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Levansucrase Lsc3 and endo-levanase BT1760: characterization and application for the synthesis of novel prebiotics

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Chair of Genetics, Institute of Molecular and Cell Biology, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on following original publications that are referred to by Roman numerals in the text:

I. Levansucrases from *Pseudomonas syringae* pv. tomato and *P. chlororaphis* subsp. aurantiaca: substrate specificity, polymerizing properties and usage of different acceptors for fructosylation. Visnapuu T*, **Mardo K***, Mosoarca C, Zamfir AD, Vigants A, Alamäe T. *J Biotechnol*. 2011 Sep 20;155(3):338–49. *Equal contribution
II. Mutational analysis of conserved regions harboring catalytic triad residues of the levansucrase protein encoded by the *lsc-3* gene (*lsc3*) of *Pseudomonas syringae* pv. tomato DC3000. **Mardo K**, Visnapuu T, Vija H, Elmi T, Alamäe T. *Biotechnol Appl Biochem*. 2014 Jan-Feb;61(1):11–22.
III. High-throughput assay of levansucrase variants in search of feasible catalysts for the synthesis of fructooligosaccharides and levan. **Mardo K**, Visnapuu T, Gromkova M, Aasamets A, Viigand K, Vija H, Alamäe T. *Molecules*. 2014 Jun 20;19(6):8434–55.
IV. A Highly Active Endo-Levanase BT1760 of a Dominant Mammalian Gut Commensal *Bacteroides thetaiotaomicron* Cleaves Not Only Various Bacterial Levans, but Also Levan of Timothy Grass. **Mardo K***, Visnapuu T*, Vija H, Aasamets A, Viigand K, Alamäe T. *PLoS One*. 2017 Jan 19;12(1): e0169989. *Equal contribution
V. First crystal structure of an endo-levanase – the BT1760 from a human gut commensal *Bacteroides thetaiotaomicron*. **Ernits K**, Eek P, Lukk T, Visnapuu T, Alamäe T. *Sci Rep*. 2019 Jun 11;9(1):8443.

Author's contribution

Author's contribution to the papers ('*' indicates minor contribution, '***' shows major contribution)

	I	II	III	IV	V
Experimental work	**	***	***	***	***
Data analysis	*	**	**	***	***
Manuscript preparation	*	**	**	**	***

LIST OF ABBREVIATIONS

BT1760 – endo-levanase from *Bacteroides thetaiotaomicron*
CBM – carbohydrate-binding module
CBM66 – carbohydrate-binding module of exo-levanase from *Bacillus subtilis*
CTAB – cetyltrimethylammonium bromide
DP – degree of polymerization
FOS – fructo-oligosaccharides
I-FOS – inulin-type FOS
L-FOS – levan-type FOS
GH – glycoside (glycosyl) hydrolase
HMW – high molecular weight
HOFs – hetero-oligofructans
INU2 – endo-inulinase from *Aspergillus ficuum*
INUE – exo-inulinase from *Aspergillus awamori*
LevB1 – endo-levanase from *Bacillus licheniformis*
LMW – low molecular weight
Lsc3 – levansucrases from *Pseudomonas syringae* pv. tomato
MsFFase – fructosyltransferase from *Microbacterium saccharophilum*
PDB – Protein Data Bank (https://www.rcsb.org/)
PUL – polysaccharide utilization locus
SacB – levansucrase from *Bacillus subtilis*
SCFA – short-chain fatty acid
Sus – starch utilization system

INTRODUCTION

Countries all over the world are establishing rules to reduce the amount of simple sugars (sucrose, glucose, fructose) added to foods and drinks, because while improving taste of the food, they also cause obesity (US Department of Agriculture 2015; Vos et al. 2017). The pyramid of food aids in choosing the proper food for staying healthy and preventing obesity-related diseases. The base of the food pyramid is composed of cereals and vegetables, leaving only minor space at the top for added sugars (Montagnese et al. 2015). Diet containing a sufficient amount of whole grain cereals and vegetables is rich in food fibre. For commercial applications those complex carbohydrate molecules are extracted from plants or synthesized enzymatically. This PhD study deals with two enzymes applicable for synthesis of novel food fibre.

Food fibre has a prebiotic function – it passes the stomach unchanged and is degraded only when it reaches the large intestine providing energy and carbon source for probiotic bacteria – bifidobacteria, lactobacilli and others (Verspreet et al. 2016). The most popular available prebiotic is inulin – a polymeric fructan extracted from plants (mostly chicory) in which fructose residues are bound by β-2,1 linkages. There is also another natural fructan – levan – which is synthesized mostly by bacteria; it has β-2,6 linkage between the fructose residues and has usually a very high molecular weight. According to several reports, levan and levan-type fructo-oligosaccharides (L-FOS) have even higher prebiotic efficiency than inulin and inulin-type fructo-oligosaccharides (I-FOS) (Gimeno-Pérez et al. 2015; Porras-Domínguez et al. 2014; Yamamoto et al. 1999).

Our workgroup contributes extensively to synthesis and development of fructose-based novel prebiotics, focusing mainly on levan-type fructans. For that, we have been using two enzymatic approaches: i) synthesis of levan-type fructans using a levansucrase, and ii) hydrolysis of levan into fructo-oligosaccharides (FOS) using an endo-levanase.

Levansucrase Lsc3 from a plant-pathogen *Pseudomonas syringae* pv. tomato is highly active and stable catalyst which we have studied and used for the synthesis of levan and FOS (Refs I–IV). Through structure prediction and mutational analysis of Lsc3 we have identified the enzyme's key catalytic amino acids and those implicated in its polymerizing function (Refs II–III).

Endo-levanase BT1760 from human gut commensal *Bacteroides thetaiotaomicron* characterized in Refs IV and V is the fastest endo-levanase described so far. Recently, we solved the crystal structure of this enzyme and of its catalytically inactive mutant (Ref V). As expected, the structures revealed a fold typical for a glycoside hydrolase family 32 (GH32) enzymes: an N-terminal β-propeller connected with a C-terminal β-sandwich module. The ligand-bound structure of the protein revealed a novel topology of the active site pocket among the fructan-acting enzymes. Ref V characterizes binding of the levan chain into the

substrate pocket, specific roles of C-terminal and N-terminal domains of the protein and deeper analysis of levan hydrolysis.

This work was financed by Estonian Research Council (grants ETF7528, ETF9072, PUT1050), and by European Regional Development Fund (grant 3.2.0701.12-0041).

I REVIEW OF LITERATURE

1.1. Probiotics

Bacterial community of the human gut contributes to biological functions crucial for health. It participates in vitamin synthesis and food fibre degradation, provides energetic metabolites for colonocytes, modulates immune response and exerts systemic effects on the host, influencing even the brain (Collins, Surette, and Bercik 2012; Hemarajata and Versalovic 2013; Sherwin et al. 2016; Thaiss et al. 2016). Disturbed gut microbiota-host homeostasis in humans is associated with several diseases and pathologies: irritable bowel disease, ulcerative colitis and Crohn's disease as well as allergies, asthma, cardiovascular disease and obesity (Carding et al. 2015; Kamada et al. 2013; Toor et al. 2019).

The concept of balancing the gut microbiota by oral consumption of beneficial (probiotic) microorganisms has intrigued humans for a long time. The term "probiotic" first appeared in 1974 and has evolved to a current-day definition: "Live microorganisms of human origin that confer a health benefit on the host when administered in adequate amounts" (Hill et al. 2014; Suez et al. 2019). Probiotic bacteria are commonly added to dairy products, snack and nutrition bars, breakfast cereals and infant formulas. Aside of food products, they are also commercialized as lyophilised bacteria in pills sold in pharmacies over the counter (Hoffmann et al. 2014). In general, probiotic bacteria are expected to reach the gut and stay and multiply there to exert positive effect on gut microbiota and human health.

Probiotic bacteria have been successfully used as preventive or therapeutic agent against (antibiotic-associated) acute diarrhoea (Feizizadeh, Salehi-Abargouei, and Akbari 2014; Van Niel et al. 2002; Szajewska et al. 2013) as well as *Clostridium difficile*-associated diarrhoea (Goldenberg et al. 2017; Shen et al. 2017), especially when administrated right after the treatment with antibiotics. Probiotics have also aided to relieve symptoms of irritable bowel syndrome, prevent neonatal sepsis and necrotizing enterocolitis (Ford et al. 2018; Olsen et al. 2016; Rao et al. 2016). They suppress pathogens in the gut, improve the barrier function of the gut epithelium and provide protection against physiological stress (Suez et al. 2019). According to some reviews, probiotics even help to reduce the severity, duration and incidence of common cold (acute respiratory infections) (Hao et al. 2011; King et al. 2019).

Among probiotic bacteria, certain strains of lactobacilli and bifidobacteria, as well as mixtures of these strains, have the longest history of marketing and safe usage (Douillard and de Vos 2019). Extensive research in the field of probiotics is currently focused on (i) identifying and selecting new strains of lactobacilli and bifidobacteria, (ii) genetically modifying already described ones and (iii) searching completely new, "next-generation" probiotic species (Douillard and de Vos 2019). These "next-generation therapeutic bacteria" are either butyrate producers such as *Faecalibacterium prausnitzii* or efficient poly-

saccharide degraders such as species of *Bacteroides* (Chassard et al. 2008; Louis and Flint 2009; Martín, Bermúdez-Humarán, and Langella 2018; Sheridan et al. 2016; Sonnenburg et al. 2010). In general, fermentation of complex dietary fibre increases the level of short-chain fatty acids (SCFA), mostly acetate, butyrate and propionate in the gut lumen, which in turn increases the solubility and absorption of calcium and expression of calcium-binding proteins (Scholz-Ahrens et al. 2007). From SCFA produced in the gut, butyrate is probably the most important: it is a key energy source for colonocytes, maintains epithelial hypoxia (<1% oxygen) protecting indigenous gut bacteria from toxic oxygen, and is an important regulator of gene expression, inflammation, differentiation and apoptosis of host cells (Hamer et al. 2008; Litvak, Byndloss, and Bäumler 2018; Louis and Flint 2009). The dominant butyrate producers in the gut are *F. prausnitzii*, *Roseburia* spp., *Eubacterium rectale*, *E. hallii* and *Anaerostipes* spp. (Louis and Flint 2009; Sheridan et al. 2016). Importantly, the polysaccharide degraders (*Bacteroides*, *Akkermansia muciniphila* and others) can cross-feed butyrate producers with fermentation products (such as acetate and lactate) promoting thereby butyrate production in the gut (Belzer et al. 2017; Mahowald et al. 2009).

As next-generation probiotic bacteria are natural gut residents, they are highly sensitive to oxygen and have longer colonisation time compared to traditional probiotics (Schmidt 2013). Therefore, their cultivation, manufacturing, preservation and administration is complicated (Broeckx et al. 2016; Fu et al. 2018). An alternative approach to keep the gut microbiota in healthy balance is to support the growth of resident probiotic bacteria with specific food ingredients – prebiotics.

1.2. Prebiotics

Diet is one of the most important drives in gut microbiota manipulation. Thus, it is possible to enhance multiplication and activity of health-beneficial (probiotic) bacteria by consuming specific food ingredients – prebiotics. According to definition, prebiotics are substrates that are selectively utilized by microbiota of the host, producing health benefits to the host (Verspreet et al. 2016). In general, they are non-digestible di-, oligo- and polysaccharides and are often called the food fibre (Khangwal and Shukla 2019). Tolerance to gastric acid and resistance to mammalian digestive enzymes are the key properties of prebiotics ensuring their safe passage through stomach and small intestine (Cummings, Macfarlane, and Englyst 2001; Verspreet et al. 2016). After reaching the colon, prebiotics are fermented by gut microbiota mainly to SCFA, but vitamins and other compounds are produced as well (Fernando et al. 2018). Health benefits of dietary fibres depend on their properties as they differ in molecular size and composition, solubility, viscosity, water-binding ability and fermentability by colon microbiota (Fuller et al. 2016; Verspreet et al. 2016). In general, prebiotics stimulate the immune system, enhance absorption and digestion of

nutrients, and reduce adhesion and growth of pathogens (Cummings et al. 2001; Gibson et al. 2017; Khangwal and Shukla 2019). Dietary fibre also increases the volume of chyme and decreases the transit time thereby alleviating constipation and reducing the colon cancer risk (Cummings and Macfarlane 2002; Fuller et al. 2016; Pranami, Sharma, and Pathak 2017; Slavin 2013).

In nature prebiotics occur in several vegetables such as leeks, onion, garlic, Jerusalem artichoke, chicory, soybeans, but also in mushrooms and cereals (Geigerová et al. 2017; Van Loo et al. 1995). Some commercially produced prebiotics are inulin and I-FOS (see next paragraph), isomalto- and galacto-oligosaccharides (Khangwal and Shukla 2019; Madsen et al. 2017; Öner, Hernández, and Combie 2016). Inulin from chicory has received an EU health claim whereas other potential prebiotics, *i.e.* galacto- and xylo-oligosaccharides, soybean oligosaccharides and some others are considered as candidate prebiotics or novel foods (Gibson et al. 2017; Verspreet et al. 2016). Prebiotics are directly extracted from plants, produced by enzymatic or chemical degradation of polymeric sugars or synthesized enzymatically (Madsen et al. 2017). Many pharmaceutical and food companies have high interest in cost-efficient production of prebiotics, and specific enzymatic degradation of polymeric substrates into prebiotic oligosaccharides is the most common way to produce present-day high-quality prebiotics (Khangwal and Shukla 2019).

1.3. Fructans as prebiotics

Fructans can be divided into two groups according to linkage between the fructose residues in polymer or oligomer. Inulin and I-FOS are fructans categorized as "Generally Recognized as Safe" for use in food since 2002 (Flores, Morlett, and Rodríguez 2016). In inulin (degree of polymerization, DP>10) or I-FOS (DP<10) molecules β-2,1 linkage is synthesised between the fructose residues and every fructan chain starts with the glucose residue (Kelly 2008; Morris and Morris 2012). Inulin is extracted from plants: chicory and dahlia roots, or Jerusalem artichoke, but it is also synthesised by some microorganisms (Ni et al. 2019; Zhu et al. 2016). Inulin from plants has typically a linear structure whereas bacterial inulin has branches connected with the main β-2,1 chain through β-2,6 linkages (van Hijum et al. 2006; Lopez, Mancilla-Margalli, and Mendoza-Diaz 2003).

As required for prebiotics, inulin and I-FOS are not degraded by mammalian digestive enzymes, but are fermented in the colon by many health-beneficial bacteria, for example lactobacilli, bifidobacteria and butyrate-producing *Roseburia inulinivorans* (Roberfroid and Slavin 2000; Scott et al. 2006). Many species of *Bacteroides* are common residents of the gut. Of those, *B. caccae*, *B. ovatus*, *B. uniformis* and *B. fragilis*, were able to degrade inulin, though *B. vulgatus* and *B. thetaiotaomicron* were not (Sonnenburg et al. 2010). Inulin has many beneficial health effects to humans: acting against obesity, diabetes and hypertension, promoting mineral absorption in colon, controlling inflam-

matory bowel disease, relieving constipation, stimulating the immune system and regulating endocrine system, glucose and lipid metabolism (Roberfroid 1993, 2007; Schaafsma and Slavin 2015; Vogt et al. 2015; Wang 2009). Recent studies have shown that I-FOS produced from inulin using endo-inulinase, have a high potential to be used in infant milk formulas to mimic human milk oligosaccharides (Akkerman, Faas, and de Vos 2019). Importantly, a sweetener difructose dianhydride, which also has a prebiotic effect, is also produced enzymatically from inulin (Zhao et al. 2011).

Levan, on the other hand, is a fructan with a backbone of β-2,6 linked fructose residues and branches added to it through β-2,1 linkage. Levan is produced from sucrose in levansucrase reaction by numerous bacteria as a biofilm component (Benigar et al. 2016; Koczan et al. 2009; Laue 2006; Sutherland 2001; Velázquez-Hernández et al. 2011), by some halophilic archaea (Kırtel et al. 2019) and by a limited number of plant species from family Poaceae (e.g. timothy, orchard, Harding and meadow soft grasses) (Arvidson, Rinehart, and Gadala-Maria 2006; Bonnett et al. 1997; Cairns et al. 1999; Kasperowicz et al. 2016; Öner et al. 2016; Vijn and Smeekens 1999). Levans from various origin differ in molecular size and structure: bacterial levans have a high molecular weight (HMW) with multiple branching points (Jakob et al. 2013; Runyon et al. 2014), while levan from plants has a rather low molecular weight (DP about 60) and has no branches (Bonnett et al. 1997; Cairns et al. 1999).

Currently, levan is permitted as functional food additive in Japan and South Korea (Kang et al. 2009) whereas it is currently not commercially produced and employed in Europe. For the production of levan at larger scale, precipitation of enzymatically synthesized levan by ethanol is used, followed by dialysis and lyophilization. For feasible commercial production, this methodology needs to be scaled up. FOS can also be purified from the levansucrase reaction mixture, because levansucrases produce both, levan and FOS with proportions depending on reaction conditions and intrinsic properties of the levansucrase (Bersaneti et al. 2018; Porras-Domínguez et al. 2015). In order to purify FOS from the levansucrase reaction mixture, the interfering sugars: glucose, fructose and residual sucrose should be removed (Adamberg et al. 2014; Öner et al. 2016).

Despite technological difficulties, levan and L-FOS have been tested for applications in food, medicine and chemical industry (Gomes et al. 2018; Kazak Sarilmiser and Toksoy Oner 2014; Liu et al. 2017; Öner et al. 2016; Srikanth, Siddartha, et al. 2015). Levan and L-FOS have shown promising effects on probiotic bacteria of human gut consortia (Adamberg et al. 2015, 2018; Cai et al. 2019; Jang et al. 2003; Marx, Winkler, and Hartmeier 2000; Porras-Domínguez et al. 2014; Visnapuu, Mardo, and Alamäe 2015). During fermentation of L-FOS, bifidobacteria, especially *Bifidobacterium adolescentis,* exhibited fastest growth and SCFA production (Marx et al. 2000). Of six studied *Bacteroides* species, only *B. thetaiotaomicron* was capable of growth on levan. Notably, *B. fragilis* that is considered a potential pathogen, could not use levan (Sonnenburg et al. 2010). This is important, because prebiotics should not support the growth of pathogenic bacteria. Aside of *Bacteroides*, levan also

enhanced the growth of *Faecalibacterium* and some other species in gut consortia of healthy humans (Adamberg et al. 2015). A study on fecal consortia of overweight children showed that levan affected microbiota composition as well as SCFA pattern positively (Adamberg et al. 2018). Bondarenko *et al.* used levan to produce levan-coated mineral nanoparticles (Bondarenko et al. 2016). They recommended combination of levan and nutritionally important micro-elements (selenium, iron and cobalt) in the form of nanoparticles to be used as food supplements. This combination adds a safe and efficient alternative delivery method of microelements to humans and supports beneficial gut microbiota with nutritional oligosaccharides. However, additional data on physiological effects of levans and L-FOS demand production of these substrates in higher amounts.

1.4. Glycosyl hydrolase family 68 and 32 enzymes synthesizing and hydrolysing fructans

The glycosyl hydrolase clan J (GH-J) is composed of two families: GH68 and GH32. Enzymes belonging to GH-J share a common 5-blade β-propeller structure of the catalytic domain (Lammens et al. 2009). The GH68 family contains two main types of bacterial enzymes producing fructans from sucrose: levansucrases (EC 2.4.1.10) and inulosucrases (EC 2.4.1.9). Aside of these two, few invertases (β-fructofuranosidases) which also have some polymerizing activity, also belong to GH68 family (Henrissat and Davies 1997). Inulosucrases covert sucrose into inulin-type fructans – these have β-2,1 linkage in the main chain and β-2,6 linkages at the branching points. Levansucrases perform in the opposite way – they synthesize β-2,6 linkages for the main chain and β-2,1 linkages to add branches. The GH32 family contains mostly fructosyltrans-ferases of plants and hydrolytic enzymes (invertases, exo/endo-inulinases and exo/endo-levanases) of mostly microbial origin (Lombard et al. 2014). This PhD work is focused on one enzyme from the GH68 family – levansucrase Lsc3 of *Pseudomonas syringae* and one enzyme of the GH32 family – the endo-levanase (EC 3.2.1.65) BT1760 of *Bacteroides thetaiotaomicron*.

1.4.1. Levansucrases: distribution in bacteria and general features

Levansucrases (alternative names: β-2,6-fructosyltransferase, sucrose 6-fructo-syltransferase) have been described from numerous bacteria. The following Gram-positive bacterial species have levansucrases: *Bacillus subtilis*, *B. megaterium*, *B. amyloliquefaciens, C. acetobutylicum, Streptococcus mutans, S. salivarius, Leuconostoc mesenteroides, L. citreum, Lactobacillus sanfranciscensis, Lb. gasseri, Lb. johnsonii* and *Lb. reuteri*. Among Gram-negative bacteria, levansucrases have been described in *Zymomonas mobilis*, *Gluconacetobacter diazotrophicus, Erwinia amylovora, Rahnella aquatilis, Halomonas smyrnensis,*

P. chlororaphis subsp. aurantiaca, *P. fluorescens* and many *P. syringae* strains ((Lombard et al. 2014); www.cazy.org).

Usually, levansucrases of Gram-positive bacteria are bigger than respective proteins of Gram-negative species. For instance, levansucrases of Gram-positive bacteria *Lb. sanfranciscensis* (879 aa), *Lb. gasseri* (768 aa), *Lb. johnsonii* (788 aa), *Lb. reuteri* (796 aa), *S. salivarius* (969 aa) and *S. mutans* (795 aa) are much larger than levansucrases of Gram-negative bacteria *Z. mobilis* (423 aa), *E. amylovora* (415 aa), *H. smyrnensis* (416 aa), *P. savastanoi* pv. phaseolicola (431 aa), *P. syringae* pv. tomato DC3000 (431 aa) and *G. diazotrophicus* (584 aa). Levansucrases of *Bacillus* (Gram-positive bacteria) are exceptions as they have quite moderate size: *B. subtilis* (473 aa), *B. megaterium* (484 aa) and *B. licheniformis* (481 aa) (UniProt Consortium 2018).

Another specific feature of levansucrases from Gram-positive bacteria is their dependence on Ca^{2+} ions, which stabilize the protein fold (van Hijum et al. 2006; Meng and Fütterer 2003). Levansucrases of Gram-negative bacteria are not Ca^{2+}– dependent. In the case of levansucrase of *G. diazotrophicus*, a disulphide bridge stabilizes the protein's conformation (Martínez-Fleites et al. 2005). Levansucrase from a halophilic bacterium *H. smyrnensis* requires NaCl for activity (Kirtel et al. 2018), while other stabilizing mechanisms for levan-sucrases have not yet been described.

1.4.2. Levansucrases: the structure

Despite the differences between levansucrases of Gram-positive and Gram-negative bacteria, they all possess a similar overall structure: a five-bladed β-propeller, which accommodates the active site in the centre of a funnel-shaped cavity (Figure 1). The first five-bladed β-propeller fold was observed in tachylectin-2 (Beisel et al. 1999). From this point, many protein structures with the same overall architecture have been determined. The first structure of levansucrase was solved in 2003 for SacB from *B. subtilis* (Table 1) (Meng and Fütterer 2003). The active site of SacB has three acidic amino acids crucial for catalytic activity: two aspartates and a glutamate, also called a catalytic triad (Meng and Fütterer 2003). The bottom of the funnel, harbouring the active site, was shown to be closed by a loop, which forces product molecules to exit the active centre through the entrance (Wuerges et al. 2015).

Table 1. Overview of crystallized levansucrases and one fructosyl transferase with similar activity

Organism and protein	Mutation	PDB ID	Ligand	Diffraction (Å)	Reference
Bacillus subtilis 168 SacB	–	1OYG	–	1.50	(Meng and Fütterer 2003)
	Acid/base catalyst E342A	1PT2	Sucrose	2.07	
	Acid/base catalyst E342A	3BYN	Raffinose	2.03	(Meng and Fütterer 2008)
Gluconacetobacter diazotrophicus SRT4 LsdA	–	1W18	–	2.50	(Martínez-Fleites et al. 2005)
Bacillus megaterium SacB	Stabilizer D257A	3OM2	–	1.9	(Strube et al. 2011)
	K373A	3OM4		1.75	
	N252A	3OM5		1.95	
	Y247A	3OM6		1.96	
	Y247W	3OM7		1.86	
Microbacterium saccharophilum MsFFase	–	3VSR	–	2.0	(Tonozuka et al. 2012)
	–	3VSS	Fructose	1.97	
	–	3WPU	Glycerol	1.60	(Ohta et al. 2014)
	T47S/F447V/F470Y/P500S	3WPV	Glycerol	1.81	
	T47S/S200T/F447V/P500S	3WPY	–	2.00	
	T47S/S200T/F447P/F470Y/P500S	3WPZ	–	2.27	
Erwinia amylovora EaLsc	–	4D47	Fructose, Glucose	2.77	(Wuerges et al. 2015)
Erwinia tasmaniensis EtLsc	–	6FRW	–	1.52	(Polsinelli et al. 2019)

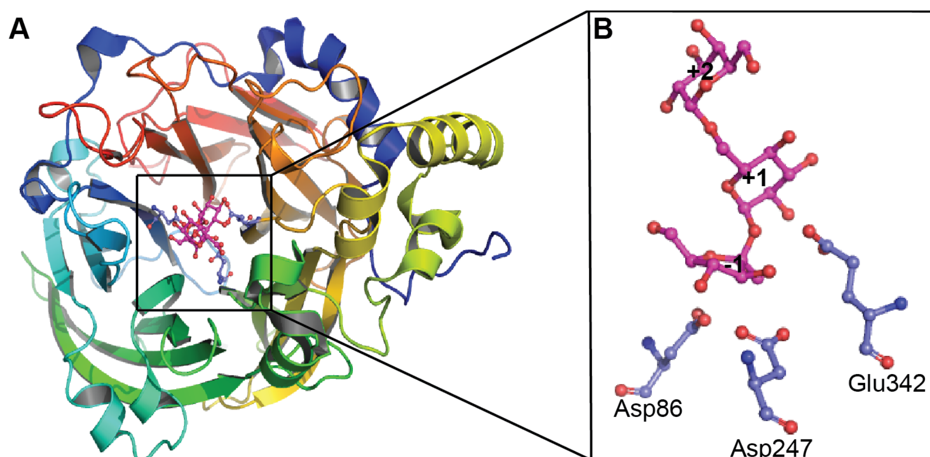

Figure 1. (A) The structure of *Bacillus subtilis* levansucrase SacB in complex with raffinose (PDB 3BYN); (B) the zoomed in active site of SacB with catalytic triad and raffinose bound at –1, +1 and +2 subsites of the substrate-binding pocket indicated. The structure was visualized using PyMOL 1.8.6.0 (Schrödinger, LLC 2015).

The function of the catalytic triad residues is following. An aspartate (Asp86 of *B. subtilis* SacB) acts as a nucleophile and is responsible for the formation of covalent fructosyl-enzyme intermediate (Lammens et al. 2009). Asp of the RDP-motif (Asp247 in SacB) acts as a transition state stabilizer, which is not directly involved in the catalysis, but supports binding of fructose to the active site *via* C3 and C4 groups. The acid/base catalyst in levansucrases is generally a glutamate – Glu342 in SacB (Meng and Fütterer 2003). The catalytic triad residues of SacB are designated in Figure 1, panel B. Functions of catalytic amino acids are described in the next section.

1.4.3. Levansucrases: substrate-binding subsites and catalytic mechanism

Levansucrase works *via* double displacement, also referred to as Ping-Pong mechanism, retaining the configuration of the anomeric carbon after the hydrolysis (Chambert and Gonzy-Treboul 1976; Hernandez et al. 1995; Martínez-Fleites et al. 2005).

The proposed catalytic reaction of levansucrase on sucrose proceeds as follows: in the first glycosylation step, a nucleophilic attack is performed on the anomeric carbon of fructose residue of sucrose which is bound at subsite –1 by the carboxylate of the nucleophile – Asp68 in the case of SacB, and fructosyl-enzyme intermediate arises (Chambert and Gonzy-Treboul 1976; Martínez-Fleites et al. 2005; Meng and Fütterer 2003, 2008). The acid/base catalyst (Glu342 in SacB) acts as a general acid and donates a proton to enable release of glucose. In the second deglycosylation step, the acid/base catalyst acts as a general base and removes a proton from the incoming fructosyl acceptor. When

the entering acceptor molecule is water (Figure 2), fructose is released as a product of hydrolysis. When the acceptor is appropriate sugar (binds at subsites +1 and further), the released product is elongated by one fructosyl unit (Figure 2) (Lammens et al. 2009). In general, molecules containing α-1,2 glycosidic linkage between glucose and fructose, such as sucrose, raffinose and stachyose are suitable substrates for levansucrases (Meng and Fütterer 2008; Öner et al. 2016; Visnapuu, Mäe, and Alamäe 2008; Visnapuu et al. 2015; Yanase et al. 2002).

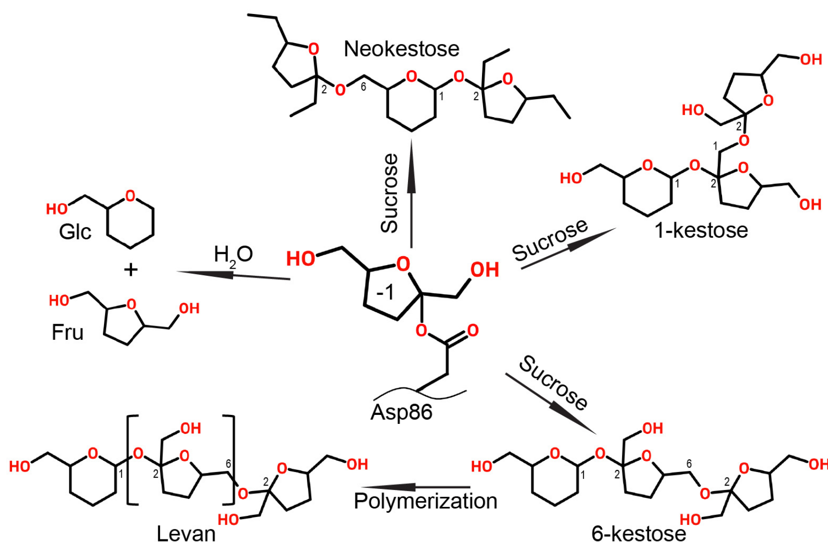

Figure 2. The products produced from sucrose by the levansucrase. Nucleophile (Asp86 in the case of levansucrase SacB of *Bacillus subtilis*) forming a covalent enzyme-fructosyl intermediate with fructose bound at –1 subsite is indicated in the centre of the figure. Reactions with various fructosyl acceptors (water and sucrose) create a variety of products: neokestose, 1-kestose and 6-kestose. The latter one is polymerized into levan in further steps of transfructosylation, parenthesis indicate multiple copies of fructose residues attached *via* β-2,6 linkage.

According to the nomenclature for sugar-binding subsites in glycosyl hydrolases proposed by Davis *et al*., hydrolysis of glycosidic linkage takes place between –1 and +1 subsites, whereas fructosyl residue is positioned into –1 subsite (Figure 1, panel B; Figure 2) (Davies, Wilson, and Henrissat 1997). Acceptor binding starts from the +1 subsite of the levansucrase (Lammens et al. 2009; Meng and Fütterer 2008; Ozimek et al. 2006). It is proposed that arrangement of catalytic amino acids in –1 subsite is conserved among GH32 and GH68 family enzymes, but the selectivity of further plus-subsites: +1, +2, +3 is quite low (Chuankhayan et al. 2010; Raga-Carbajal et al. 2018). Therefore, relaxed acceptor specificity and regioselectivity of levansucrases allow the usage of several traditional fructosyl acceptors such as kestoses, sucrose, glucose and fructose, as well as numerous non-conventional acceptors (described further in more detail).

1.4.4. Levansucrases: processive and non-processive activity

Levan and fructo-oligosaccharide synthesis can proceed either *via* processive or non-processive mechanism. Processivity is an enzyme's ability to catalyse consecutive reactions without releasing its substrate. For example, DNA polymerases are processive enzymes – they synthesize a novel DNA strand by adding up to 1000 nucleotides per second without releasing the template strand (Johansson and Dixon 2013). In cellulose hydrolysis, both processive and non-processive enzymes are involved. For instance, endo-glucanases which act non-processively, cleave cellulose chain randomly creating new free chain ends while cellobiohydrolases attack these ends and produce cellobiose processively (Várnai et al. 2014). In the case of levansucrase from *B. subtilis*, processive and non-processive synthesis has been described producing different types of levan: HMW (2 300 kDa) levan in the case of processive and low molecular weight (7.2 kDa; LMW) levan in the case of non-processive synthesis. Processive synthesis occurs when levansucrase concentration is low (0.1 U/mL), and LMW levan is synthesized at a high enzyme concentration (10 U/mL) (Raga-Carbajal et al. 2016, 2018).

Non-processive synthesis of LMW levan by SacB of *B. subtilis* proceeds through sequential elongation of earlier produced intermediate oligosaccharides taken from the reaction medium. Three stages of this process (Raga-Carbajal et al. 2018) are as follows:

i. During the early phase with duration of about 30 minutes, SacB converts about 20% of initial sucrose into glucose, fructose and various transfructosylation products, mainly neokestose, followed by 1- and 6-kestoses (Figure 2), which are elongated rapidly in further transfructosylation steps via β-2,6 linkage (Figure 2, polymerization). DP of transfructosylation products produced in the early phase reaches 30.
ii. Late phase of the LMW levan synthesis starts when about 20% of sucrose is converted and new low-DP products such as erlose, inulobiose, blastose and levanbiose arise. The two latter ones initiate blasto-FOS and oligolevan synthesis. Secondary intermediates (DP 3–20) are detectable after one hour of reaction and differ from the primary intermediates. Oligomers synthesised in the early phase are further elongated to DP higher than 40.
iii. Sucrose depletion phase starts after 2 hours of incubation (over 60% of sucrose converted): LMW levan of DP50 is produced, and the reaction mixture contains both primary and secondary intermediates and their elongated products. The concentration of neo-kestose and 6-neo-nystose is decreasing as they are used as fructosyl donors in blasto-FOS synthesis.

Synthesis of HMW levan from sucrose proceeds without accumulation of LMW levan and intermediate oligosaccharides (Raga-Carbajal et al. 2016, 2018) requiring permanent contact between the acceptor and enzyme molecule. However, dissociation of acceptor-protein complex may shift the process towards non-processive elongation, resulting in production of LMW levan. Dissociation may be triggered by many factors, for example high ionic strength of the buffer (Tanaka, Oi, and Yamamoto 1979).

Most commonly, levansucrases of bacteria produce HMW levan: *R. aquatilis* (380 kDa), *M. laevaniformans* (710 kDa) and *H. smyrnensis* (1483 kDa) (Kazak Sarilmiser et al. 2015; Yoo et al. 2004). Some bacterial levansucrases produce levan with bimodal size: LevU of *Z. mobilis* (3 000 kDa and 5 kDa) and SacB of *B. subtilis* TH4-2 (660 kDa and 6 kDa) (Byun, Lee, and Mah 2014; Porras-Domínguez et al. 2015). But, for example, the levansucrase from a plant pathogen *G. diazotrophicus* produces mostly I-FOS (1-kestose), and only a small amount of levan (Támbara et al. 1999; Trujillo et al. 2001). Still the structure and working mechanism of this enzyme is similar to levansucrases producing mostly levan.

Literature data on variety of oligo- and polymeric fructans produced by levansucrases is presented in Table 2.

Table 2. Product spectrum of levansucrases from Gram-negative and -positive bacteria

The host bacterium	Product spectrum	Substrate [C]; reaction time	Reference
Gram-negative bacteria			
Erwinia tasmaniensis	Levan; FOS DP* 2–6 (levanbiose, levantriose, 6-kestose, 6-nystose, 6,6,6-kestopentaose)	1 M Suc; 24 h	(Polsinelli et al. 2019)
Erwinia amylovora	Levan; FOS DP 2–6 (levanbiose, levantriose, 6-kestose, 6-nystose, 6,6,6-kestopentaose)	1 M Suc; 24 h	(Polsinelli et al. 2019)
Gluconacetobacter diazotrophicus	Small amount of levan; FOS DP 3–4 (1-kestotriose and 1,1-kestotetraose)	0.8 M Suc; 7 h	(Hernandez et al. 1995)
Halomonas smyrnensis AAD6T	Levan, FOS DP 2–5 (blastose, inulobiose, levanbiose, neokestose, 1-kestotriose, 6-kestotriose, 1-kestotetraose, 6-kestotetraose, GF_3, GF_4)	1.5 M Suc; NA	(Kirtel et al. 2018)
Rahnella aquatilis JCM-1683	Levan	0.29 M Suc; 48 h	(Ohtsuka et al. 2009)
Zymomonas mobilis	Levan; FOS DP 3–4	0.6 M Suc; 12 h	(Yanase et al. 2002)
Gram-positive bacteria			
Bacillus subtilis	Mainly levan	0.8 M Suc; 7 h	(Hernandez et al. 1995)
Bacillus megaterium	Levan; FOS DP 2–4 (1-kestose, blastose, 6-kestoase, neokestose, nystose)	0.5 M Suc; 19 h	(Homann et al. 2007)
Lactobacillus sanfranciscensis	Levan; 1-kestose	0.5 M Suc; 24 h	(Tieking et al. 2005)

[NA] – data not available; *FOS DP – fructo-oligosaccharides with the degree of polymerization.

1.4.5. Levansucrases: non-conventional acceptors for transfructosylation

The fructosyl acceptor site of levansucrases (the +1 subsite) accommodates a wide range of non-conventional acceptors for transfructosylation reaction, enabling synthesis of hetero-oligofructans (HOFs) (Davies et al. 1997; Meng and Fütterer 2003, 2008; Seibel et al. 2006). For example, the *B. subtilis* NCIMB 1187 levansucrase accepts and transfructosylates D-galactose, D-xylose, D-fucose, their L-isomers and also disaccharides such as cellobiose, maltose, isomaltose, melibiose and lactose (Seibel et al. 2006). The transfructosylation product is always one fructosyl longer than the acceptor molecule. The short HOFs can be further transfructosylated. So, β-fructofuranosidase of *Aspergillus niger* can elongate the HOFs synthesized by *B. subtilis* levansucrase, to produce XylF, ManF and GalF fructans with DP 3–4 (Zuccaro et al. 2008). It was shown that these newly synthesised fructans had no toxic effect nor suppressed cell growth of human epithelial cells and may act as prebiotic oligosaccharides with extended biological effect. EG-6 fructosyltransferases of *B. macerans* can transfructosylate many acceptors, of which D-xylose was the most appropriate, followed by L-arabinose, L-sorbose, D-galactose, maltose, D-mannose, lactose and raffinose (Nam et al. 2000). Levansucrase from *B. licheniformis* transfructosylates galactose, cellobiose, xylose, maltose, lactose, arabinose, and trehalose (Lu et al. 2014). Levansucrases from *M. laevaniformans* and *B. amyloliquefaciens* prefer disaccharides (cellobiose, lactose, melibiose) as fructosyl acceptors to monosaccharides such as D-arabinose, D-xylose and D-galactose (M. Li, Seo, and Karboune 2015; Park et al. 2003). The levansucrase from a halophilic bacterium *H. smyrnensis* elongates efficiently disaccharides cellobiose and lactose and monosaccharides arabinose and galactose when provided as acceptors (Kirtel et al. 2018).

One of the best-characterized HOFs is lactosucrose – a trisaccharide consisting of D-glucose, D-galactose, and D-fructose. In transfructosylation reaction, lactose and sucrose are used as a fructosyl acceptor and donor, respectively (W. Li et al. 2015). Levansucrases of *Aerobacter levanicum*, *B. natto*, *B. subtilis* KCCM 32835, *B. subtilis* NCIMB 11871, *B. methylotrophicus* SK21.002, *B. licheniformis* 8-37-0-1, *B. amyloliquefaciens, Paenibacillus polymyxa* IFO 3020, *Sterigmatomyces elviae* ATCC 18894 and *Z. mobilis* have been reported to produce lactosucrose (Avigad 1957; Choi et al. 2004; Han et al. 2009; Lee, Lim, Park, et al. 2007; Lee, Lim, Song, et al. 2007; M. Li et al. 2015; Lu et al. 2014; Park, Choi, and Oh 2005; Seibel et al. 2006; Takahama et al. 1991; Wu et al. 2015). It has been shown that lactosucrose has prebiotic properties: it enhances intestinal calcium absorption, reduces body fat and prevents obesity (Li *et al.*, 2015).

1.5. Levan: occurrence and functions in nature

Some bacterial biofilms contain levan among other components. Soil bacteria *B. megaterium* and *B. subtilis* synthesize levan-containing biofilm to protect themselves from drying (Dogsa et al. 2013; Homann et al. 2007). A Gram-negative sugarcane endophyte *G. diazotrophicus* uses levan capsule to create appropriate microaerobic environment for nitrogen fixation (Hernandez et al. 1995; Velázquez-Hernández et al. 2011). Many plant-pathogenic bacteria use levan as a virulence factor. For example, *E. amylovora* and *P. syringae* use levan to hide from plant defence mechanisms (Koczan et al. 2009; Mehmood et al. 2015). *P. syringae* pv tomato DC3000 levansucrase, which is characterized in this PhD work, causes necrotic specs of tomato leaves (Preston 2000). The bacterium enters the intercellular spaces of leaves through stomata and multiplies in the apoplast asymptomatically prior to disease's development. Since sucrose is present in the plant apoplast, extracellular levansucrase of the bacterium produces levan that acts as a morphological spacer between the plant cell wall and a bacterium, inhibiting hypersensitive response of the plant (Kasapis *et al.*, 1994; Hettwer, Gross and Rudolph, 1995).

1.6. Levan: biotechnological potential

Levans have many potential applications. Due to low viscosity (Arvidson et al. 2006), high water-binding ability (Gupta et al. 2011; Han and Clarke 1990; Srikanth, Reddy, et al. 2015) and other intrinsic properties, levans are applicable in (i) food industry as a prebiotic, stabilizer and fat substitute, (ii) in cosmetics as a whitener and moisturizer, and (iii) in pharmacy as an anti-oxidant, -inflammatory, -clotting and -cancer agent (Kim et al. 2005; Korakli et al. 2003; Moscovici 2015; Öner et al. 2016; Srikanth, Reddy, et al. 2015; Yoo et al. 2004). Importantly, according to the 'The Human Repeated Insult Patch Test', levan meets every safety criteria: it shows neither skin or eye irritation nor allergic reaction, and has no cytotoxic effect (Montana Polysaccharides Corp. 2017). Due to a high film-forming ability, biocompatibility and strong adhesivity, levan has a superb potential as a biopolymer for medical applications (Ates 2015; Kazak Sarilmiser et al. 2015; Öner et al. 2016). For example, levan from *H. smyrnensis* has been recommended as material for nano- and microcarriers for drugs (Sezer et al. 2011, 2017), an adhesive nanostructured multilayer film for new-generation bandages (Costa et al. 2013), biodegradable and temperature-responsive hydrogel for controlled drug release (Osman, Oner, and Eroglu 2017), a heparin mimetic for antithrombotic treatment (Erginer et al. 2016), and a material for tissue engineering (Avsar et al. 2018).

Prebiotic effects of levan have also been reported (Kang et al. 2009; Öner et al. 2016; Srikanth, Reddy, et al. 2015). Levan fermentation by human faecal samples supported the growth of next-generation probiotics such as species of *Bacteroides* and *Faecalibacterium* (Adamberg et al. 2015, 2018). Novel

candidates for prebiotics are most often tested on rodents first. Feeding rats with levan-supplemented food reduced obesity, hyperlipidaemia, adipocyte hypertrophy, serum triglycerides and cholesterol (Kang et al. 2004). According to another study, combination of levan and fermented ginseng reduced body and white adipose tissue weight, fasting blood glucose level and insulin resistance when fed to rats (Oh et al. 2014).

Levan has also been suggested as feed additive of farm animals to replace prophylactic growth-promoting antibiotics (Öner et al. 2016). When diet of pigs was supplemented with levan, improvement of the growth performance, digestibility and immune response of pigs was clearly visible (Li and Kim 2013). Similar results were observed in the case of chicken – levan administration improved the growth performance, decreased the concentrations of excreted ammonia, increased the levels of probiotic bacteria and decreased the pathogenic bacteria in the cecum (Zhao, Wang, and Kim 2013). When the diet of common carp was supplemented with levan, it fully survived after experimental *Aeromonas hydrophila* infection (Rairakhwada et al. 2007).

Unfortunately, only limited number of companies are producing levan commercially: Natural Polymers Inc. (using *B. subtilis*), Real Biotech Co., Ltd., (using *Z. mobilis*), Advance Co., Ltd (using *S. salivarius*) and Rahn AG (Öner et al. 2016). Currently, the main bottleneck in application of levan is its high price and limited availability.

1.7. Fructan utilization locus of *Bacteroides thetaiotaomicron*

Bacteroidetes is a predominant bacterial phylum of the human normal colonic microbiota (Moore and Holdeman 1974). In general, *Bacteroides* species stay in the gut, but at rupture of the gastrointestinal tract or after intestinal surgery, few of them (*e.g. B. fragilis*) may cause anaerobic infections (Wexler 2007). *Bacteroides* species are anaerobic, asporogenic, bile-resistant Gram-negative rods that are passed from mother to child *via* vaginal birth. Generally, mutualism is considered as the relationship between *Bacteroides* species and humans, because both partners experience increased fitness as a result (Wexler 2007).

The genome of *B. thetaiotaomicron* (6.26 Mbp, ATCC 29148) was sequenced in 2003 and the organism itself is the most studied species of *Bacteroidetes* (Xu et al. 2003). This bacterium is famous for its superior ability to degrade food fibre. The toolbox for this function comprises 269 glycoside hydrolases, 87 glycosyl transferases, 15 polysaccharide lyases, and 19 carbohydrate esterases (Lombard et al. 2014; Ravcheev et al. 2013). Aside of food fibre, *B. thetaiotaomicron* can utilize mucin-derived polysaccharides of the host and catabolize mono- and oligosaccharides present in breast milk (Wexler 2007). In comparison, the human genome (2.85 Gbp) encodes only 97 glycoside hydrolases, and no polysaccharide lyases. Eight glycoside hydrolases of humans are directly involved with digestion, and nine are possibly digestive enzymes, but the

remaining 80 enzymes have no roles in food digestion (El Kaoutari et al. 2013). Importantly, humans have no enzymes to degrade common food fibre species, for example xylan, pectin, and arabinose-containing polysaccharides, while *B. thetaiotaomicron* has 64 corresponding enzymes hydrolysing these substrates (Xu and Gordon 2003).

Genes for glycan degradation are clustered in the genome to polysaccharide utilizing loci (PULs). The *B. thetaiotaomicron* possesses 88 PULs which differ in polysaccharide specificity (Martens, Chiang, and Gordon 2008; Sonnenburg et al. 2010). The starch utilization system (Sus), described about twenty years ago, was the first PUL characterized in *B. thetaiotaomicron* (Reeves, Wang, and Salyers 1997). The system encodes eight clustered genes *SusRABCDEFG*, whereas a pair of *SusC* and *SusD* genes mostly defines the characteristics of the PUL. Outer membrane SusCD complex binds and imports starch oligo-saccharides previously hydrolysed by the cell surface amylase SusG (Bjursell, Martens, and Gordon 2006; Sonnenburg et al. 2010). We have been interested in fructan PUL of *B. thetaiotaomicron* VPI-5482 which encodes: (i) GH32 family hydrolases (SusG homologues) responsible for degradation of glycosidic linkage in a fructan; (ii) outer membrane transporter and glycan binding proteins (SusC/SusD homologues), responsible for fructan recognition and transport, and (iii) a hybrid two-component system sensor regulator that transcriptionally activates the PUL in response to fructose (Joglekar et al. 2018; Sonnenburg et al. 2006, 2010). *B. thetaiotaomicron* is constantly ready for levan degradation, meaning immediate response upon fructan arrival into the distal gut environment (Sonnenburg et al. 2010). Levan is captured by outer membrane-bound levan-binding proteins and cleaved extracellularly by the endo-levanase BT1760. The resulting FOS are actively imported by the outer-membrane transporter complex (SusC/SusD homologues). In the periplasm, the exo-fructanases liberate fructose from FOS. A small amount of fructose binds to the periplasmic two-component system sensor/regulator and upregulates the whole fructan PUL, while majority of liberated fructose is transported *via* the inner membrane transporter into the cytoplasm for fermentation (Bolam and Sonnenburg 2011; Sonnenburg et al. 2010). A recent study reveals that *B. thetaiotaomicron* 8736, a close relative of *B. thetaiotaomicron* VPI-5482 cannot utilize levan and uses inulin instead. The fructan PUL of *B. thetaiotaomicron* 8736 lacks the endo-acting fructanase on the cell surface, and inulin is probably transported unmodified into the periplasm. The SusC/SusD homologues forming outer-membrane transporter complex are responsible for specific recognition and import of inulin (Joglekar et al. 2018).

1.7.1. Endo-acting fructanases and their applications

Only scarce information is available on endo-fructanases. Table 3 summarizes the literature concerning microbial endo-inulinases, endo-levanases and their products. With regard to endo-inulinases, a recent work on a yeast *Lipomyces starkeyi* refers to the most active endo-inulinase described so far. This enzyme

has 1.5–4 times higher specific activity (2263 U/mg) than respective enzymes of filamentous fungi (Bao et al. 2019). The optimal temperature for the catalysis of *L. starkeyi* enzyme is 70 °C, that gives the enzyme a powerful advantage over other endo-inulinases used in biotechnology, since high temperature ensures solubility of inulin, prevents microbial contamination and thereby lowers the production costs (Gao et al. 2009; Rocha et al. 2006). The most valuable feature of endo-inulinase is the I-FOS production, for which the endo-inulinase from *L. starkeyi* is perfectly capable, producing FOS with DP 3–6 within 30 minutes (Bao et al. 2019). Other characterized endo-inulinases hydrolyse inulin with lower rate or require elevated concentration of the substrate (Table 3).
Even less information is available for L-FOS production using endo-levanases. Data in Table 3 indicate that the endo-levanase from *B. thetaiotaomicon* is a promising tool to produce L-FOS (Sonnenburg et al. 2010). This enzyme was further characterized in current PhD work.

Prebiotic properties of inulin-type FOS (I-FOS) with DP < 10 have been demonstrated (Gibson et al. 2004) and they are important components in industrially produced prebiotic foods (Kolida and Gibson 2007). I-FOS are commercially produced either from sucrose by using fungal fructosyl transferases, or from plant inulin using microbial endo-inulinases (Roberfroid 2007; Singh and Singh 2010). However, the production of I-FOS from plant inulin has seasonal limitations (Mussatto et al. 2012), and therefore new methods for continuous FOS production are highly valued.

Levan-type FOS (DP 2–3, levanbiose and -triose respectively) are commercially available from Megazyme (https://www.megazyme.com/), but they are extremely expensive – 1 g costs about 7 500 euros. Thus, novel low-price production methods are in high demand. So far, only little information on physiological effects of L-FOS is available and mostly they have been investigated as novel candidates for prebiotics (Adamberg et al. 2018; Marx et al. 2000; Porras-Domínguez et al. 2014). There are three possibilities to produce L-FOS: (i) bacterial levansucrases are producing high amount of levan and some L-FOS from sucrose, (ii) levan can be hydrolyzed to FOS by partial acid hydrolysis or (iii) using enzymatic hydrolysis with bacterial endo-levanase.

The endo-levanase LevB1 of *Bacillus licheniformis* was used to produce L-FOS from levan synthesized by *B. subtilis* levansucrase (Porras-Domínguez et al. 2014). The obtained FOS showed higher prebiotic potential towards probiotic bacteria *Bifidobacterium bifidum, B. longum* var. infantis NRRL 4661 and *B. breve* than levan (Porras-Domínguez et al. 2014). Probably, these probiotic strains had no enzymatic machinery for levan hydrolysis, but they can metabolize the L-FOS. Production of L-FOS has also been described for endo-levanase from *B. lehensis* G1 (Table 3). The resulting FOS enhanced the growth of lactic acid bacteria *Lb. casei* and *Lb. rhamnosus* (Fattah et al. 2018).

Strict linkage-dependence of endo-levanase has been used to determine the content of levan from fermented foods such as wheat sourdough or fava bean dough (Shi et al. 2019) and to determine the linkage type in branched fructans. For example, the endo-levanase LevB from *B. subtilis* was used to assay the

ratio of different linkage types in graminans – fructans with mixed β-2,1 and β-2,6 linkages (Jensen et al. 2016).

Table 3. Microorganisms with characterized endo-inulinases and -levanases. The spectrum of products formed at indicated conditions is presented.

Organism	Substrate	Product spectrum	Substrate [C]; time; temperature	Reference
Endo-inulinases				
Lipomyces starkeyi NRRL Y-11557	Chicory inulin	DP[a] 2–6	5 g/L; 0.5 h; 70 °C	(Bao et al. 2019)
Bacillus smithii T7	Inulin*	DP 1–8	20 g/L; 12 h; 60 °C	(Gao et al. 2009)
Arthrobacter sp. S37	Inulin*	DP 2	20 g/L; 6 h; 35 °C	(Li et al. 2012)
Xanthomonas oryzae No. 5	Chicory inulin	DP 5–7	50 g/L; 10 h; 50 °C	(Cho and Yun 2002)
Aspergillus ficuum JNSP5-06	Chicory inulin	DP 3–4	50 g/L; 24 h; 55 °C	(Chen et al. 2013)
A. niger CICIM F0620	Chicory inulin	DP 2–5	400 g/L; 8 h; 50 °C	(He et al. 2014)
A. fumigatus Cl1	Inulin*	DP 3–5	20 g/L; 24 h; 55 °C	(Chen et al. 2014)
Endo-levanases				
Bacteroides thetaiotaomicon	*Zymomonas mobilis* levan	DP 2–4	5 g/L; 1 h; 37 °C	(Sonnenburg et al. 2010)
Bacillus licheniformis Ibt1	*Bacillus subtilis* levan	DP 2–8	100 g/L; 1 h, 35 °C	(Porras-Domínguez et al. 2014)
B. subtilis 168	*Erwinia herbicola* levan	DP 2–6, levan	11 g/L; 24 h; 35 °C	(Jensen et al. 2016)
B. lehensis G1	levan*	DP 3–4	10 g/L; 2 h; 30 °C	(Fattah et al. 2018)
Paenibacillus amylolyticus	*E. herbicola* levan	DP1, low DP FOS[b], levan	2 g/L; 48 h; 30 °C	(Shi et al. 2019)
Treponema zioleckii kT	Timothy grass levan	DP 3–6 or DP 1–4	2 g/L; 24 h; 40 °C	(Kasperowicz et al. 2010)
Butyrivibrio fibrisolvens 3071	Timothy grass levan	DP 3–7, levan	2 g/L; 24 h; 40 °C	(Kasperowicz et al. 2016)

* The origin of the fructan was not specified; [a] – degree of polymerization, [b] – fructo-oligosaccharides

II AIMS OF THE STUDY

The main goal of this study was to characterize and evaluate new catalysts for the production of prebiotic fructans. For that, two biotechnologically promising enzymes: the levansucrase Lsc3 of *P. syringae* pv tomato and the endo-levanase BT1760 of *B. thetaiotaomicron*, were heterologously produced, purified and validated as catalysts for fructan synthesis.

Levansucrase Lsc3 was assayed for structure-function relationship using random and site-directed mutagenesis of the protein (Refs I–III). Catalytic properties of obtained levansucrase mutants were assayed using traditional and high-throughput methods (Ref III). Endo-levanase was thoroughly characterized using biochemical methods (Refs IV–V) and its 3D structure was solved (Ref V).

The more detailed aims of this work were following:

i. To create a panel of levansucrase mutants using random and site-directed mutagenesis. To characterize polymerizing ability, thermostability and pattern of reaction products of created mutants.
ii. To elaborate new cost-efficient and high-throughput methods for characterizing levansucrase activities.
iii. To clone and express endo-levanase of *B. thetaiotaomicron* and characterize its ability to cleave various bacterial and plant levans.
iv. To obtain crystals of the wild-type and catalytically inactive ligand-bound mutant of endo-levanases, to solve and analyze the 3D structures of respective proteins.

III RESULTS AND DISCUSSION

3.1. Characterization of the levansucrase Lsc3 using random- and site-specific mutagenesis (Ref I, II and III)

3.1.1. Lsc3 is a powerful catalyst

The levansucrase Lsc3 of *P. syringae* pv tomato DC3000 studied and characterized in this work was expressed in *Escherichia coli* and purified to homogeneity (Refs I–III). The Lsc3 is one of most active levansucrases described so far. It uses sucrose, raffinose and stachyose as a substrate (Visnapuu et al. 2008). The K_M of the levansucrase for sucrose cleavage is 18.5 mM and for raffinose, the affinity is about twice lower: the K_i for raffinose cleavage is 39.9 mM (Table 1 in Ref I). Catalytic efficiency of sucrose cleavage by Lsc3 is 27.3 mM^{-1} s^{-1}, that is higher compared to levansucrases from *B. subtilis* (20.3 mM^{-1} s^{-1}), *G. diazotrophicus* (5.5 mM^{-1} s^{-1}), *Z. mobilis* (0.2 mM^{-1} s^{-1}), but still lower than that of the *B. megaterium* levansucrase (254 mM^{-1} s^{-1}) (Refs I–III; (Martínez-Fleites et al. 2005; Ortiz-Soto et al. 2008; Strube et al. 2011; Yanase et al. 2002)).

3.1.2. The Asp62, Asp219 and Glu303 comprise the catalytic triad of Lsc3

Three key catalytic amino acids: nucleophile, transition-state stabilizer and acid/base catalyst of Lsc3 were experimentally proven to be Asp62, Asp219 and Glu303 (Ref II). Mutation of these amino acids to alanine caused drastic reduction of k_{cat} values from 5 000 to 126 000 times compared to the wild-type enzyme, while affinity of the enzyme for sucrose remained almost unaltered (Table 2 in Ref II). Similar inactivation due to alanine substitution of catalytic amino acids was observed for levansucrases of *B. subtilis, B. megaterium, Lb. reuteri* and *G. diazotrophicus* (Batista et al. 1999; Meng and Fütterer 2003; Ortiz-Soto et al. 2008; Ozimek et al. 2004). Additional Glu303Gln mutant of Lsc3 was unable to hydrolyse nor polymerize and showed seven-fold decreased affinity for sucrose (Table 1 in Ref III). In the case of *Z. mobilis* levansucrase, the acid/base catalyst substitution with aspartate caused only 30-fold decrease of the k_{cat}, while the K_M for sucrose remained the same (Yanase et al. 2002).

3.1.3. Mutations obtained through random mutagenesis reveal a great importance of His113 and Asp300 in the catalysis

Random mutagenesis of proteins is a powerful tool to reveal amino acids with a specific function. Refs I and III describe random mutants of Lsc3 obtained using chemical (ethylmethane sulfonate) mutagenesis. Three mutated genes of levansucrase, from colonies of nonmucoid phenotype on sucrose plate, revealed ten point-mutations. Respective mutations were investigated separately *in silico*

and subsequently Asp31Asn, His113Gln, Asp300Asn and Thr302Pro single point-mutations were introduced in *lsc3* (Ref I and III). The Asp31Asn mutant was thermally unstable and tended to precipitate. A homology modelling of Lsc3 shows Asp31 location in the first secondary structure element of the N-terminal part of the protein – an α-helix, lining the outer surface of Lsc3. According to our data, the Asp31Asn mutation disturbed polymerization reaction of Lsc3 more strongly than sucrose cleavage (Figure 5 in Ref III), hinting that this position may have a role in binding of the growing FOS chain. Substitution of His113 with glutamine had a strong negative effect on catalytic activity (Table 4; Figure 5 in Ref III). The sucrose cleavage and polymerization reactions were severely hampered by His113Gln mutation (Figs 5 and 6 of Ref III). To better understand the role of His113, the additional mutant – His113Ala – was constructed. Both mutants, His113Gln and His113Ala, had nearly ten times decreased affinity for sucrose, low transfructosylating ability and a very low capability to produce FOS (Table 4 and Ref III). The crystal structure of the fructose-bound fructosyltransferase MsFFase (PDB 3VSS) of a *Microbacterium saccharophilum* K-1 revealed positioning of His147 (homologous to His113 in Lsc3) in MsFFase (Figure 3). His147 of MsFFase is at H-bondage distance (2.9 Å) from C6 of fructose bound at –1 subsite (Figure 3). Predicted position of His113 in Lsc3 suggests its contribution to binding of the substrate at both donor and acceptor subsites. Thus, mutation of this residue affects both sucrose cleavage as well as polymerization. Notably, a helix harbouring His147 of MsFFase is missing in several levansucrases, for example in SacB of *B. subtilis*, but is present in levansucrases of halophilic archaea (Kırtel et al. 2019; Ortiz-Soto et al. 2019).

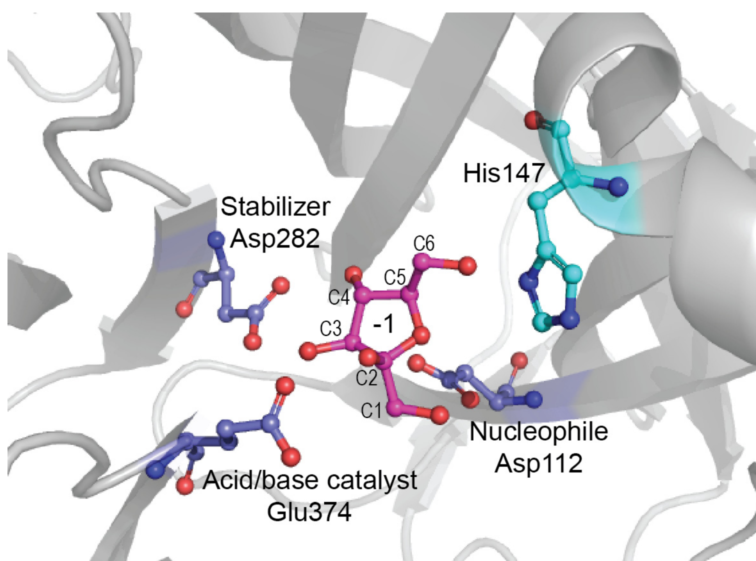

Figure 3. Catalytic center of levansucrase MsFFase of *Microbacterium saccharophilum* K-1 (PDB 3VSS). Catalytic triad (Asp112, Asp282 and Glu374) is indicated in dark blue. Fructose molecule (magenta) is residing at subsite –1. Position His147, equivalent to His113 in Lsc3, is indicated in light blue. The structure was visualized using PyMOL 1.8.6.0 (Schrödinger, LLC 2015).

Table 4. Affinity for sucrose and ability to polymerize Lsc3 and its mutants. Table is slightly modified from Ref II.

Levansucrase	**K_M increased (times)**	**TA[a] (%)**	**DP[b]**	***… continues*** **Levansucrase**	**K_M increased (times)**	**TA[a] (%)**	**DP[b]**
Lsc3	18.5 mM	74	3–7	Glu236Gln[III]	14.4	50	3–6
Asp31Asn[III]	0.8	70	3–6	Val248Ala[III]	0.8	72	3–6
Trp61Ala[II]	7.8	69	3–6	Asp300Ala[III]	1.0	58	3–8
Trp61Asn[II]	47.0	51	3–4	Asp300Asn[I]	2.7	60	3–10
Asp62Ala[II]	**1.1**	**ND**	**ND**	Gln301Ala[II]	17.0	24	3–4
Thr63Ala[II]	0.9	71	3–7	Gln301Glu[II]	1.3	45	3–5
Leu66Ala[II]	1.5	73	3–7	Thr302Met[II]	0.8	70	3–6
Trp109Ala[III]	1.6	77	3–7	Thr302Pro[I]	2.3	52	3–6
Trp109Phe[III]	0.5	74	3–7	**Glu303Ala[II]**	**1.5**	**ND**	**ND**
Trp109Arg[III]	13.5	40	3	**Glu303Gln[III]**	**7.0**	**ND**	**ND**
Glu110Asp[III]	3.1	70	3–7	Arg304Ala[II]	3.6	70	3–6
His113Ala[III]	9.2	41	3–6	Arg304Cys[II]	0.7	69	3–4
His113Gln[III]	10.3	51	3–6	His306Ala[II]	1.1	72	3–7
Glu146Gln[III]	2.2	76	3–6	His321Leu[I]	19.0	20	3
Asp219Ala[II]	**2.3**	**ND**	**ND**	His321Lys[I]	28.6	27	3–4
Pro220Ala[II]	1.3	75	3–6	His321Arg[I]	24.4	25	3–4
Asp225Ala[II]	0.7	71	3–7	His321Ser[I]	27.2	23	3–4
Asp225Asn[II]	1.0	71	3–7	Asp333Ala[III]	1.5	68	3–5
			…	Asp333Asn[III]	2.2	80	3–7

[a] – transfructosylating activity; [b] – degree of polymerization; ND – activity not detected; [I, II, III] – first discussed in Ref I, II and III, respectively. The mutants of catalytic triad are shown in bold font.

The Lsc3 mutants Asp300Asn and Thr302Pro (Refs I–III) had k_{cat}/K_M values decreased by about 4 times (Table 1 in Ref I). They differed from the wild-type enzyme mostly by their polymerizing properties producing less FOS. In addition, the size-pattern of produced FOS differed from that of the wild type. The Asp300Asn variant produced FOS with DP 3–10 while the wild-type enzyme produced FOS with DP 3–7 (Table 3 in Ref II). Intriguingly, the Asp300Asn mutant synthesized more levan (8.6 mg/mL) than the wild-type enzyme (7.2 mg/mL) (Ref I). Further investigation of levan produced by Asp300Asn showed that the mutant Asp300Asn produced only LMW levan – two size-fractions of that (16.6 kDa and 7.4 kDa) were characterized by us (Table 1 in Ref IV). Interestingly, the activity of Asp300Ala mutant was almost identical to the wild type (Table 4; Figure 5 in Ref III). The position equivalent to Asp300 of Lsc3 has been earlier mutated in levansucrase of *Z. mobilis* levansucrase: respective mutant Asp275Asn behaved like the wild-type enzyme

(Yanase et al. 2002). Levansucrases of Gram-positive bacteria stabilize their levansucrases with Ca^{2+} ion. Importantly, in levansucrase of *Lb. reuteri* 121, Asp500 that is equivalent to Asp300 in Lsc3, was assumed to participate is Ca-binding. When Asp500 was replaced by either Asn or Ala, both the catalytic activity and ability to bind calcium were reduced (Ozimek et al. 2005). The Thr302Pro mutation, also originating from random mutagenesis, caused a slight negative effect on levansucrase activity. When Thr302 was replaced with methionine to make the enzyme similar to invertases, the mutant had the activity of wild-type enzyme (Ref II–III). In the case of *B. subtilis* levansucrase, the Ile341Val mutant (Ile341 resides at position homologous to Thr302 of Lsc3), behaved like the wild-type enzyme (Ortiz-Soto et al. 2008). To sum up, random mutagenesis disclosed two positions (His113 and Asp300) in Lsc3 crucial for the catalysis (Ref I–III).

3.1.4. Mutations around the nucleophile Asp62

The catalytic triad of Lsc3 is surrounded by amino acids, which are strongly conserved among levansucrases (Figure 2 in Ref III). For example, an invariant Trp61 is located next to the nucleophile Asp62 (Ref II). Substitution of Trp61 with Asn or Ala caused drastic reduction in sucrose-splitting and polymerizing activities (Table 4). Out of those two mutants, Trp61Ala mutant retained partial activity and produced slightly more levan and FOS than the Trp61Asn mutant (Ref II). Respective position has been changed to His, Ala and Asn in other levansucrases, whereas the His and Asn variants had decreased catalytic efficiency by 700 and 94 times, respectively (Li et al. 2011; Ozimek et al. 2006). The aromatic Trp61 of Lsc3 probably forms hydrogen bonds with the fructosyl moiety bound at –1 subsite, and as substitutions in this position do not support H-bonding, binding of the substrate is hampered.

Positions Thr63 and Leu66 of Lsc3 (Ref II) have not been targeted in levansucrases before. Changing Thr63 to alanine reduced catalytic efficiency of Lsc3 ten-fold, leaving the affinity for sucrose and the FOS production unaltered (Ref II). Though leucine in position 66 is invariant among levansucrases, its mutation into alanine had only subtle negative effect on the catalysis and product formation. According to structure modelling, Thr63 and Leu66 are both buried residues and thereby may affect the enzyme's activity indirectly (Ref II).

The position of Trp109 was substituted with alanine, phenylalanine and arginine. Changes of Trp109 to alanine or arginine hindered the catalysis (Table 4 and Ref III). Intriguingly, the Trp109Phe mutant seemed to be better catalyst than the wild-type Lsc3 – increased affinity with boosted synthesis of levan. In *Z. mobilis* levansucrase, homologous Trp80Arg mutation caused disturbed HMW levan synthesis (Yanase et al. 2002). Possibly, Trp109 of Lsc3 is involved in substrate (acceptor) binding at +2 subsite and arginine or alanine at this position does not fulfil this function.

Mutant levansucrases, Glu110Asp and Glu146Gln, had 2–3 times decreased affinity for sucrose compared to the wild-type Lsc3, while the V_{max} and trans-fructosylating activities remained unchanged (Table 4 and Ref III). Glu110 is located next to a catalytically important Trp109 and is possibly required for substrate binding at +2 subsite and for levan synthesis. The Glu146Gln mutation in Lsc3 caused decrease in affinity and increase in FOS synthesis, possibly at the expense of levan. The equivalent position of Glu146 was mutated in *Z. mobilis* levansucrase previously: the Glu117Gln mutant had reduced affinity for sucrose and increased polymerizing activity (Yanase et al. 2002).

3.1.5. Mutations around the stabilizer Asp219

Pro220 is located next to the transition-state stabilizer Asp219 in Lsc3. The Pro220Ala mutant was created to mimic respective region of inulosucrases. This mutation did not cause any reduction in levansucrase activity compared to the wild-type enzyme (Table 4 and Ref II). Asp225 was mutated to alanine and asparagine to reveal catalytic significance of this acidic residue (Table 4 and Ref II). As respective mutants behaved like the wild type, Asp225 has no role in the catalysis. Intriguingly, Asp306 in *Arthrobacter globiformis* β-fructofuranosidase that is equivalent of Asp225 in Lsc3, has been erroneously predicted as a transition-state stabilizer (discussed in Ref II).

The Glu236Gln mutant showed decrease in sucrose hydrolysis and FOS production, and complete disability to produce levan (Table 4 and Ref III). Homologous position has been mutated in *Z. mobilis* levansucrase (Glu211Gln) showing similar consequences (Yanase et al. 2002). According to the crystal structure of *B. subtilis* levansucrase (PDB 3BYN), the Glu262 corresponding to Glu236 of Lsc3 is located spatially close to the stabilizer and acid/base catalyst. Respective glutamate forms hydrogen bonds to the substrate bound at –1 and +1 subsites over water molecule, thereby contributing to anchoring of the substrate (Meng and Fütterer 2008). Similarly, to Glu236Gln mutation, the Val248Ala mutation decreased the enzyme's ability to produce FOS, but the affinity for sucrose and levan-producing activity remained unchanged (Table 4 and Ref III). Interestingly the Thermofluor assay showed a decreased melting temperature for this mutant, which could point to importance of Val248 in protein folding.

3.1.6. Mutations around the acid/base catalyst Glu303

Acid-base catalyst Glu303 is surrounded with conserved amino acids such as Asp300, Gln301, Thr302 Arg304 and His306. The effect of Asp300Asn/Ala and Thr302Pro/Met mutations was discussed already in "Mutations obtained through random mutagenesis reveal a great importance of His113 and Asp300 in the catalysis" section. The Gln301Ala mutant of Lsc3 was capable of sucrose

splitting but had a very low affinity for sucrose and was defective in trans-fructosylation. While the Gln301Glu mutant was competent in sucrose splitting, it was poor in polymerization and acted mainly hydrolytically (Table 4 and Ref II). According to the structure of *B. subtilis* levansucrase, Glu340 (corresponds to Gln301 of Lsc3) locates in +1 subsite and is involved in binding of fructosyl acceptor (Meng and Fütterer 2008). Thus, Gln301 of Lsc3 most probably plays role in polymerisation. Next, no previous functional data is available for positions Arg304 and His306 of Lsc3. The mutations Arg304Ala and Arg304Cys of Lsc3 had a significant decrease of both sucrose-splitting and FOS-producing abilities (Table 4 and Ref II). The Arg304Cys mutation was constructed based on alignment of GH32 and GH86 enzymes. Most GH32 enzymes have a cysteine in respective position, so the side-chain of arginine at this position was suggested to be crucial for levansucrase activity. Changing His306 into alanine did not cause any significant changes in enzyme activity – the mutant behaved almost similarly to the wild-type Lsc3 (Ref II).

His321 in Lsc3 was mutated to lysine, leucine, arginine and serine following the previous reports (Chambert and Petit-Glatron 1991; Yanase et al. 2002). Lsc3 did not tolerate any of the above-mentioned substitutions – the affinity of His321 replacement mutants towards sucrose increased over 25 times, and catalytic efficiency decreased by over 30 times. In addition, the mutants could produce only short FOS – with DP up to four (Table 1 and Figure 2 in Ref I). This position close to +1 subsite of levansucrases was shown as determinant of transfructosylation, stabilizing glucose moiety of the donor sucrose (Meng and Fütterer 2008). His321 equivalents in levansucrase from *Z. mobilis*, *B. subtilis* and *B. megaterium* are His296, Arg360 and Arg370 respectively (Homann et al. 2007; Ortiz-Soto et al. 2008; Yanase et al. 2002). Yanase and co-workers reported similar reduction in activity and polymerization in case of *Z. mobilis* enzyme (Yanase et al. 2002), while the Arg360Ser and Arg360Lys of the *B. subtilis* levansucrase became more hydrolytic, producing only FOS and almost no levan (Ortiz-Soto et al. 2008). As suggested by (Ortiz-Soto et al. 2008), these mutants could not hold the acceptor molecule in +1 subsite strongly enough to enable efficient polymerization.

The Asp333Ala/Asn mutants were constructed to mimic Asp308Asn mutation of *Z. mobilis* levansucrase. The asparagine substitution altered the catalytic activity only slightly – it lowered the affinity of the enzyme for sucrose. Yet, the alanine substitution decreased the mutant's ability to synthesise FOS and levan (Table 4 and Ref III), therefore Asp333 of Lsc3 can be involved in substrate binding and elongation of the fructan chain.

All investigated positions and mutations mostly decreased either the enzyme's sucrose-splitting or polymerization activity, or both. Besides shaping the central cavity, amino acids surrounding the catalytic triad also assist in catalysis. Regarding FOS and levan production, the mutants Glu146Gln, Thr302Met and Asp333Asn had slightly enhanced FOS production, while Trp109Phe mutant showed enhanced levan production (Ref III).

3.2. The yield and spectrum of polymerization products of Lsc3 (Refs I, II and IV)

Levansucrases have gained attention from biotechnologists and food scientists because of the synthesis of potentially prebiotic sugars from cheap commercial substrates such as sucrose. We have thoroughly studied one of the levansucrases – Lsc3 of *P. syringae* pv tomato ((Visnapuu et al. 2009, 2015); Refs I–III of this PhD work). The most suitable substrate for the Lsc3 protein is sucrose, followed by raffinose and stachyose. Even levan can be hydrolysed by levansucrases when other substrates are not available (Méndez-Lorenzo et al. 2015). However, hydrolysis of levan by Lsc3 is low – it constitutes less than 1% of sucrose-splitting activity, and the hydrolysis produces only fructose that is not considered prebiotic (Ref I).

Lsc3 produces two types of fructans: highly polymerized levan and FOS. In Ref IV we showed that levan synthesised by Lsc3 from 1.2 M sucrose had a bimodal distribution of molecular weight: 4733 ± 125 kDa (HMW) and 10.7 ± 1.0 kDa (LMW). A similar feature has also been reported for levan produced by the levansucrase of *B. subtilis* (Ortiz-Soto et al. 2008; Porras-Domínguez et al. 2015; Raga-Carbajal et al. 2016). Using Lsc3 as a catalyst, about 13 g of levan can be produced per mg of protein (Adamberg et al. 2014). The yield of FOS (DP 3–7) per 1 mg of Lsc3 is approximately 15 g (Ref II). Aside of levan, FOS and fructose (the latter is a hydrolysis product of sucrose), residual sucrose and lots of glucose is present in the reaction mixture of Lsc3 reacted with 1.2 M sucrose at 23 °C for 20 hours (Adamberg et al. 2014). A quantitative analysis of respective FOS fraction revealed 1-kestose (37% out of total FOS, DP3), 6-kestose (9.2%; DP3), nystose (28.4%; DP4), fructosyl nystose (19.7%; DP5), DP6 FOS (7%) and DP7 FOS (3.6%) (Adamberg et al. 2014). This result clearly shows that Lsc3 produces from sucrose both I-FOS and L-FOS. We have used invertase-negative mutant of *Saccharomyces cerevisiae* to remove excess glucose and fructose from the FOS mixture, but it is time- and money-consuming (Adamberg et al. 2014; Jõgi et al. 2015). Recently, a naturally invertase-negative yeast *Hansenula polymorpha* was used to remove fructose and glucose from the FOS mixture produced by a mutant levansucrase of *B. megaterium* (Possiel et al. 2019). Absence of invertase in the microorganism used for removing glucose and fructose is crucial, because otherwise the FOS will be hydrolysed (Adamberg et al. 2014; Nobre et al. 2018; Yoon, Mukerjea, and Robyt 2003). Recently, a new promising FOS purification method with phenylboronic acid was introduced removing glucose, fructose and residual sucrose that resulted with 97% of purity (Porras-Domínguez et al. 2019). As concluded above, Lsc3 is probably not the best for L-FOS production, but the Lsc3-produced levan opens new biotechnological opportunities – it can be enzymatically hydrolysed to L-FOS using a proficient endo-levanase (Ref IV).

Hetero-oligofructans, for example lactosucrose, have exhibited high prebiotic efficiency (Ibrahim 2018; Mu et al. 2013). Our work has shown that Lsc3 can synthesize HOFs with DP up to 5 by transfructosylation of non-con-

ventional acceptors such as D-xylose, D-fucose, L- and D-arabinose, D-ribose, D-sorbitol, xylitol, xylobiose, D-mannitol, D-galacturonic acid, methyl-α-D-glucopyranoside (Ref I form this PhD work and (Visnapuu et al. 2009)).

3.3. Study of levansucrase mutants using high-throughput methods (Ref III)

If a protein is heterologously synthesized in an expression host (most often *E. coli*) that does not have respective background activity, the activity of expressed enzyme (*e.g.* maltase or levansucrase) can be studied in crude cell extract of the host (Liiv, Pärn, and Alamäe 2001; Visnapuu et al. 2008). Permeabilization of bacteria and yeasts can also be used to measure activities of intracellular enzymes (Alamäe et al. 2012; Alamäe and Järviste 1995; Jamur and Oliver 2010; Maleknia, Ahmadi, and Norouzian 2011). Though *P. syringae* levansucrases are extracellular enzymes, they are not excreted from the cell if heterologously synthesized in *E. coli* – 88% of produced protein residing in the cytoplasm (Visnapuu et al. 2008). In Ref III, permeabilization of levansucrase-expressing *E. coli* cells was used in a microplate-based high-throughput approach to evaluate enzymatic properties of Lsc3 mutants. Figure 4 illustrates evaluation of sucrose-splitting activity of Lsc3 mutants in permeabilized cells.

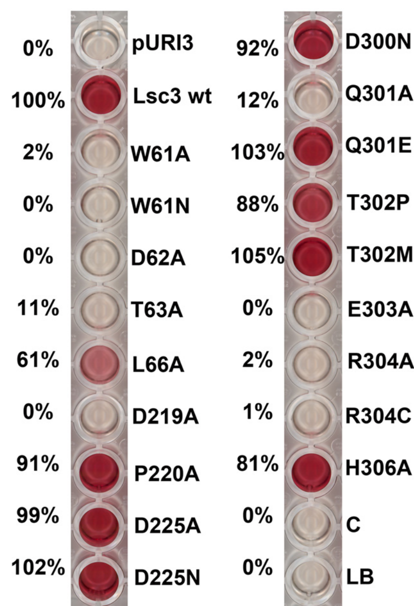

Figure 4. Sucrose-splitting activity of Lsc3 and its mutants in CTAB-permeabilized levansucrase-expressing *Escherichia coli* cells. The activity of wild-type Lsc3 is taken for 100% and permeabilized cells carrying the empty vector (pURI3), McIllvaine's buffer (C) along with uninoculated LB media (LB) are used as controls. The image is taken from Ref II.

The data from experiments with permeabilized *E. coli* cells were compared with respective results of purified proteins. However, pure recombinant proteins, especially mutated ones, often aggregate and precipitate from the solution. Thus, other methods should also be considered for the study of enzymes to over-come for example problems of solubility.

In Ref III, a panel of 36 mutants of Lsc3 along with the wild-type enzyme were characterised for (i) sucrose-splitting activity, (ii) the amount and spectrum of produced FOS, (iii) the ability and kinetics of levan synthesis, and (iv) thermal stability of the proteins. In most cases, a microplate-format was applied.

Sucrose-splitting activity of Lsc3 mutants can be evaluated using permeabilized recombinant *E. coli* cells as catalysts. Figure 5 shows good correlation between the sucrose-splitting activities measured using two methods, one of which uses purified enzymes and the other permeabilized *E. coli* cells. In this work, 0.1% cetyltrimethylammonium bromide (CTAB), that had only a minor inhibitory effect on levansucrase activity, was used for cell permeabilization (Ref III).

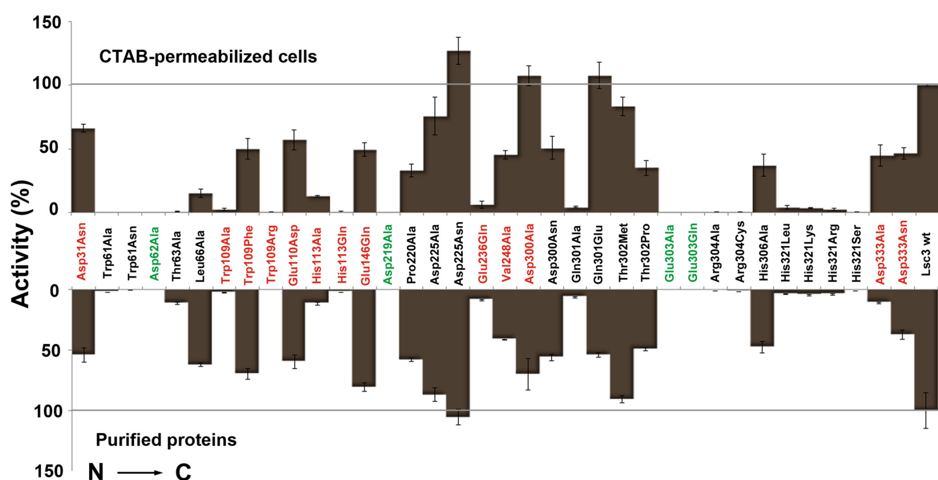

Figure 5. Sucrose-splitting activity of levansucrase Lsc3 and its mutants. The activities of thirty-seven proteins were assayed by two methods: using CTAB-permeabilized *E. coli* cells expressing levansucrase assayed on a microplate (upper panel) and using purified proteins (lower panel). Mutants of catalytic triad are shown in green; mutants characterized for the first time in Ref III are designated in red. The 100% value is the respective value of the wild-type Lsc3. The image is from Ref III.

FOS synthesis evaluated using permeabilized *E. coli* cells expressing levan-sucrase yielded similar results as the study of purified enzymes. For example, both assays indicated that the Asp300Asn mutant of Lsc3 produced FOS with extended chain length (DP up to 9–10), while the wild-type Lsc3 produced the FOS with DP up to 7 (Table 1 in Ref III). In addition, the transfructosylating activity of mutants correlated well between the two assays, indicating that the

high-throughput cost-efficient methods are feasible for the analysis of levansucrases.

Ref III additionally shows that a high-throughput differential scanning fluorimetry (also known as Thermofluor), is a suitable method to study thermal stability of levansucrase mutants. The results obtained from a Thermofluor experiment (total volume of the analysed sample only 20 µL) nicely correlated with the results of traditional inactivation assay, where the protein is kept at certain temperature and then its residual catalytic activity is measured. The melting temperature of the wild-type Lsc3 according to Thermofluor assay was 65.4 °C, which was higher than that of *E. amylovora* levansucrase (57 °C) (Ref III) (Caputi, Cianci, and Benini 2013). Some Lsc3 mutants with reduced catalytic activity such as Asp31Asn, Val248Ala and Thr302Pro had decreased T_m ranging from 44–55 °C, showing that loss of catalytic performance was probably due to improper folding causing instability of the protein.

3.4. Cleavage of bacterial and plant levans into fructo-oligosaccharides by the endo-levanase BT1760 of *B. thetaiotaomicron* (Ref IV)

To produce FOS from levan, two biotechnologically proficient enzymes are required: a levansucrase to produce levan, and an endo-levanase to 'chop' levan into FOS. We have shown that the levansucrase Lsc3 has certainly properties of a feasible biotechnological catalyst: it is highly active in sucrose splitting and levan synthesis (Refs I–III) and very stable, maintaining its full activity even after keeping the protein for 200 days at a relatively high temperature (37 °C) (Ref IV). Its high stability can be explained by extracellular nature of the enzyme in the natural host – extracellular enzymes have to tolerate harsh environmental conditions (Hettwer et al. 1995; Visnapuu et al. 2008).

The endo-levanase BT1760 was first isolated and briefly analysed by (Sonnenburg et al. 2010), Ref IV offers more detailed characterization of the enzyme. As stated in Ref IV, the BT1760 should be considered the most active endo-levanase described so far: it hydrolyses levan 300 times more rapidly than the endo-levanase LevB1 from *B. licheniformis.*

The performance of BT1760 was assayed using six different levans, including one levan of plant origin – extracted from timothy grass. All levans served as substrates for BT1760 (see further). At the same time, dahlia inulin, xylo-oligosaccharides, raffinose and stachyose were not hydrolysed by the enzyme. Activity of BT1760 was only modest on I-FOS preparations P95 and Synergy1 (Ref IV). Considering the six different levans, the enzyme had the highest affinity for levans produced by *Z. mobilis* and by mutant levansucrase Lsc3Asp300Asn, followed by levans of timothy grass and levan produced by *H. smyrnensis.* Levans produced by *P. syringae* levansucrase Lsc3 from sucrose or raffinose were less suited substrates, according to the affinity parameter (Ref IV). It seemed, that the endo-levanase prefers LMW levans as

Lsc3Asp300Asn-produced levan (16.6 kDa) and timothy grass levan (about 60 kDa) were the most suited levans for the enzyme (Kasperowicz et al. 2016; Nelson and Spollen 1987). The four other bacterial levans used in Ref IV as endo-levanase substrates had a high molecular weight, reaching megadaltons, and the catalytic efficiency of BT1760 towards these levans was lower compared to LMW levans (Table 2 in Ref IV). Branching of levan most probably also affects its endo-degradation. According to the literature, the *Z. mobilis* levan is branched, whereas the *H. smyrnensis* levan is reported as unbranched, which is quite unusual among bacterial levans (Benigar et al. 2014; Kazak Sarilmiser et al. 2015). We assume that levan synthesized by Lsc3 is branched.

Kinetics of FOS release from levans (5 g/L) was monitored during a 72-hour period. The endo-levanase BT1760 produced FOS most rapidly from LMW levans: the Lsc3Asp300Asn levan and timothy grass levan (Figure 6, panels C and F; Ref IV). It took only 15 minutes to degrade timothy grass levan into L-FOS (4.16 g/L, DP 2–9) (Figure 6, panel F; Table 3 in Ref IV). In case of Lsc3Asp300Asn levan, the highest amount of FOS (4.56 g/L, DP 2–7) was produced by 60 min of BT1760 reaction. In both cases, the major product during the rapid phase of the hydrolysis was levantriose (DP3). During prolonged incubation, the DP3 product was further hydrolyzed into DP2 oligomer (levanbiose) and fructose (Figure 6 and Ref IV). Depolymerization of *Z. mobilis* levan yielded the highest FOS amount (3.28 g/L, DP 2–9) by 180 minutes of the reaction. Levans from *H. smyrnensis* and *P. syringae* Lsc3 were much less convenient substrates for FOS production by BT1760, and the most resistant to endo-hydrolysis was levan produced by Lsc3 from raffinose, yielding 3.15 g/L of FOS (DP 2–8) by 72 h of reaction (Figure 7 in Ref IV).

During the endo-levanase reaction, levan is first hydrolysed into long FOS, which are further cleaved into smaller oligomers until levanbiose and fructose remain as final products. Accumulation of fructose and levanbiose indicates that BT1760 is an endo-acting enzyme and that levanbiose is not its substrate. Similarly, L-FOS with DP 2–8 were produced by endo-levanase LevB1 of *B. licheniformis* whereas extended incubation resulted in levanbiose as the main product (Porras-Domínguez et al. 2014). 91.2% of levan turned into FOS, when LMW Lsc3Asp300Asn-produced levan was used as a substrate. This high yield is close to 97% reported for the endo-levanase LevB1 of *B. licheniformis* (Porras-Domínguez et al. 2014). Our data (Figure 6 and Ref IV) showed that even though timothy grass levan is cleaved by BT1760 the most rapidly, this levan is not the best substrate for FOS production, because of the enhanced fructose production (Figure 6, panel F; Ref IV). It can be due to absence of branching of this LMW levan that makes it easily accessible for the endo-hydrolysis.

We consider that endo-levanases are appropriate for the production of L-FOS from polymeric levan. Both, LevB1 from *B. licheniformis* and BT1760 from *B. thetaiotaomicron* are perfect proofs of that (Ref IV, (Porras-Domínguez et al. 2014)).

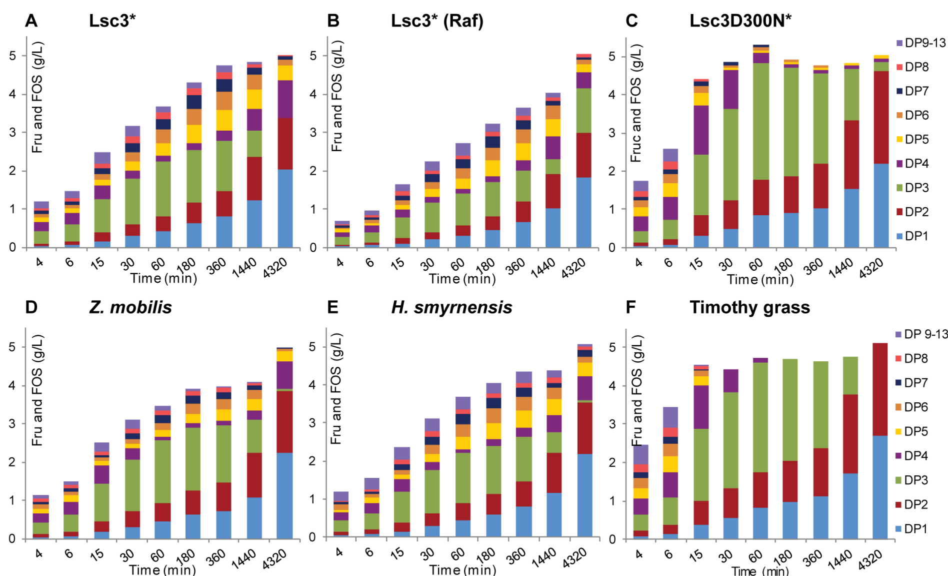

Figure 6. Time course of hydrolysis of six different levans by the BT1760. *Levans synthesized by Lsc3 from sucrose; raffinose or its mutant D300N. The figure is taken from Ref IV.

3.5. The structure of the endo-levanase BT1760 (Ref V)

The glycoside hydrolases are grouped into families and clans based on their amino acid sequence and fold. So far, eight protein structures have been determined for GH68 family members: seven levansucrases and one inulosucrase (Table 1). They all comprise a single domain with acidic active site located in the middle of the β-propeller (Figure 1). The catalytic differences between the enzymes are created by loops lining the active site influencing kinetics, product spectrum and stability of the enzymes.

The GH32 family consists of invertases, endo/exo-inulinases and endo/exo-levanases (Lombard et al. 2014). By 2019, thirteen protein structures were solved for this family: mostly invertases, but also exo- and endo-inulinases from a filamentous fungus *Aspergillus* (Lombard et al. 2014). All these proteins share a bimodular fold: a catalytic N-terminal five-bladed β-propeller connected to a C-terminal β-sandwich domain. The N-terminal domain structure is superimposable with that of GH68 proteins.

In 2019, we solved the structure of the endo-levanase of *B. thetaiotaomicron* revealing a bimodular domain arrangement as shown for other GH32 proteins (Ref V). The structure of wild-type enzyme (PDB: 6R3R) with a MES [2-(N-morpholino)ethanesulfonic acid] molecule bound to the active centre was solved at 1.65 Å resolution. The N-terminal domain was folded into 5-bladed β-propeller, every blade of which had a classical 'W' topology comprising four antiparallel β-strands (Figure 7). The negatively charged active centre was

located at the central pond-like cavity of the propeller. The C-terminal domain folded into β-sandwich, with two β-sheets formed from seven and eight antiparallel β-strands, respectively. No ligand-binding pockets were detected in the β-sandwich domain (Ref V). The BT1760 structure was solved using molecular replacement method with endo-inulinase structure (PDB 3RWK) applied as a template. The alignment of protein sequences showed that like other GH32 and GH68 family proteins, the BT1760 had three acidic key catalytic amino acids: a nucleophile Asp41, a stabilizer Asp169 and an acid/base catalyst Glu221. We experimentally proved the function of Glu221 in BT1760: the Glu221Ala mutant was catalytically inactive. In the case of acid/base catalyst mutant, substrate is expected to remain tightly bound to the enzyme. Proceeding with this knowledge, the Glu221Ala mutant was considered a suitable variant of the endo-levanase to be crystallized in ligand-bound state. The structure of endo-levanase mutant Glu221Ala with levantetraose in its active site was solved at resolution of 1.90 Å (PDB: 6R3U).

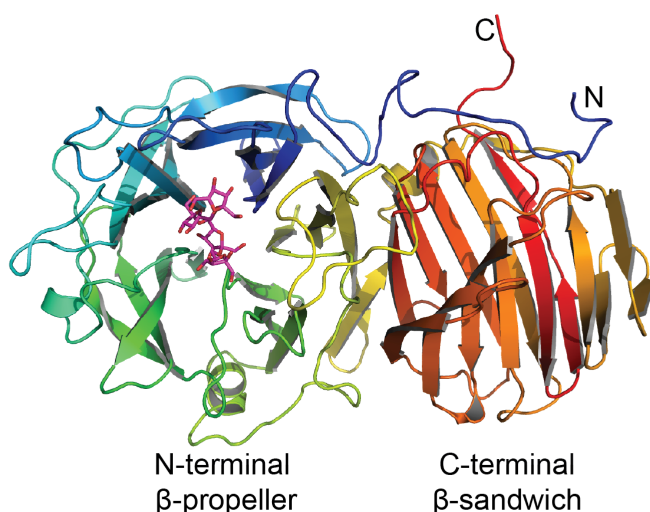

Figure 7. The overall structure of endo-levanase BT1760. A schematic view of the protein in complex with levantetraose (magenta), color is ramped from the N-terminus (blue) to C-terminus (red). The figure is taken from Ref V.

3.5.1. The N-terminal catalytic domain

In Ref V we compared the binding of substrates to the catalytic cavity of endo-inulinase, exo-inulinase and endo-levanase. The exo-inulinase INUE (of *A. awamori*) has a deep funnel-shaped active site cavity, accommodating only one fructose residue at its bottom (Nagem et al. 2004). Comparison of INUE, INU2 (endo-inulinase of *A. ficuum*) and BT1760 structures revealed different shapes for their substrate-binding cavities as well as different modes of ligand binding (Figure 8, panel B). In levantetraose-bound BT1760 structure fructose

in –1 subsite lied at the bottom of the pocket, whereas fructose residues bound at –2 and +1 subsites reached upwards along the pocket edges. The substrate-binding cavity of the endo-inulinase of *A. ficuum* binds at least three fructose residues of the ligand, kestopentaose (FFFFG), at –3, –2 and –1 subsites located at the flat bottom while fructose and glucose residues binding at +1 and +2 line the edge of the pocket (Pouyez et al. 2012).

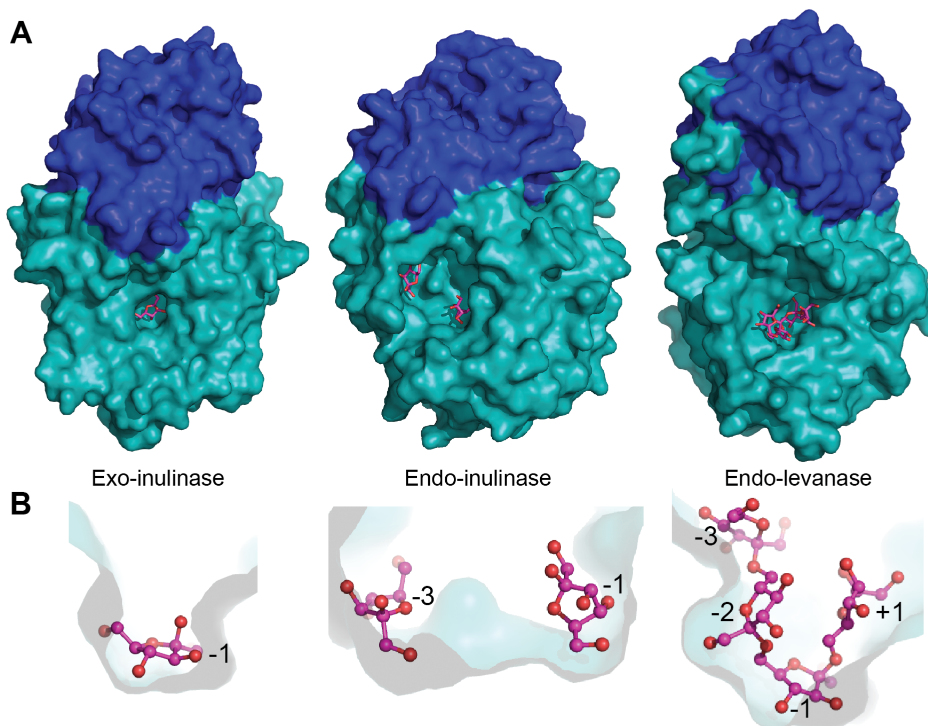

Figure 8. The structures of exo-inulinase, endo-inulinase and endo-levanase. In panel (A) the catalytic N-terminal domains are colored turquoise, while the C-terminal β-sandwich domains have dark blue color. Ligands are in magenta color. Panel (B) shows binding mode of ligands in the active site pocket: a fructose for *Aspergillus awamori* exo-inulinase (PDB 1Y9G), two fructoses for *A. ficuum* endo-inulinase (PDB 3RWK) and levantetraose for endo-levanase of *Bacteroides thetaiotaomicron* (PDB 6R3U). The figure is taken from Ref V.

Comparison of the structures of endo-inulinase INU2 and exo-inulinase INUE (Pouyez *et al.*, 2012; Nagem *et al.*, 2004) indicated that the four loops (1–4) at the edges of the active center and three tryptophan residues in loop regions shaped the active site of these proteins (Nagem et al. 2004; Pouyez et al. 2012). In exo-inulinase INUE, the positions of Trp residues narrowed the substrate-binding cavity at the bottom, whereas in endo-inulinase INU2, the tryptophans left enough space to accommodate more than one fructose residue at the bottom of the cavity. Positions of loops 1 and 4 enlarged the active site of the endo-

inulinase, while loops 2 and 3 were shown overlapping in structures of endo- and exo-inulinase (Nagem et al. 2004; Pouyez et al. 2012). When compared to endo-inulinase structure, the loops 1 and 4 of the endo-levanase were pushed even farther towards the edge of the protein, and the long loop 4 partially covered the C-terminal β-sandwich module (Figure 3 in Ref V). Notably, the active site region of the endo-levanase has no tryptophan residues at positions suggested to determine and limit the active site borders in the case of INU2 and INUE (Pouyez et al. 2012). We suggest that absence of tryptophans and wide spacing between the loops 1 and 4 in endo-levanase creates a moderately deep pond-like cavity for substrate binding required for endo-hydrolysis.

3.5.2. The C-terminal β-sandwich module

We propose that the C-terminal β-sandwich domain of endo-levanase from *B. thetaiotaomicron* is required for the correct folding, stability and solubility of the protein (Ref V). In ligand-bound structure of catalytically inactive BT1760, no electron density of the ligand in the C-terminal domain was observed (Figure 9, blue). The dissection of two modules of endo-levanase through cloning and separate expression resulted in fast precipitation of respective proteins and loss of activity. The separately expressed modules could not bind levan, but co-incubation of N- and C-terminal modules resulted in low, but clearly detectable levan-hydrolysing activity (Figure 7 in Ref V). We hypothesize that (i) the exposure of hydrophobic surfaces after dissection of the modules caused aggregation and precipitation in the aqueous solution and (ii) the modules merged through hydrophobic contacts between these surfaces, restoring some activity to the enzyme (Ref V). So far, different functions for the C-terminal domain have been proposed for GH32 enzymes. In *Thermotoga maritima* invertase the C-terminal domain most probably contributes to stabilization of the protein – no ligand was detected bound to this domain, and raffinose was bound only to the active center (Alberto et al. 2006). The endo-inulinase from *A. ficuum* was co-crystallized with kestopentaose, and again, the fructose residues of the ligand were detected only in the active site. In this work, the authors did not assign any function to the C-terminal module (Pouyez et al. 2012). In contrast to that, in fructosyl transferase of *Paenarthrobacter ureafaciens* (PDB 4FFI), both, the C-terminal domain and the active site of the β-propeller domain, bound levanbiose and -triose (Park et al. 2012). The authors proposed that the enzyme's C-terminal domain (Figure 9, grey) helps to anchor levan chain to the enzyme, and so its non-reducing end can reach the catalytic centre for the hydrolysis. Crystallization of the C-terminal carbohydrate-biding module CBM66 of the *B. subtilis* exo-levanase also showed binding of the ligand – levantriose (Figure 9, green) (Cuskin et al. 2012). The study proposes that the CBM66 confers specificity for levan through an "avidity" mechanism in which the C-terminal module and the catalytic module bind the termini of different branches of the levan molecule.

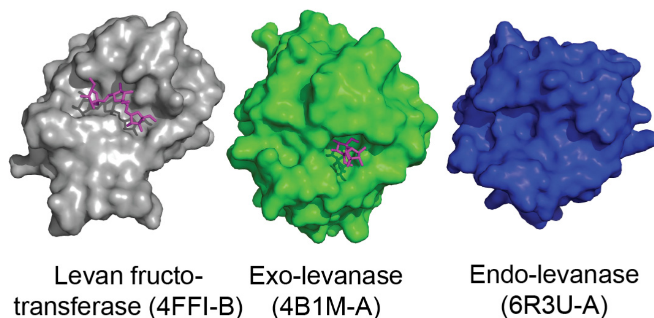

Figure 9. The C-terminal domains of three GH32 family proteins. Grey domain belongs to fructosyl transferase of *Paenarthrobacter ureafaciens* (PDB 4FFI-B) with magenta levantriose bound to its surface. Green represents the CBM66 structure (exo-levanase of *Bacillus subtilis*, PDB 4B1M-A) and the blue C-terminal domain belongs to the endo-levanase of *B. thetaiotaomicron* (PDB 6R3U-A). Image is taken from (Ref V) with slight modifications.

3.5.3. How does the endo-levanase operate?

The ligand-bound structure of endo-levanase indicated that levantetraose was bound to the enzyme at four subsites. According to binding subsites designation (Davies et al. 1997), the non-reducing end of the levan chain is bound at a '–' subsite and the reducing end at '+' subsites.

We suggest that similarly to other GH32 family proteins (Lammens et al. 2009), endo-levanase BT1760 works by retaining the configuration of the anomeric carbon of fructose using double displacement mechanism. In the first step of the reaction, a nucleophile Asp41 attacks the anomeric carbon (C2) in fructose monomer, forming a covalent fructosyl-enzyme intermediate. At the same time, Glu221 acts as a general acid and donates a proton to the leaving fructosyl group. In the second step, Glu221 functions as a general base and removes a proton from the water molecule resulting in hydrolysis of the fructosyl-enzyme intermediate. The positions of catalytically important amino acids are shown in Figure 10.

In experiments described in Ref V, we used levan from timothy grass that is linear (Kasperowicz et al. 2016; Nelson and Spollen 1987) and should provide L-FOS with uniform structure after the hydrolysis. After the reaction of timothy levan with endo-levanase BT1760 (described in Ref IV), products of different DP were isolated. Levan oligomers with DP 3–5 were used in Ref V as substrate for endo-levanase to determine the shortest L-FOS that can be hydrolysed by the enzyme and to characterize the product spectrum. Figure 11 shows that DP3 (levantriose) remained in the solution along with products from its hydrolysis: fructose and levanbiose (DP2). Levantetraose (DP4) was rapidly cleaved into fructose and levantriose, which was subsequently cleaved into fructose and levanbiose. Levanpentaose (DP5) was cleaved mostly into

levanbiose and -triose, but some fructose and levantetraose were also produced (Figure 11). In Ref IV we showed that at rapid phase of levan degradation, mostly levantriose was produced, whereas levanbiose was accumulating at the end of the reaction. Figure 11 clearly shows that levantriose is the shortest L-FOS cleaved by BT1760. We assume that at hydrolysis of L-FOS of DP ≥ 4, the DP3 oligomer is released from the levan chain bound at subsites –3 to –1 in endo-levanase active centre (Ref V).

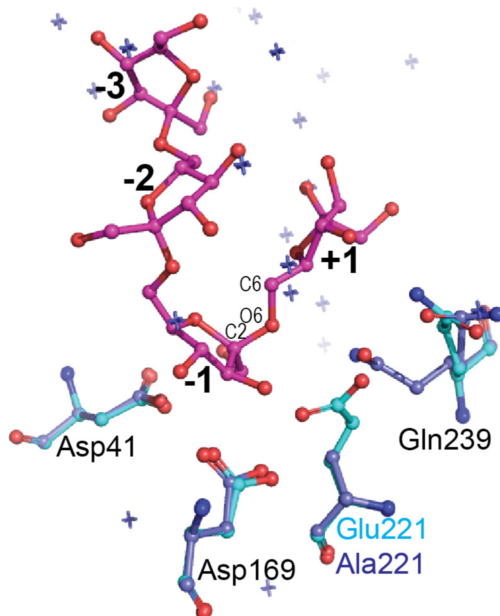

Figure 10. Superimposed apo- and ligand-bound (E221A mutant with levantetraose) structures of endo-levanase (in light and dark blue, respectively). Levantetraose (in magenta) is bound at –3, –2, –1 and +1 subsites. No conformational changes are observed in catalytic pocket region except for missing side-chain of Ala221 and Gln239 presented by two alternate rotamers in E221A model. The figure is taken from Ref V.

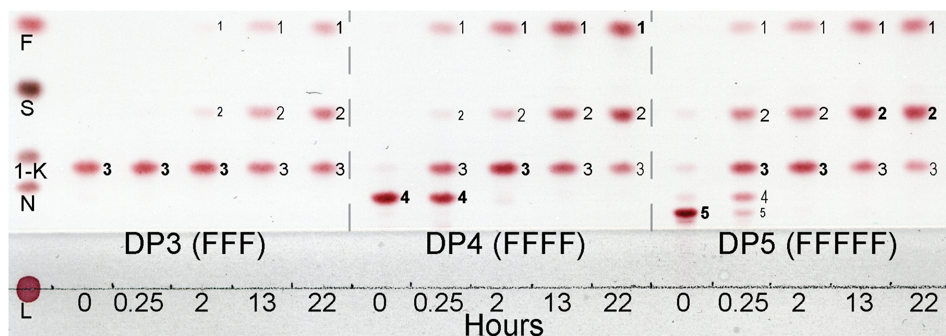

Figure 11. Hydrolysis of levantriose (DP3), levantetraose (DP4) and levanpentaose (DP5) by the endo-levanase BT1760. The mixture of fructose (F), sucrose (S), 1-kestose (1-K), nystose (N) and levan (L) was used as a marker. The numbers 1–5 indicate degree of polymerization (DP) of the fructan. The Figure is taken from Ref V.

Levans may have a very high DP and molecular weight reaching several megadaltons (Table 1 in Ref IV). Hydrolysis of that big molecule is probably not a simple task for an endo-acting enzyme. According to the theory presented in Ref V, during hydrolysis of levan, the first endo-cuts into high-DP levan chains are probably done randomly. To enable the first cuts, bending of the levan chain into the substrate-binding pocket is probably required, producing levan oligomers of moderate DP. After binding of moderate-length levan oligomers with their non-reducing ends at –3 subsite, levantriose is released. This theory explains why levantriose is a prominent product formed at the rapid phase of levan degradation by BT1760 (Refs IV and V).

CONCLUSIONS

This doctoral thesis is focused on levans, L-FOS and enzymes that can be used for their production. So far, many possible applications of levans in medicine, pharmacy, agriculture, food industry *etc.* are proposed while in the case of L-FOS, their bifidogenic effect is mostly emphasized. Levans and mixtures of I- and L-FOS are synthesised by bacterial levansucrases, while production of pure L-FOS is probably biotechnologically feasible using enzymatic endo-hydrolysis of levan. Despite many beneficial properties of levan-type fructans, costs for large-scale manufacturing yet remain unacceptable for the industry.

Here, in this thesis, two perspective catalysts for the production of levan and L-FOS are thoroughly described.

The main results of this thesis are summarized as follows:

i. Levansucrase Lsc3 from a plant pathogen *Pseudomonas syringae* is one of the most active levansucrases described so far. It uses sucrose, raffinose and stachyose as substrates producing levan with bimodal size distribution: 4 733 kDa and 10.7 kDa, as well as FOS: 1-kestose, 6-kestose, nystose, fructosyl nystose, and longer FOS, of DP 6 and 7. When alternative fructosyl acceptors are provided, Lsc3 produces hetero-oligofructans.
ii. A panel of 36 random and site-directed mutants of Lsc3 was constructed and assayed in order to specify the functions for each residue:
   a. The catalytic triad of Lsc3 – Asp61, Asp219 and Glu303 – was identified through construction and assay of alanine substitution mutants. Respective mutants were catalytically inactive.
   b. A set of mutated positions obtained from random mutagenesis, was analyzed *in silico* and four positions were selected for site-specific mutagenesis. *In vitro* analysis revealed the importance of Asp300, which was shown to participate in determination of length of the synthesized product. Levan synthesized by the Asp300Asn mutant had a low molecular weight, which turned out to be a preferred substrate for the endo-levanase. The His113 was shown to contribute to both sucrose splitting and polymerization, presumably participating in substrate binding at both –1 and +1 subsites.
   c. The Glu146Gln, Thr302Met and Asp333Asn mutants had slightly enhanced FOS production, and the Trp109Phe mutant produced more levan than the wild-type enzyme. All others investigated mutations decreased catalytic performance of the Lsc3.
iii. Cost-efficient and high-throughput methods were proven suitable for the characterization of Lsc3 and its mutants. Permeabilized cells of *E. coli* expressing Lsc3 variants could be used as ‘levansucrase preparations’ in evaluation of both sucrose-splitting and FOS-producing activities. Diffe-

rential scanning fluorimetry was shown as a feasible high-throughput method to evaluate thermal stability of levansucrases.

iv. The endo-levanase BT1760 from a human gut commensal *B. thetaiotaomicron* was heterologously expressed, purified and proven as the most active endo-levanase described so far.

v. Substrate specificity assay of BT1760 revealed that it specifically cleaved β-2,6 linkage in levan-type fructans longer than DP2. Dahlia inulin, xylo-oligosaccharides, raffinose and stachyose did not serve as substrates for BT1760. The endo-levanase BT1760 preferred low-molecular weight levans (*e.g.* timothy grass levan) to levans with high molecular weight.

vi. The first step of endo-levanase reaction on levan is random hydrolysis of levan chains into long FOS. These FOS are then further cleaved into shorter species.

vii. Glu221 was specified as acid/base catalyst of the endo-levanase BT1760. Alanine substitution of Glu221 inactivated the endo-levanase enabling co-crystallization of this mutant with a ligand – levantetraose.

viii. The structures of endo-levanase BT1760 and its catalytically inactive ligand-bound mutant were solved. The structure of BT1760 revealed a bi-modular fold of N-terminal five-bladed β-propeller connected with C-terminal β-sandwich domain. The levantetraose-bound structure exposed a novel architecture for the active site pocket among fructan-acting enzymes. The substrate binding cavity of the endo-levanase was wider compared to that of the exo-inulinase from *Aspergillus awamori*, but narrower at its bottom when compared to the active site cavity of the endo-inulinase from *A. ficuum*. No role in the catalysis was revealed for the C-terminal β-sandwich domain – it was suggested to participate in folding and stabilization of the entire protein.

SUMMARY IN ESTONIAN

Levaansukraasi Lsc3 ja endo-levanaasi BT1760 iseloomustamine ja rakendatavus uudsete prebiootikumide tootmises

Toidus sisalduvatest süsivesikutest on kiudained inimese soolebakteritele kõige sobilikumad. Need polümeersed keerulise ehitusega suhkrud toimivad prebiootikumidena: jõuavad seedumata jämesoolde ja lagundatakse inimese tervisele kasulike (probiootiliste) bakterite, nt bifidobakterite ja laktobatsillide toimel. Selle tulemusena moodustuvad kiudainetest lühikese ahelaga rasvhapped, millest näiteks võihape ehk butüraat on peamiseks energiaallikaks meie soolt vooderdavatele epiteelirakkudele.

Prebiootikumide manustamine on meie igapäevaelus üsna tavaline: neid lisatakse näiteks imikute piimasegudele, kasutatakse toidulisandina või apteegis müüdava suukaudse preparaadina. Fruktoosi polümeer inuliin ja selle hüdrolüüsil saadavad frukto-oligosahhariidid on enimuuritud prebiootikumid maailmas. Sigurist eraldatud inuliini lubatakse kasutada toidus juba 2002. aastast. Lisaks inuliinile on looduses olemas ka teise sidemetüübiga fruktoosi polümeer – levaan. Levaani sünteesivad peamiselt bakterid, kuid ka mõned taimed kõrreliste hulgast. Levaanil ja levaani-tüüpi frukto-oligosahhariididel on näidatud prebiootikumidele iseloomulikke toimeid, kuid tootmiskulude kõrge hinna tõttu on neid veel väga vähe uuritud.

Dotsent Tiina Alamäe töögrupis on tegeldud levaani-tüüpi prebiootikumide ensümaatilise sünteesiga juba üle kümne aasta. Antud doktoriväitekiri keskendub kahele biotehnoloogiliselt olulisele ensüümile: levaansukraasile ja endo-levanaasile, mille abil on võimalik neid fruktoosi polü- ja oligomeere toota tavalisest lauasuhkrust. Mõlemad ensüümid on pärit bakteritest.

Taimepatogeeni *Pseudomonas syringae* levaansukraas Lsc3 lõhustab sahharoosi, rafinoosi ja stahhüoosi molekuli ning polümeriseerib neist pärinevad fruktoosi jäägid frukto-oligosahhariidideks ja kõrgmolekulaarseks levaaniks. Bakter ise kasutab levaanuskraasi endale limase levaankapsli sünteesiks, et varjuda taime kaitsemehhanismide eest, kuid meile pakkusid huvi selle ensüümi struktuuri ja funktsiooni vahelised suhted ning kasutus prebiootiliste suhkrute sünteesiks. Lsc3 on üks efektiivsemaid levaansukraase, mis senini on kirjeldatud. Kui ta reageerib sahharoosiga, siis moodustub produktide segu: tekib inuliini- ja levaani-tüüpi frukto-oligosahhariide ning ka levaani. Ebasoovitavaks produktiks on suur kogus glükoosi, mis takistab oligo-fruktaanide segu kasutamist prebiootikumina ilma eelneva puhastuseta.

Valgu katalüüsis oluliste aminohapete kindlakstegemiseks on parim meetod mutatsioonanalüüs, mida antud töös rakendasin ka levaansukraasi Lsc3 puhul. Senikirjeldatud levaansukraaside struktuure analüüsides ennustasin Lsc3 valgu struktuuri ning katalüütilisi aminohappeid. Kohtsuunatud mutagenees kinnitas ennustuse õigsust: ensüümi katalüütilise kolmiku moodustavad kolm happelist aminohapet: Asp62, Asp219 ja Glu303. Lisaks selgitasin, et katalüütilist

kolmikut ümbritsevad aminohapped on samuti katalüüsis olulised, toetades substraadi sidumist, lõhustamist ja fruktoosijääkide polümeriseerimist. Kokku iseloomustati doktoritöös Lsc3 valgu 36 erinevat mutanti. Enamasti olid mutandid algsest valgust katalüütiliselt 'kehvemad', kuid paaril mutantsel valgul oli ka potentsiaali biotehnoloogiliseks kasutamiseks. Näiteks sünteesis Trp109Phe mutant enam levaani kui algne valk ning Asp300Asn mutandi sünteesitud levaan oli väikese molekulmassiga ning osutus väga heaks substraadiks endo-levanaasile. Seega saaks seda mutanti kasutada frukto-oligosahhariidide tootmiseks sobiva levaani sünteesimisel.

Doktoritöös kirjeldatud Lsc3 valgu 36 mutandi iseloomustamiseks kasutasin lisaks traditsioonilisele analüüsile ka uudseid säästlikke ja kiireid meetodeid. Töös näitasin, et levaansukraasi sisaldavaid kolibakteri rakke saab kasutada preparaadina levaansukraasi omaduste uurimisel. Kui kolibakteri rakumembraan keemiliselt augustada, siis pääseb levaansukraas rakust välja ning kokkkupuutel sahharoosiga toimub nii sahharoosi lõhustamine kui ka polümeriseerimine. Tõestasin, et kolibakteris 'peidus' oleva levaansukraasiga mikroplaatidel tehtud analüüsi tulemused on usaldusväärsed ja korreleeruvad hästi tulemustega, mis saadi puhastatud valke ning traditsioonilisi meetodeid kasutades.

Lsc3 produktidest on ilmselt olulisim levaan, sest levaani on lihtne lahusest sadestamisega puhastada ning selle ensümaatilisel hüdrolüüsil saaks toota ainult levaani-tüüpi sidemeid sisaldavaid frukto-oligosahhariide, millel on mitmete uuringute alusel eriti tugev prebiootiline toime. Sobivaks levaani lagundavaks ensüümiks osutus inimese jämesoolebakteri *Bacteroides thetaiotaomicron* endo-levanaas BT1760. Näitasin, et kolibakteris sünteesitud endo-levanaas BT1760 oli väga tõhus ja 'tükeldas' efektiivselt kõiki testitud levaane. Selgus, et parimaks substraadiks talle on väikese molekulmassi ja hargnemata ahelaga timutist pärinev levaan.

Levaan ei ole ensüümile lihtne substraat lagundamiseks, sest koosneb väga pikkadest ahelatest, mis on enamasti ka hargnenud. Seetõttu huvitas meid, milline näeb see ensüüm välja, milline on tema substraaditasku ja kuidas toimub levaani tükeldamine. Vastuse saamiseks otsustasime endo-levanaasi kristalliseerida, et oleks võimalik tema ehitust uurida. Koostöös Tallinna Tehnikaülikooliga see meil ka õnnestus. Selgus, et ensüümil on kaks moodulit: katalüüsi läbi viiv N-terminaalne viielabaline β-propeller, mis on ühendatud C-terminaalse β-võileiva struktuuriga. Propelleri keskel asub aktiivtsenter, kus toimub substraadi ankurdamine ja hüdrolüüs. Substraadi seostumise selgitamiseks kristalliseerisime endo-levanaasi inaktiivse mutandi koos levaantetraoosiga aktiivtsentris. Selgus, et endo-levanaasi substraaditasku meenutab sügavat kaussi, mille põhjas toimub lõike tegemine. Seega peab pikk levaaniahel painduma substraaditaskusse, et oleks võimalik esimese lõike tegemine. Selgitasin ka välja, et endo-levanaasi β-võileiva moodul ei osale ei katalüüsis ega levaaniahela sidumises ning tema rolliks on pigem terve valgu kooshoidmine.

Käesolev doktoritöö panustab kindlasti ka biotehnoloogiasse. Näitasin, et *P. syringae* levaanuskraasiga on odavast toorainest (lauasuhkrust) võimalik

sünteesida polümeerset levaani ja frukto-oligosahhariide. Sellise produktide segu kasutamine prebiootilise preparaadina ei ole hea variant, sest temas on erineva sidemetüübiga frukto-oligosahhariide ning kõrvaproduktina väga suures koguses glükoosi. Levaani-tüüpi sidemega frukto-oligosahhariide on seetõttu otstarbeks toota levaansukraasiga sünteesitud levaani ensümaatilisel hüdrolüüsil, milleks sobib väga hästi bakteri *B. thetaiotaomicron* endo-levanaas.

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