Levansucrases of a *Pseudomonas syringae* pathovar as catalysts for the synthesis of potentially prebiotic oligo- and polysaccharides

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Gut microbiota influences more physiological and developmental processes of humans and animals than earlier expected. Therefore, the possibility to shape the composition and activity of this bacterial population by prebiotics becomes especially important. Inulin, a β-2,1 linked fructan polymer, from plants and fructooligosaccharides (FOS) derived from it are recognized and already widely used as prebiotics while β-2,6 linked fructans have received much less attention from scientific community. In this mini-review, we will address β-2,6 linked fructans: levan and levan-type FOS as novel potential prebiotics and summarize the literature data on levansucrases of *Pseudomonas* bacteria which are producing these fructans. The major attention is drawn to stable and highly efficient levansucrases of *Pseudomonas syringae* pv. tomato, among which the Lsc3 protein has been most thoroughly studied using biochemical methods as well as extensive mutagenesis of the protein.

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**Introduction: food resources for colon microbiota, inulin-type fructans as prebiotics**
The microorganisms residing in and on the human body are collectively termed as human microbiota. The colon bacteria, comprising a major part of it, are involved in many functions: food digestion, food energy harvest and storage, vitamin production, mineral absorption, development and stimulation of the immune system, and maintenance of the intestinal barrier integrity [1–4]. The colon microbiota co-develops with the host, changes from birth to old age and is affected by the genome, but also lifestyle and nutritional habits of the host as well as antibiotic treatment and other factors [3–5]. Among those, food plays a key role [1,5–7]. For a fresh review on significance of food, gut microbiota and its metabolites as epigenetic effectors in humans and animals, see [8].

A novel goal established by knowledge-based food policy is functional food. This type of food should either supply the organism with *prebiotic* (beneficial, health-promoting) bacteria, vitamins and minerals and/or promote multiplication and activity of these bacteria in the intestine. Probiotic colon bacteria, such as bifidobacteria and lactobacilli, can be selectively stimulated by certain complex carbohydrates acting as *prebiotics*. According to the definition, prebiotics are selectively fermented substrates naturally present or added to the food, resulting in specific changes in
the composition and/or activity of the gastrointestinal microbiota, conferring benefits upon the host health and well-being [9,10]. Complex carbohydrates (also referred to as food fiber) are specifically suited to manipulate the human colon microbiota as they resist human digestion system, reach to the colon and serve as food for resident bacteria. Millennia of human–bacteria co-evolution have equipped the gut microbiota with potent tools for the degradation of complex carbohydrates: resistant starch, inulin, xylan, pectin [1,11,12] and some others. The colon bacteria also feed on oligosaccharides of breast milk and those attached to gut mucins [11,13]. Prebiotic potential of a complex carbohydrate depends on many factors: monomeric composition, linkage type, degree of polymerization (DP), crystallinity, solubility and association with other substrates [12]. Isomaltooligosaccharides, xyloglucans, arabinoxylans, pectin, resistant starch and β-glucans are considered as emerging prebiotics ([14–17] and references therein) whereas inulin, inulin-type fructooligosaccharides (FOS), galactooligosaccharides and lactulose have been already approved as prebiotics [10,17]. Among those, inulin and inulin-type FOS (β-2,1 linked fructans) are probably the most widely studied and applied prebiotic food ingredients [10,17,18]. These fructans have been added to yoghurt, cream cheese, juices, bread, pet food, and inulin has been also recommended to replace fat in desserts and sausages [19,20]. Many health-promoting effects of oligo- and polysaccharides on humans are mediated by bacterial metabolites. Prebiotic fibers are fermented by their target bacteria to short-chain fatty acids (SCFA): lactate, acetate, propionate and butyrate. SCFA provide energy for enterocytes and thereby improve energy harvest as well as intestinal defence mechanisms mediated by epithelial cells [21,22]. SCFA also reduce the pH in the gut lumen promoting absorption of the minerals [23].

A stimulating effect of inulin-type fructans on growth of beneficial colon bacteria has been recorded both in vitro and in vivo [9,24–30]. For example, a Q-PCR assay of gut microbiota of healthy volunteers indicated that 16 days of inulin administration significantly increased the populations of Bifidobacterium adolescentis and B. bifidum [30]. This effect is expected, because bifidobacteria possess β-fructofuranosidases specifically cleaving β-2,1 linkages ([31] and references therein). Involvement of inulin-type FOS in reduction of cholesterol and blood sugar levels, prevention of colorectal cancer and inflammatory bowel disease and stimulation of the immune system of the host has also been shown ([32–35] and references therein).

Inulin is commercially manufactured from plants, mostly from chicory. In chicory root inulin comprises about one-fifth of the fresh weight containing 55% of oligosaccharides with a DP of 2–19, 28% with a DP of 20–40 and 17% with a DP of >40 [36]. The plant-derived inulin can be enzymatically hydrolyzed to produce prebiotic FOS of varied DP as reviewed in [37]. A novel and technologically promising source of inulin-type FOS is yakan (Smallanthus sonchifolius), roots of which contain a high amount (40–70% from dry weight) of β-2,1-linked FOS with DP 3-10 ([38] and references therein). Aside of plant sources, inulin-type FOS are enzymatically produced from sucrose using microbial fructosyltransferases (EC 2.4.1.9) [39] as well as β-fructofuranosidases (EC 3.2.1.26) reacted under transfer-favoring conditions [40].

Compared to inulin, prebiotic activity of fructans containing β-2,6 linkages is yet scarcely assayed. Search of PubMed in September 2014 with key-words ‘inulin + prebiotic’ and ‘levan + prebiotic’ yielded respectively 408 and 12 publications. Still, few earlier studies [41–43] and one very recent paper [44] clearly indicate their high prebiotic potential. The literature on levane-type fructans and their enzymatic synthesis will be briefly reviewed below.

**Levan and levane-type FOS as potential novel prebiotics**

Levan differs from inulin according to the linkage type. In the main chain of levan, fructose residues are joined by β-2,6 linkages and the side chains are attached through β-2,1 linkages. As inulin, levan is synthesized from sucrose and contains a sucrose moiety as a starter unit of the chain [45]. Figure 1 shows substrates and oligosaccharidic products of levansucrases.

In addition to FOS (Fig. 1), levansucrases produce polymeric levan, DP of which can be very high, even over 100,000 [45]. The first reaction of levan synthesis is formation of 6-kestose from two molecules of sucrose: one acting as a fructosyl donor and the other as an acceptor (Fig. 2). Then, 6-kestose is further extended through numerous transfructosylation reactions.

Levan is synthesized by bacterial extracellular enzymes, levansucrases (EC 2.4.1.10) [46] that are present in bacilli (e.g. *Bacillus subtilis, B. megaterium*), lactic acid bacteria (*Lactobacillus gasseri, L. reuteri, L. sanfranciscensis, L. panis* and others), plant-associated and -pathogenic Gram-negative bacteria such as *Zymomonas mobilis, Gluconacetobacter diazotrophicus, Erwinia amylovora* and *Pseudomonas syringae* pathovars (CAZy and Brenda databases) [47,48]. Levansucrase is also present in a plant pathogen *Burkholderia cepacia* that also infects respiratory tract of cystic fibrosis patients [47,49]. Some levansucrases, for example, the LsdA of *G. diazotrophicus*, are specifically prone for FOS synthesis, producing inulin-type FOS and only a small amount of levan [50,51]. According to Tambara and coworkers [50], at a high concentration (600 g/L) of sucrose the LsdA synthesized a high amount (241 g/L) of 1-kestose. As this enzyme produces levan, it should also synthesize 6-kestose (DP 3; Figs 1 and 2) that does not accumulate as it is efficiently extended [50]. The *Z. mobilis* levansucrase also produces 1-kestose as the major oligosaccharidic product [52], but 6-kestose is also detected among the reaction products [53]. Interestingly, β-fructofuranosidases of several filamentous fungi (e.g. *Thermoascus aurantiacus*) and yeasts (e.g. *Schwanniomyces occidentalis* and *Rhodotorula dairenensis*) also produce a considerable amount of 6-kestose from sucrose [54–56]. For some levansucrases, such as the LevU of *Z. mobilis* [53], synthesis of neokestose has also been recorded. In neokestose (belongs to neo-FOS; DP 3; Fig. 1), β-2,6 linkage joins the fructose and glucose residues [57,58]. Neo-FOS are produced also from sucrose by several fungal (e.g. *Penicillium citrinum*) and yeast (e.g. *Xanthophyllomyces dendrorhous*) β-fructofuranosidases [57,59–61]. So, respective enzyme of *X. dendrorhous* produced in a 48 hours reaction with 41% sucrose 40 g/L of neokestose and 18 g/L of 1-kestose [57].

Some plants, for example timothy grass (*Phleum pratense*) and orchard grass (*Dactylis glomerata*), synthesize linear β-2,6-linked fructans which are referred to as plant levans or phleins [45,62,63]. Phleins have lower DP than bacterial levans which usually have the DP >100 [45,64]. Graminans have both, β-2,1 and β-2,6 linkages and they are present in cereals, including popular food crops wheat and barley [45]. Graminan- and levan-type fructans are specific not only for monocot plants – they have also been
described in *Pachysandra terminalis*, an evergreen frost-resistant dicot plant [65].

There are some studies concluding that FOS having β-2,6 linkages (levan-type FOS and neo-FOS) may have enhanced prebiotic properties [41–43]. A study by Kilian et al. [42] indicated that neokestose that was produced using a yeast *Pfaaffia rhodozyma*, synonyme *X. dendrorhous* (i) selectively enhanced numbers of bifidobacteria and lactobacilli in fecal slurries, (ii) acted as good growth substrate for all studied lactobacilli and bifodobacteria and (iii) promoted production of lactic and acetic acids by fecal slurries more strongly than raftilose 95, a commercial preparation of inulin-type FOS. Importantly, neo-FOS had higher resistance in the pH range of 6-10 and higher thermal stability than inulin-type FOS [66]. Effects of levan-type FOS have only scarcely been studied as these substrates are currently impossible to purchase. Sill, Marx et al. [41] showed that bifidobacteria were capable of fermenting β-2,6 linked FOS which were prepared by acid-hydrolysis of *Z. mobilis* levan. Among the bifidobacteria tested, *B. adolescentis* exhibited the best growth and SCFA production from both short- and long-chain β-2,6 linked FOS species [41]. Levan-type FOS can also be prepared by enzymatic hydrolysis of levan. Very recently, an endolevanase, LevB1 from *B. licheniformis*, was cloned, characterized and applied to produce levan-type FOS from bacterial levan [44]. In this study, the authors compared the growth of six strains...
of probiotic bacteria on levan and levan-derived FOS and showed that *L. paracasei*, *B. longum* var. *infantis* 17930 and *B. longum* had similar growth rate on both substrates. However, *B. bifidum*, *B. longum* var. *infantis* NRRL 4661 and *B. breve* grew better on FOS [44]. This study clearly confirms prebiotic potential of levan and levan-type FOS.

To the best of our knowledge, prebiotic effects of phleins purified from plants have not been studied and for graminins only one publication was found. It is highly possible that not only levan, but also phleins and graminins can act as selective food for health-beneficial bacteria in the colon. A report by Jenkins and coworkers [67] stated that certain fractions of cereal graminins were in vitro fermented by colon microbiota resulting in SCFA production comparable to that induced by oligofructose preparation. Thus, most probably those fructans can be prebiotic, and their prebiotic effect can be either direct or mediated by common gut resident bacteria. The latter possibility is supported by data from Sonnenburg et al. [68] showing that *B. thetaiotaomicron*, a human gut generalist, grows on levan and possesses an endolevanase. Among tested *Bacteroides* species, endolevanase was present only in *B. thetaiotaomicron* being absent from *B. caccae*, *B. ovatus*, *B. fragilis*, *B. vulgatus* and *B. uniformis* that coincided with their inability to grow on levan [68]. Four *Bacteroides* species tested: *B. caccae*, *B. ovatus*, *B. fragilis* and *B. uniformis* grew well or moderately on inulin [68], indicating species-specific metabolism of fructans in *Bacteroides* bacteria. Among colon microbiota, the *Bacteroidetes* bacteria are the most active and numerous degraders of plant fiber polysaccharides [11,12,69]. Also, the majority of members in gut microbiota belong either to *Bacteroidetes* or *Firmicutes* phyla [5]. We hypothesize that if a probiotic bacterium cannot degrade longer β-2,6 linked fructans, it can still be stimulated by it due to levan-degrading activity of *Bacteroides* or/and some other bacteria. Levan is degraded extracellularly [68] and thereby the degradation products of levan will be available for the bacteria that can internalize and catabolize respective FOS.

**Biochemical properties of levanases of *Pseudomonas* bacteria relevant for biotechnological applications**

According to the literature and databases in the web, many species of *Pseudomonas*, for example, *P. fluorescens*, *P. chlororaphis*, *P. poae* and numerous pathovars of *P. syringae* possess levanases. These bacteria have mostly been isolated from plant phyllosphere, some of them are endophytic, some have biocontrol abilities and several cause diseases in plants ([70–74] and references therein). *P. syringae* pathovars cause diseases in many plants [75] and they are exceptional among levanase-possessing bacteria for having multiple (two or three) copies of respective genes [70,76,77]. *P. syringae* pv. tomato DC3000 (*Pst*) encodes three levanases: Lsc1 (431 amino acids, aa), Lsc2 (415 aa) and Lsc3 (431 aa) [77]. Levanases of *P. syringae* pathovars are much smaller than respective proteins of lactobacilli and other Gram-positive bacteria [77,78]. A small size of the protein can be beneficial in case of heterologous expression – it should reduce the burden for the host and increase the expression level. In an agreement with that, a high yield, 30 mg of Lsc3 protein per 1 ml of recombinant *Escherichia coli* culture, was achieved at its overexpression [74].

Earlier studies on levanases of pseudomonads were mainly focused on genetic issues – allelic spectrum of levanase genes of *P. syringae* pathovars and expression of these genes in native hosts were studied [71,79]. Later, evolutionary origin of multiple levanase alleles in *P. syringae* pathovars was addressed [80,81]. In 1995, Hettwer et al. [82] reported on biochemical characterization of a levanase of *P. syringae* pv. phaseolicola. This enzyme was purified from the growth medium of the *P. syringae* pv. phaseolicola NCPPB 1321 and according to a sequenced fragment of the protein, it was a 431 aa levanase [82]. The levanase LscA of *P. chlororaphis* subsp. *aurantiaca* (Pca) [72,83] which is distributed by Fluka (Sigma–Aldrich, Steinheim, Germany) was characterized from the aspect of biochemical properties and polymerization products by Visnapuu et al. [84] and Alamíe et al. [85]. Literature search did not yield additional data on levanases from other species of *Pseudomonas*. Yet, there are few publications on synthesis of difructose anhydride (DFA IV) from levan by *Pca* and *P. fluorescens* [83,86]. Most producers of this prebiotic substrate are Gram-positive bacteria such as *Arthrobacter* and *Microbacterium* [86].

The Lsc2 and Lsc3 of *Pst* have been produced as heterologous proteins in *E. coli* and after the purification thoroughly biochemically characterized [74,77,84,85,87–90].

Table 1 reviews some biochemical parameters of above-mentioned levanases. It shows that the pH optima of levanases of *Pseudomonas* bacteria are rather similar, close to 6.0. All studied levanases of pseudomonads, except for the pv. phaseolicola

<table>
<thead>
<tr>
<th>Enzyme, source</th>
<th>$K_m$ (sucrose; mm)</th>
<th>$K_v$ for raffinose (mm)</th>
<th>$K_{cat}$ for sucrose (s$^{-1}$)</th>
<th>Optimal pH</th>
<th>Optimal temperature $({}^\circ$C) for transfructosylation</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lsc, <em>P. syringae</em> pv. phaseolicola</td>
<td>160</td>
<td>No/ND</td>
<td>ND</td>
<td>5.8–6.6</td>
<td>60</td>
<td>ND</td>
</tr>
<tr>
<td>Lsc3, <em>P. syringae</em> pv. <em>tomato</em></td>
<td>18.5</td>
<td>Yes</td>
<td>39.9</td>
<td>50.4</td>
<td>6.0</td>
<td>60</td>
</tr>
<tr>
<td>Lsc2, <em>P. syringae</em> pv. <em>tomato</em></td>
<td>17.1</td>
<td>Yes</td>
<td>49.0 ($K_m$)</td>
<td>328.3</td>
<td>6.0$^a$</td>
<td>50$^b$</td>
</tr>
<tr>
<td>LscA, <em>P. chlororaphis</em> subsp. <em>aurantiaca</em></td>
<td>24.1</td>
<td>Yes</td>
<td>80.8</td>
<td>7.2</td>
<td>6.0–6.6</td>
<td>60</td>
</tr>
</tbody>
</table>

$^a$His-tagged protein.$^b$Protein with no tag.$^c$Purchased from Sigma–Aldrich.
enzyme [82], cleave not only sucrose, but also raffinose and stachyose (Table 1) which all share a sucrose moiety in their structures (Fig. 1). Quite surprisingly, the enzyme of pv. phaseolicola was reported incapable of raffinose usage, while stachyose was not tested for a substrate [82]. Unfortunately, no more data on catalytic properties of levansucrases of this pathovar are available. As indicated in Table 1, sucrose is certainly a preferred substrate of levansucrases from Pseudomonas bacteria – the $K_m$ values of the enzymes for sucrose cleavage are much lower (~20 ms) than respective $K_m$ or $K_i$ values for raffinose cleavage (Table 1). Kinetic parameters ($K_m$ and $k_{cat}$) for sucrose cleavage were determined by measuring initial velocity of glucose release. In case of Lsc2 and Lsc3, raffinose is used about twice less efficiently than sucrose. For the LscA protein, raffinose is even less convenient substrate given a high $K_i$ value (~80 ms) of sucrose cleavage inhibition by raffinose (Table 1). Lsc3 and LscA proteins were shown capable of catalysis using sugar beet molasses as a cheap sucrose-rich substrate [84] that can be important if cost-efficient production of polymerization products will be initiated. Lsc2 and Lsc3 proteins of Pst are highly active catalysts, their $k_{cat}$ values of sucrose cleavage are close to 500 s$^{-1}$. The catalytic turnover value of the LscA protein is about 70 times smaller (Table 1). Respective catalytic constants of other characterized levansucrases from Gram-negative bacteria, such as Z. mobilis and G. diazotrophicus [91,92] are up to 8 times lower compared to Lsc2 and Lsc3. Though Lsc2 and Lsc3 proteins are highly active catalysts, even higher $k_{cat}$ (2272 s$^{-1}$) has been reported for the SacB protein of B. megaterium [93].

For biotechnologically relevant enzymes, stress tolerance is very important. The Lsc3 protein of Pst has been assayed for thermostability using a traditional assay of residual activity and also a high-throughput Thermofluor assay [90]. $T_m$ (melting temperature) determined for Lsc3 protein was 65.4°C and when the protein was incubated for 30 min at 60°C, it retained ~80% of its sucrose-splitting activity [90]. The protein was also characterized as highly stable during long-term storage, retaining ~50% of its activity after 140 days of incubation at 37°C [90]. Importantly, Lsc3 could perfectly react with its substrates even in distilled water [87]. Taking into account a high catalytic turnover and stress tolerance, the Lsc3 is a good candidate for biotechnological applications.

**Synthesis of FOS and levan by levansucrases of Pseudomonas bacteria**

Among Pseudomonas bacteria, the ability to produce FOS has been studied only in case of levansucrases of Pst and Pca. Levansucrases of Pst (Lsc2, Lsc3) and Pca (LscA) produce from sucrose aside of levan also FOS with DP 3-7 [74,84,85,87,89]. Polymerizing properties of two studied levansucrases of Pst, Lsc2 and Lsc3 were reported to be similar that agrees with their very high (95%) sequence identity [85,89]. Compared to Lsc3 protein, the LscA of Pca polymerized much less efficiently at low sucrose concentration [84,85].

The main conditions largely affecting the spectrum and yield of polymerization products of Lsc3 and Lsc2 proteins were substrate concentration and reaction temperature [84,87,94], but the amount of added catalyst had also an effect [94]. It is well known that all levansucrases possess two distinct activities: substrate (sucrose) cleavage and transfructosylation reactions [46,82,85]. Generally, low temperature enhances transfructosylation, whereas elevated temperature promotes sucrose cleavage [84,94]. In case of levansucrases of Pseudomonas bacteria (Lsc3 of Pst and LscA of Pca), the optimum temperature for transfructosylation was approximately 40° lower than the optimum of sucrose splitting (Table 1). When the Lsc3 protein was reacted with sucrose at 20°C, 80% of fructose residues resulting from sucrose splitting was polymerized to fructans. Respective value dropped to 67% if reaction was conducted at 60°C. In case of the LscA protein, the increase of temperature decreased transfructosylation even more [84]. An exceptionally thermoactive levansucrase has been described from Bacillus sp. TH4-2. This enzyme produced the highest amount of levan when reacted at 50°C, whereas the optimum temperature for sucrose cleavage was 60°C [95], the latter being close to respective value of levansucrases from Pseudomonas bacteria (Table 1).

The conditions of FOS and levan synthesis have been recently optimized for the Lsc3 protein of Pst [94]. Highest FOS yield was achieved if the reaction was conducted at 30°C or 37°C for 20 hours using a low amount (~1 g/ml) of the Lsc3 protein. A high sucrose concentration also promoted FOS synthesis by the Lsc3. From 410 g/L of sucrose approximately 100 g/L of FOS (15.4 g per mg of protein) was synthesized [74]. The FOS mixture produced by the Lsc3 contained 1- and 6-kestoses, nystose and longer FOS with DP up to 7. Additionally, blastose (a sucrose analog) was detected in the FOS mixture, whereas neokestose was not disclosed [96]. For respective structures, see Fig. 1. Therefore the spectrum of oligomeric reaction products of Lsc3 is similar to that of levansucrases from G. diazotrophicus, Z. mobilis and B. megaterium [50,52,53,93]. A low yield of 6-kestose and absence of neokestose formation differentiate Lsc3 [96] from yeast and fungal β-fructofuranosidases [54–57,59–61]. A highest yield of levan using Lsc3 was obtained when reaction was carried out at moderate temperature (20 or 30°C) and low (300 mM; 102 g/L) concentration of sucrose. Also, the authors recommended to conduct the reaction with a high amount of the enzyme, if the reaction time has to be shortened [94].

A procedure for the reduction of monosaccharide content of the FOS mixture produced by the Lsc3 has been elaborated applying treatment of the FOS mixture with invertase-negative Saccharomyces cerevisiae mutant [96]. Lsc3-produced levan and yeast-treated FOS preparations were used as carbon sources for B. thetaiotaomicron to study the effect of amino acids on fructan fermentation kinetics and product spectrum [96]. B. thetaiotaomicron is abundant in the human gut, has a high potential for polysaccharide degradation [11] and was earlier shown capable of levan usage [68]. The authors show [96] that in addition to levan, B. thetaiotaomicron also grows on levansucrase-synthesized FOS, producing mainly acetate, succinate and propionate as fermentation products. Surprisingly, α-lactate was also detected among the fermentation products, and its presence correlated with extensive usage of certain amino acids such as Ser, Thr and Asp [96] that are major components in gut mucins which are among crucial food sources of colonic bacteria [11].

Importantly, using a modern fully automated chip-based nanoelectrospray mass spectrometry analysis, the ability of Pseudomonas bacteria levansucrases (Lsc3 and LscA) to synthesize heterooligosaccharides was disclosed [84]. When these levansucrases were reacted with equimolar amounts (600 mM) of sucrose and a nonconventional fructosyl acceptor, for example, D-xylose,
xylobiose, xylitol, d-sorbitol, l- and d-arabinose, d-galacturonic acid, heterooligosaccharides were synthesized in addition to traditional FOS [84]. Similarly, some other levansucrases, for example the SacB of B. subtilis have been shown to transfructosylate non-conventional acceptors: mono- and disaccharides, aromatic and aliphatic alcohols [97–100]. Therefore, a broad spectrum of fructosyl acceptors and a wide variety of different transfructosylation products seems to be a common feature of levansucrases.

**Mutation of the Lsc3 and Lsc2 proteins of Pseudomonas syringae pv. tomato**

From levansucrases of *Pseudomonas* bacteria, only Lsc3 and Lsc2 proteins of *Pst* have been addressed by mutagenesis [74,84,90,101]. Key catalytic residues (a catalytic triad) of two levansucrases, Lsc3 and Lsc2 of *Pst*, have been experimentally verified [74,101]. See Fig. 3 for catalytic triad positions Asp62, Asp219 and Glu303 of the Lsc3. When catalytic triad residues of Lsc2 or Lsc3 were replaced by alanines, the resulting mutants could bind the substrate, but were practically incapable of the catalysis [74,101]. Catalytically inactive mutants of levansucrases are valuable because they can be co-crystallized with natural ligands. So, a catalytic triad mutant Glu342Ala of *B. subtilis* levansucrase SacB was co-crystallized with its substrates, sucrose (PDB: 1PT2) and raffinose (PDB: 3BYN), disclosing specific contacts between the protein and a substrate in the catalytic pocket [102,103]. Crystal structures of levansucrases of *Pseudomonas* bacteria are not available yet, but the 3D structures of the Lsc3 and Lsc2 of *P. syringae* pv. tomato have been modeled on the basis of crystal structures of either LsdA of *G. diazotrophicus* [84,85] or β-fructofuranosidase of *Arthrobacter* sp. K-1 [74]. Since publications by Visnapuu and coworkers [84] and Mardo and others [74,90] on the Lsc3 protein, the LevU of *Z. mobilis* was the only levansucrase from Gram-negative bacteria subjected to extensive mutational analysis [91,104,105]. A pioneering study by Yanase et al. [91] disclosed a number of catalysis-related positions in LevU. This paper encouraged the group working on the *Pst* Lsc3 protein to verify significance of these positions in Lsc3 by constructing and characterizing respective mutants [84,90]. In most cases, homologous mutants of these two proteins behaved similarly, for example, the Trp109Arg variant of Lsc3 and the Trp80Arg variant of LevU (Fig. 3) had both largely reduced synthesis of high-molecular levans [90,91]. The Trp80Arg mutant was isolated through random mutagenesis [91]. A number of catalysis-relevant positions of Lsc3 (i.e. Asp31, His113, Asp300 and Thr302) were also identified from random mutants [84,90]. The Asp300 and Thr302 residues of Lsc3 locate close to acid–base catalyst Glu303. The Asp300Asn mutant had gained the ability to synthesize FOS with DP 8, 9 and 10, which were absent among the products of the wild-type enzyme that produced FOS with DP up to 7. It was concluded that Asp300 has a role in polymerization and determination of the chain-length of produced FOS [84,90]. The Asp300Asn mutant of Lsc3 can find application in biotechnology for enzymatic production of long-chain FOS and levans. In case of the Thr302Pro mutant of Lsc3, sucrose cleavage was reduced by 1.5 times and transfructosylation ability was dropped from 74 to 52%. The major polymerization products of this mutant were kestose (DP 3) and nystose (DP 4). Mardo and coworkers [74] conducted a Thr302Met substitution in Lsc3 to mimic respective region of the *Z. mobilis* invertase protein [106]. The affinity of the Thr302Met mutant for sucrose cleavage was increased (Km ~ ~ 15 ms) compared to the Thr302Pro mutant and also to the wild-type Lsc3, making the Thr302Met protein in this respect more similar to invertases (see references in [74]). Arg304 and His113 also proved to be catalytically important positions of levansucrases. Arg304 locates at a position that is fully conserved in GH68 enzymes, next to the acid–base catalyst Glu303 [46,74] and His113 is also conserved in most levansucrases [90]. If Arg304 was replaced with either alanine or cysteine, the kcat value for sucrose cleavage was largely decreased. Both mutants had also severely reduced synthesis of FOS and levan, whereas the Arg304Cys mutant was more seriously affected [74]. Equivalent of Arg304 in *B. subtilis* levansucrase is Arg343 that forms a hydrogen bond with Asp247 which functions as a transition-state stabilizer [102]. The authors suggest that a positively charged polar arginine residue is crucial for that position and that Arg304 could belong to −1 or +1 subsite of Lsc3 possibly participating in binding of both, fructosyl donor and acceptor [74]. Both singly constructed mutants, His113Gln and His113Ala, had largely reduced catalytic activity and the His113Gln variant was more seriously affected (see Fig. 4). The authors suggest that His113 participates in binding of both fructosyl donor (at −1 subsite) and acceptor (at +2 subsite) with the Lsc3 protein (Fig. 3) [90].

Altogether, data on catalytic properties of 36 mutants of the Lsc3 of *Pst* have been published [90]. Figure 4 illustrates sucrose cleavage and FOS production abilities of these mutants and indicates that in case of most mutants, both Vmax of sucrose cleavage and synthesis of fructans are decreased [90]. Affinity of sucrose binding is also reduced in most Lsc3 mutants [74,90]. Thus, it will probably be very complicated to obtain novel levansucrase variants without losing at least some of the catalytic activity. Figure 4 shows that some mutants were specifically affected in polymerization. For example, in substitution mutants of His321 (a homologue of Arg360 of *B. subtilis* SacB and of His296...
of Z. mobilis LevU) [84], His113, Asp300 and Gln301, polymerizing activity of the protein was certainly more than sucrose cleavage. On the contrary, in some Lsc3 mutants (e.g. Thr302Met and Glu146Gln), the FOS synthesis was reduced less than sucrose cleavage. Levansucrases catalyze multiple reactions and according to mutant studies these reactions can be specifically affected. Quite intriguingly, assay of Lsc3 mutants has disclosed several amino acid positions in levansucrases (e.g. Leu66, Pro220, Asp225 and His306 in Lsc3) that tolerate substitutions fairly well despite of their high conservation grade [74]. If a crystal structure of the Lsc3 protein will be resolved, the mutations characterized in Lsc3 protein can be addressed using this information. Also, structure of the E. amylovora levansucrase would greatly assist to interpretation of Lsc3 mutants because of the high sequence identity (77%) between these proteins [101]. The crystals of E. amylovora levansucrase have been obtained [107], but the structure has not been resolved yet.

**Acknowledgements**

Support from grants GLOMR9072 from Estonian Research Council (ERC) and 3.2.0701.12-0041 (SLOMR12215T) from EU Regional Development Fund (ERF) is acknowledged.

**References**


