How lactobacilli synthesize inulin
Anwar, Munir Ahmad

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Chapter 1

General Introduction:
Fructosyltransferase enzymes of lactic acid bacteria: current advancements and emerging concepts

Munir A. Anwar, Slavko Kralj, Hans Leemhuis and Lubbert Dijkhuizen

Submitted for publication
1. Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of Gram positive bacteria that produce lactic acid as a major product during carbohydrate fermentation. These organisms are heterotrophic and generally have complex nutritional requirements because they lack many biosynthetic capabilities. Most species have multiple requirements for amino acids and vitamins, due to which lactic acid bacteria are generally abundant only in habitats where these nutrients are available. Examples of such habitats are mucosal membranes of man and animal (oral cavity, intestine and vagina), plants or material of plant origin, manure, and man-made habitats such as sewage, spoiled food and fermenting food (131,195). The LAB group encompasses the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Alloicoccus*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactospaera*, *Oenococcus*, *Carnobacterium*, *Tetragenococcus*, *Vagococcus* and *Weissella*. These microbes are widely used in the production of fermented food products, such as yogurt (*Streptococcus* spp. and *Lactobacillus* spp.), cheeses (*Lactococcus* spp.), sauerkraut (*Leuconostoc* spp.) and sausage (*Lactobacillus curvatus*, *Lactobacillus sakei*).

LAB are one of the industrially most important groups of bacteria. These microorganisms are used in a variety of ways, such as food production, health improvement and production of macromolecules, enzymes and metabolites (131). LAB maintain remarkable importance in food industry due to their beneficial metabolic capabilities such as carbohydrate and citrate metabolism, proteolysis, production of antimicrobials and the production of an abundant variety of homo- and heteropolysaccharides (35,36,42,69,116,180,193). These microorganisms play an important role in the fermentation of food. Their growth lowers both the carbohydrate content of the foods that they ferment, and the pH due to lactic acid production. This acidification process is one of the most desirable side-effects of their growth and associated metabolic activities, which inhibits the growth of most other microorganisms including most common human pathogens, thus increasing the shelf life of foods. The acidity also changes the texture of the foods due to precipitation of some proteins, and the biochemical conversions involved in growth enhance the flavor and, frequently, the nutritional attributes of the products (7). Also LAB antimicrobial peptides, called bacteriocins, have potential applications in food preservation (31) and preventing pathogen infections (30). There is also a growing interest in LAB and
their polysaccharides because of the health benefits related to their probiotic and prebiotic properties. A probiotic is as a viable microbial dietary supplement that beneficially affects the host through its effects in the intestinal tract (138,172). Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon (44,131). LAB can also be used to produce lactic acid (89) and B vitamins (15). To fully exploit their industrial potential, LAB have been subjected to considerable research involving metabolic pathway engineering and modeling (61,77,172). Comparative and functional genome analysis is used to obtain global insights into physiological and metabolic capabilities of LAB (100,116,154,197). To date, 25 LAB genomes have been sequenced and published (reviewed by Zhu et al. (197)), while several more are in progress. Another recent comprehensive review encompasses current knowledge about several interesting features of lactobacilli including genome-based prediction of their extracellular proteome, comparative analysis of their genes and proteins, and molecular interaction of their specific extracellular components with the host intestinal system (76).

2. Exopolysaccharides of lactic acid bacteria

Some LAB, including species of *Lactobacillus*, are known to produce extracellular polysaccharides often referred to as exopolysaccharides (EPS). These EPSs are large molecules with molecular masses varying between $10^1$ to $10^4$ kDa, and may contain 50 to 50,000 glycosyl units. Depending on the composition of constituting glycosyl units, two types of EPSs are produced by lactic acid bacteria, i.e. heteropolysaccharides, containing different types of sugar moieties, and homopolysaccharides consisting of only one type of glycopyranosyl residue.

Heteropolysaccharides are usually produced in low amounts and mostly glucose, galactose and L-rhamnose are the main constituents (34). They can further be decorated with charged groups like acetate, phosphate or glycerol phosphate. Heteropolysaccharides are produced by many LAB such as *Lactobacillus sakei* (178), *Lactobacillus casei* (80), *Lactobacillus delbrueckii* spp *bulgaricus* (21), *Lactobacillus helveticus*, *Lactobacillus paracasei*, *Lactobacillus acidophilus* (139,140), *Lactobacillus delbrueckii* (51), *Lactobacillus*
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*Pseudomonas rhamnosus* (98,99), *Streptococcus salivarius* ssp. *thermophilus* (1,22,50,103,162), and different *Lactococcus* strains (20,78,111,176). *Lactobacillus gasseri* strain DSM 20243 was also found to have a heteropolysaccharide gene cluster (2)(See also Chapter 3), but heteropolysaccharide production by this strain has not been reported yet.

Homopolysaccharides of LAB consist of either glucose (polymer: \(\alpha\)-glucan) or fructose (polymer: \(\beta\)-fructan) residues with various binding types. They are synthesized by extracellular enzymes of the sucrase type (see below), using a donor molecule, such as sucrose, and an acceptor molecule, e.g. the growing polymer chain (109,182,197). The enzymes responsible for the synthesis of glucose polymers are known as glucosyltransferases (GTFs) or glucansucrases. The sucrase enzymes responsible for the production of polymers consisting of fructose are referred to as fructosyltransferases (FTFs). In the following paragraphs we mainly focus on fructans and FTF enzymes of lactic acid bacteria and their (potential) applications.

3. Fructans

As mentioned above, fructans are homopolysaccharides comprising one type of glycopyranosyl residues, i.e. fructose. Fructans are synthesized by microorganisms and plants. Based on the type of bonds between the fructosyl residues, fructans can be categorized as inulins, levans, mixed levans and neo-series inulins and levans (189). Inulins and levans are linear or branched polymers of fructose, linked through \(\beta(2\text{-}1)\) and \(\beta(2\text{-}6)\) bonds, respectively (Fig. 1). Occasionally, branches are attached to the main polymer chain through \(\beta(2\text{-}1)\) linkages in levans and \(\beta(2\text{-}6)\) linkages in inulins (182). The fructans synthesized from sucrose contain a terminal glucose molecule (40) and are designated as GF\(_n\), where G refers to the terminal glucose unit, F refers to fructose units and n is the number of fructosyl units per fructan molecule, also designated degree of polymerization (DP). Short chain fructan oligosaccharides are usually labelled fructo-oligosaccharides (FOS).
Inulin polymers from bacteria have a DP of 20-10,000 fructosyl units, while plant inulins are relatively small polymers usually consisting of 30 to 150 fructosyl units per fructan molecule (182). Similarly, bacterial levans have a DP > 100 fructosyl residues, while plant levans usually have a DP < 100. In some cases, the molecular weight of the fructan produced is dependent on bacterial growth/incubation conditions, e.g. the temperature, salinity, and sucrose concentration used (12,58,167,168). Mixed levans, known as gramminans, are produced by plants and have both β(2-1) and β(2-6) linked fructosyl residues. Neo-series fructans are also produced by plants (189). In inulin and levan neo-series, two inulin or levan type fructosyl chains are attached to the sucrose starter unit, respectively. One chain is linked to the C1 and the other to the C6 of the glucose residue.

Fructans have a multitude of application for food and health, and in industry. Possible applications of inulin and its oligosaccharides for food and health have been
reviewed earlier (71). The probiotic role of several lactobacilli has been attributed to the synthesis of prebiotic fructans from sucrose involving FTF enzymes (4,81,117). Inulin-type fructans of higher degree of polymerization are of particular interest due to their demonstrated pronounced in vitro prebiotic effects (177). In the food industry, inulin is also used as fat replacer, and for providing texture and stability in several products, such as desserts, bakery and fermented dairy products, as well as infant formula (138). Inulin polymers also have potential application as surfactants. For instance, carbamoylated inulin reduces the interfacial tension, thus providing a biodegradable surface-active agent (161). Levan was found to counteract inflammatory reactions to skin irritants in artificially generated skin from human cell lines (3-D bio-artificial skin). Levan also exerted a cell-proliferative effect in bio-artificial skin, thereby indicating its potential applicability as a cosmeceutical agent (72). Levan also has found application as a biological glue (http://www.polysaccharides.us/levanadhesive_summary.php). Populations of Paenibacillus polymyxa are frequently found in the rhizosphere of various crop plants. High-molecular weight levan produced by these bacteria is implicated in the aggregation of root-adhering soil on wheat (14).

4. Physiological functions of fructans

Although the physiological roles of exopolysaccharides/fructans in bacteria have not been clearly established yet, the available data suggest that these are related to the bacterial ecological niches, providing protection against various environmental stresses, and serving in energy storage. Most bacteria that synthesize fructans, interact with eukaryotic hosts and their interactions with the environment are related to the presence of fructans (189). The polymers may also render protection to microbial cells in their natural environment, against desiccation, phagocytosis and phage attack, antibiotics or toxic compounds (e.g. toxic metal ions, sulfur dioxide, ethanol), predation by protozoans, and osmotic stress. They may also play a role in adhesion of cells to solid surfaces and biofilm formation, and also in cellular recognition (20). Fructans (and glucans) may also contribute to the provision of reduced oxygen tension, of strong relevance to facultatively anaerobic lactic acid bacteria, and participate in the uptake of metal ions (20). Enhanced expression of FTF enzymes was observed during growth of Lactobacillus reuteri under environmental conditions that affect
biophysical properties of the cytoplasmic membrane, indicating that fructans are involved in resistance to certain stresses (148). Accordingly, it was shown in the same study that fructans (inulin) protected cells of *Lb. reuteri* TMW1.106 during freezing and drying.

*Streptococcus mutans* and *Streptococcus sanguis* are commonly found in the oral cavity, associated with dental plaque formation, and are therefore considered causal agents of dental caries (9). Fructans (and glucans) may play a role in the adhesion and colonization of cariogenic bacteria, thus contributing to the formation of dental plaque biofilm (143). Other examples of fructans that function as adhesive agents and facilitate interactions between bacteria and their hosts are (i) levan production by the sugar cane plant root invading Gram-negative *Gluconacetobacter diazotrophicus* (164) and (ii) the persistence and virulence of *Actinomyces naeslundii* and *Actinomyces viscosus* in the mammalian oral cavity (13).

The physiological roles of microbial fructans also have been elucidated by analyzing the effects of disruption of *ftf* genes. For instance, disruption of the *ftf* gene of *Strep. mutans* strain V403 resulted in a decrease in virulence to about 70% of the cariogenic activity of the wild-type strain (147). *Erwinia amylovora* is a pathogen of Pomoideae plants such as apple, pear and rose. Studies involving disruption of the levansucrase gene of this bacterium have suggested that levansucrase activity acts as a virulence factor in this pathogen (43,79). Similarly, disruption of the levansucrase gene resulted in decrease in the severity of infection of *Pseudomonas syringae*, which is a causal agent of blight disease in soybean leaves (94). *Lactobacillus* spp. synthesizing glucan and fructan polymers are common inhabitants of the mammalian and avian gastrointestinal tracts. Gene disruption studies have led to the conclusion that the glucansucrase GtfA and inulosucrase Inu enzymes confer important ecological attributes of *Lb. reuteri* TMW1.106 and contribute to colonization of the mouse gastrointestinal tract (192).

In several *Bacillus* species and other bacterial strains within biofilms in oral cavities, fructans also serve as extracellular carbohydrate storage compounds that can be metabolized by bacteria during periods of nutrient deficiency (16,20,28,45). Also *Lactobacillus paracasei* subsp. *paracasei* 8700:2 has been shown to metabolize inulin type fructans/fructooligosaccharides producing lactic acid as the main end product (101). In another study, *Lb. gasseri* CECT5714 and *Lactobacillus fermentum* CECT5716 were found
to metabolize 1-kestose (10). In our studies with *Lb. johnsonii* NCC 533 (3) (Chapter 2) and *Lb. gasseri* strains DSM 20077, 20243, 20604, (2) (Chapter 3), we have not seen degradation of inulin polymeric material.

A diverse number of naturally-occurring *Lactobacillus* species inhabit the gastrointestinal (GI) tract of man and animals and are widely considered to exert a number of beneficial effects on human health. Among these, *Lactobacillus johnsonii* strain NCC 533 belongs to the acidophilus group of intestinal lactobacilli that have been extensively studied for their probiotic activities, pathogen inhibition, epithelial cell attachment, and immunomodulation (32,47-49,64,74,115). The food company Nestlé uses this microorganism in a yogurt-like dairy product called LC1, which, according to the company, strengthens the body's natural defenses and keeps the bowel healthy (41). *Lb. gasseri*, which is speculated to be an autochthonous human intestinal organism (8), constitutes the major part of homofermentative *Lactobacillus* species occupying the human GI tract (88). *Lb. gasseri* is associated with a variety of probiotic functions including reduction of activities of fecal mutagenic enzymes (127), adherence to intestinal tissues (29,74) stimulation of macrophages (73,171), and production of bacteriocins (65).

5. Fructan Synthesis

Fructans are synthesized by a wide variety of microorganisms and plants. Levans are mainly synthesized by diverse types of Gram-positive and Gram-negative bacteria, whereas inulin production is mainly limited to plants and a few species of lactic acid bacteria. Among lactic acid bacteria, levan production has been reported for streptococci (19,54,155), *Leuconostoc mesenteroides* (141), *Lb. reuteri* 121 (181,183), *Lb. gasseri* (2) (Chapter 3), *Lactobacillus sanfranciscensis* (82), *Lactobacillus panis* (174,191). Also *Lactobacillus frumenti*, *Lactobacillus pontis* and *Weissella confusa* were found to produce fructans, but the linkage types in the fructan products have not been determined (102,174). Synthesis of β(2-1) and/or β(2-6) linked fructooligosaccharides have also been reported in some fungi and yeasts (60,67,91,136,187). Levan production has been studied in most detail in non-LAB, *G. diazotrophicus* (56), *Zymomonas mobilis* (90,158), and *Bacillus* sp. (97,129,166). In contrast to the wealth of information about levan synthesis, till recently only a few inulosucrase enzymes were known, and no experimental evidence was available for in situ production of
inulin by lactic acid bacteria. We have identified \textit{Lb. johnsonii} NCC 533 (Anwar \textit{et al.}, 2008) and \textit{Lb. gasseri} DSM 20604 (2) to produce inulin \textit{in situ} (also see chapters 2 and 3).

6. Fructansucrase enzymes of bacterial origin

Whereas plants employ two consecutive FTF enzyme steps for fructan synthesis (137,179), bacteria use a single FTF or fructansucrase (FS) enzyme for fructan synthesis. Two types of fructan synthesizing enzymes are known, the inulosucrases, synthesizing $\beta(2\rightarrow1)$ linked polymer inulin, and levansucrases, responsible for the synthesis of $\beta(2\rightarrow6)$ linked polymer levan. Each of these enzymes is specific for the types of linkages that are synthesized in their respective FOS and polymer products.

Levansucrase genes/enzymes have been found in a large variety of Gram-positive (including LAB and non-LAB) and Gram-negative bacteria. Among LAB, levansucrase enzymes have been characterized from \textit{Strep. salivarius} (157), \textit{Strep. mutans} (153), \textit{W. confusa} (102), \textit{Lb. reuteri} (183,184), \textit{Lb. sanfranciscensis} (82,173), \textit{Lb. panis} (191), \textit{Leuc. mesenteroides} (70,110,121) and \textit{Lb. gasseri} DSM 20077 (2) (chapter 3). Non-LAB with levansucrase enzymes include \textit{Z. mobilis} (53,158), \textit{Erwinia amylovora} (43,52), \textit{G. diazotrophicus} (5), \textit{Bacillus polymyxa} (14), \textit{Bacillus amyloliquefaciens} (170), \textit{Geobacillus stearothermophilus} (97), \textit{Microbacterium laevaniformans} (Park et al 2003), \textit{Bacillus megaterium} (62) and \textit{B. subtilis} (160). In contrast to levansucrases, inulosucrases have been characterized only from a few species of Gram-positive bacteria (all lactic acid bacteria except \textit{Bacillus sp.}) including \textit{Strep. mutans} (142), \textit{Leuc. citreum} CW28 (119,120), \textit{Lb. reuteri} 121 (186), \textit{Bacillus sp.} (190), \textit{Lb. reuteri} TMW 1.106 (149), \textit{Lb. johnsonii} NCC 533 (3) and \textit{Lb. gasseri} (2)(see also chapters 2 and 3). A phylogenetic tree of known FTF proteins is shown in Fig. 2. Whereas FTFs of Gram-positive and Gram-negative bacteria are clearly separate phylogenetically, no such clear separation is observed between inulosucrase and levansucrase enzymes of Gram-positive bacteria. Nevertheless, the inulosucrases form a small but distinct group indicating that there are small differences between these two groups of closely related enzymes. Conversely, there should be additional similarities (or conserved motifs) among these inulosucrase proteins that cause them to cluster together. Analysis of such specific sequence features in inulosucrase enzymes may provide information about their specific functional properties, i.e. ability to synthesize $\beta(2,1)$ linkages.
Fig. 2. Phylogenetic tree of all bacterial fructosyltransferase (FTF) proteins (family GH68 proteins: Lev, levensucrase; Inu, inulosucrase). FTF designation was used for proteins that have not been characterized with respect to products synthesized. The FTFs characterized in this thesis are shown in
bold. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (165). Numbers at the nodes indicate confidence bootstrap percentages of 500 repeats. Scale bar indicates number of changes per amino acid. Sequences are represented by the names of the microorganisms followed by the GenBank accession number of the corresponding fructosyltransferase protein. Branches represented by more than three proteins of the same species were compressed and are composed of the following sequences: for *Pseudomonas syringae* AAC36056, AAK49951, AAK49952, AAY35812, AAY37146, AAZ36063, AAZ38051, AAC36063, AAZ37865, AAO54974, AAO55819, AAO59056; for *Zymomonas mobilis* AAA27702, AAA27695, ACV75412, ACV75413, AAG29870; for *Leuconostoc mesenteroides* AAY19523, AAT81165, ABJ62502, ABJ62503, ABJ62504. FTFs of various *Burkholderia* sp. that clustered closely were also compressed to avoid complexity in the phylogenetic tree. These include: *Burkholderia cenocepacia* ABF78985, ABK11005, CAR55261, *Burkholderia mallei* AUA46678, ABN00553, ABO03647, ABA51542, *Burkholderia pseudomallei* ABN94007, ABA51542, ABN86027, CAH38000; *Burkholderia thailandensis* ABC36154; *Burkholderia vietnamiensis* ABO57793; *Burkholderia phymatum* AAC75109. FTF sequence of *Lb. reuteri* 100-23 was obtained from http://genome.jgi-psf.org/lacro/lacro.home.html by “tblastn” using *Lb. reuteri* 121 levansucrase as query sequence and *Lb. panis* levansucrase sequence was obtained from (191); no accession numbers are available for these proteins.

7. Mechanism of fructan synthesis

Bacterial FTF enzymes belong to the Glycoside Hydrolase (GH) group of carbohydrate active enzymes. Glycoside hydrolases have been classified into 115 families (www.cazy.org) (18) on the basis of their amino acid sequence similarities, which imply both structural and mechanistic relationships (55). The inulosucrase (E.C.2.4.1.9) and levansucrase (E.C.2.4.1.10) enzymes belong to family 68 (GH 68) of carbohydrate active enzymes. Family GH68 enzymes retain the configuration at the anomeric carbon in their catalytic reactions. A ping-pong type reaction mechanism involving the formation of a transient fructosyl-enzyme intermediate has been demonstrated for the *B. subtilis* (26), *G. diazotrophicus* (56) and *Strep. salivarius* (156) FTF enzymes.
Fig 3. Schematic presentation of reactions catalyzed by FTF enzymes showing the involvement of the three catalytic residues. This mechanism has been deduced taking into account the information available at http://www.cazypedia.org/index.php/Glycoside_Hydrolase_Family_68, (133), (62), (106) and (144).

In retaining enzymes, hydrolysis with net retention of configuration is most commonly achieved via a two step, double-displacement mechanism involving a covalent glycosyl-enzyme intermediate (Fig. 3). Each step passes through an oxocarbenium ion-like transition state (http://www.cazypedia.org/index.php/Glycoside_Hydrolase_Family_68) (133,144). Three invariant residues, including two aspartates and one glutamate, have been identified essential for catalysis in family GH68 enzymes (see section 10.2.2 for details). The reaction is catalyzed by one of the aspartates (acting as nucleophile) and the glutamate.
(acid/base catalyst), while the second aspartate does not participate in the reaction but acts as a transition state stabilizer. In the first step, the nucleophile attacks the anomeric carbon while simultaneously the acid/base catalyst protonates the glycosidic oxygen forming an oxocarbenium-ion like transition state, which subsequently ends in cleavage of the glycosidic bond with the release of a glucose molecule and formation of fructosyl-enzyme intermediate (Fig. 3). In the second step, the fructosyl-enzyme is either hydrolyzed by water, or the fructosyl unit is transferred to an acceptor molecule (e.g. sucrose) via oxocarbenium-ion transition state. The transfer of a fructosyl unit to acceptors other than water (e.g. sucrose, oligosaccharides, growing polymer chain etc.) is referred to as transglycosylation activity, whereas its transfer to a water molecule results in hydrolytic activity. The sum of transglycosylation and hydrolytic activities is called total activity. FTF enzyme activities are measured by determining the amounts of glucose and fructose released from sucrose by the enzyme. The amount of glucose ($V_G$) formed reflects the total amount of sucrose utilized during the reaction (total activity). The amount of fructose ($V_F$) formed is a measure of the hydrolytic activity. The transglycosylation activity is calculated by subtracting the amount of free fructose from glucose ($V_G - V_F$).

Levansucrases also use various types of acceptor substrates other than sucrose and water. For instance, *B. subtilis* levansucrase can use monosaccharides (D-glucose, D-mannose, D-xylose, and D-galactose) as acceptor substrates to produce various di-, tri-, and tetrasaccharides (with sucrose as donor substrate) (169). Another independent study with *B. subtilis* levansucrase shows that, in addition to several monosaccharides, many disaccharides such as maltose, isomaltose melibiose, cellobiose and lactose, can also be used as acceptor substrates (150). *Lb. sanfranciscensis* levansucrase uses raffinose, maltotriose, maltose and xylose as acceptors producing a range of heterooligosaccharides (175). Levansucrase from *Leuc. mesenteroides* B-512 FMC can use maltose as an acceptor, producing erlose (maltosylfructose: Glu-Glu-Fru) (70). This trisaccharide was also produced by *B. subtilis* levansucrase using maltose as an acceptor (17,150). In addition, synthetic sucrose analogues can be used as acceptor substrates in the FTF reaction to produce novel FOS, which have the potential to be used as prebiotics or dietary fiber in food and health products, as well as synthons to synthesize glycosylated drugs or pharmaceutical ingredients (84).
8. Biochemical properties of bacterial FTF enzymes

A detailed survey of general kinetic properties of levansucrase and inulosucrase enzymes has been presented recently (189). It appears that most levansucrases are monomeric enzymes with a molecular weight of 46–73 kDa, with the exception of *Rahnella aquatilis* JMC-1683 and *Leuc. mesenteroides* B-512 FMC levansucrases that form dimers and have molecular weights of 120 kDa and 103 kDa, respectively (70,118). Optimum pH values of levansucrases range from 5.0 to 6.2, while pI values range between 2.6 and 5.5. A great variation is found in the sucrose Km values of levansucrases, which range from 4 to 160 mmol l$^{-1}$ (189). The molecular weights of inulosucrases range from 83 to 170 kDa, their pH optima range from 4.5 to 7.0 and pI values from 4.5 to 5.5 (2,3,120,186). The maximum enzyme activity of most levansucrases lies between 28 and 55 ºC, except for *Pseudomonas syringae* pv. phaseolicola and *R. aquatilis* enzymes that have temperature optima at 18 ºC and 60 ºC, respectively (189). Inulosucrases display relatively high temperature optima, i.e. from 45 ºC to 55 ºC. (2,189). No FTF enzymes have been characterized yet from acidophilic, alkalophilic, or thermophilic bacteria.

A requirement for metal ions as cofactor has been reported for the *Lb. reuteri*, *Leuc. mesenteroides*, *B. subtilis*, *Strep. salivarius*, *Lb. panis* and *Lb. gasseri* levansucrases, and *Lb. reuteri*, *Lb. johnsonii* and *Lb. gasseri* inulosucrases, all requiring Ca$^{2+}$ for optimum activity (2,3,66,108,110,123,184). In the presence of Ca$^{2+}$, Inu and Lev showed considerably enhanced activities at high temperatures, and strongly increased thermostability. The activity and stability of FTF enzymes of Gram-negative origin, such as the levansucrases of *R. aquatilis* and *P. syringae*, apparently are not affected by the presence of calcium (58,118).

Available data shows that levansucrases are relatively more hydrolytic as compared to inulosucrases: the *Lb. reuteri* and *Lb. gasseri* levansucrases for instance exhibited transglycosylation/hydrolysis ratios of 2.6 and 0.2, respectively, whereas for the *Lb. reuteri*, *Lb. johnsonii* and *Lb. gasseri* (InuG-BR) inulosucrases ratios of 4.3, 26.0 and 4.0 were measured, respectively (2,3,124). The transglycosylation/hydrolysis ratio was also found to be changed by immobilization of the enzyme on hydroxyapatite, mimicking *in vivo* conditions where levansucrases are attached to the cell wall of bacteria or to tooth surfaces. Immobilization of *B. subtilis* levansucrase shifted activity mainly to polymerization (25). For several FTF enzymes it has been observed that higher sucrose substrate concentrations favor
transglycosylation activities (121,184,185). Also the presence of low molecular-weight fructans was shown to accelerate the rate of polysaccharide formation and to increase the fructan to free fructose product ratio (37,185).

Where studied, most FTF enzymes follow Michaelis-Menten type of kinetics for the hydrolysis and transglycosylation reactions (173,184,191). Exceptions to this rule are the *Lb. reuteri* inulosucrase and levansucrase enzymes, and the *Lb. sanfranciscensis* levansucrase which cannot be saturated by their substrate sucrose (in case of the *Lb. reuteri* levansucrase only at 50 °C) (173,184,185). This phenomenon has also been observed for total and transfructosylation activities of the *Lb. johnsonii* and *Lb. gasseri* inulosucrases (2,3)(See also chapters 2 and 3). The non-Michaelian behaviour of the *Lb. reuteri* 121 and *Lb. johnsonii* inulosucrases has been attributed to the fructooligosaccharides that were formed at the early stage of reaction (125) (3). FTF activity thus results in a range of growing fructooligosaccharide chains and new fructan molecules, which in turn can be used as acceptor substrates, complicating a detailed kinetic characterization.

9. Expression, secretion and folding of FTF proteins

Based on available data, induction of *ftf* gene expression seems to be strain dependent and no uniform expression pattern has been observed among Gram-positive or Gram-negative bacteria. For instance, whereas expression of *B. subtilis* (128), *Lb. reuteri* (181) and *Strep. mutans* (96) levansucrases is reported to be induced by sucrose, the *Strep. salivarius* FTF expression is not induced by sucrose; rather, sucrose stimulates release of the FTF protein from the cell-wall (108), thus causing an increase in soluble FTF activity. A few reports indicate that FTF expression was influenced by environmental pH, growth rate and carbon source (63,75,96,194). A more recent study showed that expression of FTF was negatively regulated by the *covRS* two-component regulatory system in *Strep. mutans*. It was hypothesized that *Strep. mutans* senses the sucrose level in the environment, resulting in derepression of *ftf* transcription at low sucrose concentrations (93). Sugars like sucrose, D-glucose, D-fructose, D-glucitol, D-mannitol and xylitol may enhance the expression level of FTF enzyme in *Strep. mutans* (152). Induction of FTF enzyme expression may also be independent of the carbohydrate source as in case of *Act. naeslundii* levansucrase (13). Whereas the levansucrase gene of the Gram-negative *G. diazotrophicus* is expressed
constitutively (56,57), a growth phase-dependent regulation of levansucrase expression has been reported for another Gram-negative bacterium, *R. aquatilis* (151). Similarly, a more recent transcriptional analysis study, using quantitative real time PCR, revealed that expression of *ftfA* of *Lb. reuteri* LTH5448 was induced by sucrose, while sucrose had no effect on *gtfA* and *inu* expression of *Lb. reuteri* strain TMW 1.106 (149).

Secretion of levansucrase proteins has most extensively been investigated in *B. subtilis* and a two-step mechanism has been postulated for its translocation across the membrane (Petit-Glatron et al., 1987). Two different transient forms of the levansucrase protein, of 53,000-Mr and 50,000-Mr, were found to be located in the membrane compartment of *B. subtilis* QB112 cells induced for synthesis of the extracellular form of the levansucrase. The two step secretion mechanism postulates that the cellular events occur according to the following sequence: (i) translation of the levansucrase mRNA into a 53,000-Mr protein which is attached to the cellular membrane, (ii) covalent modification yielding a protein of 50,000-Mr, followed by (iii) secretion of the 50,000-Mr form. Processing of the 53,000 Mr to the 50,000 Mr membrane-form of the protein involves cleavage of signal peptide by signal peptidase. Pulse-labelling experiments indicated that the second step of levansucrase translocation is an active transport step and it appears to be directly linked to the influx of iron into the cell (23). Inside the cell, under cytosolic conditions (pH 7, 37 °C, and absence of free metals such as Ca$^{2+}$ and Fe$^{3+}$), the newly synthesized protein remains unfolded. After spontaneous insertion into the membrane bilayer and cleavage of the signal sequence, the protein is released extracellularly and refolded. Ca$^{2+}$ and Fe$^{3+}$ ions catalyze levansucrase folding outside the cell (130). By site-directed mutagenesis, it was also shown in the same study that the residue G366 was involved in the metal-dependent refolding of the protein after secretion by the organism.

The levansucrase (LsdA) of the Gram-negative bacterium *G. diazotrophicus* is also secreted by a two-step mechanism, which is different from the *B. subtilis* levansucrase translocation. This mechanism involves the removal of a 30-residue N-terminal signal peptide during transport to the periplasm and transfer of the folded mature protein across the outer membrane via a type II secretion system. The LsdA signal peptide has the essential features of a Sec-dependent signal peptide (6). In contrast, other Gram-negative bacteria secrete levansucrases by a signal-peptide-independent pathway (94,158,159,163).
10. Structural aspects of FTF enzymes

10.1. Three Dimensional Structures

Based on their common protein fold, some of the GH families are grouped at a higher hierarchical level described as clans. Presently, 50 GH families have been grouped into 14 clans (A to N). Among these, the glycoside hydrolase family GH68 is grouped in Clan-J together with the family GH32, which includes yeast and plant fructosyltransferases (112,132), both families having 5-fold β-propeller topology. Whereas six 3D structures have been resolved for different family GH32 proteins (reviewed by (92) and (27,188)) to date, only two 3D structures have been published for family GH 68 members. Both of these are levansucrase proteins: the \textit{B. subtilis} levansucrase and the \textit{G. diazotrophicus} levansucrase.

Initially, the high-resolution 3D structure for \textit{B. subtilis} levansucrase (PDB ID: 1OYG) and a sucrose-bound inactive mutant (E342A) (PDB ID: 1PT2) of the same enzyme were elucidated at a resolution of 1.5 Å and 2.1 Å, respectively (106). Recently, a raffinose (Frc-Glc-Gal) bound complex with a mutated levansucrase (E342A) (PDB ID: 3BYN) has also been resolved (107). The overall structure displays a rare five-fold β-propeller topology, with the active site located at its center, in a deep, negatively charged pocket surrounded by several conserved residues (Fig. 4 a). The fructosyl unit of sucrose is bound at the bottom of this pocket (subsite -1, nomenclature according to Davies et al. (33)), with three absolutely conserved acidic residues D86, D247, and E342 approaching it. The acid/base catalyst E342 forms a salt bridge with R246 and a strong hydrogen bond to the Tyr411 hydroxyl which, in turn, forms a hydrogen bond with R360. R360 is located immediately adjacent to the central pocket, in the vicinity of E342 and E340. Both R360 and E340 form tight H-bond with the glucosyl moiety in the +1 subsite (Fig. 4 b). These specific contacts in the –1 and +1 subsite lock the fructosyl and glycosyl moiety into a defined orientation, allowing catalysis. The –1 subsite is highly specific for accommodating fructose units, whereas the +1 binding site might show more flexibility, allowing binding of glucose (sucrose or raffinose as donor substrate) and fructose (sucrose or fructans as acceptor substrate) (107,124). The structure of the mutant E342A complexed with raffinose revealed that the galactosyl moiety of raffinose protruded out of the active site, while specificity-determining contacts were essentially restricted to the sucrosyl (Frc-Glc) moiety (107).
Fig. 4. The overall 3D structure of the *B. subtilis* levansucrase (1OYG), showing the three catalytic residues (a), and a closer view of the catalytic core with bound sucrose molecule (1PT2) showing +1/-1 subsite residues (b) (106).

The 3D structure of the levansucrase (LsdA) protein of the Gram-negative, non-LAB *G. diazotrophicus* (PDB ID: 1W18) displays the same five-bladed β-propeller architecture in which the catalytic residues are perfectly superimposable with their equivalents in the *B. subtilis* levansucrase (104,105). It is worthy to mention that *G. diazotrophicus* levansucrase contains E340 and R360, equivalent to N399 and H419, respectively, of *B. subtilis* levansucrase.

The specific structural features in the levansucrase and inulosucrase enzymes determining their glycosidic bond specificity remained to be determined. Only recently, a 3D structure has become available for an inulosucrase protein. In collaboration with Tjaard Pijning and Bauke Dijkstra (Biophysical Chemistry, University of Groningen) attempts to crystallize available inulosucrase proteins were made. Properly diffracting crystals were obtained with a truncated form of the *Lb. johnsonii* inulosucrase carrying a C-terminal Histag (InuJ145-708His), resulting in elucidation of a high (1.75 Å) resolution. The InuJ inulosucrase structure shows the typical five-fold β-propeller topology of the catalytic core domain (residues 210-662) present in levansucrases (Fig. 5) (see also chapter 4). Whereas
the InuJ subsite -1 is virtually identical to that of SacB and LsdA, structural differences do occur in the InuJ acceptor binding subsite architecture giving rise to an acceptor binding pocket with different properties compared to levansucrase proteins. InuJ also contains a unique helix (α7) and a unique 2-stranded antiparallel β-strand (β2-β3) packing against the side of blade 1, which are absent in SacB and LsdA (for more details, see chapter 4).

Fig. 5. The overall 3D structure of the *Lb. johnsonii* inulosucrase (InuJ), showing the three catalytic residues (see chapter 4 for more details).

### 10.2. Protein Assembly and structure-function Studies

Based on amino acid sequences, the microbial FTF enzymes can be divided into four domains of secondary structure (Fig. 6): (i) a signal peptide, (ii) an N-terminal domain, (iii) a catalytic core, and (iv) a C-terminal stretch, in some cases containing a cell-wall anchoring binding domain. Characteristics for each domain are discussed below.

#### 10.2.1. The signal peptide and N-terminal variable domain

Bacterial FTFs are extracellular enzymes, containing a secretion targeting signal sequence of about 36 amino acids at the start of the N-terminus (Fig. 6). The signal-peptide containing precursor is cleaved upon secretion of FTFs by Gram-positive bacteria (13,14,97,134,145,160,170). The N-terminal domain of FTFs is variable in length and no
function has yet been assigned to this domain. In *Lb. sanfranciscensis* TMW1.392 levansucrase, the N-terminal domain has a 16-amino-acid sequence (DNATSGSTKQESSIAN) that is repeated seven times. However, truncation of these N-terminal repeating units had no effects on the kinetic properties of the enzyme or on the product spectrum produced from sucrose (173).

![Schematic presentation of lactic acid bacterial FTF proteins showing secondary structure domain organization.](image)

**Fig. 6.** Schematic presentation of lactic acid bacterial FTF proteins showing secondary structure domain organization. The conserved amino acid sequence motifs with the 3 catalytic residues are shown in sequence logos created by using WebLogo version 2.8.2 (www.weblogo.berkeley.edu). Amino acid sequences of levansucrases of *Strep. salivarius* (AAA71925), *Strep. mutans* (AAA88584), *Lb. reuteri* 121 (AAO14618), *Lb. sanfranciscensis* (CAD48195), *Leuc. mesenteroides* (AAY19523), *Lb. gasseri* DSM 20077 (ACZ67287) and inulosucrase of *Leuc. citreum* CW28 (AAO25086), *Lb. reuteri* 121 (AAN05575), *Lb. reuteri* TMW 1.106 (CAL25302), *Lb. johnsonii* NCC 533 (AAS08734), *Lb. gasseri* DSM 20243 (BK006921) and *Lb. gasseri* DSM 20604 (ACZ67286) were aligned with ClustalX (68) for creating sequence logos.

A signal peptide at the N-terminus domain of an FTF protein of a Gram-negative bacterium has only been reported for the levansucrase of *G. diazotrophicus*. This signal peptide is located in the first 30 amino acids, which are recognized by a type II secretion system (6). With this exception, other FTF enzymes of Gram-negative origin apparently are secreted by signal-peptide-independent pathways (6,43,59,158,159,163). FTFs of Gram-
negative bacteria have a conserved motif in the N-terminal domain, with the sequence WT(R/I)ADA(L/M), which is also present in internal membrane-located proteins (163).

10.2.2. The core region

The catalytic domain of microbial FTFs consists of about 450 to 500 amino acids and contains several highly conserved motifs. The functionally more important motifs of this domain are shown in Table 1 (motifs I to IX). These motifs were deduced from amino acid alignments made with ClustalX (68) and selected on the basis of available data depicting their importance. Most FTF structure-function studies have focused on determining the roles of amino acids in the catalytic domain. The 3D structure of the *B. subtilis* levansucrase has provided important insights in functional roles of several conserved amino acid residues in this core region (106). Firstly, all the GH68 family members contain an absolutely conserved Asp residue in this domain (Table 1, motif I) corresponding to D86, which was identified as the catalytic nucleophile based on the 3D structural analysis and site directed mutagenesis of *B. subtilis* levansucrase and by the mutagenesis study of its corresponding residue (D272 Inu, D249 Lev) in *Lb. reuteri* FTFs mutants (106,126). Secondly, an RDP amino acid motif (Table 1, motif V) is present in a conserved position in the core region of FTF enzymes. Mutation of the aspartate amino acid residue in this motif of *Strep. salivarius*, *G. diazotrophicus*, and *Z. mobilis* levansucrases resulted in a dramatic decrease in catalytic activities while the affinities for their substrate sucrose remained almost unaltered (11,156,196). Analysis of the 3D structure of *B. subtilis* SacB revealed that this D247 residue in the RDP motif, although required for catalysis, apparently is not directly involved in the FTF reaction mechanism (i.e. bond making or bond breaking): It is involved in stabilization of the transition state reaction intermediate (106). Thirdly, a drastic decrease in catalytic activity of the *Z. mobilis* levansucrase was observed on mutation of E278 (196). This amino acid residue is present in motif VI (glutamate adjacent to arginine) (Table 1). Analysis of the sucrose-bound structure of a *B. subtilis* levansucrase inactive mutant (E342A) revealed that the E342 residue, corresponding to the *Z. mobilis* E278 residue, functions as a catalytic general acid. The nucleophile, general acid/base catalyst and transition state stabilizer residues have also been identified in *B. megaterium* levansucrase by site-directed mutagenesis (62).
Two further highly conserved regions (Table 1, motifs II and IV) among FTFs have been designated as “sucrose binding boxes” (146). In the E117Q mutant (motif II) of *Z. mobilis* levansucrase the catalytic turnover was similar to that of the wild-type enzyme, while greater transfructosylating activity was observed (196), indicating direct involvement of motif II in catalysis of transfructosylation. Based on the analysis of the activity of site directed mutants D312E/S/N/I/K located in motif III (Table 1) of *Strep. salivarius* levansucrase, it has been suggested that this Asp residue is most likely involved in determining acceptor recognition, or stabilization of a β-turn in the protein (156). The 3D structure of the *B. subtilis* levansucrase shows that D312 indeed forms a 180º reverse β-turn between motifs II and IV and is located on the surface of the protein, far from the active site (106).

The coordination geometry and the protein-metal distances in the *B. subtilis* levansucrase 3D structure suggest the presence of a Ca$^{2+}$ site, as expected from biochemical studies (106). The residue that is involved in Ca$^{2+}$ binding in *B. subtilis* levansucrase has been identified as D339, and equivalent residues have been proposed as D520 and D500 for the *Lb. reuteri* Inu and Lev proteins, respectively (123). Sequence alignments of GH68 proteins revealed that residues involved in calcium binding are conserved in most enzymes of Gram-positive bacteria, but are absent in proteins of Gram-negative bacteria (123,182). A disulphide bridge in FTFs of Gram-negative bacteria may serve the same purpose, as has been observed in the 3D structure of the levansucrase of *G. diazotrophicus*. It has been suggested that these features have a role in the maintenance of protein fold stability (104).
Functional roles of some other conserved amino acids present in the catalytic core of FTF enzymes have also been determined by site-directed mutagenesis. For instance, a spontaneous mutation R331H in levansucrase of a mutant B. subtilis strain QB252 yielded an enzyme with decreased polymerase activity relative to hydrolysis. Reverting this mutation (H331R) by site-directed mutagenesis restored polymerase activity to the level observed for the wild-type enzyme (24). Further, introducing the mutations R331K, R331S, and R331L at this position resulted in the loss of ability of the enzyme to synthesize levan from sucrose as a single substrate. It was only able to catalyze the first step of levan synthesis, i.e. production of the trisaccharide kestose (GF$_2$). These studies lead to the conclusion that the nature of the amino acid at this position modulates the specificity and the efficiency of the transfructosylation process (24). It should be mentioned that this R331 residue is equivalent to R360 of B. subtilis levansucrase, whose 3D structure has been elucidated (section 10.1). R360 is conserved in levansucrases from Gram-positive bacteria, whereas a histidine is present at the equivalent position in levansucrases from Gram-negative bacteria. Replacing the R360 counterpart in Z. mobilis levansucrase (H296) with arginine led to a reduction in transfructosylation activity to 20% of the wild-type level and accumulation of a small amount of oligosaccharides (196). On the basis of modeling and site-directed mutagenesis experiments it was proposed that the corresponding H296 in Z. mobilis acts as an acceptor substrate recognition and binding site (95). However, subsequently the Z. mobilis levansucrase was found to exist in two active forms - micro-fibrils and dimer, depending upon the pH and ionic strength. It was suggested that H296 might be a crucial amino acid for generating enzyme micro-fibrils associated with the capacity for levan polymerization (46). A H296Arg mutant indeed failed to form levansucrase micro-fibrils and levan polymers.

Comparison of 3D structures of G. diazotrophicus (LsdA) and B. subtilis (SacB) levansucrases revealed that both FTF enzymes possess identical active sites. Nevertheless, LsdA synthesizes a large amount of FOS (1-kestotriose) while SacB produces mainly large levan polymer (104). The mechanism underlying these differences has not been identified. Subsequent site-directed mutagenesis studies with Inu of Lb. reuteri 121 showed that amino acid residues (W271, W340 and R423) located at the (putative) -1 donor substrate sugar binding subsite (selected on basis of the 3D structure of the B. subtilis levansucrase) play a crucial role in determining size of the products synthesized (FOS versus inulin polymeric
Site-directed mutagenesis experiments with the *B. megaterium* levansucrase indicated that N252 and R370, constituting the +2 subsite, are critical for the polymer versus oligosaccharide product ratio of the enzyme (62). Recently, the crystal structure of the S164A *B. subtilis* levansucrase mutant (PDB ID: 2VDT) has been elucidated. S164 was found to be important in maintaining the position of the nucleophile in the active site. Furthermore, site-directed mutagenesis experiments showed that R360, Y429, R433 (Table 1, motifs VII and VIII) have important roles in acceptor substrate specificity. These amino acids have direct or indirect (through a water molecule) interaction with sucrose. The mutants R360S, Y429N and R433A were clearly more hydrolytic than the wild-type levansucrase and produced exclusively oligosaccharides (122). These mutations may have reduced the affinity of +2, +3 and further subsites for the growing polymer chain, in line with the proposal of Ozimek et al (125) that polymerization is a processive reaction requiring a high enzyme affinity for the polymer at higher subsites.

A more detailed understanding of structure-function relationships in FTFs would enable rational design of biocatalysts with increased specificity for the synthesis of tailored polysaccharide and FOS products for a range of different applications. In this perspective, it would be interesting to know what structural features in FTF enzymes determine a) their specificity for synthesis of either β(2-6) or β(2-1) linkages, and b) the sizes of fructan products synthesized. As levansucrase and inulosucrase enzymes of lactic acid bacteria are closely related (>60 similarity), the difference in linkages of their fructan products moast likely is based on a limited number of amino acid differences. At present, the structural and mechanistic basis of product (β(2-6) vs β(2-1)) and reaction (transglycosylation/hydrolysis ratio) specificities in these enzymes is still not known. In addition, only limited experimental data about type and size distribution of FOS products synthesized by FTF enzymes from sucrose and related substrates is available.

In analogy to FTF enzymes, glucansucrase (GTF) enzymes (belonging to family GH70) catalyze hydrolysis and transglycosylation reactions on sucrose, leading to the production of free glucose and fructose, glucooligosaccharides and glucose polymers (α-glucans) with different linkage types between glucose units (83,109,182). Among various types of lactobacterial glucan polymers, reuteran is mainly composed of α(1-4) glycosidic bonds, whereas dextran is predominantly composed of α(1-6) linkages; these glucan
polymers are synthesized by the closely related reuteransucrase and dextran sucrase enzymes, respectively (85,86). Based on amino acid sequence alignments identifying residues potentially involved in linkage type determination, and subsequent site-directed mutagenesis, a reuteransucrase was transformed into a dextran sucrases type of enzyme by rational design (87). We adopted a similar strategy with Lb. reuteri 121 Inu to determine the functional importance of amino acid residues which are well conserved in inulosucrases but not in levensucrases. These residues have never been mutated before. Our data (chapter 5) demonstrated that mutations in these Inu amino acid residues located in blade 4, including the unique helix α7 (compared with the Lb. johnsonii NC 533 InuJ 3D structure, chapter 4), had a clear effect on transglycosylation activity and the FOS product profile. Particularly, mutation of N543, which forms a hydrogen bond to the adjacent arginine R544 (present near subsites +1 and/or +2), synthesized less FOS compared to wild type (chapter 5). Introducing mutation G416E, located at the rim of the active site pocket, increased hydrolytic activity 2-fold, without significantly changing transglycosylation activity. Mutation A538S, located behind the general acid/base, increased the total activity 2.5-fold. These results demonstrated that the product specificity of Inu is easily altered by protein engineering, obtaining Inu variants with higher transglycosylation specificity, higher catalytic rates and different FOS oligosaccharide size distributions (chapter 5).

It has been speculated that addition of a fructosyl residue to sucrose by the action of a levensucrase results in the formation of 6-kestose (O-β-D-fructofuranosyl-(2-6)-β-D-fructofuranosyl-(2-1)-α-D-glucopyranoside), the first intermediate of levan biosynthesis (189). However, B. megaterium levensucrase is the only FTF for which 6-kestose has been characterized as one of the products synthesized (62), whereas 1-kestose (O-β-D-fructofuranosyl-(2-1)-β-D-fructofuranosyl-(2-1)-α-D-glucopyranoside) has been shown to be produced from sucrose by the levensucrase enzymes of Z. mobilis (Crittenden & Doelle 1993), B. subtilis C4 (38), Lb. sanfranciscensis LTH2590 (82) and Lb. sanfranciscensis TMW 1.392 (173). Interestingly, the B. megaterium levensucrase data clearly shows the consumption of 1-kestose and apparent accumulation of 6-kestose (62). Also the Lb. reuteri levensucrase utilizes 1-kestose, producing long chain FOS (125). On the basis of this data, it can be hypothesized that at least in the first step, levensucrases produce 1-kestose, which is also synthesized by inulosucrases (2,3,125). The main structural differences between
levansucrase and inulosucrases therefore maybe found in the +2, +3 or higher (if present, remain to be identified) acceptor substrate subsites, which guide the subsequent binding of fructosyl units to the growing polymer chain (FOS) in such a way that addition occurs either with a $\beta(2-6)$ or $\beta(2-1)$ bond, producing levan or inulin, respectively. This kind of structural difference between Inu and Lev has also been depicted schematically by Ozimek et al (125)(Fig. 7), which shows that the -1 subsite is specific for binding of fructosyl residues only whereas +1, +2 and +3 subsites are flexible to bind various types of acceptor substrates. As families GH68 and GH32 share similar protein folds, it would be worthy to mention here a recent report on the 3D structure of *Aspergillus japonicus* fructosyltransferase (a GH32 enzyme) with bound substrate molecules, where the residues Y404 and E405 have been shown to contribute to the +2/+3 subsites and speculated to be responsible for the formation of the inulin-type FOSs(27).

![Fig. 7. Schematic representation of bound acceptor molecule in an FTF enzyme. The differences in affinity between Inu and Lev at the +2 and +3 subsites are shown by a shallow cleft (dark grey; low affinity), and a deep cleft (light grey; high affinity), respectively. Sugar binding subsites are either shown in white (-1 subsite) reflecting specific and constant affinity for binding of fructosyl residues only, or in light/dark grey (+1, +2, +3 subsites) reflecting their ability to bind either fructosyl, glucosyl (with GFn substrate) or galactosyl (with raffinose) residues. Adopted from (125).](image)

10.2.3. The C-terminal domain

The C-terminal region of FTF enzymes is variable in length and often contain a cell-wall-binding domain. Although the function of the C-terminal region of FTFs has not been studied extensively, suggestions for a possible function for this region have been obtained in studies with the levansucrase gene of *B. subtilis* (23). For instance, read through of a stop codon enabled a downstream region to enlarge the gene at the 3’-end. Changing this stop codon into a glutamine codon yielded a mutant *B. subtilis* levansucrase protein that had
become enlarged by 3 kDa. This resulted in an enzyme that synthesized levan of molecular weight higher than $5 \times 10^6$ Da, whereas the molecular weight of levan synthesized by the native levansucrase was mainly lower than $5 \times 10^5$ Da. The increase in levan molecular weight was mainly attributed to an increase in the number of branches in the levan. Moreover, the extended enzyme was able to form an active dimer from two polypeptide chains linked by an S-S bridge (23). In contrast, the truncation of the C-terminus of inulosucrase of *Lb. reuteri* 121 showed no effect on product formation (186).

The cell-wall-binding domains in the C-terminal regions of cell-wall-anchored proteins from Gram-positive bacteria typically contain (i) a spacer region (50 to 125 amino acid residues) rich in P/G and / or T/S residues, (ii) an LPXTG sortase recognition motif, and (iii) a stretch of hydrophobic amino acids containing PXX repeats (39,114,173), and (iv) two to three positively charged amino acids (L, R, and H) at the C-terminus.

The motif LPXTG is well conserved in the cell wall associated proteins of Gram-positive bacteria (39,184,186,191). Among FTF proteins it is mostly present in the enzymes from lactobacilli (Table 1, motif IX). Although the LPXTG cell-wall anchoring motif was found in both levansucrase and inulosucrase of *Lb. reuteri* 121 (184,186), neither the presence of inulosucrase in culture supernatants nor actual cell-wall association of the inulosucrase have been demonstrated in cultures grown in sucrose containing medium. Contrarily, levansucrase was found cell-wall associated during growth of *Lb. reuteri* 121 on glucose and was found predominantly in the supernatant when grown in sucrose containing medium (181). The cell-wall associated *Strep. salivarius* FTF protein contains a C-terminal region that resembles the cell-wall anchoring motif but lacks the actual LPXTG amino acids (135). A slightly different motif (LPKAG) is found in the FTFs of *Lb. johnsonii* NCC 533 and *Lb. gasseri* DSM 20243 and 20077 (2,3). However, the inulosucrase protein of *Lb. gasseri* DSM 20604 terminated abruptly and lacked the cell wall anchoring LPXTG/LPKAG motif. The impact of lacking this motif was clearly apparent from the tendency of the enzyme to be secreted into the growth medium, in contrast to the *L. gasseri* 20077 levansucrase, which was exclusively detected associated with the cell surface (2).

The stretch of hydrophobic residues acts as a membrane-spanning region with the positively charged amino acid residues directed towards the cytosol. The protein is located outside the cytoplasmic membrane with an N-terminal signal sequence attached to the
membrane. After proteolytic cleavage of the signal sequence by a signal peptidase (SPase) the remaining N-terminal part of the protein, spaced by the P/G and/or T/S rich region, is directed outwards of the cell. Subsequently, the LPXTG motif is proteolytically cleaved between the Thr and the Gly residues by a sortase enzyme and covalently linked to the peptidoglycan layer (113,114).

An FTF with unusual structure is the inulosucrase (IslA) of Leuc. citreum CW28 (120). It is a naturally occurring chimeric enzyme resulting from the substitution of the catalytic domain of alternansucrase with that of a fructosyltransferase. Its N-terminal region therefore is similar to the variable region of GTF enzymes, its catalytic domain is similar to the core region of FTFs from various bacteria, and its C-terminal domain presents similarity to the glucan binding domain (GBD) from alternansucrase (119). Evidence concerning the role of the glucan binding C-terminal region in determining enzyme activity and stability was obtained from the characterization of the inulosucrase gene (islA) from Leuc. citreum CW28. Truncation of the C-terminal domain resulted in an increase in total activity and hydrolysis/transglycosylation ratio, and decrease in thermal stability of the enzyme (119).

11. Scope of this thesis

The studies described in this PhD thesis deal with fructansucrase or fructosyltransferase (FTF) enzymes (family GH68; www.cazy.org) from probiotic lactobacilli. At the start of these studies, only a few inulosucrase enzymes had been characterized, compared to the relatively large number of levansucrase enzymes that were known already. As a consequence, structural features responsible for the different linkage types and (sizes of the) products synthesized by levansucrase and inulosucrase enzymes, remained elusive. Moreover, a 3D structure had not been resolved for an inulosucrase protein yet, whereas two high resolution 3D structures had become available for levansucrase proteins from Gram-positive and Gram-negative bacteria, including structures with bound substrate molecules (sucrose, raffinose). Previous structure-function studies with FTF enzymes had focused on the functional roles of amino acid residues that are highly conserved in both the levansucrase and inulosucrase enzymes. The aims of the research described in this thesis therefore were (i) characterization of FTF proteins, and in particular inulosucrases, from probiotic lactobacilli, (ii) identification of conserved residues in
inulosucrase proteins, differing in levansucrase proteins, constituting targets for site-directed
mutagenesis, (iii) introduction of point mutations in selected residues and analysis of the
effects on enzyme biochemical properties, (iv) over-expression, purification and
crystallization of inulosucrase proteins to resolve their 3D structures by X-ray
crystallographic studies.

Chapter 1 reviews current knowledge about fructansucrase enzymes, their biochemical
properties, domain organization and 3D protein architecture.

Chapter 2 describes the cloning of the inulosucrase gene of *Lb. johnsonii* NCC 533. A
detailed biochemical characterization of this enzyme (InuJ) is presented. *Lb. johnsonii* NCC
533 is the first *Lactobacillus* strain proven to produce inulin and FOS *in situ*.

In Chapter 3 we describe the isolation of 3 *ftf* genes from three different *Lb. gasseri* strains
and molecular and biochemical characterization of the FTF (inulosucrase and levansucrase)
enzymes encoded by these genes. *Lb. gasseri* DSM 20604 is the second *Lactobacillus* strain
shown to produce inulin polymer and FOS *in situ*, and is unique in its distribution of FOS
synthesized, ranging from DP2 to DP13.

Chapter 4 reports the first high resolution 3D structure of native and mutant *Lb. johnsonii*
NCC533 inulosucrase InuJ proteins.

Chapter 5 presents the characteristics of mutant *Lb. reuteri* 121 inulosucrase proteins
produced by introducing mutations in amino acid residues that are highly conserved in
inulosucrases but differing in levansucrases.
References


Chapter 1


