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Comparative analysis of fructosyltransferases of lactobacilli

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Wer sich Steine zurechtlegen kann, über die er stolpert,
hat Erfolg in den Naturwisschenschaften.

Erwin Chargaff (*1905), östr.-amerikan.
Biochemiker und Schriftsteller)

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Abbrevations

°C	degree Celcius
µg	microgramme
µl	microlitre
aa	amino acid
bp	base pair(s)
BSA	bovine serum albumine
CAPS	*N*-cyclohexyl-3-aminopropanesulfonic acid
coPCR	cross over PCR
CPS	Capsular polysaccharides
DNA	desoxyribo nucleic acid
dNTP	desoxy nucleotid triphosphate
DTT	dithiothreitol
EDTA	ethylendiaminetetraacetic acid
EPS	exopolysaccharides
fig	figure
FOS	fructooligosaccharide
FPLC	free presure liquid chromatography
FTF	fructosyltransferases
g	gramme
GBD	Glucan binding domain
GOS	glucooligosaccharide
GRAS	Generally regarded as safe
GTF	glucosyltransferases
h	hours
HePS	Heteropolysaccharides
HoPS	Homopolysaccharides
iPCR	inverse PCR
IPTG	isopropyl-β-D-thiogalactopyranoside
kbp	kilo base pair(s), 1000 base pairs
l	litre
LAB	Lactic acid bacteria
M	molar, mol per litre
mA	milliampere
mg	milligramme
min	minutes
ml	millilitre
mM	millimolar, millimol per litre
mMRS	modified MRS medium
MW	molecular weight
OD	optical density
OS	oligosaccharides
PAGE	polyacrylamide gelelectrophoresis
PAS	periodic acid-Schiff staining
PCR	polymerase chain reaction
pMol	picmol per litre
rbs	ribosome binding site
rbs	ribosome binding site
rpm	rounds per minute
s	second

SAP	shrimp alkaine phosphatase
SDS	sodium dodecyl sulfate, sodium lauryl sulfate
sec	second
tab	table
TE	TRIS-EDTA buffer
TLC	thinlayer chromtaography
Tris	tris(hydroxymethyl)-aminomethane
U	units
UV	ultra violett
V	Volt
v	volume
w	weight

Content

1 Introduction

1.1 Lactic acid bacteria and food fermentation

Fresh food of various sources can serve as environment for microbial life. Microbial growth and metabolism can influence various parameters of food, namely sensorial properties, texture and shelf life. From case to case this is judged as spoilage by man or deliberately used as a process to give food new qualities. The tradition of fermenting raw materials of animal or plant source is an ancient technique of food preservation.

In many of theses fermentations, lactic acid bacteria (LAB) play a major role. They can be found in practically all relevant food matrices. Examples therefore are milk products as yoghurt (*Lactobacillus acidophilus ssp. bulgaricus*, *Streptococcus thermophilus*) and cheese (*Pediococcus sp.*), meat products as salami (*Lactobacillus sakei*, *Lactobacillus curvatus*, *Streptococcus carnosus*), vegetable fermentations as sauerkraut (*Leuconostoc sp.*) and cereal fermentations as sourdough (*Lactobacillus sanfranciscensis*, *Lactobacillus panis*). (Fig. 1)

The natural habitats of LAB are milk and decaying plant material but also the human and animal mucosae and intestinal tracts. LAB comprise the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Pediococcus* and *Bifidobacterium* forming the family of *Lactobacterioceae*. Concerning cell morphology, this group does not show much uniformity and short and long rods can be found as well as coccoid forms. Basically all LAB are gram positive, most are not motile and with the exception of *Sporolactobacillus inulinus* they do not form spores. The GC content with an average of 40% is relatively low. (Kandler and Weiss 1986)

Food relevant LAB are classified as GRAS (generally regarded as safe)-organisms which means, they are food grade and can be used as starter cultures in food fermentations since they have proven their innocuousness by a long safe use.

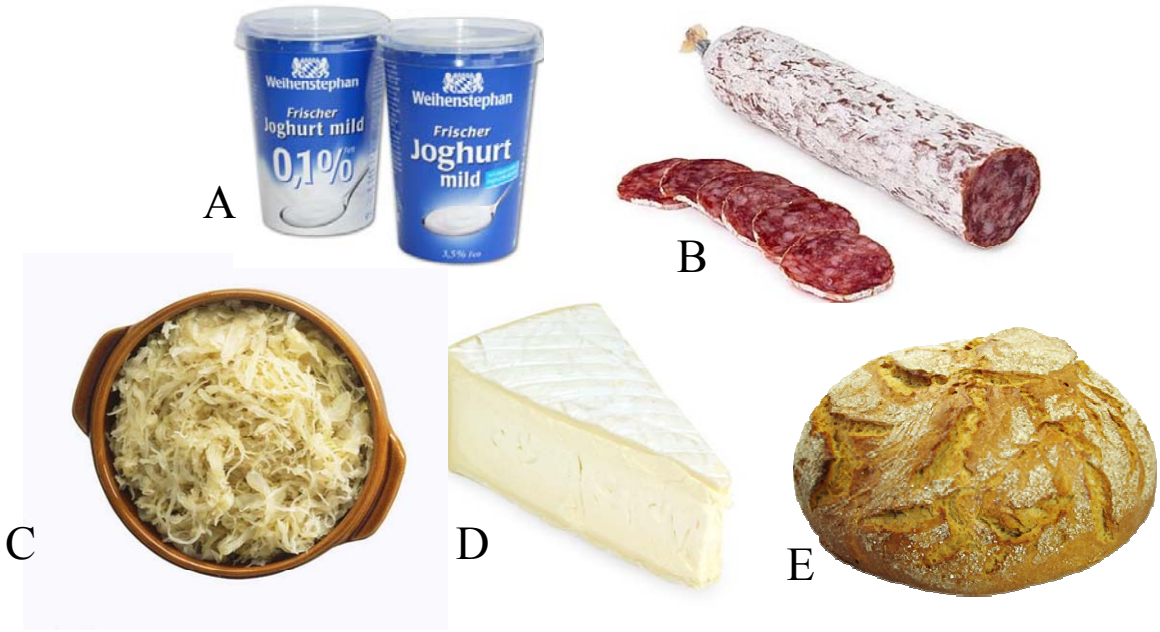


Fig.1: Lactic acid bacteria play major role in fermentation of different food products. Some examples are milk products as yoghurt (A) and cheese (D), meat products as fermented raw sausages (B) and fermentations on plant base like sauerkraut (C) and sourdough (E).

When bacterial strains are inserted in food fermentation as starter cultures, they have different basic functions: Ensure preservation and food safety, enhance sensoric properties of the product and achieve a desired textural structure. When introducing LAB strains, safety is achieved by acidification (lactic acid, acetic acid) and the resulting drop of pH and from case to case by production of bacteriocins (small proteinogenous antibiotic substances, mostly with limited target range). Beside from acidification several small metabolites are responsible for characteristic sensoric properties of the fermented product (e.g. amines formed by decarboxylation of amino acids (aa)). Structure and textural properties are mainly influenced by large compounds as exopolysaccharides. During the formation of the latter there can be formed oligosaccharides, additionally. These prebiotic carbohydrates may achieve an additional aim of starter cultures: beneficial effects on consumers´ health by added value functions.

1.2 Bacterial Exopolysaccharides

1.2.1 Basic facts about polysaccharides

Basically, Polysaccharides are chains of monosaccharides linked by osidic bondages. Polysaccharides can be formed by plant and algae, but also certain microorganisms are

capable of synthesizing a broad range of polysaccharides. In contrast to yeast and fungi, polysaccharide production seems to be a wide spread ability among bacteria. (Sutherland 1972; Sutherland 1982; Sutherland 1985)

Bacterial polysaccharides can be cell wall components as peptidoglycan, or they can be part of the lipopolysaccharide (O-antigen) in Gram negatives. However, a lot of polysaccharides exist extracellular and are called exopolysaccharides (EPS).

The expression EPS, describing microbial, extracellular polysaccharides was basically shaped by I. W. Sutherland (Sutherland 1972). Nevertheless, already in 1861 L. Pasteur could show that bacteria caused gelatinizing of sugar cane syrup and identified the responsible polysaccharide as dextran in 1874 (Monsan et al. 2001).

1.2.2 Possible benefits of microbial EPS for the producing organism

The function of EPS for the producing cell may be variable and is not completely solved up today. In contrast to intracellular polysaccharides, utilization of EPS as energy or carbohydrate source is unlikely since most organisms do not have the necessary enzymes for degrading their polysaccharides. An exception is *S. pyogenes* (MacLennon 1956). More important is their role in biofilm formation and surface adhesion. EPS produced by oral streptococci causing dental caries therefore are a good example. Also protective effects of EPS as protection against dehydration, phagocytosis or phage attacks seems to be plausible (Cerning 1990; De Vuyst and Degeest 1999).

1.2.3 Possible classifications of EPS

EPS can be divided in ultimately cell surface attached as capsular polysaccharides (CPS) and free EPS, only loosely bound to the producing cell or completely secreted to the ambient medium. (Cerning 1990; Boels et al. 2001; Kumar et al. 2007)

Further on, EPS producing stains can be distinguished in ropy and no ropy strains. That appearance does not correlate with the classification of capsule forming CPS and free EPS. Since not all unattached EPS produce ropiness, four groups can be differentiated:

Group I: capsule-forming ropy strains that produce capsules and unattached ropy EPS

Group II: capsule-forming non ropy strains that produce capsules and possibly unattached EPS

Group III: noncaspule-forming ropy strains

Group IV: strains producing no or undetectable EPS (Hassan et al. 2007)

Another possibility of EPS classification is based on the composition of the sugar chains in homo- (only one basic carbohydrate, e.g. glucose or fructose) and heteropolysaccharides (various monomers are combined to polymer) (Laws et al. 2001).

1.2.3.1 Heteropolysaccharides

Heteropolysaccharides (HePS) are linear molecules with repeating side chains of various lengths (two to eight sugar subunits) in periodic distances. They can have regular or irregular repeating units (Sutherland 1997; De Vuyst et al. 2001; Laws et al. 2001; Tieking et al. 2005c). The main chain is composed of different monosaccharides e.g. D-glucose, D-galactose, D-fructose and D-rhamnose or the sugar derivates N-acetylglucosamine and N-acetylgalactosamine. Single subunits can be linked by α- or β-glycosidic bondages (Boels et al. 2001; De Vuyst et al. 2001). The synthesis of HePS starts intracellular with construction of the side chain subunits which are proximately transported to cell surface and linked to the EPS chain or network. The process is similar to cell wall synthesis and an energy dependent process. Amounts up to 2 g/l (and for arabinan up to 5 g/l) were reported (De Vuyst and Degeest 1999; De Vuyst et al. 2001; Bergmaier et al. 2005; Tieking and Gänzle 2005; Korakli and Vogel 2006). A prominent example for an HePS is xanthane, which was the first EPS approved for the application in food, although the producing organism *Xanthomonas campestris* has in contrast to LAB no safe traditional use in food processing and is not classified as GRAS (Welman and Maddox 2003). The main chain is formed by β-(1→4) linked glucose subunits and amongst others contains derivatized mannose and glucoronic acid. (Sutherland 1997). Amongst LAB HePS are synthesized foremost by mesophilic (*L. lactis*, *L. casei*) and thermophilic (*L. acidophilus*, *S. thermophilus*) species. These EPS play an important role in the fermentation of fermented milk products, particularly drinks influencing texture, rheology and mouth feel (De Vuyst et al. 2001).

1.2.3.2 Homopolysaccharides

In contrast to complex HePS homopolysaccharides (HoPS) exhibit a simpler structure and biosynthesis. They contain only one type of monosaccharide mainly linked with a dominant bondage type. Since sucrose is the basic molecule for the synthesis of most bacterial HoPS, two major groups can be distinguished: Glucans, containing exclusively glucose subunits and fructans, constructed only with fructose monomers. Nevertheless, additional EPS from different sugar monomers with identical subunits and variable linkage types are possible. An example therefore is polygalactan. Based on the dominant linkage type, HoPS can be classified in more detail (De Vuyst and Degeest 1999). HoPS synthesis in contrast to complex

HePS synthesis is a one enzyme reaction. Glucans and fructans are synthesized extracellular by secreted proteins named glycosyltransferases or glycansucrases.

1.2.3.2.1 Glucans

Glucans can be divided in two subgroups: α-D-glucans and β-D-glucans. The latter are mainly linked by β-(1→3) osidic bondages with β-(1→2) branches. Such EPS are produced by *Streptococcus* spp. and *Pediococcus* spp. α-D-glucans are produced by a series of LAB (e.g. *Leuconostoc* spp., *Streptococcus* spp. and *Lactobacillus* spp.). Four different subtypes were described which can all be produced by Lactobacilli: The most common is dextran with α-(1→6) linked glucose subunits. In the linear molecule branching is possible at positions 2, 3 or 4. Positions 2 and 3 are used less frequently. The degree of branching is strain specific. Glucans with mainly α-(1→3) linked subunits are called mutan. A glucan with alternating α-(1→6) and α-(1→3) glucosidic linkages was called alternan. Finally there is reuteran with a majority of α-(1→4) linkages, also containing α-(1→6) glucosidic bonds and α-(1→4,6) branching points. Glycosyltransferases synthesizing glucans are named glucansucrases or glucosyltransferases (GTFs) and in more detail according to the produced products dextransucrases, mutansucrases, alternansucrases or reuteransucrases respectively. (De Vuyst and Degeest 1999; van Hijum et al. 2006)

1.2.3.2.2 Fructans

In contrast to the more variable glucans just two types of fructans has been described yet. Mainly β-(2→6) linked fructose monomers are called levan. They can casually contain β-(2→1) branches. In inulins, β-(2→1) is the dominating linkage type, β-(2→6) branching is possible. Data for molecular masses of fructans produced by bacteria are varying in a range from $2*10^4$ to $50*10^6$ Dalton. (De Vuyst and Degeest 1999; van Hijum et al. 2006)

Fructans are synthesised by glycosyltransferases called fructansucrases or fructosyltransferases (FTFs). FTFs producing levan are called levansucrases and inulin synthesising enzymes are named inulinsucrases respectively.

1.3 Glycansucrases – HoPS producing enzymes

As mentioned above, glucans and fructans are synthesized by glycosyltransferases. These enzymes, also named glycansucrases, are secreted by the EPS producing strains and can be connected to the cell surface by a C-terminal cell wall anchor.

They mainly use sucrose as a substrate for their reactions. The glycosidic bondage between the glucose and fructose unit of the sucrose molecule provides the energy for a transfer reaction of one of the sugar monomers to an acceptor while the other one is set free. GTFs

transfer the glucose unit while FTFs transfer the fructose unit respectively. Three types of that transfer reactions can be distinguished depending on the acceptor molecule:

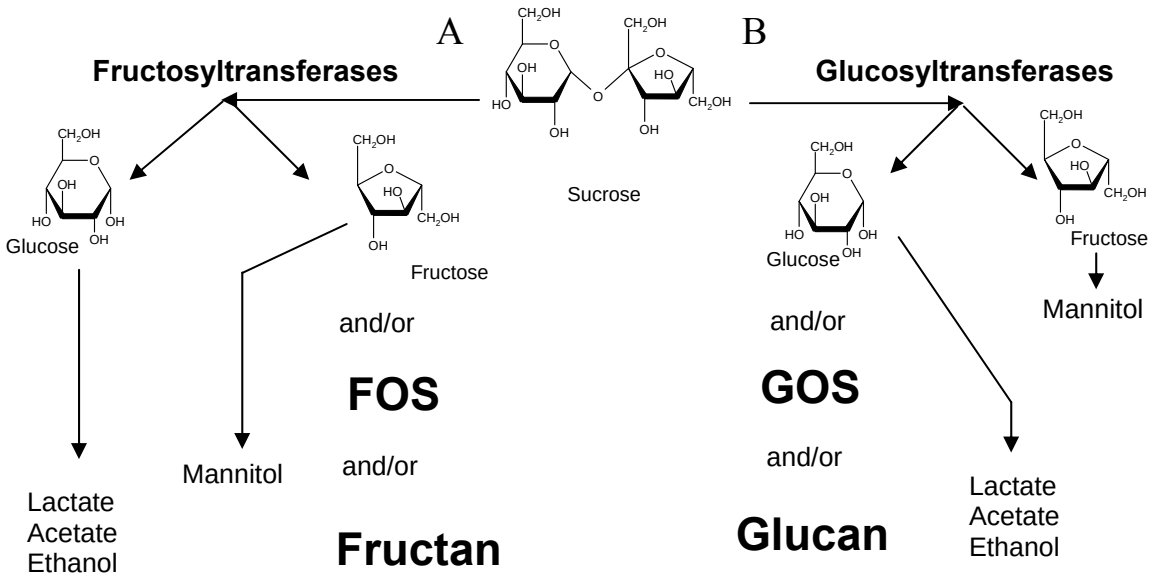


Fig. 2: Sucrose utilization by glycosyltransferases. Sucrose is used as a substrate by Glycosyltransferases. Fructansucrases set free glucose and transfer fructose to an acceptor molecule (A). Glucansucrases in contrast utilize glucose monomers for transfer to an acceptor and set free fructose (B).

(i) If a water molecule is used as acceptor, the reaction is a hydrolysis resulting in a free glucose and free fructose molecule. (ii) In a polymerization reaction, the acceptor is the growing EPS chain and the end product is a prolonged glucan or fructan molecule, respectively. (iii) The third option is to use an alternative acceptor molecule and transfer the according sugar unit in a so called acceptor reaction. Such acceptor can be a carbohydrate resulting in an oligosaccharide. This can result in a series of homooligosaccharides of different size as product of a glycosyltransferase reaction. For some glycosyltransferases the utilization of different sugar molecules as acceptor molecule is described and in consequence the formation of several heterooligosaccharides (HeOS). The possibility of the glycosylation of other molecule classes than carbohydrates, e.g. proteins has been discussed (van Hijum et al. 2006).

1.3.1 GTFs

In contrast to FTFs, GTFs are thoroughly investigated and reviewed elsewhere (e.g. van Hijum et al. 2006). So, only a short outline about GTFs is given here.

Principally two groups of glucans, α- and β-glucans are possible as mentioned above. Nevertheless, microbial GTFs exclusively synthesize α-glucan polymers. In general, GTFs

use sucrose as a glucose donor. While glucose is transferred to one or different acceptor molecules as described above, resulting in glucooligosaccharides or glucans, fructose of every cleaved sucrose molecule is set free and can be transported into and metabolized by the bacterial cell. In the acceptor reaction carbohydrates as maltose, isomaltose, *O*-α-methylglucoside or other saccharides but not sucrose can be used. The utilization of aromatic compounds and salicylic alcohol as acceptor molecules has also been observed. (Koepsell et al. 1953; Fu and Robyt 1991; Dols et al. 1997; Meulenbeld and Hartmans 2000; Arguello Morales et al. 2001; Kralj et al. 2004; Yoon et al. 2004; Kralj et al. 2005a)

In contrast to a wide spread distribution of bacterial FTFs throughout different bacterial groups, GTFs are only found in the group of LAB. A reason for that phenomenon is not known today. So, GTF genes are found in lactobacilli, leuconostoc and streptococci.

Described GTFs up to date are all classified in family GH70 of glycoside hydrolases. There is no three dimensional structure of a bacterial GTF. Nevertheless, secondary structure prediction analysis and corroborative circular dichroism experiments allow comparison with the structure of family GH13 α-amylases. These enzymes possess a $(\beta/\alpha)_8$ barrel structure. Since four conserved regions of amino acids (I to IV) described in the members of GH13 family are conserved in GH70 family members and six of seven amino acid residues completely conserved in family GH13 can be found in GH70 (His122 (Taka-amylase A numbering) is replaced by Gln in all known family GH70 GTFs), concrete conclusions can be made concerning the steric structure of GH70 family GTFs: The $(\beta/\alpha)_8$ of family GH13 can be found here as well but the motif is presumably circularly permuted and characterized by eight β-sheets alternating with eight α-helices. The β-sheets seem to be located in the core of the enzyme while α-sheets are presented on the protein surface. Due to the circular permutation, conserved region I is found C-terminal to regions II to IV. (Svensson 1994; MacGregor et al. 1996; MacGregor et al. 2001; van Hijum et al. 2006)

All GTFs are large enzymes with an average molecular mass of 160 kDa. They share a common basic structure of four domains. Since glycosyltransferases are secreted enzymes, there is a signal peptide at the N-terminus, responsible for the extracellular location of the enzyme. Its sequence is highly conserved, 32 to 34 aa long and typical for gram-positive bacteria. It is followed by a not conserved variable domain, which varies in sequence and length (200 to 700 aa). The function of that part of the enzymes is not known yet. Deletion of variable region in *S. downei* MFe28 GTF revealed no significant role whereas further N-terminal deletions drastically reduced enzyme activity (Monchois et al. 1999). The largest part is about 1000 aa long and has been identified as catalytic region including the sucrose binding

domain and the active centre of the GTF. Due to its functionality it is highly conserved. On the basis of the better known GH13 family enzymes, three potential amino acids with catalytic function can be identified. Mutational analysis approved their necessity for enzymatic activity. Catalytic nucleophile (essential role in the formation of covalent glucose-enzyme-complex) Asp1024, acid/base catalyst Glu1061 and transition state stabilizer Asp1133 (all in GTFA *L. reuteri* 121 numbering) could be identified (Knegtel et al. 1995; MacGregor et al. 1996; Devulapalle et al. 1997; Kralj et al. 2004; Kralj et al. 2005b; van Hijum et al. 2006). Site directed mutagenesis in GTFs could identify regions and amino acids responsible for glucosidic linkage type, glucan solubility and enzyme activity. By replacing of relevant amino acid residues respective glucan characteristics can be altered. (Shimamura et al. 1994; Monchois et al. 2000; Remaud-Simeon et al. 2000; Kralj et al. 2005b).

C-terminally located is a glucan binding domain (GBD) of approximately 400 aa. This domain is composed of a series of tandem repeats that can be classified in groups A, B, C and D. Number, class and distribution of these repeats is a specific characteristic for each GTF enzyme (Monchois et al. 1999; Korakli and Vogel 2006; van Hijum et al. 2006).

1.3.2 FTFs

As mentioned above, bacterial fructosyltransferases are less variable concerning the linkage types of the produced fructans. Just two variations are described: mainly β-(2→6) linked levans produced by so called levansucrases and inulin with a domination of β-(2→1) linkages synthesized by inulinsucrases. The latter are only found in LAB while levansucrase enzymes have a wide distribution in both gram-positive and gram-negative bacteria. The similarity of the levansucrases of gram-negative and gram-positive origin shows with approximately 20% only a low similarity. Generally, the FTFs of LAB are lager than the fructansucrases of non-LAB bacteria. By trend FTFs are smaller proteins than GTFs. Nevertheless, particular large enzymes as the 140 kDa levansucrase of *S. salivarius* ATCC 13419 are possible (Newbrun and Baker 1968; van Hijum et al. 2006).

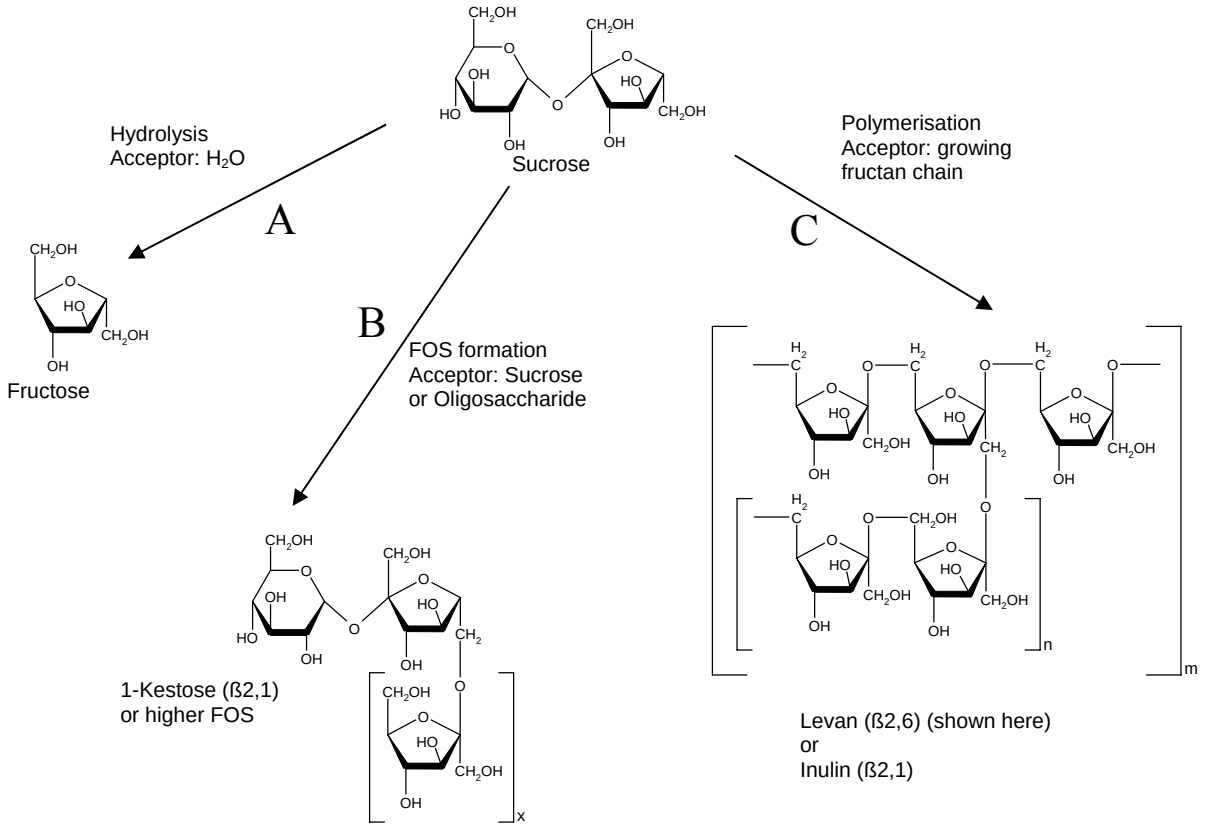


Fig. 3: Fructose splits sucrose and transfers the fructose monomer to an acceptor molecule. Dependant on the kind of acceptor, different reaction types can be distinguished. If fructose is transferred to a water molecule the reaction is called hydrolysis (A). The coupling of fructose to a sucrose, FOS or alternative molecule is named transfer reaction (B). If a growing fructan chain is elongated using the fructose monomer, the reaction is defined as polymerisation (C).

Most studies up to date concentrate on the more widely spread levansucrases. Among these enzymes from *Bacillus* spp. and *Zymomonas* spp. were in focus. Less information is available on FTFs of *Gluconobacter* spp. and LAB. Fructans and in some cases corresponding enzymes are reported for *Lactobacillus* spp. (*L. reuteri* (levan and inulin) (van Hijum et al. 2001; van Hijum et al. 2002; van Hijum et al. 2004), *L. sanfranciscensis* (levan) (Korakli et al. 2001; Korakli et al. 2002; Tieking et al. 2005c)), *Streptococcus* spp. (*S. salivarius* (levan) (Ebisu et al. 1975; Song and Jacques 1999a; Song and Jacques 1999b), *S. mutans* (inulin) (Sato and Kuramitsu 1986; Shiroza and Kuramitsu 1988; Heyer et al. 1998)) and *Leuconostoc* spp. (*L. mesenteroides* (levan) (Kang et al. 2005; Morales-Arrieta et al. 2006), *L. citreum* (inulin) (Olivares-Illana et al. 2002; Olivares-Illana et al. 2003)). Fructan production has been described for *L. frumenti* (5 strains), *L. pontis* (2 strains), *L. panis* and *Weisella confusa* (Tieking et al. 2003b) as well as for some streptococci (*S. sobrinus* (levantype) (Corrigan and Robyt 1979), *S. criceti* and *S. ratti* (inulintype) (Ebisu et al. 1975)).

Like GTFs bacterial FTFs are extracellular enzymes. Their main substrate is sucrose but in some cases also raffinose can be used as fructose donor. Using the energy of the cleaved bondage between fructose and glucose in the donor sugar, the fructose unit is coupled to an acceptor molecule in a hydrolysis (acceptor: H_2O), polymerization (acceptor: growing fructan chain) or an acceptor reaction (acceptor: sucrose, fructooligosaccharide (FOS), alternative carbohydrate (e.g. raffinose) or other molecule). Initially the polymerization and FOS production starts by a priming reaction. For this purpose, the fructose unit, achieved by cleaving a sucrose molecule, is bound to another nonreducing fructose with a free primary alcohol group at position C-2. In subsequent steps the primer can be elongated to a higher FOS or a fructan (Deonder 1966; Robyt 1998; van Hijum et al. 2006). Since reaction is initiated with a sucrose molecule, fructan chains contain a non reducing glucose unit at the end of the chain (French and Waterhouse 1993). Beside the mentioned polymers levan and inulin, various products by acceptor reactions are possible. For several bacterial FTFs the fructose transfer to different acceptors is described. Among them sucrose (in contrast to GTFs) and raffinose as well as further mono-, di-, tri- and tretrasaccharides, short chain acylalcohols and sorbitol. If sucrose is the acceptor, *Lactobacillus* FTFs form β-(2→1) linked 1-kestose (GF_2) and if possible, based on that further inulin type FOS (1,1-nystose (GF_3), 1,1,1-kestopentaose (GF_4) etc.) are synthesized (van Hijum et al. 2006).

The LAB FTFs are classified as protein family GH68 proteins. No three dimensional structure of LAB FTF has been solved yet. Nevertheless, high resolution crystal structures of the non-LAB *Bacillus subtilis* SacB levansucrase and of a sucrose bound inactive mutant of the same enzyme have been described. These structures reveal a rare five-fold β-propeller topology with a deep, negatively charged central pocket that has no consensus with the described family GH13 proteins whose structure could be adapted to family GH70 GTFs (Meng and Futterer 2003; van Hijum et al. 2006). In addition to that, a three dimensional structure of non-LAB *Gluconoacetobacter diacetrophicus* levansucrase has been presented showing the same five bladed β-propeller architecture (Martinez-Fleites et al. 2005). This accordance in combination with the highly conserved positions of the essential catalytic amino acid residues indicates a strong structural relatedness of those enzymes.

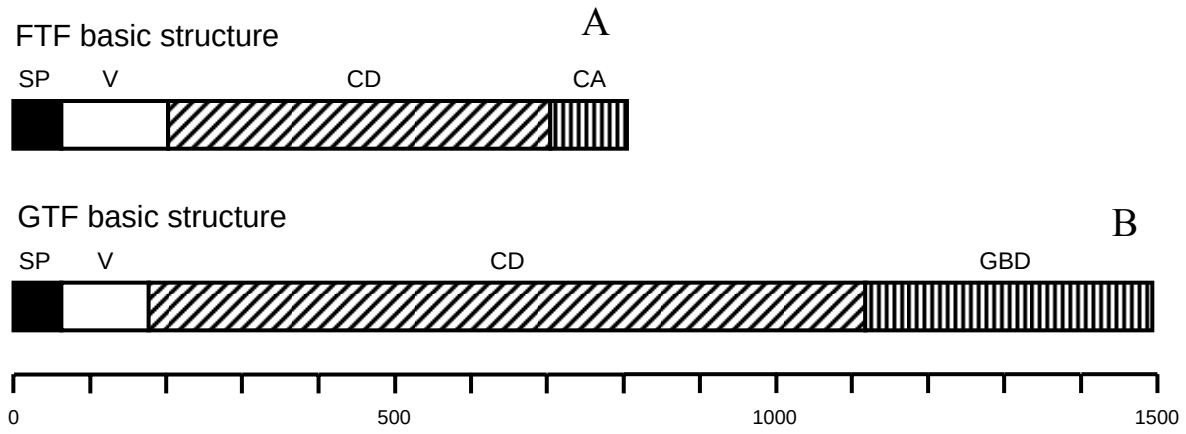


Fig. 4: Schematic basic structure of fructan-(A) and glucan-(B) sucrases. Scale represents length in amino acids. Both protein classes have and N-terminal signal peptide (SP) for extracellular location followed by a variable region (VD) that can also vary in length. For both enzyme groups the core of the protein is the enzymatic active catalytic domain (CD). Therein important regions are highly conserved. Fructosyltransferases C-terminally carry conserved sell wall anchor motive (CA). In glycosyltransferases C terminus forms a glucan binding domain (GBD).

Comparable to GTFs all LAB FTFs share a conserved sequence structure of four domains: N-terminal signal peptide, variable region, catalytic domain and C-terminal region with cell wall anchor motif. As in GTFs, FTFs have an N-terminal signal peptide for extracellular location of enzyme of 36 to 39 aa. That precursor peptide is cleaved of after secretion of the enzyme. Subsequently to the signal peptide, there is a region variable in length and sequence. This region may contain direct repeats of varying number, length and sequence. In *L. sanfranciscensis* levansucrase, a motif of 16 aa is repeated seven times, in *L. reuteri* levansucrase 14 aa are repeated 3 times and in others this region does practically not exist. The function of that region still is not known. *L. sanfranciscensis* levansucrase was cloned with and without N-terminal variable region was cloned and expressed, but no significant influence on kinetic properties could be observed (Tieking et al. 2005a). The core of the enzyme is the largest region, which is responsible for the catalytic activity. Most work concerning structure function relationships in this region is done in non-LAB bacteria. Nevertheless, based on homologies among the levansucrases the findings can be partially assigned on LAB FTFs. It is about 500 to 600 aa in length and contains several highly conserved regions, namely seven well-conserved domains containing aspartate and glutamate residues (Korakli and Vogel 2006). Among them two sections in the active site, designated as sucrose binding boxes (SBB) can be identified based on the mentioned three dimensional models and strong homologies on amino acid level. They are highly conserved in LAB fructansucrase enzymes. Also residues directly involved in sugar binding and constituting the

-1 and +1 subsites according to the nomenclature introduced by Davies et al. (Davies et al. 1997) could be designated. In addition to that a catalytic triade for a two step reaction (Sinnott 1987) has been proposed. A Ping Pong type of mechanism involving the formation and subsequent cleavage of a covalent enzyme-substrate intermediate has been reported for similar enzymes(Chambert et al. 1974; Hernandez et al. 1995; Song and Jacques 1999b). Highly conserved amino acid residues strongly conserved in FTFs, invertases (sucrose hydrolyzing enzymes) can be assigned to that triade. The thesis could be proven by mutational analysis in *L. reuteri* 121 levansucrase and inulinsucrase.

For *Lactobacillus* FTFs bivalent calcium cations have been shown to be important for enzymatic function, particularly at higher temperatures. The complexation of Ca^{2+} has been proposed to stabilize the steric structure. Based on the solved three dimensional structure of *B. subtilis* levansucrase which provides evidence for the bonding of a metal ion, five well conserved amino acid residues are suspected to be involved in the calcium chelate formation. Exchange of that residues resulted in a decreased optimum temperature and loss of affinity for Ca^{2+} ions (Ozimek et al. 2005).

By directed mutagenesis, several highly conserved amino acids of different LAB FTFs have been modified confirming the function of theses residues described above (van Hijum et al. 2006).

C-terminal domain may be responsible for size of produced fructans and /or the specificity of the fructansucrase. A hint for that functionality are experiments with modified *B. subtilis* levansucrase with an enlarged C-terminus producing a more branched and for this reason larger fructose polymer (Chambert et al. 1992). A second function is probably the connection of the fructansucrase protein to the cell surface since e.g. in all *Lactobacillus* FTFs and also in *S. salivarius* ATCC 25975 levansucrase (Rathsam and Jacques 1998) there is a conserved LPXTG cell wall anchoring motif. There are various potential functions for such proteins presented on surface of bacterial cell. In *S. aureus* proteins displayed on the cell surface are amongst others responsible for the infection process in humans (Ton-That et al. 1997) . In urogenital *Lactobacillus* spp. surface proteins are described to mediate adhesion to tissue cells and increase maintenance of beneficial urogenital flora (Howard et al. 2000; Sillanpaa et al. 2000). So this is comparable to the functionality of cell-associated HoPS produced by cell bound glycansucrases, playing a role in adherence to and colonization of tissue surfaces as teeth and intestinal mucosa (Rozen et al. 2001).

It is remarkable that in Lactobacillus FTFs nearly the complete C-terminus comprises of a series of PXX repeating units.

1.4 Application of Bacterial EPS in food

Some bacterial EPS are already used in food industry where they can enhance product textural quality, replace artificial additives as hydrocolloids or plant and algae polysaccharides used as gelling or thickening agents or insert added value functions in food products. This follows a growing consumers´ demand for less artificial additives and more original food.

1.4.1 LAB HePS in milk products

There is a well established application of LAB HePS in fermented milk products like cheese and yoghourt where they can be produced by the LAB in the starter culture. There are effects on the structure formation of fermented milk, e.g. by modifying the formation of casein aggregates. Other factors affected by EPS production during fermentation are the rheology, texture and syneresis of the products. By selection of appropriate starter strains and fermentation conditions, mentioned parameters can be adjusted to a desired level. In special applications the quality of problematic products can be significantly enhanced. An example is the application of EPS or EPS forming starter strains in reduced fat cheddar cheese. Beneficial effects in this case are due to an increase of moisture retention by a better water binding capacity and therewith an enhancement of textural and functional properties in reduced-fat cheese (Hassan et al. 2007). Since the targeted and commercial application of EPS in milk products is the field of furthest progress in EPS application in food, this is just one example of a broad field.

1.4.2 LAB HoPS in sourdough products

The use of HoPS is not so common yet. However, sourdough products are an interesting field for such applications. Here, homopolysaccharides produced by Lactobacillus strains and closely related species are dominating. First efforts have been made:

A good example therefore is the established application of *Leuconostoc mesenteroides* dextran (an α-(1→6) linked glucan) in an Italian sweet bread called panettone. This product is extensively consumed in Italy during short Christmas period. Therefore, production has to start months before consummation. However, panettone stays fresh for months due to high dextran content produced by *L. mesenteroides* starter strain also responsible for typical crumb structure with large lengthy holes. During traditional fermentation, sucrose content is successively increased in several refreshment steps. In the end, up to 25% dextran in dry matter can be achieved. The process is claimed by a patent hold by Puratos® Company. Based on that traditionally fermented dough, pumpable and even dried doughs for industrial applications can be produced without loss of dextran functionality (Decock and Cappelle 2005; Lacaze et al. 2007).

Molecular mechanisms of action of HoPS in sourdough and baking process appeared to be complex as shown by existing studies. In the case of dextran, a coherence of chain length and effect on dough rheological parameters as well as on bread structure could be shown. Since EPS are suspected to act as hydrocolloids, different bacterial homopolysaccharides has been compared to alternative hydrocolloids of variable sources revealing that an individual adjustment of EPS additive and desired effect in respective product is necessary to obtain an optimal result. Recently it was shown that the fructose polymer levan has similar influences on dough rheology and bread quality as glucose based dextran. Nevertheless levan turned out to be less effective in the performed experiments (Ross et al. 1992; Rosell et al. 2001; Guarda et al. 2004; Kaditzky 2008).

However, the addition of bacterial polysaccharides, in particular fructans and fructooligosaccharides may bring an additional value. These carbohydrate structures have been described to have beneficial influence on human intestinal flora. This effect is based on bifidogenic properties, meaning a selective stimulation of growth and propagation on bifidobacteria species. The latter has been proven to have positive effects on intestinal health and immunostimulating effects (Dal Bello et al. 2001; Korakli et al. 2002; Corsetti and Settanni 2007). For levan, even a tumour reducing activity is described (Yoo et al. 2004). This added value functions are of special interest since consumers demand for more healthy foods and there is a growing market of functional food products.

1.4.3 Problems in HoPS application in food

The use of bacterial HoPSs in different kinds of food can be favourably due to different reasons: They have the potential to provide an original alternative to already used food additives as hydrocolloids, used in sourdough applications. In addition to that, they can open new facets of food quality enhancement concerning aspects in production (e.g. influencing rheological properties) or in the end product (increased structural parameters, prolonged shelf life). Particularly in case of fructans and FOSs, the insertion of these carbohydrates offers the potential for added value functions of food. This is due to prebiotic effects on intestinal bifidobacteria and other beneficial effects on (mainly) intestinal health.

LAB, namely the group of Lactobacilli, plays an important role in the production of fermented foods as milk or sourdough products. They have a GRAS status and can be introduced in foods without legal limitations. Therefore, the selection of appropriate starter strains in food fermentations is a good possibility to introduce process or product improvements by in situ production of bacterial HoPSs as it is clearly demonstrated in panettone production, or the addition of added value functions. Nevertheless, metabolic activities of the starter culture (e.g.

acidification by acetate formation) may negatively influence the beneficial effects of fructans as has been demonstrated (Kaditzky and Vogel 2008). So, the application of purified EPS would be necessary when indicated. Although the addition of purified bacterial glucans and fructans as well as oligosaccharides would have to be labelled, an addition of a product or food related substance will have a better consumers´ acceptance than chemical or less related additives.

So there will be a demand for the effective production of defined and pure HoPS and FOS. Therefore it is important to have a good knowledge about the producing GTF and particularly FTF enzymes to be able to design and perform economic production processes with corresponding, if necessary optimized enzymes and/or production strains.

As shown a lot of basic work concerning bacterial fructosyltransferases has been done. Several postulations about potential functionality and applications of this enzyme group have been made based on only few experimental data. Nevertheless, a clear picture of the potential of LAB FTF enzymes for applications does not exist since only few LAB FTF enzymes are described yet and comparative analysis of these enzymes are lacking.

1.5 Aim of this study

For application in industrial food production, purified fructans and FOS could be useful. Therefore FTF enzymes with clearly defined product ranges and optimal yields are necessary. The construction of customized proteins based on natural occurring FTFs is thinkable and can be advantageous.

For a profound valuation if lactobacilli FTF do carry these potentials, a basic comparison of as many related enzymes as possible is needed.

Due to that, in this work FTF enzymes of the group of lactobacilli are explored. New enzymes are described and a detailed comparison of FTFs concerning sequence and functional data should reveal the potentials of the native enzymes and potential regions of interest for molecular modelling. First approaches in changing domains in between two *Lactobacillus* levansucrases are made.

2 Material and Methods

2.1 Materials

2.1.1 Devices

Major devices used in this work are listed in table 1 by alphabetical order.

device	model	Manufacturer
agarose gel chamber	Easy Cast electrophoresis system	Owl Separation Systems, Portsmouth, NH, USA
autoclaves	2540 ELV	Systec GmbH, Wettenberg, G
	Varioklav	H + P Labortechnik, Oberschleißheim, G
blotting oven	MINI 10	MWG Biotech AG, Ebersberg, G
breeding/incubation	Certomat BS-1	B. Braun Biotech International, Melsungen, G
	Hereaus B5042E	Heraeus Instruments, Hanau, G
	Memmert INB series	Memmert GmbH & Co. KG, Schwabach, G
centrifuges	Sigma 1 K 15	Sigma Labortechnik, Osterrode am Harz, G
	J-6	Beckman, Palo Alto, CA, USA
	J-2	Beckman, Palo Alto, CA, USA
	Hermle Z383 K	Hermle Labortechnik, Wehningen, G
	Hermle Z382 K	Hermle Labortechnik, Wehningen, G
	Sigma 112	Sigma Labortechnik, Osterrode am Harz, G
	Hermle Z233 MK	Hermle Labortechnik, Wehningen, G
Electroblotting	HEP-1 The Panther TM	Owl Separation Systems, Portsmouth, NH, USA
FPLC system	Biologic HR Controller	Bio-Rad Laboratories, Hercules, CA, USA
	Biologic HR Workstation	Bio-Rad Laboratories, Hercules, CA, USA
	Modell 2128 Fraction Collector	Bio-Rad Laboratories, Hercules, CA, USA
HPLC-column oven	Thermostat column compartement TCC-100	Dionex GmbH, Idstein, G
	K5	Gynkotek Gesellschaft für den Bau wiss. Geräte, Germering, G
HPLC-degasser	DG503	Gynkotek Gesellschaft für den Bau wiss. Geräte, Germering, G
HPLC-EC detector	ED40 Electro chemical detector	Dionex GmbH, Idstein, G
HPLC-pumps	M480	Gynkotek Gesellschaft für den Bau wiss. Geräte, Germering, G
	P680ISO	Dionex GmbH, Idstein, G
	G550 Gradient pump	Dionex GmbH, Idstein, G
HPLC-RI detectors	RI-71	Gynkotek Gesellschaft für den Bau wiss. Geräte, Germering, G
	RI-101	Showa Denko K.K., Kanagawa, Japan
HPLC-sampler	GINA 50	Gynkotek Gesellschaft für den Bau wiss. Geräte, Germering, G
	AS 50 Autosampler	Dionex GmbH, Idstein, G
HPLC-UV detectors	UV160S	Gynkotek Gesellschaft für den Bau wiss. Geräte, Germering, G
	Ultimate 3000 Variable wavelength detector	Dionex GmbH, Idstein, G
Incubation hood	Certomat H	B. Braun Biotech International, Melsungen, G
laminar flow sterile work bench	HERA safe	Heraeus Instruments, Hanau, G
PCR-cycler	Primus 96 plus	MWG Biotech AG, Ebersberg, G
	Mastercycler gradient	Eppendorf AG, Hamburg, G
pH determination	InLab 412, pH 0-14	Mettler-Toledo, Gießen, G

(electrode)		
pH determination (measuring)	Knick pH 761 Calimatic	Knick elektroische Geräte, Berlin, G
photometer	Novaspec II	Pharmacia Biotech
pipettes	Pipetman	Gilson-Abomed, Langenfeld, G
plate readers	TECAN SPECTRAFlour	TECAN Deutschland GmbH, Crailsheim, G
	TECAN SUNRISE	TECAN Deutschland GmbH, Crailsheim, G
Power supplies	MPP 2 x 3000 Power Supply	MWG Biotech AG, Ebersberg, G
	Electrophoresis Power Supply EPS 3000	Pharmacia Biotech
	2197 Power Supply LKB Bromma	LKB Bromma
	EPS 3501 XL	Pharmacia Biotech
	Power Supply PPS 200-1D	MWG Biotech AG, Ebersberg, G
pure water	Euro 25 and RS 90-4/UF pure water system	SG Wasseraufbereitung GmbH, Barsbüttel, G
SDS-PAGE	Mini Protean III-System	Bio-Rad Laboratories, Hercules, CA, USA
shaking	Certomat R	B. Braun Biotech International, Melsungen, G
	Vortex 2 Genie	Scientific Industries Inc., Bohemia, NY, USA
stirring	RCT-Basic	Mettler-Toledo, Gießen, G
thermo block	Techne DRI-Block DB3	Thermo-Dux Gesellschaft für Laborgerätebau mbH, Wertheim, G
ultra sonic water bath	Sonorex Super RK103H	Bandelin electronic, Berlin, G
ultra sonification	UP 200S	Dr. Hielscher GmbH, Teltow, G
	SONOPLUS/SH70G	Bandelin electronic, Berlin, G
UV table	Herolab UVT 28M	Herlab GmbH Laborgeräte, Wiesloch, G
water bath	Lauda BD	LAUDA Dr. D. Wobser GmbH & Co., Lauda-Königshofen, G

Tab. 1: List of devices used for this work by alphabetical order.

Specific consumable supplies of interest are mentioned in the methods part of this work.

2.1.2 Chemicals

Chemicals and enzymes used in this work are listed in tab. 2 by alphabetical order. Kits are listed in tab. 3.

Chemicals	purity	Manufacturer
1,1,1-kestopentaose	~95%	Megazyme International Ireand Ltd., Bray, I
1,1-kestotetraose/nystose	>95%	Megazyme International Ireand Ltd., Bray, I
1-kestose	>98%	ABCR GmbH & Co. KG, Karlsruhe, G
acetic acid	HPLC-grade	Mallinkrodt Baker B. V., Deventer, NL
acetonitrile	HPLC-grade	Mallinkrodt Baker B. V., Deventer, NL
acrylmide/bis 30%	high purity	Gerbu Biotechnik GmbH, Gaiberg, G
agar	European agar	Difco, BD Sciences, Heidelberg, G
Agarose	Seakem® LE Agarose	Cambrex Biosciences Rockland Inc., Rockland, USA
ampicillin sodium salt	93,30%	Gerbu Biotechnik GmbH, Gaiberg, G
Anti-Digoxigenin-AP	-	Rockland Immunochemicals Inc., Gilbertsbille, PA, USA

anti-His-antibody	-	Rockland Immunochemicals Inc., Gilbertsbille, PA, USA
APS	electrophoresis grade	SERVA, Heidelberg, G
arabinose	>98%	SIGMA-Aldrich, Steinheim, G
BCIP	-	Gerbu Biotechnik GmbH, Gaiberg, G
Bio-Rad assay solution	-	Bio-Rad Laboratories, München, G
blocking solution	-	Roche Diagnostics GmbH, Mannheim, G
bromphenol blue	for electrophoresis	SIGMA-Aldrich, Steinheim, G
BSA	fraction V for biochemical use	Merck, Darmstadt, G
butanol	p.a.	Merck, Darmstadt, G
$CaCl_2*2H_2O$	p.a.	Merck, Darmstadt, G
CAPS	-	SIGMA-Aldrich, Steinheim, G
chloroform	p.a.	Merck, Darmstadt, G
cobalamine	p.a.	SIGMA-Aldrich, Steinheim, G
cysteinhydrochloride*H_2O	for biochemical use	Merck, Darmstadt, G
dATP	-	Roche Diagnostics GmbH, Mannheim, G
dCTP	-	Roche Diagnostics GmbH, Mannheim, G
dGTP	-	Roche Diagnostics GmbH, Mannheim, G
DIG Easy Hyb	-	Roche Diagnostics GmbH, Mannheim, G
DIG labelled dUTP	-	Roche Diagnostics GmbH, Mannheim, G
DIG uTP	-	Roche Diagnostics GmbH, Mannheim, G
DTT	high purity, for molecular biology	Gerbu Biotechnik GmbH, Gaiberg, G
dTTP	-	Roche Diagnostics GmbH, Mannheim, G
EDTA	for molecular biology	SIGMA-Aldrich, Steinheim, G
ethanol	HPLC-grade	Mallinkrodt Baker B. V., Deventer, NL
ethanol vergällt	99% with 1% methylethylketone	Chemikalien und Laborbedarf Nierle, Freising, G
ethidium bromide	1% in H_2O for electrophoresis	Merck, Darmstadt, G
folic acid	p.a.	SIGMA-Aldrich, Steinheim, G
fructose	HPLC-grade	Merck, Darmstadt, G
galactose	HPLC-grade	Merck, Darmstadt, G
glucose	for biochemical use	Merck, Darmstadt, G
glycine	BioChemika Ultra 99.5%	SIGMA-Aldrich, Steinheim, G
glycine	p.a.	Merck, Darmstadt, G
HCl	reinst, pHEur	Merck, Darmstadt, G
imidazole	for biochemical use	SIGMA-Aldrich, Steinheim, G
inulin from chicory root	-	SIGMA-Aldrich, Steinheim, G
inulinase	-	Fluka Biochemika, Steinheim, G
IPTG	p.a.	Gerbu Biotechnik GmbH, Gaiberg, G
isopropanol	p.a.	Scharlau Chemie S. A., Sentmenat, Spain
K_2HPO_4	p.a.	Merck, Darmstadt, G
kalium acetate	p.a.	Merck, Darmstadt, G
KCl	p.a.	Merck, Darmstadt, G

KH_2PO_4	p.a.	Merck, Darmstadt, G
Kodak®GBX developer	-	SIGMA-Aldrich, Steinheim, G
Kodak®GBX fixer and replisher	-	SIGMA-Aldrich, Steinheim, G
lactose	pharmaceutical grade	Gerbu Biotechnik GmbH, Gaiberg, G
lysozyme	-	SERVA, Heidelberg, G
maleic acid	for synthesis	Merck, Darmstadt, G
maltose	HPLC-grade	Merck, Darmstadt, G
mannose	>98%	Appli Chem, Darmstadt, G
meat extract	for microbiology	Merck, Darmstadt, G
methanol	HPLC-grade	Mallinkrodt Baker B. V., Deventer, NL
$MgCl_2$	for synthesis	Merck, Darmstadt, G
$MgSO_4 * 7\ H_2O$	p.a.	Merck, Darmstadt, G
$MnCl_2$	p.a.	Merck, Darmstadt, G
$MnSO_4 * 4\ H_2O$	p.a.	Merck, Darmstadt, G
MOPS	for molecular biology	Gerbu Biotechnik GmbH, Gaiberg, G
NaCl	p.a.	Merck, Darmstadt, G
NaOH	p.a.	Merck, Darmstadt, G
NBT	-	Gerbu Biotechnik GmbH, Gaiberg, G
NH_4Cl	p.a.	Merck, Darmstadt, G
nicotinic acid	p.a.	SIGMA-Aldrich, Steinheim, G
panthothenic acid	p.a.	SIGMA-Aldrich, Steinheim, G
Pepton from Casein	for microbiology	Merck, Darmstadt, G
perchloric acid 70%	p.a.	SIGMA-Aldrich, Steinheim, G
periodic acid	>98%	SIGMA-Aldrich, Steinheim, G
phenol	for DNA isolation	Carl Roth GmbH & Co. KG, Karlsruhe, G
phosphoric acid 85%	-	Mallinkrodt Baker B. V., Deventer, NL
pyrridoxal-HCl	p.a.	SIGMA-Aldrich, Steinheim, G
rabit-anti-mouse-antibody	-	Rockland Immunochemicals Inc., Gilbertsbille, PA, USA
raffinose	research grade	SERVA, Heidelberg, G
RbCl	p.a.	Merck, Darmstadt, G
Ready-to-use CSPD	-	Roche Diagnostics GmbH, Mannheim, G
rhamnose	HPLC-grade	Fluka Biochemika, Steinheim, G
SAP	-	MBI Fermentas GmbH, St. Leon-Rot, G
Schiff´s reagent	-	SIGMA-Aldrich, Steinheim, G
SDS	research grade	SERVA, Heidelberg, G
sodium acetate * 3 H_2O	p.a.	Merck, Darmstadt, G
sodium bisulfite	65,20%	SIGMA-Aldrich, Steinheim, G
sodium citrate * 3 H_2O	HPLC-grade	Merck, Darmstadt, G
sucrose	HPLC-grade	Gerbu Biotechnik GmbH, Gaiberg, G
sulfuric acid	p.a.	Merck, Darmstadt, G
T7 ligase	-	MBI Fermentas GmbH, St. Leon-Rot, G
TEMED	p.a.	Merck, Darmstadt, G

thiamine HCl (vit B1)	-	SIGMA-Aldrich, Steinheim, G
trehalose	HPLC-grade	Fluka Biochemika, Steinheim, G
trichloroacetic acid	p.a.	Merck, Darmstadt, G
Tris	ultra pure	MP Biomedicals Solon, Ohio, USA
Tris base	ultra pure	ICN Biomedicals, Inc., Ohio, USA
Tris-HCl	p.a.	Merck, Darmstadt, G
Tween 20	Ph. Eur.	Merck, Darmstadt, G
Tween 80	-	Mallinkrodt Baker B. V., Deventer, NL
urea	for biochemical use	Merck, Darmstadt, G
xylose	HPLC-grade	Fluka Biochemika, Steinheim, G
yeast extract	for microbiology	Merck, Darmstadt, G
$ZnCl_2$	p.a.	Merck, Darmstadt, G

Tab. 2: Chemicals used in this work in alphabetical order.

Kit	**manufacturer**	**type**
DNA isolation	Omega Bio-Tek Inc., Norcross, GA, USA	E.Z.N.A. bacterial DNA kit
gel extraction	PEQLAB Biotechnologie GmbH, Erlangen, G	peqGOLD gelextraction kit
Glucose/Fructose kit	r-biopharm, Darmstadt, D	D-glucose/D-fructose UV method kit
KOD hot start polymerase	Novagen, EMD chemicals Inc., San Diego, CA, USA	KOD hot start DNA polymerase
PCR purification kit	Qiagen GmbH, Hilden, G	QIAquick PCR purification kit
Plasmid midiprep kit	Promega, Madison, WI, USA	Pure Yield plasmid midiprep system
Plasmid miniprep kit	PEQLAB Biotechnologie GmbH, Erlangen, G	peqGOLD plasmid miniprep kit
Taq polymerase	MP Biomedicals Solon, Ohio, USA	Taq DNA polymerase

Tab. 3: Kits used in this work.

2.1.3 Bacterial strains

Lactobacillus strains used in this work are from TMW strain collection and a list of them is presented in tab. 6.

For cloning and expression two strains of *E. coli* K12 strain are used: *E. coli* K12 DH5α for cloning and long time preparations, *E. coli* K12 JM109 for expression experiments.

2.1.4 Primer

Oligonucleotides for PCR and sequencing experiments are produced by MWG Biotech AG, Ebersberg, Germany. All used oligonucleotide primers are listed in tab. 4.

PCR screening

Primer	Sequence (5´ to 3´)	Use
LevF	GAYGTI TGGGAYTCITGG	PCR
LevR	TCITYYTCRTCISWIRMCAT	PCR
lev_for	GAYGTITGGGAYTCITGG	PCR
lev_rev	CIGGIACIGCRTARTAIG	PCR
lev_forsignalp	AARRAICAYAARAARATITM	PCR

Plasmid sequencing

Primer	Sequence (5´ to 3´)	Use
T7	TAATACGACTCACTATAGGG	plasmid sequencing
T7 term	GCTAGTTATTGCTCAGCG	plasmid sequencing
pET-RP	CTAGTTATTGCTCAGCGG	plasmid sequencing

***L. panis* ftf**

Primer	Sequence (5´ to 3´)	Use
levseq1_for	GCTAATGGTGCTCTTGGTATT	inverse PCR
648seq1_rev	CATCATTGCGATTACCAGTTG	inverse PCR
648seq2_rev	CGTTTCATTAGCAGTATTTGCC	inverse PCR
648seq2_revinv	GAGGCAATACTGCTAATGAAAC	inverse PCR
648seq2a_for	AATGCTGCAAATAGCGCTGCGCTACCT	inverse PCR
648seq2a_rev	GCGTAAACTGTTTGCGTTTCATTAGCAGTA	inverse PCR
648seq3_for	TGTCCTTGACCAGAATGC	inverse PCR
648seq3_rev	GTTTGATCAGTTACCGTTG	inverse PCR
648seq4_rev	TAAGTTACCTGCGTCAATC	inverse PCR
levpan_fw	TATATCTAGAAGGAGATATACATATGGCTGATCAAGTTG AGGCAAATACT	PCR and cloning
levpan_rev	TATAGGATCCATGGCCATCATTATTGTCTGACAC	PCR and cloning
levpanmitte	CAACAATTTGCTAACGGAAACG	plasmid sequencing
levpanmitte2	GCGACTGCAACTCTGCACCTAAATG	plasmid sequencing

***L. frumenti* ftf**

Primer	Sequence (5´ to 3´)	Use
levfruseq1_rev	GCAGACTTCGAGAAATAAATC	inverse PCR
levfruseq1_for	AACCGAAGTGCTGGTTTG	inverse PCR
levfruseq2_rev	CATTAATATGCGCATTGTTAACAGCC	inverse PCR
levfruseq2_for	AGCAACCAAATAATAAACCGGGTAC	inverse PCR
levfrumfw-xba1	TATATCTAGAAGGAGATATACATATGGCCGACCAAGTTA CTACTAATAGT	PCR and cloning
levfrumrev-bamh	TATAGGATCCATTAGGGGTTTCACGAGGGTTTAG	PCR and cloning

***L. reuteri* 1.1274 ftf**

Primer	Sequence (5´ to 3´)	Use
1274seq1_for	GCCGTCCCATCATAGCGAT	inverse PCR
1274seq1_rev	TAGCCGATAGTCTTACAGATCC	inverse PCR
1274seq2_for	CCATAGCATTTTTATCCATAAG	inverse PCR
1274seq2_rev	TAGTCTGGTGTTACTTGTTAC	inverse PCR
1274seq2_revinv	TAACAAGTAACACCAGCACTAG	inverse PCR
1274seq3_rev	GATTCATTATATCCAAATATAGATCC	inverse PCR
1274seq3_for	TGATGATACAACTATGGTATTAGC	inverse PCR
1274sondfw	CCAAGAATGGTCGGTTCTG	Southern blot probe
1274sondfw	CGTTCAAGTTCATCACTAACCTA	Southern blot probe

***L. acidophilus* ftf**

Primer	Sequence (5´ to 3´)	Use
987seq2_for	GATCATTTGACACACGGATATG	inverse PCR
987seq1_rev	ATCCCCATCATGGCAATAAG	inverse PCR
987seq1_for	AGGTATTTTGAAGTTAAGTGGAG	inverse PCR
987seq3_for	CACACGGATATGTTCCTTTA	inverse PCR
987seq6_for	CATTTAGCGTTAATTTAGTTCCGTCTTCTGGATTCCC	inverse PCR
987seq5_rev	CTGTAAGTTCAATGCTTGGTGCAATGGGTTTAGC	inverse PCR
987seq4_for	CTTCTGGATTCCCTTCAAATG	inverse PCR

lacfttfsond_for	GTCGGGATCTATCTTTGGTTATG	Southern blot probe
lacfttfsond_rev	TAAGAAATTACGGACGTTGAACTTATC	Southern blot probe

***L. gasseri* ftf**

Primer	Sequence (5´ to 3´)	Use
ftfgas_fw	ATATCTAGAAGGAGATATACATATGGCTACACTAATGCAGACAAC	coPCR, PCR and cloning
ftfgas_rev	TATACAGACTGTATAGGATCCTTCTGATTGAGTTGTCTTCTTAACTGA	coPCR, PCR and cloning
CO-PCR-GAS-fw	GACTAACCAAGGTGACTGGATTTGGGATGACACTAG	coPCR
CO-PCR-GAS-rev	CTAGTGTCATCCCAAATCCAGTCACCTTGGTTAGTC	coPCR

***L. sanfranciscensis*-*L. panis* hybrids**

Primer	Sequence (5´ to 3´)	Use
SFklon_fw	TATATCTAGAAGGAGATATAATGGCTGATGCTGTTGAG	coPCR, PCR and cloning
SFnterm1_rev	CTGCTTTAGCTGACTATTATCATTTTCTGTG	coPCR
SFnterm2_rev	CAGGTTAGTCTGAGTAGCAGCTGATG	coPCR
PANmitte1_fw	GAAAATGATAATAGTCAGCTAAAGCAGAATACAAC	coPCR
PANmitte2_fw	TCAGCTGCTACTCAGACTAACCTGAG	coPCR
PANklon_rev	TATAGGATCCATGGCCATCATTATTGTCTG	coPCR, PCR and cloning
SFmitte_fw	GTGATGAACAAACACAATTAAAGCAAACTAATAATG	coPCR
SFmitte_rev	TTCATCAGCTGCTTGATGAGGCTTTAA	coPCR
SFcterm_fw	AAGCCACACCGCCAGTTAACCCAATG	coPCR
SFklon_rev	TATAGGATCCCCGTTGGTCCACAAAATTAGT	coPCR, PCR and cloning
PANklon_fw	TATATCTAGAAGGAGATATAATGGCTGATCAAGTTGAGG	coPCR, PCR and cloning
PANnterm_rev	GTTTGCTTTAATTGTGTTTGTTCATCACTGTTAG	coPCR
PANmitte_rev	GGTTAACTGGCTGGTGTGGCTTC	coPCR
PANcterm_fw	AAGCCTCATCAAGCAGCTGATGAACC	coPCR

Tab. 4: All primers used in this work. Code for unspecific bases used in degenerated primers is presented in tab. 5.

symbol	specific bases	description
R	A or G	pu**r**ines
Y	C or T (U)	p**y**rimidines
W	A or T (U)	**w**eak hyrogen bonds
S	G or C	**s**trong hydrogen bonds
M	A or C	a**m**ino group
K	G or T (U)	**k**eto group
H	A, C or T (U)	not G (**H** following G in alphabet)
B	G, C or T (U)	not A (**B** following A in alphabet)
V	G, A or C	not T (U) (**V** following U in alphabet)
D	G, A or T (U)	not C (**D** following C in alphabet)
N	G, A, C or T (U)	a**n**y

Tab. 5: Code for unspecific nucleobases used in degenerated primers in tab. 4.

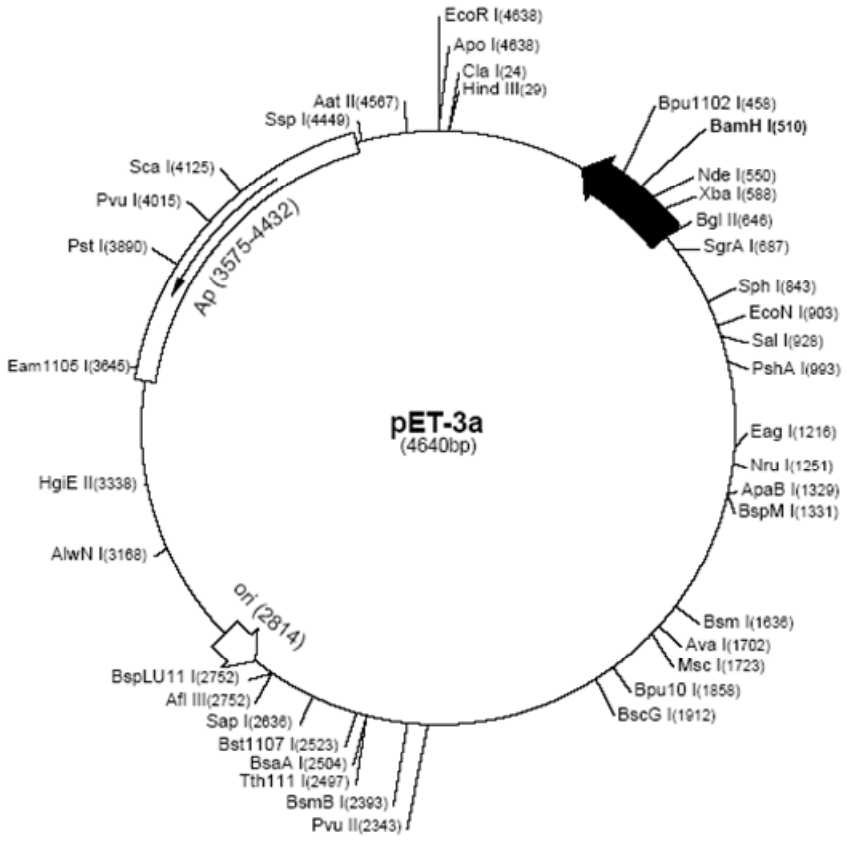
2.1.5 Restriction enzymes

All restriction enzymes used in this work are provided by MBI Fermentas GmbH, St. Leon-Rot, Germany and applied as recommended in manufacturer's instructions. If available, Fastdigest enzymes are used to reduce incubation times.

2.1.6 Plasmids

Native *ftf* genes, modified *L. gasseri ftf* gene and artificial *ftf* gene hybrids are cloned in a pET3a expression plasmid vector (provided by Novagen and Merck, Darmstadt, Germany) using XbaI and BamHI endonuclease restriction sites. Plasmid harbouring can be selected by its ampicillin resistance. Expression of inserted genes is possible by the lactose analogon IPTG since inserts are introduced behind a lacZ promoter.

A



B

Fig. 5: Schematic map of pET3a plasmid vector. Topology is presented including endonuclease restriction sites (A). Sequence section of multiple cloning site has been altered by insertion of code for 6 His residues behind BamHI restriction site (red arrow) (B).

The plasmid is additionally upgraded with base triplets for 6 N-terminal His residues (CAC CAT * 3) enabling an affinity purification of the target protein as described for cloning, expression and purification of *L. sanfranciscensis* levansucrase by Tieking (Tieking et al. 2005a). The plasmid provides binding sites for T7, T7rev and pET-RP primers to check inserts by sequencing. An overview of original pET3a plasmid with restriction sites is presented in fig. 5.

For sequence identification DNA fragments was cloned in a pBluescript II KS+ plasmid (Agilent/Stratagene, Böblingen, Germany). This plasmid enables blue-white selection when inserts are cloned inside the LacZ operon and metabolism of X-Gal is disabled. As a consequence, clones containing inserts appear white while clones with an uninterrupted LacZ cassette are blue. Inserts can be analyzed by standard primers binding on the plasmid (e.g. T7 primer). A schematic vector map is presented in fig. 6.

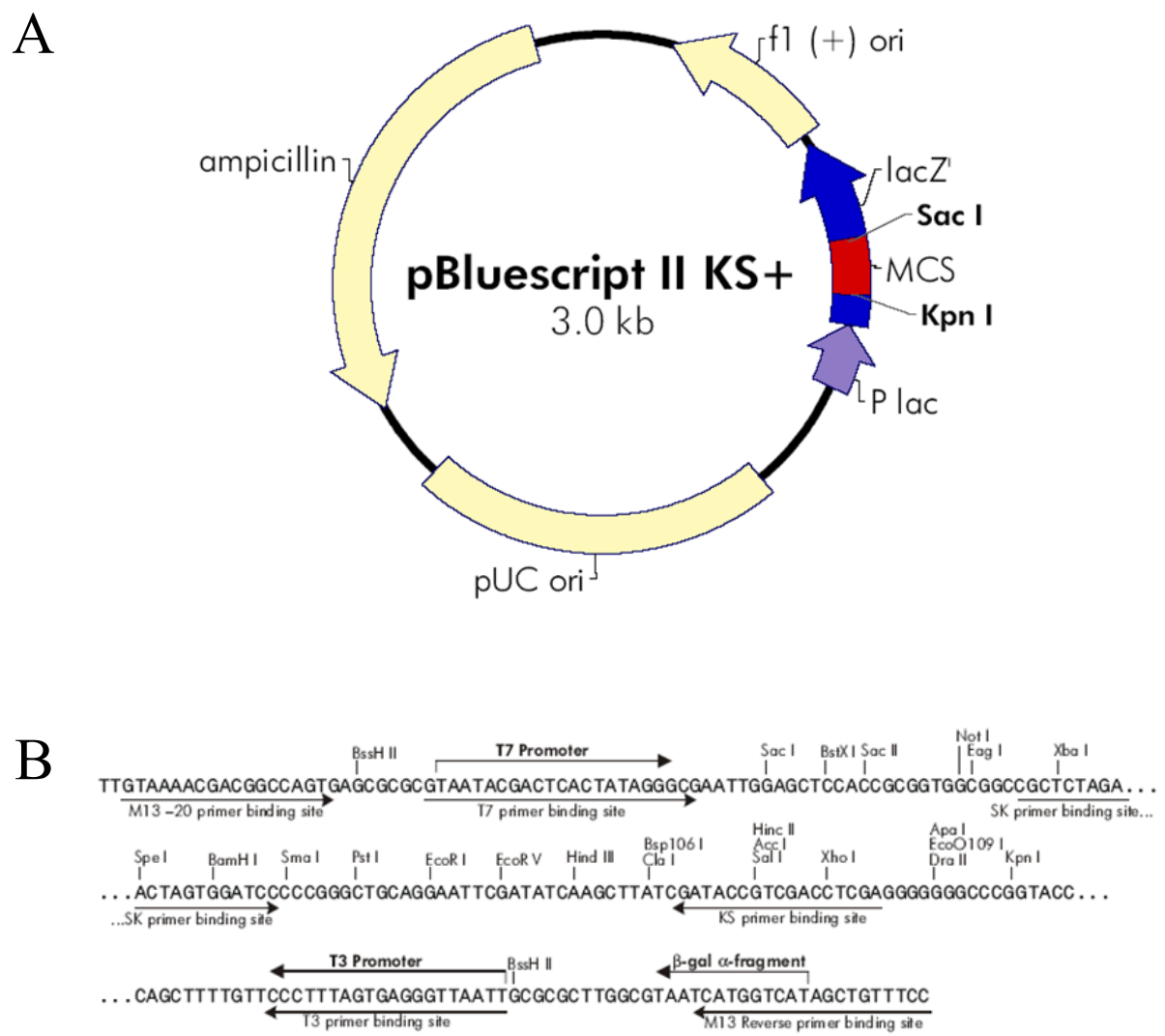


Fig. 6: Topology (A) and multiple cloning site (B) of pBluescript II KS+ plasmid vector.

2.2 Methods

2.2.1 Microbiological methods

2.2.1.1 Media

All lactobacilli were cultivated in modified MRS (mMRS) medium. Basic medium contained 10 g/l pepton from casein, 5 g/l meat extract, 5 g/l yeast extract, 2.6 g/l K_2HPO_4, 4.0 g/l KH_2PO_4, 3.0 g/l NH_4Cl, 0.5 g/l cysteinhydrochlorid and 1.0 ml/l Tween 80. Magnesium and manganese was added as 1000 fold stock solution (end concentrations in medium: $MgSO_4$ * 7 H_2O 100 mg/l, $MnSO_4$ * 4 H_2O 50 mg/l). This components were dissolved in 750 ml H_2O_{dest} resulting in a pH of 6.2 (if necessary, pH was adjusted with NaOH or HCl). Sugar components were dissolved in 250 ml H_2O. Basic medium and carbohydrate solution were autoclaved (121°C, 20 min) separately to avoid browning by Maillard reactions and mixed after cooling. 1 ml sterile filtrated (diameter 0.2 µm) vitamin solution was added. Vitamin solution was composed as follows: 10 mg cobalamine, 10 mg folic acid, 10 mg nicotinic acid, 10 mg panthothenic acid, 10 mg pyrridoxal-HCl and 10 mg thiamine dissolved in 50 ml of H_2O_{dest}. Vitamin solution aliquots of 1 ml were stored at -20°C.

Different mMRS variations concerning the carbohydrate source were used. mMRS-MFG10 means the basic medium containing 10 g/l maltose, 10 g/l fructose and 10 g/l glucose. mMRS-Sac80 contained beside the components of the basic medium 80 g/l sucrose. mMRS4 contained 10 g/l maltose, 10 g/l fructose and 10 g/l glucose as well as 80 g/l sucrose.

E. coli strains were grown in LB medium containing 10 g/l pepton, 5 g/l yeast extract and 5 g/l NaCl. Components were dissolved in H_2O_{dest} and pH is adjusted to 7.5 with NaOH. Medium was sterilized by autoclaving. For working with pET vectors, 1 ml/l of 100 mg/ml sterile filtrated (pore diameter 0.2 µm) ampicillin stock solution were added after cooling.

For production of agar plates, independently of the medium, 17 g/l agar were added to liquid medium before autoclaving. Agar dissolved during sterilization process. Medium was cooled to approximately 60°C before adding temperature sensitive components as vitamins or antibiotics and casting about 20 ml in sterile plastic Petri dishes. The solid agar plates were stored at 4°C.

2.2.1.2 Cultivation parameters

Lactobacilli were cultivated at 30°C or 37°C depending on the strain. Liquid cultures were incubated in tight closed bottles or plastic tubes and not shaken. Agar plates were incubated anaerobically by use of Anaerocult (Merck, Darmstadt, Germany) system in heat-sealed plastic bags (one to four plates) or airtight incubation containers (up to 12 plates). Incubation

time was irregularly and depended on the strain as well as mass and condition of cells inoculated. To obtain a visual observable amount of cells it took one to three days.

E. coli strains were normally cultivated at 37°C. Liquid cultures were incubated in Erlenmeyer flasks (0.5 l medium in 1 l flask, 1 l medium in 2 l flask etc.) or test glasses caped with Kapsenberg caps. Liquid cultures were shaken at 200 to 220 rounds per minute (rpm). Plates were incubated aerobically. If not mentioned otherwise, *E. coli* cultures were grown over night for 13 to 17 hours (h).

2.2.1.3 Screening for EPS formation

The ability of different *Lactobacillus* strains to produce EPS from sucrose was tested. Therefore cultivation methods were used. Strains were grown on mMRS Sac80 agar plates containing sucrose as single carbohydrate and on sucrose free mMRS MFG10 plates as control. EPS formation was judged by colony appearance: A slimy, ropy and wet appearance of colonies on MRS Sac80 indicated EPS formation if less slimy and ropy but more dry colonies were found on sucrose free control plates.

For a more sensitive detection of EPS and to gain EPS material for further experiments, strains were also grown in liquid mMRS Sac80 medium. Liquid cultures of the same strains in mMRS MFG10 broth served as control. Cells were removed by centrifugation (5000 rpm, 30min). EPS was detected by ethanol precipitation from culture supernatant and further characterized as described in EPS treatment section.

2.2.1.4 DNA isolation from lactobacilli

For isolation of DNA from lactobacilli, two methods were used:

E.Z.N.A. Bacterial DNA kit (Peqlab) was used according the kit description. For the isolation 2 ml of a 15 ml culture in mMRS MFG10 medium were used.

Since the kit yield in some cases was not satisfying, a conventional protocol was used, too:

Therefore, 15 ml culture in mMRS MFG10 medium were grown, cells were harvested by centrifugation (5000 rpm, 20 min). Used medium was discarded and cells were washed with 5 to 10 ml of TE buffer (10 mM Tris, 1 mM ethylendiaminetetraacetic acid (EDTA), pH 8.0) and centrifuged as described above. Cells were resuspended in 2 ml of TE buffer and a pinch of lysozym was added. Lysis of cell wall was performed during incubation at 4°C over night (13 to 17 h) or alternatively at 37°C for 1 h. For control of lysis a drop of cell suspension was mixed with a drop of 25 % SDS solution on a microscope slide. A successful lysis is indicated by clearing of the suspension and increased ropiness. 25 % SDS solution was now added to lysozym treated cell suspension to a final concentration of 2 % SDS and heated to 60°C for 10 min. 5 M NaCl solution was used to get a salt concentration of 1 M in the sample. One sample

volume of phenol:chloroform (1:1) was added to the suspension and mixed moderately for 10 min. Phases were separated by centrifugation (5000 rpm, 10min). Upper (water) phase containing dissolved DNA was carefully transferred to a new tube and mixed with one sample volume of chloroform for another 10 min. Phases were separated by centrifugation and upper (water) phase was transferred in a new tube, again. 3 M sodium acetate (pH 5.2) was added in a ratio of 1:8 (sodiumacetate:sample) and the solution was carefully overlaid with 250 % volume of ethanol (-20°C). Careful mixing resulted in first white cords, an incubation of several hours at -20°C completely precipitated chromosomal DNA that could be coiled and transferred to a 1.5 ml reaction tube with a pipette tip. DNA was washed by addition of 1 ml of ethanol and careful mixing. Supernatant ethanol was removed and DNA is air dried at 37°C over night. Dried DNA was carefully dissolved in TE buffer and stored at 4°C for further use. Result of DNA isolation was monitored by analytical agarose gel electrophoresis.

2.2.1.5 Production of chemical competent cells and transformation protocol

For transformation of plasmid DNA in *E. coli* JM109 and *E. coli* DH5α competent cells and a heat shock transformation protocol was used.

For competent cells 200 ml of LB broth in a 1 l Erlenmeyer flask were inoculated with 2 ml of a over night culture of the respective *E. coli* strain and incubated at 37°C (shaking: 200 rpm) until an OD_{600nm} of 0.3 to 0.5 (3 to 4 h). When respective cell mass is reached, cultures were cooled down on ice and transferred to four 50 ml plastic centrifuge tubes. Cells were harvested by centrifugation (3000 rpm, 15 min, 4°C) in a precooled rotor. Supernatant was disposed and pellets were resuspended in 50 ml of precooled (on ice) RF1-solution (12 g/l RbCl, 9.9 g/l $MnCl_2$, 1.5 g/l $CaCl_2*2H_2O$, 2.9 g/l potassium acetate, 121 ml/l glycerol (87 %) in water, pH adjusted to 5.8 with acetic acid, sterile filtration (pore diameter 0.2 µm)). Cells were retrieved by centrifugation as described. Subsequently pellets were resuspended in 8 ml of precooled (on ice) RF2-solution (2.1 g/l MOPS, 1.2 g/l RbCl, 11 g/l $CaCl_2*2H_2O$, 121 ml/l glycerol (87 %) in water, pH adjusted to 6.8 with NaOH, sterile filtration (pore diameter 0.2 µm)). Chemical competent cells were now aliquoted in portions of 200 µl. They could be used for transformation immediately or be frozen in liquid nitrogen and stored at -80°C.

For transformation frozen competent cells were thawed slowly on ice. Plasmid solution up to 0.25 % of volume of competent cell suspension was added and mixed. After an incubation on ice for 20 min, heat shock was performed (42°C, 90 sec) and cells are put back on ice for 2 min. 4 volumes of LB medium were added and cells were incubated at 37°C (careful shaking up to 100 rpm). 100 µl of cell suspension were plated on an LB agar plate containing

respective antibiotic. The residual cells were pelleted by short centrifugation and also plated on an LB agar plate with antibiotic. Plates were breed at 37°C over night.

2.2.2 EPS treatment

2.2.2.1 EPS precipitation

EPS in solution produced by bacterial metabolism or in vitro enzyme reactions had to be removed from solution to get rid of residual carbohydrates and other media components as proteins and to get increased EPS concentrations for further analysis.

Therefore ethanol precipitation was performed. EPS containing media or buffer is mixed with two sample volumes of precooled (-20°C) ethanol followed by an incubation at 4°C at least over night (13 to 17 h). Precipitated EPS was harvested by centrifugation (5000 rpm to 7000 rpm, 30 to 45 min, 4°C). Supernatant was removed and EPS pellet was air dried at maximum 37°C. Dried EPS could be stored at room temperature or be resolved in H_2O_{bidest} for further analysis. EPS solutions were stored at 4°C for short time or -20°C for storage more than one week.

No complete recovery of EPS can be expected by this method. (Azeredo and Oliveira 1996)

2.2.2.2 EPS hydrolysis and inulinase digest

For analysis of EPS composition, EPS was hydrolyzed by acidic treatment. Therefore, 5 µl of perchloric acid (70 %) were added to 200 µl of EPS solution and incubated at 96°C for 3 h. If EPS could not be hydrolyzed by this protocol as it may be the case for some glucans, sulphuric acid can be used instead of perchloric acid.

Sugar monomers in hydrolyzed samples were analyzed by HPLC or could be stored at -20°C.

Two types of fructan concerning the linkage type are known: levan (β-(2→6)) and inulin (β-(2→1)). To identify the linkage type of an unknown fructan, the fructose polymer was treated with inulin specific inulinase. This enzyme hydrolyzes β-(2→1) glycosidic bondages present in inulin but not β-(2→6) linkages typical for levan. So, detectable free fructose was only present if inulinase treated fructan was inulin. For the test, 500 µl of a 1 %(w/v) fructan solution in 10 mM sodium acetate buffer, pH 4.1 were incubated with 500 µl of inulinase solution (5 U/ml) were mixed and incubated for 30 min at 37°C. The disappearing of fructan could be visualized on TLC plates. Alternatively produced fructose could be detected by HPLC methods when there was sample in abundant amounts.

2.2.2.3 EPS dialysis

Since small molecules as sugar monomers can contaminate precipitated EPS, for critical experiments these contaminations were removed by dialysis. Therefore EPS had to be

dissolved and solution was transferred in Viskis® dialysis tubing 20/32 (SERVA, Heidelberg, Germany) and dialyzed thrice against 2 l of H_2O_{bidest} for 5 to 15 h at 4°C and careful stirring. If necessary, EPS could be precipitated after dialyses with ethanol as described above.

2.2.3 Molecular biologic methods

2.2.3.1 Sequence analysis and bioinformatics

DNA and aa sequence alignments were performed with an online version of ClustalW software available on different web pages (e.g. http://clustalw.ddbj.nig.ac.jp/top-k.html or http://www.ebi.ac.uk/Tools/clustalw2/index.html (Thompson et al. 2002)).

For search of homologue DNA and aa sequences, BLAST algorithm (Altschul et al. 1997) was used accessed via pubmed server.

Relationship of DNA or aa sequences could be visualized by dendrograms. These are calculated by TreeTop software (http://www.genebee.msu.su/services/phtree_reduced.html). Alternatively, phylogenetic trees could be calculated with ClustalW software directly.

Free Webcutter 2.0 tool was used for analysis of endonuclease restriction sites inside DNA sequences. This tool is accessible via http://rna.lundberg.gu.se/cutter2/ or http://users.unimi.it/~camelot/tools/cut2.html and delivered a restriction map of the DNA fragment as well as a listing of enzymes not cutting the respective sequence.

The conversion of DNA sequences in reverse, complementary or reverse-complementary sequence was done using an online tool provided on the sequence manipulation suite (http://www.bioinformatics.org/SMS/index.html) (Stothard 2000). For translation of DNA sequences to aa sequences the translation tool of expasy server tools collection (http://www.expasy.ch/tools/) "Translate" was used. All possible six reading frames were comprised in the analysis and the output style could be varied. Reverse translates from aa to DNA sequence (e.g. for design of degenerated primers) were performed by software provided on http://arbl.cvmbs.colostate.edu/molkit/.

Cloning experiments were designed using Clone Manager 5.0 software. Therewith plasmids and DNA fragments can be virtually treated with DNA restriction endonucleases or relegated to new constructs. A modification of sequences or a restriction analysis is possible, too.

Sizes of target protein based on DNA sequence length were estimated using an online tool provided on http://molbiol.ru/ger/scripts/01_06.html.

Bacterial signal peptides of the sec-pathway could be found in sequences using SignalP 3.0 software accessible on http://www.cbs.dtu.dk/services/SignalP/ (Lund et al. 2002; Bendtsen et al. 2004).

For aa sequences of fructansucrase a simplified molecular modelling using online tools was performed. These software tools can be found on tools listing of expasy server (see above) and use an alignment with similar protein sequences for which a 3-dimensional structure (based on x-ray analysis of protein crystals) has been published. Trials were made with 3D-jigsaw (http://bmm.cancerresearchuk.org/~3djigsaw/) (Bates and Sternberg 1999; Bates et al. 2001; Contreras-Moreira and Bates 2002) and CPHmodels (http://www.cbs.dtu.dk/services/CPHmodels/) (Lund et al. 2002) modelling programs. Received data files including 3 dimensional structure proposals were viewed and handled with free PyMOL pre-1.0 software (download: http://delsci.com/rel/099/).
Data of kinetic studies were processed with MS Excel and Sigmaplot 9.0 software.

2.2.3.2 Agarose gel electrophoresis and gel extraction of DNA fragments

Visualization of DNA, e.g. for monitoring of DNA isolation, PCR, restriction digest and plasmid miniprep experiments was done by agarose gel electrophoresis.
1 to 1.2 %(w/v) agarose were dissolved in 1x TAE buffer (stored as 50x TAE buffer (0.1 M EDTA, 1 M acetic acid (100 %), 2 M Tris, pH 8.2) and diluted 1:50 for use) by cooking in a microwave oven. Agarose solution was cooled down on a magnetic stirrer to a temperature of approximately 60°C and casted in the skid of agarose gel gadget. Combs were inserted and agarose solution was let cool down for gelling. Gel was transferred to electrophoresis chamber and the latter is filled with 1x TAE buffer till gel was overlaid. Combs were removed.
DNA samples were mixed with loading dye (50 mM EDTA, 40 % (w/v) sucrose, 0.001 % (w/v) bromphenol blue (Mülhardt 2003)) in ratio of 5:1 (sample:loading dye) and applied in Gel cavities. 5 to 15 µl sample were used for analytical gel electrophoresis, up to 100 µl in respective cavities were applied for preparative gel electrophoresis. Different ready-to-use DNA size standards were applied (10 µl in small cavities, 15 µl in large cavities) to determine DNA fragment sizes by comparison.
Separation was performed by application of voltage of 90 to 120 V for 1 to 1.5 h. Progress of separation could be monitored by location of bromphenol blue contained in the loading dye.
Gel was incubated in an ethidium bromide-water solution (1 µl/ml) for 15 to 45 h and subsequently washed in water. DNA was visualized by UV light (wavelength 320nm) and documented by video camera and Intas® GDS equipment and software.
DNA fragments could be recovered in preparative gel electrophoresis. Therefore respective DNA fragments were excised with a scalpel and transferred to a 1.5 ml reaction tube. DNA was extracted from agarose gel using Peqlab agarose gel extraction kit following

manufacturer's instructions. Result of gel extraction was checked by analytical gel electrophoresis.

2.2.3.3 PCR screening for *ftf* genes with degenerated primer

Different *Lactobacillus* strains were screened for the presence of family GH68 *ftf* genes by the use of degenerated primer deduced from homologous aa regions in FTF sequence alignments. One pair of primer (levV and levR) resulting in a approximately 560 bp PCR product has been constructed by Tieking et al. (Tieking et al. 2003b) another pair of screening primer (lev_for and lev_rev) for a PCR fragment of around 1 kbp was newly constructed. Therefore aa sequences of available lactobacillus and LAB FTF proteins were aligned with ClustalW algorithm and two regions containing highly conserved amino acids in maximal distance to each other were identified. The forward primer could not be significantly improved concerning position and sequence and so just one of them was used in the further experiments. Degenerated primers were designed considering all possibilities provided by degenerated DNA code for each amino acid and in consequence a mixture of different primers was produced. In positions where all four DNA bases were possible, inosine was used since this special nucleotide pairs with every other nucleotide.

Fig. 7: Section of alignment of known FTF aa sequences of lactobacillus origin (FTF of *L. johnsonii* (ftfLBJS), inulinsucrase of *L. reuteri* (inuLBREUT), levansucrase of *L. sanfranciscensis* (levLBSF) and levansucrase of *L. reuteri* (levLBREU)) to demonstrate position of degenerated primer pairs. Respective locations are also conserved in other FTF proteins from LAB.

PCR reactions were composed as follows: 0.3 to 0.5 µl of DNA, 0.6 µl of forward primer, 0.6 µl of reverse primer, 2.5 µl of 10x PCR buffer containing MgCl, 0.5 µl dNTPs, 0.3 µl Taq DNA polymerase and 20.0 to 20.2 µl H_2O_{PCR} (free of DNA and nucleases) to an end volume of 25 µl. All components and completed reaction samples are kept on ice.

PCR is performed in Eppendorf and Primus PCR-cycler-machines. In a first step, genomic DNA was denatured (94°C, 90 sec). Then amplification cycle with denaturation (94°C, 30 sec), primer annealing (52°C, 30 sec) and elongation (72°C, 30 sec (levV + levR) or 60 sec (lev_for + lev_rev)) was repeated 30 times. In the end a final elongation step (72°C, 300 sec) finished PCR.

Presence of PCR fragments in the expected size was checked by agarose gel electrophoresis. If only a single PCR product could be detected this was isolated from PCR mix using a Qiagen PCR purification kit following manufacturer's instructions. If several bands appear, fragments of correct size were excised from a preparative agarose gel and extracted as described above. Sequences of fragments were obtained by automated sequencing reactions performed by Sequiserve GmbH, Vaterstetten, Germany.

2.2.3.4 Discovering complete *ftf* genes

Since PCR with degenerated primer only covers maximum half of the complete gene sequence further experiments had to be performed to complete sequence information. Different strategies are possible:

A degenerated primer lev_forsignalp was designed based on a conserved aa motif in the signal peptide of different FTF proteins and PCR in combination with levR and lev_rev was done as described above. Different annealing temperatures and elongation times were tried.

A further method was inverse PCR (iPCR). Therefore genomic DNA was cut with a restriction endonuclease not cutting inside the known sequence: 5 to10µl DNA solution were mixed with 7.5 µl of buffer (compatible to restriction endonuclease used), 4 µl of restriction endonuclease and nuclease free water to a total volume of 75 µl and incubated at 37°C over night.

The fragmented DNA was ligated with T4 ligase: 5 µl of 10x ligation buffer, 5µl T4 DNA ligase, 5 to 35 µl of restricted DNA solution with water to a total volume of 50µl were mixed and incubated at 4°C over night (13 to 17 h). Taq-PCR was performed using primers binding on the outer regions of the known sequence in outward direction. Different annealing temperatures and elongation times had to be tested. If DNA fragments including the known part of the *ftf* gene ligated with themselves to a circular product, with these primers a PCR product could be amplified and analyzed revealing new sequence information (See figure 8).

Since this basic method did not always lead to fast results, different approaches were made to increase prospects of success.

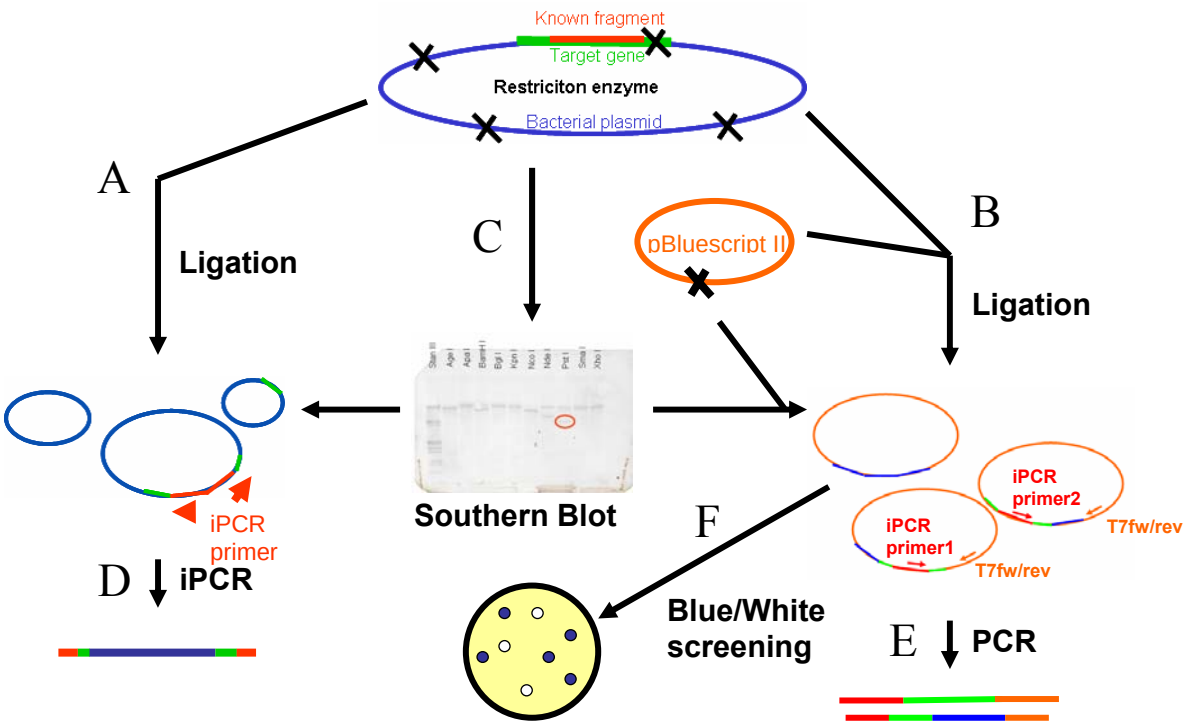


Fig. 8: Schematic presentation of different strategies of exploration of complete *ftf* gene sequences based on an initial fragment. For simple inverse PCR genomic DNA was fragmented using a restriction endonuclease not cutting inside the known sequence, ligation to circular DNA molecules (A) and iPCR (D). Alternatively, restricted DNA could be ligated in a respectively digested plasmid (B) and PCR with primers on known sequence and on plasmid DNA could be performed (E). For both methods fragmented DNA could be examined by Southern blot (C) and only positive fragments are inserted in iPCR or ligation in plasmid to increase probability of PCR products carrying *ftf* gene fragments. Southern blot supported pBluescript constructs could alternatively be cloned in *E. coli* and screened for insertion of fragment in plasmid by blue/white screening on agar plates containing IPTG and X-Gal.

One possibility was to constrain DNA fragments before ligation. Identification of restriction fragments carrying *ftf* gene elements was done by southern blot method using a probe specific for the known sequence fragment. The probe was labelled with Digoxigenin (DIG). For probe PCR and southern blot protocol was adapted from DIG application manual provided by Roche Diagnostics GmbH, Mannheim, Germany. A DIG labelled probe was produced by PCR. Using Taq polymerase the reaction setup was pipetted as follows: 0.5 µl template DNA, forward and reverse Primer 5 µl each, 5 ml reaction buffer, 1.5 µl $MgCl_2$ 100 mM, 3.5 ml dNTP-mix and 1.0 µl DIG labelled dUTP, 1.0 µl of Taq DNA polymerase and 36.5 µl H_2O_{PCR} for a final volume of 50 µl. dNTP-mix was composed of dATP, dCTP, dGTP 20µl each as

well as 13 µl if dTTP, diluted in 427 µl H_2O_{PCR}. dNTP-mix was stored at -20°C. PCR was performed with an initial denaturation step (90 s, 95°C), a cycle (denaturation (30 s, 95°C), annealing (90 s, 55°C) and elongation (35 s, 72°C)) repeated 30 times and a final elongation step (5min, 72°C). For southern blot, genomic DNA of organism carrying the target *ftf* gene was digested with DNA restriction endonucleases as described above. Digested DNA and a DIG labelled molecular weight marker were separated by an agarose gel. The gel was not stained with ethidium bromide. For control of separation quality, a second identical agarose gel with the same samples was done in parallel and could be stained with ethidium bromide. Before blotting separated DNA was depurinated by incubation for 10 to 20 min at room temperature in 250 mM HCl. Progress of diffusion of HCl in gel could be observed by colour change of bromphenol blue from blue to yellow. Then, gel was rinsed with H_2O_{bidest}. Subsequently DNA was denatured by incubating (gently shaking, 15 min, room temperature) twice in denaturation solution (0.5 M NaOH, 1.5 M NaCl).

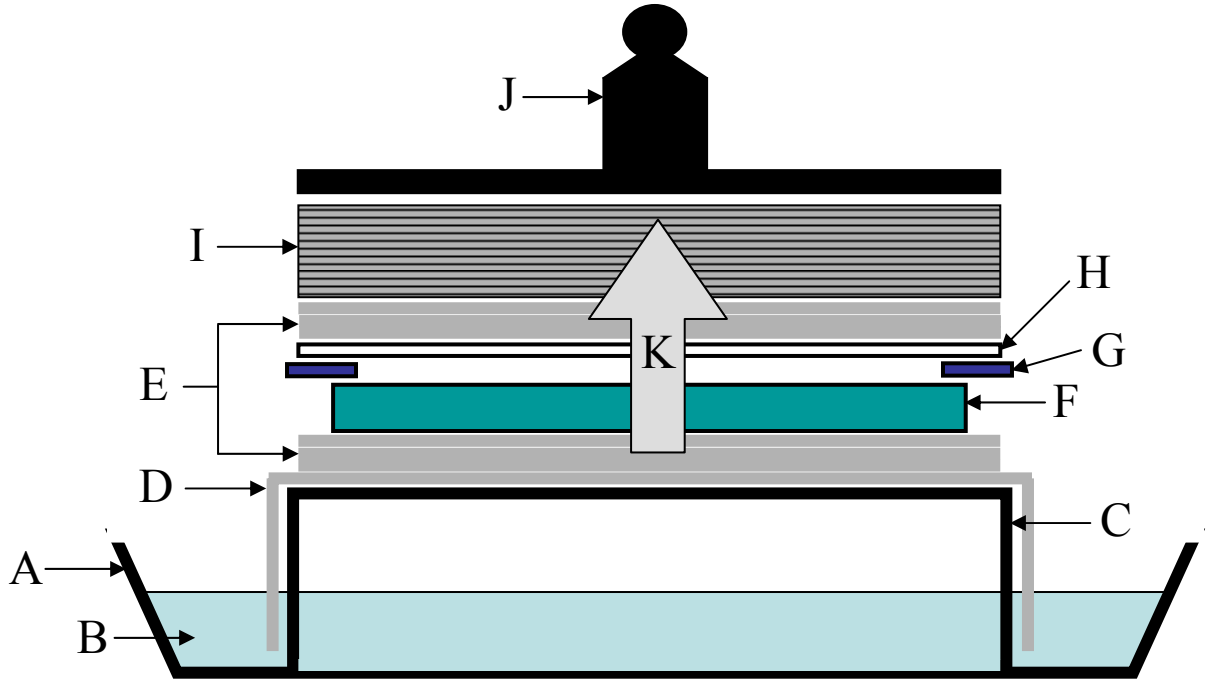


Fig. 9: Schematic view of Southern blot assembling: In a tank (A) filled with 10x SSC buffer (B), a plastic bridge (C) provided the platform for the blot sandwich. A bridge of 10x SSC buffer soaked filter paper (C) was the connection between blot and blotting buffer. On another three layers of buffer soaked filter paper (E), the agarose gel with separated DNA fragments (F) was applied. A frame of Parafilm (G) sealed the areas around the gel avoiding a shortcut of capillary buffer transfer beside the buffer moisten nylon membrane (H) applied on the gel. Another three layers of 10x SSC soaked filter paper (E) were applied and the sandwich was covered with a stack of dry paper towels (I) and compressed with a glass plate and a weight (J). Blot was driven by capillary force drawing buffer from the tank through the blot into the dry paper towels. Blotting direction is indicated by light grey arrow (K).

Gel was rinsed again with H_2O_{bidest} before submerging in neutralization solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) and incubating twice for 15 min at room temperature. Subsequently gel was equilibrated in 20x SSC (3 M NaCl, 300 mM sodium citrate, pH 7.0) for at least 10 min. Blot was assembled as shown in fig. 9 and DNA was transferred to nylon membrane by capillary force over night (Mülhardt 2003).

DNA was fixed to the still moist membrane by UV crosslinking (256nm, 3min) and dried subsequently. For prehybridization, blot was placed in a 50 ml plastic centrifugation tube and incubated at hybridization temperature (40°C for TMW 1.1274 probe) in 10 ml of DIG Easy Hyb for 30 min in a rotating blot incubation oven. Probe was prepared for hybridization as follows: 10µl of probe was diluted with 50µl of H_2O_{PCR} and boiled for 5 min to denature probe DNA. Then, probe was chilled immediately on ice. Denatured probe was added to 10 ml of prewarmed DIG Easy Hyb which subsequently was applied on blot membrane. Blot was incubated with probe at hybridization temperature over night (at least 6 h). Probed blot then was incubated in low stringency buffer (2x SSC containing 0.1 % SDS) at room temperature or five minutes. This step was repeated once. High stringency buffer (0.5x SSC containing 0.1 % SDS) was preheated to 65°C. Used low stringency buffer was removed, high stringency buffer was applied on blot and the blot was incubated twice for 15 min at 65°C. Visualization of results was realized by chemiluminescence. Therefore blot was washed with washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5; 0.3 % (v/v) Tween 20) for 2 min. All steps in detection protocol were carried out at room temperature. After discarding the washing buffer, 40 ml of blocking solution were added and blocking was performed for 30 min. Blocking solution was discarded and 20 ml of freshly prepared antibody solution (Anti-Digoxigenin-AP 1:10000 (75 mU/ml) in 20 ml Blocking solution) were added and incubated 30 min. Then, membrane was washed twice with 40 ml of washing buffer. After that, membrane was equilibrated in 20 ml of detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). Washed membrane was applied on a plastic foil and ready-to-use CSPD was applied drop by drop on the surface. A second plastic foil was used to cover the blot and spread the reagent over the entire membrane. After an incubation of 5 min, Excess liquid was squeezed out and membrane was heat sealed in the plastic foils. Damp membrane was incubated for 10 min at 37°C. Subsequently an X-ray film was exposed to the blot in a light excluding cassette for 15 to 20 min and developed with Kodak photo developer and fixing reagents following manufacturer's instructions. By the help of DNA standard, size of fragments carrying (partial) *ftf* gene could be estimated. From a preparative agarose gel the corresponding fragments of genomic DNA digest could be isolated and inserted in ligation and iPCR procedure.

Alternatively it was tried to ligate isolated fragments of distinct size into a pBluescript KS plasmid cut with blunt end cutting SmaI. Plasmids were transformed in chemical competent DH5α *E. coli* and plated on LB agar plates supplemented with ampicillin, IPTG and X-Gal. Cells containing a plasmid without insert could metabolize the lactose analogue X-Gal resulting in blue stained colonies whereas plasmids containing an insert fragment had a white appearance since X-Gal metabolism was inhibited. Plasmids of these clones could be isolated and inserts could be sequenced using primers positioned on the plasmid.

Another alternative was the ligation of DNA fragments from genomic DNA digest in a plasmid of known sequence: pBluescript II KS plasmid was treated with respective restriction endonuclease and dephosphorylated with SAP to avoid religation of the plasmid, according to the enzymes manufacturer's instructions. Restricted genomic DNA was ligated to the plasmid DNA using T4 DNA ligase following the protocol provided with the enzyme. For PCR beside the iPCR primer two primers binding on the plasmid DNA in direction to inserted DNA fragment were introduced. PCR was done with one primer for inverse PCR and respectively one primer binding on the known plasmid. All four combinations were performed. If a fragment carrying (part of) *ftf* gene was now contained in the plasmid, the region outside the known sequence could be amplified and analyzed (see fig. 8).

Also a combination of Southern blot and ligation in a plasmid is possible.

2.2.3.5 Cloning of *ftf* genes in pet 3a plasmid

When complete sequence of an *ftf* gene was known, it could be cloned in a plasmid vector for heterologous expression in *E. coli* cells.

pET3a vector system was used as described for levansucrase of *L. sanfranciscensis* (Tieking et al. 2005a). The original pET3a plasmid has been supplemented with a sequence section coding for six histidine residues located at the C-terminus of the target protein called His-tag. As base for cloning new *ftf* genes the plasmid containing levansucrase gene of *L. sanfranciscensis* was used (Tieking et al. 2005a). This gene was excised using *Xba* I and *Bam*H I restriction sites, keeping the coding sequence for the His-tag but removing ribosome binding site (rbs). Endonuclease restriction digest of plasmid was performed using regular *Xba* I and *Bam*H I enzymes or respective fast digest enzymes following manufacturer's instructions. Restricted plasmid DNA was purified by a preparative agarose gel electrophoresis to get rid of incomplete digested plasmid molecules. The use of two different restriction sites theoretically eliminates the option of a religation of the plasmid molecule without attaching an insert molecule. Nevertheless, restricted plasmid was dephosphorylated using shrimp alkaline phosphatase (SAP) considering manufacturer's protocol. SAP was

inactivated by a heat step (15 min, 75°C). Plasmid ready for insert ligation could be stored at -20°C.

Inserted fragment carrying target *ftf* gene was produced by specific PCR. Forward primer oligonucleotide started with a nonsense sequence TATA for improving restriction digest, followed by *Xba* I restriction site (TCTAGA). Subsequently the last three bases AGA of restriction site were completed to a functional *E. coli* ribosome binding site (rbs) AGAAGGAGA. This precursor was used for cloning of all *ftf* genes and respective constructs in this work. It was followed by a specific sequence of at least 15 bp, matching to the original *ftf* gene sequence. *ftf* genes were cloned without N-terminal signal peptide. Reverse primer concluded only 5´ nonsense TATA followed by *Bam*H I restriction sequence GGATCC and a stretch of target gene specific sequence of at least 20 bp. End of cloned fragment was defined by LPXTG cell wall anchoring motif. KOD hot start DNA polymerase (Novagen) was used for amplification of approximately 2 kb insert fragment since this polymerase in contrast to Taq polymerase performs proof reading activity while Taq polymerase has an error rate of 1 in 1 kb. Following manufacturer's instruction reaction setup was arranged as follows: 5 µl of 10x reaction buffer, 3 µl of provided 25 mM $MgSO_4$ solution, 5 µl provided dNTPs (2 mM each), 1 ml of KOD polymerase (1 U/ml) 1.5 ml of each oligonucleotide primer, 0.5 to 1 µl of respective lactobacillus DNA and H_2O_{PCR} to and total volume of 50µl. Cycling conditions also was set according to provided protocol: 95°C, 2 min for denaturation of genomic DNA and activation of hot start KOD polymerase, 30 to 35 repeats of cycle (95°C, 20 s for denaturation, 10 sec at optimized annealing temperature, 70°C for adjusted extension time (20 s/kb)) and a final elongation step of 70°C, 2 min. Annealing temperature and elongation time had to be fit for each PCR product separately. PCR results were monitored by analytic agarose gel electrophoresis. If only one PCR product of correct size appears, it could be isolated using PCR purification kit. If there were additional products of various sizes, desired DNA fragment was obtained by preparative agarose gel electrophoresis and gel extraction using Peqlab kit. Purified insert fragments were treated with restriction endonucleases *Xba* I and *Bam*H I as described for plasmid. Regular restriction endonucleases could be heat inactivated (70°C, 15 min) whereas fast digest enzymes had to be removed using PCR purification kit. Insert fragments did not have to be dephosphorylated.

Insert and plasmid DNA were assembled by T4 ligation. The ligation setup is prepared following the protocol provided by manufacturer: 2 µl ligation buffer, 2 µl T4 DNA ligase (1 U/µl), 6 µl nuclease free water and 10 µl mixture of plasmid and insert DNA. Ration of plasmid insert DNA was varied from 1:1 to 1:50 (v:v). Ligation reaction was carried out either

at 4°C over night (13 to 17 h) or at 22°C for 1 h. T4 DNA ligase was terminally inactivated (65°C, 10 min) before transformation in *E. coli* DH5α and breeding over night (37°C).
Colonies of transformants were picked with a sterile wood toothpick and transferred to a new agar plate. For testing if cloning was successful, a trace of colony material was applicated in a PCR reaction tube. A master mix containing all PCR components expect template DNA was added and PCR was performed. If the respective clone harboured insert containing plasmid there was a PCR product in size of the insert fragment. An alternative method of clone screening was cultivation of 5 to 10 ml of over night culture in a reagent glass, plasmid isolation using Peqlab plasmid miniprep kit following the kit instructions and restriction of isolated plasmids with *Xba* I and *Bam*H I enzymes. By analytical agarose gel electrophoresis plasmid containing inserts of correct size could be identified.

2.2.3.6 Base Exchange by crossover PCR in *ftf gasseri*

Since database search revealed an *ftf* gene in *L. gasseri* strain TMW 1.1173 which turned out not to produce a functional gene product, for the exchange of a single base pair to remove a point mutation responsible for a stop codon inside the gene, crossover PCR (coPCR) method was used.

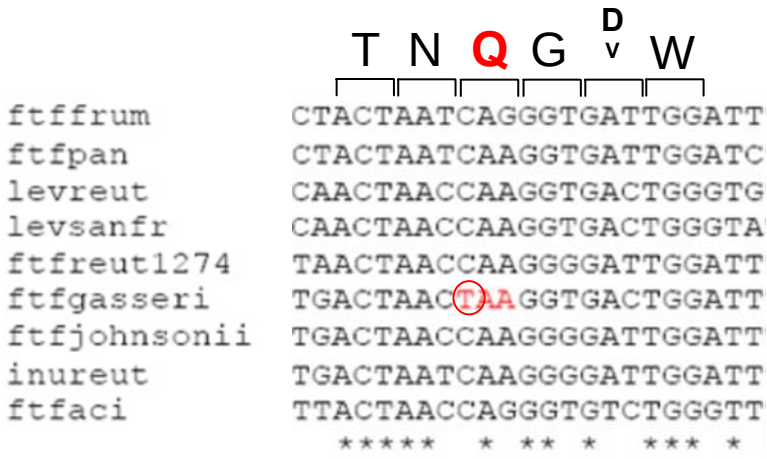


Fig. 10: Section of alignment of several *ftf* gene sequences of *Lactobacillus* origin (*L. frumenti* (ftffrum), *L. panis* (ftfpan), *L. reuteri* levansucrase (levreut), *L. sanfranciscensis* levansucrase (levsanfr), *L. reuteri* TMW1.1274 *ftf* gene fragment (ftfreut1274), *L. gasseri* (ftfgasseri), *L. johnsonii* (ftfjohnsonii), *L. reuteri* inulinsucrase (inureut) and *L. acidophilus ftf* gene fragment (ftfaci). This alignment reveals the point mutation T to C in *L. gasseri ftf* gene causing a stop codon in region highly conserved among lactobacillus *ftf* genes. A correction was possible by using coPCR method.

Therefore in addition to regular cloning primers ftfgass_fw and ftfgass_rev (see above) two additional primers ftfcopcr_fw and ftfcopcr_rev were designed. They were positioned around

the target T base and their sequence was identical to template DNA except the target base which was changed to the designed C nucleotide in the primer. Both primers were identical beside their orientation which was contrary to each other. First step of coPCR were PCRs with ftfgass_fw and ftfcopcr_rev and ftfgass_rev and ftfcopcr_fw using KOD hot start DNA polymerase and *L. gasseri* genomic DNA as template, resulting in two PCR products. These were used as templates for a further KOD PCR with regular cloning primers ftfgass_fw and ftfgass_rev. The result was one PCR product carrying the mutation of T to C in the target position which means the replacement of the stop codon to a glutamine codon which is conserved in all known functional *ftf* genes of lactobacilli.

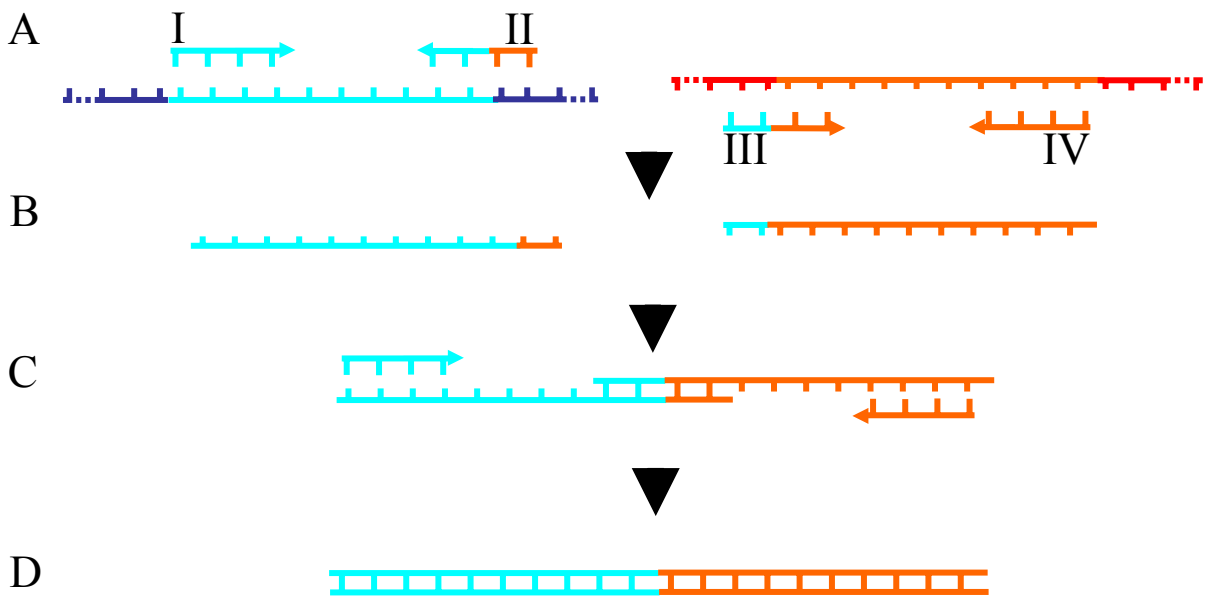


Fig. 11: Cross Over PCR for construction of hybrid proteins and targeted insertion of point mutations. In a first step gene fragments to be assembled were amplified using primers which are completely specific at the future edges of the hybrid genes (I, IV) and for each fragment a primer composed of a specific main part and a tail specific to the sequence to be connected (II, III) was used for PCR (A, B). The resulting PCR fragments served as a template for a subsequent PCR with primers I and IV (C) resulting in a fusion of the two fragments (D).

2.2.3.7 Domain change by crossover PCR

The same technique was used to change domains of *lev* genes of *L. sanfranciscensis* and *L. panis*. Fig. 12 presents the constructs made. Since the exact beginning of the enzymatic active domain is not known, two versions of *L. sanfranciscensis lev* N-terminal domain with *L. panis lev* central and C-terminal domain were made.

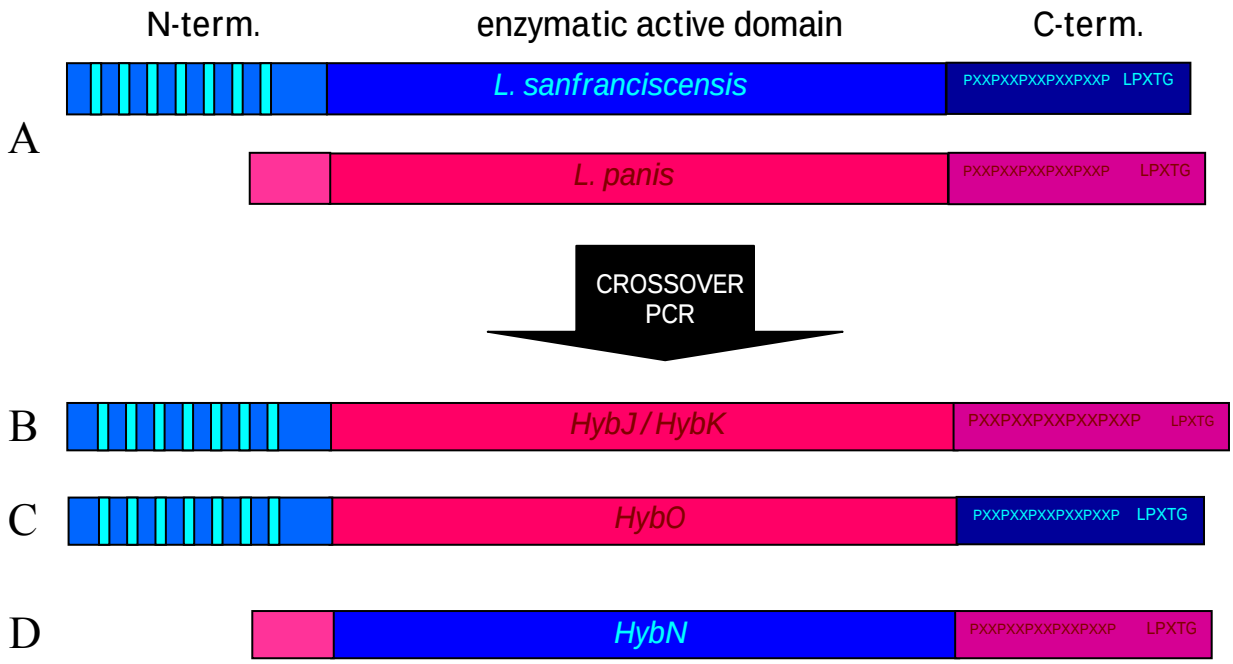


Fig. 12: Schematic view of *ftf* gene hybrid constructs based on *L. sanfranciscensis* levansucrase and *L. panis ftf* gene (A). Constructs HybJ and HybK are composed of enzymatic active and C-terminal domain of *L. panis ftf* gene, short N-terminal was replaced by direct repeat containing N-terminus of *L. sanfranciscensis* levansucrase. Since exact beginning of catalytic domain is not known, two different versions were constructed (B). HybO hybrid was planned as catalytic domain of *L. panis* origin with N- and C- terminus of *L. sanfranciscensis* (C). HybN was developed as counterpart to HybO with *L. sanfranciscensis* levansucrase enzymatic active domain and short N-terminus and C-terminal region of *L. panis ftf* gene.

With two primer sets, N-terminus, central domain and C-terminus of the two levansucrase genes were amplified in respective PCRs with KOD hot start DNA polymerase. The primers on the outer parts (N-terminus forward primers and C-terminus reverse primers) were constructed as cloning primers (see description above). Primers inside the genes were composed of a specific sequence section and an attachment complementary to start of next region of the other gene as shown in fig. 12. The fragments were used as template DNA for two steps of coPCR resulting in the genes for hybrid levansucrases that were subsequently cloned and expressed heterologously in *E. coli*.

2.2.4 Protein chemical methods

2.2.4.1 Expression

E. coli JM 105 strain was transformed with the designated plasmid and cultivated on an agar plate over night. 1 l LB medium containing the respective antibiotic in a 2 l Erlenmeyer flask was inoculated with the cell material of half of the transformation agar plate. The cells were grown up to an optical density at 600nm (OD_{600nm}) of 0.5 at 37°C, 220 rpm. Required cell

amounts were reached after 4 to 6 h. Subsequently expression of target protein was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) addition to a final concentration of 1 mM by adding 1 ml of 1 M sterile filtrated (pore diameter 0.2 µm) stock solution in water (stored at -20°C).

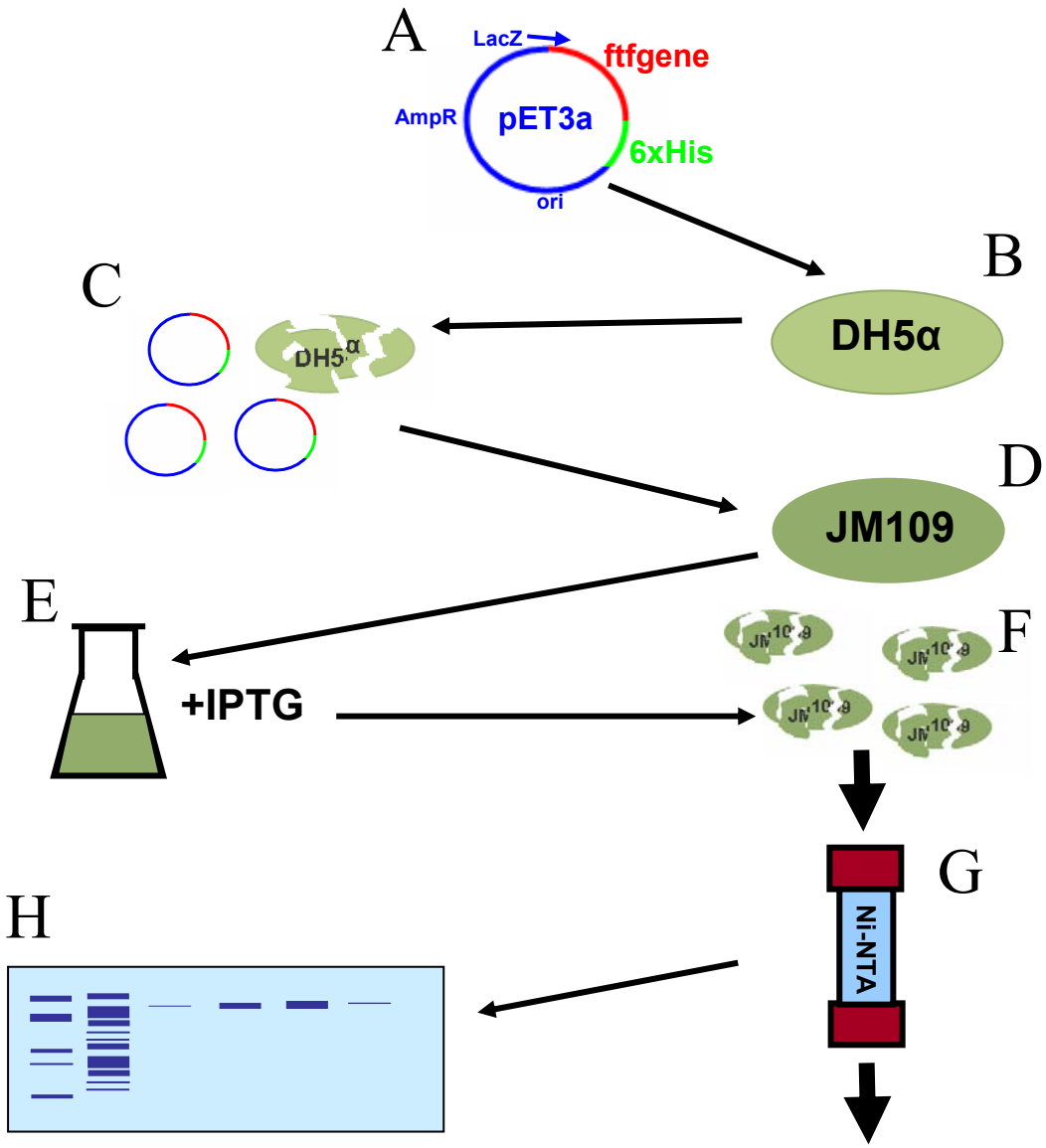


Fig. 13: Flowchart of *ftf* gene cloning and expression strategy: *ftf* gene is ligated in a modified pET3a plasmid vector with an origin of replication, an ampicillin resistance gene, an ITPG inducible LacZ promoter site and an additional C-terminal (6x) His-Tag using BamHI and XbaI restriction sites (A). Plasmids are transformed in chemical competent *E. coli* DH5α cells and positive clones are selected by growth on ampicillin containing agar (B). For expression plasmids checked for correct inserts are gained by plasmid preparation (C) and transferred into chemical competent *E.coli* JM109 cells (D). These transformants are cultivated in 1 l liquid medium; expression is induced by addition of IPTG (E) and harvested by centrifugation. Cell extract is obtained by breaking washed cells using ultrasonification (F) and His-tagged target protein is separated by Ni-NTA affinity FPLC (G). Success of purification is monitored by SDS-PAGE and Coomassie staining (H)

Incubation was progressed at 37°C for 2 h, 220 rpm, then temperature was decreased to 30°C and culture was shaken at 220 rpm over night (13 to 17 hours).

For L. reuteri inulinsucrase cloned in pBAD plasmid, vector DNA was transformed in E.coli Top10 cells. Culture was grown as described above. Expression of cloned gene was induced by adding 1 mM raffinose to culture with OD600nm of 0.5 and further performed as for IPTG induced pET3a clones.

2.2.4.2 Cell harvest and disruption

Cells were harvested by centrifugation at 500 rpm for 30 min at 4°C. Supernatant was discarded. Cells were washed by resuspension in 50 ml of precooled buffer A (application buffer) of His-Trap protocol and centrifugation. Again, supernatant was discarded and cells were resuspended in 10 ml of buffer A. Cell extract was obtained by sonification of cell suspension (cycle 0.5, 90%, 30 sec) in three to four repeats with breaks of at least one minute. During sonification and in between sonification, the suspension was consequently kept on ice. Progress and final result of cell disruption was checked by phase contrast microscopy. Cell fragments were separated from cell extract by centrifugation in two steps: 30 min, 7000 rpm in 15 ml centrifuge tubes and 45 min, 13000 rpm in 2 ml tubes. The clear supernatant was pooled (approximately 10 ml) and stored on ice.

2.2.4.3 FPLC

For FPLC (free pressure liquid chromatography) all buffers and solutions were degassed and sterilized by filtration (pore diameter 0.2 µm) using a vacuum pump. Two 1 ml HisTrap HP affinity columns (Amersham Biosciences) were coupled and equilibrated with at least 10 column volumes of buffer A (application buffer) (20 mM NaH_2PO_4, 500 mM NaCl, 50 mM imidazole, pH 7.4). Cell extract was manually applied in portions of 5 ml (each loading step 10 ml by 1 ml/min). While target protein was bound to column particles due to the His-Tag, residual proteins were flushed out in a wash step of ten column volumes of buffer A (2 ml/min). A gradient from 100% buffer A and 0% buffer B to 0% buffer A to 100% buffer B (elution buffer) (20 mM NaH_2PO_4, 500 mM NaCl, 500 mM imidazole, pH 7,.4) (four column volumes, 2 ml/min) released the target protein of the His-Trap column. A wash step of ten column volumes of buffer B (2 ml/min) regenerated the column by removing of any residual protein. A steep gradient from 0% buffer A, 100% buffer B to 100% buffer A, 0% buffer B with 2 ml at 2 ml/min ends the elution. In the end, the column was equilibrated again by washing with six column volumes of buffer A. To store the His-Trap columns buffer was replaced by 20% ethanol.

During the FPLC process, eluate was collected in 1 ml fractions and process was monitored by UV detection.

Although residual imidazole was contained in the samples, enzyme was applied in further assays as eluted from HisTrap column since imidazole could not be shown to have any influence on enzyme activity. Additionally, when dialyzed there was a strong decrease of protein concentration due to sample volume expansion and up to 90 % of protein was lost when concentrated with Amicon centrifugation tubes after dialysis.

2.2.4.4 Determination of Protein concentration

The fractions potentially containing the target protein were further analyzed by protein concentration determination with Bio-Rad protein assay. Therefore Bio-Rad assay solution was 1:5 in H_2O_{bidest} and filtrated with a fluted filter. For calibration a dilution series of bovine serum albumin (BSA) with concentrations of 0.01 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.075 mg/ml, 0.1 mg/ml, 0.25 mg/ml and 0.5 mg/ml were prepared. Normally protein samples were diluted 1:10 in water. For measurement 200 µl of sample or standard was mixed with 1 ml of prepared assay solution in plastic semi micro cuvettes and incubated for 5 min at room temperature. As reference 200 µl of H_2O_{bidest} was inserted in the assay. OD_{595nm} was determined in relation to reference ($OD_{595nm} = 0$). A straight calibration line was calculated with MS excel software based on the linear range of standard values. By using the resulting slope protein concentrations of the samples were calculated.

2.2.4.5 SDS-PAGE

Protein size and pureness were checked by denaturation, one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Two 12 % polyacrylamide gels were produced as follows. For separating gel, 1.35 ml H_2O_{dest}, 2.5 ml Tris(hydroxymethyl)aminomethane (Tris)-HCL (1.5 M, pH 8.8), 40 µl of 25 % SDS solution and 4 ml of acrylamide/bis 30 % were mixed. For stacking gel 3.05 ml H_2O_{dest}, 1.25 ml Tris (0.5 M, pH 6.8), 40 ml of 25 % SDS solution and 665 µl of acrylamide/bis 30 % were mixed separately. Glass slides were cleaned with 70 % ethanol, assembled and fixed in casting apparatus. 50 µl ammonium persulfate (APS) 10 % in water and 12 µl tetramethylethylendiamine (TEMED) were added to separating gel solution and mixed thoroughly immediately. Gels were casted using 1 ml transfer pipette and overlaid with isopropanol. When gel was polymerized after 45 min isopropanol was removed and APS (50µl) and TEMED (12 µl) were added to stacking gel solution and mixed. Gel was applied on gelled separating gel, comb was inserted in casting sandwich and gel was let gelling for other 45 min.

10 µl of protein samples were mixed with 10 µl of Laemmli buffer (4.58 ml H_2O_{dest}, 1 ml Tris (0.5 M, pH6.8), 920 µl glycerol 87 %, 700 µl SDS 25 %, 400 µl bromphenol blue 1 % and 1.5 g dithiothreitol (DTT)) and incubated at 96°C to 100°C in a heating block for 20 min.

Gel sandwiches were mounted in electrophoresis apparatus, combs were removed and 1x electrophoresis buffer (5x concentrated stock solution: 9 g Tris base, 43.2 g glycine, 3 SDS disodium salt in 600 ml H_2O_{dest}, pH adjusted to 8.3) was added. 10 to 15 µl of samples were applied in gel cavities. For protein size determination a different unstained protein ladders were used.

Separation was started at 60 Volt for 15 min, then voltage was increased to 120 V and gel was run for 90 to 120 min.

Proteins were visualized by a rapid Coomassie staining method (http://www.proteinchemist.com/tutorial/coomassie.htm): Gel was covered in a plastic container with staining solution (40 % methanol, 10 % acetic acid, 50 % H_2O_{dest}, 0.1 %(w/v) Coomassie Brilliant Blue R250) and heated on maximum in a microwave oven for approximately one minute. Than gel was gently shaken on a for at least 10 min. Staining solution was poured out and abundant amount of de-staining solution (40 % methanol, 10 % acetic acid, 50 % H_2O_{dest} or alternatively 20% ethanol, 20 % acetic acid, 50 % H_2O_{dest}) was applied. De-staining could be pushed on by addition of Kim Wipes that take the colour very quickly resulting in visible results after a few minutes. For complete de-staining about two hours of shaking was required. Then gel was washed with H_2O_{dest} and digitalized by scanning for documentation.

2.2.4.6 Western blot

Samples were alternatively checked by Western blot analysis with anti-His-tag antibodies. Therefore and SDS-PAGE was made as described above but a prestained Fermentas (St. Leon-Rot, Germany) protein ladder is used as size marker and gels are not stained.

Separated gels were incubated in precooled (on ice) blotting buffer (10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer, 10 % methanol, pH 11.0) for 20 min at 50 rpm. A nitrocellulose and four slices of filter paper were prepared and equilibrated in blotting buffer. Blotting sandwich was assembled as shown in fig. 14 and semidry blotting apparatus was closed. Proteins were transferred from gel to membrane by a voltage of 20 V and a current of 50 mA (0.8 to 1 mA/cm^2) in 1.5 h.

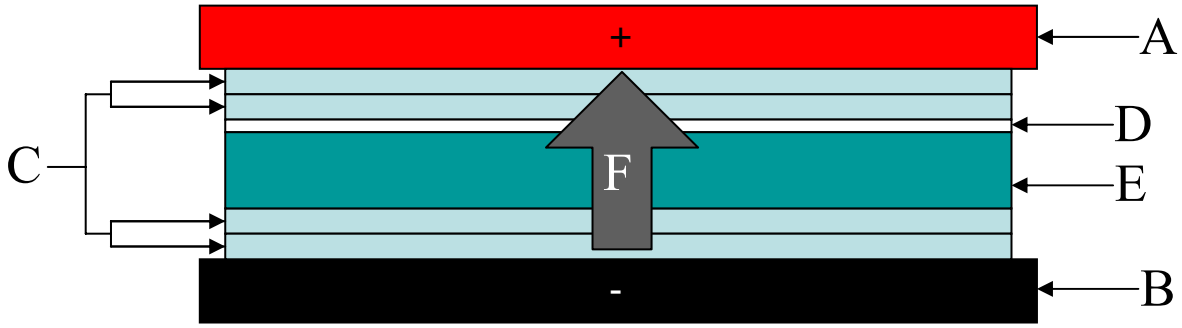


Fig. 14: Schematic view of Western blot assembling: Two layers of blot buffer soaked filter paper (C) bigger than gel and blotting membrane were applied on the cathode plate (B). Polyacrylamide gel with separated protein fractions (E) was placed on these papers avoiding air inclusions. Nitrocellulose membrane (D) tinctured in blotting buffer was applied bubble free on the gel and covered with another two layers of blot buffer soaked filter paper. The blot was closed by the lid of the blotting apparatus containing the anode (A) and tightened with appropriate screws by hand before applying current. Grey arrow (F) is indicating blotting direction.

Subsequently, membrane was washed in TBS buffer (20 mM Tris-HCl, 50 mM NaCl, pH 7.5) for one minute and then incubated in blocking buffer (20 mM Tris-HCl, 150mM NaCl, pH 7.5, 1% BSA) for 1 h at 37°C in a rotating incubation oven, followed by an incubation step in 20 ml TBS buffer containing 5 µl (dilution 1:2000) of primal anti-His-antibody (mouse origin) for 1.5 h at 37°C and three wash steps in TBS-T (20 mM Tris-HCl, 50 mM NaCl, 0.05 % Tween 20, pH 7.5) at room temperature for five minutes each. 12.5 µl (dilution 1:2000) secondary antibody (rabbit-anti-mouse, coupled with alkaline phosphatase) were applied in 25 ml of TBS buffer at 37°C for 1.5 h. Membrane was washed three times in TBS-T and once in TBS (each step 5 min at room temperature) and incubated in 10 ml staining solution (1 M Tris-HCl, 2 M NaCl, 2M $MgCl_2$) containing 66 ml nitro blue tetrazolium chloride (NBT) and 66 µl 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) solution. Development of stained protein bands (30 s to 120 s) was observed visually and reaction was stopped with 3 % trichloroacetic acid (2 min).

2.2.4.7 Renaturating SDS-PAGE and EPS activity staining

For proofing EPS forming activity of purified proteins a renaturating SDS-PAGE method with following periodic acid-Schiff staining (PAS) was used.

Therefore normal 12 % polyacrylamide gels were casted as described above. 10 µl of samples were mixed with 10 µl of Laemmli buffer but, in contrast to regular SDS-PAGE sample preparation, were incubated at 37°C for 2 h resulting in a reversible denaturation of proteins. Gel was run at 4°C at maximum 30 mA to avoid irreversible denaturation during electrophoresis.

For subsequently PAS the gel was washed thrice in sodium acetate buffer (20 mM sodium acetate, 0.3 mM $CaCl_2$, 0.1 % Tween 80, pH 5.3) at 4°C for 10 min to renature proteins by removal of SDS. EPS were produced during incubation in sodium acetate buffer (see above) supplemented with sucrose (10 %) for 72 h at 30°C on a shaker with approximately 50 rpm.
For staining of produced carbohydrate polymers, gel was shaken (55 rpm) in methanol:acetic acid (5:1) for 30 minutes at room temperature and washed in H_2O_{dest} for 30 min (55 rpm). Then gel was treated with periodic acid solution (1% periodic acid, 3% acetic acid) for 45 min at room temperature on a shaker (55 rpm) followed by another washing step (H_2O_{dest} for 2 h, room temperature, 55 rpm). Staining was performed with Schiff's reagent for 8 min. A destaining step with sodium bisulphite (0.5 % sodium bisulphite in water) for 30 min finished the process. Gel was scanned for documentation.

2.2.5 Chromatographic methods

2.2.5.1 TLC

Fructose containing carbohydrates were separated by thin layer chromatography (TLC) and stained by a fructose specific urea-phosphoric acid staining method as described before. (Trujillo Toledo et al. 1996; Ozimek et al. 2006a)
For each sample, 1 µl was applied on a line on TLC plate and dried. Fructose, sucrose, 1-kestose, 1,1-nystose, 1,1,1-kestopetaose and purified *L. sanfranciscensis* levan were used as standards. TLC plate was run twice with butanol:ethanol:H_2O (5:5:3) in a TLC glass container. That means plate was run once, dried and subsequently run once again at least as far as the first run. The procedure took about seven to nine hours.
Dried plate was sprayed with staining spray. Staining spray was produced as follows: 3 g urea were dissolved in 100 ml phosphoric acid in water saturated butanol. 5 ml of ethanol were added to remove water phase appearing when urea is dissolved.
Sprayed plate was incubated at 100°C to 110°C several minutes, until fructose containing sugars appear as blue-black spots. TLC plates were scanned for documentation immediately since colour of staining changes with time.

2.2.5.2 HPLC

Different HPLC columns and protocols were used to separate and detect carbohydrates and organic acids. Experiments were performed with Gynkotek and Dionex HPLC equipment. Carbohydrates were detected with a Shodex RI-101 or a Gynkotec RI detector. Organic acids could be visualized with a Gynkotek or Dionex UV detector.

Chromatograms were recorded and edited by Chromeleon 6.6 software. For identification and quantification of separated substances, external standards in different concentrations were used.

2.2.5.2.1 Merck OAKC column

For detection of single sugars, organic acids and alcohols a Merck OAKC anion exchange column was used. Mobile phase was 5 mM sulphuric acid which was degassed by flushing with helium gas. Column was heated on 75°C. A Varian (Varian Deutschland GmbH, Darmstadt, Germany) guard cartridge Metacarb H Plus (A5215GC) was used to protect the separation column. Constant flow rate was set to 0.4 ml/min resulting in a backpressure of approximately 80 bars. Normally 20 ml of sample were applied. A run takes about 50 min. Column could be cleaned by washing with the regular mobile phase in the reversed direction and slow flow rate of 0.05 to 0.1 ml/min.

Samples for OAKC separation were prepared by a precipitation with perchloric acid. Therefore 15 µl of perchloric acid were ad to 1 ml of sample and incubated at 4°C over night (13 to 17 h). Subsequently samples were centrifuged at 13000 rpm, 4°C for 45 min and supernatant was carefully transferred to HPLC vials. Transfer of potentially invisible pellet material had to be avoided.

Due to perchloric acid treatment and hot sulphuric acid as mobile phase, sucrose and FOS were hydrolyzed and can not be quantified. Maltose appeared to be more stable.

2.2.5.2.2 Shodex NH2P-50 column

For detection and quantification of various carbohydrates a Shodex NH2P-50 column was used. 75 % acetonitrile was used as a mobile phase. Column was heated to 25°C and constant flow rate was set to 1 ml/min resulting in a backpressure of 100 to 120 bars. Mobile phase was degassed by a Gynkotek online degasser. The separation column was protected by a Phenomenex (Phenomenex, Ltd. Aschaffenburg, Germany) Security guard filter cartridge and a short precolumn containing the same material as the separation column. As a general rule, 20ml of sample were applied. Runtime was approximately 40 min. If backpressure increased column could be cleaned by washing with following protocol: 5 ml of H_2O_{bidest} followed by 60 ml of 0.1 M perchloric acid ($HClO_4$), another 5 ml of H_2O_{bidest} and subsequently 60 ml of 0.1 M NaOH. 10 ml of H_2O_{bidest} finished the cleaning. For enhanced separation performance, column should be flushed with 100 mM aqueous ammonium acetate solution pH 9.3 to have a maximum ratio of protonated amino groups.

Samples for NH2P-50 separation were prepared by ethanol precipitation: 600 µl of sample was blended with 400 ml of ethanol and incubated at 4°C at least over night (13 to 17 h).

Precipitated samples were centrifuged at 13000 rpm, 4°C for 45 min and supernatant was carefully transferred to HPLC vials without transferring potential pellet fragments.
Organic acids and alcohols could not be separated by this column. Various single and higher sugars could be detected by using the NH2P50 column in combination with an IR detector.

2.2.5.3 Gel filtration

To analyze differences in molecular weights of fructans, a Superdex 200 column in combination with a RI detector was used. As running buffer a 50 mM Sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl was prepared, sterile filtrated and degassed. Column was stored at 20 % ethanol. So, column had to be equilibrated with running buffer before use. Samples (50 µl each) were applied and eluted with a constant flow rate of 0.5 ml/min resulting in a backpressure of maximal 15 bar. Detection of carbohydrate components was carried out with RI detection.
Samples were prepared by centrifugation (13000 rpm, 45min).

2.2.6 Protein characterization

For exploring characteristic differences in FTF protein activity, different experiments were done.

2.2.6.1 In vitro EPS production

EPS was produced in vitro by adding purified FTF protein to an acetate buffered sucrose solution (500 mM sucrose, 50 mM sodium acetate, 1mM $CaCl_2$ pH 4.0 to 4.5 depending on enzyme optimum). Incubation was carried out between two and ten days at room temperature or 30°C. Produced fructan then could be precipitated with ethanol and harvested by centrifugation as described above.

2.2.6.2 Determination of optimum conditions for enzymatic activity

For new FTF proteins of *L. panis*, *L. frumenti* and modified *L. gasseri* FTF, the optimal temperature and pH was determined.
For examination of pH influence buffers of different pH were used. Since the effective range of a buffering substance was limited, different types of buffer were used to cover a broad pH range. Sodium citrate buffer was used for pH 2.3 to pH 6.6 in the steps pH 2.3, pH 3.2, pH 4.3, pH 5.4 and pH 5.6. Sodium acetate buffer covered the interval of pH 3.8 to pH 5.8 with pH 3.8, pH 4.3, pH 4.6, pH 5.2 and pH 5.8. For pH 6.8 to pH 8.7 Tris buffer was used with samples adjusted to pH 6.8, pH 7.3, pH 7.8, pH 8.1 and pH 8.7. MOPS covered pH values between pH 6.4 and pH 7.6 in steps of pH 6.4, pH 6.7, pH 7.2, pH 7.4 and pH 7.6. Buffering

substance was concentrated 50 mM in assay, sucrose concentration was set to 250 mM and assay buffers contained 1 mM $CaCl_2$.

For assay, 250 µl sucrose containing buffer were prewarmed in a water bath (45°C). 5 µl of 3 M NaOH were prepared in cavities of a PCR plate.

Reaction was started by addition of up to 15 µl of enzyme and thoroughly mixing. First sample (t=0 min) of 50 µl was taken and pipetted into a NaOH containing PCR plate cavity. Low pH due to NaOH immediately stops FTF reaction while reaction products were not affected. The assay was put back in 45 °C water bath as fast as possible. Further samples were taken after 5, 10 and 20 min and stopped the same way.

Glucose and fructose concentration of the samples were determined using R-Biopharm glucose/fructose enzyme kit. This kit enables quantification of D-glucose and D-fructose by a series of enzyme reactions. In a first step the monosaccharides were phosphorylated by the enzyme hexokinase (HK) and the cofactor adenosine-5´-triphosphate (ATP) resulting in adenosine-5´-diphosphate (ADP) and D-fructose-6-phosphate (F-6-P) and D-glucose-6-phosphate (G-6-P) respectively:

$$\text{D-glucose / D-fructose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P / F-6-P} + \text{ADP}$$

Only G6P could be oxidized by nicotineamide-adenine dinucleotide phosphate (NADP) and the catalysator enzyme glucose-6-phosphate dehydrogenase (G6P-DH). Reaction products were D-gluconate-6-phosphate and reduced nicotinamide-adenine dinucleotide phosphate (NADPH):

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

NADPH increased light absorbance at 340 nm and so could be measured by a UV spectrophotometer. Since the amount of NADPH generated by the reaction was stoichiometric to the amount of G-6-P and therewith to the primal amount of glucose, the latter could be determined using a calibration curve.

For determination of fructose, F-6-P has to be converted to G-6-P, a reaction performed by the enzyme phosphor-glucose isomerase (PGI):

$$\text{F-6-P} \xleftrightarrow{\text{PGI}} \text{G-6-P}$$

Since all produced G-6-P was oxidized as described above immediately, in the end all F-6-P was converted and a stoichiometric amount of NADPH was produced. Due to that a further light absorbance at 340 nm could be measured corresponding to original fructan concentration.

Since the amount of available sample was low and many measurements had to be performed, manufacturer's protocol using semi micro cuvettes with an assay volume of 1 l, was scaled down for measuring in microtiter plates. So, 10 µl of sample were mixed with 100 µl of solution one, containing NADP, ATP and magnesium sulphate, buffering the assay to a pH of approximately 7.6. NaOH contained in sample could not negatively influence assay pH. The solution was diluted with 190 µl of H_2O_{bidest}. After incubation of 3 minutes at room temperature, basic OD_{340nm} was measured in a Tecan sunrise plate reader. Reaction was started by adding 2 µl of the first enzyme solution containing HK and G6P-DH in each cavity. OD_{340nm} was measured in 1 min intervals for 35 min to make sure that oxidation of present G-6-P was complete. The last value was relevant for determination of glucose concentration. Subsequently, 2 µl of PGI suspension were added to each assay and OD_{340nm} was monitored as described for another 35 min. The last value was used to determine fructose concentration.

Since the length of the light path was not known when the experiment was performed in a microtiter plate, for determination of absolute concentrations a calibration curve had to be compiled from a dilution series of glucose.

For calculation of glucose concentration, the basic OD_{340nm} value (before addition of enzymes) was subducted from last OD_{340nm} value, for fructose concentration the last value of glucose determination was the basic OD_{340nm} value that was subducted from the last OD_{340nm} value of the second measurement. The resulting difference was divided through the slope of the linear section of calibration curve. Concentrations produced this way were plotted against reaction time resulting in an activity curve of the enzyme. The slope of the linear section of that curve represents the comparable activity of the enzyme.

Activity calculation based on glucose concentration resulted in the over all activity of fructansucrases since the amount of utilized sucrose substrate molecules represents all hydrolase and transferase activities of the enzyme. Based on fructose concentrations, hydrolase activity could be quantified separately since only fructose from hydrolysis could be determined using the enzymatic assay. The residual fructose had been transferred to acceptor molecules forming FOS or fructan polymer. Consequently, the transfer activity could be determined calculating on the difference of determined glucose and fructose concentrations.

Tecan software collected data in an MS Excel file. Subtractions were performed using MS Excel software, slope calculations were performed with SigmaPlot 8.0 software.
All experiments were done at least twice for calculation of mean and standard deviation. These calculations were also done using SigmaPlot 8.0 software.
Also negative controls without the addition of enzyme were measured.

2.2.6.3 Determination of Michaelis Menten kinetic parameters

Michaelis Menten (MM) kinetic and parameters were determined similar to pH and temperature optimum. In a reaction buffer (50mM sodium acetate, optimal pH corresponding to enzyme, 2 mM $CaCl_2$) different concentrations of sucrose were inserted (500 mM, 250 mM, 100 mM, 50 mM, 25 mM, 10 mM, 2 mM, 2.5 mM, 1 mM). Reaction was started by addition of 10 µg of FTF enzyme and further performed as described above at optimal temperature. Glucose and fructose concentrations in relation to reaction time are determined.
Calculation of enzymatic activity values was carried out similar to pH and temperature optimum experiments and resulting figures were plotted against substrate (sucrose) concentration. By the help of SigmaPlot 8.0 software, data were fit to a MM equation and parameters K_M and V_{max} were calculated for overall activity, hydrolysis and transfer activity.

2.2.6.4 Dependency of Ca^{2+} and influence of alternative metal cations

FTFs of *Lactobacillus* origin are described to complex bivalent Ca^{2+} cations (Ozimek et al. 2005) stabilizing their sterical structure and so playing an important role for function and temperature stability. Available FTF enzymes were tested on their dependency of calcium ions and if Ca^{2+} can be replaced by alternative bivalent metal cations.
Therefore 10 to 20 µl (depending on concentration) were mixed with 50 µl of aqueous 125 mM EDTA solution and another volume of enzyme was mixed with H_2O_{bidest}. All samples were incubated for 5 min at room temperature. Then 250 µl of buffer (50mM sodium acetate, pH optimal for enzyme) containing 100 mM of the test cation (Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, K^{+}) are added. Buffer without any additional cations served as a control. Cations were applied as chlorides and pH of buffer was adjusted with HCl and NaOH, since sodium was contained in every buffer due to the insertion of acetate as sodium salt.
The mixture was incubated for another 5 minutes at room temperature. Then enzyme reaction was started by adding 100 µl of 1 M sucrose solution and activity was determined as described for pH and temperature optimum experiments above.

2.2.6.5 Different reaction products and their ratios

The influence of the different factors substrate concentration, pH and reaction temperature on products formed by FTF enzymes from sucrose and their ratios was examined.

300µl samples were prepared. Standard conditions (50mM sodium acetate, pH 4.6, 2 mM $CaCl_2$, 500 mM sucrose, 45°C) were varied as follows:

Sucrose concentrations: 50 mM , 100 mM, 250 mM, 500 mM

pH: 3.8, 4.3, 4.6, 5.2

Incubation temperature: 30°C, 37°C, 45°C

Samples were prepared and reaction was started by addition of 3.6 µg of FTF enzyme. To negative control samples, nothing was added. Samples were incubated for three days at the respective temperature.

The samples were subsequently analyzed by TLC (specific staining of fructose containing sugars and HPLC (NH2P-50 column)). Concentrations were quantified by calculation of peak areas and a dilution series of sucrose, fructose and glucose as well as dilution series of 1-kestose, which deliver a calibration curve. Sample peak area was divided through slope of respective calibration curve to obtain concentration. For absolute sugar concentration this value was multiplicated with dilution factor 1.5 derived from sample preparation (EPS precipitation by ethanol addition). Based on a simplified reaction equation, the amount of fructose moieties bound in fructan is defined as difference between the amount of free glucose and the sum of free fructose and 1-kestose:

[Fructose (fructan)] = [Fructose (total utilized)] – ([Fructose (free)] + [Fructose (FOS)])

with: $x\text{Sucrose} \xrightarrow{\text{FTF}} y_1\text{Sucrose} + y_2\text{Glucose} + a\text{Fructose} + b\text{FOS} + c\text{Fructan}$

and: $x = y_1 + y_2$

$y_2 = a + b + c$

[Fructose (total utilized)] = [Glucose (free)]

[Fructose (FOS)] = [Fructose (kestose)] + [Fructose (nystose)]

[Fructose (fructan)] = [Glucose (free)] – ([Fructose (free)] + [Fructose (kestose)] + [Fructose (nystose)])

Since FTF enzymes do not show identical activity at different experimental conditions, fructose containing reaction products were used in relation to total sucrose utilized, represented by concentration of free glucose for comparison.

2.2.6.6 Alternative acceptor molecules and raffinose utilization

The formation of heterooligosaccharides of sucrose and various acceptor carbohydrates was examined. Therefore, to a standard assay buffer (50mM sodium acetate, pH 4.6, 2 mM $CaCl_2$, 250 mM sucrose) 250 mM of different sugars were added. These were maltose, xylose, arabinose, galactose, lactose, mannose, trehalose and rhamnose were offered as acceptor molecules in FTF reaction. Negative controls without enzymes were performed in parallel.

For monitoring of raffinose utilization as fructose donor, 250 mM raffinose was added as single carbohydrate to a standard assay buffer (50mM sodium acetate, pH 4.6, 2 mM $CaCl_2$).

Reaction in 500 µl samples was started by addition of approximately 6 µg FTF enzyme and incubated at 42°C for three days.

Samples were analyzed by TLC and specific staining of fructose containing carbohydrates.

3 Results

3.1 EPS production in various lactobacillus strains and screening for *ftf* genes

33 *Lactobacillus* strains from the TMW strain collection were involved in metabolic screening for EPS production from sucrose. On agar plates containing sucrose as a sole carbon source, effective fructan production could be detected for *L. sanfranciscensis* (1.392), *L. suebicus* (1.44), *L. kunkeei* (1.1258), *L. frumenti* (1.666), *L. mucosae* (1.81) and *L. panis* (1.648). Although presenting the slimiest appearance on agar plates, in contrast to the other EPS positive strains, EPS production by *L. mucosae* could not be proven by gel filtration experiments based on liquid cultures in mMRS Sac80 broth. Further analysis of metabolic products by HPLC for selected strains was performed and hydrolysis rates were calculated as quotient of produced mannitol and metabolized fructose. Results are shown in tab. 6.

Strain	TMW	EPS-Production	Hydrolysis rate
L. sanfranciscensis	1.392	+++	38%
L. frumenti	1.103	-	60%
L. frumenti	1.666	+	80%
L. reuteri	1.1274	-	100%
L. panis	1.648	-	96%
L. fructosus	1.8	-	40%

Tab. 6: Hydrolysis rates calcuated on basis of HPLC analysis of metabolic products. Hydrolysis rates are quotients of produced mannitol and metabolized fructose.

The PCR screening with degenerated primer pairs levV and levR or lev_for and lev_rev respectively included 45 *Lactobacillus* strains from TMW strain collection (see tab. 7).

Species	TMW	Origin	Lev_for + Lev_rev	LevV + LevR	Physiology
L. acetotolerans	1.1244	DSM 20749	/	800bp	fak.hetero.
L. acidipiscis	1.1245	JMC 10692	/	/	fak.hetero.
L. agilis	1.803	DSM 20509	/	/	fak.hetero.
L. alimentarius	1.11	DSM 20249	830bp	1000bp	fak.hetero.
L. animalis	1.806	DSM 20602	/	/	fak.hetero.
L. bifermentas	1.1249	DSM 20003	/	/	fak.hetero.
L. casei	1.14	DSM 20011	/	/	fak.hetero.
L. coleohominis	1.1207	DSM 14060	/	/	fak.hetero.
L. coryniformis	1.1251	DSM 20001	/	/	fak.hetero.
L. coryniformis	1.1167		/	/	fak.hetero.
L. hamsteri	1.1254	DSM 5661	/	/	fak.hetero.
L. intestinalis	1.1256	DSM 6629	/	/	fak.hetero.
L. kimchii	1.257	DSM 13961	/	/	fak.hetero.

L. murinus	1.1260	DSM 20452	/	/	fak.hetero.
L. paralimentarius	1.256	DSM 13238	/	/	fak.hetero.
L. pentosus	1.10	DAM 20314	/	/	fak.hetero.
L. plantarum	1.9		/	/	fak.hetero.
L. sakei	1.1189		/	600bp	fak.hetero.
L. spicheri	1.262	DSM 15429	/	/	fak.hetero.
L.brevis	1.57	DSM 20054	/	/	hetero.
L. parabuchneri	1.429		/	2x<500bp(weak)	hetero.
L. fructivorans	1.1253	DSM 20203	/	/	hetero.
L. fructivorans	1.59		/	/	hetero.
L. fructosus	1.8	DSM 20349	1000bp	800bp	hetero.
L. frumenti	1.666		/	/	hetero.
L. hilgardii	1.45	DSM 20176	/	/	hetero.
L. kefiri	1.36	DSM 20587	/	/	hetero.
L. kunkeei	1.1258	DSM 12361	/	/	hetero.
L. lindneri	1.88	DSM 20690	/	/	hetero.
L. mucosae	1.81	LTH 3566	940bp	/	hetero.
L. oris	1.1143	DSM 4864	/	/	hetero.
L. panis	1.648	DSM 6035	800bp	1000bp	hetero.
L. pontis	1.397		/	/	hetero.
L. reuteri	1.1274	4020	1000bp	800bp	hetero.
L. reuteri	1.693	DSM 20016	/	1000bp	hetero.
L. sanfranciscensis	1.392		830bp	800bp	hetero.
L. vaginalis	1.1144	DSM 5837	/	/	hetero.
Ln. mesenteroides	2.48	DSM 20484	/	/	hetero.
L. suebicus	1.44		/	/	hetero.
L. spec.(pastorianus)	1.71	DSM 20197	/	/	hetero.
L. acidophilus	1.18		/	/	homo.
L. algidus	1.1246	Japan 10491	/	/	homo.
L. amylolyticus	1.487		/	/	homo.
L. amylophilus	1.428	DSM 20553	1000bp+570bp	1500bp+570bp	homo.
L. amylovorus	1.694	DSM 20531	<500bp	<500bp	homo.
L. arizonensis	1.1248	DSM 13273	820bp	830bp	homo.
L. crispatus	1.1144	C16	/	/	homo.
L. delbrückii	1.72	DSM 20074	/	/	homo.
L. farciminis	1.68	DSM 20184	/	/	homo.
L. helveticus	1.1176	DSM 20075	/	/	homo.
L. helveticus	1.1176		/	/	homo.
L. jensenii	1.1257	DSM 20557	/	/	homo.
L. johnsonii	1.1179	DSM 10533	/	/	homo.
L. manihotivorans	1.1259	DSM 13343	/	/	homo.
L. manihotivorans	1.126		/	/	homo.
L. ruminis	1.1261	DSM 20403	/	/	homo.
L. ruminis	1.804		/	/	homo.
L. salivarius	1.810	DSM 20554	/	/	homo.
L. sharpeae	1.1262	DSM 20505	/	>500bp	homo.
L. vesmoldensis	1.1215	DSM 14857	/	/	homo.
L. bavaricus	1.5		3000bp	2500bp	homo.

Tab. 7: Results of PCR screening for *ftf* genes with two pairs of degenerated primers

In 9 strains PCR products in the expected size of 600 to 1000 bp for a homologous *ftf* gene were amplified. These first results could only be repeated for 6 strains, namely *L. reuteri* TMW 1.1274, *L. panis* TMW 1.648, *L. mucosae* TMW 1.81, *L. parabuchneri* TMW 1.429, *L. frumenti* TMW 1.103 and *L. sakei* 1.1189. For *L. reuteri* 1.1274, *L. frumenti* 1.103 and *L. panis* 1.648, sequence analysis and BLAST search revealed that the amplified fragments share strong homologies with known genes coding for fructosyltransferases in lactobacilli and other LAB, showing more similarity with levansucrases than inulinsucrase genes.

Database search also revealed a fragment of an *ftf* gene in further *Lactobacillus acidophilus* TMW 1.987 isolated from duck faeces, and a complete *ftf* gene in the genome sequenced *L. gasseri* strain ATCC 33323 comprised in TMW strain collection with number TMW 1.1173. Translation of this gene with Expasy translation tool revealed a stop codon inside this gene and so no functional gene product could be expressed.

3.2 Exploration of new *ftf* gene sequences of lactobacillus origin and sequence analysis

Since sequence fragments of *L. panis* 1.648, *L. frumenti* 1.103 and *L. reuteri* 1.1247 obtained by PCR with degenerated primer pairs revealed high homologies to known *ftf* gene sequences, iPCR to gain knowledge of complete gene sequences was performed.

Extended *ftf* gene sequences for *L. panis* and *L. frumenti* could be obtained by this method.

For *L. reuteri* TMW 1.1274 and *L. acidophilus* TMW 1.987, described Southern blot and plasmid-insertion methods were established but did not lead to a successful expansion of respective *ftf* gene.

Amino acid sequences of known FTFs of lactobacilli and new, completed sequences as well as aa sequences of *Leuconostoc citreum* inulinsucrase and non LAB *B. subtilis* levansucrase SacB are aligned with BLAST algorithm and alignment is reduced to a segment covered by all sequences for calculation of a phylogenetic tree shown in fig. 15 (for alignment see appendix). Levansucrase sequence of *B. subtilis* forms an isolated branch while enzymes of LAB origin have a common branch start. Nevertheless at 0.1 this stem is branched between *L. citreum* and the proteins of lactobacilli. Uncharacterized *L. johnsonii* FTF forms a cluster with inulin producing FTF of *L. gasseri* and less closely related with inulinsucrase of *L. reuteri*. Next close relationship was found to the fragment of *L. reuteri* TMW 1.1247 FTF. A second big group is formed by the closely related couples of *L. sanfranciscensis* and *L. reuteri* levansucrases and *L. frumenti* and *L. panis* levan producing enzymes. The fragment of *L. acidophilus* FTF is next closely related to that cluster. The basic tree topology and the grades of relationship of the proteins does not change if complete sequences without the two short

fragments of *L. acidophilus* and *L. reuteri* TMW 1.1247 FTFs are compared (data not shown). Nevertheless it has to be mentioned that the protein relations became more far when longer sections are compared.

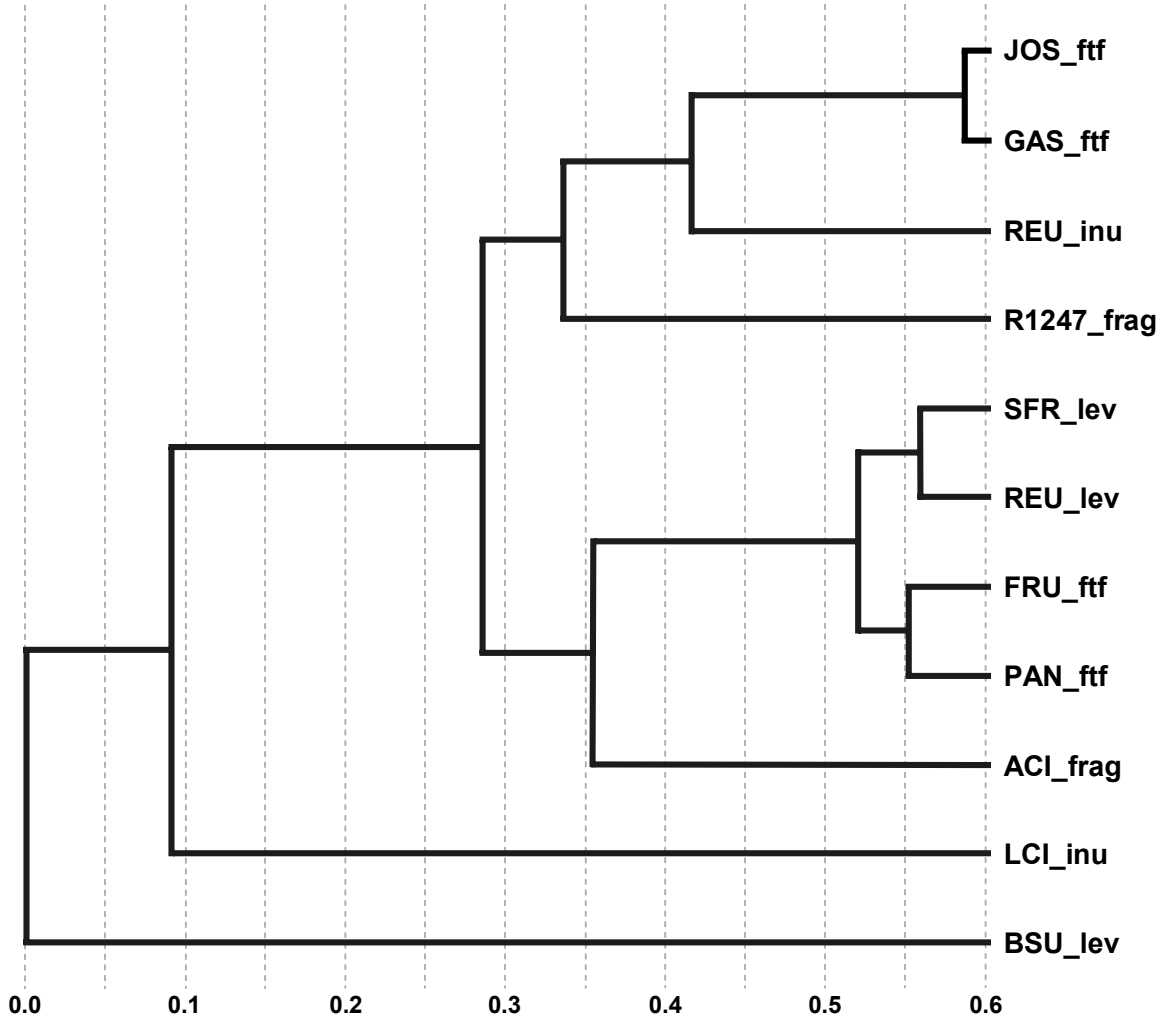


Fig. 15: Phylogenetic tree calculated on basis of an alignment of a central section of aa sequences of different FTF enzymes. Known lactobacillus FTFs *L. reuteri* inulin- (REU_inu) and levansucrase (REU_lev) as well as *L. sanfranciscensis* levansucrase (SFR_lev) and uncharacterized *L. johnsonii* FTF (JOS_ftf) and inulin producing *L. gasseri* enzyme (GAS_ftf) are compared with new complete sequences of *L. panis* (PAN_ftf) and *L. frumenti* (FRU_ftf) FTFs and fragments of *L. acidophilus* (ACI_frag) and *L. reuteri* TMW 1.1247 (R1274_frag) FTF fragments. For further comparison alignment also includes *Leuconostoc citreum* inulinsucrase (LCI_inu) and non LAB *B. subtilis* levansucrase (BSU_lev) sequences.

For *L. panis* a 763 bp core sequence of the *ftf* gene was obtained with degenerated primers Lev_for and Lev_rev from chromosomal DNA of *L. panis*. The complete sequence of the *ftf* gene of 2424 bp was obtained after two rounds of inverse PCR based on a core sequence, revealed with degenerated primers, and sequencing of the PCR products. The *ftf* gene encodes a putative 808 amino acid (aa) protein with a predicted relative molecular weight (M_W) of

87.802 kDa and a predicted pI of 4.50. Blast research showed highest similarities with a levansucrase from *L. sanfranciscensis* (70% identities and 79 % positives in 845 aa).

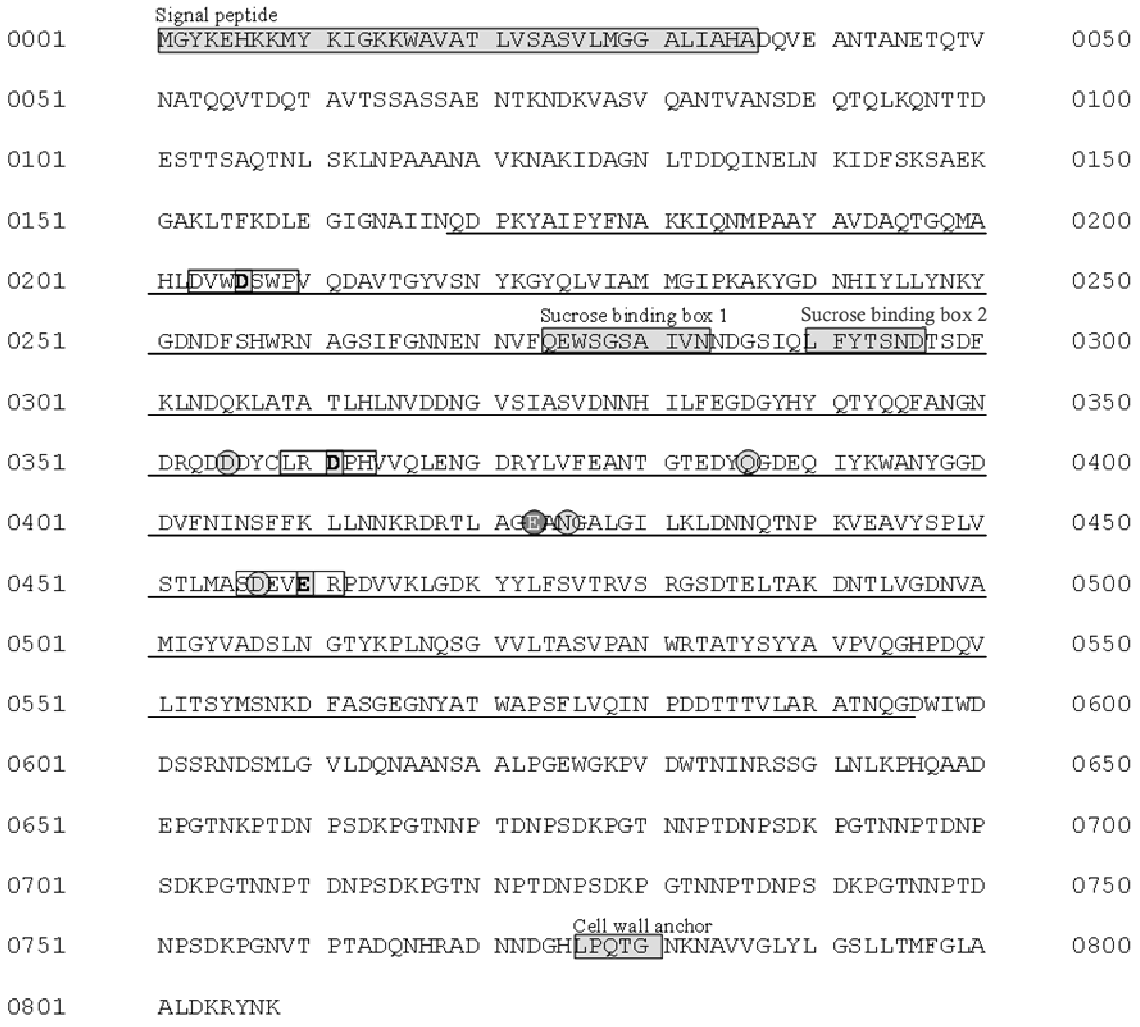


Fig. 16: Amino acid sequence of *L. panis* FTF open reading frame. In the sequence the signal peptide, the two sucrose binding boxes (conserved in sucrose utilizing enzymes) and the LPXTG-motive are marked with big grey boxes. The three amino acids belonging to the catalytic triad (Asp206, Asp361 and Glu460) are marked with small grey boxes. The regions around (white boxes) are strongly conserved in fructosyltransferases of lactobacilli. Residues potentially complexing a Ca^{2+} ion are marked with circles. The light grey marked residues (Asp355, Gln386, Asn425 and Asp475) are conserved in all known *lactobacillus* fructosyltransferases. The aberrant residue Glu423 is marked in a dark grey circle. Underlined region is used in tertiary structure prediction presented in fig. 17.

An overview over amino acid sequence of *L. panis* FTF is presented in fig. 16. Based on the aa sequence of this protein four regions were determined: (1) SignalP software could clearly identify an N-terminal signal peptide (Bendtsen et al. 2004). (2) A small variable region of about 50 amino acids followed by (3) the enzymatic active domain which is very similar to the ones of known levansucrases. BLAST software could identify two sucrose binding boxes

conserved among sucrose utilizing enzymes. Alignments with further FTF sequences reveal the presence of the known conserved domains and the potential residues of the catalytic triade could be identified (catalytic nucleophile: Asp206, transition state stabilizer: Asp361 and acid base catalyst: Glu460). In addition to that four of five aa residues proposed to complex a bivalent calcium cation are conserved (Asp355, Gln386, Asn425 and Asp475). Finally (4) a C-terminal cell wall anchoring region containing PXX repeats and a conserved LPXTG motif. Based on this sequence a tertiary structure prediction is carried out with CPHmodel software. After alignment 428 aa (see fig. 16) are adapted to a fold recognition/homology based 3 dimensional model presented in fig. 17.

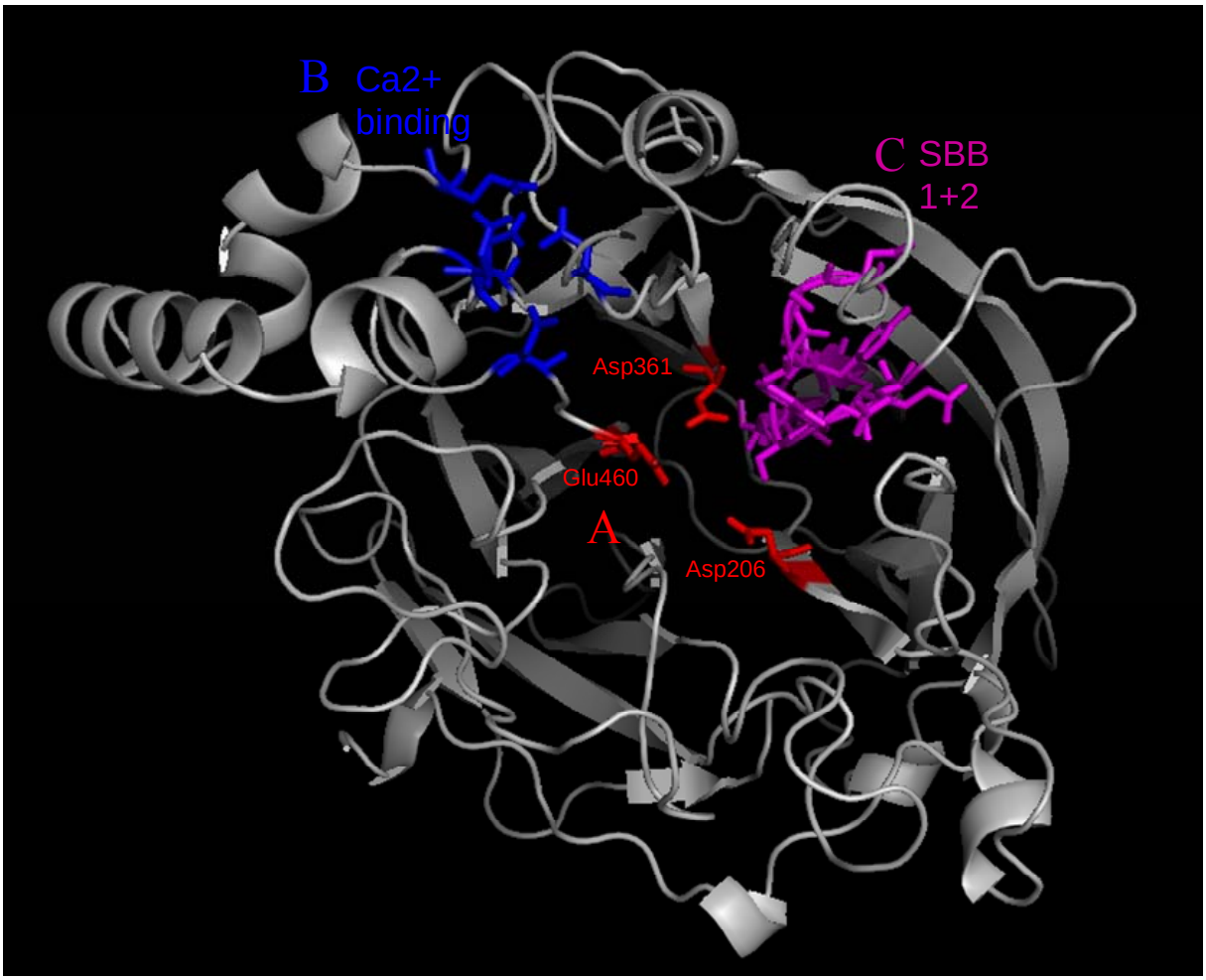


Fig. 17: Tertiary structure model of *L. panis* FTF calculated with CPHmodel software. A five bladed β-propeller topology with a deep cavity is the basic structure. Inside the cavity three aa residues being part of the catalytic triade are presented as red stick models (A): catalytic nucleophile Asp206, transition state stabilizer Asp361 and acid base catalyst Glu460. Blue coloured aa are potentially involved in complexion of a Ca^{2+} cation (B). Purple residues are identified being part of sucrose binding boxes 1 and 2 (C).

Molecular modelling excludes the short N-terminal variable domain and part of N-terminal residues of the catalytic core as well as a section of the C-terminal catalytic domain and the complete C-terminus. The model locates the aa residues involved in catalytic triade deep inside a cavity formed by a five bladed β-propeller topology that is closed at the bottom by a loop without a recognizable secondary structure. Dimension of cavity and distances between catalytic residues would enable binding of a sucrose molecule (not shown). Sucrose binding boxes 1 and 2 are located close together also inside the cavity. Although potential Ca^{2+} complexing aa residues are not found side by side in primary structure, they form a compact domain in 3 dimensional structure model. C-terminal aa form an α-helix structure in *B. subtilis* levansucrase x-ray model (see fig. 20-D). That secondary structure element can not be reconstructed using *L. panis* aa sequence as a template.

```
0001      ADQVTTNSDN AVVTNTASQP TGSSSDVTSN TSTSPSTITG AVQVQLKQST      0050
0051      NVDTVPSENN QKKLNPSAAQ AVNNAHINVN NLTEDQINEL NKINFSKSAE      0100
0101      KGAKLTFKDL EGIGNAIVKQ DPRYTIPYFN AKEINNMPAA YAVDAQTGKM      0150
0151      AHLDVWDSWP VQDAVTGYVS NYKGYQLVIA MMGIPNTPYG DNHIYLLYNK      0200
                                  Sucrose binding box 1   Sucrose binding box 2
0201      YGDNDFSHWR NAGSIFGNKE NNVYQEWSGS AIVNNDGSIQ LFYTSNDTSD      0250
0251      FKLNDQKLAT ATLHLNVDDN GVSIASVDNN HVIFEGDGYH YQTYQQFAEG      0300
0301      KDRKDDYQL RDPHVVQLTN GDRYLVFEAN TGKEDYQSDE QIYNWANYGG      0350
0351      DDAFNIKSFF KLLNNKKDRE LAGLANGSLG ILKLNNNQDN PEVDEVYSPL      0400
0401      VSTLMASDEV IERPDIVKLGG KYYLSSVTRV SRGSDTELTQ KDNATVGDNV      0450
0451      AMIGYVADNL MGPYKPLNNS GVVLTASVPA NWRTATYSYF AVPVEGHPDQ      0500
0501      VLITSYMSNK DFASGKGNYA TWAPSFLVQI NSDNTTMVLA RATNQGDWIW      0550
0551      DNSSRNDKML GVLDKNAANS AALPGEWGKP VDWSLINRSA GLGLKPHQGA      0600
0601      DPSEQPNNKP GTNPGNKPGT NPDNKPGDKP GTNPDNKPGD KPGTNPDNIP      0650
0651      GNNPGLNPRE TPN
```

Fig. 18: Amino acid sequence of *L. frumenti* FTF. In the sequence the two sucrose binding boxes (conserved in sucrose utilizing enzymes) are marked with big grey boxes. The three amino acids belonging to the catalytic triad (Asp157, Asp312 and Glu411) are marked with small grey boxes. The regions around (white boxes) are strongly conserved in fructosyltransferases of lactobacilli. Residues potentially complexing a Ca^{2+} ion are marked with light grey circles. Underlined region is used in tertiary structure prediction presented in fig. 19.

Trying different methods of gene identification, sequence *L. frumenti ftf* gene could only be extended by iPCR resulting in a 1994 bp fragment covering the important regions of the gene coding for a protein of 663 aa with a predicted M_W of 72.374 kDa and a predicted pI of 4.83 in an uninterrupted reading frame. In BLAST search the fragment showed highest similarities with *L. reuteri* levansucrase (79% identities, 86% positives in 622 aa).

Further expansion of sequence information was unsuccessful. The identified sequence did not contain a signal peptide or an LPXTG cell wall anchor structure. Nevertheless, the essential residues of the catalytic triade could be identified (catalytic nucleophile: Asp157, transition state stabilizer: Asp312 and acid base catalyst: Glu411) inside highly conserved sections, as well as five conserved residues possibly involved in Ca^{2+} complexion (Asp306, Gln337, Leu374, Asn377 and Asp408).

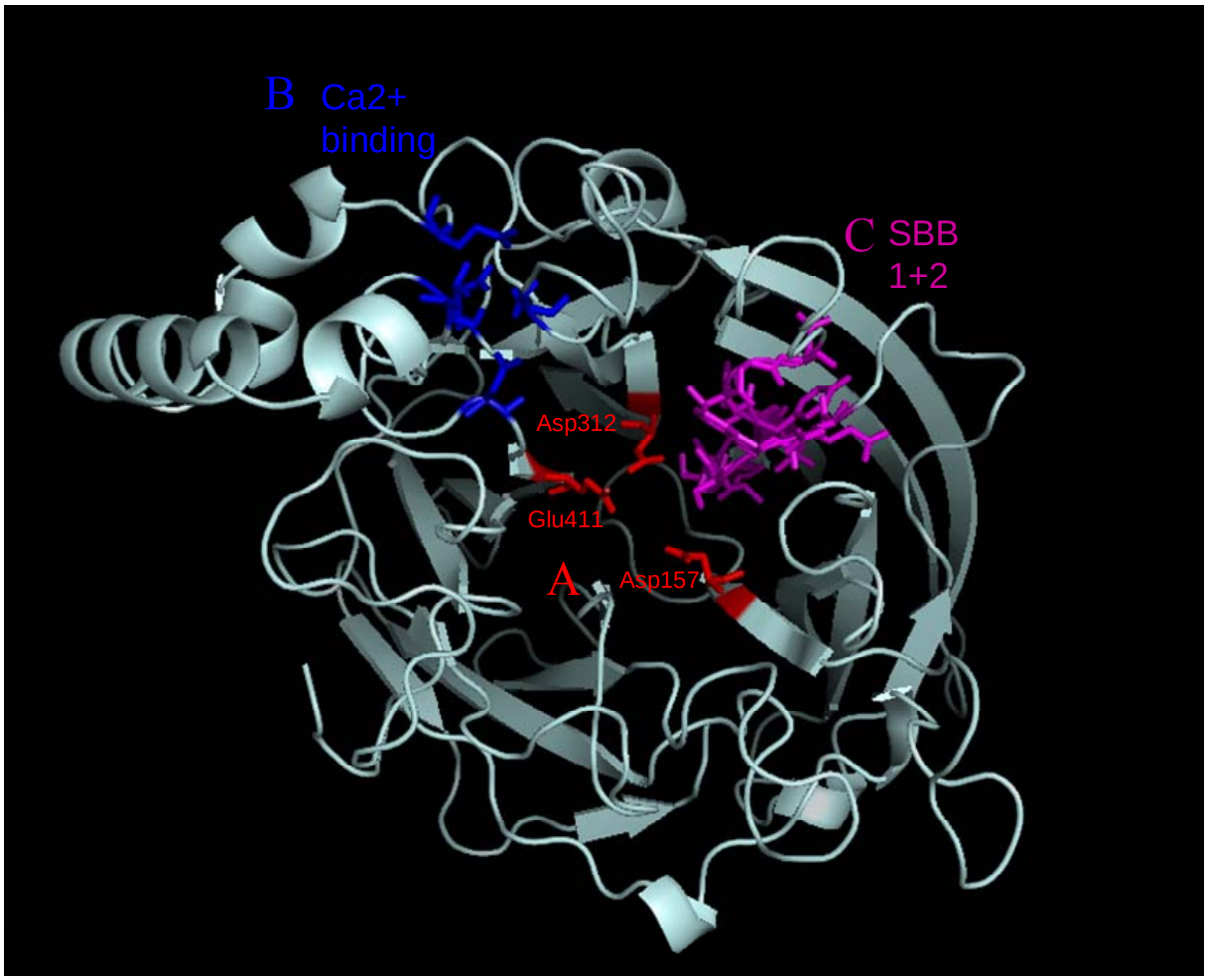


Fig 19: Three dimensional model of *L. frumenti* FTF calculated with CPHmodel software. A five bladed β-propeller topology with a deep cavity is the basic structure. Aa residues being part of the catalytic triade are presented as red stick models inside the cavity (A): catalytic nucleophile Asp157, transition state stabilizer Asp312 and acid base catalyst Glu411. Blue coloured aa are potentially involved in complexion of a Ca^{2+} cation (B). Purple residues represent sucrose binding boxes 1 and 2 (C).

Both sucrose binding boxes are present without significant modifications from the conserved consensus sequence. An overview of the sequence fragment is shown in fig. 18.

Since alignments with known *ftf* genes of lactobacilli revealed that the established sequence starts directly behind a potential, unknown signal peptide and ends in a prolin rich region comparable to the one of the other enzymes, the complete sequence was cloned and expressed in pET3a plasmid system.

Tertiary structure prediction using CPHmodel software resulted in a similar structure to *L. panis* FTF. Sequence fragment of *L. frumenti* could be folded to a five fold β-propeller topology with the amino acids of catalytic triade inside a cavity that additionally hosts both sucrose binding boxes that can be found a close steric conformation. A potential binding site for bivalent calcium cations also could be identified.

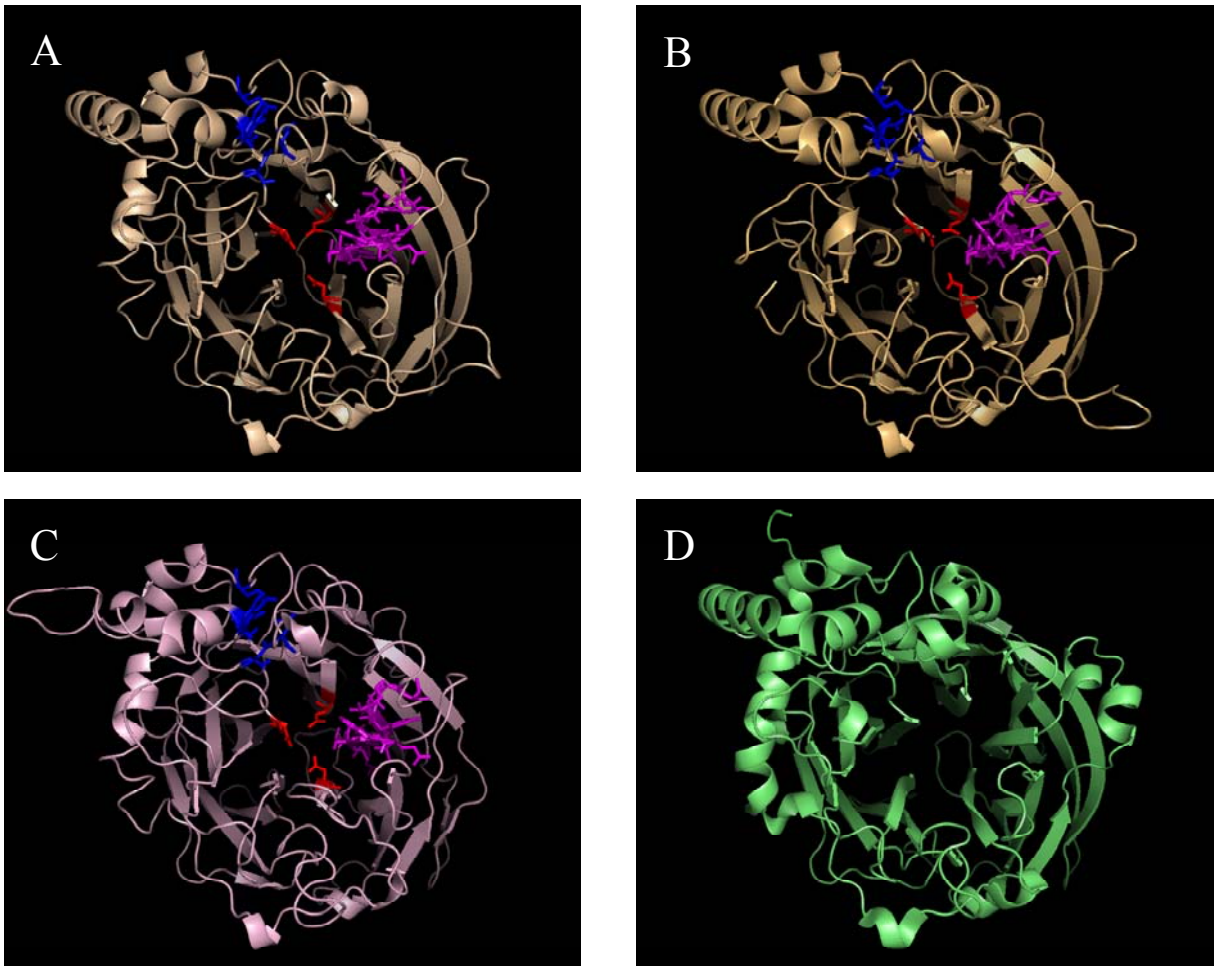


Fig. 20: Tertiary structure models of *L. reuteri* inulinsucrase (A), *L. sanfranciscensis* levansucrase (B) and *L. gasseri* modified FTF (C) developed with CPH-model software. As in figures 17 and 19, respective aa residues which are part of the catalytic triade are presented as red stick models, sucrose binding boxes are in purple and residues potentially involved in Ca^{2+} complexion are in blue. The green model (D) is the x-ray based model of *B. subtilis* levansucrase.

In analogy to *L. panis* FTF C-terminal aa do not form an α-helical secondary structure as found in *B. subtilis* levansucrase structure (fig. 20-D). Tertiary structure model is presented in fig. 19.

Tertiary structure prediction using CPH model software was also performed for already known aa sequences of *L. reuteri* inulinsucrase, *L. sanfranciscensis* levansucrase and the modified *L. gasseri* FTF delivering similar results as for *L. panis* and *L. frumenti* ftf (fig. 20). As for the models described above, only a central section of the sequence could be used for molecular modelling in comparison with x-ray structure of *B. subtilis* levansucrase. In all cases the basic five bladed β-propeller topology could be constructed harbouring the aa residues for the catalytic triade and the two sucrose binding boxes in very similar configuration. In addition to that the aa residues for Ca^{2+} complexion are positioned sterically close together. The C-terminal α-helix found in *B. subtilis* levansucrase can be reconstructed for the inulin producing enzymes of *L. reuteri* and *L. gasseri* but not with *L. sanfranciscensis* levansucrase sequence. Therefore, in *L. gasseri* protein a further helix structure element close to Ca^{2+} binding site does not occur in the calculated model. The respective sequence section is presented as an unstructured loop protruding from the globular structure of the protein. In addition to that, the β-sheet motif on the opposite site of that loop is smaller in *L. gasseri* FTF than in the other proteins.

3.3 Cloning and heterologous expression of *ftf* genes of *L. panis*, *L. frumenti* and a modified *L. gasseri ftf* gene

ftf genes of *L. panis*, *L. frumenti* and *L. gasseri* were cloned in pET3a plasmid vector system by using XbaI and BamHI restriction sites. *L. sanfranciscensis* levansucrase ftf gene in pET3a plasmid was already was existing and *L. reuteri* inulinsucrase was supplied by a Dutch work group cloned in raffinose inducible pBAD plasmid and transformed in *E.coli* Top10 cells (van Hijum et al. 2002). A Tag of six His residues was fused to the proteins within cloning. For *L. gasseri* ftf an artificial coPCR product with a modified base was cloned since expression of native gene did not produce a functional gene product. The original T 1894 was changed to a C to eliminate a stop codon inside the open reading frame. Correct insertion of the inserts was proven by sequencing with T7 and T7rev standard primers located on the pET3a plasmid after restriction digest of isolated vector DNA with BamHI and XbaI resulting in fragments of the expected sizes.

Expression of genes in *E. coli* JM109 expression strain resulted in an apparent protein peak when target protein is eluted from the His Trap column. The collected fractions of that peak reach protein concentrations up to 2 mg/ml. In different repetitions of expression experiments the yield of protein showed strong variations.

Coomassie stained SDS-PAGE of elution fractions revealed a significant amount of protein at the expected molecular weight. Nevertheless, in addition to that several smaller proteins are stained although less intensive, indicating a lower concentration of these contaminations.

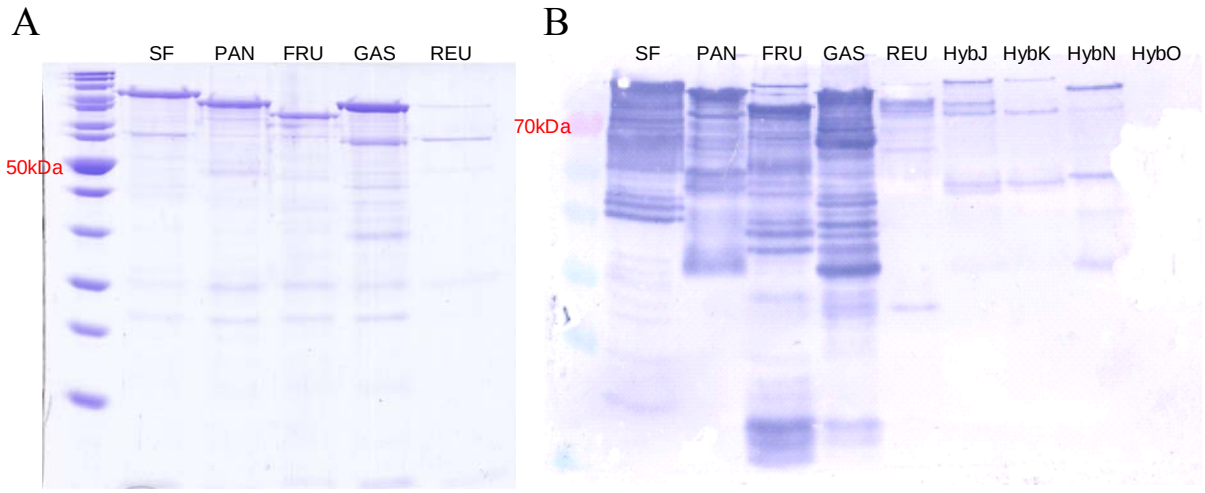


Fig. 21: Coomassie stained SDS-PAGE (A) and His-tag specific Western blot (B) of His-trap purified heterologously expressed FTF proteins of *L. sanfranciscensis* levansucrase (SF), *L. panis* FTF (PAN), *L. frumenti* FTF (FRU), *L. gasseri* modified FTF (GAS), *L. reuteri* inulinsucrase (REU) and *L. sanfranciscensis*-*L. panis* hybrid FTF proteins HybJ, HybK, HybN and HybO. As MW standards Page Ruler™ Unstained Protein Ladder (MBI Fermentas GmbH, St. Leon-Rot, Germany) (A) and PageRuler™ Prestained Protein Ladder (MBI Fermentas GmbH, St. Leon-Rot, Germany) (B) are used.

Western blot analysis also shows contamination of target protein with smaller protein fractions.

Schiff stained EPS produced by separated and renatured proteins in polyacrylamide gels proved activity of FTF enzymes at correct molecular weight whereas smaller proteins do not produce sugar polymers from sucrose.

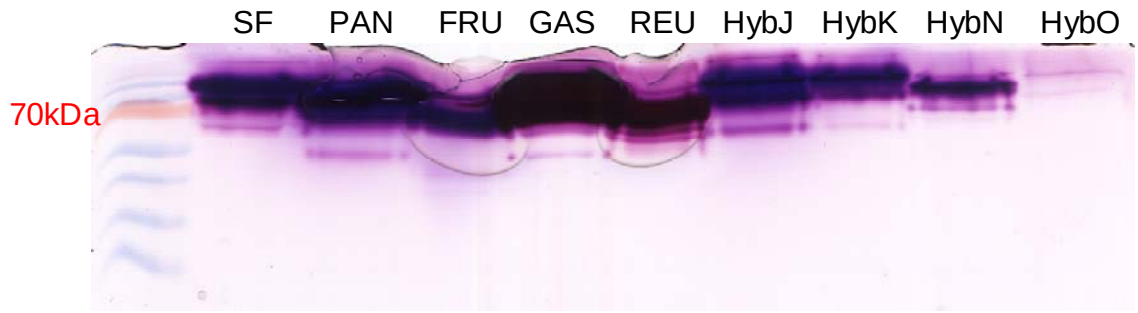


Fig. 22: Image section of activity stained renaturing SDS-PAGE of *L. sanfranciscensis* levansucrase (SF), *L. panis* FTF (PAN), *L. frumenti* FTF (FRU), *L. gasseri* modified FTF (GAS), *L. reuteri* inulinsucrase (REU) and *L. sanfranciscensis*/*L. panis* hybrid FTF proteins HybJ, HybK, HybN and HybO. As MW standard PageRuler™ Prestained Protein Ladder (MBI Fermentas GmbH, St. Leon-Rot, Germany) is used.

3.4 Construction, cloning and expression of *ftf hybrid* genes

By a coPCR strategy, four different combinations of ftf gene of *L. panis* and ftf gene of *L. sanfranciscensis* were designed. Two of them were designed as *L. panis* ftf gene with N-terminal domain replaced by *L. sanfranciscensis* N-terminal domain. Since the beginning of the catalytic active domain is not known exactly, two variations of that domain exchange were produced (*hybJ, hybK*). The third hybrid was planned as catalytic domain of *L. sanfranciscensis ftf* gene flanked by N-terminal and C-terminal domain of *L. panis ftf* gene (*hybN*) while the fourth construct was constructed the other way round: catalytic domain of *L. panis* origin with N- and C-terminal domain of *L. sanfranciscensis* (*hybO*).

hybJ and *hybK* DNA fragments were produced in one coPCR. For *hybN* and *hybO* two coPCR steps were needed: Assembling of respective catalytic domain with N-terminal domain of different origin as first step, followed by addition of respective C-terminus.

These fragments were cloned in pET3a plasmids, fusing a His-tag to the hybrid gene construct and expressed as described for native *ftf* genes cloned in pET3a plasmid system. Nevertheless, expression experiments were about factor ten less effective than with original unmodified genes. For HybJ, HybK and HybN, protein concentrations were between 0.1 and 0.3 mg/ml in FPLC fractions containing maximum protein. On SDS PAGE, similar to unmodified FTFs, additional protein contamination smaller than the target protein can be seen and in Western blot experiments these proteins can partly be detected by His-tag specific antibodies. For gene product of *hybO* practically no protein in affinity chromatography elution fractions could be detected by SDS-PAGE or Western blot.

Activity staining in native SDS-polyacrylamide gels supported these observations since in lanes of HybO sample barley any stainable EPS was produced whereas for HybN, HybK and HybN EPS producing activity in these experiments could be shown.

Also with TLC, HPLC, enzymatic reaction product determination and EPS precipitation no activity in HybO samples can be detected.

3.5 Functional analysis of *ftf* and *ftf hybrid* gene products

3.5.1 EPS produced

Fructans were produced *in vitro* by purified enzymes. The fructan type was determined indirectly by digest with β-(2→1) linkage specific inulinase. By TLC analysis, it could be shown that fructans of *L. reuteri* and *L. gasseri* were hydrolyzed by inulinase. All other original (*L. sanfranciscensis*, *L. panis* and *L. frumenti*) FTFs as well as *L. sanfranciscensis*-*L. panis* hybrid (HybJ, HybK, HybN) FTFs produced fructans that could not be affected by inulinase. Since HybO practically shows no activity, there was not enough material to prove a resistance against inulinase treatment.

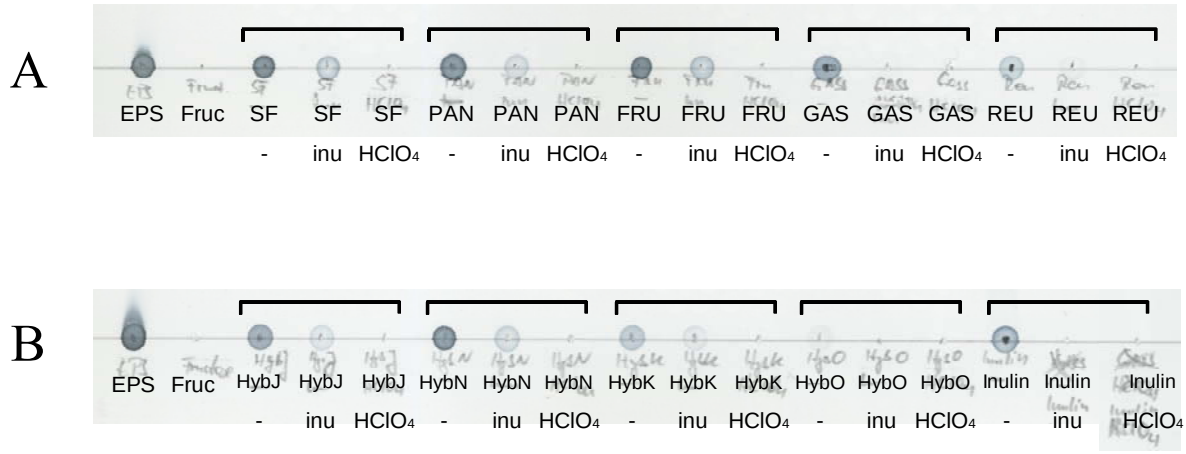


Fig. 23: Visualization of hydrolysis of fructan produced by different TFT enzymes on TLC plates. Hydrolysis is performed unspecifically chemically with $HClO_4$ and enzymatically with β-(2→1) specific inulinase. Concentration of negative controls is double than in samples. *L. sanfranciscensis* levan (EPS) and fructose (Fruc) are applied as standards. Upper row (A) shows results for FTF enzymes of *L. sanfranciscensis* (SF), *L. panis* (PAN), *L. frumenti* (FRU), *L. gasseri* (modified) (GAS) and *L. reuteri* (REU). On lower line (B) *L. sanfranciscensis*-*L. panis* hybrid FTF protein products are applied: *L. panis* enzyme with *L. sanfranciscensis* N-terminus in two variations ((HybJ) and (HybK)), the *L. sanfranciscensis* catalytic domain with N- and C-terminus of *L. panis* origin (HybN) and the *L. panis* enzymatic core with *L. sanfranciscensis* N- and C-terminus (HybO). Chemically pure inulin is used as positive control and also applied in lower line (B).

3.5.2 pH and temperature optima

Analysis of pH and temperature optima was performed for new FTFs of *L. panis*, *L. frumenti* and modified enzyme of *L. gasseri* by determination of overall activity in including all hydrolysis and transfer reaction activities, monitored by generated free glucose.

Detailed results of pH experiments are shown in fig. 24.

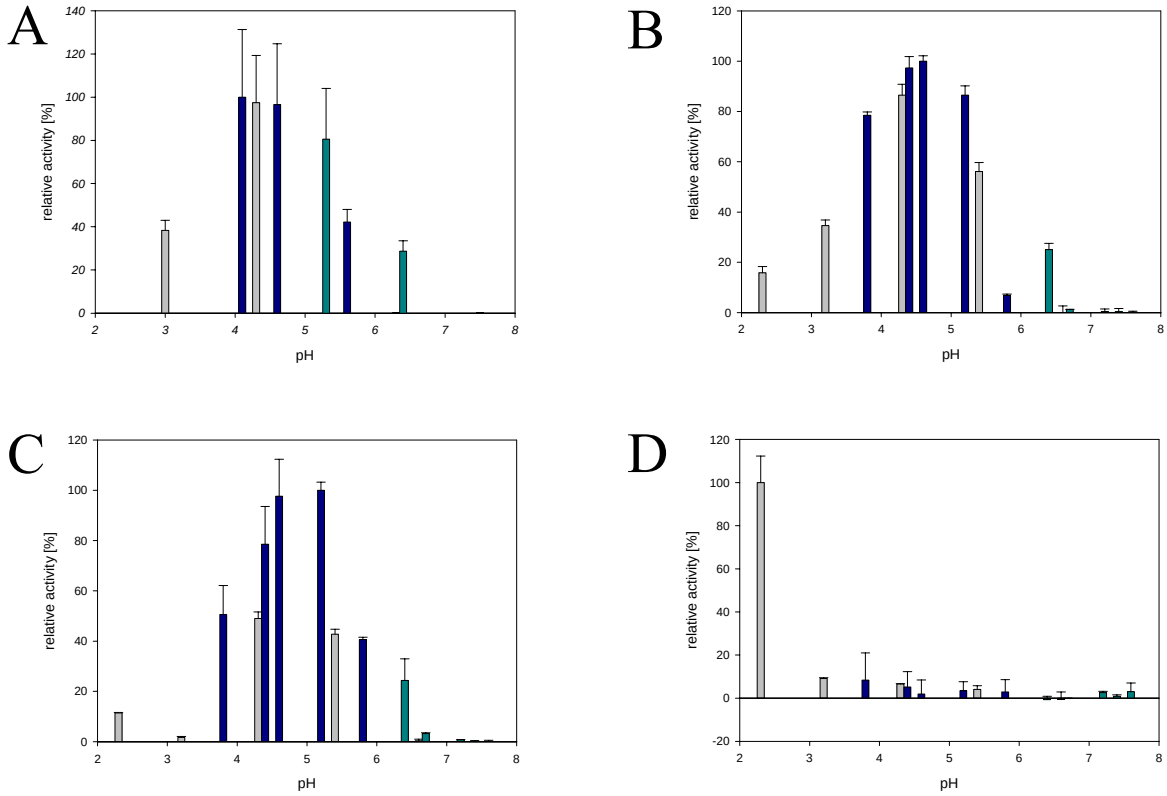


Fig. 24: Influence of pH and different buffer substances on the activity of different FTFs of lactobacillus origin: *L. panis* (A), *L. frumenti* (B) and modified *L. gasseri* FTF (C). Negative controls (D) show a significant influenced of pH on experiment only at pH lower than 2.5.

L. panis FTF showed best over all activity in a range of 4.0 to 5.0. At pH higher than 5.5 or less than 3.0 more than 50 % of the activity is lost. In ranges where the buffering capacity of the different buffering substances overlays, it could be seen that there is no significant influence of the buffering substance on enzymatic activity. In the neutral and weak alkaline pH range adjusted with Tris buffer no activity is detected.

L. frumenti FTF showed a similar behaviour to *L. panis* enzyme. Optimum range turned out to be within one pH unit (pH 4.2 to 5.2). 50 % of activity were reached around pH 3.5 and 5.5. Activity practically was lost at pH 5.8 when buffered with acetate. Nevertheless, there was around 35 % of activity at pH 6.4 when MOPS buffer is used. Beside that, activity was not significantly influenced by the buffering substance used. No activity could be detected using Tris buffer within a pH range of pH 6.8 to 8.7.

L. gasseri FTF pH optimum range lies in acetate buffered range of pH 4.6 to 5.2. Around pH 4.3 the enzyme seems to have less activity in citrate than in acetate buffer. 50 % activity was observed at pH 3.8 and around pH 5.5. The enzyme was practically inactive at pH 6.7 and in

the more alkaline range. So, also in this case no activity in Tris buffered samples could be observed.

For all enzymes optimum ranges are similar when regarded transfer activity and hydrolysis (data not shown).

Control sample showed a significant amount of glucose only in one sample, adjusted with citrate to pH 2.3 due to chemical hydrolyses of sucrose. That has to be taken in account regarding the apparent FTF enzyme activity below pH 3.0.

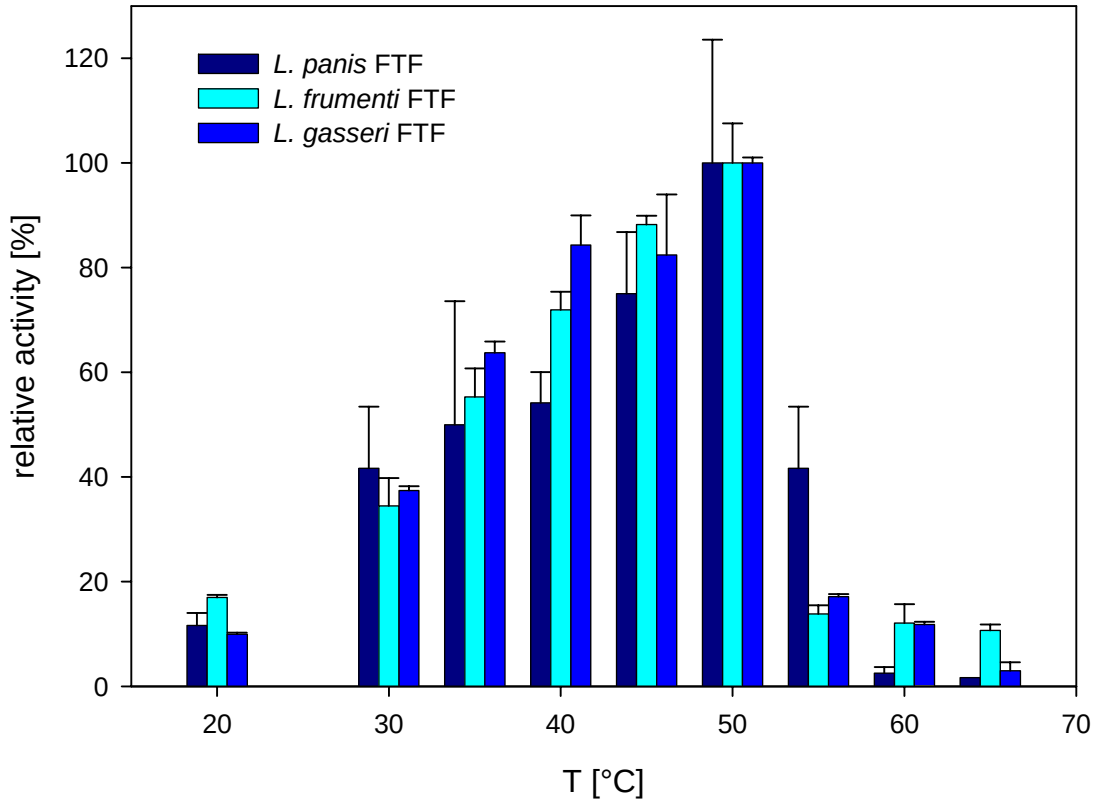


Fig. 25: Temperature optimum of over all activity of different FTFs from lactobacilli: *L. panis*, *L. frumenti* and *L. gasseri* modified FTF.

Concerning the temperature influence on over all activity, the three enzymes behaved very similar (see fig. 25). Maximum activity was reached at 50°C for all samples. At 45°C about 20 % of the activity is lost. 50 % activity was observed at 35°C. At 20°C only about 10% of maximum activity could be detected. When temperature is increased to more than 50°C, activity loss is relatively drastic. At 55°C *L. panis* FTF still showed 40% of maximum activity but for *L. gasseri* and *L. frumenti* FTF less than 20% of possible activity can be seen. When temperature is raised, further activity is hardly detectable since no exact time to glucose concentration plots can be calculated and drops beneath 20 %. Results are shown for total

glucose release representing over all activity. Temperature influence is similar for hydrolysis and transfer reactions (data not shown). Control did not show a significant proportion of chemical hydrolysis to measured activities at the given conditions.

3.5.3 MM kinetics

Influence of substrate (sucrose) concentration on the enzymatic activity of the functional gene products of various *ftf* genes cloned was examined, analyzing the possibility to fit the resulting activity/concentration plot to a MM equation and the parameters resulting from that curve fitting.

For newly identified or first time expressed FTFs MM parameters were determined at optimum conditions. For *L. panis* sodium acetate buffer of pH 4.8, for *L. frumenti* pH 4.5 and for *L. gasseri* derived enzyme pH 4.8 was used. Temperature was optimal for all enzymes at 48°C.

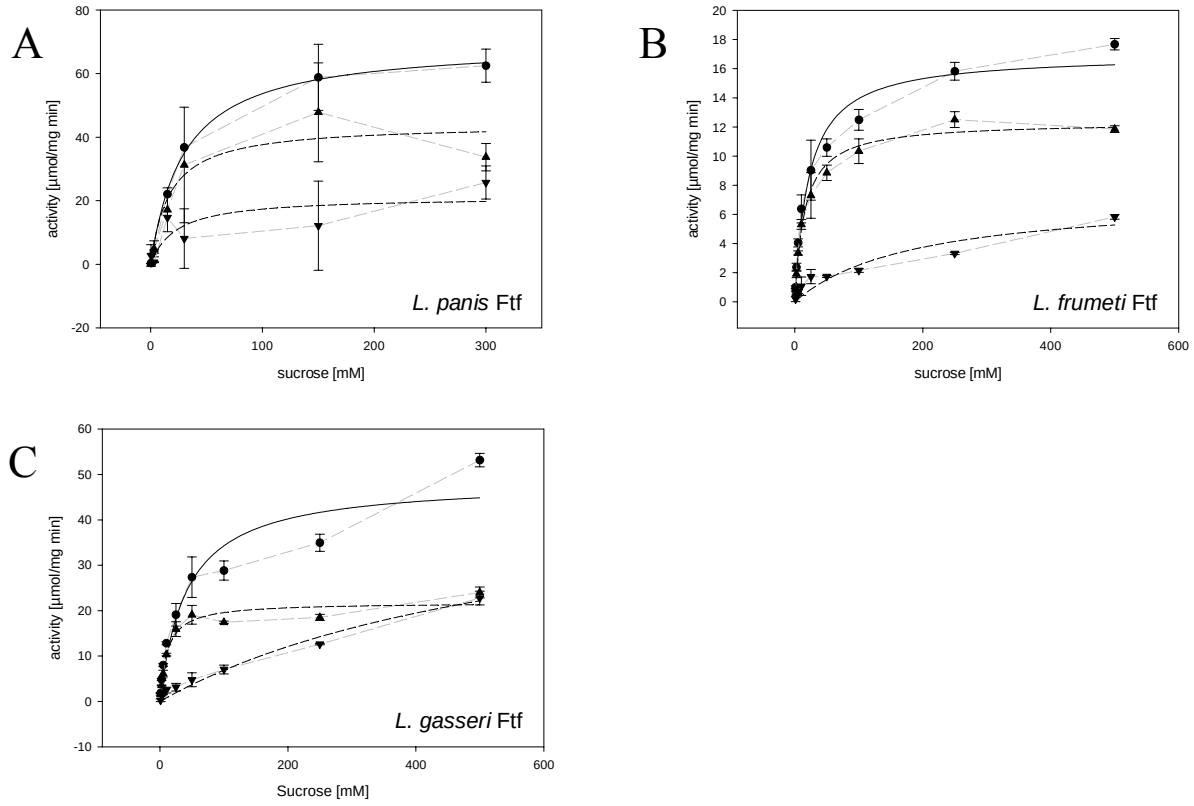


Fig 26: Plots of MM-kinetics for FTF proteins of *L. panis* (A), *L. frumenti* (B) and modified FTF protein of *L. gasseri* (C) at optimal reaction conditions. Over all activity is symbolized by closed circles (●), partial activities are symbolized by triangles (hydrolysis (▲) and transfer reactions (▼)). Solid black lines represent ideal MM kinetic fitted to over all activity. Fitting curves for hydrolysis and transfer reactions are drawn with black dashed lines.

Kinetic parameters and R^2 values indicating quality of curve fitting can be found in tab. 8. Most data can be fit well to MM equation. Concerning the R^2 value of transfer activity of FTF from *L. panis* origin, this partial reaction does not follow a MM kinetic.

FTF origin	type of activity	R^2	V_{max} [µmol/mg min]	K_M [mMol]
L. panis	over all	0,997	69,74	29,89
	hydrolysis	0,907	44,08	17,10
	transfer reactions	0,662	21,26	22,51
L. frumenti	over all	0,971	16,97	21,69
	hydrolysis	0,988	12,34	15,31
	transfer reactions	0,885	7,26	187,90
L. gasseri	over all	0,923	48,61	41,79
	hydrolysis	0,985	21,79	11,31
	transfer reactions	0,971	50,05	628,40

Tab. 8: R^2 values and kinetic parameters for FTF enzymes of *L. panis*, *L. frumenti* and *L. gasseri* origin derived at optimal reaction conditions.

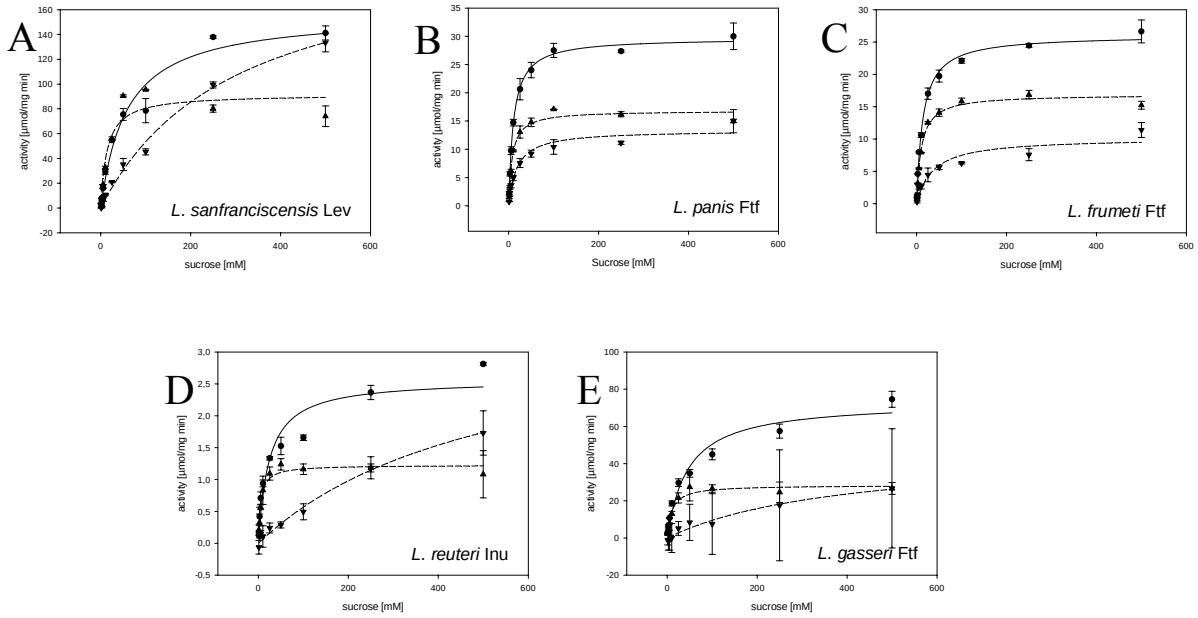


Fig. 27: Graphic plots of data achieved with various FTF enzymes with *Lactobacillus* origin. All reactions with levan producing enzymes of *L. sanfranciscensis* (A), *L. panis* (B) and *L. frumenti* (C) as well as inulin producing FTFs from *L. reuteri* (D) and *L. gasseri* (E) are carried out at identical pH 4.6 and 45°C for comparison. Over all activity is symbolized by closed circles (●), partial activities are symbolized by triangles (hydrolysis (▲) and transfer reactions (▼)). Solid black lines represent ideal MM kinetic fitted to over all activity. Fitting curves for hydrolysis and transfer reactions are drawn with black dashed lines.

For comparison of different enzymes influence of substrate concentration was examined at fixed pH and temperature conditions for described, newly found and artificial hybrid FTF

enzymes. Used pH of 4.6 and a temperature of 45°C are chosen as compromise for different optima of the different enzymes. Graphical plots of non hybrid FTF protein activities are displayed in fig. 27.

The very flat slope in the kinetics of transfer reactions for enzymes derived from *L. sanfranciscensis*, *L. reuteri* and *L. gasseri* is noticeable. Tab. 9 contains the corresponding kinetic parameters and R^2 values for experiments at comparison conditions. A quite good fitting of MM kinetics was achieved for all experimental data. The low V_{max} values of *L. reuteri* inulinsucrase are remarkably. They are about two orders of magnitude below the ones of *L. sanfranciscensis* levansucrase which delivered highest V_{max} values.

FTF origin	type of activity	R^2	V_{max} [µmol/mg min]	K_M [mMol]
L. sanfranciscensis	over all	0,970	157,10	57,77
	hydrolysis	0,907	91,74	14,00
	transfer reactions	0,993	213,70	300,00
L. panis	over all	0,996	29,70	10,55
	hydrolysis	0,979	16,84	7,86
	transfer reactions	0,954	13,36	18,48
L. frumenti	over all	0,993	26,05	13,70
	hydrolysis	0,988	16,89	10,29
	transfer reactions	0,902	10,17	35,85
L. reuteri	over all	0,921	2,57	23,65
	hydrolysis	0,961	1,23	5,14
	transfer reactions	0,987	3,59	531,40
L. gasseri	over all	0,962	73,39	45,57
	hydrolysis	0,965	28,37	10,09
	transfer reactions	0,962	46,82	392,20

Tab. 9: R^2 values and MM parameters for non hybrid FTF enzymes at comparison conditions (pH 4.6, 45°C).

MM kinetic data was also obtained from *L. sanfranciscensis* and *L. panis* FTF hybrid enzymes HybJ and HybK (*L. panis* catalytic core with N-terminus of *L. sanfranciscensis* FTF), HybN (*L. sanfranciscensis* catalytic domain with N- and C-terminus of *L. panis* origin) and HybO (*L. panis* catalytic core with N- and C-terminus of *L. sanfranciscensis* origin), although the purification of the latter did not show significant amounts of target protein and only weak activity could be detected by polymer staining in renaturing SDS-PAGE. Experiments were performed at the comparison conditions mentioned above (ph 4.6, 45°C). Resulting plots are presented in fig. 28.

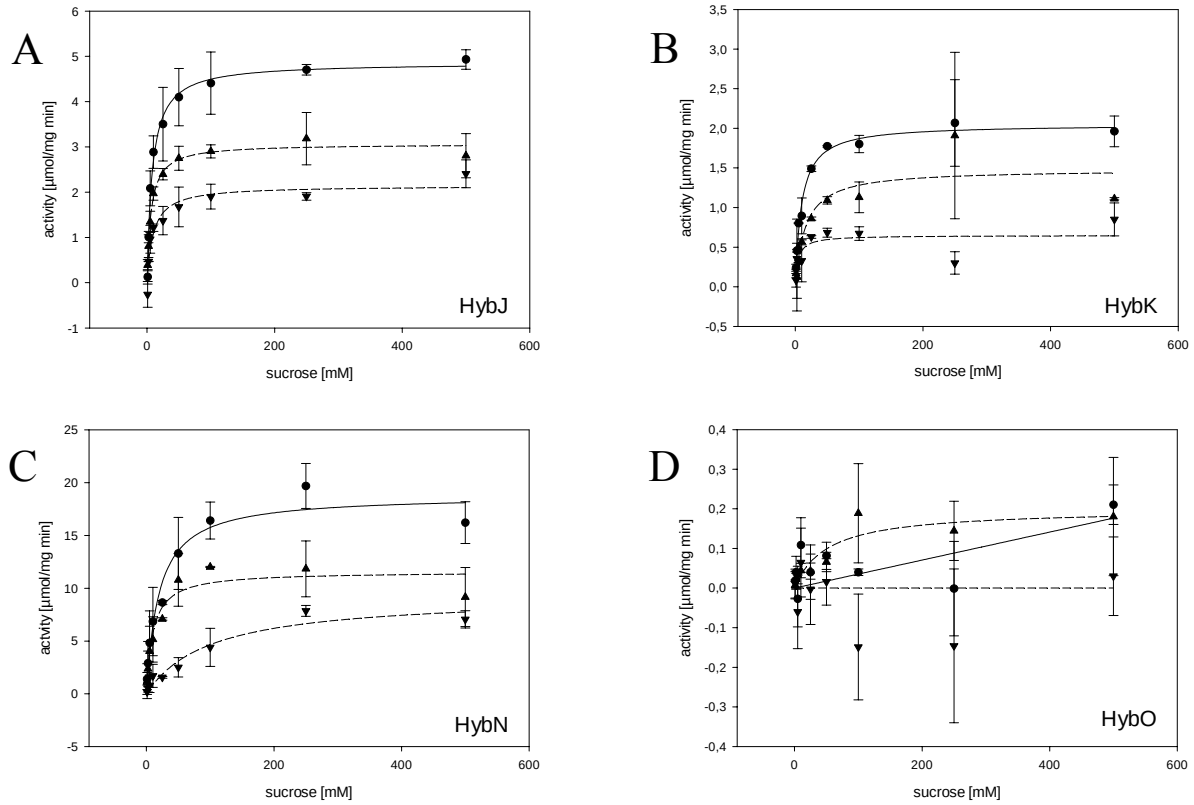


Fig 28: MM plots of *L. sanfranciscensis-L. panis* FTF hybrid constructs: The *L. panis* enzyme with *L. sanfranciscensis* N-terminus in two variations (HybJ (A) and HybK (B)), the *L. sanfranciscensis* catalytic domain with N- and C-terminus of *L. panis* origin (HybN (C)) and the *L. panis* enzymatic core with *L. sanfranciscensis* N- and C-terminus (HybO (D)). Data are obtained at standard conditions (pH 4.6, 45°C). Over all activity is symbolized by closed circles (●), partial activities are symbolized by triangles (hydrolysis (▲) and transfer reactions (▼)). Solid black lines represent ideal MM kinetic fitted to over all activity. Fitting curves for hydrolysis and transfer reactions are drawn with black dashed lines.

FTF origin	type of activity	R^2	V_{max} [µmol/mg min]	K_M [mMol]
HybJ	over all	0,985	4,86	8,15
	hydrolysis	0,987	3,07	6,31
	transfer reactions	0,917	2,14	9,81
HybK	over all	0,986	2,05	9,59
	hydrolysis	0,829	1,48	15,59
	transfer reactions	0,587	0,65	4,40
HybN	over all	0,958	18,81	19,41
	hydrolysis	0,921	11,57	10,29
	transfer reactions	0,948	9,30	99,89
HybO	over all	0,169	0,09	12,31
	hydrolysis	0,853	0,20	51,09
	transfer reactions	0,000	0,00	95,46

Tab. 10: R^2 values and MM parameters for *L. sanfranciscensis-L. panis* hybrid FTF enzymes at comparison conditions (pH 4.6, 45°C).

Construct HybK only showed weak activity and with HybO samples practically no activity could be detected. A good fitting was possible for HybJ and HybN constructs. All in all hybrid proteins were significantly less active than the original proteins including modified *L. gasseri* FTF. These facts also can be read from the respective kinetic parameters which are shown in tab. 10.

3.5.4 Dependency of Ca^{2+} cations

The dependence and influence of bivalent metal cations on FTF protein activity was examined by adding different cations to regular assays with untreated proteins and to assays for which proteins have been treated with EDTA to remove complexed metal cations. Newly identified *L. panis* and *L. frumenti* FTFs are examined as well as modified FTF of *L. gasseri* origin and the known *L. sanfranciscensis* levansucrase. Results are graphically presented in fig. 29.

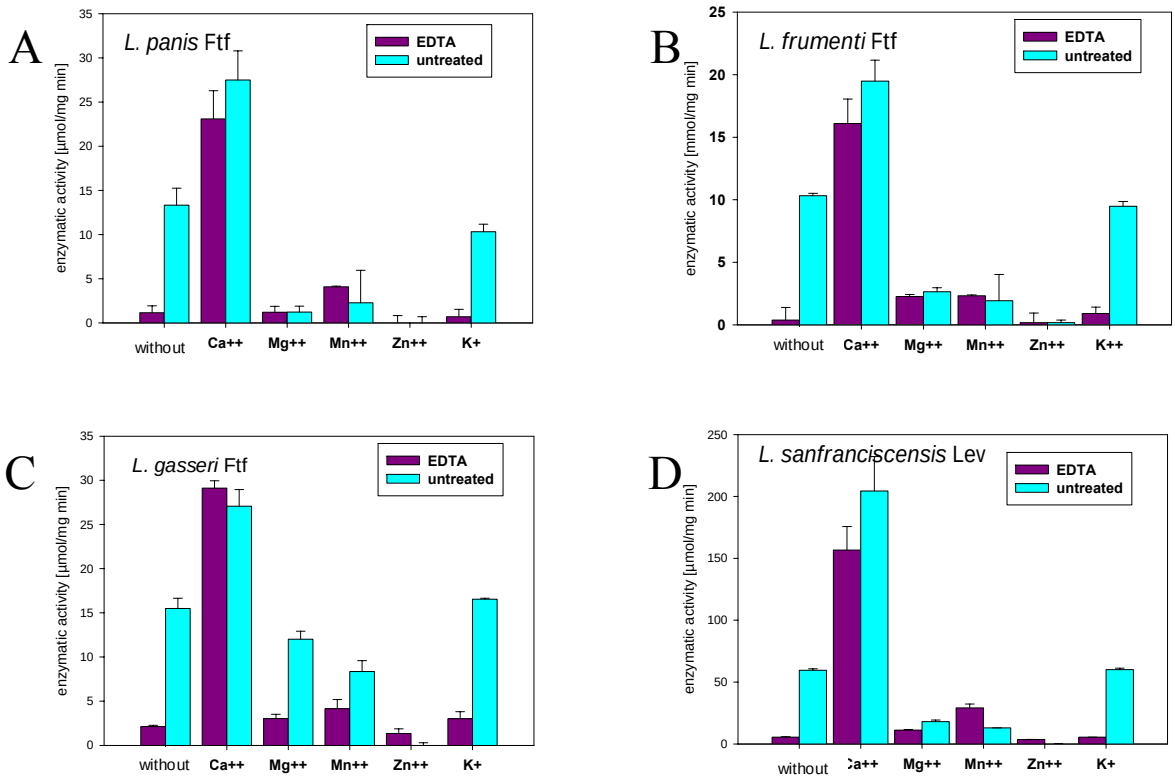


Fig. 29: Influence of EDTA, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and K^{+} on the activity of selected FTF enzymes: *L. panis* FTF (A), *L. frumenti* FTF (B), *L. gasseri* FTF (C) and *L. sanfranciscensis* levansucrase (D). Values for EDTA treated samples are coloured in violet, samples not treated with EDTA are represented by turquoise columns.

In original enzymes, Ca^{2+} ions increased activity of untreated enzymes by a factor 2 and higher (see *L. sanfranciscensis* levansucrase). Mg^{++} ions added to untreated enzymes decreased enzyme activity. The effect was just slightly seen in case of *L. gasseri* FTF but

quite drastic in case of *L. panis*, *L. frumenti* and *L. sanfranciscensis* FTFs. *L. panis* FTF activity practically was blocked completely. Mn^{++} ions reduced *L. gasseri* FTF activity to 50 % relative to an assay without the addition of any cation to untreated enzymes. Again, the other three proteins were more drastically affected and showed similar results to Mg^{++} addition. Zn^{++} in all cases completely delayed enzymatic activity. Monovalent K^{+} ions did not have significant effects on the over all activity of the untreated FTF enzymes.

When the FTF proteins were treated with EDTA and no cation is applied, practically no enzymatic activity could be detected. However, *L. gasseri* FTF seems to be a bit less affected by the chelating agent. The addition of Ca^{++} ions to EDTA treated *L. gasseri* FTF completely restored enzyme activity to a level similar to untreated enzyme supplied with the same amount of Ca^{++}. Also the activity of *L. panis*, *L. frumenti* and *L. sanfranciscensis* FTF enzymes was restored by addition of Ca^{++}. Nevertheless theses enzymes did not reach the activity level of enzyme not treated with EDTA and supplied with Ca^{++}. Mg^{++} could not perform a significant activity increase for *L. gasseri*, *L. panis* and *L. sanfranciscensis* FTF incubated in EDTA. For *L. frumenti* FTF a slight increase of activity after Mg^{++} addition could be observed. However, activity is not more than 10 % of activity measured after Ca^{++} addition. A similar effect could be seen after Mn^{++} addition but not only for *L. frumenti* FTF but also for proteins of *L. panis*, *L. sanfranciscensis* and *L. gasseri* origin. The effect was less distinct for *L. gasseri* FTF enzyme. In no case Zn^{++} could increase any activity in EDTA treated FTFs. It rather tends to reduce basal activity of *L. gasseri* FTF. As on untreated enzymes monovalent K^{+} cations did not have any effect on the reaction.

3.5.5 Influence of pH sucrose concentration and incubation temperature on product ratios

For *L. panis* FTF detailed information about influence of substrate concentration, pH and temperature were obtained. Lowest pH and highest temperature samples delivered extreme results due to chemical hydrolysis of sucrose. A trend of an enhanced production of FOS and fructan was recognizable at lower temperatures, higher pH and elevated sucrose concentrations. Kestose is the only FOS produced by that enzyme detectable with HPLC analysis. Results are displayed in fig. 30.

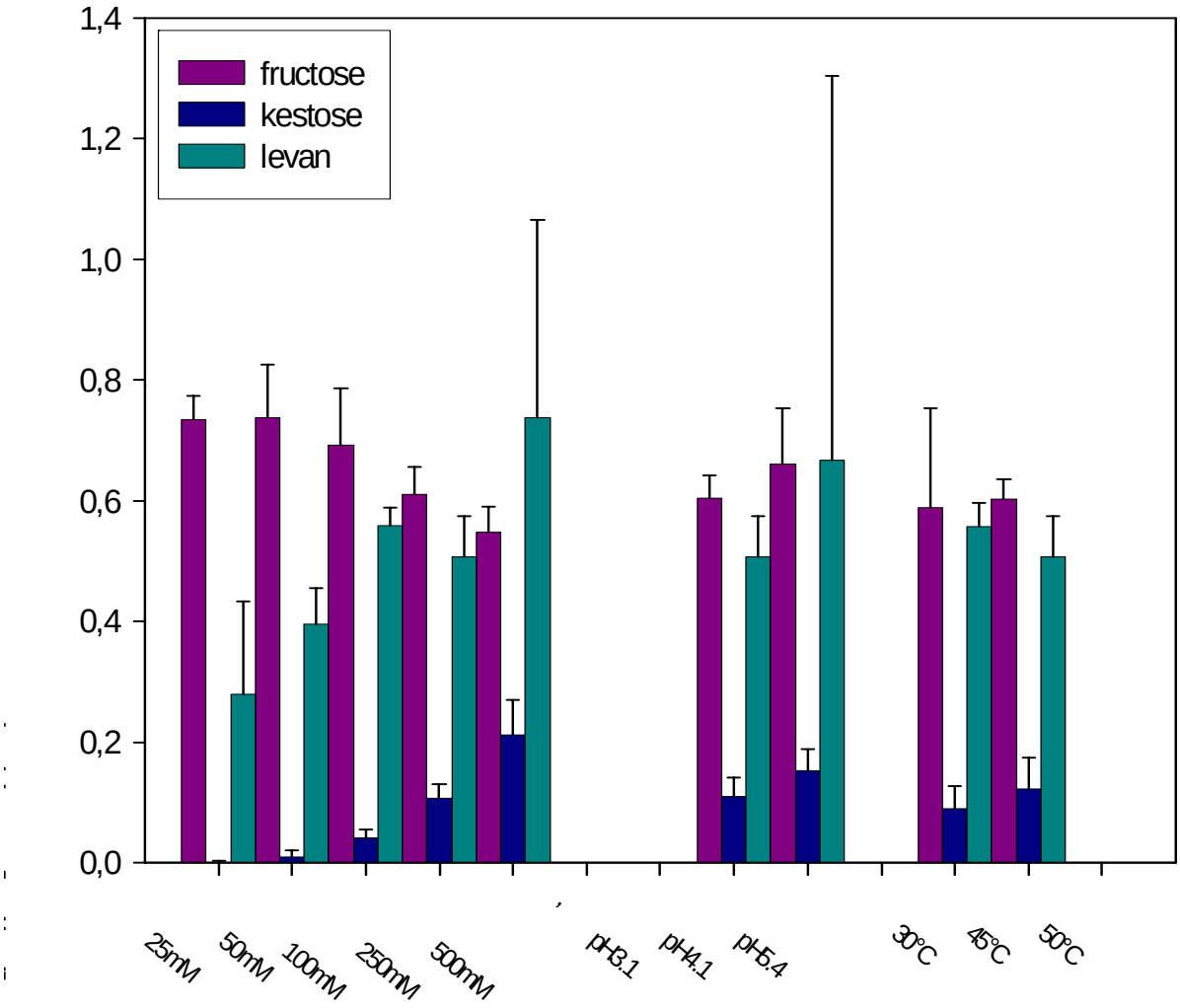


Fig. 30: Influence of sucrose concentration, pH and temperature on product ratios of *L. panis* FTF. Concentration of free fructose (purple columns) and kestose (blue columns) were determined by HPLC analysis; fructose moieties bound in levan (green columns) were calculated. The product concentrations were referenced to free glucose which was also quantified by HPLC analysis. All experiments were performed three times to obtain mean value and standard deviation. At pH 3.1 and 50°C, chemical hydrolysis of sucrose overlaid enzymatic activity.

For comparison of native FTF enzymes among each other and with the newly constructed hybrid proteins, similar experiments were carried out in smaller scale and not in double. TLC analysis reveals that all active FTF enzymes produce EPS and the FOS 1-kestose from sucrose. *L. sanfranciscensis* levansucrase, *L. reuteri* inulinsucrase and *L. gasseri* modified FTF enzyme in addition to kestose produce higher FOS in detectable amounts up to GF_8 (see Fig. 31).

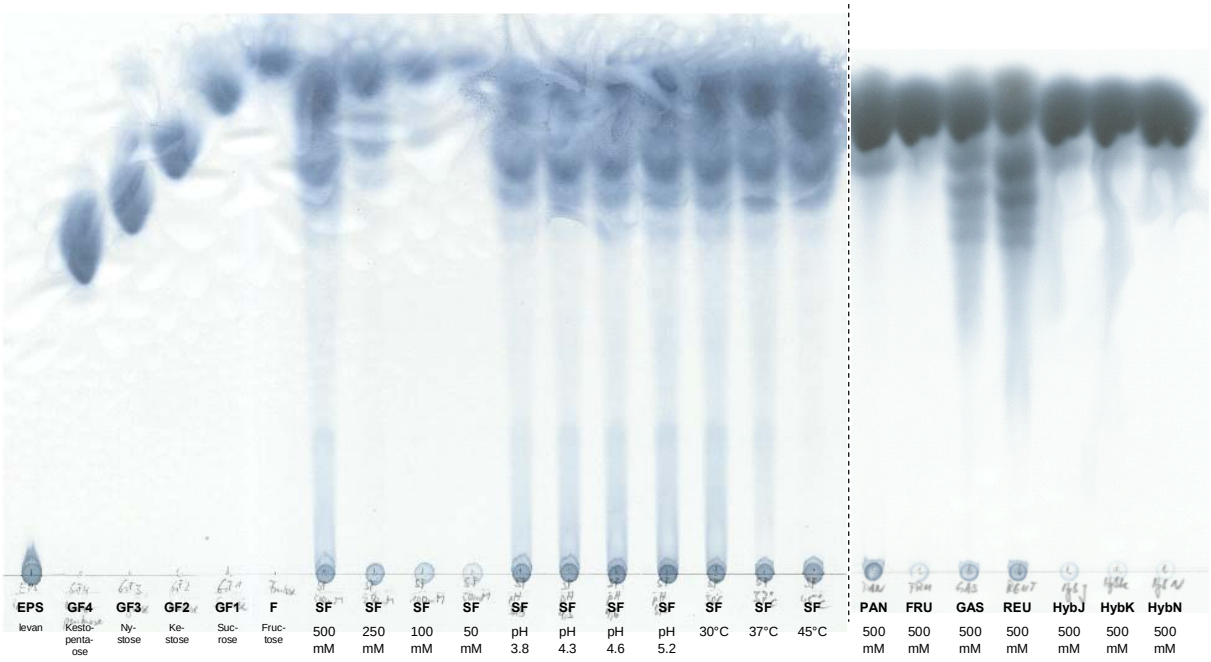


Fig. 31: TLC analysis of oligosaccharide production of FTF enzymes from sucrose. Lane 1 to 6 show standard sugars: *L. sanfranciscensis* produced levan (EPS), 1,1,1-kestopentaose (GF4), 1,1-nystose (GF3), 1-kestose (GF2), sucrose (GF1) and fructose (F). For *L. sanfranciscensis levansucrase* (SF) different sucrose concentrations (500 mM, 250 mM, 100 mM, 50 mM), different pH (pH 3.8, pH 4.3, pH 4.6, pH 5.2) and different temperature (30°C, 37°C, 45°C) experiments are shown. Since TLC does not reveal big differences when pH and temperature is modified and differences with different sucrose concentrations are not quantifiable, only 500 mM sucrose samples of residual FTFs derived from *L. panis* (PAN), *L. frumenti* (FRU), *L. gasseri* (GAS) and *L. reuteri* (REU) as well as *L. sanfranciscensis*-*L. panis* hybrid constructions HybJ (HybJ), HybK (HybK) and HybN (HybN) are shown. For HybO sample no activity could be shown by TLC method.

Since TLC method does not enable quantification, influence of pH, temperature and substrate concentration variations are analyzed by HPLC analysis of samples. Residual sucrose, produced fructose and glucose as well as 1-kestose can be detected and quantified. Detectable amounts of kestose in these experiments were only produced by *L. sanfranciscensis* levansucrase, *L. reuteri* inulinsucrase and *L. gasseri* modified FTF enzyme. In these samples, an additional peak indicating a higher oligosaccharide (1-nystose) is also present. In *L. panis* and *L. frumenti* FTF samples no significant kestose production could be observed by HPLC methods, although 1-kestose can be detected by more sensitive staining on TLC plates.

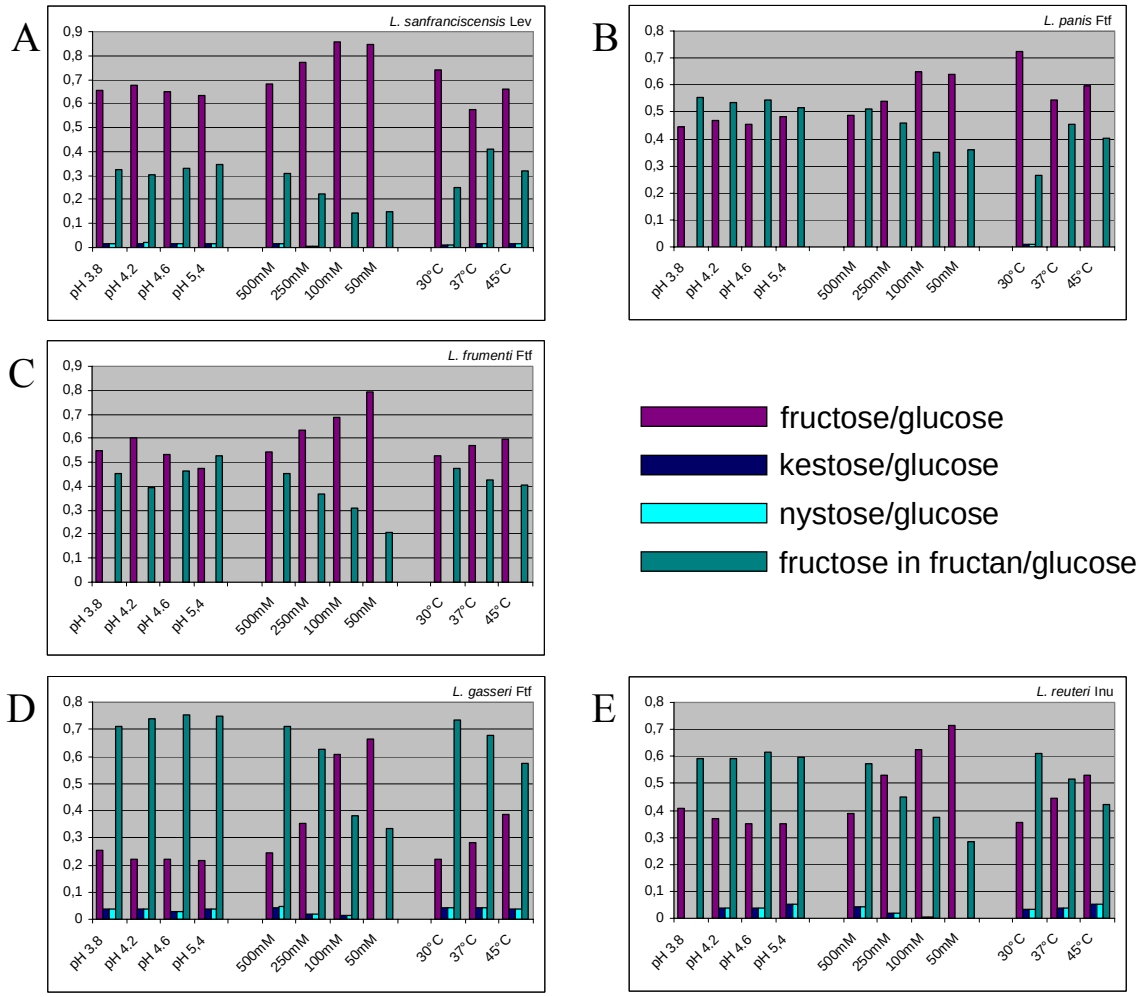


Fig 32: Product ratios of different FTF enzymes of different lactobacillus origin. Amounts of produced free fructose, kestose and nystose are quantified by HPLC and presented in relation to produced free glucose representing amount of utilized sucrose. Amount of fructose moieties bound in produced levan are calculated based on these data and also relativated to amount of free glucose. These data are shown for levan producing enzymes from *L. sanfranciscensis* (A), *L. panis* (B) and *L. frumenti* (C) as well as for inulin producing enzymes of *L. gasseri* (D) and *L. reuteri* (E) origin.

Relative data for fructose containing products of FTF enzymes show no significant influence of pH on the ratio of free fructose and transfer or polymerisation products for *L. sanfranciscensis* levansucrase, and *L. panis* FTF enzymes. For *L. reuteri* and *L. gasseri* modified FTF enzyme pH steps show a trend to a preference of polymerization reactions at higher pH values. This trend can be seen for *L. frumenti* FTF, too. Nevertheless, in this case at pH 3.8, polymerisation is on a similar level as at pH 4.6.

More significant is the influence of concentration of the fructose donor molecule sucrose on the relation of the produced products. For all enzymes it could be observed that higher substrate concentration increase polymerisation and if detectable FOS producing transfer reactions. Hydrolysis of sucrose is preferred at lower substrate concentrations.

For *L. frumenti*, *L. reuteri* and *L. gasseri* enzymes, a trend to relatively more hydrolysis reactions with increasing temperature can be observed. For *L. sanfranciscensis* and *L. panis* FTFs in this series of experiments the same trend seems to occur when concerning results from 37° and 45°C samples. But in both cases at 30°C higher hydrolysis rates and lower polymerisation rates are detected. However, FOS production rates in *L. sanfranciscensis* levansucrase show a very slight tendency to grow with increasing temperature. In contrast to that in *L. panis* samples FOS are exclusively detectable by HPLC in the sample incubated at lowest temperature (30°C). Although, the FOS amounts in this samples are very close to the detection limit.

Comparing the complete data obtained from different FTFs in an overview, for *L. sanfranciscensis* levansucrase activity hydrolysis reactions are dominating. For the other FTFs producing inulinase resistant fructans (FTF from *L. frumenti* and *L. panis*), data show an over all balanced ratio of hydrolysis and polymerization that is mainly affected by variation of substrate concentration. In inulinase sensitive fructan producing enzymes from *L. reuteri* and *L. gasseri* polymerization reactions are dominating. Among them, *L. gasseri* FTF shows highest relative polymerization activity. Transfer reactions resulting in FOS are only present in *L. sanfranciscensis* levansucrase, *L. reuteri* inulinsucrase and *L. gasseri* FTF, whereupon the relative transfer activity is approximately doubled in the latter. For all three, relative transfer activity is at least one order of magnitude beyond hydrolysis and transfer reactions.

L. sanfranciscensis-*L. panis* hybrids HybJ, HybK and HybN in contrast to HybO produced detectable amounts of products during the reaction period. Fructose, glucose and residual sucrose could be detected and quantified. No kestose or – in accordance to TLC plates – higher FOS appeared as peaks. In HybO samples practically only major sucrose peaks appeared. pH variations did not show a noticeable trend in experiments with hybrids HybJ and HybN. Nevertheless, results for HybK revealed a clearly increased hydrolysis and reduced polymerization activity at pH 3.8 and a growing preference for polymerization activity in samples with increased pH. There was no significant difference in activity ratios in samples with pH 4.6 and 5.4. Influence of sucrose concentration on distribution of relative activity ratios is not significant for HybJ protein samples, only a trend to enhanced polymerisation activity at higher substrate concentrations could be seen in the data. This tendency was much more distinct in HybK protein: At 50mM sucrose hydrolysis and polymerisation activity practically were equal whereas there was dominating polymerisation activity at 500mM. HybN showed a similar trend but here, hydrolysis and polymerisation were balanced at

500mM sucrose and hydrolysis activity was more then double of polymerisation at 50mM sucrose.

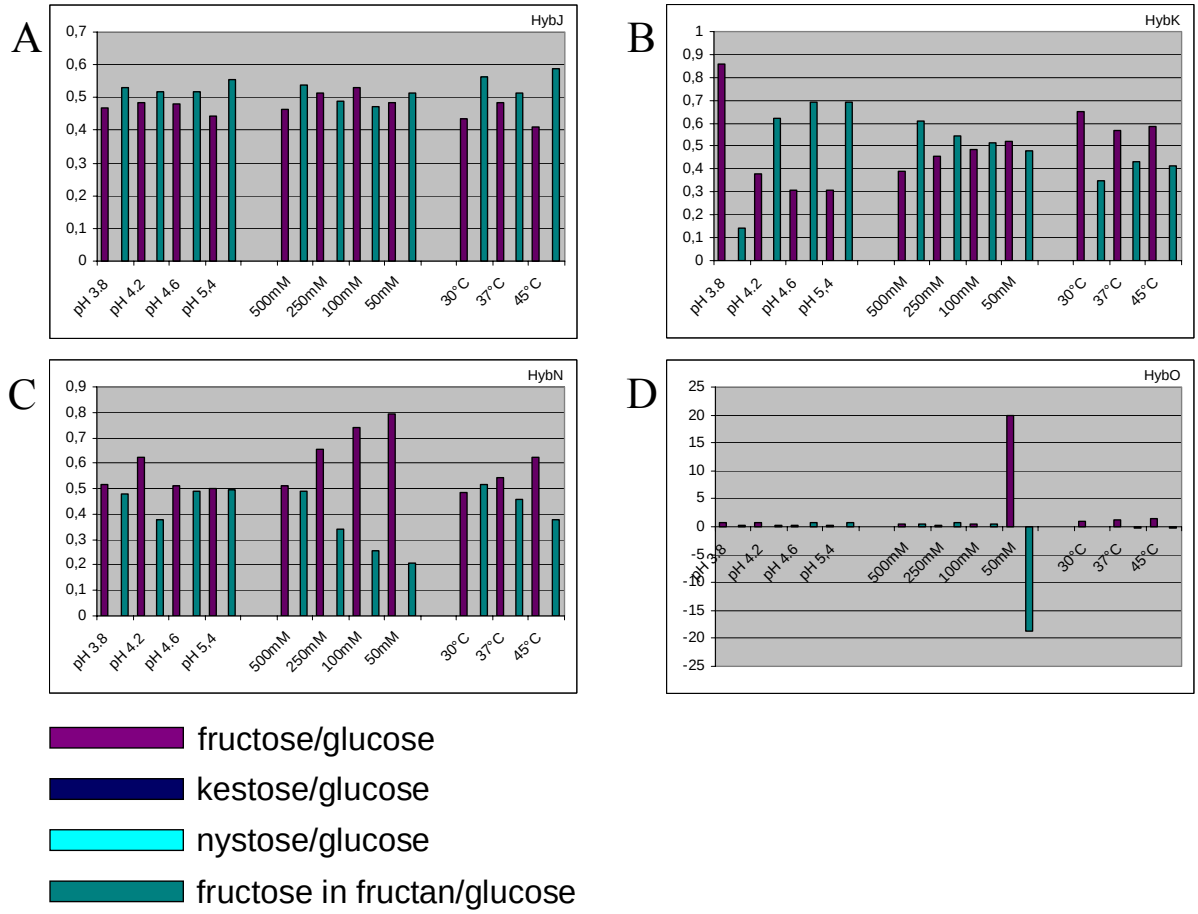


Fig 33: Product ratios of artificial *L. sanfranciscensis*-*L. panis* hybrid FTF enzymes: The *L. panis* enzyme with *L. sanfranciscensis* N-terminus in two variations (HybJ (A) and HybK (B)), the *L. sanfranciscensis* catalytic domain with N- and C-terminus of *L. panis* origin (HybN (C)) and the *L. panis* enzymatic core with *L. sanfranciscensis* N- and C-terminus (HybO (D)) Amounts of produced free fructose, kestose and nystose are quantified by HPLC and presented in relation to produced free glucose representing amount of utilized sucrose. Amount of fructose moieties bound in produced levan are calculated based on these data and also relativized to amount of free glucose.

This construct also had a clear trend concerning temperature influence: At 30°C polymerization and hydrolysis activity appeared nearly equalized and with increasing temperature, hydrolysis became dominant. For the constructs HybJ and HybK a clear effect of temperature could not be observed: In HybJ samples activities at 37°C seemed balanced and polymerization was slightly dominating at 30°C and 45°C. For HybK hydrolysis is bigger then polymerisation activity at every temperature but difference is bigger at lowest temperature (30°C). Comparing the three active hybrid constructs from *L. sanfranciscensis* levansucrase and *L. panis* FTF, HybJ turned out not to be very sensitive against pH,

temperature or substrate concentration influences and has very similar hydrolysis and polymerisation levels. In contrast to that, activity ratios of HybK could be influenced by varying pH and sucrose concentration. In HybN substrate concentration was the critical parameter in modifying activity levels. A slight but clear trend in temperature influence could be detected for that protein.

To reveal possible differences in MW of fructan chains at different conditions, samples of experiments with native and hybrid FTFs are analyzed with gel permeation chromatography using a Superdex200 column. These analyses were difficult for fructans produced by *L. sanfranciscensis*-*L. panis* hybrid proteins since the amounts of fructose polymer produced were very low.

For native proteins pH had only small effects on the MW of fructan chains produced by *L. sanfranciscensis*, *L. frumenti*, *L. gasseri* and *L. reuteri* enzymes. Only a slight trend to an increased percentage of lower MW with elongated elution time could be observed for lower pH samples. Peak shape did not alter in any of these chromatograms and time for peak maximum did not shift. In contrast to that in *L. panis* samples a clear peak shape alteration can be seen: with decreasing pH peaks develop a significant tailing indicating a growing percentage of lower MW fructan molecules. However time point for peak maximum does not shift significantly fig. 34.

Applied sucrose concentration had a bigger and more differentiated influence on size of fructan chains: In *L. sanfranciscensis* only a slight tendency to higher MW chains at higher sucrose concentrations could be observed (fig. 35 (A)) since peak maximum appears after shorter elution time with increasing substrate availability. In contrast to that in *L. panis* samples no increase of MW of fructan could be found (fig. 35 (B)) but an alteration of peak shape showing that in addition to bigger fructan molecules more and more fructans with lower MW are produced when more substrate can be permuted. *L. frumenti* protein does neither change peak shape nor maximum elution time depending on sucrose concentration (not shown) whereas *L. gasseri* derived enzyme has a clear trend to lower MW fructan when there is more substrate. This can be seen in a shift of the peak maximum to longer elution times while peak shape does not change much (fig. 35 (C)). *L. reuteri* samples show a similar trend (fig. 35 (D)).

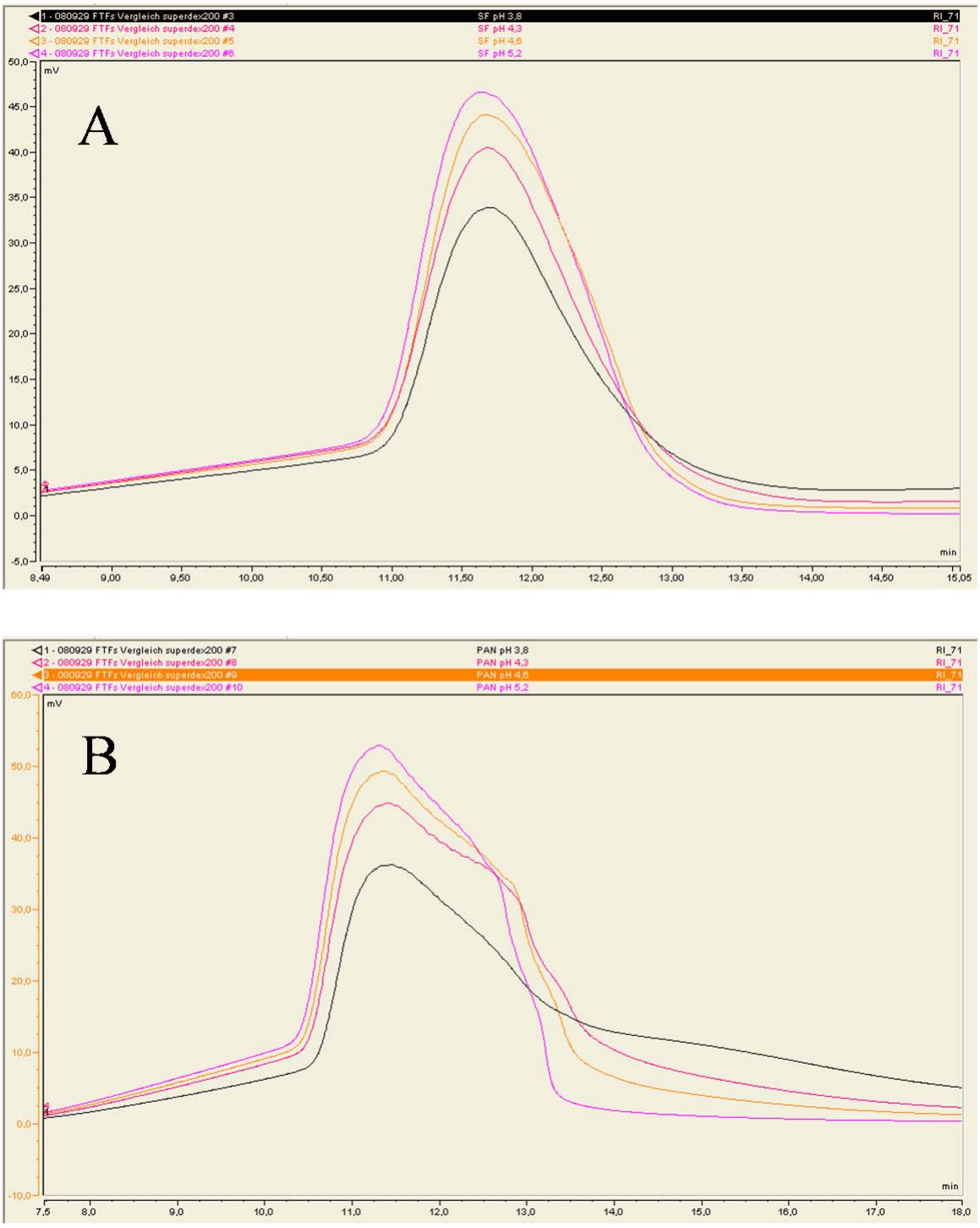


Fig. 34: Superdex analysis of levan produced by *L. sanfranciscensis* (A) and *L. panis* (B) at different pH conditions (pH 3.8 (black), pH 4.3 (purple), pH 4.6 (orange) and pH 5.2 (pink)).

Temperature only has marginal influence on size of fructan molecules produced. However, a trend could be observed: At higher temperatures peak maxima appear after longer elution time than at lower temperatures indicating a decrease in MW of fructan produced when temperature is increased.

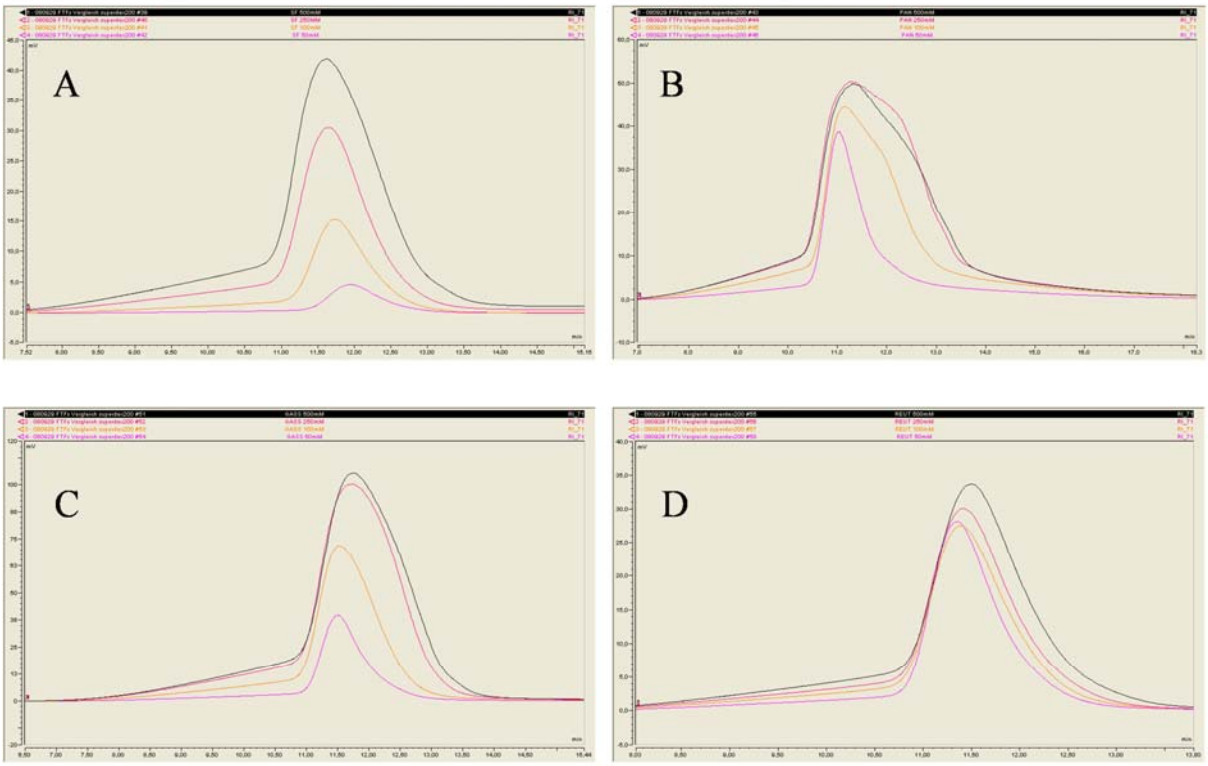


Fig. 35: Effect of sucrose concentration (500 mM (black), 250 mM (purple), 100 mM (orange), 50 mM (pink)) on MW of fructans produced by levansucrases of *L. sanfranciscensis* (A) and *L. panis* (B) and inulinsucrases of *L. gasseri* (C) and *L. reuteri* (D).

As mentioned above, analysis of fructans produced by artificial hybrid FTF enzymes was difficult due to low fructan levels in the samples. Nevertheless, the basic trends to higher MW fructans at lower pH, high sucrose concentrations and lower temperature could be observed for HybJ, HybK and HybN, too.

3.5.6 FOS and HeOS production and the use of raffinose as alternative fructose donor

The presence of FOS produced by fructose transfer is monitored on TLC plates due to the high sensitivity of that method.

For *L. sanfranciscensis* levansucrase, *L. reuteri* inulinsucrase and modified *L. gasseri* FTF the production of inulintype FOS GF_2 (1-kestose), GF_3 (1,1-nystose), GF_4 (1,1,1-kestopentaose) and occasionally smaller amounts of GF_5 (1,1,1,1-kestohexaose, mainly produced of *L. gasseri* FTF) from sucrose can be detected. An example for that can be seen in above in fig. 31. For *L. frumenti* and *L. panis* exclusively 1-kestose is produced as single FOS from sucrose.

The newly constructed FTF hybrid proteins of *L. panis* and *L. sanfranciscensis* FTFs hybN and hybJ and HybK only showed 1-kestose production. The amounts of kestose produced by

hybK are relatively low. HybO protein did not show the production of any oligosaccharide but also no other activity could be detected.

For all native and hybrid proteins except HybO EPS production could clearly be seen on TLC plates by stainable, not migrating carbohydrate fractions as spots at application position.

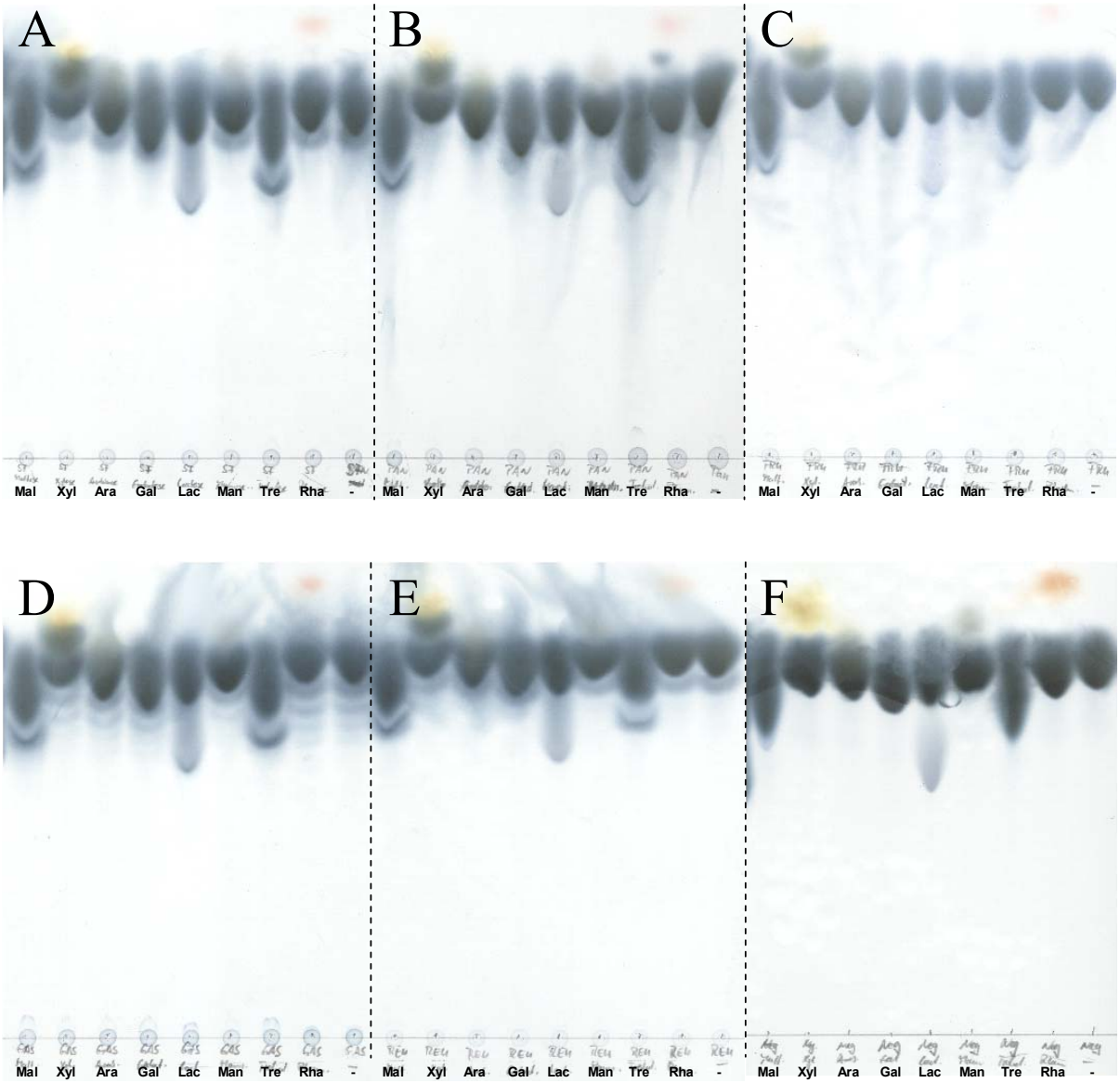


Fig. 36: TLC analysis of potential HeOS production by different FTF enzymes from *L. sanfranciscensis* (A), *L. panis* (B), *L. frumenti* (C), *L. gasseri* (modified) (D) and *L. reuteri* (E). A negative control without any enzyme is also shown (F). In addition to sucrose maltose (Mal), xylose (Xyl), arabinose (Ara), galactose (Gal), lactose (Lac), mannose (Man), Trehalose (Tre) and rhamnose (Rha) are inserted in the samples. For comparison, there is a control with sucrose as sole carbohydrate (-). In no case there is an additional spot that can not be traced back on exclusive sucrose utilization.

The production of HeOS based on sucrose as fructose donor and various carbohydrates as acceptor molecules is examined with various sugars. For none of the native or hybrid FTF

enzymes traces of HeOS production could be detected. On TLC plates no additional spots to the known spots, appearing when only sucrose is added to the reaction mix could be found. Negative controls and samples for some sugars showed a weak stainability of most of the potential acceptor molecules. Colour of the stained carbohydrates can be different from fructose containing sugars which appear as blue black spots: Maltose similar to glucose is not stained at all, xylose appears as a red to yellow spot with longer migration range than fructose spots. Arabinose is approximately on the height of sucrose and appears scarcely stained in brown. Galactose, lactose and mannose are stained in blue similar to fructose containing sugars but less intensive. Galactose is found approximately at the separation range of sucrose, lactose much lower and mannose migrates further than fructose. Trehalose with the performed staining method can not be detected at all and rhamnose appears in red with a comparably long migration range.

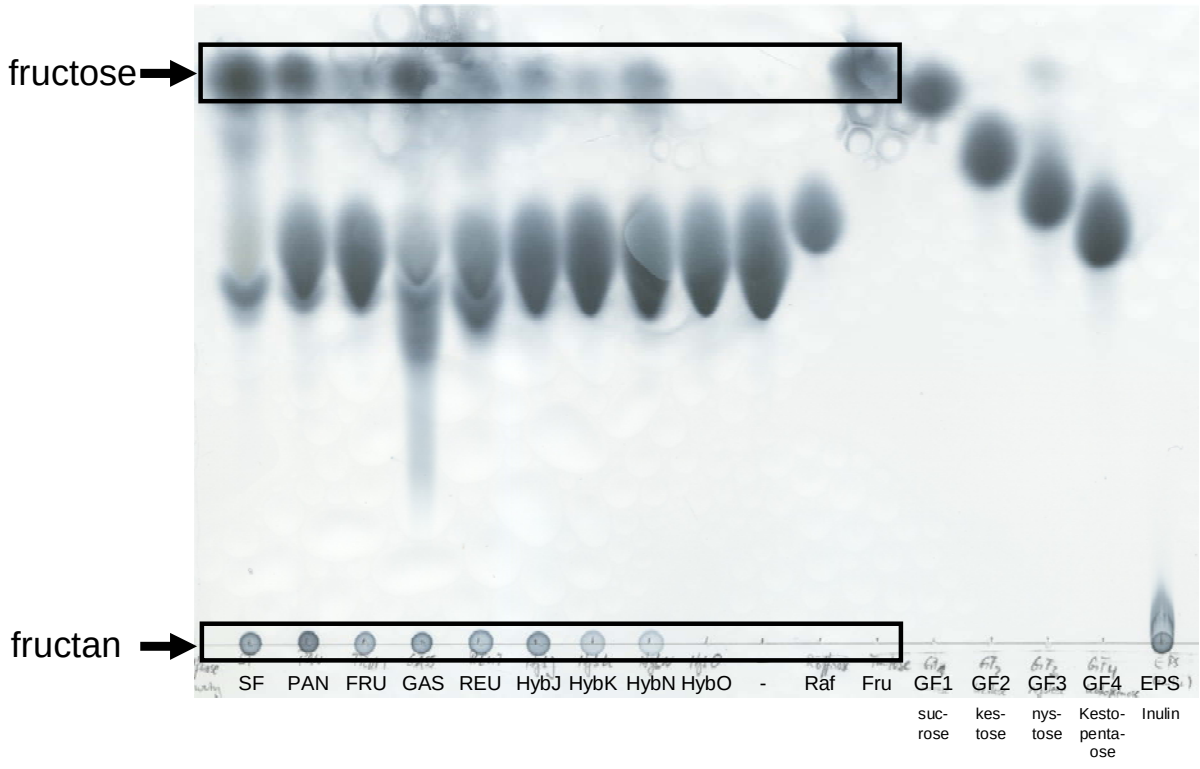


Fig. 37: Analysis of utilization of raffinose by different FTF enzymes on TLC plate. Levan producing enzymes of *L. sanfranciscensis* (SF), *L. panis* (PAN), and *L. frumenti* (FRU) and inulin producing FTFs of *L. gasseri* (GAS) and *L. reuteri* (REU) as well as *L. sanfranciscensis*-*L. panis* hybrids (HybJ, HybK, HybN and HybO) are incubated with raffinose as single sugar substrate. Raffinose (Raf), fructose (Fru), sucrose (GF1), 1-kestose (GF2), 1,1-nystose (GF3), 1,1,1-kestopentaose (GF4) and inulin (EPS) are applied as standard. Raffinose utilisation is proofed by production of fructan and free fructose.

Raffinose can be detected well, since being a fructose containing sugar. On TLC plates this carbohydrate appears as black blue spot in a separation range between GF_3 and GF_4. All FTF enzymes used showed indices for utilisation of raffinose as fructose donor: In contrast to negative control, enzyme containing samples show fructan traces retained at application spot, free fructose and partly traces of levan and in case of *L. reuteri* inulinsucrase and *L. gasseri* modified FTF higher oligosaccharides. The only exception is the artificial hybrid FTF enzyme HybO. In this sample no signs of activity were detected.

4 Discussion

Aim of this work was to provide an overview over characteristic data of different Lactobacillus FTFs as bases for an analysis of the potentials for application of these enzymes for production fructans and FOS for application in food. Therefore it is important to gain comparable data, particularly about the different possible products, biochemical characteristics and information about structure function relation as basis for potential protein engineering.

A critical review of the results described above is needed to draw differentiated conclusions about these questions.

4.1 Screening experiments and sequence identification

Although EPS production is described as a wide spread potential in *Lactobacilli* isolated from sourdoughs or intestine (Tieking et al. 2003a), the screening in the presented work revealed an effective fructan production only for a few strains. The newly identified and modified *ftf* genes also belong to organisms from these environments. (*L. panis* and *L. frumenti* from sourdough, *L. gasseri* isolated from human intestine). So fructan production seems to be an advantage for occupying these ecological niches and if necessary search for further *ftf* genes should be performed among respective organisms.

4.2 Cloning of native genes and artificial hybrid *ftf* genes

Correct cloning processes were proven by sequencing experiments, and the expression of active gene products in case of *L. sanfranciscensis* levansucrase, *L. reuteri* inulinsucrase and the *ftf* genes of *L. panis*, *L. frumenti* and the modified gene of *L. gasseri*. For the first two clones this has been described before (van Hijum et al. 2001; Tieking et al. 2005a). As expected the genes could be cloned in the same plasmid system as *L. sanfranciscensis* levansucrase, correctness of inserts was checked by sequencing with primers located on the plasmid sequence (T7, petRP) and expression resulted in functional gene products with exception of unmodified *L. gasseri* gene. However, the modification T to C did success in a functional protein. So it is proven that the native strain only carries a non-functional mutated gene and so cannot produce inulin or inulin type FOS from fructose.

Yields of protein purification were sufficient for the following experiments. Nevertheless, Coomassie stained SDS-PAGE showed a number of smaller protein fractions in the purified protein pools. These proteins could also be found on His-tag specific Western blot. Therefore, a possible explication is a protein degradation of the target protein, resulting in a set of smaller fragments also carrying the His-tag and being detectable by an anti-His-tag antibody.

However activity staining in renaturing SDS-PAGE clearly shows that only the fractions at the expected size have polymer producing activity. So despite the contamination with smaller protein fragments, these samples can be used for subsequent experiments. Although it has to be taken in account that a determination of amount of enzymatically active target protein can hardly be determined. Due to non target protein contaminants, results of Bradford assays cannot represent the amount of functional, non-degraded and active FTF protein in the sample.

4.3 Comparison of FTF from different lactobacilli

4.3.1 Sequence analysis and classification of newly identified FTF enzymes

ClustalW sequence analysis reveals a close relationship of FTF proteins of lactobacilli. Among all completely known sequences the typical structure including a signal peptide followed by a variable domain and a subsequent catalytic core C-terminally terminated by a proline rich sequence and a LPXTG cell wall anchoring motif are present. Especially the catalytic region in the centre appears to be highly conserved among all (known and newly identified) enzymes. In the active centre of all new enzymes eight amino acids that has been described to be highly conserved among GH68 and many GH32 enzymes and be involved in sucrose binding in the active site (according to studies of available structural information (Ozimek et al. 2006a)), can be identified: Three residues forming a catalytic triade (Meng and Futterer 2003; Ozimek et al. 2004) (see fig. 17, 19 and 20), an arginine residue identified as part of acceptor binding site (Chambert and Petit-Glatron 1991) and four other residues (three of them has been examined by site-directed mutagenesis (Ozimek et al. 2006a)) completely conserved among the *Lactobacillus* FTF sequences. These results are in accordance with the reviews of van Hijum and Korakli (Korakli and Vogel 2006; van Hijum et al. 2006). The close relations of the newly identified enzymes and fragments is not surprising since screening for further enzymes is based on the homologue regions of the enzymes. Phylogenetic tree calculation shows a clustering of FTFs with lactobacillus origin in comparison to other LAB FTFs. Within this group, there is a branching: In the first group, there can be found *L. reuteri* inulinsucrase (van Hijum et al. 2001) and *L. johnsonii* FTF recently described to form β-(2→1) linked inulin (Anwar et al. 2008). In addition to that protein derived from expression of modified *L. gasseri* ftf gene also can be found in that cluster and in this work, was proven to likewise producing an inulinase sensitive fructan. Clearly separated on a second branch there are the described levansucrases of *L. sanfranciscensis* and *L. reuteri* (van Hijum et al. 2004; Tieking et al. 2005a) together with newly identified FTFs of *L. panis* and *L. frumenti*, both producing inulinase resistant fructans. The combination of these facts allows the classification

of *L. gasseri* FTF as an inulinsucrase and *L. panis* and *L. frumenti* enzymes as levansucrases. Further on, phylogenetic analysis enables a prediction for the fructan structure produced by *L. reuteri* 1.1274 FTF (inulin) and *L. acidophilus* FTF (levan) which gene and protein sequences are only known fragmentary. Unfortunately both strains did not produce enough fructan in liquid culture to confirm this hypothesis.

Based on the sequence comparison it can be further stated that within the group of FTFs of lactobacillus origin the homology is very high since proteins do not primary cluster in species groups (e.g. a cluster containing exclusively *L. reuteri* enzymes) but due to the product specificity. That means that the critical difference between inulinsucrases and levansucrases lies within the primary sequence as proposed before (Korakli and Vogel 2006). Since phylogenetic analysis of only a short central section leads to similar clustering, the differences may probably be located within this section.

Analysis of tertiary structure models supports that thesis, since only a central part is used for molecular modelling (for example in *L. panis* model only about half of the complete sequence is used). Nevertheless, that is sufficient forming a globular protein with a deep cavity harbouring the aa residues necessary for catalytic triade as well as a sucrose binding site and structure for complexing a Ca^{2+} cation. In principle, models obtained by that modus of molecular modelling which bases upon an alignment with known structures, are limited in their information. One reason lies in the fact, that modelling is directed to approach an already explored structure, in this case, a five bladed β-propeller topology as described for *B. subtilis* levansucrase (Meng and Futterer 2003). Due to that detailed information as steric angels of distinct amino acids are not accessible. Secondly, input data are reduced in length as mentioned above. That means interesting regions as N-terminal variable regions with directed repeats are not part of the model. Nevertheless, despite a certain difference in primary structure tertiary structure developing a five bladed β-propeller seems to be very probable. The model is supported by a reasonable arrangement of the aa residues involved in catalytic triade as well as a expedient position of the sucrose binding boxes and a supposable conformation of the aa residues potentially complexing a Ca^{2+} cation as presented in fig. 17, 19 and 20. Regardless these basic aspects, striking differences between the models as missing secondary structure elements like certain α-helices, must not be overvalued. To sum up the information obtained by molecular modelling, it can be stated that models with a high plausibility were developed, since they are close to the known structure of a related protein and important aa residues are located at reasonable positions. Nevertheless there cannot be found structural characters supporting the classification of phylogenetic analysis or revealing

further information about structure function relationship. That is due to the fact that the used modelling method does not allow drawing conclusions about detailed structural information.

4.3.2 Functional analyses

4.3.2.1 Fructans produced

As described above, inulinase digest in combination with sequence alignments and phylogenetic examination, the fructans produced by the heterologously expressed FTFs of *L. panis*, *L. frumenti* and *L. gasseri* (modified by elimination of a stop codon inside the original gene) can be classified. Thus, *L. panis* and *L. frumenti* FTF enzymes produce levans whereas *L. gasseri* FTF enzyme has product specificity for inulin. Since up to date only two types of fructans has been described with β-(2→1) linked inulinase sensitive inulin and β-(2→6) linked levan, insensitive against hydrolysis by inulinase, performed assay obtains strong evidence concerning the linkage type of the examined fructans. Results are supported by sequence data which clusters *L. gasseri* enzyme with inulin producing enzymes of *L. reuteri* and *L. johnsonii*. *L. panis* and *L. frumenti* derived fructans in the phylogenetic tree are on one branch with levansucrases from *L. reuteri* and *L. sanfranciscensis*. Nevertheless in last consequence the existence of alternative linked fructans can not be excluded and results would have to be proven by NMR in last consequence to give a definitive answer on the question about linkage types in produced fructans.

4.3.2.2 pH and temperature influences

The selection of the buffering system turned out to have only little effect on the activity of the tested proteins. The pH optima for over all activity of the three newly cloned and heterologously expressed FTFs (*L. panis*, *L. frumenti* and *L. gasseri* origin) are in a comparable range between pH 4 and 5. Nevertheless *L. gasseri* inulin producing enzyme tends to be less active at pH 4 than the levan producing enzymes. Theses optimal ranges are about one pH unit below the described optima for *L. sanfranciscensis* levansucrase (optimum at 5.4, more than 50% of optimal activity between 4.0 to 6.2 (Tieking et al. 2005a)) and *L. reuteri* levansucrase (optimum at pH 5.4 (van Hijum et al. 2004)) and inulinsucrase (optimum range pH 5.0 to 5.5 (van Hijum et al. 2002)). For *L. johnsonii* inulin producing FTF even a considerably higher optimum at pH 7 has been described, however 85% and more activity of that enzyme can be detected in a range of pH 4.5 to 6.0 (Anwar et al. 2008). Other related enzymes with comparably high pH optima can be found, e.g. levansucrase (LevC) from *Leuconostoc mesenteroides* (optimum between pH 6.5 o 7.0 (Olvera et al. 2007)). Since *L. gasseri* inulin producing enzyme is closely related to *L. johnsonii* protein this is particularly

remarkable. In this context, it has to be mentioned that *L. gasseri* modified enzyme in comparison with examined levan producing FTFs shows higher pH optima and a drastic loss of activity at pH 4 as mentioned above. Nevertheless it has to be mentioned that most FTF enzymes have a broad range of activity that is similar to the environmental conditions where lactobacillus strains are found.

In contrast to that, temperature optima clearly deviate from the growth optima of the original organisms (mostly around 37°C). Experimental data received in this work reveal a temperature optima for glucose release of *L. panis*, *L. frumenti* and *L. gasseri* derived enzymes of 45°C to 50°C. Similar data are published for levansucrase and inulinsucrase of *L. reuteri* (50°C both) (van Hijum et al. 2002; van Hijum et al. 2004) and for *L. johnsonii* inulin producing enzyme with 55°C even a slightly higher temperature optimum was explored (Anwar et al. 2008). Only the reported optimum of *L. sanfranciscensis* levansucrase (a range of 35°C to 45°C) is close to the growth optima of thermophilic *Lactobacillus* strains. The phenomenon of an increased temperature optimum of enzymes compared to the growth optimum of their original organism is not unique. Similar observations can be made for many catalytic proteins of various sources.

4.3.2.3 Influence of substrate concentration

The influence of substrate (sucrose) concentration was investigated by using MM kinetic model. Parameters obtained by experiments in this work are problematic regarding some aspects: On the one hand, experimental data has to fit quite well in the MM function to deliver reliable values for the respective parameters. Not all plots derived do fulfil this criterion as can be seen in the R^2 values in tab. 8 and 9. In addition to that saturation is not reachable in the experiments for some plots. This particularly affects kinetics of transfer reactions. A further problem is that absolute concentration of active enzyme is not known since there is significant contamination with smaller proteins and peptides as shown by SDS-PAGE and Western blot experiments. However this information is needed to achieve reliable V_{max} values. So all V_{max} values presented in this work could only be suitable for a direct comparison among each other under the assumption that the grade of contamination of FTF preparations is similar for all enzymes. If that is not the case, they may act as an indicator for quality of purification of heterologously expressed FTF enzyme. K_M values are not dependent to enzyme concentration and so can be compared to literature data of other enzymes. Therefore, MM kinetics are developed for the newly cloned FTF enzymes of *L. panis*, *L. frumenti* and *L. gasseri* at optimum pH and temperature conditions (see tab. 8). The obtained values are higher than already published data for similar enzymes; however they are in the

same order of size. K_M values for over all activity obtained at optimum conditions are reported for *L. sanfranciscensis* levansucrase over all activity (13.1±3.4 mM) (Tieking et al. 2005a) and *L. reuteri* levansucrase (21.0±.41 mM) (van Hijum et al. 2004). For the latter also data for K_M of hydrolysis (13.0±1.4 mM) are available. For *B. subtilis* SacB enzyme (which structure serves as basis for molecular modelling) K_M for over all activity and hydrolysis has been reported with 4.0±0.4 mM (Chambert and Petit-Glatron 1991). The significance of the observed differences is limited since a close look to the plots and fitted curves reveals a certain degree of impreciseness which can also be presumed for the experiments concerning the published data.

For a better comparison, experiments examining the influence of sucrose concentration on activity of FTF enzymes, a series of measurements with all enzymes available in this work has been carried out in parallel at identical temperature and pH conditions. Results are presented in fig. 27 and tab. 9. Problems and impreciseness as described above do also affect these experiments, nevertheless do these experiments allow a direct comparison. Very striking are the different V_{max} values which differ up to one magnitude among different enzymes. The reason for that with high probability lies in the different content of active FTF enzyme in the different preparations as discussed above. Since the amount of total protein inserted in the assay was equalized, these values only show that the used purification fraction of *L. reuteri* inulinsucrase was distinctly less successful than the one of *L. sanfranciscensis* levansucrase. A further eye-catching observation regarding the plots and K_M values are very high K_M values for transfer activity of *L. sanfranciscensis* levansucrase, *L. reuteri* inulinsucrase and *L. gasseri* derived inulin producing enzyme. Separately monitored hydrolysis and transfer reactions (in contrast to over all activity based on glucose release) at optimum conditions are reported not to follow MM kinetics (Tieking et al. 2005a). That cannot be approved by these experiments at suboptimal comparison conditions. Regarding the R^2 values, all experimental data could quite well be fit to MM equation. Nevertheless, it has to be mentioned, that for the respective curves of transfer activities saturation was not achieved in the experiment. In addition to that, standard deviation is quite high for several values of hydrolysis and transfer activity data in measurements with *L. gasseri* derived inulin producing FTF. A direct connection to similarities in aa sequence among the three proteins, compared to *L. panis* and *L. frumenti* levan producing enzymes and therewith a structure function relationship, cannot be found. However it is a striking phenomenon that such obviously different behaviour can be observed among very closely related *L. sanfranciscensis* and *L. panis* levansucrases.

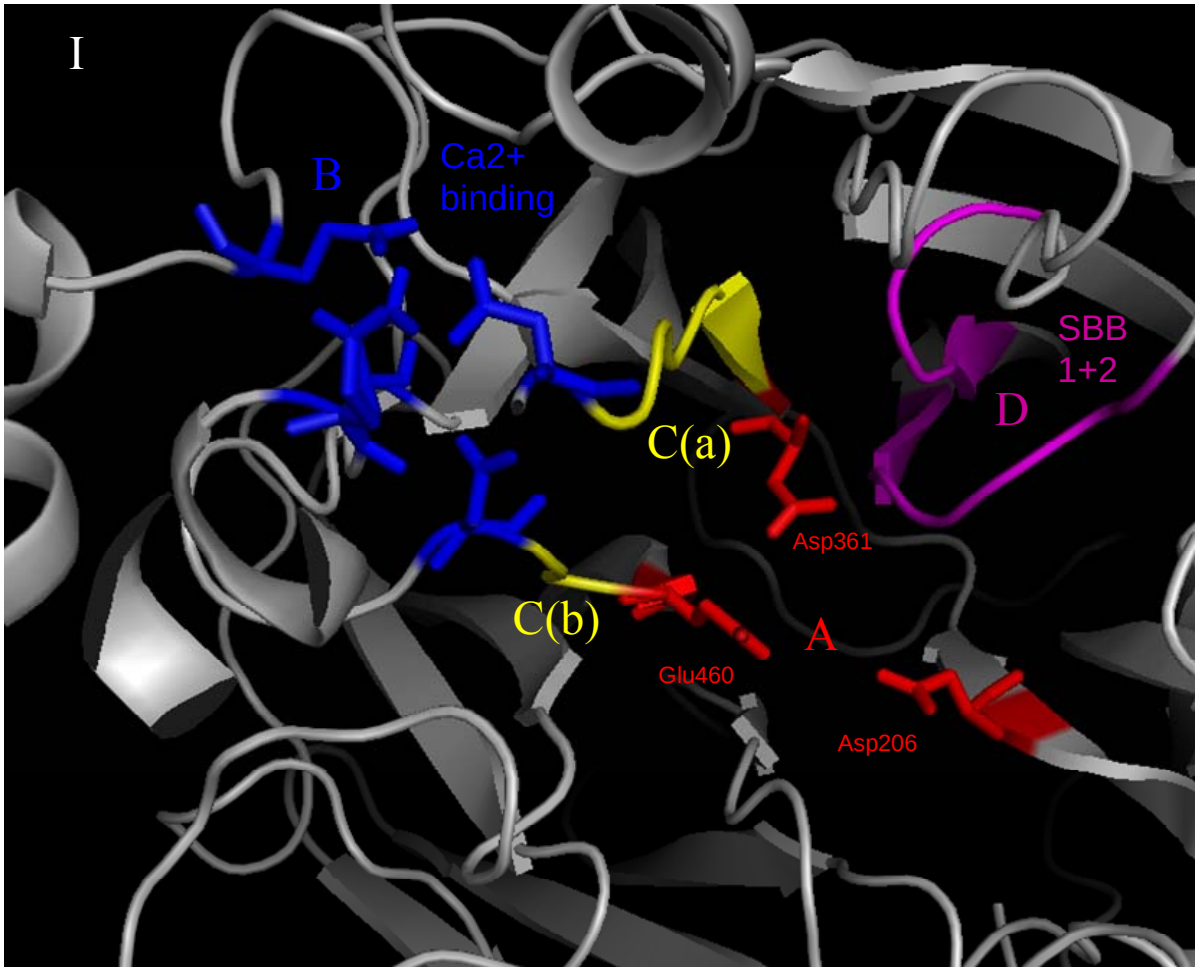
4.3.2.4 Role and influence of Ca^{2+} and alternative bivalent metal cations

Further comparative experiments on functionality of different of lactobacilli were performed on the influence of metal cations. The necessity of bivalent calcium cations for proper functionality of these enzymes has been perceived and widely studied for *L. reuteri* fructosyltransferases (levansucrase and inulinsucrase) (Ozimek et al. 2005). An improved temperature stability of the tertiary structure by complexion of Ca^{2+} ions is proposed in this context. In this work the influence and the possibility of replacement of Ca^{2+} by alternative (bivalent) cations is examined. Levansucrases of *L. sanfranciscensis*, *L. panis* and *L. frumenti* and modified inulinsucrase of *L. gasseri* were involved in the tests. An interesting observation is the influence of Mg^{2+} and Mn^{2+} ions on the enzymes: As expected, if calcium cations are added in an assay with heterologously expressed and purified FTF enzyme a significant increase of activity (around 100 %) was observed in all tested enzymes. The effect seems a bit stronger in *L. sanfranciscensis* levansucrase, but standard deviation of the value obtained with Ca^{2+} addition has to be taken in concern. In contrast to that the addition of Mg^{2+} and even stronger Mn^{2+} reduces the activity of all of the FTF enzymes. Nevertheless, while the effect of both was quite drastic in levansucrases (less affected was *L. frumenti* enzyme which lost approximately 75 % activity compared to control), Mg^{2+} and Mn^{2+} only reduced total activity of *L. gasseri* inulinsucrase approximately 25 % and 50 % respectively.

EDTA practically totally delays activity of FTF enzymes a very low significant residual activity could only be detected for *L. gasseri* inulinsucrase. For this enzyme Ca^{2+} cations can completely restore activity. So the conclusion can be drawn that the negative effect of EDTA on this inulin producing enzyme is due to removal of Ca^{2+} ions bound to the enzyme. In contrast to that, activity of levansucrases is nearly completely restored but in all three cases there is a significant difference to the maximum derived with Ca^{2+} addition without a previous EDTA treatment.

cation	ionic radius [pm]
Na^{+}	98
K^{+}	133
Ca^{2+}	**106**
Mg^{2+}	78
Mn^{2+}	91
Zn^{2+}	83

Tab. 11: Ionic radii of different cations used in experiments. Ionic radii are given in pm. Optimal cation Ca^{2+} is accentuated in bold letters.



II

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SFR_lev   RKNDDYCLRDPHV 469   MASDEVERVNVVK 571
REU_lev   RENDDYCLRDPHV 407   MASDEVERPNVVK 509
FRU_ftf   RKDDDYCLRDPHV 333   MASDEVERPDIVK 435
PAN_ftf   RQDDDYCLRDPHV 364   MASDEVERPDVVK 466
JOS_ftf   KGADNIAMRDAHV 428   MVSDEIERPDVVK 530
GAS_ftf   KGADNIAMRDAHV 423   MVSDEIERPDVVR 525
REU_inu   KGADNIAMRDAHV 427   MVSDEIERPNVVK 529
          :  *: .:**.**       *.***:** ::*:
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C(a) C(b)

Fig 38: I: Structural detail of *L. panis* FTF active site. Aa of catalytic triade are presented as stick models and labelled in red (A), aa involved in bivalent cation complexion are presented as stick models in blue (B). Shortest connections between cation complexion site and aa of catalytic triade are coloured in yellow (C). Transition state stabilizer Asp361 is connected via 5 aa to D355 (C (a)) and acid base catalyst Glu460 is connected via two aa to D457 (C (b)) of the complexion site. For better orientation sucrose binding boxes one and two are coloured in purple (D).

II: Alignment of according sequence sections of completely known FTFs (levansucrases of L. *sanfranciscensis* (SFR_lev), *L. reuteri* (REU_lev), *L. frumenti* (FRU_ftf) and *L. panis* (PAN_ftf) and inulinsucrases of *L. johnsonii* (JOS_ftf), *L. gasseri* (GAS_ftf), *L. reuteri* (REU_ftf)). Colours are according to structure details in part I of this figure; bold sequences belong to enzymes involved in experiments exploring cation influence.

Accordingly, EDTA must be suspected to directly affect the protein, too. The different extent to dependence of Ca^{2+} cations concerning levan and inulin producing enzymes has been previously reported (Ozimek et al. 2005).
Neither Mg^{2+} nor Mn^{2+} did show much potential to restore activity. A reason for the effects of the different cations may be based in the different ionic radius (see table 11).

A possible explanation for the negative effect of alternative bivalent cations is their lower ionic radius compared to Ca^{2+}. Since this ion is described to stabilize tertiary structure when complexed, alternative ions may also be complexed due to their charge (the charge seems to be of importance since Na^{+} or K^{+} did not show any influence). When bivalent cations of lower ionic radius replace Ca^{2+} they might squeeze the ion complexion site and in consequence slightly modify the tertiary structure of the entire molecule.
This would be of minor interest if the complexation site would not be connected closely to two of the three aa of the catalytic triade, namely the transition state stabilizer Asp361 and acid base catalyst Glu460 (*L. panis* FTF numbering). For *L. panis* levansucrase this is demonstrated in fig. 38, I. It is worth to mention that respective spacer sequences are specific for levan- and inulinsucrases (fig 38, II). A small flexion of the spacer aa strand due to complexion of a bivalent cation of wrong ionic radius might modify the angle of critical aa residues of the catalytic triade and therewith significantly influence enzymatic activity. The fact that Mn^{2+} with an ionic radius of 91 pm of the chosen ions is closest to Ca^{2+} radius (106 pm) tends to bring back more activity than Mg^{2+} of Zn^{2+} supports that thesis. However ionic radius analysis cannot explain why in all cases Zn^{2+} does affect the activity mostly effective and proposes there are further factors that must be considered.

4.3.2.5 Product spectrum and product ratios of different heterologously expressed FTFs at different environmental conditions

For two variations (containing and not containing N-terminal variable domain) heterologously expressed *L. sanfranciscensis* levansucrase, data about the products of enzyme reactions at different pH, temperature and sucrose concentrations have been published (Tieking et al. 2005a). Full length and truncated version did not show differences concerning these experiments. For *L. panis* levansucrase similar detailed data are obtained in that work (see fig. 30). With increasing temperature for *L. sanfranciscensis* enzymes hydrolysis reactions are preferred, transfer and polymerisation reactions are reduced. In data presented here for *L. panis* enzyme a decrease of polymerization reactions is also observed. In contrast to *L. sanfranciscensis* levansucrase, kestose ratios apparently increase with raising temperature. A

clear trend to increased fructose release due to preferred hydrolysis cannot clearly be approved by this data due to standard deviation (theses are not given for *L. sanfranciscensis* levansucrase by Tieking (Tieking et al. 2005a)). In addition to that, these conclusions for *L. panis* enzymes are based on only two temperature points since in the experiments carried out here, chemical hydrolysis of sucrose was clearly dominating at 50°C. Similar problems were observed at low pH values (pH 3.1) so a direct comparison with published data for *L. sanfranciscensis* levansucrase is not useful. Concerning sucrose concentrations trends for both enzymes were clear and identical concerning fructose release by hydrolysis and kestose production: higher sucrose concentrations decrease hydrolysis and boosts kestose production. But while this is combined with decreasing polymerization rates for *L. sanfranciscensis* levansucrase, for *L. panis* enzyme kestose and levan production increase in parallel with increasing substrate availability. Over all, the striking difference in comparing these data is a domination of kestose production compared to polymerization reactions for *L. sanfranciscensis* enzyme while *L. panis* levansucrase shows significantly higher levan production rates compared to transfer reaction rates.

That phenomenon could not be reproduced in a series of similar experiments in smaller scale that were carried out in that work with five different enzymes (levansucrases of *L. sanfranciscensis*, *L. panis* and *L. frumenti* and inulinsucrases derived from *L. gasseri* and *L. reuteri*) in parallel to achieve a good comparability (see fig. 32). In these experiments produced FOS merely could not be detected and quantified with HPLC methods. Nevertheless it could be shown that *L. sanfranciscensis* levansucrase was the most effective producer of inulintype FOS among levansucrases supported by TLC results, which showed a production of higher FOS (GF3, GF4 etc.) for *L. sanfranciscensis* levansucrase but not for *L. panis* and *L. frumenti* levan producing enzymes (see fig. 30) . Inulin producing enzymes are more effective in FOS production than levansucrases. However, the obtained data reveals similar trends of all enzymes when pH is altered: at increased pH levels less free fructose but higher polymer and FOS are detected. Compared to the parameters substrate concentration and temperature the effect of pH is relatively weak. In addition to that, it must be considered that there is an influence of chemical environment on reaction products, which means an increasing hydrolysis of fructans and FOS with decreasing pH resulting in free fructose units. So, a clear influence of pH on the product ratios of different *Lactobacillus* FTFs can hardly be proven by this series of experiments.

Temperature modification could have similar effects on fructans and FOS: High temperatures would support chemical hydrolysis of polymer- and oligosugars under release of free fructose. That trend can be observed for both inulinsucrases and *L. frumenti* FTF. In levansucrases of *L. sanfranciscensis* and *L. panis* data does not support that development completely. However repeated measurements possibly could confirm this trend here, too. Although more significant than pH influence, it cannot be resolved by this experiments if changes in product ratios observed at different temperature steps are due to effects of temperature on the enzymatic product specificity or just chemical phenomena affecting the produced fructans and FOS.

However an influence on product ratios by affecting enzymatic product specificity can be stated for substrate concentration: Basically for all FTF enzymes tested data show with few exceptions that high sucrose concentrations lead to increased transfer and polymerization ratios. As mentioned above, this phenomenon has been observed for *L. sanfranciscensis* levansucrase before (Tieking et al. 2005a). There is no obvious mechanism proposed for the effect of substrate concentration on product ratios.

Formation of FOS has been well examined for *L. reuteri* inulin- and levansucrases. A comparison of *L. reuteri* inulin- and levansucrase revealed a preference of levansucrase for fructan production. With raising sucrose concentration saturation could be achieved. In contrast to that, inulinsucrase mainly produces inulintype FOS and saturation could not be reached by increasing substrate concentrations (Ozimek et al. 2006b). For inulinsucrase of *L. reuteri* this kinetic behaviour could be reproduced for transfer reactions (see fig. 27 D). Inulin producing enzyme of *L. gasseri* origin tended to a comparable transfer reaction kinetic and also FOS producing reactions of *L. sanfranciscensis* levansucrase did not reach saturation. Nevertheless it could not be approved that FOS production is dominating in inulinsucrases in this study, although they significantly produce more and higher FOS than levansucrases. Based on the observations to *L. reuteri* FTFs Ozimek (Ozimek et al. 2006b) proposed a theoretical mechanism for fructose transfer on acceptor molecules in *Lactobacillus* FTFs (Nomenclature according to (Davies et al. 1997)):

Sucrose is bound in the central pocket of FTF protein and glucose is released from +1 position, while fructose monomer stays fixed in the active site (-1 position) as covalent fructosyl-enzyme intermediate. Subsequently water or an alternative acceptor molecule can enter and fructosyl residue is transferred. The more effective FOS production in inulinsucrases that by trend is approved by experiments in this work is explained by a higher affinity of levansucrases +2 and +3 subsites for growing fructan chain. Effective FOS production is also

named as reason for the more efficient fructose utilization of inulinsucrases (less hydrolysis activity than in levansucrases) that was also observed in the present studies.
Mutational analysis of three amino acids suspected to be involved in sucrose binding has been performed: W (205) and W (276) as part of -1 subsite and R (461) as revealed in -1 and +1 subsites. These experiments could proof the necessity of these residues for FOS formation but also revealed that further regions of protein are responsible for product specificity (Ozimek et al. 2006a). Concluding, inulinsucrases are supposed to highly efficiently produce kestose and higher FOS while in levansucrases polymer production and hydrolysis are preferred, kestose production plays a minor role and higher oligosaccharides are practically not formed. This is in contrast to observations made by Tieking (Tieking et al. 2005a; Tieking et al. 2005b; Tieking et al. 2005c) by HPLC analysis for *L. sanfranciscensis* levansucrase and approved in this work by TLC. Nevertheless, *L. sanfranciscensis* is the only levansucrase comprised in this study producing detectable amounts of higher oligosaccharides. In addition to that oligosaccharide rates were significantly below the ones of the tested inulinsucrases. Since *L. sanfranciscensis* levansucrase product rate profiles apart from that, share much similarity to other levansucrases in this study, all in all the results presented here support the proposed reaction mechanism assuming that *L. sanfranciscensis* levansucrase +2 and +3 subsites have a greater affinity for FOS as acceptor molecules than the other levansucrases comprised in this study.

The influence of pH and temperature on the MW of the fructans produced was rather weak. Generally percentages of higher MW fructan appear at higher pH and lower temperatures. So, it can be speculated that there is no effect on the enzyme itself, but chemical hydrolysis of the fructan produced. Such chemical reactions will be favoured at increased temperatures and low pH. The differences between fructans produced by different FTF enzymes may be due to characteristics of the fructans themselves. The ratio of branching could be a crucial factor, here. Unfortunately, information about the branching in fructans could not be obtained in this work.
Sucrose concentration did not influence fructan size produced by *L. frumenti* and only slightly for *L. sanfranciscensis*. In contrast to that fructans produced by *L. panis*, *L. gasseri* and *L. reuteri* enzymes have a tendency to an increased percentage of lower MW polymers when substrate concentration is higher. A reason for this alteration of fructan composition by modification of substrate concentration might be found in the affinity to the fructan produced. If affinity is equal for higher MW and lower MW polymer molecules the probability for

elongation of every chain is equal, too. This could lead to a symmetric distribution of fructan molecules as observed for *L. frumenti* levansucrase and similar to *L. sanfranciscensis* levansucrase. For the latter a higher affinity of acceptor binding site for longer chains can be assumed leading to an increase of higher MW levan molecules when more sucrose substrate is available. In contrast to that, for *L. panis* levansucrase, and inulinsucrases of *L. gasseri* an *L. reuteri*, the affinity of the acceptor binding site for higher MW fructans seems lower resulting in a growing percentage of lower MW fructan molecules with increasing substrate concentration. This would explain the asymmetric peak development observed (fig. 35).

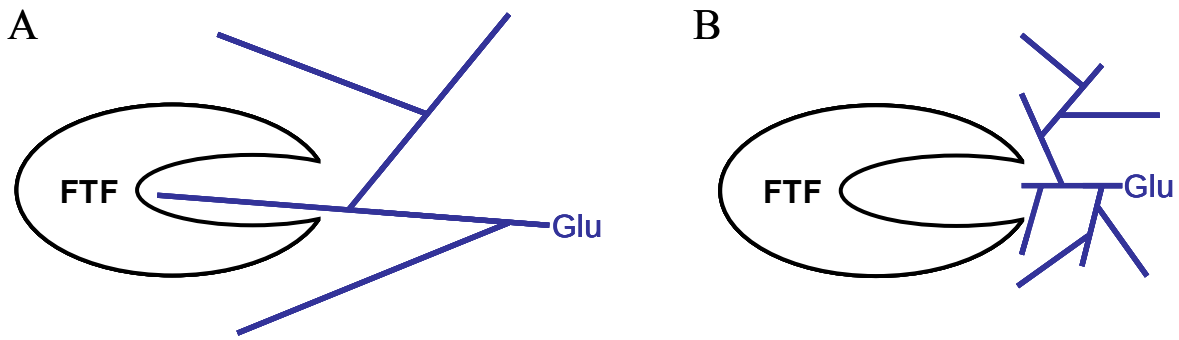

Fig. 39: Model for possible correlation of fructan branching on MW distribution. Despite less reducing ends in the lowly branched fructan (A) molecule can grow to higher MW since reducing ends sterically can fit in the active centre of the FTF enzyme and can be elonged. In contrast to that, highly branched fructan (B) cannot bind to acceptor binding site inside FTF protein due to steric hindrance and so does to grow to higher MW despite more reducing ends for elongation are present in the molecule. When sucrose concentration is higher more FTF in fig. B will start to produce more molecules while FT F in fig. A can use the additional sucrose for elongation of existing chains.

As suspected for the influence of temperature and pH on the MW of fructans produced the grade of branching could be an important factor here, too: Interaction of enzyme and fructan could be influenced by the structure of the polymer. It is imaginable that long chains with few branches are not limited in entering the enzyme active site while highly branched molecules sterically might not fit inside the enzyme to reach the acceptor binding site (fig. 39). A surplus of sucrose might lead to a further elongation of existing molecules when they are only occasionally branched. In contrast to that, enzymes producing highly branched polymers will have to use additional substrate for producing more but smaller fructan molecules since elongation of existing chains is not possible.

4.3.2.6 Capability of formation of HeOs and acceptance of raffinose as fructose donor

In addition to production of kestose and nystose, *L. sanfranciscensis* levansucrase has been suspected to produce HeOS by fructose transfer from sucrose to various alternative sugar

molecules contained in wheat flour when HeOS production was observed in sourdough fermentation by the original levansucrase positive *L. sanfranciscensis* strain (Tieking et al. 2005c). Although this thesis was supported by the absence of HeOS in the dough when wheat flour was fermented by levansucrase knockout mutant, HeOS formation could not be reproduced by *in vitro* utilization of heterologously expressed and purified enzyme in this work.

Neither *L. sanfranciscensis* levansucrase nor any of the other enzymes showed capability of HeOS formation by TLC (see fig. 36) or HPLC analysis (data not shown) when sucrose and different alternative sugar acceptor molecules were inserted in the assays. As seen above for FOS production, for HeOS formation the basic question is about the configuration of the +2 and +3 substrate binding subsites. Generally the subsites in levansucrases has been proposed to be preferentially specific for growing levan chains while inulinsucrase less specific substrate binding subsites are more efficient in binding sucrose and inulintype FOS as target molecules for fructose transfer. So, for inulinsucrases there is higher exception of HeOS forming capability. However, these did neither show any tendency to utilize alternative sugar types as acceptors. Most of the sugars do obviously not affect FOS production (for example see fig 36 D) and so probably cannot even be bound to acceptor sites, but some sugars have negative effects on separation and staining performance on TLC and their exact effects cannot be estimated.

Nevertheless, the general possibility of HeOS production of these enzymes may not be excluded since in this context, it must be mentioned that for structurally related *L. mesenteroides* levansucrase the synthesis of erlose by fructosyltransfer to maltose was reported (Kang et al. 2005). Additionally for *B. subtilis* levansucrase the production of sucrose analogues and HeOS production by use of various glycopyranosides and disaccharides as acceptors has been described and the existence of only one acceptor site identical to the substrate site is proposed (Biedendieck et al. 2007). In that study for the examined enzyme, also the use of distinct sucrose analogues as fructose donors is reported.

For the enzymes comprised in this work only raffinose has been tested for its capability to serve as fructose donor in *Lactobacillus* FTFs. The ability to use this trisaccharide as substrate has been reported before for *L. sanfranciscensis* levansucrase (Tieking et al. 2005c; van Hijum et al. 2006). The results obtained clearly show a utilization of raffinose as fructose source for fructose production and release of free fructose for all active FTF enzymes involved in the experiments. Raffinose is the only fructose-containing agent in the assays and

fructose or fructose containing sugars beside raffinose cannot be detected in negative control. So, this is sufficient evidence for FTF activity based on raffinose as fructose donor substrate.

4.4 Hybrid proteins from *L. sanfranciscensis* and *L. panis* levansucrases

Beside lacking detail information for structure function relationships in the catalytic centre of *Lactobacillus* FTFs that could best be solved by experiments exploring details about sterical relationship of crucial aa residues in the catalytic site in the centre of the protein, significant differences in sequences of the closely related lactobacillus FTFs are in the N-terminal variable domain. In addition to that relationship among FTFs is less close regarding the C-terminus.

Nevertheless the function of the not conserved N-terminal region, that in some cases can be very large including series of direct repeats and in others be merely existent, is still totally unclear (van Hijum et al. 2006). By cloning *L. sanfranciscensis* levansucrase, Tieking could not observe significant differences in characteristics of full length and truncated protein lacking N-terminal directed repeats (Tieking et al. 2005a). Also, functionality of C-terminal domain is not known yet. It can be speculated since in analogy to GTFs there may be an influence of this protein section on product size and/or enzyme specificity. This thesis is supported by experiments observing lager fructan polymers in non-LAB *B. subtilis* FTF when C-terminus is enlarged (Chambert et al. 1992).

In this work, artificial hybrid FTF proteins have been constructed, combining long, repeat-containing *L. sanfranciscensis* levansucrase N-terminus with *L. panis* FTF, which originally has a short N-terminal variable region without any direct repeats (HybJ, HybK). In addition to that catalytic domains of *L. panis* and *L. sanfranciscensis* levan producing enzymes are combined with the N- and C-terminus of the respectively other FTF (HybN, HybO).

With coPCR, required DNA constructs could be obtained, and cloning was carried out in analogy to unmodified *ftf* genes. Nevertheless, heterologous expression turned out to be less effective than with native genes. *L. panis* FTF catalytic core in combination with *L. sanfranciscensis* levansucrase N- and C-terminus (HybO) could not be produced and purified in a detectable amount at all. Just poor activity traces are observed in activity stained renaturing SDS-PAGE. There is no obvious reason for the impossibility to express and purify this hybrid construct since sequencing of produced plasmid did not show errors in respective regions with high quality sequencing results. However, it was impossible to completely sequence the entire plasmid construct, so mutations in the basic plasmid regions cannot be excluded, although this is not very probable. It must not be forgotten that purification yields of

all hybrid proteins in this study was lower than the ones of native proteins for an unidentified reason.

Comparing results of kinetic studies of hybrid FTFs and native levansucrases of *L. sanfranciscensis* and *L. panis*, it is observed that activity levels reached with hybrid proteins are significantly lower than the ones of native proteins. Since expression levels are dissatisfactory, contaminants with smaller protein and peptide fragments may play a more important role here. And problematic aspects in context with this experiments discussed above do even stronger affect theses results since the activity is lower and statistical errors have a stronger influence. Nevertheless, similarities between *L. panis* FTF and HybJ and HybK and *L. sanfranciscensis* levansucrase and HybN respectively can be observed. As compared to native *L. panis* enzyme, transfer activity of HybJ and HybK does achieve saturation at relatively low substrate concentrations while for HybJ a saturation of transfer activity was not reached within the sucrose concentration range tested. However, saturation seems not as unattainable as for *L. sanfranciscensis* levansucrase there is a clear difference to the distinct plateau in HybJ and HybK kinetic.

Consequently it can be stated that the sequence information in the central catalytic domain of FTFs is responsible for distribution of transfer- hydrolysis and polymerization reactions as described and discussed above (Tieking et al. 2005a; Korakli and Vogel 2006; Ozimek et al. 2006a; Ozimek et al. 2006b; van Hijum et al. 2006).

This is supported by data obtained by quantification of products obtained with different sucrose concentrations. Although differences are more distinctive regarding *L. sanfranciscensis* and *L. panis* native enzymes, hydrolysis rates are dominating in HybN and are significantly lower for HybJ and HybK. Product ratios at different temperature do not differ much for native enzymes and so do not for hybrids. So comparison of these data does not bring further information. That is similar to the results of experiments exploring the influence of pH on product ratio. But there is one exception: While, in analogy to native enzymes, HybJ and HybN balanced hydrolysis and polymerization rates are only weakly affected by pH changes, for HybK there is a drastic domination of hydrolysis at ph 3.8 while polymerization clearly dominate at increased pH. Due to the fact that HybJ and HybK only differ in few aa residues, this is surprising and suggest that modification of the respective aa residues introduce a sensitivity against H^+ concentrations because neither *L. sanfranciscensis* native levansucrase not *L. panis* FTF enzyme showed a similar behaviour. But in this context, it must be reminded that enzymatic activity of artificial hybrid FTFs is quite weak and so

amounts of products are relatively small. However, quantification of such small amounts of substances with HPLC turned out to be not as exact as desired.

All in all results of experiments with domain change constructs between *L. sanfranciscensis* and *L. panis* levan producing proteins only brings limited information that supports the theories about structure function relationships in *Lactobacillus* FTF enzymes already presented before. Main problems in this context are the bad expression and purification yields that even eliminated construct HybO from the studies and weak activity levels. Further, crucial parameters as detailed fructan analysis by NMR were not available for experiments in this work.

5 Conclusions

In the presented work, two new *ftf* genes of lactobacilli could be identified in *L. panis* and *L. frumenti*. Both could be cloned and heterologously expressed in *E. coli*. Analysis of sequence and produced fructans allow the classification of both FTFs as levansucrases. An inactive *ftf* gene of *L. gasseri* could be cloned in an active form repairing the open reading frame by introducing a base exchange. The heterologously expressed protein could be classified as inulinsucrase fitting sequence analytical data.

These three new enzymes were compared with known *L. sanfranciscensis* levansucrase and *L. reuteri* inulinsucrase concerning their sequences, biochemical properties, kinetic data and reaction products. Molecular modelling revealed a close structural relationship of these enzymes. Hybrid proteins of *L. sanfranciscensis* and *L. panis* levansucrases have been constructed, expressed and examined.

This work represents the largest comparative analysis of fructosyltransferases of lactobacilli. The studies concerning EPS confirm the existence of two different fructan types that are formed by similar enzymes. However, experiments with the available enzymes could not support postulated potential for formation of various HeOS and only limited the formation of higher FOS. Inulinsucrases have shown to be more suitable for the production of those inulintype FOS.

Data obtained in the work confirm hypothesis about structure/function relationships postulated before. But since the comparison of sequence data including molecular modelling of known and new FTFs does not clearly reveal critical regions for product specificity, the enhancement of enzymatic performance by protein engineering must be judged as difficult. Nevertheless, beside the published aspects about structure/function relation and the artificial protein models, construction and functional analysis of hybrid FTFs shows a concentration of main enzymatic functions in a relatively small active centre region. Based on these catalytic cores the construction of simplified tailor made proteins seems possible.

Especially detailed structural information about the fructans produced by the newly heterologously expressed enzymes should be aim of future studies and could reveal new aspects about product specificity based in the primary structure of family GH68 enzymes. Further on, crystalline structures of selected lactobacillus FTFs with bound substrate and acceptor molecules are desirable to identify crucial protein domains and aa residues influencing product specificity.

All in all particularly the introduction of three new FTFs is an important step for an establishment of these enzymes in an industrial scale process to produce fructans and FOS for application in food.

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7 Appendix

7.1 Figures

7.2 Tables

7.3 Alignment of FTF aa sequences of lactobacilli

```
SFR_lev         ---MTKEHKKMYKAGKYWAVATLVSASILMEV-GVTTHADAVENNKYDGTANVNIDCQAN 56
REU_lev         --MEYKEHKKMYKVGKNWAVATLVSASILMGG-VVTAHADQVESNNYNGVAEVNTERQAN 57
FRU_ftf         ------------------VATIVSTSVLMGG-VVSAHADQVTTNSDNAVVTNTASQPTG 40
PAN_ftf         --MGYKEHKKMYKIGKKWAVATLVSASVLMGG-ALIAHADQVEANTANETQTVNATQQVT 57
ACI_frag        ------------------------------------------------------------
JOS_ftf         -MLENKNHKKISLSGKSLLMGTLSTAAIVLSASTANAATINADNVNENQTVEVTAS-SVN 58
GAS_ftf         KMLENKNHKKMSLSGKSLLMGTLSTAAIVLSASTVNAATTNADNVNENKTVAVSTTTSAN 60
REU_inu         -MLERKEHKKMYKSGKNWAVVTLSTAALVFGATTVNASADTNIENNDSSTVQVTTG--DN 57
R1274_frag      ------------------------------------------------------------

SFR_lev         ----VDGKIISTDDNATSGSTKQESSIANDNATSGSTKQESSIANDNATSGSTKQESSIA 112
REU_lev         GQIGVDGKIISANSNTTSGSTNQESSATNN---------------------------- 87
FRU_ftf         -----------SSSDVTSNTSTSPSTITG---------------------------- 58
PAN_ftf         -----------DQTAVTSSASSAENTKNDK---------------------------- 76
ACI_frag        ------------------------------------------------------------
JOS_ftf         NENNKQVTEKDSADKSTSDVAEDANTKKSN---------------------------- 88
GAS_ftf         NKNNNQVN--SSSEKSVDTKAEKATTVTS---------------------------- 87
REU_inu         DIAVKSVTLGSGQVSAASDTTIRTSANANS---------------------------- 87
R1274_frag      ------------------------------------------------------------

SFR_lev         NDNATSGSTKQESSVANDNATSGSTKQESSVANDNATSGSTKQESSVANDNATSGSTKQE 172
REU_lev         ----------TENAVVNESKNTNNT-----------------ENAVVNENKNT------ 113
FRU_ftf         ------------------------------------------------------------
PAN_ftf         ------------------------------------------------------------
ACI_frag        ------------------------------------------------------------
JOS_ftf         --------------ENTETTEKNTQTVVTNAPVSDVKNTNTVTAETPVDKVVNNSDQKT 133
GAS_ftf         -----------------AATKEVKADAVNTSPVNNVKAATTSTTTT--KETVDGTEKTP 127
REU_inu         --------------ASSAANTQNSNSQVASSAAITSSTSSAASSNNTDSKAAQENTNTA 132
R1274_frag      ------------------------------------------------------------

SFR_lev         SSVANDTKTAVVDESKNTSNTENDNSQLKQTNNEQPSAATQANLKKLNHEAAKAVQNAKI 232
REU_lev         ----NNTENAVVNENKNTNNTENDNSQLKLTNNEQPSAATQANLKKLNPQAAKAVQNAKI 169
FRU_ftf         ---------------------AVQVQLKQ-STNVDTVPSENNQKKLNPSAAQAVNNAHI 95
PAN_ftf         ----------VASVQANTVANSDEQTQLKQNTTDESTTSAQTNLSKLNPAAANAVKNAKI 126
ACI_frag        ------------------------------------------------------------
JOS_ftf         TNAATTDTKKDDVKQVEKKDSVDKTNAEENK-DSSVKPAENATKAELKGQVKDIVEESGV 192
GAS_ftf         VNSSADVKKNDAVKQDEKAATSFKTNTEEKANETSTKTASNDNKAELKGQIKDIVKESDV 187
REU_inu         KNDDTQKAAPANESSEAKNEPAVNVNDSSAAKNDDQQSSKKNTTAKLNKDAENVVKKAGI 192
R1274_frag      ------------------------------------------------------------

SFR_lev         DAGSLTDEQINELNKINFSKSAEKGAKLTFKDLEGIGNAIVKQDPQYAVPYFNAKEIKNM 292
REU_lev         DAGSLTDDQINELNKINFSKSAEKGAKLTFKDLEGIGNAIVKQDPQYAIPYFNAKEIKNM 229
FRU_ftf         NVNNLTEDQINELNKINFSKSAEKGAKLTFKDLEGIGNAIVKQDPRYTIPYFNAKEINNM 155
PAN_ftf         DAGNLTDDQINELNKIDFSKSAEKGAKLTFKDLEGIGNAIINQDPKYAIPYFNAKKIQNM 186
ACI_frag        ------------------------------------------------------------
JOS_ftf         DTSKLTNDQINELNKINFSKEAKSGTQLTYNDFKKIAKTLIEQDARYAIPFFNASKIKNM 252
GAS_ftf         DTSKLTNDQINELNKINFSKEAKSGTQLTYSDFKKIAKTLIEQDARYAIPFFNASKIKNM 247
REU_inu         DPNSLTDDQIKALNKMNFSKAAKSGTQMTYNDFQKIADTLIKQDGRYTVPFFKASEIKNM 252
R1274_frag      -------------------AAKYGTQITYNDFQKTADTLIKQDGRFTVPFFKASEIKNM 40

SFR_lev         PASYTVDAQTGKMAHLDVWDSWPVQDP-TGYVSNYKGYQLVIAMMGIPNTPNGDNHIYLL 351
REU_lev         PATYTVDAQTGKMAHLDVWDSWPVQDPVTGYVSNYKGYQLVIAMMGIPNSPTGDNHIYLL 289
FRU_ftf         PAAYAVDAQTGKMAHLDVWDSWPVQDAVTGYVSNYKGYQLVIAMMGIPNTPYGDNHIYLL 215
PAN_ftf         PAAYAVDAQTGQMAHLDVWDSWPVQDAVTGYVSNYKGYQLVIAMMGIPKAKYGDNHIYLL 246
ACI_frag        -------------------AWPVQDPITGYVSNYKGYQLVIAMMGMPKK--NDNHIYLL 38
JOS_ftf         PAAKTLDAQSGKVEDLEIWDSWPVQDAKTGYVSNWNGYQLVIGMMGVPNV--NDNHIYLL 310
GAS_ftf         PAAKTMDAQTGKVEDLEIWDSWPVQDAKTGYVSNWNGYQLVVGMMGVPNT--NDNHIYLL 305
REU_inu         PAATTKDAQTNTIEPLDVWDSWPVQDVRTGQVANWNGYQLVIAMMGIPNQ--NDNHIYLL 310
R1274_frag      PAATTKDAQTNTIEPLDVWDSWPVQDPVTGYVANWNGYQLVIAMMGRPHH--EDNHIYLL 98
                                :*****  ** *:*::*****:.*** *:    *******
```

```
SFR_lev         YNKYGDNDFSHWRNAGSIFGTNENNVYQEWSGSAIVNDNGTIQLFYTSNDTSDYKLNDQR 411
REU_lev         YNKYGDNDFSHWRNAGSIFGTKETNVFQEWSGSAIVNDDGTIQLFFTSNDTSDYKLNDQR 349
FRU_ftf         YNKYGDNDFSHWRNAGSIFGNKENNVYQEWSGSAIVNNDGSIQLFYTSNDTSDFKLNDQK 275
PAN_ftf         YNKYGDNDFSHWRNAGSIFGNNENNVFQEWSGSAIVNNDGSIQLFYTSNDTSDFKLNDQK 306
ACI_frag        YNKYNDNNFSHWRNAGSIFGYEETPDLQEWSGSAIVNKDDSIQLFYTRNDTSNGKINDQQ 98
JOS_ftf         YNKYGDNDFNHWKNAGPIFGLG-TPVIQQWSGSATLNKDGSIQLYYTKVDTSDNNTNHQK 369
GAS_ftf         YNKYGDNNFNNWKNAGPIFGLG-TPVIQQWSGSATLNKDGSIQLYYTKVDTSDNNTNHQK 364
REU_inu         YNKYGDNELSHWKNVGPIFGYNSTAVSQEWSGSAVLNSDNSIQLFYTRVDTSDNNTNHQK 370
R1274_frag      YNKYGDNDFSHWRNAGSIFGYNESPLTQEWSGSAIVNSDNSIQLFYTINDTN-NAINHQK 157
                ****.**::.:*:*.*.***   .   *:***** :*.:.:***::*  **.    *.*:

SFR_lev         LATATLNLDVD--DNGVAIKSVDNYHILFEGDGFHYQTYDQFANGKDRKNDDYCLRDPHV 469
REU_lev         LATATLNLNVD--DNGVSIKSVDNYQVLFEGDGFHYQTYEQFANGKDRENDDYCLRDPHV 407
FRU_ftf         LATATLHLNVD--DNGVSIASVDNNHVIFEGDGYHYQTYQQFAEGKDRKDDDYCLRDPHV 333
PAN_ftf         LATATLHLNVD--DNGVSIASVDNNHILFEGDGYHYQTYQQFANGNDRQDDDYCLRDPHV 364
ACI_frag        LATANLKLRVD--NNGVSIVSVDNDHVIFIGDSKKYQTYDQFANGINRNKDNYTLRDPHV 156
JOS_ftf         LASATVYLNLEKDQDKISIAHVDNDHIVFEGDGYHYQTYDQWKETN-KGADNIAMRDAHV 428
GAS_ftf         IASATVYLNLEKDQDKISIAHVDNDHIVFEGDGYHYQTYNQWKKTN-KGADNIAMRDAHV 423
REU_inu         IASATLYLTD--NNGNVSLAQVANDHIVFEGDGYYYQTYDQWKATN-KGADNIAMRDAHV 427
R1274_frag      LASATMYLTA--DNDGVHINNVENNHVVFAGDGYHYQTYDQWKAAN-SFVDNYTLRDGHV 214
                :*:*.: *     :. : :  * * :::* **.  ****:*:        *:  :** **

SFR_lev         VQSENGDRYLVFEANTGMEDYQSDDQIYNWANYGGDDAFNIKSFFKLLNNKNDRELASLA 529
REU_lev         VQLENGDRYLVFEANTGTEDYQSDDQIYNWANYGGDDAFNIKSFFKLLNNKKDRELAGLA 467
FRU_ftf         VQLTNGDRYLVFEANTGKEDYQSDEQIYNWANYGGDDAFNIKSFFKLLNNKKDRELAGLA 393
PAN_ftf         VQLENGDRYLVFEANTGTEDYQGDEQIYKWANYGGDDVFNINSFFKLLNNKRDRTLAGEA 424
ACI_frag        VEEENGDRYLVFEANTGSDNYQGDNQVYNWTNYGGNDKFNVRNFLDYFDNDNDKALASAA 216
JOS_ftf         IDDDNGNRYLVFEASTGTENYQGDDQIYQWLNYGGTNKDNLGDFFQILSNSDIKDRAKWS 488
GAS_ftf         IDDKDGNRYLVFEASTGTENYQGADQIYQWLNYGGTNKDNLGDFFQILSNSDIKDRAKWS 483
REU_inu         IEDDNGDRYLVFEASTGLENYQGEDQIYNWLNYGGDDAFNIKSLFRILSNDDIKSRATWA 487
R1274_frag      VQMPNGDRYLVFERNTGTENYQGEDQLYNWSNYGGNDRFNIKSLFHLLSNDVDYKKAIFA 274
                ::  :*:****** .** ::**. :*:*:* **** :  *: .::  :.*.     *  :

SFR_lev         NGAIGILKLNNDQTNPKVE--EVYSPLVSTLMASDEVERVNVVKLGDKYYLFSATRVSRG 587
REU_lev         NGALGILKLTNNQSKPKVE--EVYSPLVSTLMASDEVERPNVVKLGDKYYLFSVTRVSRG 525
FRU_ftf         NGSLGILKLNNNQDNPEVD--EVYSPLVSTLMASDEVERPDIVKLGGKYYLSSVTRVSRG 451
PAN_ftf         NGALGILKLDNNQTNPKVE--AVYSPLVSTLMASDEVERPDVVKLGDKYYLFSVTRVSRG 482
ACI_frag        NGALGILKLSGEQNNPIVEPENVYSPLVTSLMA-------------------------- 249
JOS_ftf         NAAIGIIKLNDDVKNPSVA--KVYSPLISAPMVSDEIERPDVVKLGNKYYLFAATRLNRG 546
GAS_ftf         NAAIGIIKLNNDTKNPGVE--KVYTPFISSPMVSDEIERPDVVRLGNKYYLFAATRLNRG 541
REU_inu         NAAIGILKLNKDEKNPKVA--ELYSPLISAPMVSDEIERPNVVKLGNKYYLFAATRLNRG 545
R1274_frag      NGALGIIKLTNDEKNPQVE--EVYTPLVTSNMVSDELERPNVVKLGDKYYLSSATRLSRG 332
                *.::**:**  :  :* *    :*:*:::: *.

SFR_lev         SDRELNAKDITIVGDNVAMIGYVSDNLMGKYKPLNNSGVVLTASVPANWRTATYSYYAVP 647
REU_lev         SDRELTAKDNTIVGDNVAMIGYVSDSLMGKYKPLNNSGVVLTASVPANWRTATYSYYAVP 585
FRU_ftf         SDTELTQKDNATVGDNVAMIGYVADNLMGPYKPLNNSGVVLTASVPANWRTATYSYFAVP 511
PAN_ftf         SDTELTAKDNTLVGDNVAMIGYVADSLNGTYKPLNQSGVVLTASVPANWRTATYSYYAVP 542
ACI_frag        ------------------------------------------------------------
JOS_ftf         SNDDAWMATNKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAVP 606
GAS_ftf         SNDDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAVP 601
REU_inu         SNDDAWMNANYAVGDNVAMVGYVADSLTGSYKPLNDSGVVLTASVPANWRTATYSYYAVP 605
R1274_frag      TNIDTLNKANKVVGDNVAMIGYVADSLTDPYKPLNGSGIV------------------- 372


SFR_lev         VEGHPDQVLITSYMSNKDFASGEGNYATLAPSFIVQINPDDTTTVLARATNQGDWVWDDS 707
REU_lev         VAGHPDQVLITSYMSNKDFASGEGNYATWAPSFLVQINPDDTTTVLARATNQGDWVWDDS 645
FRU_ftf         VEGHPDQVLITSYMSNKDFASGKGNYATWAPSFLVQINSDNTTMVLARATNQGDWIWDNS 571
PAN_ftf         VQGHPDQVLITSYMSNKDFASGEGNYATWAPSFLVQINPDDTTTVLARATNQGDWIWDDS 602
ACI_frag        ------------------------------------------------------------
JOS_ftf         VEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLAKMTNQGDWIWDDS 666
GAS_ftf         VEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLAKMTNQGDWIWDDT 661
REU_inu         VAGKDDQVLVTSYMTNRNGVAGKGMDSTWAPSFLLQINPDNTTTVLAKMTNQGDWIWDDS 665
R1274_frag      ------------------------------------------------------------
```

```
SFR_lev         SRNDNMLGVLKEGAVNSAALPGEWGKPVDWSLINRSSGLGLKPHQPVNPSQPTTPATPVN 767
REU_lev         SRNDNMLGVLKEGAANSAALPGEWGKPVDWSLINRSSGLGLKPHQPVQPKIDQPDQQPS- 704
FRU_ftf         SRNDKMLGVLDKNAANSAALPGEWGKPVDWSLINRSAGLGLKPHQGAD------PSEQPN 625
PAN_ftf         SRNDSMLGVLDQNAANSAALPGEWGKPVDWTNINRSSGLNLKPHQAADEPGTNKPTDNPS 662
ACI_frag        ------------------------------------------------------------
JOS_ftf         SENPDMMGVLEKDAPNSAALPGEWGKPVDWDLIG---GYNLKPHQPVTP-IPNVPTTPET 722
GAS_ftf         SENDDMMGVLKKDAPNSAALPGEWGKPVDWDLIG---GYNLKPHQPVTP-IPNVPTTPEK 717
REU_inu         SENLDMIGDLD-----SAALPGERDKPVDWDLIG----YGLKPHDPATPNDPETPTTPET 716
R1274_frag      ------------------------------------------------------------


SFR_lev         PSQPTTPATPVNPSQPTTPATPVNPSATTTPATPVNPSATTTPAKPVNPSQPTTPAKPVQ 827
REU_lev         -GQNTKNVTPGNGDKPAGKATPDN----TNIDPSAQPSGQNTNIDPSAQPSGQNTKNVTP 759
FRU_ftf         NKPGTN-----PGNKPGTN-PDNKPGDKPGTN-PDNKPGDKPGTN-----PDNIPGNN-- 671
PAN_ftf         DKPGTNNPTDNPSDKPGTNNPTDNPSDKPGTNNPTDNPSDKPGTNNPTDNPSDKPGTNNP 722
ACI_frag        ------------------------------------------------------------
JOS_ftf         PTTPDKPEVPTTPEVPTTPETP----------TPEAPKNPVKKTSQSKLPKAGDKNSFA- 771
GAS_ftf         PNTP---TTPNTPDTPHNPTKP----------TSEVPTTSVKKTTQSELPKAGAKNGIV- 763
REU_inu         PETPNTPKTPKTPENPGTPQTPNTPNTPEIPLTPETPKQPETQTN-NRLPQTG-NNANK- 773
R1274_frag      ------------------------------------------------------------


SFR_lev         AG---QATATNFVDQRLPQTGEN-----------------------------------NS 849
REU_lev         GN---EKQGKNTDAKQLPQTG-------------------------------------NK 779
FRU_ftf         -------PGLN-PRETPNE---------------------------------------- 682
PAN_ftf         TDNPSDKPGTNNPTDNPSDKPGTNNPTDNPSDKPGNVTPTADQNHRADNNDGHLPQTGNK 782
ACI_frag        ------------------------------------------------------------
JOS_ftf         ------------------------------------------------------------
GAS_ftf         ------------------------------------------------------------
REU_inu         ------------------------------------------------------------
R1274_frag      ------------------------------------------------------------


SFR_lev         QSQTMSFIGILLAMFGSLLGFLGIKKRRND- 879
REU_lev         SGLAGLYAGSLLALFG----LAAIEKRHA-- 804
FRU_ftf         -------------------------------
PAN_ftf         NAVVGLYLGSLLTMFG----LAALDKRYNK- 808
ACI_frag        -------------------------------
JOS_ftf         -AVVLGAVSSILGAVG----LTGVSKRKRNN 797
GAS_ftf         -AAIVGAVSSMLGVIG----LAGVSKRKRNN 789
REU_inu         -AMIGLGMGTLLSMFG----LAEINKRRFN- 798
R1274_frag      -------------------------------
```

Aa sequence alignment of FTFs of lactobacilli. Aa forming catalytic triade are accentuated by red bold letters. Aa beeing probably involved in Ca^{2+} complexion are accentuated by blue bold letters. Aa which are part of sucrose binding box 1 and 2 are accentuated by pink bold letters.

7.4 FTF *L. panis* sequence

```
gaggggttatagcggctcaaaaagacgtgcggttgggccacaaagagcggggtgaatat
 E  G  V  I  A  A  Q  K  D  V  R  L  G  H  K  E  R  G  E  Y
caagccattttccgtgagtagtttatcaatgatgcggcggttgaaattattaactgaaac
 Q  A  I  F  R  E  -  F  I  N  D  A  A  V  E  I  I  N  -  N
ttgaaagtatgaagacagactgatcccttggtatcacagggcatattttaattaatttaa
 L  K  V  -  R  Q  T  D  P  L  V  S  Q  G  I  F  -  L  I  -
tcatttagcttgctgttaacagcgaaactaaccgtttcggcaaaaatgtgaagaactgat
 S  F  S  L  L  L  T  A  K  L  T  V  S  A  K  M  -  R  T  D
tcatcatctgaataatttgggctcttcttgcataaacttcatgaaagaccgccttttttat
 S  S  S  E  -  F  G  L  F  L  H  K  L  H  E  R  P  P  F  Y
atcgcactaaaataccgaattgaaatttttaaaagaaactttacttttctatttttga
 I  A  L  K  Y  R  I  E  I  F  -  K  K  L  Y  F  F  Y  F  -
tgttattttaatgattgttaaacgtttatcaaaaagaaagggaagttaataatggggtat
 C  Y  F  N  D  C  -  T  F  I  K  K  K  G  K  L  I  M  G  Y
aaagaacataaaaag atg tataagattggcaaaaaatgggcagttgcaactttagtgtct
 K  E  H  K  K  K  M  Y  K  I  G  K  K  W  A  V  A  T  L  V  S
gcttcggttttaatgggtggggcattgattgctcatgctgatcaagttgaggcaaatact
 A  S  V  L  M  G  G  A  L  I  A  H  A  D  Q  V  E  A  N  T
gctaatgaaacgcaaacagttaacgctacgcaacaggtaactgatcaaactgctgtcact
 A  N  E  T  Q  T  V  N  A  T  Q  Q  V  T  D  Q  T  A  V  T
agtagtgcttcttctgctgagaatactaagaatgacaaagtagcttcagtacaagcaaat
 S  S  A  S  S  A  E  N  T  K  N  D  K  V  A  S  V  Q  A  N
actgttgctaacagtgatgaacaaacacagctaaagcagaatacaacagacgaaagtact
 T  V  A  N  S  D  E  Q  T  Q  L  K  Q  N  T  T  D  E  S  T
acaagtgcacagactaacctgagtaagttaaatccagctgcagctaacgcagtaaagaac
 T  S  A  Q  T  N  L  S  K  L  N  P  A  A  A  N  A  V  K  N
gctaagattgacgcaggtaacttaactgatgatcaaattaacgaattaaacaagattgat
 A  K  I  D  A  G  N  L  T  D  D  Q  I  N  E  L  N  K  I  D
ttttccaaatcagcagaaaagggtgctaaattaacttttaaggatttggaaggtattggt
 F  S  K  S  A  E  K  G  A  K  L  T  F  K  D  L  E  G  I  G
aatgcaattattaaccaagatccaaagtatgctattccttactttaatgccaagaagatt
 N  A  I  I  N  Q  D  P  K  Y  A  I  P  Y  F  N  A  K  K  I
cagaacatgccagctgcatatgcagttgatgcacaaactggtcaaatggcccacttggat
 Q  N  M  P  A  A  Y  A  V  D  A  Q  T  G  Q  M  A  H  L  D
gtatgggactcttggccagttcaagatgctgttaccggctacgtttctaactataagggc
 V  W  D  S  W  P  V  Q  D  A  V  T  G  Y  V  S  N  Y  K  G
taccaactggtaatcgcaatgatgggaattccgaaggccaagtatggtgacaaccatatt
 Y  Q  L  V  I  A  M  M  G  I  P  K  A  K  Y  G  D  N  H  I
taccttctgtacaacaagtatggtgacaatgatttttcccattggcgcaatgccggttcc
 Y  L  L  Y  N  K  Y  G  D  N  D  F  S  H  W  R  N  A  G  S
atttttggaaataacgaaaacaatgtctttcaagaatggtcaggttccgcaatcgttaac
 I  F  G  N  N  E  N  N  V  F  Q  E  W  S  G  S  A  I  V  N
aatgatggctcaatccaattattctacacttcaaacgatacatctgattttaagcttaat
 N  D  G  S  I  Q  L  F  Y  T  S  N  D  T  S  D  F  K  L  N
gatcaaaaattagcgactgcaactctgcacctaaatgttgacgataatggtgtatcaatt
 D  Q  K  L  A  T  A  T  L  H  L  N  V  D  D  N  G  V  S  I
gcgagtgttgataataaccatatcctgtttgagggtgatggttaccactaccaaacttac
 A  S  V  D  N  N  H  I  L  F  E  G  D  G  Y  H  Y  Q  T  Y
caacaatttgctaacggaaacgatcgtcaagatgatgactactgcttacgtgatccccat
 Q  Q  F  A  N  G  N  D  R  Q  D  D  D  Y  C  L  R  D  P  H
gttgttcagttagaaaatggtgatcgttacttggtatttgaagccaatacaggtaccgaa
 V  V  Q  L  E  N  G  D  R  Y  L  V  F  E  A  N  T  G  T  E
gactaccaaggtgatgagcaaatttacaagtgggctaattatggtggtgacgatgtcttt
 D  Y  Q  G  D  E  Q  I  Y  K  W  A  N  Y  G  G  D  D  V  F
aatattaatagcttctttaagttattaaacaataagcgagaccgcacccttgctggtgag
 N  I  N  S  F  F  K  L  L  N  N  K  R  D  R  T  L  A  G  E
gctaatggtgctcttggtattttaaagcttgataataatcaaactaatccaaaggttgaa
 A  N  G  A  L  G  I  L  K  L  D  N  N  Q  T  N  P  K  V  E
gctgtatactcaccattagtatcaactttaatggcaagtgacgaagttgaacgtccagat
 A  V  Y  S  P  L  V  S  T  L  M  A  S  D  E  V  E  R  P  D
```

```
gttgtgaaacttggtgataagtactatctcttctcagtaactcgggttagtcgtggatca
 V  V  K  L  G  D  K  Y  Y  L  F  S  V  T  R  V  S  R  G  S
gatactgaattaacggctaaggataacactcttgttggtgataatgttgctatgatcggc
 D  T  E  L  T  A  K  D  N  T  L  V  G  D  N  V  A  M  I  G
tatgttgcagatagcttgaacggtacttacaagccgttgaaccagtctggagttgtcctg
 Y  V  A  D  S  L  N  G  T  Y  K  P  L  N  Q  S  G  V  V  L
actgcttctgtacctgctaactggcggactgcaacctactcctattacgccgttcctgtt
 T  A  S  V  P  A  N  W  R  T  A  T  Y  S  Y  Y  A  V  P  V
cagggacacccagatcaagttttaattacttcatacatgagtaacaaggactttgcttct
 Q  G  H  P  D  Q  V  L  I  T  S  Y  M  S  N  K  D  F  A  S
ggagaaggtaattatgctacctgggcaccaagtttcttggttcaaattaatccagatgac
 G  E  G  N  Y  A  T  W  A  P  S  F  L  V  Q  I  N  P  D  D
acaacaacggttctggcacgggctactaatcaaggtgattggatctgggatgattccagt
 T  T  T  V  L  A  R  A  T  N  Q  G  D  W  I  W  D  D  S  S
cgtaatgacagcatgttaggtgtccttgaccagaatgctgcaaatagcgctgcgctacct
 R  N  D  S  M  L  G  V  L  D  Q  N  A  A  N  S  A  A  L  P
ggtgaatggggtaagccggtcgactggacaaatattaaccggagtagtggcttaaacctg
 G  E  W  G  K  P  V  D  W  T  N  I  N  R  S  S  G  L  N  L
aagccacaccaggcagctgatgaaccgggtactaataaaccaacggataacccgagtgat
 K  P  H  Q  A  A  D  E  P  G  T  N  K  P  T  D  N  P  S  D
aagccaggcaccaacaacccaacggataacccgagcgacaagccaggtactaataaccca
 K  P  G  T  N  N  P  T  D  N  P  S  D  K  P  G  T  N  N  P
acggataacccaagtgataagccaggtactaataatccaacggataacccaagtgataag
 T  D  N  P  S  D  K  P  G  T  N  N  P  T  D  N  P  S  D  K
ccaggtaccaacaatccaacggataacccaagtgataagccaggtaccaacaatccaacg
 P  G  T  N  N  P  T  D  N  P  S  D  K  P  G  T  N  N  P  T
gataatccgagtgacaagccaggtaccaacaatccaacggataatccgagtgataagccg
 D  N  P  S  D  K  P  G  T  N  N  P  T  D  N  P  S  D  K  P
ggtaccaacaatccaacagataacccaagtgataagccaggtaatgttaccccaacagca
 G  T  N  N  P  T  D  N  P  S  D  K  P  G  N  V  T  P  T  A
gatcaaaatcatcgtgcagacaataatgatggccatttaccgcaaactggtaataagaat
 D  Q  N  H  R  A  D  N  N  D  G  H  L  P  Q  T  G  N  K  N
gctgttgttggtttataccttggttcattattgacaatgttcgggcttgctgctttagat
 A  V  V  G  L  Y  L  G  S  L  L  T  M  F  G  L  A  A  L  D
aagcgttacaacaagtaaattcaaagtgaccccaattaataaataaattagtattaaata
 K  R  Y  N  K  -  I  Q  S  D  P  N  -  -  I  N  -  Y  -  I
tagccttagaactaagaggcgcggaccaccaggatacagtggtccgcgccttttgtatta
 -  P  -  N  -  E  A  R  T  T  R  I  Q  W  S  A  P  F  V  L
ttcatcttcaccaataattttaaagccatagtcgcgaatgtggtttttcagcagttggat
 F  I  F  T  N  N  F  K  A  I  V  A  N  V  V  F  Q  Q  L  D
atattcttgcccaagctggctgataacggtgtgctggtggtgg
 I  F  L  P  K  L  A  D  N  G  V  L  V  V
```

Sequence identified from *L. panis* including open reading frame of ftf gene (black letters). Underlined section was cloned in pET3a plasmid and heterologously expressed in *E. coli*. For detailed comments to sequence see fig. 16.

7.5 FTF *L. frumenti* sequence

```
atgccgaccaagttactactaatagtgataatgctgttgtgaccaatactgctagtcaacct
   A  D  Q  V  T  T  N  S  D  N  A  V  V  T  N  T  A  S  Q  P
actgggtccagctctgatgtaacaagtaacaccagcactagccctagtacaattactggt
 T  G  S  S  S  D  V  T  S  N  T  S  T  S  P  S  T  I  T  G
gccgttcaagttcaattaaaacaaagcactaatgttgatacagtacctagtgaaaacaat
 A  V  Q  V  Q  L  K  Q  S  T  N  V  D  T  V  P  S  E  N  N
caaaaaaagttgaacccctctgctgctcaggctgttaacaatgcgcatattaatgttaat
 Q  K  K  L  N  P  S  A  A  Q  A  V  N  N  A  H  I  N  V  N
aacttgacagaagaccaaattaatgagctgaataagattaatttctcgaagtctgctgaa
 N  L  T  E  D  Q  I  N  E  L  N  K  I  N  F  S  K  S  A  E
aaaggtgctaagttaaccttcaaagatttagaaggcattggcaatgcaattgttaagcaa
 K  G  A  K  L  T  F  K  D  L  E  G  I  G  N  A  I  V  K  Q
gatccacggtatacaattccttattttaacgctaaggaaattaataatatgccagctgct
 D  P  R  Y  T  I  P  Y  F  N  A  K  E  I  N  N  M  P  A  A
tatgctgtggatgcacaaacgggtaaaatggcgcaccttgacgtgtgggattcctggcca
 Y  A  V  D  A  Q  T  G  K  M  A  H  L  D  V  W  D  S  W  P
gtacaagatgctgttacaggatatgtttcaaattacaagggctaccagctagtaatcgct
 V  Q  D  A  V  T  G  Y  V  S  N  Y  K  G  Y  Q  L  V  I  A
atgatggggattcctaatacaccctatggcgacaaccatatttatctcttgtacaacaag
 M  M  G  I  P  N  T  P  Y  G  D  N  H  I  Y  L  L  Y  N  K
tatggtgacaatgacttctcacactggcgtaatgcgggttcaatctttggtaacaaagaa
 Y  G  D  N  D  F  S  H  W  R  N  A  G  S  I  F  G  N  K  E
aacaatgtctaccaagaatggtcgggttctgccattgtgaacaacgatggttcaattcaa
 N  N  V  Y  Q  E  W  S  G  S  A  I  V  N  N  D  G  S  I  Q
ttattctatacttctaatgatacttctgattttaagttaaatgatcaaaagctggctaca
 L  F  Y  T  S  N  D  T  S  D  F  K  L  N  D  Q  K  L  A  T
gctaccctgcacttaaacgttgatgataatggggtatcaatcgctagtgttgataataat
 A  T  L  H  L  N  V  D  D  N  G  V  S  I  A  S  V  D  N  N
catgttatctttgaaggtgatggctatcattaccagacctaccaacaatttgccgaggga
 H  V  I  F  E  G  D  G  Y  H  Y  Q  T  Y  Q  Q  F  A  E  G
aaagatcgtaaagatgatgattactgcttgcgcgatccacatgtagtgcaattaacaaat
 K  D  R  K  D  D  D  Y  C  L  R  D  P  H  V  V  Q  L  T  N
ggtgatcgctacttagtatttgaagccaatactggtaaagaagattaccaaagtgatgag
 G  D  R  Y  L  V  F  E  A  N  T  G  K  E  D  Y  Q  S  D  E
caaatttataactgggctaattacggtggcgatgatgcctttaatattaagagcttcttt
 Q  I  Y  N  W  A  N  Y  G  G  D  D  A  F  N  I  K  S  F  F
aaacttttaaataacaagaaagatcgtgaattagctggactagccaatggctcgctaggg
 K  L  L  N  N  K  K  D  R  E  L  A  G  L  A  N  G  S  L  G
atcttgaaattaaataacaatcaagacaatccagaggtagatgaagtctactcaccatta
 I  L  K  L  N  N  N  Q  D  N  P  E  V  D  E  V  Y  S  P  L
gtatctacattgatggcaagtgatgaagttgaacggccggatattgtaaaacttggtggt
 V  S  T  L  M  A  S  D  E  V  E  R  P  D  I  V  K  L  G  G
aaatattatctttcctccgtaacgcgtgttagtcggggatcagatactgaactgactcaa
 K  Y  Y  L  S  S  V  T  R  V  S  R  G  S  D  T  E  L  T  Q
aaagataatgcaactgtcggggacaatgtcgccatgatcggttatgttgcggataattta
 K  D  N  A  T  V  G  D  N  V  A  M  I  G  Y  V  A  D  N  L
atgggtccttacaagccattaaacaactctggggttgtattgactgcttctgttcctgct
 M  G  P  Y  K  P  L  N  N  S  G  V  V  L  T  A  S  V  P  A
aattggcgaaccgccacttattcatacttcgctgtaccagttgaaggacacccagatcaa
 N  W  R  T  A  T  Y  S  Y  F  A  V  P  V  E  G  H  P  D  Q
gttctgattacttcatatatgagtaacaaagattttgcttctggtaaaggtaattatgct
 V  L  I  T  S  Y  M  S  N  K  D  F  A  S  G  K  G  N  Y  A
acctgggcacctagtttcttggttcaaattaattcagacaatactacaatggtattggca
 T  W  A  P  S  F  L  V  Q  I  N  S  D  N  T  T  M  V  L  A
cgagctactaatcagggtgattggatttgggataattcaagccgcaatgataaaatgctc
 R  A  T  N  Q  G  D  W  I  W  D  N  S  S  R  N  D  K  M  L
ggtgtgcttgataagaatgctgctaacagtgctgctttaccgggtgaatggggcaagcca
 G  V  L  D  K  N  A  A  N  S  A  A  L  P  G  E  W  G  K  P
gttgattggagtttaattaaccgaagtgctggtttgggattaaagcctcatcagggagct
 V  D  W  S  L  I  N  R  S  A  G  L  G  L  K  P  H  Q  G  A
```

```
gatccatcagagcaaccaaataataaaccgggtactaatccaggcaataagccgggtact
 D  P  S  E  Q  P  N  N  K  P  G  T  N  P  G  N  K  P  G  T
aaccctgacaacaaaccaggtgataagccaggtactaaccctgacaacaaaccaggtgat
 N  P  D  N  K  P  G  D  K  P  G  T  N  P  D  N  K  P  G  D
aagccaggtactaatcctgataatattcccggaaataatccaggactaaaccctcgtgaa
 K  P  G  T  N  P  D  N  I  P  G  N  N  P  G  L  N  P  R  E
acccctaatg
 T  P  N
```

Identified fragment of *L. frumenti* ftf gene. Fragment is missing an expected signal peptide and an end of open reading frame containing LPXTG motif. So, complete fragment was cloned in pET3a and heterologously expressed in *E. coli*. Resulting gene product performed FTF activity. For comments to sequence details see fig. 18.

7.6 Modified FTF *L. gasseri* sequence

ctgtaagatgaaatgttaaacgcttagcatgtaggaggagaaaag**atg**ttggaaaataaa
L - D E M L N A - H V G G E K **M** L E N K
aatcataagaagatgtctttaagcggaaaatctttattaatgggaaccttgtctacagca
N H K K M S L S G K S L L M G T L S T A
gcaattgtattaagtgcttccactgtaaatgctgctactactaatgcagacaacgttaat
A I V L S A S T V N A A T T N A D N V N
gaaaacaaaactgtagcagtaagtactactacttcagcaaataataaaaataataatcaa
E N K T V A V S T T T S A N N K N N N Q
gtaaattcaagctcagaaaagagtgttgatactaaagcagaaaaagctactactgtgact
V N S S S E K S V D T K A E K A T T V T
tcagcagcaactaaagaagttaaagcagatgctgttaatacttcaccagtaaataatgtt
S A A T K E V K A D A V N T S P V N N V
aaagctgctaccacctctacaacaactactaaagaaacagtagatggtactgagaagaca
K A A T T S T T T T K E T V D G T E K T
cctgttaattcttctgctgatgttaaaaagaacgatgcagtaaaacaagatgaaaaagca
P V N S S A D V K K N D A V K Q D E K A
gctacttcatttaagacaaatactgaagaaaaagctaacgaaacttcaacaaaaacagca
A T S F K T N T E E K A N E T S T K T A
tcaaacgataacaaagctgaattaaagggtcaaattaaagatattgttaaagaatctgat
S N D N K A E L K G Q I K D I V K E S D
gtagataccagtaaattaaccaatgatcaaattaatgaattaaataagattaacttttct
V D T S K L T N D Q I N E L N K I N F S
aaagaagcaaagagcggtactcaattaacttacagtgactttaagaaaattgctaaaact
K E A K S G T Q L T Y S D F K K I A K T
ttaattgaacaagatgctcgttacgctattccttttctttaatgcaagtaaaattaagaac
L I E Q D A R Y A I P F F N A S K I K N
atgcctgcagcaaaaactatggatgcccaaacaggaaaagtagaagacttagaaatttgg
M P A A K T M D A Q T G K V E D L E I W
gattcatggccagttcaagacgcaaaaactggttatgtttctaactggaatggttatcaa
D S W P V Q D A K T G Y V S N W N G Y Q
ttagtagttggaatgatgggagttccaaatactaatgacaatcatatttatcttctttac
L V V G M M G V P N T N D N H I Y L L Y
aacaagtacggtgacaataactttaataattggaagaatgctggtcctatttttggctta
N K Y G D N N F N N W K N A G P I F G L
ggtactccagttattcaacaatggtctggctcagcaactttaaataaagatggttcaatc
G T P V I Q Q W S G S A T L N K D G S I
caactttactatactaaggttgatacaagtgataacaacactaaccaccaaaagattgca
Q L Y Y T K V D T S D N N T N H Q K I A
agtgcaactgtctacttaaatcttgaaaaggatcaagacaagatttctattgcacacgtc
S A T V Y L N L E K D Q D K I S I A H V
gataatgaccacatcgtttttgagggtgatggctaccattaccaaacttacaatcaatgg
D N D H I V F E G D G Y H Y Q T Y N Q W
aagaagaccaacaagggtgcagataatattgcaatgcgtgatgcacacgtaattgatgat
K K T N K G A D N I A M R D A H V I D D
aaagatggtaatcgttaccttgtctttgaagcaagtactggtacagagaattatcaaggt
K D G N R Y L V F E A S T G T E N Y Q G
gctgaccaaatttatcaatggttaaattacggtggcactaacaaagataatttaggtgat
A D Q I Y Q W L N Y G G T N K D N L G D
ttcttccaaatcttgtctaactctgatattaaagatagagcaaaatggtctaatgctgca
F F Q I L S N S D I K D R A K W S N A A
atcggtattattaagttaaacaatgatactaagaaccctggtgttgagaaggtctacaca
I G I I K L N N D T K N P G V E K V Y T
ccatttattagttctccaatggtaagtgatgaaattgaacgtcctgatgtagttcgtttg
P F I S S P M V S D E I E R P D V V R L
ggcaacaagtattacttatttgctgctactagattaaaccgtggaagtaacgacgatgca
G N K Y Y L F A A T R L N R G S N D D A
tggatggctgctaataaagcagttggtgataacgttgcaatgattggttacgtttctgat
W M A A N K A V G D N V A M I G Y V S D
aacctaactcatggatacgttccattaaatgaatctggagtagttttaactgcttctgtt
N L T H G Y V P L N E S G V V L T A S V

```
ccagcaaactggcgtactgcaacttactcatactatgcagttccagtagaaggaagagat
 P  A  N  W  R  T  A  T  Y  S  Y  Y  A  V  P  V  E  G  R  D
gatcaattattgattacttcttatatcactaaccgtggtgaagttgctggaaagggtatg
 D  Q  L  L  I  T  S  Y  I  T  N  R  G  E  V  A  G  K  G  M
cacgcaacttgggcaccaagtttcttgttacaaattaatccagataatactactactgtt
 H  A  T  W  A  P  S  F  L  L  Q  I  N  P  D  N  T  T  T  V
ttagctaaaatgactaaccaaggtgactggatttgggatgacactagcgaaaacgatgat
 L  A  K  M  T  N  Q  G  D  W  I  W  D  D  T  S  E  N  D  D
atgatgggtgttcttaaaaaagatgcaccaaatagtgctgctcttcctggtgaatggggc
 M  M  G  V  L  K  K  D  A  P  N  S  A  A  L  P  G  E  W  G
aaaccagttgattgggacttaatcggtggatataacttgaagccacatcaacctgtaact
 K  P  V  D  W  D  L  I  G  G  Y  N  L  K  P  H  Q  P  V  T
ccaattccaaatgttccaactactccagagaaacctaatacaccaacaacaccaaatact
 P  I  P  N  V  P  T  T  P  E  K  P  N  T  P  T  T  P  N  T
cctgatactccgcataatccaactaaaccaacttcagaagttccaacaacttcagttaag
 P  D  T  P  H  N  P  T  K  P  T  S  E  V  P  T  T  S  V  K
aagacaactcaatcagaacttccaaaagctggagctaaaaatggtattgttgcagctata
 K  T  T  Q  S  E  L  P  K  A  G  A  K  N  G  I  V  A  A  I
gtaggtgctgtaagttcaatgcttggcgttatcggcttagctggtgtttctaagcgtaaa
 V  G  A  V  S  S  M  L  G  V  I  G  L  A  G  V  S  K  R  K
cgtaataactaaattgattaaaaaggatgagctttgaaactcatccttttttttgctata
 R  N  N  -  I  D  -  K  G  -  A  L  K  L  I  L  F  F  A  I
```

Open reading frame of modified *L. gasseri ftf gen* (black letters). Region cloned in pET3a plasmid and heterologously expressed in *E. coli* is underlined. Modified base is accentuated in red with yellow background. Original base triplet “taa” is a stop codon. The product of modified gene showed FTF activity.

7.7 Sequence of *ftf gene* fragment of *L. reuteri* TMW 1.1274

caataagctgcaaagtatggtacccaaattacttataatgatttccaaaagactgctgat
 Q - A A K Y G T Q I T Y N D F Q K T A D
acgttaatcaaacaagatggtcggttcacagttccattctttaaagcaagtgaaatcaaa
 T L I K Q D G R F T V P F F K A S E I K
aatatgcctgccgctacaactaaagatgcacaaactaatactattgaacctttagatgta
 N M P A A T T K D A Q T N T I E P L D V
tgggattcatggccagttcaagatccagtgactggttatgttgctaattggaatggctat
 W D S W P V Q D P V T G Y V A N W N G Y
caacttgtgatcgctatgatgggacggcctcatcacgaagataatcatatttatttactt
 Q L V I A M M G R P H H E D N H I Y L L
tataataagtatggtgataatgatttttcacactggcgaaatgcaggatctatatttgga
 Y N K Y G D N D F S H W R N A G S I F G
tataatgaatctccactaacacaagaatggtctgggtctgctattgttaattctgacaac
 Y N E S P L T Q E W S G S A I V N S D N
tctattcaactcttttatactatcaatgatacaaataatgctattaaccatcaaaaatta
 S I Q L F Y T I N D T N N A I N H Q K L
gcaagtgctactatgtacttaacagccgacaatgatggtgtccatattaataatgtagag
 A S A T M Y L T A D N D G V H I N N V E
aataatcatgtggtatttgcaggtgatggttatcattaccaaacttatgatcaatggaaa
 N N H V V F A G D G Y H Y Q T Y D Q W K
gctgcaaatagttttgttgacaactacactttgcgggatggacatgttgtacaaatgcca
 A A N S F V D N Y T L R D G H V V Q M P
aatggtgatcggtatttagtatttgaaagaaatactggaactgaaaattatcaaggtgaa
 N G D R Y L V F E R N T G T E N Y Q G E
gatcaattatataattggtcaaattatggtggtaacgatcgctttaatattaaaagcttg
 D Q L Y N W S N Y G G N D R F N I K S L
tttcatcttttaagtaatgatgttgattataaaaaagcgatctttgctaacggggcactt
 F H L L S N D V D Y K K A I F A N G A L
ggaattattaaactaacgaatgatgaaaagaacccgcaagtagaagaagtatatactcca
 G I I K L T N D E K N P Q V E E V Y T P
ttagttacatcaaatatggttagtgatgaacttgaacgtcctaatgttgttaaacttgga
 L V T S N M V S D E L E R P N V V K L G
gataagtattatctatcctcggctacacgtttaagcagaggaactaatatcgatactctt
 D K Y Y L S S A T R L S R G T N I D T L
aataaagctaataaagttgtcggagataatgttgcaatgattggttacgtagccgatagt
 N K A N K V V G D N V A M I G Y V A D S
cttacagatccatataaaccattaaatggttcagggattgtag
L T D P Y K P L N G S G I V

L. sanfranciscensis/*L.panis* hybrid FTF sequences

7.7.1 coPCR strategy

For construction of *L. sanfranciscensis*/*L.panis* hybrid constructs, three steps of coPCR had to be performed using KOD polymerase, elongation times were adapted following kit instructions.

I

product	primer	template	size	annealing temperature
A	SFklon_fw + SFnterm1_rev	*L. sanfranciscensis*	492 bp	68°C
B	SFklon_fw + SFnterm2_rev	*L. sanfranciscensis*	534 bp	68°C
C	PANmitte1_fw + PANklon_rev	*L. panis*	2061 bp	61°C
D	PANmitte2_fw + PANklon_rev	*L. panis*	2019 bp	60°C
E	SFmitte_fw + SFmitte_rev	*L. sanfranciscensis*	1632 bp	60°C
F	SFcterm_fw + SFklon_rev	*L. sanfranciscensis*	264 bp	61°C
G	PANklon_fw + PANnterm_rev	*L. panis*	171 bp	68°C
H	PANmitte1_fw + PANmitte_rev	*L. panis*	1677 bp	61°C
I	PANcterm_fw + PANklon_rev	*L. panis*	384 bp	68°C

II

product	primer	templates	size	annealing temperature
J	SFklon_fw + PANklon_rev	A + C	ca. 2500 bp	64°C
K	SFklon_fw + PANklon_rev	B + D	ca. 2500 bp	64°C
L	PANklon_fw + SFmitte_rev	E + G	ca. 1800 bp	61°C
M	SFklon_fw + PANmitte_rev	A + H	ca. 2200 bp	60°C

III

product	primer	templates	size	Annealing temperature
N	PANklon_fw + PANklon_rev	L + I	ca. 2000 bp	58°C
O	SFklon_fw + SFklon_rev	M + F	ca. 2400 bp	57°C

7.7.2 HybJ

```
tatatctagaaggagatataatggctgatgctgttgagaacaataaatacgatggcactgct
                    M  A  D  A  V  E  N  N  K  Y  D  G  T  A
aacgttaacattgattgccaagctaatgttgatgggaagattatcagtactgatgataat
 N  V  N  I  D  C  Q  A  N  V  D  G  K  I  I  S  T  D  D  N
gcaaccagtggctcgacaaagcaagaatcatctattgctaatgataatgcaaccagtggc
 A  T  S  G  S  T  K  Q  E  S  S  I  A  N  D  N  A  T  S  G
tcgacaaagcaagaatcatctattgctaatgataatgcaaccagtggctcgacaaagcaa
 S  T  K  Q  E  S  S  I  A  N  D  N  A  T  S  G  S  T  K  Q
gaatcatctattgctaatgataatgcaaccagtggctcgacaaagcaagaatcatctgtt
 E  S  S  I  A  N  D  N  A  T  S  G  S  T  K  Q  E  S  S  V
gctaacgataatgcaaccagcggttcgacaaagcaagaatcatctgttgctaacgataat
 A  N  D  N  A  T  S  G  S  T  K  Q  E  S  S  V  A  N  D  N
gcaaccagtggttcgacaaagcaagaatcatctgttgctaacgataatgcaaccagtggt
 A  T  S  G  S  T  K  Q  E  S  S  V  A  N  D  N  A  T  S  G
tcgacaaagcaagaatcatctgttgctaatgatactaaaactgctgttgttgacgaaagt
 S  T  K  Q  E  S  S  V  A  N  D  T  K  T  A  V  V  D  E  S
aaaaatactagcaacacagaaaatgataatagtcagctaaagcagaatacaacagacgaa
 K  N  T  S  N  T  E  N  D  N  S  Q  L  K  Q  N  T  T  D  E
agtactacaagtgcacagactaacctgagtaagttaaatccagctgcagctaacgcagta
 S  T  T  S  A  Q  T  N  L  S  K  L  N  P  A  A  A  N  A  V
aagaacgctaagattgacgcaggtaacttaactgatgatcaaattaacgaattaaacaag
 K  N  A  K  I  D  A  G  N  L  T  D  D  Q  I  N  E  L  N  K
attgatttttccaaatcagcagaaaagggtgctaaattaacttttaaggatttggaaggt
 I  D  F  S  K  S  A  E  K  G  A  K  L  T  F  K  D  L  E  G
attggtaatgcaattattaaccaagatccaaagtatgctattccttactttaatgccaag
 I  G  N  A  I  I  N  Q  D  P  K  Y  A  I  P  Y  F  N  A  K
aagattcagaacatgccagctgcatatgcagttgatgcacaaactggtcaaatggcccac
 K  I  Q  N  M  P  A  A  Y  A  V  D  A  Q  T  G  Q  M  A  H
ttggatgtatgggactcttggccagttcaagatgctgttaccggctacgtttctaactat
 L  D  V  W  D  S  W  P  V  Q  D  A  V  T  G  Y  V  S  N  Y
aagggctaccaactggtaatcgcaatgatgggaattccgaaggccaagtatggtgacaac
 K  G  Y  Q  L  V  I  A  M  M  G  I  P  K  A  K  Y  G  D  N
catatttaccttctgtacaacaagtatggtgacaatgatttttcccattggcgcaatgcc
 H  I  Y  L  L  Y  N  K  Y  G  D  N  D  F  S  H  W  R  N  A
ggttccatttttggaaataacgaaaacaatgtctttcaagaatggtcaggttccgcaatc
 G  S  I  F  G  N  N  E  N  N  V  F  Q  E  W  S  G  S  A  I
gttaacaatgatggctcaatccaattattctacacttcaaacgatacatctgattttaag
 V  N  N  D  G  S  I  Q  L  F  Y  T  S  N  D  T  S  D  F  K
cttaatgatcaaaaattagcgactgcaactctgcacctaaatgttgacgataatggtgta
 L  N  D  Q  K  L  A  T  A  T  L  H  L  N  V  D  D  N  G  V
tcaattgcgagtgttgataataaccatatcctgtttgagggtgatggttaccactaccaa
 S  I  A  S  V  D  N  N  H  I  L  F  E  G  D  G  Y  H  Y  Q
acttaccaacaatttgctaacggaaacgatcgtcaagatgatgactactgcttacgtgat
 T  Y  Q  Q  F  A  N  G  N  D  R  Q  D  D  D  Y  C  L  R  D
ccccatgttgttcagttagaaaatggtgatcgttacttggtatttgaagccaatacaggt
 P  H  V  V  Q  L  E  N  G  D  R  Y  L  V  F  E  A  N  T  G
accgaagactaccaaggtgatgagcaaatttacaagtgggctaattatggtggtgacgat
 T  E  D  Y  Q  G  D  E  Q  I  Y  K  W  A  N  Y  G  G  D  D
gtctttaatattaatagcttctttaagttattaaacaataagcgagaccgcacccttgct
 V  F  N  I  N  S  F  F  K  L  L  N  N  K  R  D  R  T  L  A
ggtgaggctaatggtgctcttggtattttaaagcttgataataatcaaactaatccaaag
 G  E  A  N  G  A  L  G  I  L  K  L  D  N  N  Q  T  N  P  K
gttgaagctgtatactcaccattagtatcaactttaatggcaagtgacgaagttgaacgt
 V  E  A  V  Y  S  P  L  V  S  T  L  M  A  S  D  E  V  E  R
ccagatgttgtgaaacttggtgataagtactatctcttctcagtaactcgggttagtcgt
 P  D  V  V  K  L  G  D  K  Y  Y  L  F  S  V  T  R  V  S  R
ggatcagatactgaattaacggctaaggataacactcttgttggtgataatgttgctatg
 G  S  D  T  E  L  T  A  K  D  N  T  L  V  G  D  N  V  A  M
atcggctatgttgcagatagcttgaacggtacttacaagccgttgaaccagtctggagtt
 I  G  Y  V  A  D  S  L  N  G  T  Y  K  P  L  N  Q  S  G  V
```

```
gtcctgactgcttctgtacctgctaactggcggactgcaacctactcctattacgccgtt
 V  L  T  A  S  V  P  A  N  W  R  T  A  T  Y  S  Y  Y  A  V
cctgttcagggacacccagatcaagttttaattacttcatacatgagtaacaaggacttt
 P  V  Q  G  H  P  D  Q  V  L  I  T  S  Y  M  S  N  K  D  F
gcttctggagaaggtaattatgctacctgggcaccaagtttcttggttcaaattaatcca
 A  S  G  E  G  N  Y  A  T  W  A  P  S  F  L  V  Q  I  N  P
gatgacacaacaacggttctggcacgggctactaatcaaggtgattggatctgggatgat
 D  D  T  T  T  V  L  A  R  A  T  N  Q  G  D  W  I  W  D  D
tccagtcgtaatgacagcatgttaggtgtccttgaccagaatgctgcaaatagcgctgcg
 S  S  R  N  D  S  M  L  G  V  L  D  Q  N  A  A  N  S  A  A
ctacctggtgaatggggtaagccggtcgactggacaaatattaaccggagtagtggctta
 L  P  G  E  W  G  K  P  V  D  W  T  N  I  N  R  S  S  G  L
aacctgaagccacaccaggcagctgatgaaccgggtactaataaaccaacggataacccg
 N  L  K  P  H  Q  A  A  D  E  P  G  T  N  K  P  T  D  N  P
agtgataagccaggcaccaacaacccaacggataacccgagcgacaagccaggtactaat
 S  D  K  P  G  T  N  N  P  T  D  N  P  S  D  K  P  G  T  N
aacccaacggataacccaagtgataagccaggtactaataatccaacggataacccaagt
 N  P  T  D  N  P  S  D  K  P  G  T  N  N  P  T  D  N  P  S
gataagccaggtaccaacaatccaacggataacccaagtgataagccaggtaccaacaat
 D  K  P  G  T  N  N  P  T  D  N  P  S  D  K  P  G  T  N  N
ccaacggataatccgagtgacaagccaggtaccaacaatccaacggataatccgagtgat
 P  T  D  N  P  S  D  K  P  G  T  N  N  P  T  D  N  P  S  D
aagccgggtaccaacaatccaacagataacccaagtgataagccaggtaatgttacccca
 K  P  G  T  N  N  P  T  D  N  P  S  D  K  P  G  N  V  T  P
acagcagatcaaaatcatcgtgcagacaataatgatggccatggatcctata
 T  A  D  Q  N  H  R  A  D  N  N  D  G  H
```

PCR product of HybJ *L. sanfranciscensis*/*L. panis* hybrid FTF. Sections in red letters are from *L. sanfranciscensis*, blue sections are of *L. panis* origin.

7.7.3 HybK

```
tatatctagaaggagatataatggctgatgctgttgagaacaataaatacgatggcactgct
                    M  A  D  A  V  E  N  N  K  Y  D  G  T  A
aacgttaacattgattgccaagctaatgttgatgggaagattatcagtactgatgataat
 N  V  N  I  D  C  Q  A  N  V  D  G  K  I  I  S  T  D  D  N
gcaaccagtggctcgacaaagcaagaatcatctattgctaatgataatgcaaccagtggc
 A  T  S  G  S  T  K  Q  E  S  S  I  A  N  D  N  A  T  S  G
tcgacaaagcaagaatcatctattgctaatgataatgcaaccagtggctcgacaaagcaa
 S  T  K  Q  E  S  S  I  A  N  D  N  A  T  S  G  S  T  K  Q
gaatcatctattgctaatgataatgcaaccagtggctcgacaaagcaagaatcatctgtt
 E  S  S  I  A  N  D  N  A  T  S  G  S  T  K  Q  E  S  S  V
gctaacgataatgcaaccagcggttcgacaaagcaagaatcatctgttgctaacgataat
 A  N  D  N  A  T  S  G  S  T  K  Q  E  S  S  V  A  N  D  N
gcaaccagtggttcgacaaagcaagaatcatctgttgctaacgataatgcaaccagtggt
 A  T  S  G  S  T  K  Q  E  S  S  V  A  N  D  N  A  T  S  G
tcgacaaagcaagaatcatctgttgctaatgatactaaaactgctgttgttgacgaaagt
 S  T  K  Q  E  S  S  V  A  N  D  T  K  T  A  V  V  D  E  S
aaaaatactagcaacacagaaaatgataatagtcaattaaagcaaactaataatgaacaa
 K  N  T  S  N  T  E  N  D  N  S  Q  L  K  Q  T  N  N  E  Q
ccatcagctgctactcagactaacctgagtaagttaaatccagctgcagctaacgcagta
 P  S  A  A  T  Q  T  N  L  S  K  L  N  P  A  A  A  N  A  V
aagaacgctaagattgacgcaggtaacttaactgatgatcaaattaacgaattaaacaag
 K  N  A  K  I  D  A  G  N  L  T  D  D  Q  I  N  E  L  N  K
attgatttttccaaatcagcagaaaagggtgctaaattaacttttaaggatttggaaggt
 I  D  F  S  K  S  A  E  K  G  A  K  L  T  F  K  D  L  E  G
attggtaatgcaattattaaccaagatccaaagtatgctattccttactttaatgccaag
 I  G  N  A  I  I  N  Q  D  P  K  Y  A  I  P  Y  F  N  A  K
aagattcagaacatgccagctgcatatgcagttgatgcacaaactggtcaaatggcccac
 K  I  Q  N  M  P  A  A  Y  A  V  D  A  Q  T  G  Q  M  A  H
ttggatgtatgggactcttggccagttcaagatgctgttaccggctacgtttctaactat
 L  D  V  W  D  S  W  P  V  Q  D  A  V  T  G  Y  V  S  N  Y
aagggctaccaactggtaatcgcaatgatgggaattccgaaggccaagtatggtgacaac
 K  G  Y  Q  L  V  I  A  M  M  G  I  P  K  A  K  Y  G  D  N
catatttaccttctgtacaacaagtatggtgacaatgatttttcccattggcgcaatgcc
 H  I  Y  L  L  Y  N  K  Y  G  D  N  D  F  S  H  W  R  N  A
ggttccatttttggaaataacgaaaacaatgtctttcaagaatggtcaggttccgcaatc
 G  S  I  F  G  N  N  E  N  N  V  F  Q  E  W  S  G  S  A  I
gttaacaatgatggctcaatccaattattctacacttcaaacgatacatctgattttaag
 V  N  N  D  G  S  I  Q  L  F  Y  T  S  N  D  T  S  D  F  K
cttaatgatcaaaaattagcgactgcaactctgcacctaaatgttgacgataatggtgta
 L  N  D  Q  K  L  A  T  A  T  L  H  L  N  V  D  D  N  G  V
tcaattgcgagtgttgataataaccatatcctgtttgagggtgatggttaccactaccaa
 S  I  A  S  V  D  N  N  H  I  L  F  E  G  D  G  Y  H  Y  Q
acttaccaacaatttgctaacggaaacgatcgtcaagatgatgactactgcttacgtgat
 T  Y  Q  Q  F  A  N  G  N  D  R  Q  D  D  D  Y  C  L  R  D
ccccatgttgttcagttagaaaatggtgatcgttacttggtatttgaagccaatacaggt
 P  H  V  V  Q  L  E  N  G  D  R  Y  L  V  F  E  A  N  T  G
accgaagactaccaaggtgatgagcaaatttacaagtgggctaattatggtggtgacgat
 T  E  D  Y  Q  G  D  E  Q  I  Y  K  W  A  N  Y  G  G  D  D
gtctttaatattaatagcttctttaagttattaaacaataagcgagaccgcacccttgct
 V  F  N  I  N  S  F  F  K  L  L  N  N  K  R  D  R  T  L  A
ggtgaggctaatggtgctcttggtattttaaagcttgataataatcaaactaatccaaag
 G  E  A  N  G  A  L  G  I  L  K  L  D  N  N  Q  T  N  P  K
gttgaagctgtatactcaccattagtatcaactttaatggcaagtgacgaagttgaacgt
 V  E  A  V  Y  S  P  L  V  S  T  L  M  A  S  D  E  V  E  R
ccagatgttgtgaaacttggtgataagtactatctcttctcagtaactcgggttagtcgt
 P  D  V  V  K  L  G  D  K  Y  Y  L  F  S  V  T  R  V  S  R
ggatcagatactgaattaacggctaaggataacactcttgttggtgataatgttgctatg
 G  S  D  T  E  L  T  A  K  D  N  T  L  V  G  D  N  V  A  M
atcggctatgttgcagatagcttgaacggtacttacaagccgttgaaccagtctggagtt
 I  G  Y  V  A  D  S  L  N  G  T  Y  K  P  L  N  Q  S  G  V
```

```
gtcctgactgcttctgtacctgctaactggcggactgcaacctactcctattacgccgtt
 V  L  T  A  S  V  P  A  N  W  R  T  A  T  Y  S  Y  Y  A  V
cctgttcagggacacccagatcaagttttaattacttcatacatgagtaacaaggacttt
 P  V  Q  G  H  P  D  Q  V  L  I  T  S  Y  M  S  N  K  D  F
gcttctggagaaggtaattatgctacctgggcaccaagtttcttggttcaaattaatcca
 A  S  G  E  G  N  Y  A  T  W  A  P  S  F  L  V  Q  I  N  P
gatgacacaacaacggttctggcacgggctactaatcaaggtgattggatctgggatgat
 D  D  T  T  T  V  L  A  R  A  T  N  Q  G  D  W  I  W  D  D
tccagtcgtaatgacagcatgttaggtgtccttgaccagaatgctgcaaatagcgctgcg
 S  S  R  N  D  S  M  L  G  V  L  D  Q  N  A  A  N  S  A  A
ctacctggtgaatggggtaagccggtcgactggacaaatattaaccggagtagtggctta
 L  P  G  E  W  G  K  P  V  D  W  T  N  I  N  R  S  S  G  L
aacctgaagccacaccaggcagctgatgaaccgggtactaataaaccaacggataacccg
 N  L  K  P  H  Q  A  A  D  E  P  G  T  N  K  P  T  D  N  P
agtgataagccaggcaccaacaacccaacggataacccgagcgacaagccaggtactaat
 S  D  K  P  G  T  N  N  P  T  D  N  P  S  D  K  P  G  T  N
aacccaacggataacccaagtgataagccaggtactaataatccaacggataacccaagt
 N  P  T  D  N  P  S  D  K  P  G  T  N  N  P  T  D  N  P  S
gataagccaggtaccaacaatccaacggataacccaagtgataagccaggtaccaacaat
 D  K  P  G  T  N  N  P  T  D  N  P  S  D  K  P  G  T  N  N
ccaacggataatccgagtgacaagccaggtaccaacaatccaacggataatccgagtgat
 P  T  D  N  P  S  D  K  P  G  T  N  N  P  T  D  N  P  S  D
aagccgggtaccaacaatccaacagataacccaagtgataagccaggtaatgttacccca
 K  P  G  T  N  N  P  T  D  N  P  S  D  K  P  G  N  V  T  P
acagcagatcaaaatcatcgtgcagacaataatgatggccatggatcctata
 T  A  D  Q  N  H  R  A  D  N  N  D  G  H
```

PCR product of HybK *L. sanfranciscensis*/*L. panis* hybrid FTF. Sections in red letters are from *L. sanfranciscensis*, blue sections are of *L. panis* origin.

7.7.4 HybN

```
tatatctagaaggagatataatggctgatcaagttgaggcaaatactgctaatgaaacgcaa
                    M  A  D  Q  V  E  A  N  T  A  N  E  T  Q
acagttaacgctacgcaacaggtaactgatcaaactgctgtcactagtagtgcttcttct
 T  V  N  A  T  Q  Q  V  T  D  Q  T  A  V  T  S  S  A  S  S
gctgagaatactaagaatgacaaagtagcttcagtacaagcaaatactgttgctaacagt
 A  E  N  T  K  N  D  K  V  A  S  V  Q  A  N  T  V  A  N  S
gatgaacaaacacaattaaagcaaactaataatgaacaaccatcagctgctactcaagca
 D  E  Q  T  Q  L  K  Q  T  N  N  E  Q  P  S  A  A  T  Q  A
aacttaaagaaattaaatcacgaagcagctaaagccgttcaaaatgctaagattgatgct
 N  L  K  K  L  N  H  E  A  A  K  A  V  Q  N  A  K  I  D  A
ggtagtttaacagatgaacaaattaatgaattaaataaaattaacttctctaagtctgct
 G  S  L  T  D  E  Q  I  N  E  L  N  K  I  N  F  S  K  S  A
gaaaaaggtgcaaaattaacttttaaggacttagaaggaattggtaatgcaattgtaaaa
 E  K  G  A  K  L  T  F  K  D  L  E  G  I  G  N  A  I  V  K
caagacccacaatatgccgttccttactttaatgctaaggaaatcaagaatatgcctgcc
 Q  D  P  Q  Y  A  V  P  Y  F  N  A  K  E  I  K  N  M  P  A
tcatatactgtcgatgcccaaactggcaaaatggctcatcttgatgtttgggattcttgg
 S  Y  T  V  D  A  Q  T  G  K  M  A  H  L  D  V  W  D  S  W
ccagtacaagatcccacaggttatgtgtctaattacaagggttatcaactagttattgcg
 P  V  Q  D  P  T  G  Y  V  S  N  Y  K  G  Y  Q  L  V  I  A
atgatgggtattcctaatacaccaaacggtgataatcatatttaccttctttacaataaa
 M  M  G  I  P  N  T  P  N  G  D  N  H  I  Y  L  L  Y  N  K
tatggtgataacgacttttcacattggcgtaatgcgggctcaatttttggtactaacgaa
 Y  G  D  N  D  F  S  H  W  R  N  A  G  S  I  F  G  T  N  E
aataacgtataccaagaatggtccgggtcagctattgtaaatgataatggcacaattcaa
 N  N  V  Y  Q  E  W  S  G  S  A  I  V  N  D  N  G  T  I  Q
ttattctacacttcaaacgatacctctgattacaagttaaacgatcaacgacttgctact
 L  F  Y  T  S  N  D  T  S  D  Y  K  L  N  D  Q  R  L  A  T
gcaacactaaatcttgatgtagatgataatggtgtagcaattaagagtgttgacaattac
 A  T  L  N  L  D  V  D  D  N  G  V  A  I  K  S  V  D  N  Y
catattttgtttgaaggtgatggtttccactatcagacctatgaccaatttgcaaacggg
 H  I  L  F  E  G  D  G  F  H  Y  Q  T  Y  D  Q  F  A  N  G
aaggatcgtaagaacgacgattattgtctccgagatcctcacgttgtgcaatcagaaaat
 K  D  R  K  N  D  D  Y  C  L  R  D  P  H  V  V  Q  S  E  N
ggtgatcgttacttagtatttgaagccaatacgggaatggaagattaccaaagtgatgat
 G  D  R  Y  L  V  F  E  A  N  T  G  M  E  D  Y  Q  S  D  D
caaatttataattgggctaactatggcggcgatgatgcttttaatatcaagagtttcttt
 Q  I  Y  N  W  A  N  Y  G  G  D  D  A  F  N  I  K  S  F  F
aaacttctgaataacaagaatgaccgtgaattagccagtttagctaatggggctattggt
 K  L  L  N  N  K  N  D  R  E  L  A  S  L  A  N  G  A  I  G
atcctaaagcttaacaatgaccaaaccaatccaaaggttgaagaggtttattcaccatta
 I  L  K  L  N  N  D  Q  T  N  P  K  V  E  E  V  Y  S  P  L
gtatccacattaatggctagtgacgaagttgaacgagtaaacgtagttaagcttggtgat
 V  S  T  L  M  A  S  D  E  V  E  R  V  N  V  V  K  L  G  D
aagtattatctcttctccgcaactcgtgtaagtcgtggttccgatcgtgaattaaacgct
 K  Y  Y  L  F  S  A  T  R  V  S  R  G  S  D  R  E  L  N  A
aaagatatcacaatcgttggtgataatgttgctatgatcggttatgtttctgataacttg
 K  D  I  T  I  V  G  D  N  V  A  M  I  G  Y  V  S  D  N  L
atgggtaagtacaagccattaaataactcaggtgtcgtattaactgcatcagtacctgca
 M  G  K  Y  K  P  L  N  N  S  G  V  V  L  T  A  S  V  P  A
aactggcgtactgctacttattcctactatgcagtacctgtagaaggtcaccctgatcaa
 N  W  R  T  A  T  Y  S  Y  Y  A  V  P  V  E  G  H  P  D  Q
gtactaattacttcttacatgagtaacaaggactttgcttcaggtgaaggaaactatgca
 V  L  I  T  S  Y  M  S  N  K  D  F  A  S  G  E  G  N  Y  A
actttggcaccaagtttcatagtacaaatcaatccagatgacacgacaactgtattagca
 T  L  A  P  S  F  I  V  Q  I  N  P  D  D  T  T  T  V  L  A
cgtgcaactaaccaaggtgactgggtatgggacgactctagtcggaacgataatatgctt
 R  A  T  N  Q  G  D  W  V  W  D  D  S  S  R  N  D  N  M  L
ggtgttcttaaagaaggtgcagttaacagtgccgccttaccgggtgaatggggtaagccg
 G  V  L  K  E  G  A  V  N  S  A  A  L  P  G  E  W  G  K  P
```

```
gttgactggagtttgattaacagaagttctggcttaggcttaaagcctcatcaagcagct
 V  D  W  S  L  I  N  R  S  S  G  L  G  L  K  P  H  Q  A  A
gatgaaccgggtactaataaaccaacggataacccgagtgataagccaggcaccaacaac
 D  E  P  G  T  N  K  P  T  D  N  P  S  D  K  P  G  T  N  N
ccaacggataacccgagcgacaagccaggtactaataacccaacggataacccaagtgat
 P  T  D  N  P  S  D  K  P  G  T  N  N  P  T  D  N  P  S  D
aagccaggtactaataatccaacggataacccaagtgataagccaggtaccaacaatcca
 K  P  G  T  N  N  P  T  D  N  P  S  D  K  P  G  T  N  N  P
acggataacccaagtgataagccaggtaccaacaatccaacggataatccgagtgacaag
 T  D  N  P  S  D  K  P  G  T  N  N  P  T  D  N  P  S  D  K
ccaggtaccaacaatccaacggataatccgagtgataagccgggtaccaacaatccaaca
 P  G  T  N  N  P  T  D  N  P  S  D  K  P  G  T  N  N  P  T
gataacccaagtgataagccaggtaatgttaccccaacagcagatcaaaatcatcgtgca
 D  N  P  S  D  K  P  G  N  V  T  P  T  A  D  Q  N  H  R  A
gacaataatgatggccatggatcctata
 D  N  N  D  G  H
```

PCR product of HybN *L. sanfranciscensis*/*L. panis* hybrid FTF. Sections in red letters are from *L. sanfranciscensis*, blue sections are of *L. panis* origin.

7.7.5 HybO

```
tatatctagaaggagatataatggctgatgctgttgagaacaataaatacgatggcactgct
                    M  A  D  A  V  E  N  N  K  Y  D  G  T  A
aacgttaacattgattgccaagctaatgttgatgggaagattatcagtactgatgataat
 N  V  N  I  D  C  Q  A  N  V  D  G  K  I  I  S  T  D  D  N
gcaaccagtggctcgacaaagcaagaatcatctattgctaatgataatgcaaccagtggc
 A  T  S  G  S  T  K  Q  E  S  S  I  A  N  D  N  A  T  S  G
tcgacaaagcaagaatcatctattgctaatgataatgcaaccagtggctcgacaaagcaa
 S  T  K  Q  E  S  S  I  A  N  D  N  A  T  S  G  S  T  K  Q
gaatcatctattgctaatgataatgcaaccagtggctcgacaaagcaagaatcatctgtt
 E  S  S  I  A  N  D  N  A  T  S  G  S  T  K  Q  E  S  S  V
gctaacgataatgcaaccagcggttcgacaaagcaagaatcatctgttgctaacgataat
 A  N  D  N  A  T  S  G  S  T  K  Q  E  S  S  V  A  N  D  N
gcaaccagtggttcgacaaagcaagaatcatctgttgctaacgataatgcaaccagtggt
 A  T  S  G  S  T  K  Q  E  S  S  V  A  N  D  N  A  T  S  G
tcgacaaagcaagaatcatctgttgctaatgatactaaaactgctgttgttgacgaaagt
 S  T  K  Q  E  S  S  V  A  N  D  T  K  T  A  V  V  D  E  S
aaaaatactagcaacacagaaaatgataatagtcagctaaagcagaatacaacagacgaa
 K  N  T  S  N  T  E  N  D  N  S  Q  L  K  Q  N  T  T  D  E
agtactacaagtgcacagactaacctgagtaagttaaatccagctgcagctaacgcagta
 S  T  T  S  A  Q  T  N  L  S  K  L  N  P  A  A  A  N  A  V
aagaacgctaagattgacgcaggtaacttaactgatgatcaaattaacgaattaaacaag
 K  N  A  K  I  D  A  G  N  L  T  D  D  Q  I  N  E  L  N  K
attgatttttccaaatcagcagaaaagggtgctaaattaacttttaaggatttggaaggt
 I  D  F  S  K  S  A  E  K  G  A  K  L  T  F  K  D  L  E  G
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 L  D  V  W  D  S  W  P  V  Q  D  A  V  T  G  Y  V  S  N  Y
aagggctaccaactggtaatcgcaatgatgggaattccgaaggccaagtatggtgacaac
 K  G  Y  Q  L  V  I  A  M  M  G  I  P  K  A  K  Y  G  D  N
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 H  I  Y  L  L  Y  N  K  Y  G  D  N  D  F  S  H  W  R  N  A
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 G  S  I  F  G  N  N  E  N  N  V  F  Q  E  W  S  G  S  A  I
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 V  F  N  I  N  S  F  F  K  L  L  N  N  K  R  D  R  T  L  A
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 G  S  D  T  E  L  T  A  K  D  N  T  L  V  G  D  N  V  A  M
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 I  G  Y  V  A  D  S  L  N  G  T  Y  K  P  L  N  Q  S  G  V
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gtcctgactgcttctgtacctgctaactggcggactgcaacctactcctattacgccgtt
 V  L  T  A  S  V  P  A  N  W  R  T  A  T  Y  S  Y  Y  A  V
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 P  V  Q  G  H  P  D  Q  V  L  I  T  S  Y  M  S  N  K  D  F
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 A  S  G  E  G  N  Y  A  T  W  A  P  S  F  L  V  Q  I  N  P
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 D  D  T  T  T  V  L  A  R  A  T  N  Q  G  D  W  I  W  D  D
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 S  S  R  N  D  S  M  L  G  V  L  D  Q  N  A  A  N  S  A  A
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 L  P  G  E  W  G  K  P  V  D  W  T  N  I  N  R  S  S  G  L
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 N  L  K  P  H  Q  P  V  N  P  S  Q  P  T  T  P  A  T  P  V
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 A  T  P  V  N  P  S  A  T  T  T  P  A  T  P  V  N  P  S  A
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 T  T  T  P  A  K  P  V  N  P  S  Q  P  T  T  P  A  K  P  V
caggctggtcaagcgacggctactaatttgtggaccaacgggatcctata
 Q  A  G  Q  A  T  A  T  N  F  V  D  Q  R
```

PCR product of HybN *L. sanfranciscensis*/*L. panis* hybrid FTF. Sections in red letters are from *L. sanfranciscensis*, blue sections are of *L. panis* origin.

7.8 Summary

Distinct lactobacilli are capable of production of fructan polymers and fructooligosaccharides in food fermentations. These substances may cause useful alteration in fermented foods (e.g. sourdough bread) or can have beneficial prebiotic effects.

The synthesis of these fructose containing carbohydrates is catalyzed in a one enzyme reaction from sucrose without the need of additional cofactors by so called fructosyltransferases (FTFs). This is of special interest in developing processes for production of these substances in economically useful scale. For estimating the potentials of these enzymes concerning economic applications and possible enhancement of enzyme performance by protein engineering, a comparative analysis of as many as possible FTFs from *Lactobacilli* is needed.

FTF enzymes of different lactobacillus strains have been described and first efforts in exploring structure function connections in detail have been made.

In the presented work two new FTFs from *L. panis* and *L. frumenti* are identified and characterized. An ftf gene, inactive in original *L. gasseri* strain could be cloned functionally in *E. coli* by elimination of a point mutation causing a stop codon. A functional protein could be expressed and characterized. Further, hybrid FTF proteins of L. panis and L. sanfranciscensis levansucrases are constructed and analyzed.

Sequence analysis revealed a close relationship among the FTFs and phylogenetic tree construction clustered levan and inulinsucrases. Molecular modelling approaches resulted in a five bladed β-propeller structure for all proteins used. Characterization included determination of pH and temperature optima and MM kinetic analysis. Fructan analysis revealed that *L. panis* and *L. frumenti* enzymes produced levan while *L. gasseri* derived FTF synthesized inulin. Ca^{2+} cations turned out to be essential for activity of FTFs and could not be replaced by alternative bivalent metal cations. Raffinose was accepted as fructose donor of all FTFs but no alternative carbohydrates could serve as acceptor molecules for heterooligosaccharides production. Kestose was produced by all FTFs while higher fructooligosaccharides were only produced in significant amounts by *L. sanfranciscensis* levansucrase and *L. reuteri* and *L. gasseri* inulinsucrases. pH, temperature and sucrose concentration could influence the product ratios. Among these factors the substrate concentration turned out to be the most effective.

Presented results approved existing theories for structure function relationship and description of new FTFs provides a good basis for further research. Nevertheless, product spectrum of these enzymes appears to be limited to levan, inulin and some inulin type FOS.

7.9 Zusammenfassung

Bestimmte Laktobazillen sind in der Lage in Lebensmittelfermentationen Fruktanpolymere und Fruktooligosaccharide herzustellen. Dieser Verbindungen können nützliche Veränderungen in fermentierten Lebensmitteln (z.B. in Sauerteigbrot) bewirken oder vorteilhafte prebiotische Wirkung haben.

Die Synthese dieser fruktosehaltigen Kohlenhydrate wird in einer Reaktion aus Saccharose ohne weitere Kofaktoren von nur einem Enzym, so genannten Fruktosyltansferasen (FTFs) katalysiert. Das ist in Hinsicht auf die Entwicklung von Prozessen für die Herstellung dieser Substanzen in wirtschaftlich sinnvollen Maßstab interessant. Um das Potential dieser Enzyme einschätzen hinsichtlich wirtschaftlicher Anwendungen und einer möglichen Leistungsverbesserung durch Protein Engineering Methoden einschätzen zu können, ist eine vergleichende Analyse möglichst vieler solcher Laktobazillus FTFs notwendig.

FTF Enzyme verschiedener Laktobazillusstämme wurden bereits beschrieben und erste Schritte in der Aufklärung detaillierter Struktur-Funktionsbeziehungen wurden gemacht.

In der vorliegenden Arbeit wurden zwei neue FTFs aus *L. panis* und *L. frumenti* identifiziert und charakterisiert. In im ursprünglichen *L. gasseri* Stamm inaktives ftf Gen konnte durch Eliminierung einer Punktmutation, die ein Stopcodon verursachte funktionell in *E. coli* kloniert werden. Ein funktionelles Protein konnte exprimiert und charakterisiert werden. Des Weiteren wurden hybride FTFs aus den Levansucrasen von *L. panis* und *L. sanfranciscensis* konstruiert und analysiert.

Die Sequenzanalyse zeigte eine enge Verwandtschaft unter allen FTFs und die Erstellung eines phylogenetischen Stammbaumes ergab die Gruppierung von Levan- und Inulinsucrasen. Ansätze von Molecular Modelling resultierten für eine fünfblättrige β-Propeller Struktur für alle verwendeten Proteine. Die Charakterisierung umfasste die Bestimmung der pH und Temperaturoptima und Erstellung von MM-Kinetiken. Die Analyse der Fruktane ergab, dass die Enzyme von *L. panis* und *L. frumenti* Levan synthetisieren während *L. gasseri* FTF inulin produziert. Ca^{2+} Kationen waren essentiell für die Funktionalität der FTFs und konnten nicht durch andere bivalente Metallkationen ersetzt werden. Raffinose wurde von allen FTFs als Fruktosedonor angenommen, aber kein alternatives Kohlenhydrat konnte als Akzeptor zur Synthese von Heterooligosacchariden dienen. Von allen FTFs wurde Kestose produziert, aber signifikante Mengen höherer Fruktoologosaccharide wurden nur von der Levansucrase aus *L. sanfranciscensis* und den Inulinsucrasen von *L. gasseri* und *L. reuteri* synthetisiert. pH, Temperatur und Saccharosekonzentration konnten die Produktverhältnisse beeinflussen. Unter diesen Faktoren stellte sich die Substratkonzentration als am wirkungsvollsten heraus.

Die vorliegenden Ergebnisse unterstützen die existierenden Theorien zur Struktur-Funktionsbeziehung und die Beschreibung der neuen FTFs liefert eine solide Grundlage für weitergehende Forschungen. Trotzdem zeigten die Enzyme ein eher enges Produktspektrum, das sich auf Levan, Inulin und inulinartige FOS beschränkt.

7.10 Kurzfassungen

Vergleichende Analyse von Fruktosyltransferasen aus Laktobazillen

Die Haltbarmachung von Lebensmitteln mittels bakterieller Fermentation ist eine sehr alte Technologie. Unter den verwendeten Organismen spielen Milchsäurebakterien, darunter Laktobazillen eine besondere Rolle. Sie tauchen in der Fermentation verschiedenster Lebensmittelrohstoffe wie Milch (Käse, Joghurt), Fleisch (Salami), Pflanzenmaterial (Sauerkraut) und Getreide (Sauerteige) auf.
Manche von ihnen sind in der Lage, aus Saccharose und ähnlichen Kohlenhydraten Exoploysaccharide (EPS) und Oligosaccharide zu bilden, die aus Zucker Monomeren bestehen. Man kann die aus unterschiedlichen Untereinheiten aufgebauten Heteropolysaccharide und die aus identischen Monomeren bestehenden Homopolysaccharide unterschieden. Die wichtigsten davon sind Glukane (Glukoseuntereinheiten) und Fruktane (Fructoseuntereinheiten). Diese Kohlenhydrate werden durch eine Ein-Enzym-Reaktion extrazellulär synthetisiert. Die fruktanbildenden Enzyme nennt man Fruktosyltransferasen (FTF). Diese Enzyme nutzen in erster Linie Saccharose als Fruktose-Donor und überragen den Fruktoseteil auf verschiedene Zielmoleküle. Bei einer Polymerisationsreaktion wird die Fruktose auf eine wachsende Fruktankette übertragen (i), in einer Hydrolyse ist Wasser das Akzeptormolekül (ii) und durch eine Transferreaktion auf ein alternatives Zuckermolekül (z.B. Saccharose) entstehen Oligosaccharide (iii). Der Verknüpfungstyp für das Fruktan ist durch das Enzym festgelegt und entsprechend kennt man Levansucrasen (Levan: β-(2→6)) und Inulinsucrasen (Inulin: β-(2→1)). Grundlegende Daten über Reaktionsmechanismus und Struktur-Funktionsbeziehung dieser Enzymgruppe (Familie der GH68 Transferasen) sind bekannt oder postuliert.
Fruktane sind wegen ihrer strukturverbessernden Eigenschaften (z.B. in Sauerteigbroten) und FOS wegen ihrer präbiotischen Aktivitäten für den Einsatz in Lebensmitteln interessant. Um jedoch das Potential der FTFs hinsichtlich ihrer Anwendung in wirtschaftlichen Größenordnungen und die Möglichkeiten der Effizienzsteigerung durch Protein-Engineering abschätzen zu können ist die Kenntnis möglichste vieler FTFs und eine funktionelle Vergleichsstudie dieser Enzyme notwendig.

Mittels saccharosehaltiger Agarplatten und saccharosehaltigem Flüssigmedium wurden verschiedene Laktobazillenstämme auf ihr Fruktanbildungsverhalten hin gescreent. Weiterhin

erfolgte ein molekulares Screening auf Vorhandensein von FTF-Genen mittels degenerierter Primer.
Für die Stämme die in der PCR mit diesen Primern ein Fragment aufwiesen wurde mittels inverser PCR-Techniken die komplette Gensequenz aufgeklärt.
Anhand der Aminosäuresequenz wurden Tertiärstrukturmodelle für die Enzyme auf Basis der bekannten Strukturen verwandter Enzyme errechnet.
Die FTF-Gene eines *L. panis* und eines *L. frumenti* Stammes wurden über ein pET-Vektorsystem in *E. coli* kloniert. Ein in *L. gasseri* gefundenes FTFgen mit defektem Leserahmen wurde modifiziert (durchgehender Leserahmen) in *E. Coli* kloniert. Diese Gene so wie die bekannten FTFs aus *L. sanfranciscensis* (Levansucrase) und *L. reuteri* (Inulinsucrase) wurden heterolog exprimiert und über His-Tag-Affinitätschromatografie aufgereinigt.
Artifizielle Hybride aus den Levansucrasen von *L. panis* und *L. sanfranciscensis* wurden konstruiert und ebenfalls in *E. coli* heterolog exprimiert.
Die EPS-Produktion durch die aufgereinigten Enzyme wurde in einem renaturierendem SDS Gel gezeigt. Produzierte EPS wurden mit HPLC und gelchromatographischen Methoden analysiert. Der Einfluss von pH, Puffersubstanz, Temperatur und Substratkonzentration auf die Enzymdynamik und das Produktspektrum, sowie die Notwendigkeit und Ersetzbarkeit von Ca^{2+} Ionen wurde anhand der freigesetzten Glukose (enzymatische Quantifizierung) untersucht. Die Bildung von Fructoologosacchariden (FOS) und Heterooligosacchariden wurde mit HPLC und TLC Techniken erfasst.

Die Sequenzen der bislang unbeschriebenen FTFs aus *L. panis* und *L. frumenti* konnten durchgehend aufgeklärt, kloniert und exprimiert werden. Die Sequenzen der neu identifizierten FTFs sind stark homolog zu den bekannten und in einem phylogenetischen Stammbaum clustern Inulinsucrasen und Levansucrasen. Die im Herkunftsstamm von *L. gasseri* inaktive FTF konnte durch einen einzelnen Basenaustausch funktionell exprimiert werden. Eine Expression funktioneller Hybridproteine aus *L. panis* und *L. sanfranciscensis* Levansucrasen war nur eingeschränkt möglich.
Aufgrund der Sequenzanalysen und der Analyse der gebildeten Fruktane wurden die FTFs aus *L. panis* und *L. frumenti* wurden als Levansucrasen, die FTF aus *L. gasseri* als Inulinsucrase klassifiziert.
Der optimale pH Bereich der Enzyme lag zwischen pH 4 und pH 5, die optimale Temperatur um 50°C. Mit Variation der Substratkonzentration wurden Michaelis Menten Kinetiken

ermittelt. Ca^{2+} Ionen erwiesen sich für alle untersuchten FTFs als notwendig für die Funktion. In der Regel führten höhere Saccharosekonzentrationen, höherer pH und niedrigere Temperaturen zu relativ mehr Polymerisations- und Transferreaktionen.
Raffinose konnte von allen untersuchten FTFs als Fruktosedonor genutzt werden. Das FOS Kestose wurde von allen Enzymen gebildet, höhere FOS nur von den untersuchten Inulinsucrasen und der Levansucrase von *L. sanfranciscensis*. Signifikante, detektierbare Mengen von Heterooligosacchariden auf Basis von verschiedenen Akzeptorzuckern wurden von keinem der untersuchten Proteine gebildet.

Wie erwartet sind sich die neuen und bereits bekannten FTFs hinsichtlich ihrer Sequenzen sehr ähnlich und auf Basis der beschriebenen Röntgenstruktur der *Bacillus* Levansucrase lassen sich plausible Tertiärstrukturmodelle mit einer fünfblättrigen β-Propellerstruktur errechnen. Die erhaltenen Werte für Temperatur und pH Optimum für die neuen FTFs entsprechenden den in der Literatur beschriebenen für die bekannten Enzyme. Die erhaltenen kinetischen Parameter sind angesichts von Unreinheiten der Präperation nur beschränkt nützlich. Die in der Literatur für diese Enzyme beschriebene spezifische Abhängigkeit von Ca^{2+} Kationen konnte bestätigt werden und strukturelle Überlegungen legen einen Zusammenhang mit dem Ionenradius nahe. Durch die Experimente werden die bekannten Fakten über die Struktur-Funktionszusammenhänge bestätigt.
Der Einfluss von pH, Temperatur und Substratkonzentration auf das Spektrum der gebildeten Produkte ähnelt grundsätzlich dem, für die Levansucrase von *L. sanfranciscensis* beschriebenen. Allerdings zeigen die durchgeführten Experimente, dass die Mengen an gebildeten höheren FOS relativ gering sind. Die in der Literatur postulierte Bildung von zahlreichen Heterooligosacchariden auf Basis verschiedener Akzeptorzucker konnte durch diese Arbeit nicht bestätigt werden.

Grundsätzlich stehen diese Enzyme nun zur Herstellung von Fruktanen und FOS für Lebensmittelanwendungen zur Verfügung. Zudem zeigt dieser umfassende Vergleich, dass das Potential ein konkurrierendes, breites Produktspektrum an Oligosacchariden zu bilden eingeschränkt ist. Aufgrund der bisherigen Erkenntnisse und der in dieser Arbeit ermittelten Daten ist ein Protein-Engineering als Möglichkeit der Ertragsverbesserung der Enzyme möglich.

Comparative analysis of fructosyltransferases of lactobacilli

Food preservation by bacterial fermentation is an ancient technology of man. Among the used microorganisms, lactic acid bacteria (LAB) and among them lactobacilli play an important role. They appear in fermentation of different food matrices as milk (cheese, yoghurt), meat (salami), plant material (sauerkraut) and cereals (sourdough).

Some of them are capable of the production of exopolysaccharides (EPS) and oligosaccharides from sucrose and similar carbohydrates. EPSs are long chains of sugar monomers. Heteropolysaccharides built up from different sugar subunits and homopolysaccharides composed of identical subunits can be distinguished. Among the latter the most important are glucans (glucose subunits) and fructans (fructose subunits). Those carbohydrates are synthesised by a one enzyme reaction extracellularly. Fructan producing enzymes are called fructosyltransferases (FTF). These enzymes mainly utilize sucrose as a fructose donor and transfer the fructose unit to different acceptor molecules. In a polymerization reaction, fructose is transferred to a growing fructan chain (i), in a hydrolysis reaction water is used an acceptor molecule (ii) and by a transfer reaction to an alternative sugar molecule (e.g. sucrose) oligosaccharides are produced (iii). The linkage type of the fructose subunits in the fructan chain is depending on the producing FTF and respectively, levansucrases (levan: β-(2→6)) and inulinsucrases (inulin: β-(2→1)) are known. Basic data about reaction mechanism and structure function relationship of these enzymes (family GH68 transferases) are known or postulated.

Because of their structure enhancing properties (e.g. in sourdough breads) and FOS due to their prebiotic potential are interesting for food applications. Nevertheless, for a clear knowledge about their potentials in production of EPS and fructose containing carbohydrates in economic scale and the possibilities for an increase of enzyme performance by protein engineering, as many as possible FTFs must be described and functionally compared.

Different *Lactobacillu*s strains are screened for fructose production by cultivation on sucrose containing agar plates and in sucrose containing broth. Further on, the strains are screened for presence of *ftf* genes by PCR with degenerated primers.

For strains producing PCR fragments in this experiment, complete *ftf* gene sequence was discovered by inverse PCR techniques.

Based on amino acid sequence, for these enzymes tertiary structure models are calculated based on known x-ray structures of closely related proteins.

The *ftf* genes of an *L. panis* and an *L. frumenti* strain are cloned in *E. coli* using a pET-vector system. An *ftf* gene with disrupted reading frame discovered in an *L. gasseri* strain was modified (corrected reading frame) and cloned in *E. coli*. Beside these genes, the known *ftf* genes of *L. sanfranciscensis* (levansucrase) and *L. reuteri* (inulinsucrase) was heterologously expressed and proteins purified by His-tag affinity chromatography.
In addition to that, artificial hybrids based on *L. sanfranciscensis* and *L. panis* levansucrase genes are constructed and heterologously expressed in *E. coli*.
EPS production by purified enzymes was shown by renaturating SDS-PAGE technique. EPS produced was analyzed by HPLC and gel chromatography methods. Influence of pH, buffering substance, temperature and substrate concentration on enzyme dynamics and product spectrum as well as necessity and replaceability of Ca^{2+} ions was examined by enzymatic detection (quantitative) of produced glucose. Formation of fructooligosaccharides (FOS) and heterooligosaccharides was monitored by HPLC and TLC techniques.

Sequences of unknown FTFs of *L. panis* and *L. frumenti* could be identified completely, cloned and heterologously expressed in *E. coli*. The sequences of the newly described FTFs show much homology to known FTFs and in a phylogenetic tree, there is a cluster for inulinsucrases and another for levansucrases. The originally inactive FTF of *L. gasseri* could be functionally expressed after a single base exchange. Expression of *L. panis – L. sanfranciscensis* hybrid FTF proteins was only partly successful.
Due to sequence analysis and analysis of produced EPS, FTFs from *L. panis* and *L. frumenti* were characterized as levansucrases, the FTF from *L. gasseri* was characterized as inulinsucrase.
The pH optimum for the enzymes was between pH 4 and pH5, and optimal temperature lays around 50°C. By variation of substrate concentrations Michaelis Menten kinetics were determined. Ca^{2+} cations appeared to be necessary for functionality of all analyzed enzymes.
All in all it could be stated that increased sucrose concentration, elevated pH level and low temperatures relatively favour polymerization and transfer reactions.
Raffinose could be utilized as fructose donor by all enzymes examined. The FOS kestose was produced by all FTFs involved in the experiments. Bigger FOS was only produced by the analyzed inulinsucrases and *L. sanfranciscensis* levansucrase. Significant and detectable amounts of heterooligosaccharides by fructose transfer to different acceptor sugars could not be shown for any of the enzymes.

As expected, new and known FTFs show much similarity concerning their sequences. Based on described X-ray structure of a *Bacillus* levansucrase, plausible models of a tertiary structure containing a five bladed β-propeller structure were calculated. Values for pH and temperature optima of newly described FTFs were in a similar range compared with literature data for known *lactobacillus* FTFs. Due to impure preparation of FTF proteins, kinetic parameters are of limited quality. Dependence of Ca^{2+} cations described in literature was further proven by the presented experiments and structural aspects indicate a relation to the ionic radius. Known facts about structure function relationship are supported by data obtained in this work.

Influence of pH, temperature and substrate concentration on product spectrum tends to be similar to the data published for *L. sanfranciscensis* levansucrase. Nevertheless, experiments in this work show relatively small amounts of FOS produced. Formation of various heterooligosaccharides on basis of different acceptor sugars as postulated in literature could not be stated in this work.

This work shows the possibility of production of EPS and FOS by these enzymes for food applications. Furthermore, this comparative analysis shows that the competitive production of a large number of various oligosaccharides as reported for other enzymes is limited.

Combination of the facts known up to date and on the data obtained in this work provides the basis for a possible enzyme performance increase by protein engineering.