (B) after incubation (3 h) in 0.1 M Na-acetate buffer (pH 5.0 + 0.1 M sucrose). Numbered bands were excised and analyzed *via* LC-MS/MS and subsequent Mascot/*de novo* search. L: Protein pre-stained ladder; DTT: Dithiothreitol.

Both strains secreted one major protein into the used buffer system (A1 ~ 100 kDa, B1 ~ 45 kDa; Fig. 16), respectively. Mascot and *de novo* searches of A1 yielded no reliable peptide fragments, whereas B1 was identified as levansucrase with highest similarity to a known levansucrase sequence of *Ga. xylinus*. Based on these detected peptide fragments (appendix A6), primers were constructed, which finally allowed the complete amplification of the respective levansucrase nucleotide sequence of this strain. Consequently, the genome of *K. baliensis* DSM 14400 contains at least 2 levansucrase-coding sequences, one of which does not seem to be secreted or to be active (interrupted form, 3.3.4). The

secreted levansucrase of *K. baliensis* shares highest sequence similarity with the one of *Ga. xylinus* (Fig. 17; 98%) and is phylogenetically located in cluster 1 of levansucrases from AAB. Since protein A1 secreted by *Gluconobacter species* TMW 2.1191 reflected the approximate twofold molecular weight of the expected putative monomeric protein (~ 48 kDa), an equivalent sample was treated with excessive DTT to possibly reduce the dimeric form (Fig. 17A). In this way, protein A3 was detectable *via* SDS-PAGE reflecting the approximate molecular weight of the putative monomeric form. Mascot/de novo searches of proteins A2 and A3 revealed A3 to be the putative levansucrase of *Gluconobacter species* TMW 2.1191 (accession number: HE717100, appendix A7). No reliable peptide fragments again could be calculated for protein A2, which actually seems to be the dimeric protein due to its weaker appearance after excessive reduction with 1 M DTT and subsequent Coomassie staining.

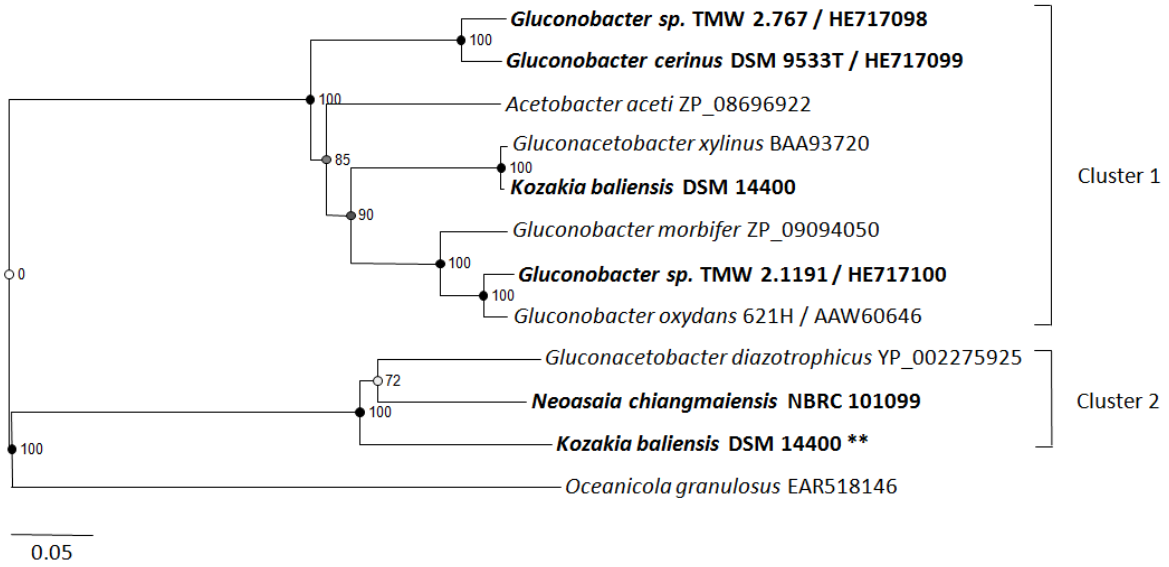

Fig. 17: Phylogenetic tree including all so far identified levansucrases from AAB species. Similarities were calculated using the neighbour-joining method. Numbers following species names represent the accession numbers of the corresponding proteins as deposited in GenBank. Newly identified FTFs are highlighted in bold. The scale bar represents number of changes per amino acid. The levansucrase protein of *Oceanicola granulosus* (EAR51814) was used as an outgroup. Bootstrap values indicated at nodes are derived from 1000 replications. ** For phylogenetic calculations the interrupted levansucrase sequence (PTV...; 519 amino acids) of *K. baliensis* DSM 14400 (3.3.4) was additionally used.

3.4.3 Enzymatic *vs*. conventional levan production using *Gluconobacter species* TMW 2.1191

Since high amounts of secreted levansucrases could be detected in fermented buffered solutions of *Gluconobacter species* TMW 2.1191 (3.4.2), an enzyme containing solution (0.1 M Na-acetate buffer + 0.1 M sucrose; cell incubation time: 3 h; OD_{600} (NaG) = 2.5) of this strain (200 mL) was used for enzymatic production of levan. The enzymatic solution was, therefore, mixed with 800 mL Na-acetate buffer (0.1 M; + 0.1 M sucrose) and incubated for 24 h without stirring at 30 °C. In this way, a completely whitish solution exhibiting a strong Tyndall effect was obtained. This solution (1 L) was proven to be cell-free (plating, microscopy), dialyzed for 48 h (removal of salts and sugars),

lyophilized and subsequently (structurally) analyzed. The obtained results are compared with results obtained using the conventional EPS production method using *Gluconobacter species* TMW 2.1191 (fermentation of 1 L NaG medium supplemented with 80 g/L sucrose (0.23 M, 2.2.1) (table 5).

Table 5: Comparison of enzymatic (cell-free) and conventional EPS production by *Gluconobacter species* TMW 2.1191. Spectra derived from NMR and AF4-MALS-RI are depicted in the appendix (A11-A13).

Parameters	**Enzymatic EPS production**	**Conventional EPS production**
EPS identity (NMR)	linear levan	linear levan
M_W (Da)	$8.26 * 10^9$	$4.2 * 10^7$
R_G (nm)	428	69
M_W/M_N	1.006	1.523
ν_G	0.28	0.44
Incubation time cells (h)	3	48
Total sucrose (g/L)	34.23	80
EPS amount (g/L)	4	9
Ethanol for precipitation (L)	0	2
Production time (days)	5	10

Cell-free, enzymatically produced (linear) levan exhibited a distinctly higher molecular weight and particle size and was determined as almost uniform polymer (M_W/M_N = 1.006). Moreover, cell-free synthesized levan molecules exhibited a highly compact spherical shape (ν_G = 0.28, Fig. 18A and B) in contrast to conventionally produced levan molecules (ν_G = 0.44). While cells, which had been pre-cultivated in a volume of 200 mL NaG to the mid-exponential phase (OD_{600} = 2.5), were solely incubated for 3 h in the presence of sucrose, a similar conversion yield (EPS amount/total sucrose ~ 11%) compared to 48 h cell incubation in 1 L fermentation medium was obtained. The enzymatic EPS production procedure is considerably faster (5 *vs*. 10 days) and less material consuming (0 *vs*. 2 L ethanol) than the conventional one, since there is no need of EPS separation from complex media components by selective alcoholic precipitation (table 5).

Moreover, this method was exemplary applied for the production of dextran (α-(1→6)-linked glucose-polymer). The water-kefir isolate *Leuconostoc mesenteroides* TMW 2.1073 was pre-cultivated in mMRS medium (diluted 1:1 with 0.2 M sucrose) to a final OD_{600} = 0.5 and afterwards incubated in a 0.1 M Na-acetate buffer (+ 0.1 M sucrose) for 2 h. In contrast to *Gluconobacter species* TMW 2.1191, *L. mesenteroides* TMW 2.1073 had to be pre-cultivated in mMRS medium in the presence of sucrose for secretion of dextransucrases into the buffer supernatant. The cell-free buffer supernatant was used for the production of dextran (24 h in 0.1 M Na-acetate buffer + 0.1 M sucrose, 30 °C), which was subsequently isolated as described above. In this way, 35 g dextran/L cells were obtained. The dextran identity was determined by ^{13}C-NMR spectroscopy (appendix A14). A method for evaluation of the molecular weight of this dextran has still to be established. The aqueous dextran-solution recovered after 24 h of incubation was not turbid/whitish (levan), but indeed transparent and highly viscous. Since enzymatic dextran production was developed at the end of this work, secreted enzymes of *Leuconostoc mesenteroides* were not investigated in more detail.

3.4.4 Microscopy of levans and of levan producing *Gluconobacter* cells

Due to its high detected R_G value (428 nm, table 5) the enzymatically produced, isolated levan was microscopically investigated at thousandfold magnification using a conventional microscope. Furthermore, levan/cell mixtures obtained after 3 h of incubation of *Gluconobacter species* TMW 2.1191 in sucrose supplemented Na-acetate buffer (pH 5.0) were microscoped.

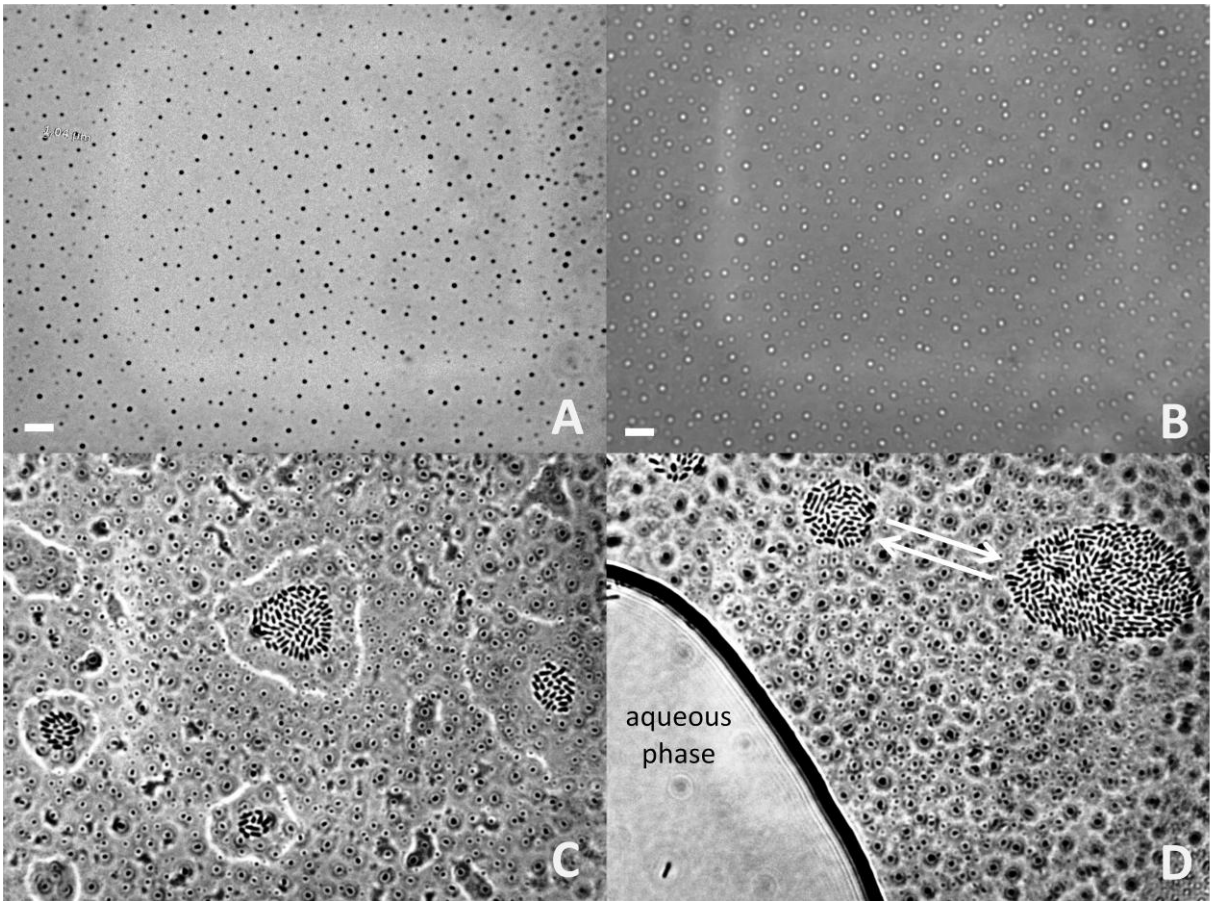

Fig. 18: Microscopic images (1000 x magnification) of isolated levan recorded at different phase contrast modes (A, B; 1 g/L) and levan/cell mixtures after 3 h hours of incubation of *Gluconobacter species* TMW 2.1191 in 0.1 M Na-acetate buffer supplemented with 0.1 M sucrose (C, D). Cells in (C) and (D) are surrounded by predominantly aggregated levan molecules, which tend to form a jellylike matrix. Cells in (C) are entrapped and immobilized in the gel matrix. White arrows in (D) indicate the directed movement of two movable cell agglomerates towards each other. In (D) the two phases are depicted, which are microscopically observable. While cells are moving irregularly in the aqueous phase, coordinated cell behaviour is observable in the "levan phase". Scale bar = 5 µm.

As expected from AF4-MALS-RI, levan molecules in fact exhibited a spherical molecular shape (ν_G = 0.28, table 5), which could be microscopically observed due to the enormous size of these molecules (Fig. 18A and B). Interestingly, levansucrase-secreting cells of *Gluconobacter species* TMW 2.1191 show a distinct coordinated behaviour in the levan-rich phase, which can be macroscopically seen on the used glass slide as small, "semi-dry" spots. In the surrounding aqueous phase cells of this strain were moving irregularly as usually seen for most bacteria. In some cases, individual cells could be watched moving quite slowly and controlled in the levan-rich phase. At a certain point, cells

commonly and directively (< 1 s) moved to different centres and agglomerated. At this point no individual cells were present in the levan-rich phase anymore. The agglomerated cell structures again moved like “flexible protozoa” towards each other and associated, as long as they were completely immobilized by final gelatinization of the surrounding levan matrix. The agglomerates could be dissolved into individual, irregularly moving cells by carefully pipetting a drop of water below the cover glass.

3.5 Baking experiments

To get first insights into the functionality and usefulness of levans from AAB for practical applications, the isolated levans of *Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533, *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400, which had been investigated regarding their structural features (3.2), were used as additives for baking of wheat breads. As mentioned in section 3.1, levan of *Gluconobacter species* TMW 2.1191 was not used for baking experiments. Breads were subsequently analyzed regarding their loaf volumes and crumb hardness/staling as delineated in sections 3.5.1 and 3.5.2.

3.5.1 Volume analysis of breads with and without added levans

Two dosages per isolated levan were used (1% and 2% w/w flour) to investigate their influence on the volume of wheat breads. The results are summarised in table 6. Volume values are averages of eight baked breads. To demonstrate the volume increasing effect (%) after adding a specific levan in a certain dosage (% w/w flour), eight breads without additive were baked simultaneously and taken as control. Significant values of volume increase ($p < 0.05$; bilateral homoscedastic t-test) are indicated by stars (*).

Table 6: Effect of levan addition from selected AAB strains on the loaf volume of wheat breads.

Fructan	Dosage (w/w flour)	Volume control breads (mL)	Volume levan breads (mL)	Increased volume (%)
G. species 2.767	1%	500.2 ± 8.8	514.4 ± 16.4	**2.8 ***
	2%	515.7 ± 6.0	518.7 ± 7.8	**0.6**
G. cerinus	1%	518.8 ± 10.4	525.9 ± 12.1	**1.4 ***
	2%	506.3 ± 6.8	522.7 ± 15.0	**3.2 ***
N. chiangmaiensis	1%	517.9 ± 13.1	531.2 ± 12.9	**2.6 ***
	2%	501.3 ± 9.1	534.3 ± 10.1	**6.6 ***
K. baliensis	1%	498.2 ± 6.5	513.5 ± 9.3	**3.1 ***
	2%	494.9 ± 4.7	522.3 ± 9.9	**5.5 ***

The addition of each tested levan in two dosages caused an average increase of volume compared to the baked control breads. Except for levan from *Gluconobacter species* TMW 2.767, the twofold amount of added levan (2%) caused a nearly twofold increase of volume compared to the lower tested dosage (1%). The greatest effects were observed by addition of 2% sugar polymer from *K. baliensis* (5.5%) and *N. chiangmaiensis* (6.6%). Furthermore, after addition of only 1% fructan of these two

strains, similar values of increased volume (3.1% and 2.6%, respectively) were detected as measured as the highest values of increased volume for levans from *Gluconobacter species* TMW 2.767 (1% dosage: 2.8%) and *G. cerinus* (2% dosage: 3.2%). Consequently, levans from *N. chiangmaiensis* and *K. baliensis* seem to have a greater functional effect on the volume of wheat breads than levans from the tested *Gluconobacter* strains.

3.5.2 Texture profile analysis (TPA) of breads with and without added levans

The staling of wheat breads with added levan of the corresponding strain in two dosages was measured as crumb hardness (N) on day 0, day 1, day 4 and day 7 after bread baking and is shown in Fig. 19. All breads (with or without additive) showed the expected increase in crumb hardness over a time period of one week (Fig. 19). Except for *Gluconobacter species* TMW 2.767 (day 7) and *G. cerinus* (day 1, 1% fructan), breads with added levan were distinctly softer than the corresponding control breads.

In table 7 the percentaged differences of crumb hardnesses of levan supplemented breads and their respective control breads are depicted. Values in brackets indicate that the averaged crumb hardness of the levan breads was higher than that of their corresponding control breads. Significant differences of crumb hardness ($p < 0.05$; bilateral homoscedastic t-test) are indicated by stars (*). For all tested levans, the highest percentaged difference of crumb hardness was measured on day 0 (day of baking, table 7). By trend these differences decreased continuously from day 0 to day 7, respectively. The highest differences were observed after addition of 2% sugar polymer from *N. chiangmaiensis*. Furthermore, levans from this strain and *K. baliensis* seem to retard the staling of wheat breads during a storage time of one week more effectively than levans from the tested *Gluconobacter* strains, since their values of percentaged differences were mostly comparatively higher (especially on day 4 and day 7). On day 0, however, the softening effect of all tested levans in two dosages with the exception of *N. chiangmaiensis* (2%) was quite similar (18-27%, table 7). Remarkably, the texture profile of the wheat breads was already clearly influenced by the addition of only 1% levan of each test strain.

Table 7: Effect of levan addition from selected AAB strains on staling of wheat breads

Fructan	**Dosage (w/w flour)**	**Percentaged difference of crumb hardness (levan bread/control bread)**			
		day 0	**day 1**	**day 4**	**day 7**
K. baliensis	1%	20.8 *	17.9 *	16.7 *	14.8 *
	2%	26.9 *	15.3 *	20.9 *	8.6
N.chiangmaiensis	1%	25.8 *	8.2 *	13.7 *	10.6 *
	2%	38.1 *	25.1 *	25.3 *	15.5 *
G. species 2.767	1%	18.7 *	11.9 *	9.0 *	(4.0)
	2%	22.0 *	15.0 *	4.2	(0.9)
G.cerinus	1%	23.9 *	(4.6)	5.8	15.9 *
	2%	21.1 *	16.5 *	7.1	4.0

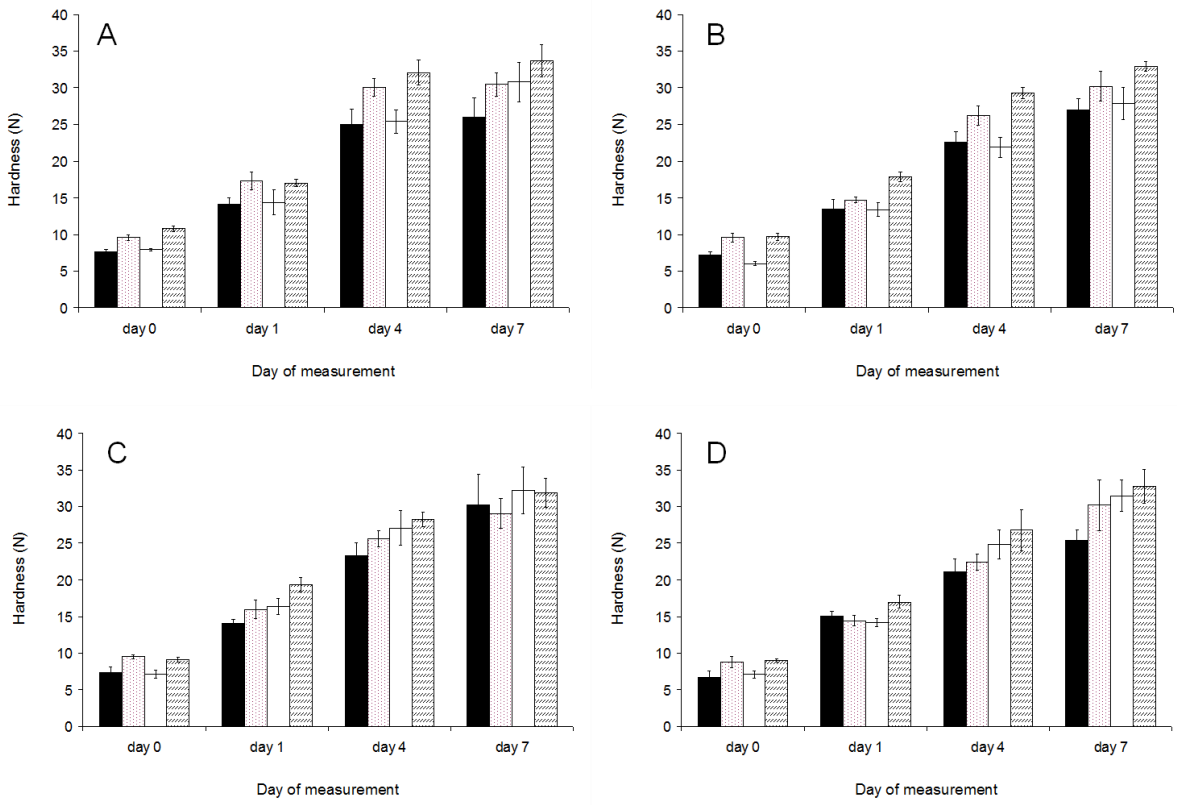

Fig. 19: Comparative staling of control and levan breads baked with levans produced by selected AAB strains. (■) 1% levan addition, (::) control breads 1% test series, ( ) 2% levan addition, (//) control breads 2% test series; levans were isolated from *K. baliensis* (A), *N. chiangmaiensis* (B), *Gluconobacter species* TMW 2.767 (C) and *G. cerinus* (D).

4. DISCUSSION

The present work highlights the potential of AAB to produce functional fructans and gives new insights into the genetic and enzymatic background of fructan biosynthesis in/by AAB.

Fructan-overproducing strains of the genera *Gluconobacter*, *Neoasaia* and *Kozakia* were identified (3.1), whose isolated fructans were used for baking experiments. It could be demonstrated, that addition of fructans from AAB to wheat doughs significantly increased volume and retarded staling of wheat breads (3.5). Furthermore, these fructans were shown to be similar (linear levans) according to their basic linkage types (NMR, 3.2.1), but to be different regarding their molecular weight distributions (AF4-MALS-RI, 3.2.2). This enables the establishment of a structure/function relationship, referring molecule structures of levans (3.2) to their observed effects on dough systems and breads (3.5).

Diverse PCR methods were used to detect ORFs coding for levansucrases in selected fructan-overproducing AAB strains (3.3.1, 3.3.4). *Gluconobacter* levansucrases could be actively expressed in *E. coli* (3.3.3), while heterologous expression of an ORF coding for a putative *N. chiangmaiensis* levansucrase in different (optimized) *E. coli* cells yielded accumulation of inclusion bodies in these recombinant hosts (3.3.4). Fermented culture supernatants of *N. chiangmaiensis* and *K. baliensis* were shown to exhibit levansucrase activities, while peptide sequencing of selected secreted proteins of these strains yielded no positive levansucrase sequences (3.3.5). Since no complete levansucrase ORF could be detected in *K. baliensis* by genetic methods, a unique system was developed (3.4), which enabled the recovery and characterization of a native levansucrase from *K. baliensis* DSM 14400 in (buffered) solutions (3.4.2). Moreover, this system is highly effective using cells of *Gluconobacter species* TMW 2.1191, as it secreted high amounts of its dimeric levansucrase into Na-acetate buffer, which could be used to produce unique levans in a time- and material-saving procedure (3.4.3). Finally, coordinated behaviour of levan-producing *Gluconobacter* cells could be microscopically observed (3.4.4), which points out the biological role/function of levan as biofilm material.

The following sections delineate and explain these findings in more detail and should enhance the current knowledge about the functionality and biomolecular background of fructans from AAB, with respect to the global context of microbial fructan biology/biotechnology.

4.1 Structural characterization of isolated fructans

The isolated fructans of *Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533, *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400 were analyzed regarding their linkage types and molecular weight distributions by using different NMR techniques and AF4-MALS-RI. In this way, each investigated fructan was identified as levan, which correlates with the known

distribution of levansucrases among several AAB (1.8). Whereas ^{13}C-NMR has proven useful for identification of levans in several previous works, assignment of carbon atoms and bound hydrogens in levans can be more precisely obtained from *COSY* and *HMQC* as demonstrated in this work and by Tajima et al. (1998). The usage of AF4-MALS-RI for characterization of levans has not been established before and seems to be well suited for this class of polymers because of their unique features to be readily soluble in water and to cause low intrinsic viscosities even at high concentrations in spite of their unusual high molecular weight (Arvidson et al., 2006). These known rheological characteristics of microbial levan implicate the more globular-like molecular shape of individual molecules, which should not tend to interact *via* hydrogen bonds among each other due to their relatively low surface. Consequently, high molecular weight levan molecules do not strongly aggregate nor significantly increase the viscosity of an aqueous solution in moderate working concentrations (e. g. 1 g/L) and are, therefore, well separable by their molecular weight following the physical principles of field flow fractionation as demonstrated in this work. The use of coupled high performance MALS detection allowed absolute determination of M_Wi and R_Gi of individual molecules, which again is known to be more accurate for globular molecules due to the principles of light scattering.

K. baliensis produced a levan with an M_W of 2,466 MDa, which is to my knowledge the largest fermentatively recovered fructan polymer reported so far. Commercially sold high molecular weight levan has a M_W of about 900 MDa (Augsten, 2008). About 75% of levan particles from *K. baliensis* had a molecular mass in the range of 1,000-2,000 MDa (Fig. 5A). Furthermore, the hydrodynamic coefficient ν_G for levan of *K. baliensis* was 0.27, which indicated the highly compact structures of these molecules. Similar ν_G values (derived from MALS detection) were reported for slightly branched (<5-7% β-(2→6)-linkages), *in vitro* synthesized high molecular weight inulins that adopt globular conformations in aqueous solutions (Wolff et al., 2000). Additional signals of carbon atoms involved in creating further linkage types could not be detected in the ^{13}C-NMR spectra of the investigated levans. These levan molecules, therefore, appeared to be linear or to have too few branches to be detected using NMR spectroscopy. Similar results (no additional carbon signals in ^{13}C-NMR spectra that indicate potential β-(2→1)-/β-(2→6)-linked branches in levans and inulins, respectively) were obtained for linear inulins produced by recombinant inulosucrases of *Lactobacillus gasseri* (Anwar et al., 2008).

The predicted linearity was not necessarily associated with linear conformations of these levan molecules in aqueous solution. In fact, ν_G values between 0.26 (*K. baliensis*) and 0.52 (*G. cerinus*) were found in the separated levan fractions (table 4). ν_G values can be obtained from AF4-MALS using equation (5) (2.4.2) to characterize the shape of a macromolecule in solution. For a sphere $\nu_G \sim$ 0.33, for a rod ν_G is close to 1 and for a random-coil macromolecule $\nu_G \sim 0.5 - 0.6$ (Nilsson, 2012).

Therefore, only levan particles of relatively low molecular weight (*Gluconobacter species* TMW 2.767 fraction 1, *G. cerinus* fraction 2; table 4) tended to adopt a more random coil conformation. Particles of higher molecular weight (levan fractions 3-7) tended to exhibit a more compact and spherical like one. These results were in agreement with previous findings reporting a spherical molecular shape of levans in aqueous solutions (Arvidson et al., 2006; Newbrun et al., 1971). Molecular-weight dependent conformational changes of levan (Stivala & Zweig, 1981) and inulin molecules (Kitamura et al., 1994) were observed in aqueous solutions, respectively. Polymer chains with molecular weights $<10^5$ Da exhibited random-coil conformations, whereas molecules with molar masses $>10^5$ Da adopted globular conformations. These findings were explained by strong intramolecular interactions of more distantly located fructose residues among each other, which can occur at certain molecular weights. Consequently, polymer chains with critical molecular weights collapse into compact coils with an overall globular shape, in which monomers have fixed positions and in which rotations about the bonds of the backbone are severely restricted (Kitamura et al., 1994). Similar tendencies of conformational changes could be observed in the present work for each isolated levan: highest v_G values were detected in levan fractions, which were composed of polymer chains with the corresponding lowest molecular weights. Moreover, v_G values tended to decrease from lower to higher molecular weight fractions (table 4). This again supports the findings and explanations of Stivala & Zweig (1981) and Kitamura et al. (1994) that conformations of both levan and inulin molecules in aqueous solutions strongly depend on their molecular weight. The observed increasing compactness of levan molecules in parallel with their increasing molecular weight (decreasing slope values v_G, table 4) could furthermore be explained by strong intramolecular forces such as hydrogen bonds. These intramolecular forces could continuously increase in parallel with increasing *DP* yielding more tightly packed molecules due to more intramolecular contacts of fructose residues.

Taken together, it can be assumed, that the structures of all isolated levans (no detectable branches in each polymer) were highly similar among each other and that the altering molecular shapes of these molecules were primarily influenced by their molecular weight.

4.2 Relation of structural features to hydrocolloid function

Levans of *Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533, *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400, which were structurally examined as described in section 4.1, were used for baking experiments to demonstrate their applicability as hydrocolloids in foods (3.5). After addition of these levans to wheat doughs (1% and 2% w/w flour) different effects on volume, texture and shelf-life of breads were observed. Each levan caused an increased volume, a softer crumb and a retarded staling of wheat breads during one week storage at the lowest tested dosage, demonstrating the high functional potential of these fructans. Volume increase, crumb softening and antistaling of wheat breads were significantly higher after addition of the isolated levans of *N.*

chiangmaiensis and *K. baliensis* as compared to added levans of *Gluconobacter species* TMW 2.767 and *G. cerinus* (3.5). As shown in the current study, levans of *K. baliensis* and *N. chiangmaiensis* were composed of particles with significantly higher molecular weight and more compact molecular shape. Otherwise, levan types and proposed degrees of branching were highly similar in all investigated fructans (4.1). Consequently, higher molecular weight of these levans suggests itself as main trigger to positively influence the properties of wheat breads in terms of volume increase and retardation of staling, suggesting their hydrocolloid role in improved water retention.

Studies about the influence of different molecular weight fractions of the same polysaccharide type on the textural properties of breads are actually rare. To my knowledge, solely Skendi et al. (2009) investigated the rheological properties of wheat doughs supplemented with β-glucans of different molecular weight, whereas the effects of β-glucan addition on finally baked breads were not determined in that work. Furthermore, only few investigations are available on the impact of fructans on wheat breads so far. Inulin was shown to exhibit limited positive technological effects on wheat and gluten-free breads (Hager et al., 2011). Though, these findings are not comparable to the present study, as the added inulin mainly consisted of short-chain oligosaccharides. The use of high molecular weight levan produced by a sourdough strain of *Lactobacillus sanfranciscensis* for preparing clean label products was patented (Vincent et al., 2005) due to its potential positive technological features such as an antistaling agent (Kaditzky, 2008a). However, a structure-function relation has not been established for this levan type as well.

Regarding positive effects such as volume increase, crumb softening and antistaling of wheat breads, the chemically modified cellulose-derivative HPMC seems to be the exclusive, commonly used food hydrocolloid, that exhibits similar effects as observed for the isolated levans structurally examined in the present work (Guarda et al., 2004; 3.5). HPMC is a good gelling agent in forming intermolecularly stabilized networks, which consist of multiple polymer chains entrapping high amounts of solvents such as water (Tritt-Goc et al., 2005). Furthermore, this polymer was shown to enhance the hydration and water binding capacities of gluten (Barcenas et al., 2009) and proposed to inhibit starch retrogradation (Collar et al., 2001; Guarda et al., 2004). Due to these different physicochemical properties, HPMC avoids extensive moisture loss during baking and, therefore, is supposed to soften the crumb and retard staling of wheat breads. Its ability to increase the loaf volume of breads is hardly understood and was explained by somehow increasing the gas retention during baking (Guarda et al., 2004).

Unlike HPMC, levan does not form gels from intermolecular interactions of different polymer chains in aqueous solutions and exhibits low intrinsic viscosities even at high molecular weight being typical for spherical particles (Arvidson et al., 2006). In further contrast to HPMC the levans in this study did

not adopt expanded molecular structures in aqueous solutions (Wittgren & Wahlund, 1997). As described above (4.1), molecular-weight dependent, conformational changes seem to be typical for both high molecular weight inulins and levans in aqueous solutions. Kitamura et al. (1994) calculated from viscosity measurements that individual high molecular weight inulin globules entrapped about 90% water within their volumes. The authors pointed out that in contrast to expanded molecules such as modified celluloses small "microgels" can be formed from individual globular inulin molecules. Consequently, the ability of the isolated levans to bind water should not result from intermolecular interactions of different elongated polymer chains. In fact, intramolecular interactions of individual levan molecules have to be considered to effectively bind water and, therefore, to act as hydrocolloids. In this context, the following considerations could differentiate the observed influences of different isolated levans on improved water retention during baking and storage of breads.

About 77% and 75% of levan particles of *N. chiangmaiensis* and *K. baliensis* had molecular weights in the range of 22-98 MDa (table 4) and 1000-2000 MDa (calculated from data involved in Fig. 5A), respectively. Moreover, about 20% of levan particles of *N. chiangmaiensis* and *K. baliensis* had molar masses in the range of 98-575 MDa and 576-1000 MDa, respectively (table 4). Therefore, distinctly more individual levan particles involved in EPS from *N. chiangmaiensis* were inserted into the respective doughs adding equal dosages (e. g. 10 g levan) during baking to obtain similar positive effects (see above and 3.5). Consequently, the higher number of molecules with $M_W \sim 10^8$ Da (*N. chiangmaiensis*) compensated the functional effects of the comparatively lower number of molecules with $M_W \sim 10^9$ Da (*K. baliensis*) and fewer molecules of highly compact molecular shape (v_G = 0.38/0.27 for levans of *N. chiangmaiensis*/*K. baliensis*, respectively) avoided moisture loss during baking and storage similarly effective. Furthermore, the relatively low numbers of high molecular weight levan particles from *K. baliensis* and *N. chiangmaienis* caused more positive functional effects than the comparative large number of lower molecular weight molecules of *Gluconobacter species* TMW 2.767 (64%: 4-6 MDa, 33%: 6-22 MDa) and *G. cerinus* (78%: 6-22 MDa, 21%: 22-98 MDa) inserted during baking using equal EPS amounts. v_G values of the main levan fractions of *Gluconobacter species* TMW 2.767 (64% of molecules) and *G. cerinus* (78% of molecules) were calculated as 0.46 and 0.52, respectively (table 4). Therefore, the main part of the isolated levan fractions from both *Gluconobacter* strains was composed of distinctly less compact particles in contrast to levans from *N. chiangmaiensis* and *K. baliensis*. Consequently, a significant proportion of levan molecules from *Gluconobacter species* TMW 2.767 and *G. cerinus* probably did not exhibit the efficient molecular weight/conformation for (comparatively) effective water retention. However, both levans contained enough levan particles, which were at least able to cause positive functional effects at the lowest tested dosage (3.5).

More tightly packed particles, therefore, could entrap water more effectively due to stronger intramolecular forces. Water retention hereby should have been mainly caused by levan particles acting as "microgels" themselves, rather than by interactions with flour particles such as gluten and starch. Due to its spherical shape, levan was shown to be effective in resisting interpenetration by other polymers leading to phase-separation phenomena (Kasapis et al., 1994). As all isolated levans increased the volume of wheat breads and as levans are reported to be excellent oxygen diffusion barriers (Montana Polysaccharides Corp., www.polysaccharides.us/aboutlevan_technical.php), they could have (additionally to water) prevented extensive gas loss during baking. Still it has to be considered, that baking could induce thermal deformation or degradation of molecules. Whereas the mechanisms influenced by putatively deformed/degraded levan particles to retain water cannot be interpreted due to their unpredictable structure, it is obvious, that fewer (potentially deformed/partially degraded) particles of (initially) higher molecular weight would have made a bigger contribution for effective water retention in the complex dough system. Therefore, it could be assumed that more tightly packed levan particles were more resistant against extensive thermal deformation/degradation processes due to stronger intramolecular forces. However, Kasapis et al. (1994) could not observe any conformational changes of densely packed levan molecules during heating up to 100 °C, which cannot be surpassed in the crumb of bread as long as water is present.

Taken together, these data suggest that the increasing molecular weight of a levan particle enforces intramolecular interactions to reach the structural compactness of a microgel with hydrocolloid properties. In situ formation of (potentially prebiotic; 1.7) microgels by food-grade strains such as AAB or LAB during natural food fermentations, therefore, could enhance the quality of bakery and other food products and replace commonly used hydrocolloids such as HPMC without the need of declaration as consumer-unfriendly food additives.

4.3 Detection of levansucrase encoding genes

A PCR based approach was chosen to identify unknown levansucrase gene sequences of the investigated levan-overproducing AAB strains. For *Gluconobacter* strains, this strategy was useful, since at least some levansucrase sequences of related organisms (*Z. mobilis, Ga. xylinus and G. oxydans*) were initially available in public databases. Newly identified levansucrase sequences could subsequently be used to create further degenerate primers, thereby targeting more specifically against conserved levansucrase gene regions of AAB species. Moreover, primers Z1F/Z1R and DgAlbF/DgAlbR were deduced from alignments, which included nucleotide instead of amino acid sequences because of possible similar codon usages among AAB strains, to possibly enhance specific binding during degenerate PCR reactions. Whereas the first core-sequence detection (*Gluconobacter species* TMW 2.767) required extensive optimization of PCR conditions due to relatively unspecific binding of degenerate primers Av and Ar (3.3.1), a partial fragment of the last identified levansucrase

gene (*Gluconobacter species* TMW 2.1191) could be amplified performing solely one PCR approach. Hence, these newly identified nucleotide sequences might help to quickly obtain parts of potential similar levansucrase genes of further levan-producing *Gluconobacter* strains.

For core sequence identifications of *N. chiangmaiensis* and *K. baliensis* levansucrases, a new degenerate primer set had to be developed, as usage of *Gluconobacter* core primers yielded no positive amplicons. In this way, degenerate primers DgAAB_F and DgAAB_R were constructed, whose nucleotide sequences had been deduced from motifs EWSGS (sucrose box 1) and NDQTER (catalytic centre) being conserved in levansucrases of several AAB species. Sequencing of PCR amplicons obtained from PCR with this primer set yielded expected core sequences coding for levansucrases. Whereas a complete ORF coding for a levansucrase in *N. chiangmaiensis* was obtained after two rounds of inverse PCRs, the identification of *K. baliensis* levansucrase was more time-consuming due to its interruption by an IS5 element. As the interrupted sequence could not be expected to be actively expressed, extracellular supernatants of *K. baliensis* (3.3.5) were investigated regarding extracellular, secretory proteins. In contrast to *N. chiangmaiensis,* no proteins with residual levansucrase activities could be recovered from fermented nutrient broths. Therefore, a method was developed (3.4), which yielded one secreted protein, that was finally identified *via* Mascot and PepNovo searches as levansucrase, being highly similar to a known levansucrase of *Ga. xylinus*. The detected peptide fragments finally allowed the construction of primers for amplification of the respective gene (reverse genetics). Since the second serine of primer DgAAB_F (EWSG**S**) was chosen as "TCN"-codon during primer construction, the levansucrase core sequence of the interrupted gene was obtained. If this codon had been selected as the alternative serine codon ACY, the core nucleotide sequence of the secreted levansucrase of *K. baliensis* would have been initially detected.

4.4 Diversity of fructans and fructosyltransferases among AAB

Fructan production in/on sucrose containing media was detected for 14 of a total of 22 test strains, while 5 strains were identified as strong levan producers. Noticeably, all investigated *Gluconobacter* strains (12) were able to produce fructan from sucrose, whereas none of the tested *Gluconacetobacter* strains (7) formed macroscopic observable, mucous polysaccharide from sucrose. Because some strains of *Ga. diazotrophicus* and *Ga. xylinus* were previously shown to be effective fructan producers (Tambara et al., 1999; Kornmann et al., 2003; Tajima et al., 1998), fructan synthesis seems to be a sporadically detectable, metabolic feature among this genus. Similarly, solely few studies report about fructan synthesis in the genus *Acetobacter* (Paul et al., 2011; Sakurai et al., 2011), while most *Acetobacter* genomes lack genes coding for FTFs. On the contrary, all so far genome-sequenced and otherwise genetically investigated *Gluconobacter* strains possess complete genes coding for levansucrases (Fig. 9). Consequently, fructan synthesis seems to be widely spread among strains of the

genus *Gluconobacter*, which could be interpreted as an adaptive physiological effort for survival in specific sugar-rich environments.

Gluconobacter strains are specialized on the incomplete oxidation of a variety of sugars and polyols, whereas *Acetobacter* and *Gluconacetobacter* strains prefer alcohols as substrates for incomplete oxidation (Deppenmeier & Ehrenreich, 2009). After secretion of fructosyltransferases into sugar-rich environments the substrate sucrose will be cleaved, while a free glucose moiety is released, which could subsequently be used for energy generation by incomplete oxidation to gluconic acids. Due to the missing ability for oxidation of acetate to CO_2, *Gluconobacter* strains are naturally confronted with higher amounts of residual acetic acid, which can additionally (to membrane-bound alcohol-/aldehyde dehydrogenases) be produced by strains of this genus *via* decarboxylation of pyruvate and subsequent oxidation of acetaldehyde to acetate (Peters et al., 2013a). Therefore, the simultaneous formation of polymeric fructans could protect cells against environmental stress factors such as osmotic pressure (high sugar/alcohol concentrations), desiccation (osmosis, heat, solar irradiation on rottening fruits) and decreasing pH (own and co-flora metabolism) *via* generation of slimy/gelling biofilms/pellicles (Velazquez-Hernandez et al., 2011). While biofilm mediated acid resistance has also been reported for different individual strains of *Lactobacillales* (Kubota et al., 2008/2009), formation of protecting/self and co-microbiota stabilizing pellicles by AAB (cellulose) and LAB (dextran) is common in mother of vinegar/tea fungus and water/milk kefir, respectively. Since fructan-overproducing strains such as *Gluconobacter species* TMW 2.767 and *Gluconobacter species* TMW 2.1191 were isolated from water kefir, but do not seem to be stably abundant in this fermented beverage (Gulitz et al., 2011/2013), they could have randomly colonized this sucrose-rich environment. Whereas secreted fructosyltransferases in water kefir are not known to participate in formation of kefir granules, which mainly consist of dextrans, fructans produced by AAB or LAB could – according to their prebiotic character - stimulate the growth of co-existing microorganisms such as bifidobacteria, which were previously shown to be highly abundant in kefir (Gulitz et al., 2013). Though, the essential role of AAB in kefir has still to be seen critically due to their relative low abundance in this food system.

In fact, all so far known levansucrases of the genus *Gluconobacter* share highest sequence similarity with ethanologenic strains of *Zymomonas mobilis* (α-proteobacteria, Sphingomonadaceae), both of which are supposed to have evolved from a common ancestor due to incomplete glycolytic pathways/tricarboxylic acid cycles, functional Entner-Doudoroff pathways and the same ubiquinone types 10 (Swings & de Ley, 1977). As observed for *Gluconobacter*, each (published) genome-sequenced or genetically investigated strain (source: NCBI) of *Zymomonas mobilis* contains levansucrase genes indicating the wide distribution of fructan synthesis among this species, while no other fructosyltransferases than levansucrases have been detected in genomes of *Zymomonas mobilis* as well as *Gluconobacter* so far. Since strains of *Zymomonas mobilis* and *Gluconobacter* are usually

detected in sugar-rich environments like nectars, fruit saps or plant juices, similar mechanisms to compete in their osmotically harsh environment could have been adapted, which help cells to degrade/metabolize preferred sugars and/or to build up (protective) networks of self-organized microbial communities. The widely distributed ability of *Gluconobacter* and *Zymomonas mobilis* strains to utilize fructosyltransferases for sucrose metabolization, anyway, seems to be an important physiological and/or ecological profit for these organisms.

Furthermore, the type strains of *K. baliensis* DSM 14400 (Lisdiyanti et al., 2002) and *N. chiangmaienis* NBRC 101099 (Yukphan et al., 2005) have been shown to be effective fructan producers during this study. Lisdiyanti et al. (2002) already observed the formation of mucous, "levan-like" polysaccharides from sucrose by different isolates of *K. baliensis*, while same authors did not investigate the chemical composition of these produced sugar polymer types. In contrast to my findings the type strain of *N. chiangmaiensis* was not reported to synthesize macroscopic observable polysaccharide from sucrose (Yukphan et al., 2005). Both strains possess (fragments of) levansucrases, which share highest similarity to those of plant-associated/N_2-fixing *Ga. diazotrophicus* and *Burkholderia* strains and form an independent cluster of AAB levansucrases based on their sequence similarity. Therefore, *N. chiangmaiensis* and *K. baliensis* might have (partially) adopted levansucrase-mediated sucrose utilization in natural environments, which are different from those of *Gluconobacter* strains. Though, *K. baliensis* could be shown to secrete a levansucrase, which is classified in cluster 1 of "typical" AAB levansucrases and differs from the cluster 2-like (interrupted) one (Fig. 17). Since *K. baliensis* represents the first AAB strain, which is known to contain (at least parts of) more than one levansucrase gene and which produces a unique heteropolysaccharide consisting of glucose, galactose and fructose in the absence of sucrose as carbon source, this strain seems to be physiologically highly adapted for (possibly essential) polysaccharide synthesis. Due to the fact, that *K. baliensis* produces pure levan in sucrose supplemented media, it prefers to use its obviously sucrose-inducible levansucrase-expression/secretion mechanism as well as the bond energy of the disaccharide sucrose to compass its (possibly) more energy-consuming machinery for heteropolysaccharide production. It remains unclear, if the physicochemical and physiological properties of certain levan fractions/amounts could/should replace those of the produced heteropolysaccharide or if these corresponding, possibly varying properties are naturally used independently from each other in different life-style situations, which are not detectable *in vitro*.

4.5 Genetic organization of levansucrase encoding genes

A total of 6 (partially) levansucrase encoding sequences have been detected during this study. 4 putatively translated levansucrases (*Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533, *Gluconobacter species* TMW 2.1191 and *K. baliensis* DSM 14400) are phylogenetically located in cluster 1 of AAB levansucrases (Fig. 17), while two levansucrases (*N. chiangmaiensis* NBRC 101099,

K. baliensis DSM 14400: interrupted form) are located in cluster 2 of AAB levansucrases, which was so far composed of solely one AAB levansucrase expressed by N_2-fixing *Ga. diazotrophicus* and several similar levansucrases expressed by N_2-fixing *Burkholderia* strains. These principal differences in putative protein sequences are furthermore reflected in the respective codon usage; while genes of cluster 1 levansucrases exhibit a similar codon usage among each other and solely few codons, which are known to be rarely used by *E. coli* (nihserver.mbi.ucla.edu/RACC/), genes of cluster 2 levansucrases contain many codons rarely used by *E. coli*. Therefore, heterologous expression of cluster 2 levansucrase genes in *E. coli* yields insufficient results as observed in this work (inclusion bodies, data not shown). On the contrary, the identified nucleotide sequences of cluster 1 levansucrases (*Gluconobacter* strains) could be expressed in *E. coli* as active proteins. While the gene coding for the secreted levansucrase of *K. baliensis* has not been cloned into *E. coli* during this work, the highly similar nucleotide sequence of *Ga. xylinus* (97% identity) was shown to be actively expressed by *E. coli* leading to macroscopic observable slime production (Tajima et al., 2000). Since *K. baliensis* possesses two (partial) genes coding for levansucrases, which significantly differ from their codon usages for same amino acids, a horizontal uptake of foreign DNA during evolution could have been occurred. The interrupted levansucrase nucleotide sequence of *K. baliensis* could (i) have been integrated (randomly) into its genome, (ii) be located on a (mobile, randomly taken up) plasmid without any (contemporary proposed) function for *Kozakia*, (iii) have been secondary disrupted and been initially actively expressed in *Kozakia*, (iv) be actively expressed *via* unknown molecular mechanisms, (v) have been actively rearranged and been expressed contemporary (even by other strains), if *Kozakia* stayed in its natural environment. Since there is actually no evidence for any (initial) functionality of the interrupted levansucrase, it seems likely that a random event occurred for integration/uptake of this gene sequence.

Otherwise, mobile genetic elements were previously reported to rearrange levansucrases in plant-pathogenic *Pseudomonas* strains, which contain more than one levansucrase coding sequence (Srivastava et al., 2012). It was demonstrated, that upstream sequences of two actively expressed levansucrases in these strains are composed of highly conserved phage associated promoter elements (PAPEs), which are identically found in N- termini of cellulases. Interestingly, the first 16 amino acids of the respective, actively expressed levansucrases are encoded by these PAPEs, which furthermore harbor the necessary promoter sequences for transcription, while a third levansucrase ORF lacks these PAPEs and was shown not to be expressed due to a missing functional promoter. While there is solely one ORF in *K. baliensis* interrupted levansucrase, which contains all known conserved motifs among levansucrases of gram-negative bacteria, but comprises a stop codon (TAG) at aa position 12, active expression *via* unknown mechanisms seems to be rather unlikely, although predicted amino acids 13-19 (TELCNRA) are identically encoded in the starting sequences of two *Burkholderia* "group 1" glycosyltransferases. Therefore, a "similar", possibly not completed rearrangement of this

levansucrase as described for *Pseudomonas* strains could be assumed, which would have been (inter)-mediated by an integrated IS5 element. Otherwise, this levansucrase gene sequence seems to be "simply interrupted" due to a detected 30 aa signal peptide beginning from the hypothetical initial start codon, a putative rho-independent transcriptional terminator as well as a downstream located GspE gene fragment, which was demonstrated to be involved in an operon coding for type II dependent levansucrase translocation in *Ga. diazotrophicus*. A similar genetic organization was detected in *N. chiangmaiensis* putative levansucrase, which, however, is flanked downstream by a gene fragment coding for BfpH. While this gene is also incorporated in the respective type II-secretion operon of *Ga. diazotrophicus*, both GspE (*K. baliensis*) and BfpH (*N. chiangmaiensis*) are not located next to the levansucrase nucleotide sequence in *Ga. diazotrophicus.* On the contrary, cluster 1 levansucrases lack signal peptides (as typically known for levansucrases of gram-negative bacteria) and associated operons, which would indicate a specific known, general protein secretion pathway.

Taken together, there are two known types of levansucrases among AAB (cluster 1+2), whose respective amino acids are encoded *via* different codon usages and which are genetically organized in different manners. As *K. baliensis* was shown to contain a functional cluster 1 and an interrupted cluster 2 levansucrase, horizontal gene transfer in specific ecological niches seems to have occurred in this strain.

4.6 Characterization of secreted proteins

While levansucrases of four *Gluconobacter* strains were actively expressed by recombinant *E. coli* strains and, therefore, can be principally processed to active enzymes, even codon-optimized *E. coli* Rosetta strains accumulated inclusion bodies after induction of *N. chiangmaiensis* levansucrase expression. Furthermore, at this point in time, solely the interrupted levansucrase nucleotide sequence of *K. baliensis* was detected, from which no "real" ORFs could be deduced. Therefore, supernatants of fermented (modified) NaG media were investigated regarding secreted, extracellular proteins to possibly identify parts of the corresponding levansucrases *via* mass spectra based peptide sequencing. The used NaG media were intentionally supplemented with a specific nitrogen source (casein acid hydrolysate), respectively, which is composed of short peptides and single amino acids, to avoid the isolation of numerous "background" proteins of similar molecular weight. Moreover, preliminary experiments revealed *N. chiangmaiensis* to be a constitutive levansucrase producer (residual activities in fermented NaG supernatants without supplemented sucrose, but too low protein contents after ammonium sulfate precipitation) and high initial sucrose concentrations to harden the isolation of proteins *via* out-salting due to simultaneous precipitation of polymeric substances. Therefore, NaG media were supplemented with low amounts of sucrose (0.1%) to (preferably) effectively induce levansucrase secretion without extensive formation of levan. In this way, isolated protein fractions of

both strains exhibited relatively high residual levansucrase activities. Electrophoretic separation of recovered protein fractions, however, yielded completely different patterns.

Whereas *N. chiangmaiensis* secreted only few proteins into the extracellular space, numerous protein bands could be visualized after staining of *K. baliensis* protein fractions. Though, both strains secreted solely few proteins in higher amounts (*N. chiangmaiensis*: 1, *K. baliensis*: 4), which were subsequently checked for potential residual levansucrase activities (reactivation after reverse staining). Protein A1 secreted by *N. chiangmaienis* (Fig. 14), which reflected the approximate molecular weight of its identified putative levansucrase without signal peptide, in fact, exhibited residual levansucrase activity, while several attempts of peptide sequencing yielded no reliable results for this protein. Indeed, secreted major proteins of *K. baliensis*, which yielded no residual activities, could be identified as chaperone DnaK and elongation factor Tu. Interestingly, Berrier et al. (2000) reported these two proteins to be secreted in high amounts into the periplasm of *E. coli via* a mechanosensitive channel upon osmotic downshock (*in vitro* induced by sucrose). Finally, these proteins are released into the extracellular space by diffusion through membrane-associated porins. Consequently, a similar reaction of *K. baliensis* could be assumed for maintenance of its osmotic balance in osmotically harsh environments. A concomitant levansucrase-mediated metabolization/partial polymerization of sucrose could furthermore contribute to a numerous reduction of osmolytes.

Since several weak protein bands were detected after electrophoretic separation of protein extracts from *K. baliensis*, the corresponding levansucrase could not be identified in this manner. Parts of the secreted levansucrase of *K. baliensis* were rather detected after secretion of the respective enzyme into Na-acetate buffer. Therefore, one of the weak bands recovered from fermented supernatants of fermented NaG media (Fig. 14B) might correspond to this secreted levansucrase. While peptide fragments of this monomeric enzyme were quickly detectable *via* peptide sequencing, sequencing of the dimeric form of *Gluconobacter species* TMW 2.1191 levansucrase recovered after secretion into Na-acetate buffer yielded no positive results. The monomeric protein obtained after reduction of the dimeric form (*Gluconobacter species* TMW 2.1191) with excessive DTT again could be sequenced. As proteins have to be properly reduced and subsequently alkylated for effective tryptic digests and subsequent peptide sequencing (Hustoft et al., 2012), negative results obtained during this study could be explained as insufficiently reduced proteins, which are inter-/intramolecularly stabilized by disulfide-bridges.

Although *N. chiangmaiensis* levansucrase was electrophoretically separated as putatively monomeric protein, which could not be sequenced, the existence of an intramolecular disulfide-bridge, as shown for the similar levansucrase of *Ga. diazotrophicus* (Betancourt et al., 1999; Martinez-Fleites et al., 2005), was calculated for this enzyme. Generally, such disulfide-bridges are known to occur especially

in cell-wall associated, soluble-secreted and extracellular matrix proteins exhibiting diverse functions such as maintenance of thermodynamic/mechanical stability, protection against proteases and redox regulation of protein functions in extracellular environments (Fass, 2012). As seen in the present study, dimeric levansucrases of *Gluconobacter species* TMW 2.1191 could be solely partially reduced to their respective monomers using excessive amounts of DTT (1 M) (3.4.2), which indicates their high stability and effective resistance against reducing conditions. Since these dimeric levansucrases could not be sequenced in contrast to the monomeric proteins, the dimeric enzyme seems to be additionally protected against digestion by prevalent proteases such as trypsin. While actually an involvement of these disulfide bonds in the catalytic properties of *Gluconobacter species* TMW 2.1191 levansucrase cannot be predicted, related levansucrases of *Zymomonas mobilis*, which exhibit a respective conserved cysteine residue in the catalytic motif "sucrose box 1" (EWSGC; Fig. 10; appendix A10), were shown to exist as dimers at neutral pH, which mainly catalyze sucrose hydrolysis, and to adapt ordered, microfibrillar structures at lower pH values and higher ionic strength, which again promote levan polymerization (Goldman et al., 2008). Due to the fact, that *Gluconobacter species* TMW 2.1191 levansucrase was re-dissolved in water and neutral sample buffers, it could be assumed that monomeric proteins of *Gluconobacter species* TMW 2.1191 and *Zymomonas mobilis* are intermolecularly linked among each other similarly. Furthermore, it was demonstrated, that substitutions of cysteine by serine residues in *Z. mobilis* levansucrases abolish levan polymerization, but only have sucrose hydrolysis activities (Senthilkumar et al., 2003). Consequently, even an involvement of cysteine residues in higher oligomerization of these types of levansucrases (> 2), which again was reported to be necessary for levan polymerization (Goldman et al., 2008), can be assumed.

A contribution of intramolecular disulfide bonds for proper protein folding and substrate binding was so far exclusively demonstrated for *Ga. diazotrophicus* levansucrase (Martinez-Fleites et al., 2005) and is supported by identification of *N. chiangmaiensis* as well as *K. baliensis* (interrupted) levansucrases, both of which possess conserved cysteine residues at the respective expected positions for disulfide formation (appendix A5). While nothing is known about the existence of any conserved/functional disulfide bonds in GH 68 FTFs from gram-positive bacteria, intramolecular disulfide bonds in cluster 2 AAB levansucrases are found in regions, which are structurally equivalent to the Ca^{2+}-cofactor-binding sites of levansucrases from *B. subtilis* (Meng & Fütterer, 2003) (Fig. 15). Moreover, amino acids involved in formation of the Ca^{2+}-binding pocket are conserved among most members of gram-positive bacteria, while they are absent in gram-negative bacteria (Van Hijum et al., 2006) indicating a group-specific distribution of different mechanisms for FTF stabilization. Taken into account, that most FTFs from gram-positive bacteria are supposed to be located at the cell surface or even known to contain conserved motifs for cell-wall anchoring in their respective C-terminal regions (Van Hijum et al., 2006), gram-positive-specific, cell-wall associated teichoic acids could

function as an ideal source for divalent cations such as Ca^{2+}, which accumulate in gram-positive cell walls due to the anionic character of these acids (negatively charged phosphate groups; Lambert et al., 1975). Therefore, a controlled localization of Ca^{2+}-dependent FTFs in cofactor-rich cell wall structures could maintain the corresponding enzymatic activities. On the contrary, FTFs from gram-negative bacteria are supposed to be secreted into the extracellular environment and lack (known) motifs involved in cell wall anchoring (Velazquez-Hernandez et al., 2009). As secreted enzymes are not associated with cells of their secreting microorganisms and are, therefore, globally confronted with the surrounding environment, the mechanistic properties of disulfide bonds, as discussed above and by Fass (2012) could maintain the structural and catalytic properties of secreted FTFs from certain gram-negative bacteria. However, secretion of disulfide containing levansucrases into supernatants by gram-negative bacteria still should not be considered as a general feature among this group of microorganisms, since there is – in contrast to *Gluconobacter species* TMW 2.1191 and *N. chiangmaiensis* - actually no further evidence for strict conservation of cysteine residues at specific positions in these FTFs. Moreover, to date there are no biochemical data available about potential intra-/intermolecular disulfide bonds in natively recovered FTFs. In this context it should be noted, that the secreted monomeric levansucrase of *K. baliensis* could be sequenced without any excessively reducing pretreatment while containing two cysteine residues at, actually, non-conserved positions.

4.7 Secretion of native levansucrases into buffered solutions

4.7.1 State of the art

Several previous studies reported about severe limitations, which usually occur during isolation of native GH 68 FTFs and GH 70 GTFs, both of which are either complexed to produced macromolecules or not selectively precipitable due to simultaneous formation of polymeric substance. There are few reports about efficient strategies for native GTF/FTF recovery such as simultaneous polymer cleavage by commercially available inulinases during levan production (Vigants et al., 2003), polyethylene glycol mediated purification of dextransucrases *via* phase separation (Nigam et al., 2006), precipitation of FTFs/GTFs with the respective pre-isolated fructans/glucans (Reese & Avigad, 1966; Dols et al., 1998) or mild ethanol concentrations for selective precipitation of produced sugar polymers (Arrieta et al., 1996). However, all of these methods need further (chromatographic) steps to obtain a finally purified protein, which can be unpredictably impaired in its initial activity due to the use of different materials and chemicals during isolation.

As a way out, most researchers recombinantly express known nucleotide sequences of (often modified/tagged) GTFs or FTFs and investigate these proteins regarding their biochemical properties after purification *via*, in most cases, affinity chromatography. As seen in the present study, expression strains such as *E. coli* can be suitably manipulated for expression of certain FTFs, while other FTFs are not actively expressed being entrapped in inclusion bodies, possibly due to differing codon usages

or to essential intramolecular disulfide bonds, whose formation is hardly catalyzed in the reducing cytoplasm of *E. coli* (Stewart et al., 1998).

As discussed in section 4.6, there are principal differences regarding the extracellular character of these enzymes. While proteins such as some dextransucrases from LAB (Dols et al., 1998) or levansucrases from some gram-negative bacteria are secreted into culture supernatants (Arrieta et al., 1996), cell-wall anchored enzymes are hardly released from cell surfaces thereby not allowing an effective purification process. Therefore, at least culture supernatants (without supplemented sucrose) of strains, which secrete FTFs/GTFs constitutively into the extracellular environment, could be used for enzyme purification, thereby avoiding the problem of simultaneous polymer formation.

During the present study, solely *N. chiangmaiensis* and *Gluconobacter species* TMW 2.1191 secreted levansucrases constitutively into NaG media (levan formation in cell-free supernatants), whereas residual activities of concentrated protein fractions were too low for further analyses. Since FTFs/GTFs undergo extreme pH fluctuations during a classical fermentation with AAB or LAB and their corresponding enzymatic activities (hydrolysis, polymerization) are generally reported to be pH dependent, fermentatively produced EPS are usually not uniform (monodisperse) with respect to their molecular weight as demonstrated in this work. Polymer fractions of the same fructan type, but of different molecular weight (distributions) again exhibit different techno-functional properties (4.1 + 4.2). Consequently, it is a big challenge to find conditions for recovery of untreated, native FTFs/GTFs and for constant (*in situ*) production of desired functional EPS.

4.7.2 Behaviour of *Gluconobacter species* TMW 2.1191 in buffered solutions

A recovery of native enzymes in buffered solutions as demonstrated in the present work has not been reported yet, possibly due to the fact, that a metabolic activity of microorganisms in such a system is commonly not expected due to lacking nutrients. More surprisingly, *Gluconobacter species* TMW 2.1191 secreted solely its obviously dimeric levansucrases into Na-acetate buffer (pH 5.0) during aerobic incubation for a few hours. However, this enzyme could not be recovered without simultaneous production of levan, as sucrose (e. g. 0.1 M) was necessary for induction of levansucrase secretion, although the residual supernatant of pre-cultivation medium (NaG without supplemented sucrose) could be used to produce levan. Constitutive levansucrase expression, therefore, seemed to be at least down-regulated or stopped, since no secreted enzymes could be detected after incubation of cells in 0.1 M Na-acetate buffer (pH 5.0) without supplemented sucrose. Sucrose itself was proven not the sole inducing factor for enzyme secretion, since this strain did not secrete levansucrases into 0.1 M Na-phosphate buffers (pH 7.0, with or without 0.1 M sucrose). Moreover, secretion was growth-dependent or rather most effective using cells from the mid to late exponential growth phase. Glucose or fructose alone (or combinations thereof, each 0.1 M) again did not induce levansucrase secretion.

Consequently, inducible secretion of FTFs by *Gluconobacter species* TMW 2.1191 seemed to occur as a response of a certain cell population on an acidic, sucrose-containing environment.

It could be assumed, that levansucrases were still secreted constitutively to a constantly low (not detectable) extent at the beginning of incubation in sucrose-supplemented Na-acetate buffer, while a simultaneous accumulation of levan molecules as well as the availability of free glucose/fructose/sucrose and their (possible) respective metabolic products in unknown ratios influenced the respective cell populations to react on varying environmental, acidic conditions by a controlled, enhanced secretion of FTFs. The (obviously regulatory) decrease in/the low tendency for ongoing constitutive levansucrase secretion in Na-acetate buffer without supplemented sucrose supports this hypothesis, since it would make no sense for *Gluconobacter species* TMW 2.1191 itself to pre-accumulate constitutively expressed FTFs, which are intended to bind/cleave sucrose, and to subsequently secrete a second sucrose-inducible set of FTFs, while the first set would have been able to metabolize the inducer sucrose anyway. Since enhanced FTF secretion was dependent on the acidic environment, the reaction of these cell populations could be interpreted as a secondary induced "boost" response to efficiently degrade/metabolize sucrose and to simultaneously produce sufficient amounts of levan for protective biofilm formation in acidic, sucrose-rich environment as already discussed in section 4.4. Otherwise, constitutive levansucrase secretion by *Gluconobacter species* TMW 2.1191 could be considered as a passive, "mild" metabolic activity, which might help itself and other organisms to metabolize sucrose during invasion of sucrose-containing micro-niches, while a direct contact to sucrose in higher amounts (inducing concentrations) in acidic environments would trigger *Gluconobacter species* TMW 2.1191 to actively up-regulate its levansucrase expression/secretion machinery for active, enhanced metabolization of sucrose. Moreover, it remains unclear, if *Gluconobacter species* TMW 2.1191 levansucrases are expressed from one nucleotide sequence, or if this strain contains more copies of actively/differentially expressed levansucrases in its genome (see also 4.5). To my knowledge, nothing is known about the differential regulation of constitutive and sucrose-inducible secretion of GTFs/FTFs in one strain. Sucrose-inducible levansucrase expression was intensively investigated in *Bacillus subtilis* and was shown to function as a complex anti-termination mechanism on transcriptional level (Daguer et al., 2004). However, levansucrase expression mechanisms are supposed to be differentially regulated in different bacterial species (Velazquez-Hernandez et al., 2009). The influence of an acidic environment on regulatory expression/secretion of these enzymes, as demonstrated in the present work, has so far not been taken into account.

The importance of levansucrases for infection/colonization of eukaryotic hosts (mainly sucrose-containing plants) by different plant-associated bacteria (*Erwinia spp*., *Streptococcus spp*., *Bacillus spp*. *Paenibacillus spp*., *Pseudomonas spp*.) was demonstrated in several previous works (Velazquez-

Hernandez et al., 2009). A contribution of levan in biofilm formation was reported for biofilms of *Pseudomonas syringae* (Laue et al., 2006) and for those of *Ga. diazotrophicus* (Velazquez-Hernandez et al., 2011). However, development of such biofilms is commonly documented macroscopically on mainly abiotic surfaces, while biofilms of bacteria, which do not secrete excessive amounts of polysaccharide, are supposed to consist of different extracellular polymeric substances, such as polysaccharides, proteins, lipids and nucleic acids (Flemming & Wingender, 2010).

The present work demonstrates for the first time the directed aggregation and immobilization of bacterial cells in a polysaccharide matrix, which is adhered to an abiotic (glass) surface, microscopically. Interestingly, the movement of individual cells in levan-rich areas appeared regular in contrast to the aqueous phase. The sudden common and simultaneous aggregation of individual cells to cell bundles occurred almost too quick (< 1 s) for a comprehensible observation by naked eye. The subsequent directed movements of cell bundles towards each other again were well visible and resembled the movement of protozoa. These actively moving, multicellular agglomerates were embedded in "elastic" material (possibly attached levan), which seemed to serve as a flexible envelope for coordinative movement towards each other. Cell bundles were finally immobilized by gelatinization of levan. Cells seemed to commonly react on increasing water deprivation by their synthesized levan matrix and to use the respective physicochemical properties of the produced polysaccharide for formation of sessile microcolonies, which could be re-activated by addition of water. These observations gain impressive insights into the coordinated formation of bacterial biofilms and point out the biological role of high molecular weight levan, whose formation is widely distributed among members of this domain. Locally high concentrations of levan can be used to move commonly along surfaces, to associate and to settle down due to the gelling properties of levan. Water bearing levan matrices again could maintain the physiological requirements for survival in ecologically harsh environments.

4.7.3 Biotechnological impact of this system

The possibility for recovery of pure, native levansucrases in (buffered) solutions enables the implementation of a multitude of biotechnological applications. First of all, natively secreted, untreated enzyme solutions can be used to produce levan in a uniquely controllable, time- and cost-saving procedure. Moreover, this method can alternatively be used for synthesis of industrially relevant dextrans (Naessens et al., 2005b). However, *L. mesenteroides* TMW 2.1073 had to be "pre-induced" with sucrose, which implies the prinicipal flexibility and explanatory power of this system. Produced polysaccharides are of high purity due to the lack of contaminating media components and do not have to be selectively precipitated. This again enables the recovery of totally produced polysaccharides without any major losses. Furthermore, tailor-made polysaccharides can be synthesized by addition of a second, substrate containing (buffered) solution to an enzymatic solution,

whose compositions can be pre-adjusted, respectively. Moreover, activities of enzymes can be almost fully exploited by - compared to classical fermentations - simple identification/combination of the ideal solution compositions and incubation conditions. Enzymatically produced levans were shown to reach levels of extraordinarily high molecular weight, which were so far not expectable regarding natural polysaccharides. Obtained, uniform polymer fractions again are commonly preferred in industrial applications due to concomitant constant rheological properties. Levans or dextrans, which were produced by this method, could be used – in accordance to their already/partially established applications (Freitas et al., 2011) - in (i) food industry ((hydro-)colloids, thickening, gelling, emulsifying, stabilizing agents; controlled release of vitamins or aroma compounds), (ii) pharmaceutics (drug delivery, biological degradable materials), (iii) chemical industry (molecular weight standards, initiator molecules for further processing, column materials for chromatographic techniques), (iv) cosmetic industry (bioadhesive substances, ingredients in e. g. creams, tooth paste), (v) environmental engineering (artificial biofilms), (vi) biomedicine (blood plasma volume expanders), (vii) paper/materials/polymer industry (surface smoothener, structural improver, firmness improver).

The efficiency of this method can be explained by stabilization of untreated enzymes in a pure system (constant pH pre-adjusted to the expected approximate activity optima of the respective enzymes) and by their transitional complexation to the produced polysaccharides, which were reported to stabilize FTFs/GTFs (Bekers et al., 2003; Dols et al., 1998). Levansucrases of *Gluconobacter species* TMW 2.1191 polymerized fructose in an obviously organized manner due to the uniformity of the synthesized polymer, which could gain new insights into the mechanistic/structural functionality of these enzymes (4.6). In this context, a unique activity definition could be established for FTFs/(GTFs) referring to a uniform product, which is principally expected in enzymatic activity definitions.

In this context, it should be noted, that extracellular levansucrases of *Gluconobacter species* TMW 2.1191 exhibited a significantly higher polymerization activity in this system in comparison to fermentation broths. This again reveals the importance of constant and “noisefree” conditions for efficient production of uniform, biologically active (4.7.2) levans. Knowledge about constitutive *vs.* inducible FTF/GTF secretion obtained from this system (4.7.2) can be adopted for *in situ* food fermentations, which could be optimized by proper addition of sucrose at suitable time points. Furthermore, the principal idea of this system could be transferred to other (recombinant) strains for recovery of further industrial relevant, natively secreted, untreated enzymes, such as e. g. proteases, lipases or glycosidases. Recent advances in efficient genetic manipulation of *Gluconobacter* strains (Peters et al., 2013a/b; Kostner et al., 2013) could be used for studies on FTF gene/operon replacement by genes coding for proteins of unknown or desired functions. In this way, new expression strains could be generated for the possible easy recovery of extracellular proteins.

5. SUMMARY

Acetic acid bacteria (AAB) are traditionally used in several food applications, such as vinegar, bionade, kombucha, kefir or cocoa production. Moreover, especially strains of the genus *Gluconobacter* have gained much interest in biotechnology due their unique ability to incompletely, stereo- and regioselectively oxidize a wide range of sugars and polyols *via* membrane-bound dehydrogenases. The concomitant accumulation and easy recovery of specific, oxidized products in fermentation broths is actually exploited in diverse pharmaceutical and chemical sectors.

Several members of AAB produce the exopolysaccharide (EPS) cellulose, which is commercially recovered from strains of *Gluconacetobacter xylinus* and is used as alternative for plant cellulose in textile, paper, food and pharmaceutical industries. Further known EPS from AAB are strain specific heteropolysaccharides and dextrans, both of which are barely investigated due to their sporadic detection.

In addition, some AAB strains produce extracellular fructans, such as levans, or possess genes coding for levansucrases, which use sucrose as substrate for levan synthesis. While little is known about a general distribution of fructan synthesis among AAB, nitrogen-fixing strains of *Gluconacetobacter diazotrophicus* were intensively investigated regarding the enzymatic properties of their secreted levansucrases, which are actually considered as model enzymes for understanding the biochemical properties of glycoside hydrolase (GH) 68 fructosyltransferases (FTFs: levan- and inulosucrases).

However, microbial fructans are actually rarely applied in industrial applications. On the contrary, plant fructans (inulins) are widely used in foods as low-calorie, non-cariogenic sweeteners, which are additionally considered as representative prebiotics. Although sugar polymers such as fructans principally exhibit water-binding capacity, the hydrocolloid character of fructans, for e. g. technological improvement of foods, has so far not been exploited due to lacking systematic studies on the functionality and applicability of these polymers. Fructans are actually rather considered as nutritionally valuable additives.

Since AAB are (food-grade) microorganisms, which are widely used and applicable in diverse (food) biotechnological sectors, and the functionality of microbial fructans has generally not been investigated in dependence of their chemical structure, the present work should highlight the (so far non-exploited) potential of AAB to produce functional fructans. In this way, a basis for biotechnological exploitation of these sugar polymers should be provided.

For these purposes, different fructan producing AAB strains should at first be identified/selected, whose synthesized fructans should be isloated and investigated regarding their chemical identity and molecular weight distributions. Some of these fructans should be exemplary used as additives for baking experiments to possibly demonstrate their applicability as hydrocolloids and to concomitantly establish comparative structure-function relationships. Moreover, fructan-producing enzymes should be identified using different genetic/enzymatic methods and be compared among each other regarding their principal genetic/enzymatic diversity. In this way, the general knowledge about fructan biosynthesis in AAB should be enhanced and be integrated into the global context of fructan biology/biotechnology.

Five AAB strains of the genera *Gluconobacter* (*G.*) (3), *Neoasaia* (*N.*) (1) and *Kozakia* (*K.*) (1) were identified as strong fructan producers among a total of 22 AAB strains. Whereas all tested *Gluconobacter* strains (12) produced fructan from sucrose with different activities, no fructan producing strains could be identified after screening of different *Gluconacetobacter* (7) and *Acetobacter* (1) strains. Therefore, fructan synthesis seems to be widely distributed among strains of the genus *Gluconobacter*, which are known to be specialized on the consumption of sugars and polyols in contrast to *Acetobacter*/*Gluconacetobacter* strains, which again prefer alcohols as energy sources for basic metabolism. Consequently, fructan synthesis among *Gluconobacter* strains could be interpreted as an adaptive physiological effort for survival in sucrose-rich environments.

Fructans of *Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533T, *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400 were isolated from fermented fermentation broths (5 L), respectively, and subsequently structurally analyzed. All fructans were identified as linear levans because of lacking additional signals in the respective ^{13}C NMR spectra, which would indicate other linkage types than β-(2→6)-linkages. On the contrary, the molecular weight distributions and particle sizes of these levans, derived from asymmetric flow field-flow-fractionation coupled to multi-angle light scattering (AF4-MALS), differed significantly among each other. While levans of *Gluconobacter species* TMW 2.767 and *G. cerinus* exhibited molecular weights in the range of 10^7 Da, levans of *N. chiangmaiensis* and *K. baliensis* exhibited molecular weights in the range of 10^8 and 10^9 Da, respectively. Moreover, in aqueous solutions the size of levan molecules present in all isolated levans continuously increased with their molecular weight and they tended to adopt a more compact, spherical-like molecular shape.

These structurally analyzed fructans were used in two dosages (1% and 2% w/w flour) for baking of wheat breads. Breads were evaluated in terms of their loaf volumes and crumb hardnesses. The influences of these levans on staling of wheat breads were documented over a time period of one week. All isolated levans caused increased volumes, softer crumbs and retarded staling of wheat

breads at the lowest tested dosage (1%) demonstrating their high technological potential for improvement of foods. Since levans of *N. chiangmaiensis* and *K. baliensis* were more effective regarding volume increase and retardation of staling, higher molecular weight of these levans suggested itself as main trigger to positively influence the properties of wheat breads. The correlation between detected molecular weight distributions, influences on wheat breads and existing literature about rheological properties of microbial fructans suggested levan molecules from AAB to function as individual microgels, which can more effectively bind and retain water with increasing molecular weight and concomitant, increasing compactness of individual molecules. The calculated globular-like shape of individual, high-molecular weight levan molecules was confirmed, as spherical levan molecules could be microscopically observed (1000 x magnification) due to their enormous size (up to 1 µm in diameter).

Since the selected AAB strains produced levans, several PCR methods were applied for identification of unknown levansucrase genes in five levan-overproducing AAB strains. A total of six (partially) levansucrase encoding sequences have been detected during this study. Four putatively translated levansucrases (*Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533, *Gluconobacter species* TMW 2.1191 and *K. baliensis* DSM 14400) are phylogenetically located in cluster 1 of AAB levansucrases, while two levansucrases (*N. chiangmaiensis* NBRC 101099, *K. baliensis* DSM 14400: interrupted/incomplete levansucrase) are located in cluster 2 of AAB levansucrases, which was so far composed of solely one AAB levansucrase expressed by N_2-fixing *Gluconacetobacter diazotrophicus* strains and several similar levansucrases expressed by nitrogen-fixing *Burkholderia* strains. Cluster 2 levansucrases possess signal peptides (and downstream located genes), which indicate their translocation to the extracellular space *via* a type II dependent protein secretion pathway. On the contrary, cluster 1 levansucrases exhibit the typical modular topology of GH 68 FTFs from gram-negative bacteria and lack motifs, which would indicate secretion *via* any known protein secretion pathways. Therefore, two different types of levansucrases are actually found in AAB, which are differentially organized on enzymatic and genetic level, respectively.

To verify, if the identified levansucrase gene sequences can be processed to active proteins, complete open reading frames were cloned into *Escherichia (E.) coli* expression strains. *Gluconobacter* levansucrases could be heterologously expressed in *E. coli*, which produced mucous polysaccharides after induction of levansucrase expression. However, recombinant *E. coli* cells harbouring the levansucrase of *N. chiangmaiensis* accumulated inclusion bodies after induction of levansucrase expression. These differences in levansucrase expression could be due to differing codon usages between cluster 1 and cluster 2 levansucrases and to the potential existence of an essential intramolecular disulfide-bridge in *N. chiangmaienis* levansucrase, which was calculated by protein modelling and is located in a structural region being equivalent to the Ca^{2+}-cofactor binding site in

Bacillus subtilis levansucrase. While an extracellular protein with residual levansucrase activity could be identified in *N. chiangmaiensis* and fermented culture supernatants of *K. baliensis* exhibited levansucrase activities, analysis of selected secreted proteins *via* peptide sequencing yielded no levansucrase sequences for both strains, respectively.

Therefore, a system was developed, which enabled the recovery and identification of a native cluster 1 levansucrase from *K. baliensis* DSM 14400 in (buffered) solutions. Consequently, the genome of *K. baliensis* DSM 14400 contains at least two levansucrase-coding sequences, one of which does not seem to be secreted or to be active (incomplete cluster 2 levansucrase). This system was highly effective for *Gluconobacter species* TMW 2.1191, which secreted high amounts of pure, dimeric levansucrases into sodium-acetate buffer, which are possibly stabilized by an intermolecular disulfide bridge. These enzymatic solutions could be used to produce uniform levans, which exhibit unique high molecular weight, in a time- and material saving procedure. New insights about constitutive and inducible FTF secretion were obtained from this system, which can be used for e. g. optimization of *in situ* food fermentations. Furthermore, cells of *Gluconobacter species* TMW 2.1191 showed coordinated behaviour in this system as microscopically observed. They used the physicochemical properties of levan to move commonly along surfaces, to associate and to settle down. In this way, the biological role of levan as extracellular biofilm material was pointed out.

In conclusion, the results presented in this work provide the basis for (food) biotechnological exploitation of fructans from AAB. Structural features of linear levans were identified and correlated with their hydrocolloid function. Unique, spherical levan molecules can be recovered more easily using an improved method for EPS production. The diversity of levan synthesis among different genera of AAB was pointed out. Moreover, the biological function of levan was demonstrated and the general knowledge about microbial (enzymatic) fructan biosynthesis was considerably enhanced.

6. ZUSAMMENFASSUNG

Essigsäurebakterien (ESB) werden traditionell zur Herstellung/Prozessierung mehrerer Lebensmittel (Essig, Bionade, Kombucha, Kefir, Kakaobohnen) verwendet. Darüber hinaus haben sich insbesondere Stämme der Gattung *Gluconobacter* als äußerst interessant für biotechnologische Anwendungen erwiesen, da sie in der Lage sind, auf einzigartige Weise verschiedenste Zucker und Polyole mittels membrangebundener Dehydrogenasen unvollständig, sowie stereo- und regioselektiv zu oxidieren. Die damit verbundene, gleichzeitige Anreicherung und leichte Gewinnung spezieller oxidierter Produkte in Fermentationsmedien wird derzeit in diversen pharmazeutischen und chemischen Unternehmensbereichen genutzt.

Mehrere ESB produzieren das Exopolysaccharid (EPS) Zellulose, welches kommerziell aus Stämmen von *Gluconacetobacter xylinus* gewonnen und als Alternative für pflanzliche Zellulose in der Textil-, Papier-, Lebensmittel- und der pharmazeutischen Industrie eingesetzt wird. ESB bilden stammspezifisch weitere EPS, wie Heteropolysaccharide und Dextrane, die jedoch aufgrund vereinzelter Nachweise bislang nicht näher untersucht wurden.

Zudem synthetisieren einige ESB Stämme extrazelluläre Fruktane (Levane: β-(2→6)-verknüpfte β-D-Fruktose-Polymere) oder besitzen Gene, die für Levansucrasen codieren, welche Saccharose als Substrat zur Levanbildung nutzen. Während bislang wenig darüber bekannt ist, inwieweit Fruktanbildung unter ESB verbreitet ist, wurden stickstofffixierende ESB Stämme von *Gluconacetobacter diazotrophicus* intensiv hinsichtlich der enzymatischen Eigenschaften ihrer sekretierten Levansucrasen untersucht, die derzeit als Modellenzyme zum Verständnis der biochemischen Eigenschaften von Glykosid-Hydrolasen (GH) 68 (Levan- und Inulosucrasen) betrachtet werden.

Mikrobiell gebildete Fruktane werden jedoch aktuell kaum in industriellen Anwendungen eingesetzt. Im Gegensatz dazu werden pflanzenbürtige Fruktane (Inuline) vielfältig in Lebensmitteln als kalorienarme, nicht-kariogene Süßstoffe verwendet und gelten zudem als representative Prebiotika. Obwohl Zuckerpolymere wie Fruktane prinzipiell Wasserbindevermögen aufweisen, wurde der Hydrokolloidcharakter von Fruktanen zur beispielsweise technologischen Verbesserung von Lebensmitteln bislang nicht genutzt, was auf fehlende systematische Studien zur Funktionalität und Anwendbarkeit dieser Polymere zurückzuführen ist. Fruktane werden derzeit vielmehr als ernährungsphysiologisch wertvolle Zusatzstoffe betrachtet.

Da ESB (lebensmittelassoziierte) Mikroorganismen sind, die weitläufig genutzt/anwendbar in verschiedenen (lebensmittel)biotechnologischen Anwendungen werden/sind, und da die Funktionalität mikrobiell gebildeter Fruktane generell noch nicht in Abhängigkeit von ihrer chemischen Struktur

untersucht worden ist, sollte die vorliegende Arbeit das (bislang nicht ausgeschöpfte) Potenzial von ESB zur Produktion funktionaler Fruktane aufzeigen. Auf diese Weise sollte eine Basis zur biotechnologischen Nutzung dieser Zuckerpolymere geschaffen werden.

Hierfür sollten zunächst Fruktan-produzierende ESB Stämme identifiziert und selektiert werden, deren gebildete Fruktane isoliert und hinsichtlich ihrer chemischen Identität und Molekulargewichts-verteilungen untersucht werden sollten. Einige dieser Fruktane sollten modellhaft in Backversuchen eingesetzt werden, um eventuell ihre Anwendbarkeit als Hydrokolloide demonstrieren und um gleichzeitig vergleichende Struktur-Wirkungsbeziehungen erarbeiten zu können. Darüber hinaus sollten Fruktan-bildende Enzyme mittels diverser genetischer/enzymatischer Methoden identifiziert und schließlich untereinander hinsichtlich ihrer prinzipiellen genetischen/enzymatischen Diversität verglichen werden. Auf diese Weise sollte das generelle Wissen über die Biosynthese von Fruktanen in ESB erweitert und in den globalen Kontext der Biologie/Biotechnologie von Fruktanen integriert werden.

Fünf ESB Stämme der Gattungen *Gluconobacter* (*G.*) (3), *Neoasaia* (*N.*) (1) und *Kozakia* (*K.*) (1) wurden als starke Fruktan-Produzenten unter insgesamt 22 ESB Stämmen identifiziert. Während alle getesteten *Gluconobacter* Stämme (12) Fruktane aus Saccharose mit unterschiedlichen Aktivitäten bildeten, konnten keine Fruktan-produzierenden *Gluconacetobacter* (7) und *Acetobacter* (1) Stämme identifiziert werden. Daher scheint Fruktanbildung unter Stämmen der Gattung *Gluconobacter* weit verbreitet zu sein, die – im Gegensatz zu *Acetobacter/Gluconacetobacter* Stämmen, welche bevorzugt Alkohole als Energiequelle für den Primärmetabolismus nutzen – auf die Verwertung von Zuckern und Polyolen spezialisiert sind. Folglich könnte Fruktanbildung durch *Gluconobacter* Stämme als adaptiver, physiologischer Zusatznutzen in Saccharose-reichen Habitaten interpretiert werden.

Fruktane aus *Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533T, *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400 wurden aus Fermentationsmedien (5 L) isoliert und anschließend strukturell untersucht. Alle Fruktane wurden als lineare Levane identifiziert, da in den jeweiligen ^{13}C NMR Spektren keine Signale nachweisbar waren, die auf andere Verknüpfungstypen als β-(2→6)-Verknüpfungen hinwiesen. Im Gegensatz dazu unterschieden sich die Molekulargewichts- und Patrikelgrößenverteilungen dieser Levane, welche mittels asymmetrischer Fluss Feld-Fluss Fraktionierung gekoppelt mit Mehrwinkellichtstreudetektion (AF4-MALS) vermessen wurden, deutlich voneinander. Demnach wiesen Levane aus *Gluconobacter species* TMW 2.767 und *G. cerinus* Molekulargewichte im Bereich von 10^7 Da auf, wohingegen Levane aus *N. chiangmaiensis* und *K. baliensis* Molekulargewichte von jeweils 10^8 und 10^9 aufwiesen. Zudem stieg die Größe der jeweiligen Levanmoleküle in wässeriger Lösung mit steigendem Molekulargewicht kontinuierlich an. Levanmoleküle nahmen dabei tendenziell eine kompaktere, sphärische molekulare Struktur an.

Diese strukturell analysierten Levane wurden in zwei Dosen (1% und 2% w/w Mehl) zum Backen von Weizenbroten verwendet. Die Brote wurden anschließend hinsichtlich ihrer Laibvolumina und Krumenhärten untersucht. Zudem wurde der Einfluss dieser Levane auf das Altbackenwerden von Weizenbroten über einen Zeitraum von einer Woche dokumentiert. Alle isolierten Levane verursachten erhöhte Volumina, weichere Krumen und verzögertes Altbackenwerden von Weizenbroten bereits ab der niedrigsten eingesetzten Dosis (1%), was deren hohes technologisches Potenzial zur Verbesserung von Lebensmitteln demonstriert. Da sich Levane aus *N. chiangmaiensis* und *K. baliensis* als vergleichsweise effizienter zur Volumenerhöhung und Verzögerung des Altbackenwerdens von Weizenbroten erwiesen, konnte angenommen werden, dass die unterschiedlichen Molekulargewichte der isolierten Levane für die unterschiedlichen Effekte auf Weizenbrote verantwortlich waren. Der Zusammenhang zwischen ermittelten Molekulargewichts-verteilungen und Einflüssen auf Weizenbroten sowie existierender Literatur über rheologische Eigenschaften mikrobieller Fruktane suggeriert, dass Levanmoleküle aus ESB als individuelle Mikrogele funktionieren, die Wasser mit ansteigendem Molekulargewicht und einhergehender zunehmender Kompaktheit der Moleküle effizienter binden und zurückhalten können. Die kalkulierte globuläre Molekülgestalt einzelner, hochmolekularer Levanmoleküle wurde dadurch bestätigt, dass sphärische Levanmoleküle bei tausendfacher Vergrößerung aufgrund ihrer enormen Größe (bis zu 1 µm) mikroskopiert werden konnten.

Da die selektierten ESB Stämme Levane produzierten, wurden mehrere PCR-Methoden zur Identifizierung unbekannter Levansucrase-Gene in fünf Levan-überproduzierenden ESB Stämmen angewandt. Es wurden insgesamt sechs (teilweise) Levansucrase-codierende Nukleotidsequenzen während dieser Arbeit detektiert. Vier putativ translatierte Levansucrasen (*Gluconobacter species* TMW 2.767, *G. cerinus* DSM 9533, *Gluconobacter species* TMW 2.1191 and *K. baliensis* DSM 14400) sind phylogenetisch in der Gruppe 1 von Levansucrasen aus ESB lokalisiert, wohingegen zwei Levansucrasen (*N. chiangmaiensis* NBRC 101099, *K. baliensis* DSM 14400: unter-brochene/unvollständige Levansucrase) in die Gruppe 2 von Levansucrasen aus ESB klassifiziert werden können, welche sich bislang aus der Levansucrase verschiedener stickstofffixierender *Gluconacetobacter diazotrophicus* Stämme und mehrerer ähnlicher Levansucrasen aus stickstofffixierenden *Burkholderia* Stämmen zusammensetzte. Gruppe 2 Levansucrasen besitzen Signalpeptide (und im Downstream-Bereich lokalisierte Gene), die darauf hinweisen, dass sie über einen Typ II abhängigen Proteinsekretionsweg ins extrazelluläre Milieu transportiert werden. Im Gegensatz dazu weisen Gruppe 1 Levansucrasen den typischen modularen Aufbau von GH 68 Fruktosyltransferasen aus gram-negativen Bakterien auf. Zudem fehlen Gruppe 1 Levansucrasen Motive, die auf bekannte Proteinsekretionswege rückschließen lassen. Daher werden gegenwärtig zwei unterschiedliche Typen von Levansucrasen in ESB gefunden, die auf enzymatischer und genetischer Ebene unterschiedlich organisiert sind.

Um zu bestätigen, dass die identifizierten Levansucrase-Gene zu aktiven Proteinen prozessiert werden können, wurden vollständige, offene Leserahmen in *Escherichia (E.) coli* Expressionsstämme kloniert. *Gluconobacter* Levansucrasen konnten heterolog in *E. coli* exprimiert werden, welche nach Induktion von Levansucrase-Expression muköse Polysaccharide bildeten. Rekombinante *E. coli* Stämme, die die klonierte Levansucrase aus *N. chiangmaiensis* besaßen, akkumulierten jedoch nach Induktion von Levansucrase-Expression Einschlusskörper in ihren Zellen. Diese Unterschiede in rekombinanter Levansucrase-Expression könnten auf unterschiedliche Codon Usages zwischen Gruppe 1 und Gruppe 2 Levansucrasen als auch auf die potenzielle Existenz einer intramolekularen Disulfidbrücke in der Levansucrase aus *N. chiangmaiensis* zurückzuführen sein. Diese Disulfidbrücke wurde mittels Proteinmodellierungen errechnet und ist in einer strukturellen Region lokalisiert, welche der Ca^{2+}-Kofaktorbindungsstelle der Levansucrase aus *Bacillus subtilis* äquivalent ist. Die Analyse selektierter, sekretierter Proteine aus *N. chiangmaiensis* und *K. baliensis* mittels Peptidsequenzierung lieferte keine Levansucrase-spezifischen Sequenzen für beide Stämme, wobei in *N. chiangmaiensis* ein extrazelluläres Protein mit Levansucrase-Aktivität identifiziert werden konnte und fermentierte Kulturüberstände von *K. baliensis* Levansucrase-Aktivität aufwiesen.

Daher wurde ein System entwickelt, welches die Gewinnung und Identifizierung einer nativen Gruppe 1 Levansucrase aus *K. baliensis* in (gepufferten) Lösungen ermöglichte. Folglich weist das Genom von *K. baliensis* mindestens zwei Levansucrase-codierende Sequenzen auf, von denen eine nicht sekretiert wird oder aktiv zu sein scheint (unvollständige Gruppe 2 Levansucrase). Dieses System erwies sich als hocheffizient für Zellen von *Gluconobacter species* TMW 2.1191, die hohe Mengen purer, dimerer Levansucrasen, welche wahrscheinlich über intermolekulare Disulfidbrücken stabilisiert sind, in Natriumacetat-Puffer sekretierten. Diese enzymhaltigen Lösungen konnten dazu genutzt werden, einheitliche Levane von einzigartig hohem Molekulargewicht in einem zeit- und kostensparenden Prozess zu produzieren. Es wurden zudem neue Erkenntnisse über konstitutive und induzierbare Fruktosyltransferase-Sekretion von diesem System erhalten, welche beispielsweise zur Optimierung von *in situ* Lebensmittelfermentationen genutzt werden können. Darüber hinaus wiesen Zellen von *Gluconobacter species* TMW 2.1191 in diesem System koordiniertes Verhalten bei mikroskopischer Betrachtung auf. Sie nutzten die physikochemischen Eigenschaften von Levan, um sich gemeinsam entlang von Oberflächen zu bewegen, sich zu vereinen und sich abzusetzen. Auf diese Weise konnte die biologische Funktion von Levan als extrazelluläres Biofilm-Material aufgezeigt werden.

Die vorliegende Arbeit schafft aufgrund der erzielten Ergebnisse die Basis zur (lebensmittel)biotechnologischen Nutzung von Fruktanen aus ESB. Es wurden strukturelle Eigenschaften linearer Levane identifiziert und mit ihrer Hydrokolloidfunktion korreliert. Einzigartige, sphärische Levanmoleküle können unter Verwendung einer verbesserten Methode zur EPS Produktion

einfacher gewonnen werden. Es wurde die Diversität der Levansynthese unter verschiedenen Gattungen von ESB aufgezeigt. Darüber hinaus wurde die biologische Funktion von Levan demonstriert und das generelle Wissen über die mikrobielle (enzymatische) Fruktan-Biosynthese deutlich erweitert.

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8. APPENDIX

atg gcc att aag tct tat aaa gcg cgt aaa aat ctt ATA att ttc gca gca att gct act gga att acg agc tct CCC ttc tgc ctt gct caa aca aca tca cct gcg ctt CGA CGA gaa gca cag cgt gtt tca CCC gct CCC ggg gtg atg ccg AGA ggc gct cca tta ttt tta gga cgt tca ctt gca acg gtt tcc ggc ttt gcg agc CCC agt att cat acg caa caa gcc tat aat CCC caa tca aat ttt act gcc cgg tgg acg cgt gcg gat gcg atc cag atc aag gcc cat ttc gat CCC aat gtg gcg gcg cgg cag aat tcc CTA CCC ttc caa ttg acg atg ccg gct ATA ccg gca aac ttt cct tcc atc agc cca gat gta tgg att tgg gat acc tgg aca CTA att gac aaa cag gcg aac caa ttc agt tat aat ggg tgg gaa gtg att ttt tct ctc act gca gat aaa aac gca ggg tac aca ttt gac gac cgc cat att cac gcc cgt ATA ggg tat ttc tat cgc cgt gcc gga ATA ccg gca tat cag cgc cct gca aat ggc ggg tgg ATA tat ggc gga ttt tta ttc CCC aac gga gct agc gca aaa gtt tat gca ggc acg acg tat acg aac cag gcc gaa tgg tcc gga tcg acc cgg ctt atc gat gta acc gga aat aaa att tcc gtt ttt tat act aac CTA gca ttc aat CGA agt gcc agc ggc gga aat ATA aca CCC CCC gta gcc acc atc acc caa tct ctc ggc act ATA cat tcg gat ttc cag cat gtc tgg ttt acc ggc ttc aac aat cat acg cca ctg ctc gta cct gat gga act tat tat cag acg ggc caa caa aac gag ttt tac agt ttt cgt gat cca ttc act ttt gaa gac ccg gac cat cct ggc gta aat tac atg gtc ttt gaa ggt aat acg gca ggt aat cgc ggt gtc ccg tcc tgt gat gct gcg gat ctt gga tat cgc ccg aat gac ccg cat gcc gag acg ctt cag caa gtc att gac ggc ggg gct tat tat cag aaa gcg aat atc ggc ctt gcc att gca cag aac agt gct ctt tca aaa tgg aag ttt ctt cct CCC ttg att tcc gcg aat tgc gtc aac gat caa acc gaa cgg cct cag gtc tat ATA aag aac gga aaa tac tat att ttc aca atc agt cac cgc acc aca ttt gcg gca ggc atg gat ggg ccg gat ggc gtc tat ggt ttt gtc ggc aat ggc att cgt agt gat ttc cag cca atg aat tac ggt agt ggg ctt gta ttg ggt aat cca acg gat ctt aac acg ccg gca gga aca gac ttt gag gct aat ccg gat caa aat ccg aac gca ttt cag tcc tac tct cac tat gtc atg ccg aac ggc CTA gtt gaa tcg ttt atc gat acg att gat ggc gta cgg ggc gga aca ctt gcg ccg acg gtg aaa ctc AGA att acg cgc gcg tca tcg gcg atc gat ctc gct tat ggc acc aac gga ttg ggt gcc tat gga aac att cca gca aat cgc gca gac atc aat att gcc gct ctg atc gct gat ctc ttg gga caa cgc tcg acg gcc tcg tct CTA ttg agt cag gta aat ggg ctg agt cag ggg agt atg tcc cca tca gtg gcg caa atc agt aat ttt ttg caa taa

Appendix A1: Complete nucleotide sequence (ORF) of *N. chiangmaiensis* levansucrase. Colored triplets are rarely used by *E. coli* (http://nihserver.mbi.ucla.edu/RACC/).

MAIKSYKARKNLIIFAAIATGITSSPFCLAQTTSPALRREAQRVSPAPGVMPRGAPLFLG
RSLATVSGFASPSIHTQQAYNPQSNFTARWTRADAIQIKAHFDPNVAARQNSLPFQLTMP
AIPANFPSISPDVWIWDTWTLIDKQANQFSYNGWEVIFSLTADKNAGYTFDDRHIHARIG
YFYRRAGIPAYQRPANGGWIYGGFLFPNGASAKVYAGTTYTNQAEWSGSTRLIDVTGNKI
SVFYTNLAFNRSASGGNITPPVATITQSLGTIHSDFQHVWFTGFNNHTPLLVPDGTYYQT
GQQNEFYSFRDPFTFEDPDHPGVNYMVFEGNTAGNRGVPSCDAADLGYRPNDPHAETLQQ
VIDGGAYYQKANIGLAIAQNSALSKWKFLPPLISANCVNDQTERPQVYIKNGKYYIFTIS
HRTTFAAGMDGPDGVYGFVGNGIRSDFQPMNYGSGLVLGNPTDLNTPAGTDFEANPDQNP
NAFQSYSHYVMPNGLVESFIDTIDGVRGGTLAPTVKLRITRASSAIDLAYGTNGLGAYGN
IPANRADINIAALIADLLGQRSTASSLLSQVNGLSQGSMSPSVAQISNFLQ

Appendix A2: Protein sequence of *N. chiangmaiensis* putative levansucrase. Green: predicted signal peptide (Petersen et al., 2011).

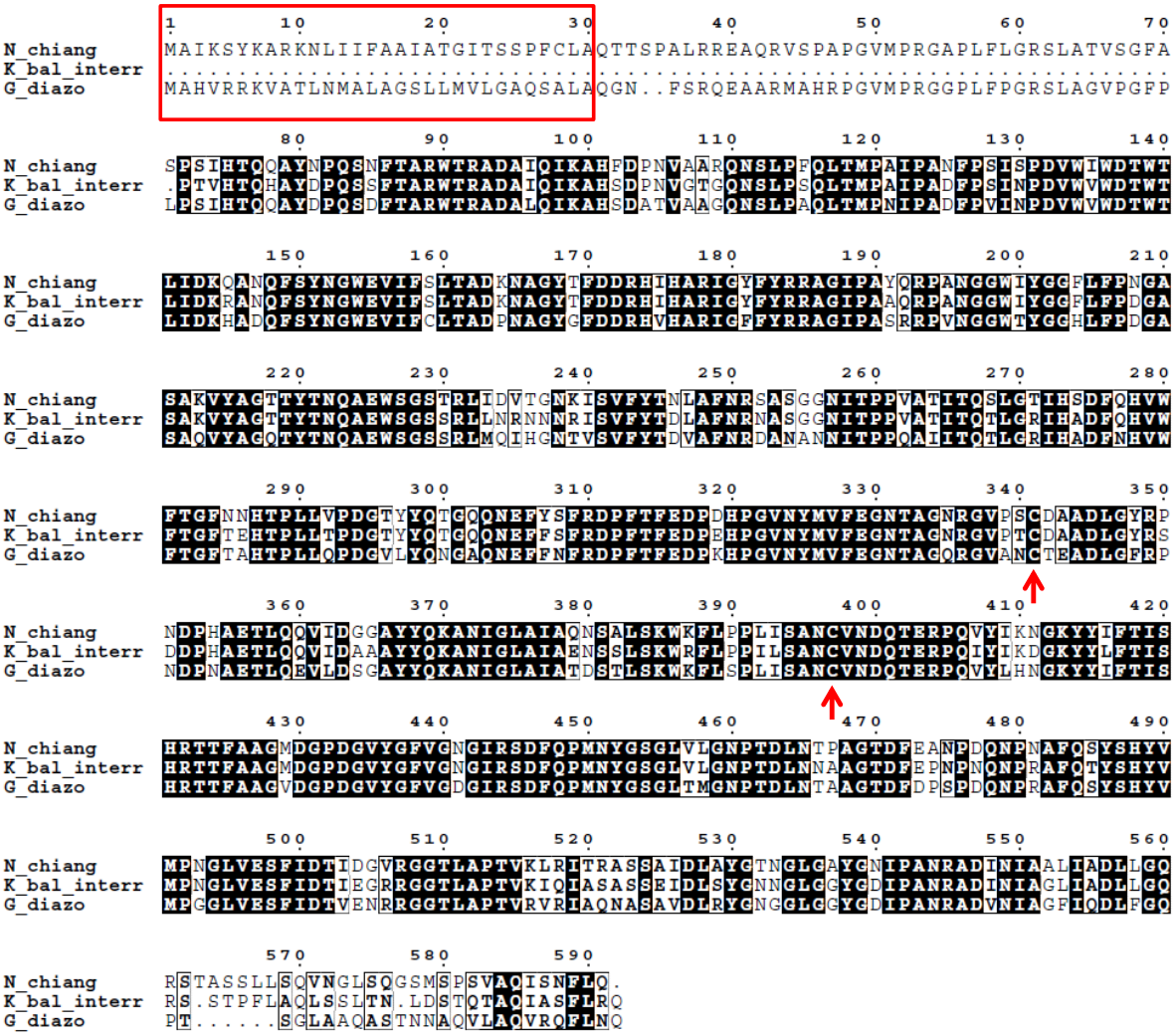
ATGTCCAAGAAGAGATGTGGAATCTTAGTAATGTCGACAcTTCTCGCAggaAGTTCGaCAaTTGCTTTT
TGCTTTCCCaATTCATCATTTGCTCAGCCtgtATCATCCTTtgTTCAACGTGAGGCACGCCGGgtGCCGCg
tgGACCTGGTTTAATGCCTTCAGGCAGTAATATTGGCCCGCTTGCAAgTGTTCCTGGTTTTGCTGGCC
CTACCGGCTTtgTTGCGTAAATGCTCGGAGTGGATTCACGGAATGAATCCAGACTGGTAGCTGGGAG
TG**ATGAGCAAGCCGAAAGCCACGCGATACCGTACGACGAACTGGTCCTCCTACAACGCCGCG**
CTGAAGAAGCGCGGCTCTCTGACGGTATGGTTTGACCCATCCATGAATTGGGAGGGCCTTCC
GACGGGACGTCGAGGACGGCGGCAGAATTACAGCGACGCCGCGATCCAGAGTTGCCTGACG
CTGAAAGTCCTGTTTGGCTTTGCGCTGCGACAGACGACGGGCTTTGTCGAAAGTCTCCTTCG
TCTGGCTGGGCTGTCGTGGTCCGTGCCGGATTTCAGCACGCTGAGCCGTCGGCAGAAATCCC
TGACGGTCGATATTCCGTATCGTAGTTCGGGCGGTCCGCTCCATCTCCTGATCGACAGCACC
GGCATCAAGGTCGAGGGAGAAGGCGAATGGCACACCCGCAAACACGGGGCGTCAAAGCACA
GGATCTGGCGCAAACTTCATATCGCGATCGATGAAGGATCACTGGAAATCAGGGCTGTTGAG
ATCACGAAAAACGATGTCGGTGATGCCCCAGTCCTACCGGCGCTTCTGGAACAGATCCCCGA
TGAagaGGACATCGCCTGCGTCACTGCTGATGGCGCGTATGATACAAGaAAATGCcCcGAAACC
ATCGCCAACCGTGGCGCACaGGCTGTCATACcgCCTCgGAAAAACgcaaaACCATGGTGCCccACA
TCACCAGGAGCCATCGCCCGCAATGAAGCCCTGCGGACGTGCGGGCATctCGGGCGGGCTAT
CTGGAGACGGTGGAGTGGTTACCATCGGCGAAGCCGCGTCGAGACAAAGATGCACTGCATC
AAGCTGCTGGGCCAGCGTCTGACAGCACGCGATTTCGACCGCCAGATCACCGAAGTTCACAT
CCGTGTGGCTATCCTCAATCGCTTCACGGCGCTCGGAATCCCTCTCACACACGCGGTCGCGT
A

ATGGAAAACTCTATAGCGGCCtcgTCACGCCAATAGACCGAATTGTGCAACAGAGCCGGCCCTACCG
TTCATACGCAACATGCTTATGACCCACAATCGTCCTTTACAGCACGTTGGACGCGTGCAGACGCCA
TACAAATCAAAGCCCATTCCGATCCTAACGTCGGAACAGGACAAAATTCGCTCCCCAGCCAACTCA
CCATGCCTGCAATTCCGGCGGATTTTCCATCGATCAACCCAGACGTTTGGGTTTGGGACACGTGGA
CGCTGATCGACAAGCGTGCCAATCAATTTAGCTATAATGGATGGGAGGTTATTTTCAGCCTCACAG
CCGACAAGAACGCGGGGTATACGTTCGATGACCGCCACATTCATGCGCGGATAGGATATTTCTATC
GCCGAGCCGGAATTCCCGCCGCTCAACGACCAGCTAATGGTGGATGGATTTATGGCGGCTTTTTAT
TCCCGGATGGGGCCAGTGCAAAGGTTTACGCGGGTACAACATACACGAATCAGGCCGAATGGTCA
GGTTCCAGCCGCTTGCTCAATAGGAACAACAATAGAATTTCCGTTTTCTATACAGACTTAGCATTTA
ATCGCAATGCAAGCGGCGGCAATATTACGCCTCCTGTCGCAACCATAACGCAAACCTTGGGCAGAA
TTCATGCTGACTTCCAGCATGTATGGTTTACAGGATTTACGGAACACACACCGCTTTTAACGCCGGA
TGGGACCTATTATCAGACTGGACAACAAAACGAATTTTTCAGCTTCCGCGATCCATTTACATTTGAG
GATCCTGAACATCCCGGTGTAAATTATATGGTCTTCGAAGGTAACACCGCCGGAAATCGAGGCGTT
CCAACATGCGATGCTGCAGATCTCGGATATCGTTCCGATGATCCCCATGCAGAAACGCTTCAGCAG
GTCATAGACGCAGCCGCCTATTACCAAAAGGCCAACATCGGGTTAGCTATCGCAGAAAATTCTAGT
CTTTCGAAATGGAGATTTCTGCCACCAATTCTTTCCGCGAACTGTGTCAACGATCAGACTGAACGA
CCTCAGATATATATTAAGGATGGAAAATACTATCTTTTCACAATAAGCCATCGCACGACTTTTGCG
GCTGGCATgGATGGTCCGGACGGTgTTTACGGGTTTGTTGGCAATGGTATCCGCAGCGACTTCCAGC
CCATgAATTACGGCagtGGTTTgGTCTTGGGCAACCCTACCGATTTGAATAATGCGGCTgGTACGGATT
TCGAGCCCAACCCCAATCAAAATCCTCGGGCTTTCCAGACCTATTCGCATTATGTCATGCCTAATGG
CTTGGTAGAGTCGTTTATCGATACAATCGAAGGACGTCGTGGTGGGACACTTGCGCCTACTGTAAA
AATTCAAATTGCCAGCGCAAGTTCAGAAATTGACCTGAGCTATGGAAATAATGGTCTTGGCGGCTA
TGGCGATATTCCCGCGAACCGCGCAGATATCAATATTGCAGGCCTGATTGCGGACCTACTTGGACA
ACGTTCTTCTACTCCTTTCCTTGCTCAACTTAGTAGCCTAACCAATCTCGACAGTACGCAAACTGCA
CAGATCGCGAGTTTTCTACGTCAATAA

Appendix A3: Nucleotide sequence of *K. baliensis* interrupted levansucrase; blue: levansucrase nucleotide sequence; black (bold): ORF IS5 element; black (rest): flanking region IS5 element; red: duplicated sequence; blue arrow: start (ATG) of hypothetical ORF with stop codon TAG (green); red arrow: start (CCT) of expected levansucrase sequence (PTV…); yellow: codons rarely used by *E. coli* (http://nihserver.mbi.ucla.edu/RACC/).

MENSIAASSRQ*TELCNRAGPTVHTQHAYDPQSSFTARWTRADAIQIKAHSDPNVGT
GQNSLPSQLTMPAIPADFPSINPDVWVWDTWTLIDKRANQFSYNGWEVIFSLTADKN
AGYTFDDRHIHARIGYFYRRAGIPAAQRPANGGWIYGGFLFPDGASAKVYAGTTYTN
QAEWSGSSRLLNRNNNRISVFYTDLAFNRNASGGNITPPVATITQTLGRIHADFQHVW
FTGFTEHTPLLTPDGTYYQTGQQNEFFSFRDPFTFEDPEHPGVNYMVFEGNTAGNRG
VPTCDAADLGYRSDDPHAETLQQVIDAAAYYQKANIGLAIAENSSLSKWRFLPPILSA
NCVNDQTERPQIYIKDGKYYLFTISHRTTFAAGMDGPDGVYGFVGNGIRSDFQPMNY
GSGLVLGNPTDLNNAAGTDFEPNPNQNPRAFQTYSHYVMPNGLVESFIDTIEGRRGG
TLAPTVKIQIASASSEIDLSYGNNGLGGYGDIPANRADINIAGLIADLLGQRSSTPFLAQ
LSSLTNLDSTQTAQIASFLRQ

Appendix A4: Hypothetical levansucrase of *K. baliensis* including IS5 element derived amino acids (red). *: stop codon (TAG).



Appendix A5: Alignment of *N. chiangmaiensis*/*K. baliensis* partial (PTV…)/*Ga. diazotrophicus* levansucrases. Red box: predicted signal peptides (Petersen et al., 2011); red arrows indicate the conserved cysteine residues possibly being involved in intramolecular disulfide formation.

MNLGISAKNQDVRTSSHWTIADAMKVHADDPTTTMPVIDYNFPVIDQDVWQWDTGALRAIT
GETVKFNDWYVMWALVANRADTGDTVEGWHNRNAFAYIGFYYSRNGIDWTFGGRLLQKS
ADLRPDEWSGSLVMRAGTKNTVDMFYTSVNTDINQSVPSCSTGKIFSNDEKVWFEGFSKTVE
MFSADGVNYANAEEDQYFDFRDPHPFLNPADGKIYCLFEGNVPGMRGKFTLHDTEIGAVPPG
YTPAAGAQYGAAAIGIARLTDGAYEKGDFSRHNWTLLPALVTALGVNDQTERPHVVFKDNY
TYIFTISHHSTYTGDSTGPDGVYGFVSENGIFGPYEPLNASGLVLGNPSSAPYETYSHFVDPDG
YVQSFIDTLPAPGSTDPQNPAAYRIGGTLAPTVRLVLEGHRTFLTEIHAYGQIFANKEWNS

Appendix A6: Secreted levansucrase of *K. baliensis*; red: identified peptides of protein B1 (Fig. 16)

MNVVSSTQSAVQSGSRWTIADAMKIRSDDPTTTMPVIDYAFPVIDSDVWQWDTWLLRDIH
GKTVTFKGWYVMFALVADRAATGDTTEGWHSRNNYSYIGYYYSRTGNGADWKFGGRVIKE
GANSRSWEWSGCAVMRENSANTVDLFYTSVNDTPSESVPSYTTGRILADATGVWFEGFDV
CTDMFQADGVNYANIVEDAYWDFRDPHIFRNPDDNQIYALFEGNVPGMRGNFTIGSDEMG
PVPPATTVPAGAQYGAAAIGIARLTSDSTRGDFSKWEMLPALVTALGVNDQTERPHVVFQ
DGLTYLFTISHHSTFTGNSTGPDGVYGFVSRNGIFGPYAPLNGSGLVLGNPSAAPYETYS
HFVDPAGYVQSFIDTLPQPGSADPQDPATYRIGGTLAPTVKIVLEGERTYLTEVHAYGQV
YAQGVWPTSSAFDKRS

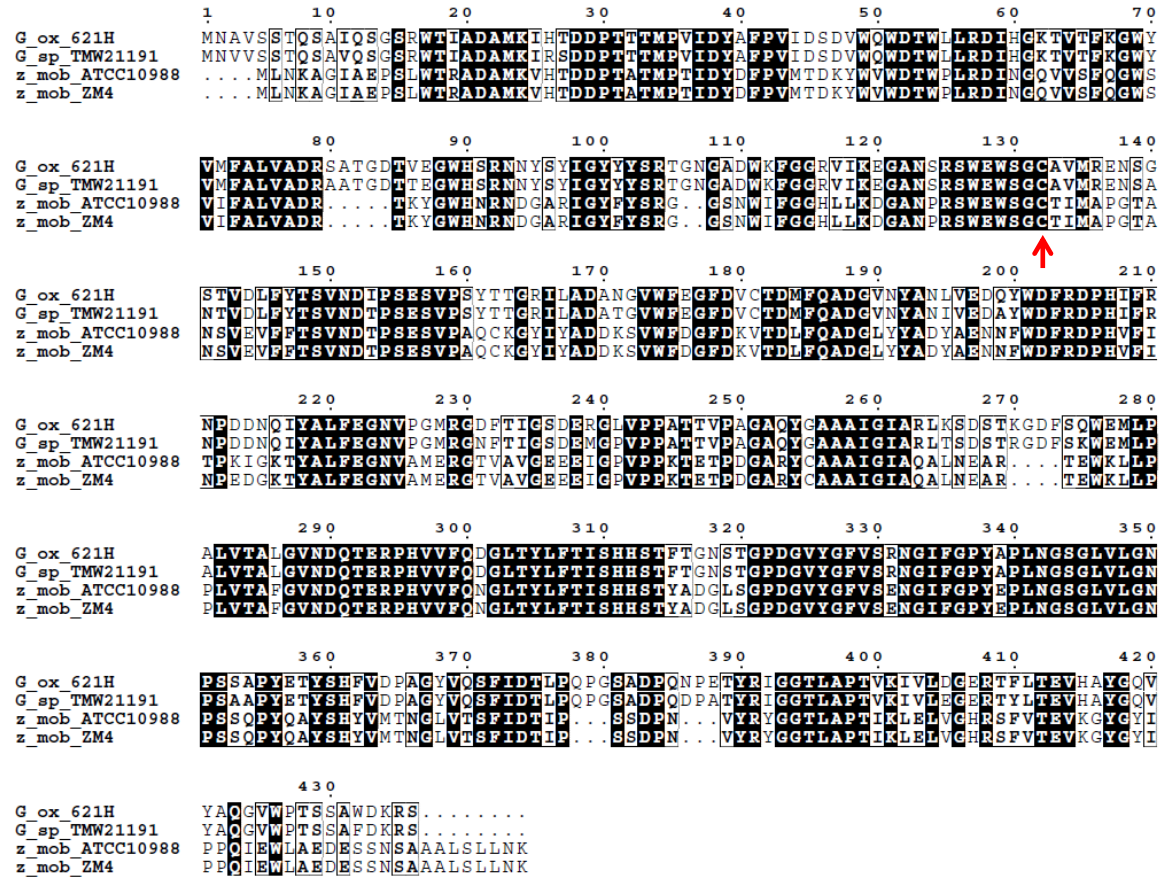
Appendix A7: Secreted monomeric (reduced) levansucrase of *Gluconobacter species* TMW 2.1191; red: identified peptides of protein A3 (Fig. 16).

Makakfertkphcnigtighvdhgktsltaaitkvlaktggatysaydqidkapeerargitistahveyetadrhyahvdcpghadyvknmit
gaaqmdgailvvsaadgpmpqtrehillarqvgvpalvvflnkvdqvddpellelvemevrellssyqfpgddipivkgsalvtledgdpsig
edrvlelmtqvdayipqperpvdrpflmpiedvfsisgrgtvvtgrvergvvnvgdeveivglkdtvkttvtgvemfrklldrgeagdnigalvr
gtkredvergqvlakpgsitphkkfkaeayiltkeeggrhtpfftnyrpqfyfrttdvtgvvtlpegtemvmpgdnvamdveliapiamdeglr
faireggrtvgagvvssita

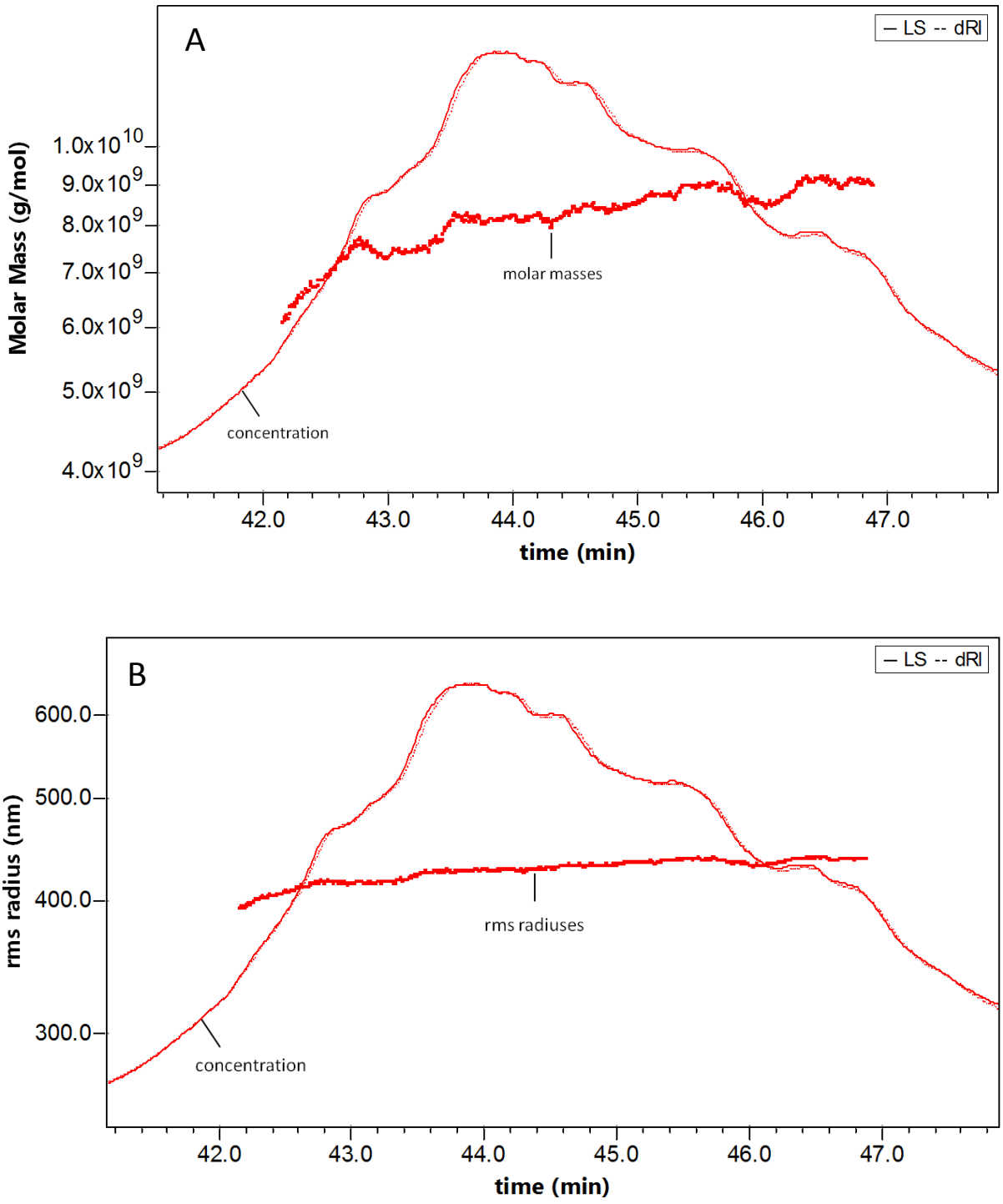
Appendix A8: Elongation factor Tu (*G. oxydans* 621H); red: identified peptides of protein B1 (Fig. 14).

mskvigidlgttnscvavregdetkviensegarttpsmvaftdngerlvgqaakrqavtnpantlyavkrligrryddptvqkdkemvpyaiv
rgdngdawveargekyapsqiaayvlgkmketaesylgetvsqavitvpayfndaqrqatrdagkiaglevlriineptaaalayglgkrdsgt
vavydlgggtfdvsileisdgvievkstngdtflggdfdnriigfladefkkdqgidlrgdklalqrlkeaaekakielsssketeinlpfitadasgp
khlvvklsrakleslvddliqrtlgpcraaikdasvsaneidevilvggmtrmpkvietvkeffgkdparnvnpdevvaigaavqgavlkgdvk
dvllldvtplslgietlggvftrlidrnttiptkksqtfstaednqnavtikvyqgeremaadnkllgnfdlqgiapaprgvpqievtfdidangivnv
sakdkatnkeqqikiqasgglsdadidrmvkdaeanasadkakrelvelrnsteslihqteksiteggdkvpaadkseaeaaiaearealggenl
dtlkaasekltqaamkvgqgvyqagqgaeggaapegekkdenvvdadfedleddskkh

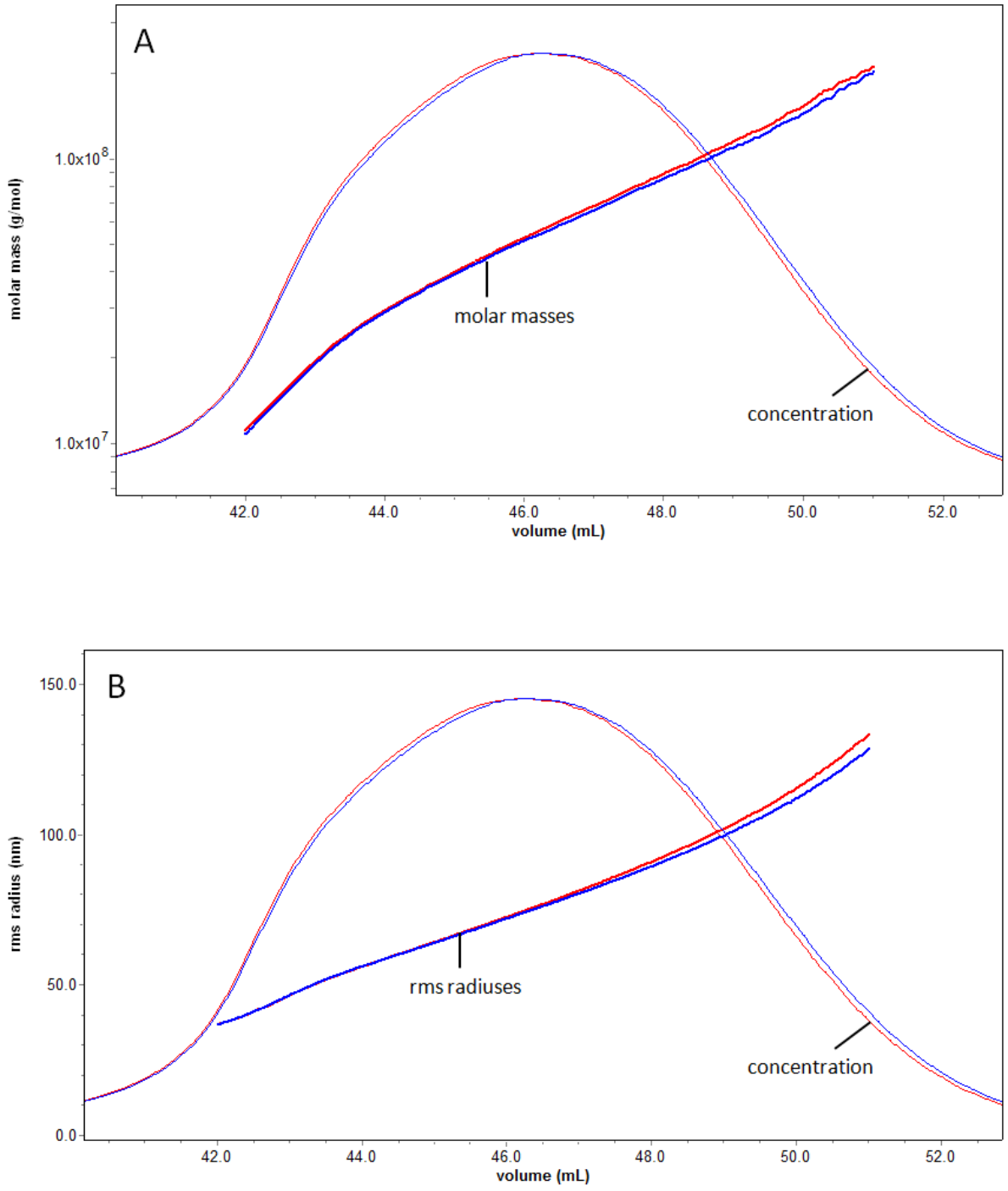
Appendix A9: Chaperone DnaK (*G. oxydans* 621H); red: identified peptides of protein B2 (Fig. 14).



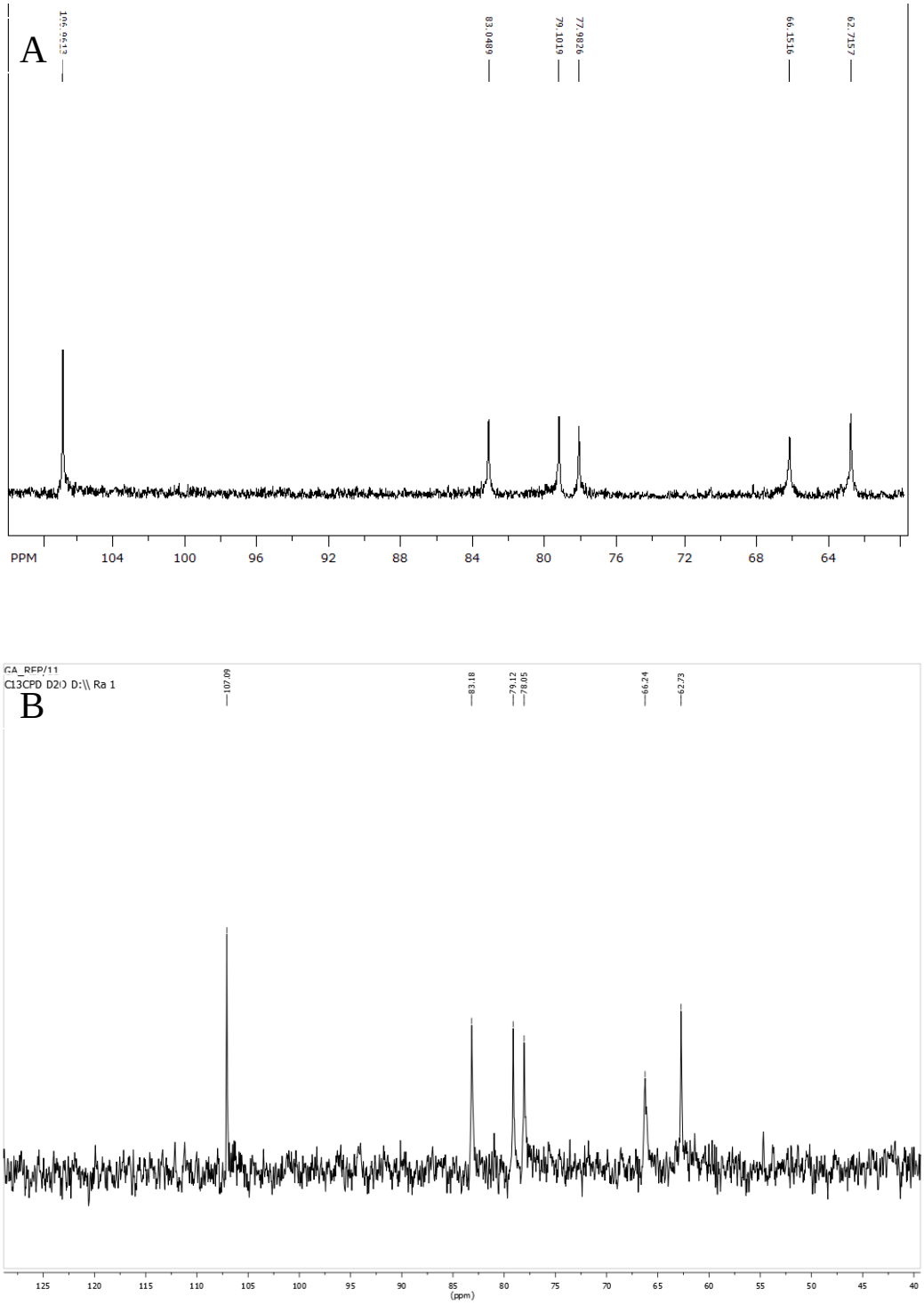
Appendix A10: Alignment of *G. oxydans* 621H/*Gluconobacter species* TMW 2.1191/*Z. mobilis* ATCC10988 (accession number: AAA27695)/*Z. mobilis* ZM4 (accession number: YP_162109) levansucrases; the red arrow indicates the conserved cysteine residue located in sucrose box 1.



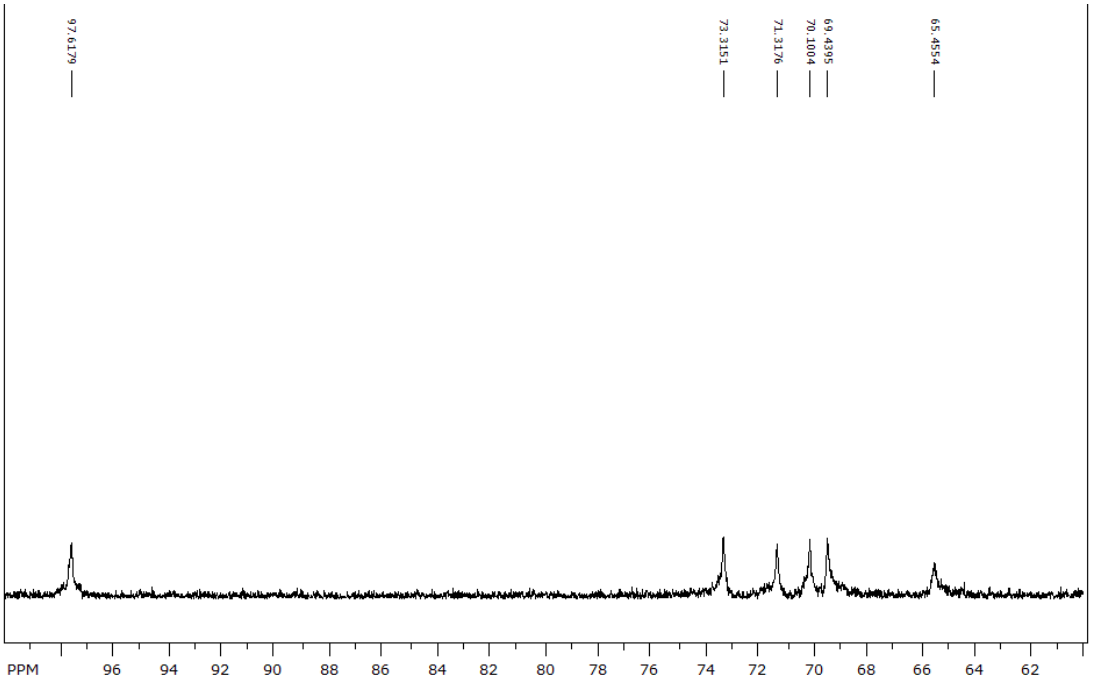
Appendix A11: Elugrams of enzymatically produced levans (*Gluconobacter species* TMW 2.1191) obtained from AF4-MALS-RI showing the relative concentrations of detected molar masses (A) and of radiuses of gyration (B).



Appendix A12: Elugrams of fermentatively produced levans (*Gluconobacter species* TMW 2.1191) obtained from AF4-MALS-RI showing the relative concentrations of detected molar masses (A) and of radiuses of gyration (B).



Appendix A13: *[13]C* NMR spectra of enzymatically (A) and fermentatively (B) produced fructan (*Gluconobacter sp.* TMW 2.1191). The chemical shifts (ppm) of the 6 main carbon signals are (A/B) 62.72/62.73 (C1), 106.96/107.09 (C2), 79.10/79.12 (C3), 77.98/78.05 (C4), 83.05/83.18 (C5) and 66.15/66.24 (C6) and are characteristic for levans (3.2).



Appendix A14: ^{13}C NMR of glucan produced by *Leuconostoc mesenteroides* TMW 2.1073. The ^{13}C NMR chemical shifts (ppm) of the 6 main carbon signals are 97.62 (C1), 71.32 (C2), 73.32 (C3), 69.44 (C4), 70.10 (C5) and 65.46 (C6) and are characteristic for dextrans (Shukla et al., 2011).

9. LIST OF PUBLICATIONS DERIVED FROM THIS WORK

Peer-reviewed journals

Jakob F., Steger S., Vogel R.F. (2012). Influence of novel fructans produced by selected acetic acid bacteria on the volume and texture of wheat breads. *European Food Research and Technology* 234: 493-499.

Jakob F., Meißner D., Vogel R.F. (2012) Comparison of novel GH 68 levansucrases of levan-overproducing Gluconobacter species. *Acetic Acid Bacteria* 1: e2.

Jakob F., Pfaff A., Novoa-Carballal R., Rübsam H., Becker T., Vogel R.F. (2013). Structural analysis of fructans produced by acetic acid bacteria reveals a relation to hydrocolloid function. *Carbohydrate Polymers* 92: 1234-1242.

Magazine contribution

Jakob F. & Vogel R.F. (2012). Hohes funktionelles Potenzial – Exopolysaccharide und deren Bedeutung in der Lebensmittelproduktion. *Lebensmitteltechnik* 7-8: 34-35.

Patent

Jakob F. & Vogel R.F. (2013). Method for producing exopolysaccharides, products and uses thereof. European Patent Application (25.07.2013); Applicant: Technische Universität München.

Oral presentations

Jakob F., Kaditzky S., Meißner D., Vogel R.F. (2010). Identification of a novel fructosyltransferase from the water kefir isolate Gluconobacter frateurii TMW 2.767. *3rd Joint Conference of the DGHM and VAAM*, Hannover.

Jakob F., Meißner D., Vogel R.F. (2012). Comparison of novel GH 68 levansucrases of levan-overproducing Gluconobacter species. *3rd International Conference on Acetic Acid Bacteria*, Cordoba (Spain).

Jakob F. & Vogel R.F. (2012). Exopolysaccharide un deren Bedeutung in der Lebensmittelproduktion. *Symposium der Gesellschaft Deutscher Lebensmitteltechnologen „Hydrokolloide VII“*, Bremerhaven.

Jakob F. & Vogel R.F. (2013). Hochmolekulare Fruktane – strukturverbessernde Mikrogele mit gesundheitsförderndem Potenzial. *64. Tagung für Getreidechemie*, Detmold.

Oral presentations (co-author, speakers are underlined)

Hermann M., **Jakob F.**, Vogel R.F. (2012). Exopolysaccharide von Essigsäurebakterien und deren Potenzial für die Verbesserung von Backwaren. *63. Tagung für Getreidechemie*, Detmold.

Jakob F., Rübsam H., Becker T., Pfaff A., Novoa-Carballal R., Müller A.H.E, Vogel R.F. (2012). Influence of different high-molecular weight levans produced by selected acetic acid bacteria on the volume and texture of breads. *3rd International Conference on Acetic Acid Bacteria*, Cordoba (Spain).

Vogel R.F., Steger S., Hermann M., Pfaff A., Novoa-Carballal R., Müller A.H.E., Rübsam H., Becker T., **Jakob F**. (2012). The potential of acetic acid bacteria for cereal fermentations. *V Symposium on Sourdough*, Helsinki.

Vogel R.F., Rühmkorf C., Hermann M., Rübsam H., Becker T., Pfaff A., Novoa-Carballal R., **Jakob F.** (2013). Reine Formsache – Mikrobielle Polysaccharide für Backwaren. *2. Frühjahrstagung des Weihenstephaner Instituts für Getreideforschung*, Freising.