university of groningen

University Medical Center Groningen

University of Groningen

Fructosyltransferases of Lactobacillus reuteri

van Hijum, Sacha Adrianus Fokke Taco

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2004

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
van Hijum, S. A. F. T. (2004). *Fructosyltransferases of Lactobacillus reuteri: characterization of genes, enzymes, and fructan polymers*. [Thesis fully internal (DIV), University of Groningen]. [s.n.].

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 18-04-2025

1

General introduction

Preface

This introduction reviews current knowledge of the topics addressed in this thesis. The first section reviews polysaccharides and their applications. Exopolysaccharides can, based on their monosaccharide composition and biosynthetic pathway, be divided into two classes, heteropolysaccharides and homopolysaccharides. Homopolysaccharides consisting of fructosyl units (fructans), and their structures, are described in more detail. The second section focusses on fructan production by plants, fungi, yeast, and bacteria, with emphasis on lactic acid bacteria. *Lactobacillus reuteri* 121, the strain used for the research described in chapters 2 - 5, is introduced. The limited biochemical information available on sucrase enzymes from various organisms producing fructan polymers (fructosyltransferases, FTFs) is presented in the third section. Section four contains a brief overview of protein anchoring systems in lactic acid bacteria focussing on a cell-wall anchoring system observed in the amino acid sequences of FTFs described in chapters 2 and 4. Finally, an outline of this thesis is presented.

S.A.F.T. van Hijum, S. Kralj, L. Ozimek, L. Dijkhuizen, G.H. van Geel-Schutten, "Homopolysaccharides and lactic acid bacteria: enzymes involved and applications", submitted for publication.

1. Carbohydrates

Carbohydrates are widely distributed on Earth, occur in many different forms, and are present in a wide variety of substances and materials. Carbohydrates play a crucial role in photosynthesis, which is the process in which the sun's energy is converted into chemical energy. During this process carbon dioxide and water are combined to form glucose and oxygen. The carbohydrates produced by photosynthizing organisms are in their turn degraded by non-photosynthesizing organisms during respiration and glycolysis. Production and conversion of carbohydrates are essential parts of the carbon cycle of our planet. It is estimated that yearly about 3.4 x 10^{14} kg of carbohydrates are synthesized by plants and photosynthesing microorganisms. They make up an important part of our diet and form more than half of the calories consumed daily.

The general formula for carbohydrates is $C_n(H_2O)_n$. In the nineteenth century it was thought that they consisted of hydrates of carbon, hence the name carbohydrates. The exact nature, however, is that they contain hydroxyl and carbonyl groups and are polyhydroxy aldehydes or ketons. Carbohydrates can be divided in structural and non-structural components of organisms. Structural carbohydrates are found as components of the cell-wall and cytoskeleton. Non-structural carbohydrates are known as energy-rich compounds used for metabolism and energy storage compounds. The research described in this thesis focusses on the non-structural bacterial carbohydrates in the form of common and special fructose polymers, also called fructans. Before describing details on these fructans, a short overview of some (non-) structural carbohydrates, is given.

1.1 Structural carbohydrates

Chitin is a polymer composed of D-glucopyranose units with an N-acetylamino group at the C2 position of the glucopyranosyl ring. It can be found in anything from the shells of beetles to webs of spiders. Chitin is very similar to cellulose. Cellulose contains a hydroxy group, while chitin contains acetamide. When applied to human wounds and surgical cloths, it accelerates the skin healing process. An acidic mixture of chitin, when applied to burns, also accelerates the healing process. Left on for a few days, it can heal a third-degree bun completely. Chitosan is a molecule that is chemically derived from chitin by strong alkali treatment. This process is also called "N-deacylation", converting the chitin amide group to an amine group. Chitosan is used to dye shirts and jeans in the clothing industry. Because chitosan cannot be digested in the intestinal tract, it can be used within the human body to regulate diet programs.

Murein (peptidoglycan) is a cell-wall structure in bacteria. G. Gram invented a staining method for murein using the dye "crystal violet". Gram-positive bacteria can be stained with this dye, whereas Gram-negative bacteria can not be stained due to the presence of an impermeable outer membrane. Murein is a polymer consisting of the hexoses N-acetylglucosamin and N-acetyl-muramic acid linked via a $\beta(1\rightarrow4)$ bond, crosslinked by short oligopeptides. Although there are other kinds of carbohydrates in the bacterial cell-wall, murein is the major structural component. The other cell-wall carbohydrates are more diverse and distinctive in various bacteria.

Pectins are large molecules typically consisting of 100,000 units. They occur primarily in plant cell-walls, albeit in relatively low amounts (1-5% of total carbohydrates). In fruits, however, they are more abundant (10-30% of total carbohydrates). Pectins act as

intracellular cementing material, giving body to fruits, helping them to keep shape [149]. Pectin consists of stretches of partially methylated poly-α(1→4)-D-galacturonic acid residues occasionally interrupted with alternating α(1→2)-L-rhamnosyl-α(1→4)-D-galacturonosyl sections. These sections contain branch-points with mostly neutral side chains (1 - 20 residues) of mainly L-arabinose and D-galactose but may also contain other residues such as D-xylose, L-fucose and D-glucuronic acid. Pectins are mainly used as gelling agents, but can also act as thickener, water binder and stabilizer.

Cellulose is an abundant carbohydrate of commercial and biological importance, found in all plants as the major structural component of the plant cell-wall. It consists of unbranched polymers of β(1→4) linked D-glucopyranose residues arranged in linear chains, where every other residue is rotated approximately 180°. Intermolecular hydrogen bonds, van der Waals interactions, and the β(1→4) linkages make that cellulose forms a very strong matrix for the cell-walls of plants.

1.2 Non-structural carbohydrates

Glucose and fructose are carbohydrates found naturally in every fruit and vegetable. These sugar molecules are the major products of photosynthesis. Glucose is a building block of cellulose, starch, sucrose (Fig. 3A), raffinose (Fig. 3B), lactose, glucans, and α,α-trehalose. It is also found in a free state in a number of materials such as honey, grapes, and raisins. Glucose plays an important role in the blood of all animals, where it serves as an immediate source of energy and as a stabilizer of the osmotic pressure of the blood. It further serves as a precursor for the formation of glycogen and fat. Fructose is found in polymeric forms (fructans) in plants, fungi, and bacteria.

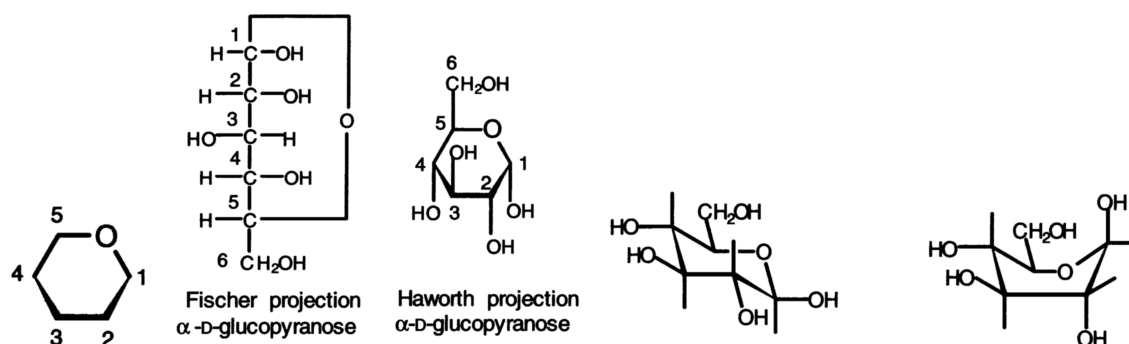


Figure 1. α-D-glucopyranose (D-glucose) projections. From left to right are drawn (i) a pyranose ring with carbon atom numbering, (ii) a Fischer projection with carbon atom numbering, (iii) a Haworth projection (commonly used to depict glucose) with carbon atom numbering, (iv) a chair conformation, and (v) a boat conformation. From [149].

Glucose is a hexose belonging to the D-family of aldoses. It is a straight chain polyhydroxylated aldehyde (aldose) that forms a pyranose ring composed of 5 carbon atoms (Fig. 1). Fructose is a hexose of the D-family of ketoses. It is a straight chain polyhydroxylated ketone (ketose) that forms a furanose ring of 4 carbon atoms (Fig. 2). In aldoses and ketoses, carbon atom numbering starts at the terminal carbon atom closest to the aldehyde or ketone group (Figs. 1 and 2). The spatial relationships of the atoms of the furanose and pyranose ring structures are more correctly described by the two conformations

identified as the the more stable chair- and the boat forms; for glucose both forms are depicted in Figure 1. Constituents of the ring that project above or below the plane of the ring are axial and those that project parallel to the plane are equatorial. In the chair conformation, the orientation of the hydroxyl group of the anomeric carbon (carbon atom 1) is axial in α-D-glucose and equatorial in β-D-glucose.

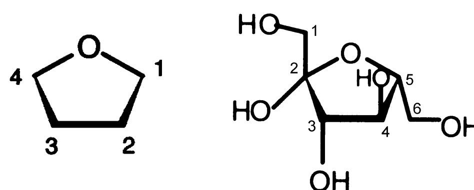


Figure 2. β-D-fructofuranoside (D-fructose) projections. Left: a furanose ring with carbon atom numbering; right: a Haworth projection (commonly used to depict fructose) with carbon atom numbering. From [149].

Disaccharides. Sucrose, α,α-trehalose and lactose are the only naturally occurring disaccharides. Sucrose (Fig. 3A) is a non-reducing heterodisaccharide composed of α-D-glucopyranose and β-D-fructofuranose. Sucrose, or as it is commonly named, 'sugar', is present in small amounts in all plants; high amounts of sucrose are present in sugar cane and sugar beet, both used as a source for table sugar [149]. Lactose is a naturally occurring heterodisaccharide of a β-D-galactopyranosyl and α-D-glucopyranosyl linked via an α(1→4) bond. It can be found in the milk of mammals and serves as the principal source of carbohydrate and energy for their young. α,α-Trehalose is also a naturally occurring non-reducing disaccharide composed of two α-D-glucopyranosyl units linked via an α(1→1) bond. It can be found in yeasts and the spores and fruiting bodies of fungi. Another source of α,α-trehalose is the hemolymph fluid of insects, where it serves as a source of energy. Its function in insects is similar to that of glucose in mammalian blood.

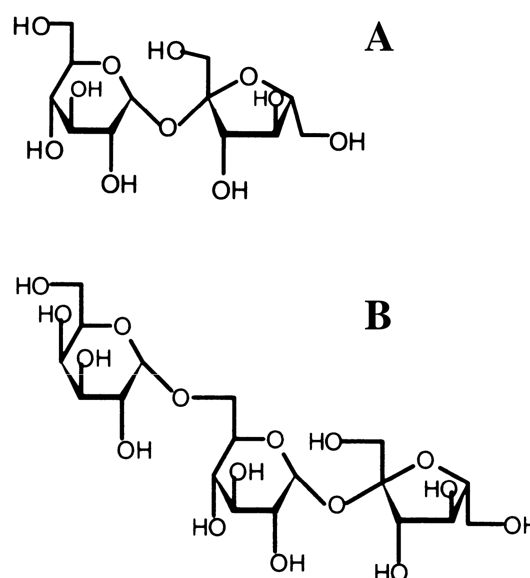


Figure 3. Sucrose (A) consists of an α-D-glucopyranosyl unit linked 1→2 to a β-D-fructofuranoside. Raffinose (B) is a non-reducing trisaccharide of α-D-galactopyranose linked 1→6 to the glucose part of sucrose. From [149].

Trisaccharides. Raffinose (Fig. 3B) is a non-reducing trisaccharide composed of an 1,6 linked α-D-galactopyranosyl unit to the glucose part of sucrose (Fig 3A). It is found in lesser amounts primarily in sugar beets. Other naturally occurring trisaccharides are melezitose, melibiose, turanose, and panose.

Starch is next to cellulose the most abundant carbohydrate. It constitutes the main form for storage of energy in plants. The starch granule is composed of two polymers: amylose and amylopectin. The amylose molecules are essentially linear α(1→4) linked glucan chains containing low amounts of α(1→4) linked glucose residues. Amylopectin molecules are α(1→4) linked glucose polymers with on average 5% α(1→6) linked glucose branches. Amylopecting often contains small amounts of covalent bound phosphate.

Glycogen is the major form of stored carbohydrate in animals. It is a homopolymer of α(1→4) linked glucose residues with every 8 - 10 residues an α(1→6) linked glucose branch.

Glycogen is a very compact structure that results from the coiling of the polymer chains. This compactness allows large amounts of carbon energy to be stored in a small volume, with little effect on cellular osmolarity.

Fructans. Most plants store starch or sucrose as reserve carbohydrates, but about 15% of all flowering plant species store fructans. Among the plants storing fructans are many of significant economic importance, such as cereals (barley, wheat and oat), vegetables (chicory, onion, lettuce), ornamentals (tulip, dahlia), and forage grasses [70]. More details on fructans will be given in the next section.

1.3 Exopolysaccharides

Intracellular storage polysaccharides are produced by plants (starch, inulin) and animals (glycogen) and, with fewer examples, by microorganisms (e.g. glycogen). Extracellular polysaccharides (exopolysaccharides; EPS) occur widely among bacteria and microalgae and, less frequently, among yeasts and fungi [42, 57, 142]. Bacterial EPS are molecules with molecular masses varying between 10 to 10^4 kDa (approximately 50 to 50,000 glycosyl units). The degree of polymerization (DP; number of residues) describes the number of monomeric residues making up a carbohydrate polymer molecule. Depending on their composition and mechanism of biosynthesis, bacterial EPSs can be divided into two classes: heteropolysaccharides and homopolysaccharides.

Bacterial heteropolysaccharides

Heteropolysaccharides are composed of a variety of sugar residues, mostly glucose, fructose, galactose and rhamnose. In some cases charged groups such as acetate, phosphate or glycerolphosphate are present. Heteropolysaccharides are usually produced in amounts below 100 mg/l. Heteropolysaccharides are synthesized at the cytoplasmic membrane by (a series of) glycosyltransferases, using intracellularly formed energy-rich precursors. These energy-rich precursors provide the energy for the polymerization reaction. The biosynthetic pathways of heteropolysaccharides show some similarities with the biosynthesis of the cell-wall components such as lipopolysaccharides, peptidoglycan and teichoic acid. Sugar nucleotides serve as precursors in the biosynthesis of different cell-wall components as well as in the biosynthesis of heteropolysaccharides [42, 43, 127]. Several enzymes involved in the biosynthesis of these sugar nucleotides are not necessarily unique to EPS formation, but may also play important roles in other metabolic pathways, such as metabolism of sugars. Heteropolysaccharides are mainly produced by lactic acid bacteria such as *Bifidobacterium adolescentis* [78], *Lactobacillus sake* [200], *Lactobacillus casei* [91], *Lactobacillus delbrueckii* spp *bulgaricus* [27, 60], *Lactobacillus rhamnosus* [55], *Streptococcus salivarius* ssp *thermophilus* [185], and various *Lactococcus* strains [25, 91, 198].

Bacterial homopolysaccharides

Bacterial homopolysaccharides are composed of only one type of glycopyranosyl residue. They may consist of either glucose (polymer: glucan) or fructose (polymer: fructan) residues with varying binding types and branching degree. They are synthesized on the outside of the cell by enzymes of the sucrase type in the presence of a donor molecule, e.g. sucrose, and an acceptor molecule, e.g. the growing polymer molecule, sucrose, raffinose, or water. Major differences with respect to the biosynthetic pathway of heteropolysaccharides are that only a single sucrase enzyme is involved in homopolysaccharide synthesis and that

sucrose instead of activated sugars is used as substrate. Differences between bacterial homopolysaccharides and heteropolysaccharides are listed in Table 1.

Some of the sucrase enzymes can also be used to synthesize oligosaccharides such as fructo-oligosaccharides and glucooligosaccharides. These oligosaccharides may contain several types of linkages between the glucose- or fructose moieties depending on the enzyme and conditions used for synthesis. There is a growing interest in oligosaccharides for instance for prebiotic purposes [125, 182]. In the case of glucooligosaccharides, the synthesis reaction of the oligo (also referred to as acceptor reaction) is different from the polysaccharide synthesis reaction: in addition to sucrose, the substrate of sucrases, an acceptor molecule such as maltose is required for the acceptor reaction to proceed. Depending on for instance the nature of the acceptor molecule and sucrose concentration, different oligosaccharides can be obtained [51, 113].

Glucans

Bacterial glucans are polymers of glucose residues synthesized by sucrase type of enzymes (glucansucrases). These enzymes cleave sucrose (Fig. 3A) and use the energy released (between the glucose and fructose; Figs. 1 and 2) to couple a glucosyl unit to a growing glucan (polyglucose) chain [112]. There is considerable variation in glucans due to differences that occur in the type of linkages, degree and type of branching, length of the glucan chains, molecular weight**,** and conformation of the polymers. As a result, there are large variations in solubility and other physical characteristics of the glucans. These differences reflect the complexity of extracellular polysaccharide synthesis from sucrose [112]. Many factors, including the growth media, incubation time, sucrose concentration used, and the presence of polysaccharide degrading enzymes**,** influence the molecular weight, structure, and physical characteristics of the polymers synthesized. Almost all glucans produced by lactic acid bacteria are composed of α-glucopyranosyl moieties; the various type of glucan differs in their linkages [112].

Table 1 Differences in synthesis and composition of bacterial homopolysaccharides and heteropolysaccharides

	Heteropolysaccharides	Homopolysaccharides
Quantity produced	low ($mg.l^{-1}$)	high ($g.l^{-1}$)
Building blocks	several per polymer rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, glucuronic acid	one per polymer glucose, fructose
Source	activated sugars	sucrose
Enzymes involved	several intracellular membrane associated	one extracellular

Examples of glucan production by lactic acid bacteria are (i) a glucan with α(1→6), α(1→4), and α(1→6→4) glucosidic bonds branching points (*Lactobacillus reuteri* 121) [204], (ii) dextran with contiguous α(1→6) linked glucopyranosyl units in the main chain and α(1→6→2), α(1→6→3), and / or α(1→6→4) branching points (*Leuconostoc mesenteroides,* and *Lactobacillus hildegardii)*, (iii) mutan, a linear unbranched polyglucose with α(1→3) linkages (various streptococci), and (iv) alternan with alternating α(1→6) and α(1→3) linked D-glucopyranosyl units in the main chain and α(1→6→3) branches (*Ln. mesenteroides)* [25, 47, 63, 108, 137, 168, 172].

Fructans

Bacterial fructans (Fig. 4) are polymers of fructose residues synthesized by a second class of sucrase type of enzymes, the fructosyltransferases (FTF). These enzymes cleave sucrose (Fig. 3A) and use the energy released (between the glucose and fructose; Figs. 1 and 2) to couple a fructosyl unit to a growing fructan (polyfructose) chain, sucrose, or water. In many cases, FTFs also use raffinose (Fig. 3B) as substrate. There are at least two types of FTFs: (i) those from plants and (ii) those from microorganisms.

In plants, two FTF enzymes are involved in fructan synthesis. The first FTF cleaves sucrose and couples the fructosyl unit to the fructose part of another sucrose, thereby making a primer for the second FTF, which couples a fructosyl unit of sucrose to this primer molecule [210].

Bacterial FTFs synthesize fructans from sucrose in a similar way, but use only one enzyme that generates the primer molecules and subsequently elongates these to synthesize the fructan. Because sucrose is used as acceptor in the initial primer reaction, both plant and bacterial fructans contain a non-reducing sucrose unit at the end of the chain [54].

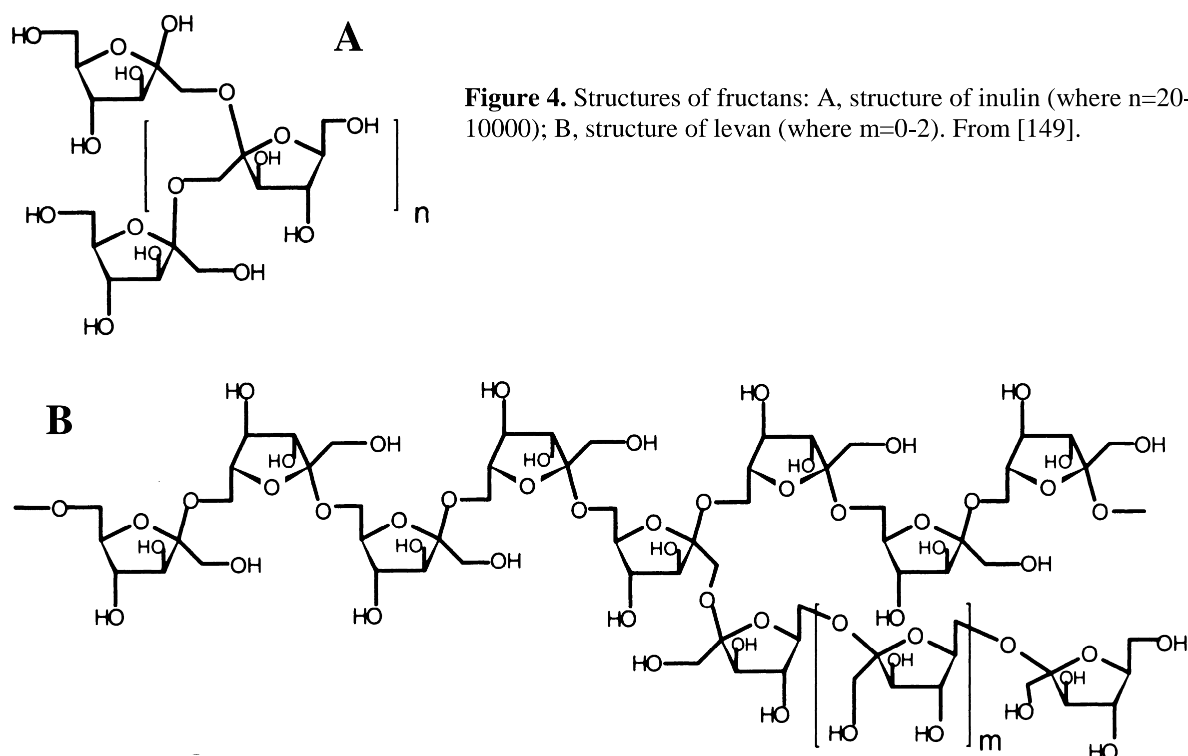


Figure 4. Structures of fructans: A, structure of inulin (where n=20-10000); B, structure of levan (where m=0-2). From [149].

Fructans may contain linkage types β(2→1), β(2→6), and β(2→1→6) branching points in any combination (Fig. 4). Fructans containing primarily (in the backbone) β(2→6) linked fructosyl units are referred to as levans; fructans containing primarily β(2→1) linked fructosyl units are referred to as inulins [210]. Plants predominantly produce fructans of the inulin type with a relatively low molecular weight (typically 4 – 10 kDa), whereas bacteria produce mostly high molecular weight fructans (typically 10 – 10^4 kDa) of the levan and inulin types [210].

Levan and inulin production by bacteria

Levan production has been frequently studied in *Gluconacetobacter diazotrophicus* [161], *Zymomonas mobilis* [95, 179], and *Bacillus* sp. [102, 135, 190]. However, limited information is available on levan production by lactic acid bacteria: it has only been described for streptococci [22, 66, 172], *Ln. mesenteroides* [150], *L. reuteri* 121 [204], and is described in **Chapters 3 and 4** of this thesis. Also *Lactobacillus sanfranciscensis* was found to produce fructans, but the fructan binding types have not been determined [93, 94].

Inulin production has only been described for several *Streptococcus mutans* subspecies, i.e. *S. mutans* Inbritt [151] and *S. mutans* GS-5 [168], and recently *Leuconostoc citreum* [131]. In **Chapters 2** and **5** of this thesis the first inulosucrase and its encoding gene from a *Lactobacillus* species is introduced. Furthermore, in **Chapter 2**, first observations of inulin oligosaccharide production from *L. reuteri* are described.

1.4 Physiological function of EPS

The physiological role of EPS in bacteria has not been clearly established, and is probably diverse and complex. EPS are thought to play a role in the protection of the microbial cell 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, osmotic stress, adhesion to solid surfaces and biofilm formation, and also in cellular recognition (e.g. via binding to a lectin) [25]. It is not likely that EPS serves as a food reserve for the bacteria producing them, because most slime-forming bacteria are not capable of metabolizing the EPS they produce [25]. The combination of a biosynthetic and a degrading enzyme has been reported in *Lactobacillus plantarum* [120], *Paenibacillus polymyxa* [17, 18], *Z. mobilis* [62], and *S. mutans* [20, 162]. EPS may also contribute to the provision of reduced oxygen tension and participate in the uptake of metal ions [25]. Furthermore, EPS may function as adhesive agents and facilitate interactions between plants and bacteria, i.e. levan production by the root of the sugar cane invading *G. diazotrophicus* [71]. Homopolysaccharides (glucans and fructans) formed by oral streptoccocci have a major influence on the formation of dental plaque. They are involved in adherence of bacteria to each other and to the tooth surface, modulating diffusion of substances through plaque and serving as extracellular energy reserves [154]. Furthermore, fructans aid in the adhesion of bacterial cells to plant cell surfaces [71].

1.5 Applications

A probiotic is a mono- or mixed culture of living microorganisms which, applied to animal or man, beneficially affects the host by improving the properties of the indigenous population of gastrointestinal microorganisms [67]. Probiotic effects are considered to include inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activity, increase of the immune response and reduction of cholesterol levels [45]. Bifidobacteria and lactobacilli have been used as probiotics to control intestinal disorders such as lactose intolerance, acute gastroenteritis due to rotavirus and other enteric pathogens, adverse effects of pelvic radiotherapy, constipation, inflammatory bowel disease, and food allergy [155, 158, 169]. These beneficial effects of lactobacilli have been attributed to their

ability to suppress the growth of pathogenic bacteria, possibly by secretion of antibacterial substances such as lactic acid, peroxide and bacteriocins [158]. Their survival during the passage through the human gut, when administered in fermented milk products, has been investigated intensely in recent years. Well-controlled, small-scale studies on diarrhea in both adults and infants have shown that probiotics are beneficial and that they survive in sufficient numbers to affect gut microbial metabolism. Survival rates have been estimated at 20-40% for selected strains, the main obstacles to survival being gastric acidity and the action of bile salts. Examples of probiotic food enchancers are: (i) Yakult (Yakult, Japan) with *L. casei* Shirota; (ii) Vivit (Mona, The Netherlands) with *L. casei* GG; (iii) Actimel (Danone, France) with *L. casei* Immunitas; (iv) Fysiq (Mona, The Netherlands) containing both prebiotic additives and *Lactobacillus acidophilus* Gilliland; and (v) *L. reuteri* (http://www.reuteri.com).

Prebiotics are defined as enhancers of probiotic strains or of beneficial endogenic strains of the gastrointestinal tract. Certain oligosaccharides and polysaccharides (lactulose and fructans) for instance are resistant to digestion by enzymes of the gastro-intestinal tract, because of the presence of fructose units in the β-form. They can, however, be fermented by beneficial microorganisms**,** such as bifidobacteria. Prebiotics therefore, selectively stimulate the growth of these microorganisms [24, 158]. Examples of fructo-oligosaccharides added to food are: (i) Nutrilon and Omneo baby-food (Numico, The Netherlands) to improve digestion and increase natural resistance; (ii) Fysiq (Mona, The Netherlands) contains inulin and a *Lactobacillus* strain; and (iii) LC1 Go (Nestlé, France).

Many food grade microorganisms, in particular lactic acid bacteria [25, 26], propionibacteria [26] and bifidobacteria [1, 147] produce EPS. Most of the EPS-producing lactic acid bacterial strains studied in more detail have been isolated from dairy products, e.g. Scandinavian ropy fermented milk products [53, 105, 118, 195], various yoghurts [6, 27, 28] and fermented milks [55], and kefir grains [97, 137]. Also cheese [91] and fermented meat and vegetables [200] served as source of EPS-producing lactic acid bacteria.

EPS can be used as viscosifying, stabilizing, emulsifying, gelling or water binding agent in the food as well as in the non-food industry [90, 187, 188, 211]. It is also used as a prebiotic (for description: see below) additive to food products [24]: some polysaccharides may contribute to human health, either as non-digestible food fraction, or because of their antitumoral, antiulcer, immunomodulating or cholesterol lowering activity [42, 50]. Various fructo-oligosaccharides are non-digestible oligosaccharides (a prebiotic), passing through to the mammalian gut system without being digested [10, 37, 153], except by some probiotic organisms such as bifidobacteria [207]. Synbiotic mixtures of *L. acidophilus* 74-2 and fructo-oligosaccharides gave rise to an increase of bifidobacteria in an artificial human intestinal microbial ecosystem [59]. Addition of fructo-oligosaccharides also caused an increase in the number of bifidobacteria [130]. A beneficial effect observed was that the number of lactic acid bacteria increased dramatically upon administration of honey (honey contains in decreasing order fructose, glucose, maltose, and sucrose) to rats [167].

Also medical uses of plant and / or bacterial EPS have been reported. It was shown that orally applied inulin reduces the colitis (inflammatory bowel disease) in rat intestine [209]. It was also shown that the bacterial flora of a prebiotic yoghurt containing *Streptococcus thermophilus*, *L. acidophilus*, enriched with *L. casei* GG did not influence the vitamin B1, B2 and B6 status of humans [49]. In short, fructans (fructo-oligosaccharides and inulin) are of increasing interest to clinical nutritionists as functional food additives.

Several patent applications have been filed for fructans, their production and applications. Examples are (i) Novel fructosyltransferases from *L. reuteri* 121 (US patent no: 09/604,958) [206]; (ii) Branched fructo-oligosaccharides, methods to obtain them and the use of products containing them (US Patent no: 5,659,028); (iii) Production of fructan (levan)

polymers using *P. polymyxa* (US Patent no: 5,547,863); (iv) Fructan-containing baby food compositions and methods therefor (US Patent no: 5,840,361); and (v) Fructosyltransferase enzyme from *G. diazotrophicus*, method for its production and DNA encoding the enzyme (US Patent no: 5,731,173).

Several companies are specialized in the use of fructans in food (both as nutrient and as pharmaceuticals; nutraceuticals). Examples are (i) Vitafoods International Ltd. (UK; http://www.vitafoods.co.uk/), (ii) Nutraceutix (USA; http://www.nutraceutix.com), (iii) Arla Foods Amba (Denmark; http://www.arlafoods.com), (iv) Biogaia (Sweden; http://www.biogaia.com), and (v) Degussa Bioactives (Germany; http://www.degussa-bioactives.com).

2. Organisms producing fructans

A large variety of organisms produce fructan polymers from the naturally occurring substrates sucrose or (in some cases) raffinose. In plants these polymers are produced intracellularly, whereas bacteria and fungi produce them extracellularly. In plants a two-component enzyme system is used for the production of fructans. Bacteria and fungi produce fructans with only one enzyme. Before extensively describing bacteria that produce fructans, other organisms producing fructans will be discussed briefly.

2.1 Eukaryotic organisms

Plants

Fructan synthesis in plants is modulated by light, affecting the availability of sucrose in the cell [210]. The substrate for plant fructan synthesis is sucrose, which is synthesized in the cytoplasm, but stored in the plant vacuole. In some cases raffinose, widely distributed in plants, is used as substrate. Fructans are produced in the vacuole by the fructosyltransferase enzymes using the stored sucrose as substrate [44]. Fructan accumulation in roots rather than leaves was found for *Taraxacum officinale* (Dandelion, Lion's tooth) [202]. The fructosyl chain length of fructans in plants varies greatly from 30 to 50 residues in general to occasionally 200 residues. Plant fructans are much smaller than bacterial fructans. Plant fructans can be divided into five major classes of structurally different polymers: inulin, levan, mixed levan, inulin neoseries, and levan neoseries [210]. Plant and bacterial inulin and levan share the same structure (see below). They are found in plants belonging to *Asterales* and some grasses. Mixed levan is composed of both $\beta(2\rightarrow1)$ and $\beta(2\rightarrow6)$ linked fructosyl units. This type of fructan is found in most plant species belonging to the *Polaes*, such as wheat and barley [23]. The inulin neoseries are linear $\beta(2\rightarrow1)$ linked fructosyl units linked to both C1 and C6 of the glucose moiety of the sucrose molecule. These fructans are found in plants belonging to the *Liliaceae* (onion and asparagus). The levan neoseries are polymers of predominantly $\beta(2\rightarrow6)$ linked fructosyl residues on either end of the glucose moiety of the sucrose molecule. These fructans are found in a few plant species belonging to the *Polaes* [210]. Although most plant fructan molecules consist of fructosyl residues linked to sucrose, also fructan molecules containing only $\beta(2\rightarrow1)$ linked fructose molecules have been isolated from species of the *Asteraceae* [210].

Yeasts

Thusfar no fructan production or fructan hydrolysis has been reported for yeast. There are, however, reports on the presence of sucrose hydrolyzing activities (invertase of glycoside hydrolase family 32; see below) in various yeasts: *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Schwanniomyces occidentalis* (website: http://afmb.cnrs-mrs.fr/~cazy/CAZY/GH_32.html). Yeast is frequently used as host for the heterologous expression of fungal FTFs [75, 77, 144].

Fungi

Fructan production and hydrolysis has been reported for several fungi. They produce inulin polymers which resemble plant inulin with respect to binding types and the low degree of polymerization (resulting in low molecular weight inulin polymers). FTF genes of fungi were recently identified in *Aspergillus sydowi* [75], *Aspergillus niger* [96], *Aspergillus foetidus* [144], and *Penicillium roquefortii* [84]. These genes share a higher sequence homology with plant 1-SST than with bacterial FTFs (Fig. 7).

2.2 Bacteria

In general, bacteria produce fructans consisting mainly of $\beta(2\rightarrow6)$ linked fructosyl residues, occasionally containing $\beta(2\rightarrow1)$ linked branches. These fructans are called levans and can reach a degree of polymerization of more than 100,000 fructosyl units, resulting in fructan molecules with molecular weights of about 20 MDa (Table 2). Some bacteria, such as *Z. mobilis*, *G. diazotrophicus* (Table 2), and *L. reuteri* (Table 2; **Chapter 2**) additionally produce oligosaccharides. Bacterial inulin production ($\beta(2\rightarrow1)$ linked fructosyl residues in the backbone of the polymer) has been observed only in some dental pathogenic *S. mutans* strains, *Ln. citreum* (Table 2), and *L. reuteri* (Table 2; **Chapters 2 and 5**). Fructan production has been reported more often for Gram-positive bacteria, although fructan production in Gram-negative bacteria appears to involve a larger variety of bacterial species (Table 2).

Gram-negative bacteria

Gram-negative bacteria are known to produce levan polymers when incubated with sucrose. The physiological function of the production of these fructan polymers are in some cases attributed to: (i) the adhesion to sugar cane roots enabling *G. diazotrophicus* to invade the plant roots [71], and (ii) the persistence and virulence of *Actinomyces naeslundii* and *Actinomyces viscosus* in the mammalian oral cavity [16]. The function of fructan production in soil bacteria such as *Pseudomonas* is not yet known. The sizes of the fructans produced (if determined) show a large variation from 1×10^5 to 50×10^6 Da (Table 2). *G. diazotrophicus* and two *Z. mobilis* strains show besides the production of fructan polymer also production of the oligofructan kestose (a fructosyl unit coupled to the fructosyl part of sucrose; Fig. 3A). This might be caused by fructotransferase activities converting the fructans into kestose. Fructotransferase enzymes produce oligo-fructans from levan (levan fructotransferase; no E.C. number assigned) or from inulin (inulin fructotransferase; E.C. 2.4.1.200). Examples are found in *Arthrobacter* sp. [156, 157, 178] and *Microbacterium* sp. [29].

Table 2. Bacterial fructan production and FTFs identified.

Strain	Fructan	Fructan size (Da)	Oligo	M_r FTF (kDa)	Gene	Reference
Arthrobacter sp. K-1	levan$^{\Phi}$	n.d.	n.d.	63	*bff*	AB062134
Bacillus amyloliquefaciens ATCC 23350	levan$^{\Phi}$	n.d.	n.d.	50	-	[106]
Bacillus amyloliquefaciens ATCC 23844	levan$^{\Phi}$	n.d.	n.d.	50	*sacB*	[192]
Bacillus circulans	levan$^{\Phi}$	n.d.	n.d.	52	-	[135]
Bacillus natto	90% $\beta(2\rightarrow6)$ 10% $\beta(2\rightarrow1\rightarrow6)$	2.5×10^6	-	n.d.	-	[80]
Bacillus subtilis BS5C4	levan$^{\Phi}$	$> 10\times10^6$	-	50	-	[35]
Bacillus subtilis QB112	levan$^{\Phi}$	n.d.	n.d.	50	*sacU*	[32]
Bacillus subtilis var. *saccharolyticus*	levan$^{\Phi}$	2×10^4	n.d.	n.d.	-	[190]
Bacillus subtilis W168	levan$^{\Phi}$	n.d.	n.d.	52	*sacB*	[183]
Clostridium acetobutilicum	levan$^{\Phi}$	n.d.	n.d.	53	*ftf*	[126]
Geobacillus stearothermophilus ATCC 12980	levan$^{\Phi}$	n.d.	n.d.	50	*surB*	[102]
Lactobacillus reuteri **121**	$\beta(2\rightarrow6)$	97% 2×10^4 3% $(3\text{-}4)\times10^6$	-	90	*lev*	**Ch 3,4**
Lactobacillus reuteri **121**	$\beta(2\rightarrow1)$	$> 1\times10^7$	1-kestose	90	*inu*	**Ch 2,5**
Lactobacillus sanfranciscensis	fructan$^{\Phi}$	n.d.	n.d.	n.d.	-	[93, 94]
Leuconostoc citreum	> 95% $\beta(2\rightarrow1)$	n.d.	n.d.	170	-	[131]
Paenibacillus polymyxa CF43	levan$^{\Phi}$	$> 5\times10^6$	kestose$^{\Psi}$	52	*sacB*	[17]
Paenibacillus polymyxa NRRL B-18475	88% $\beta(2\rightarrow6)$ 12% $\beta(2\rightarrow1\rightarrow6)$	2×10^6	-	52	-	[64, 65]
Streptococcus mutans AHT	inulin$^{\Phi}$	n.d.	n.d.	-	-	[47]
Streptococcus mutans BHT	90% $\beta(2\rightarrow1)$ 10% $\beta(2\rightarrow1\rightarrow6)$	n.d.	n.d.	-	-	[47]
Streptococcus mutans GS-5	inulin$^{\Phi}$	$(60\text{-}90)\times10^6$	-	88	*ftf*	[74, 162]
Streptococcus mutans Ingbritt	inulin$^{\#}$	n.d.	n.d.	70-94	-	[3]
Streptococcus mutans JC1	95% $\beta(2\rightarrow1)$ 5% $\beta(2\rightarrow1\rightarrow6)$	n.d.	n.d.	-	-	[47]
Streptococcus mutans JC2	94% $\beta(2\rightarrow1)$ 6% $\beta(2\rightarrow1\rightarrow6)$	$> 2\times10^6$	n.d.	-	-	[22, 151]
Streptococcus mutans OMZ 176	levan$^{\forall}$	n.d.	n.d.	-	-	[36]
Streptococcus salivarius ATCC 13419	levan$^{\Phi}$	20×10^6	n.d.	-	-	[123]
Streptococcus salivarius ATCC 25975	70% $\beta(2\rightarrow6)$ 30% $\beta(2\rightarrow1)$	$(20\text{-}100)\times10^6$	-	100	*ftf*	[47, 177]
Streptococcus salivarius HHT	90% $\beta(2\rightarrow6)$ 10% $\beta(2\rightarrow1\rightarrow6)$	n.d.	n.d.	-	-	[47]
Streptococcus salivarius S1	86% $\beta(2\rightarrow6)$ 14% $\beta(2\rightarrow1\rightarrow6)$	n.d.	-	-	-	[66]
Streptococcus salivarius SS2	70% $\beta(2\rightarrow6)$ 30% $\beta(2\rightarrow1)$	n.d.	n.d.	-	-	[172]
Actinomyces naeslundii WVU45	levan$^{\Phi}$	n.d.	-	68	*ftf*	[16]
Actinomyces viscosus T14	>50% $\beta(2\rightarrow6)^{\#}$ <50% $\beta(2\rightarrow1)^{\#}$	50×10^6	-	240*	-	[133]
Aerobacter levanicum	levan$^{\Phi}$	50×10^6	n.d.	22	-	[46]
Erwinia amylovora Ea7 / 74	levan$^{\Phi}$	n.d.	n.d.	44	*lsc*	[56, 61]
Gluconacetobacter diazotrophicus SRT4	levan$^{\Phi}$	$> 2\times10^6$	1-kestose	58	*lsdA*	[7, 71]
Gluconacetobacter xylinum NCI 1005	$\beta(2\rightarrow6)$	n.d.	n.d.	47	*lsxA*	[189]
Pseudomonas syringae						
pv. glycinea PG4180	levan$^{\Phi}$	n.d.	-	44	*lsc/B/C*	[73]
pv. phaseolicola NCPPB 1321	levan$^{\Phi}$	$(1\text{-}100)\times10^5$	-	44	*lsc*	[72, 73]
Pseudomonas aurantiaca	levan$^{\Phi}$	n.d.	n.d.	47	*lscA*	AF306513
Rahnella aquatilis ATCC 33071	$\beta(2\rightarrow6)$	n.d.	-	43	*lsrA*	[166]
Rahnella aquatilis JCM-1683	levan$^{\Phi}$	n.d.	n.d.	62	-	[129]
Zymomonas mobilis ATCC 10988 / ZM1	$\beta(2\rightarrow6)$	n.d.	1-kestose	47	*levU*	[179, 180]
Zymomonas mobilis IFO 13756 / Z6	levan$^{\Phi}$	n.d.	kestose	56	*sucE2*	[95, 215]
Zymomonas mobilis NRRL B806	levan$^{\Phi}$	n.d.	n.d.	47	*sacB*	[62]

Table 2. *Continued.*

*	M_r of this multi-subunit enzyme was determined by gel-filtration.
#	Binding types were identified by mouse antibodies raised against plant inulin polymers.
Φ	The binding types of these fructans have not been reported, nor were the standard methods used (methylation, NMR, or antibodies).
∀	Binding types were determined by partial fructan hydrolysis and comparison with inulin and levan standards on TLC.
Ψ	Minor amounts were detected.
n.d.	Not determined.

Bacilli

Fructan production has been reported for several *Bacillus* strains, i.e. *B. amyloliquefaciens*, *B. circulans*, *B. subtilis, P. polymyxa* and *B. natto* (Table 2). All of the fructans produced have been identified as levan polymers; only in the case of *B. natto* the binding type analysis was detailed enough to report the presence of 10% β(2→1→6) branching points. These organisms are associated with soil. A possible function of fructan production for these bacteria might be extracellular energy storage, enabling the organism to store carbohydrates in a soluble form, but inaccessible to organisms lacking fructan degrading activities. Levanase (EC 3.2.1.65) activities have been reported for *P. polymyxa* [18], and fructan degrading genes were reported for *L. plantarum* [120], providing further support for this hypothesis. Another function of fructan polymers in bacilli may be the attachment of cells to soil particles, but no reports providing clear experimental evidence have been published.

Streptococci

Limited information is available about fructan synthesis by lactic acid bacteria. Most attention has been focussed on oral streptococci because of their role in dental caries formation [11]. Both glucans and fructans formed by oral streptococci apparently have a major influence on the formation of plaque. They are involved in adherence of bacteria to each other and to tooth surface, modulating diffusion of substances through plaque and serving as extracellular energy reserves [154]. In the case of glucan synthesis, the fructose moiety of sucrose is fermented [9]. Streptococci produce both fructans of the levan type with β(2→6) linked fructosyl units, and of the inulin type with ß(2→1) linked fructosyl units. *S. mutans* JC2 for instance produces a fructan consisting mainly of ß(2→1) linked fructosyl units with 6% ß(2→6) branches [47, 151]. The fructan produced by *S. mutans* Ingbritt contains only ß(2→1) linked fructosyl units [3]. In some streptococci, the fructose moiety of sucrose can be used for polymer synthesis. In this way, levan and inulin-like polymers are formed.

Lactobacilli - general

Members of the genus *Lactobacillus* are facultatively anaerobic Gram-positive microorganisms that produce lactic acid as the major end product during the fermentation of carbohydrates. Lactobacilli are found in habitats where rich, carbohydrate containing substrates 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 [213]. Together with the genera *Streptococcus*, *Lactococcus, Leuconostoc*, *Pediococcus, Aerococcus, Alloicoccus, Dolosigranulum, Enterococcus, Globicatella, Lactospaera, Oenococcus, Carnobacterium,*

*Tetragenococcus***,** *Vagococcus* and *Weissella*, lactobacilli belong to the lactic acid bacteria [184] (Fig. 5).

Historically, the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* form the core of the lactic acid bacteria group [9]. They possess the GRAS (Generally Regarded As Safe) status and are known to produce an abundant variety of homo- and heteropolysaccharides [42]. Members of these genera play an essential role in the fermentation of food (for instance cheeses, milks, breads, wines, pickles and meats) and feed. Lactic acid bacteria contribute to the natural preservation of the fermented products by lowering the pH due to the production of lactic acid, and in some cases by the production of antimicrobials. In addition to providing this effective form of natural preservation, they are of influence on the flavor, texture and, frequently, the nutritional attributes of the products [9]. The biochemical processes of lactic acid bacteria, which are of technological importance, are carbohydrate and citrate metabolism, proteolysis, and production of antimicrobials and exopolysaccharides [38].

Lactobacillus polysaccharides are of special interest because they may also contribute to human health as prebiotics [24], or because of their antitumoral [41], antiulcer [128], immunomodulating [164], or cholesterol-lowering [146] activity. Moreover, some strains (e.g. *L. reuteri*) have been designated as probiotics. They may have beneficial effects on the host by improving the properties of the indigenous population of gastrointestinal micro-organisms [24, 58, 67]. Such beneficial properties of lactobacilli may be based on the polysaccharides produced.

Lactobacilli - physiology

The general metabolism and physiology of lactobacilli reflects their adaptation to niches rich in nutrients and energy sources. They have dispensed with biosynthetic capability and developed very efficient transport systems, which enable them to quickly take up the necessary solutes. Carbohydrates are metabolized very rapidly via fermentation [9], which, coupled to substrate level phosphorylation, is an essential feature of the metabolism of lactobacilli.

Transport and phosphorylation of glucose may occur by transport of the free sugar and phosphorylation by an ATP-dependent glucokinase. However, most species use the phosphoenolpyruvate sugar phosphotransferase system (PTS) for transport and concomitant phosphorylation of sugars. In either case, a high-energy phosphate bond is required for the activation of the sugar [9]. Utilizing glucose or another hexose as a carbon source, lactobacilli may be either homofermentative or heterofermentative. Homofermentative strains only produce lactic acid from hexoses, whereas equimolar amounts of lactate, CO_2 and ethanol (and / or acetic acid) are produced by heterofermentative strains. In general, hexoses are metabolized via the Embden-Meyerhoff-Parnas (glycolysis) pathway in homofermentative lactobacilli, whereas the 6-phosphogluconate / phosphoketolase pathway is used in heterofermentative strains. Various compounds (e.g. citrate, malate, tartrate, quinolate, nitrate, nitrite, etc.) may be metabolized, and used as energy sources (e.g. by generating a proton motive force) or as electron acceptors [9].

Depending on the mode of transport, disaccharides enter the cell either as free sugars or as sugar phosphates. In the former case, the free disaccharides are split by specific hydrolytic enzymes to monosaccharides, which enter the major pathways described above. When a PTS is involved, specific phosphohydrolases split the disaccharide phosphate into free monosaccharide and monosaccharide phosphates.

Sucrose fermentation mediated by a permease system is initiated by the cleavage of the sugar by sucrose hydrolase to yield glucose and fructose, which enter the major pathways. In some lactococci sucrose is transported by sucrose-PTS and a specific sucrose 6-phosphate

hydrolase cleaves the sucrose-6-phosphate into glucose-6-phosphate and fructose. The sucrose-PTS and sucrose-6-phosphate hydrolase are induced by the presence of sucrose.

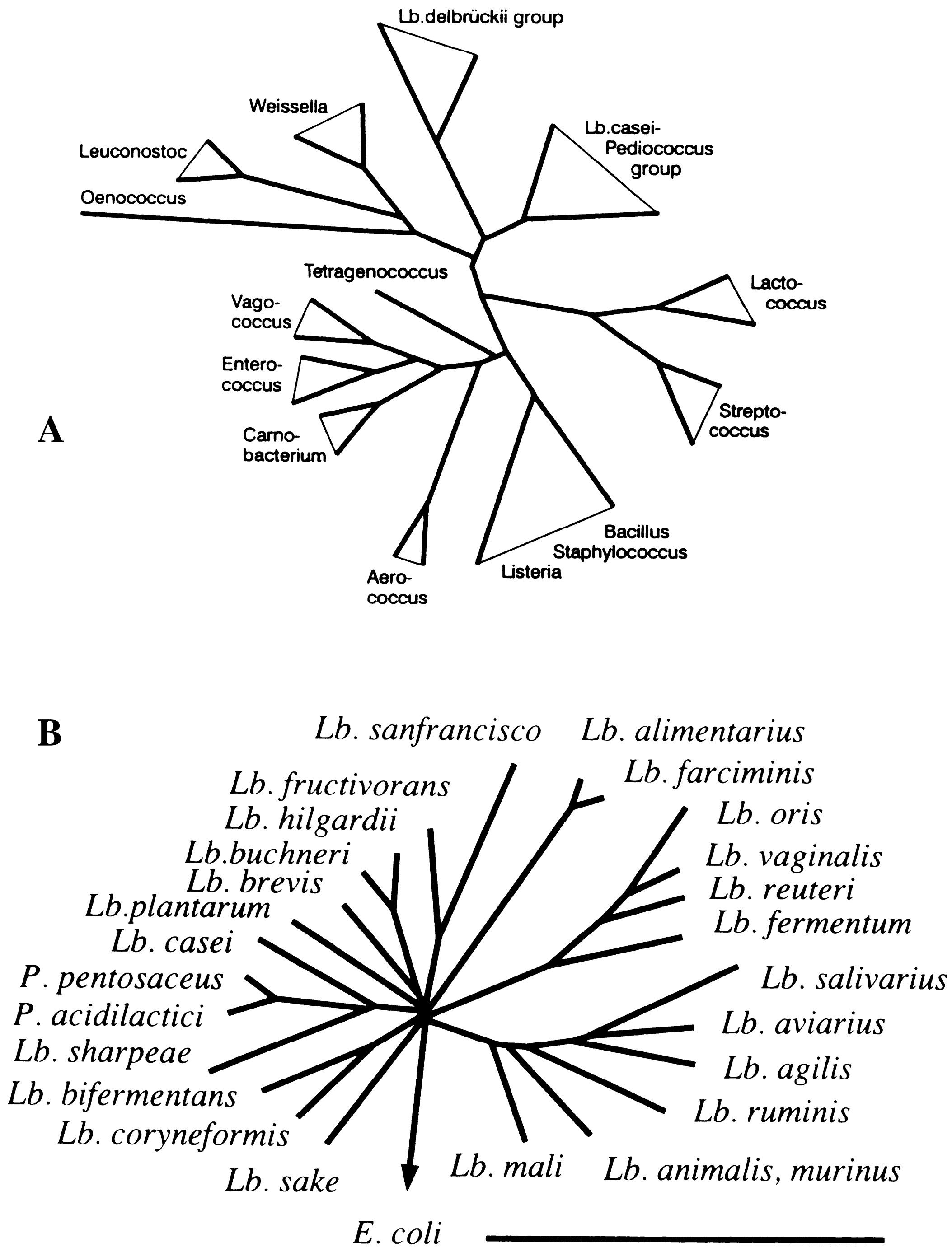


Figure 5. Schematic, unrooted phylogenetic tree of lactic acid bacteria, including some aerobic and facultative anaerobic Gram-positive bacteria of the low G + C subdivision (A). From [9]. Phylogenetic tree of the *Lactobacillus casei-Pediococcus* group to which *L. reuteri* is related (B). *L. sanfransisco* was renamed to *L. sanfranciscensis*. The horizontal bar indicates 10% expected divergence. From [165].

Sucrose may also act as a donor of monosaccharides for exopolysaccharide (EPS) formation in certain lactic acid bacteria. Recently, the molecular structure of the *L. plantarum* sucrose utilization locus was published, providing *Lactabacillus* genes to utilize various sugars including sucrose and raffinose. The locus showed to be highly similar to the plasmid oriented sucrose utilization locus of a *Pediococcus* strain, which is normally not able to catabolize sucrose [120]. Both loci contained sucrose degradative enzymes belonging to the glycoside hydrolase family 32 (see below).

Lactobacillus reuteri

L. reuteri belongs to the obligately heterofermentative lactobacilli. The species was originally isolated from human breast milk [88]. Its ecosystem is the gastrointestinal tract of a broad spectrum of hosts, including humans, mammals, and birds. It is the major component of the gut lactobacilli and the only enterolactobacillus known to be indigenous to such a broad spectrum of hosts [24].

An exclusive property of *L. reuteri* is the production and secretion of the antimicrobial metabolite reuterin (3-hydroxypropanal), which is synthesized in the presence of glycerol. Reuterin is active against a broad spectrum of microorganisms, including Gram-negative and Gram-positive bacteria, yeasts, fungi, and protozoa [24, 184]. Although there are no reports on the possible effects of reuterin on host cells, reuterin is thought to act against sulfhydryl enzymes. It was shown to be an inhibitor of the substrate binding subunit of ribonucleotide reductase, thereby interfering with DNA synthesis [132].

Because of this reuterin production, *L. reuteri* strains are of interest for various applications. For instance, [48] suggested that reuterin, or *L. reuteri* together with glycerol, could be applied either as a biopreservative or as a tool for decontamination to enhance the safety of raw meat. Another bacteriocin synthesized by *L. reuteri* is reutericin 6. This bacteriocin is produced by a strain isolated from human infant faeces and has antibacterial activity against some food-borne pathogenic bacteria. The purified substance also showed lytic activity against *L. delbrueckii* subsp. *bulgaricus* [86].

Lactobacillus reuteri *and pre- and probiotics*

The efficacy of some strains of *L. reuteri* as a probiotic has been demonstrated in various animals, such as poultry, and in humans [24]. There is even an internet site devoted to *L. reuteri* (http://www.reuteri.com). Administration of *L. reuteri* to pigs for instance, resulted in significantly lower serum total and LDL-cholesterol levels. Pigs are frequently selected as experimental animals because their digestive tract and circulation systems are comparable to those of humans [45]. In humans, *L. reuteri* is, for example, effective as a therapeutic agent against acute rotavirus diarrhea in children. Administration of *L. reuteri* to patients hospitalized with diarrhea resulted in shortening and amelioration of acute diarrhea. These therapeutical benefits were observed within 24 hours of the onset of treatment [169]. The effectiveness of *L. reuteri* as a probiotic apparently is very broad: [24] have proposed that strains of *L. reuteri* can manifest a probiotic effect on their hosts when these are challenged by bacterial, viral, or protozoal stressors.

L. reuteri 's mode of action as a probiotic remains to be determined. A particular *L. reuteri* strain bound specifically to a glycoconjugate present on mucosal cell surfaces [115]. These authors concluded that the carbohydrate-binding ability of this *L. reuteri* might be responsible for the adhesion to the mucosal surface of the intestine. Preliminary studies indicate that gut colonization by *L. reuteri* may be of importance in (i) immunopotentiating the host's cell-mediated and humoral immune responses and (ii) the regulation and maintaining of a balance among the numerous members of the gastrointestinal microorganisms, thereby optimizing this line of resistance to an assortment of diseases that

can be spawned within the enteric ecosystem [24]. For some strains, the mode of action as a probiotic may reside in the ability to produce polysaccharides. In these strains exopolysaccharides are possibly playing a role in the gut colonization. Furthermore, the exopolysaccharides produced by the strain could act as prebiotic substrates.

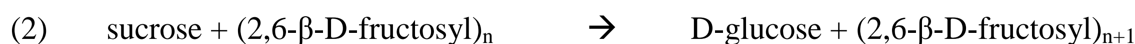
L. reuteri has already been selected as an additional probiotic culture to supplement the more commonly used *L. acidophilus* and *Bifidobacterium* species. Milk to which the three cultures were added, named "BRA" milk, has been introduced on the Swedish market [181]. A "probiotic enhanced" (symbiotic) yogurt (SymBalance, ToniLait AG, Bern, Switzerland) has recently been introduced on the market. It contains four human probiotic bacteria, including *L. reuteri***,** and inulin as a prebiotic, that selectively stimulates bifidobacteria growth in the human colon [24]. *L. reuteri* strain SD2112 is commercially exploited as a health promoting strain by BioGaia (Raleigh, North Carolina, USA) [160].

Only recently, the identification of a levan producing *Lactobacillus* strain (*L. reuteri* 121) [204] has been reported. In **Chapters 3** and **4** the isolation of the levansucrase protein and its encoding gene are described. In **Chapter 2** we describe the isolation of an inulosucrase enzyme synthesizing inulin and inulin fructo-oligosaccharides. Furthermore, inulin-oligosaccharide production was observed for *L. reuteri* (**Chapter 2**). Because bacterial fructan production has been reported only for lactic acid bacteria involved in dental decay, the identification of a (prebiotic) fructan-producing lactic acid bacterium with potential probiotic properties is significant. These properties of *L. reuteri* were also filed in a patent titled: Novel fructosyltransferases from *L. reuteri* 121 (US patent no: 09/604,958) [206].

3. Enzymes synthesizing fructans

In bacteria, inulin is synthesized by inulosucrase (sucrose: 2,1-β-D-fructan 1-β-D fructosyltransferase; E.C. 2.4.1.9; reaction 1) and levan is synthesized by levansucrase (sucrose: 2,6-β-D-fructan 6-β-D fructosyltransferase; E.C. 2.4.1.10; reaction 2), according to the following reactions:

(1) sucrose + $(2,1\text{-}\beta\text{-D-fructosyl})_n$ → D-glucose + $(2,1\text{-}\beta\text{-D-fructosyl})_{n+1}$



(2) sucrose + $(2,6\text{-}\beta\text{-D-fructosyl})_n$ → D-glucose + $(2,6\text{-}\beta\text{-D-fructosyl})_{n+1}$

These enzymes are commonly referred to as fructosyltransferases (FTFs). The only naturally occurring donor molecules are sucrose and in some cases raffinose. Unlike enzymes producing heteropolysaccharides, enzymes using sucrose (raffinose) as donor molecule (FTFs and enzymes producing polyglucose polymers), don't require activated sugars to provide the energy needed for the polymerization reaction (Table 1). This energy comes from the cleavage of the osidic bond between the glucose and fructose residue in sucrose (for FTFs and GTFs) or raffinose (some FTFs). In the initial reaction of FTFs, the fructose of a sucrose molecule is coupled by the enzyme to another fructose moiety of a sucrose or raffinose molecule. This is also referred to as the primer reaction. In subsequent steps, the enzyme elongates the primer.

A schematic representation of the various FTF catalyzed reactions is presented in Figure 6. All known bacterial FTFs catalyze the fructosyl transfer from sucrose (or raffinose) to a number of acceptors other than fructan polymer. Examples of possible acceptors are water (hydrolysis of sucrose), sucrose or raffinose (yielding a tri- or tetrasaccharide, respectively), short chain acylalkohols and various mono- and disaccharides [33]. A phylogenetic tree of FTFs, invertases, and fructan degrading enzymes (Fig. 7) shows a clustering of FTFs from Gram-positive (group II) and Gram-negative bacteria (group I). Exceptions in group 1 are: (i) *A. viscosus* levansucrase; (ii) *Z. mobilis* SacC invertase showing very high homology to other *Z. mobilis* FTFs; and (iii) *Arthrobacter* Bff. Exception in group II is the protozoal *Leishmania major* FTF. Group III represents a mixture of plant FTFs, invertases (from yeast, fungi, and bacteria), and fructan degrading enzymes for various origins. Within group III, plant FTFs form a separate cluster. Both the inulosucrase (**Chapters 2 and 5**) and the levansucrase (**Chapters 3 and 4**) from *L. reuteri* 121 clearly cluster with FTFs from lactic acid bacteria (Fig. 7).

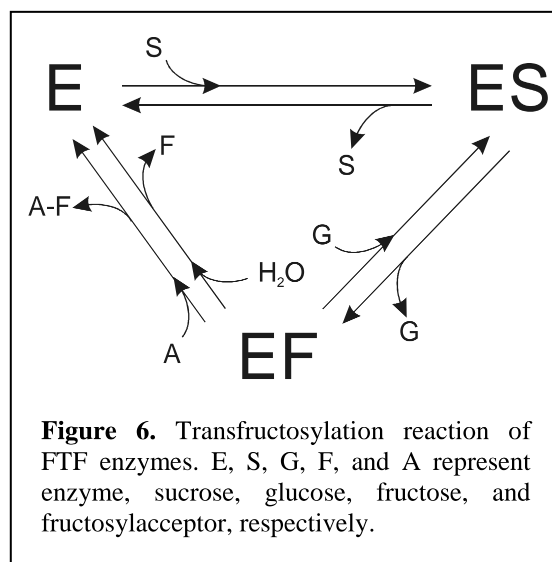


Figure 6. Transfructosylation reaction of FTF enzymes. E, S, G, F, and A represent enzyme, sucrose, glucose, fructose, and fructosylacceptor, respectively.

3.1 Eukaryotic enzymes

Plants

The fructan biosynthetic enzymes are evolutionarily related to invertases, enzymes that hydrolyze sucrose [201]. Several different enzymes are involved in plant fructan biosynthesis. A general model of fructan biosynthesis in plants was proposed based on the activity of cloned fructosyl transferases [210]. In this model sucrose is the starting point for fructan biosynthesis. Sucrose:sucrose 1-fructosyltransferase (1-SST) initiates *de novo* fructan synthesis by catalyzing the transfer of a fructosyl residue of sucrose to another sucrose molecule, resulting in 1-kestose (precursor for levan and inulin synthesis) and glucose. A second enzyme, fructan: fructan 1-fructosyltransferase (1-FFT), transfers fructosyl residues from fructan (DP $\geq$ 3) to another fructan molecule or sucrose [210]. The resulting inulins are relatively small polymers usually consisting of 20 to 50 fructosyl units per fructan molecule (resulting in fructans with M_r's of 3,000 to 10,000).

Other plant fructans are neokestose, levan, and mixed levan. Neokestose is synthesized from 1-kestose and sucrose by fructan:fructan 6G-fructosyltransferase (6G-FFT). Elongation of neokestose by 1-FFT results in fructans of the inulin neoseries type. The enzyme 6-SFT synthesizes bifurcose from 1-kestose and sucrose. Elongation of bifurcose by 1-FFT or 6-SFT results in a mixed type of levan. If only sucrose is available 6-SFT produces 6-kestose, and elongation by 6-SFT resulted in the production of levan type of fructans [210]. Most FTF enzymes display standard Michaelis-Menten kinetics, but both 1-SST and 1-FFT enzymes show unusual kinetics: these enzymes cannot be saturated by their substrate sucrose [92].

Fungi

A number of genes encoding FTFs have been reported for *Aspergillus* sp. [75, 144]. These genes encode extracellularly located FTFs. Expression of these genes in heterologous hosts showed that these enzymes produced 1-kestose from sucrose. These enzymes as well as plant 1-SST enzymes synthesize identical products (1-kestose). However, a comparison of the biochemical properties of fungal FTFs, plant FTFs, and bacterial FTFs remains to be carried out.

3.2 Bacterial enzymes

FTFs have been reported for a number of Gram-negative and Gram-positive bacteria (Table 2). Bacterial FTFs mostly produce levan polymers, the only exceptions are inulosucrases from *S. mutans*, *Ln. citreum* (Table 2), and *L. reuteri* (**Chapter 2**). Information on the binding types and molecular weights of the fructan polymers are diverse and usually incomplete. M_r's of bacterial levans and inulins show a large variation, those for bacterial inulins are much higher than those reported for plant fructans. Based on the phylogenetical distance, bacterial FTFs can be subdivided into FTFs from (i) bacilli, (ii) lactic acid bacteria, (iii) Gram-negative bacteria (Fig. 7).

FTFs from Gram-negative bacteria are proteins with an average M_r of 50,000 (Table 2). The limited data available on fructans produced by Gram-negative FTFs show that they are without exception levan polymers ($\beta(2\rightarrow6)$ linked fructosyl units) with a large variety in the percentage of branches ($\beta(2\rightarrow1\rightarrow6)$ linked fructosyl units) present. Their M_r's are around 1,000,000 and only in a few cases (*G. diazotrophicus* and *Z. mobilis*) kestose production was reported. The *ftf*'s from *G. diazotrophicus* and *Z. mobilis* have been studied most intensively.

FTFs from bacilli form a separate group from the streptococcal FTFs. This is not only based on primary sequence data (Fig. 7), but also on the sizes of enzymes (Table 2). An exception is the unique multi-subunit levansucrase from *A. viscosus* with a M_r of 240,000 (consisting of 4 sub-units; Table 2). Bacilli have been found to produce levan polymers. In lactic acid bacteria, levan synthesis has been found only in *S. salivarius*, *S. mutans*, and recently *L. reuteri* (Table 2). Bacterial inulosucrase genes have been isolated from *S. mutans* GS5 (Table 2) and *L. reuteri* (**Chapter 2**). Until now no detailed biochemical data is available on bacterial inulosucrases. In **Chapter 5**, we present the first biochemical characterization of a bacterial inulosucrase.

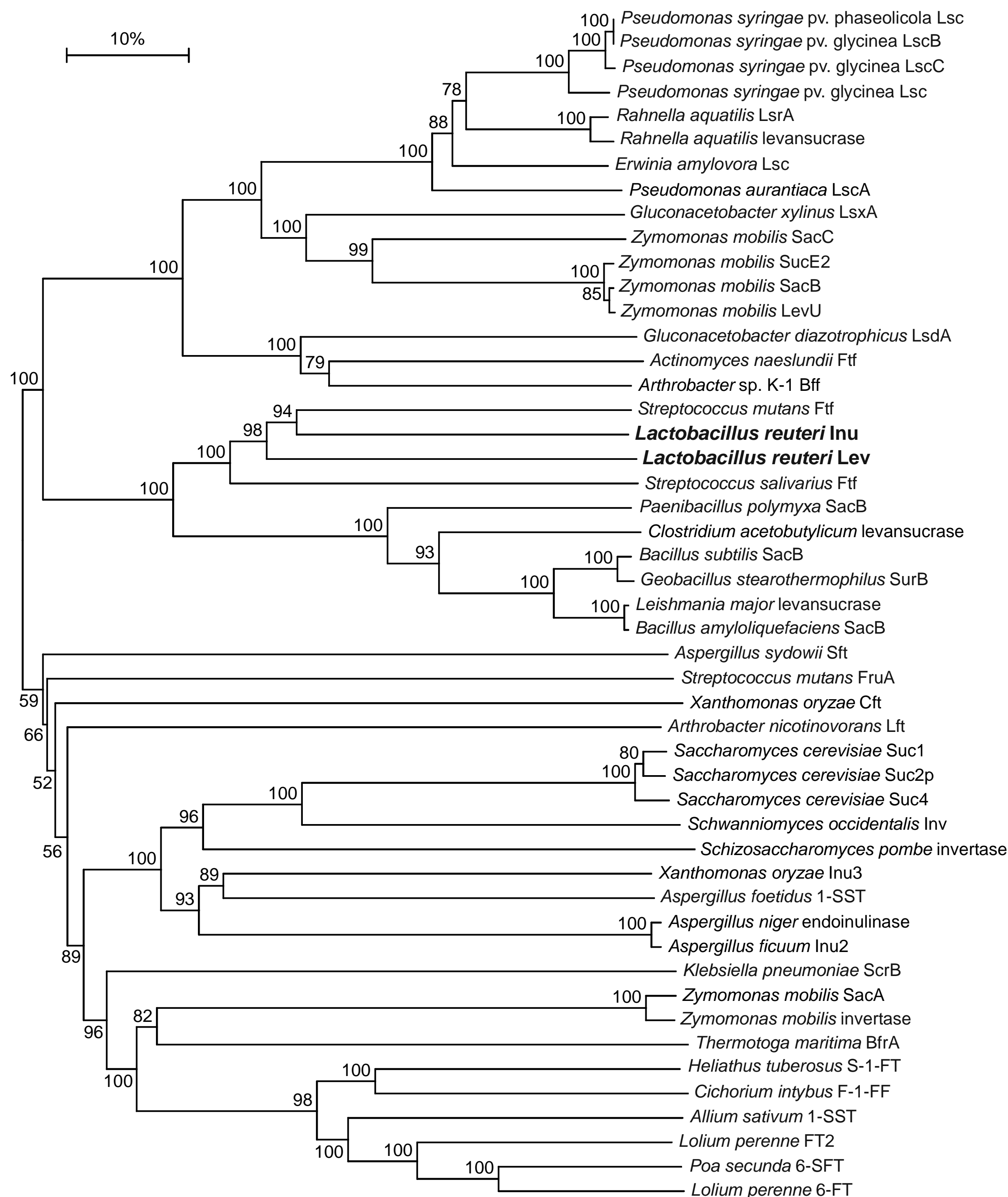


Figure 7. Unrooted phylogenetic tree of FTFs and some invertases from bacteria, plants, fungi, and a protozoa. The bar indicates 10% difference at the amino acid level. Alignments were made with ClustalW 1.74 [194] using a gap opening penalty of 30 and a gap extension penalty of 0.5. Dendrogram construction was done with TreeCon 1.3b [199] using the neighbour joining method with no correction for distance estimation. Bootstrap values (in percentage) are indicated at the branching points. FTFs from Gram-positive bacteria: *A. naeslundii* Ftf (AF228582); *Arthrobacter* sp. K1 Bff (AB062134); *C. acetobutylicum* levansucrase (AAK79737.1); *S. mutans* Ftf (M18954); *S. salivarius* Ftf (L08445); *P. polymyxa* SacB (AJ133737); *B. subtilis* SacB (X02730); *G. stearothermophilus* SurB (U34874); *B. amyloliquefaciens* SacB (X52988); *L. reuteri* Inu and Lev (**Chapters 2 and 4**). Fungal FTFs: *A. sydowii* Sft (ASY289046), and *A. foetidus* 1-SST (CAA04131). FTFs from Gram negative bacteria: *P. syringae* pv. phaseolicola Lsc (AF052289); *P. syringae* pv. glycinea LscB (AF345638), LscC (AF346402), and Lsc (AF037443); *R. aquatilis* LsrA (U91484), and levansucrase (AY027657); *E. amylovora* Lsc (X75079); *P. aurantiaca* LscA (AF306513); *G. xylinus* LsxA (AB034152); *Z. mobilis* SucE2 (D17524), SacB (L33402), and LevU (AF081588); *G. diazotrophicus* LsdA (L41732). Levansucrase from the

Figure 7. *Continued.*

protozoal eukaryotic parasite *L. major* (CAC00286). Some plant FTFs: *C. intybus* F-1-FF (AAD00558); *H. tuberosus* S-1-FT (CAA08812); *A. sativum* 1-SST (AAM21931); *L. perenne* FT2 (AAL92880) and 6-FT (AAM14603); *P. secunda* 6-SFT (AF192394-1). Fructan degrading enzymes: *S. mutans* FruA (Q03174); *A. nicotinovorans* Lft (AB001984); *A. niger* endoinulinase (AF435736); *A. ficuum* Inu2 (AJ006951); *X. oryzae* Cft (AY077612), and Inu3 (AY077613). Invertases: *Z. mobilis* invertase (JU0460), SacA (M62718), and SacC (AAC36942); *T. maritima* BfrA (O33833); *K. pneumoniae* ScrB (S62332); *S. cerevisiae* Suc1 (X07570), Suc2p (NP012104), and Suc4 (X07572); *S. occidentalis* Inv (X17604); *S. pombe* invertase (CAA07622). The tree is divided into groups: (i) from Gram-negative bacteria, with exceptions of *Arthrobacter* Bff, *Z. mobilis* SacC, and *Actinomyces* Ftf; (ii) from Gram-positive bacteria, with exception of *L. major* levansucrase; (iii) contains FTFs, invertases, and fructan degrading enzymes from various origins.

3.3 Biochemical properties of FTFs

Limited information is available on the biochemical properties of bacterial and plant FTFs. Based on the reaction catalyzed they were assigned to the family 68 of glycoside hydrolases, but due to a lack of structural protein data an exact family could not be assigned (website: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). A group of enzymes, closely related to the FTFs, are the glucansucrase (Glucosyltransferase; GTF) enzymes. These enzymes produce glucose polymers from sucrose. They belong to the family 70 of glycoside hydrolases with a circularly permuted $(\beta/\alpha)_8$ barrel structure [104]. Typically, this motif consists of 8 β-strands located in the core of the protein alternated with 8 α-helices located at the surface of the protein. Because of the limited information available on FTFs, and the common use of sucrose as donor molecule, we will briefly discuss some basic properties of GTFs prior to discussing biochemical data on FTFs.

Glucansucrases

GTFs are extracellular enzymes synthesizing various glucans from their only natural substrate sucrose. As in FTFs, the energy necessary to catalyze the reactions is provided by the cleavage of the osidic bond in sucrose. Glucans are produced by numerous lactic acid bacteria, e.g. by various species of *Leuconostoc* and *Streptococcus*, lactococci and by some species of *Lactobacillus* [170] including *L. reuteri* 121 [204]. An example is dextran, that is synthesized by dextransucrase (sucrose: 1,6-α-D-glucan 6-α-D glucosyltransferase, E.C. 2.4.1.5) according to the following reaction:

$$\text{sucrose} + (1,6\text{-}\alpha\text{-D-glucosyl})_n \rightarrow \text{D-fructose} + (1,6\text{-}\alpha\text{-D-glucosyl})_{n+1}$$

GTFs are large proteins with average M_r's of 160,000. Because more biochemical work has been done on GTFs than on FTFs, more is known about the reaction mechanism of GTFs.

Reaction mechanism glucansucrases

The catalytic mechanism of glucansucrase is very complicated and still not fully understood. There are several aspects complicating the elucidation of the reaction mechanism. As outlined above, various glucan structures can be synthesized as well as oligosaccharides. These oligosaccharides are produced in a separate acceptor reaction when other carbohydrates are present in addition to sucrose, acting as donor substrate. A broad

spectrum of carbohydrates can be used as acceptors. Also a range of non-carbohydrate substrates can be used as acceptor substrates [193, 214]. GTF enzymes are also capable of hydrolyzing sucrose without glucan synthesis. Here, water serves as an acceptor for the transfer of a glucosyl moiety of sucrose. Furthermore, branching reactions can also occur, indicating that one enzyme is capable of synthesizing different kinds of linkages [112].

α-Amylase type of enzymes are closely related to glucansucrases but belong to family 13 of glycoside hydrolases. In this type of enzymes, a catalytic triad consisting of two Aspartate and one Glutamate amino acid residue, plays a key role in catalysis [186]. As in α-amylase enzymes, glucan synthesis in glucansucrases occurs via the formation of covalent glucosyl- and glucanyl-enzyme complexes [114]. The glucosyl residue from sucrose is then transferred to the reducing end of the glucan chain in glucan synthesis or, in the acceptor reaction, to the non-reducing end of the acceptor molecule. The formation of the covalent glucosyl-enzyme complex is the only reversible step in the catalytic reaction [148]. For the *Streptococcus sobrinus* GTF enzyme, a covalent glucosyl-enzyme complex has been isolated. An Aspartate residue was found to be covalently attached to the glucosyl unit of sucrose [114]. Furthermore, additional Aspartate, Glutamate, and Histidine amino acid residues were found to be vital for enzyme GTF activity [110, 111]. However, no three-dimensional structure of a glucansucrase protein has been reported. Another, and closely related group of enzymes are amylosucrases. They synthesize amylose glucans, and belong to the family of α-amylases. Site-directed mutagenesis revealed that the amylosucrase from *Neisseria polysaccharea* also contains an Aspartate amino acid residue frunctioning as catalytic nucleophile, a Glutamate amino acid residue as general acid base catalyst, one Aspartate, and two histidine amino acid residues essential for catalytic activity [161, 174]. Recently, a three-dimensional structure of the same amylosucrase protein was reported showing an active site mutant in complex with its substrate sucrose [109, 175].

Reaction mechanism FTFs

Although the reactions performed by FTFs and GTFs are similar with respect to the use of sucrose as donor- and acceptor molecule, the proteins involved do not share the same overall structure. Until now, nucleotide sequences of 19 bacterial *ftf* genes have been described. Based on amino acid sequence alignments, levansucrases (E.C. 2.4.1.10) belong to the glycoside hydrolase family 68 (of levansucrases and invertases). Plant FTFs belong to the glycoside hydrolases family 32 (of invertases and fructan degrading enzymes). However, bacterial inulosucrases (E.C. 2.4.1.9) have not yet been assigned to a family. No common tertiary structure has been identified and only a low-resolution (3.8 Å) three-dimensional protein structure of a *B. subtilis* levansucrase is available [99]. Comparison of the amino acid sequences of glycoside hydrolase families 43 (of bifunctional beta-xylosidases and alpha-L-arabinofuranosidases) and 68 revealed that they are related in a region in the central part of the sequences. An invariant Glutamate residue showing up in the alignments between members of both families was considered to be a component of their active centers [119]. For a related group of enzymes, family 32 (of invertases and fructan degrading enzymes), a three-dimensional protein structure was modelled by using common prediction software. This structure consisted of a β-propeller fold [139]. A sequence-structure compatibility search using TOPITS, SDP, 3D-PSSM, and SAM-T98 programs identified a similar β-propeller fold with scores above the confidence threshold, thus indicating a structurally conserved catalytic domain in FTFs of diverse origin and substrate specificity. The predicted fold appeared related to those of neuraminidase and sialidase, of glycoside hydrolase families 33 and 34, respectively [138]. More conclusive evidence concerning the validity of these predicted three-dimensional structure models could be obtained by protein crystals and subsequent protein structure determination. Crystals diffracting at 1.5 Å, but not yet a three-dimensional

protein structure, were obtained for the exo-inulinase (glycoside hydrolase family 32) enzyme of *Aspergillus awamori* [5]. A detailed biochemical and structure / function characterization of FTFs is complicated by the fact that during the reaction FTFs generate new fructan molecules, which in turn can be used as acceptor molecule.

A proposed mechanism of catalysis for FTFs is a two-step mechanism, as in enzymes from the α-amylase family, involving a bifunctional catalysis in which an acidic group and a nucleophilic group of the protein are important for transfructosylation [173]. A stabilized enzyme-fructosyl complex of the levansucrase of *B. subtilis* and its substrate sucrose was obtained by quenching the enzyme with its substrate and subsequent chemical analysis of the amino acid covalently bound to the fructosyl unit. A β-carboxy group of an Aspartate amino acid residue had been identified as the nucleophilic group binding to the fructosyl group of sucrose [30]. The position and identity of the Aspartate residue in the protein, however, remains unknown. In the *S. salivarius* levansucrase, mutation of an Aspartate residue into a Serine residue in an Arginine / Aspartate / Proline amino acid motif (RDP) resulted in a catalytically inactive enzyme [176]. Mutation of the corresponding Aspartate residue to an Asparagine residue in the *G. diazotrophicus* levansucrase yielded an enzyme with a 75-fold decrease in catalytic activity (K_{cat}) [15]. These findings support the assumption that FTFs, although not sharing a common overall protein structure with family 13 and 70 enzymes, make use of a reaction mechanism involving similar amino acid residues as found in family 13 and 70 enzymes.

An interesting question is what determines the specificity of β(2→6) versus β(2→1) linkages and the sizes of fructans produced. A shift from high molecular weight fructan to the formation of fructo-oligosaccharides was found after mutating an Arginine residue into a histidine (Arg331His) [33]. Analysis of the *B. subtilis* levansucrase gene sequence showed that after the native stop codon, an in-frame DNA sequence was present encoding an additional stretch of amino acids followed by another stop codon. Deletion of this stop codon sequence resulted in an increased M_r of the levansucrase protein from 50,000 to 53,000. Analysis of the fructan products of the C-terminally enlarged levansucrase revealed an increase in fructan M_r. This C-terminally enlarged protein synthesized a fructan with a higher M_r, due to an increase in the number of branches present in the fructan [34].

Thus far, no mutants of bacterial inulosucrases have been reported. By comparing detailed biochemical data on (mutated) inulosucrase enzymes with (new) data available for levansucrase enzymes, more insight could be given in structure / function relationships of FTF enzymes. Questions to be answered are (i) which factors determine the fructan binding type specificity and (ii) what determines the fructan molecular weight. Subsequently, tailor-made enzymes could be created, producing (hybrid) fructans with specific sizes and / or containing specific binding types.

4. Anchoring of proteins to lactic acid bacteria

When characterizing the *L. reuteri* FTFs we discovered that these enzymes share a common C-terminal LPXTG cell-wall anchoring motif (**Chapters 2 and 4**). These LPXTG cell-wall anchoring motifs are well-known for a variety of proteins from many Gram-positive organisms such as *Staphylococcus aureus* [107], *Listeria monocytogenes* [21], and *Streptococcus pyogenes* [12]. Thus far, no LPXTG cell-wall anchoring motif had been reported for FTFs. Some FTFs are attached to their natural hosts, a few examples are *L. reuteri* levansucrase [204], *S. salivarius* FTF [143], the periplasmic *Pseudomonas syringae* LscC [101], and *A. viscosus* levansucrase [133]. Only for the *S. salivarius* FTF a putative

cell-wall binding domain was found in its amino acid sequence. For the other cell-associated FTFs no amino acid sequences involved in the cell-attachment process have been reported. Therefore, a summary of different protein anchoring systems is given with emphasis on the LPXTG cell-wall anchor.

Proteins displayed on the bacterial surface may have various functions for the bacterial cell. For the pathogens *S. aureus* and *Enterococcus faecium*, surface proteins are thought play a major role in the infection process in humans [196]. Surface proteins from lactobacilli mediate the adhesion with tissue cells [79, 171] and play a role in the maintenance of a healthy urogenital microflora. One strain, *Lactobacillus fermentum* RC-14, releases surface-active components which can inhibit adhesion of uropathogenic bacteria [68]. Anchoring of proteins to the cell surface of bacteria raises many questions, such as: (i) what underlies the process of displaying proteins at the cell surface, (ii) what are the interactions between bacteria and its environment, (iii) what are biotechnological applications of protein anchoring (oral vaccination), and (iv) how could we use protein anchoring to cell-walls to target bacterial cells to pathogens for therapeutic purposes (e.g. antibiotic therapy).

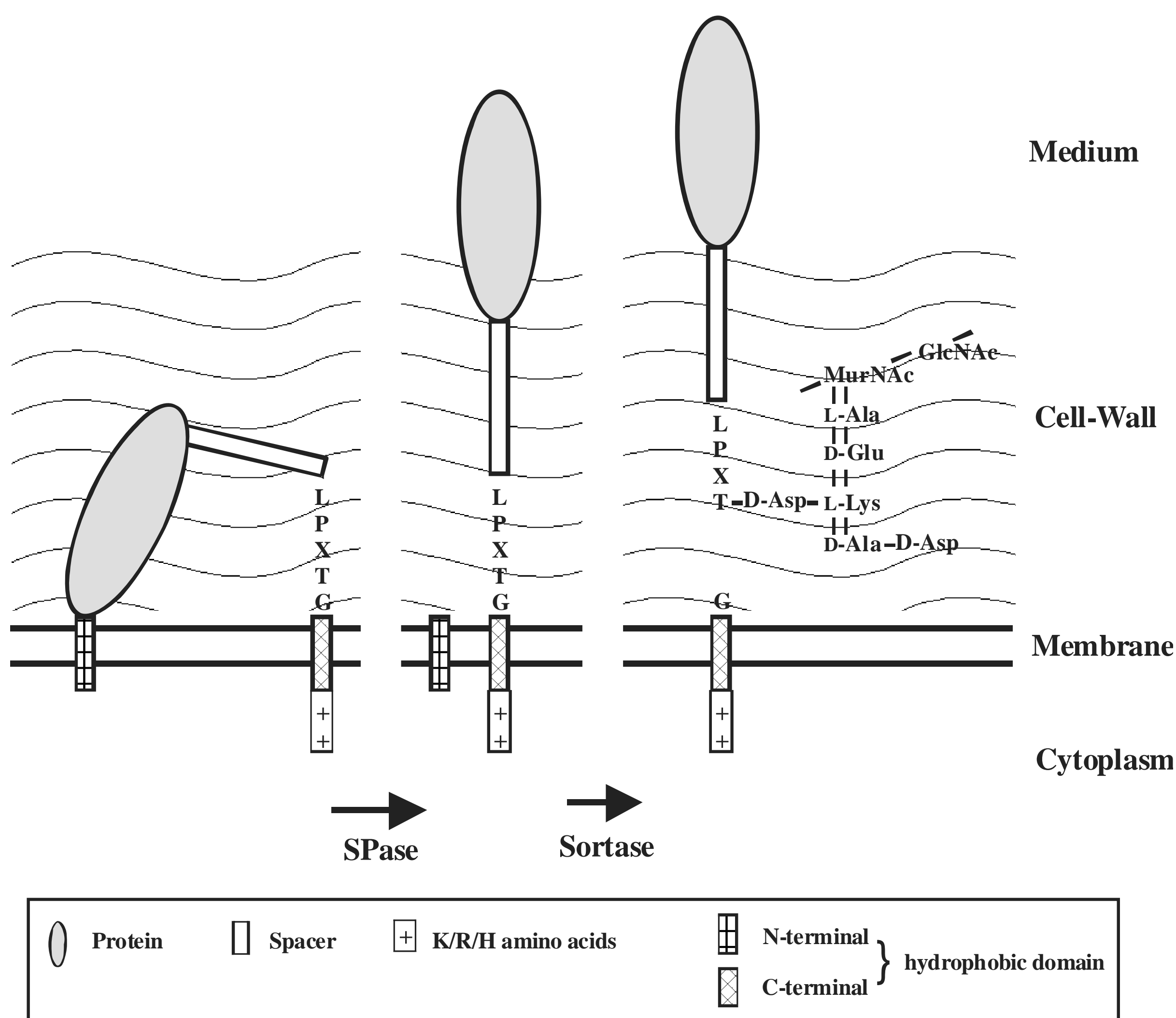


Figure 8. Schematic representation of the consecutive steps in linking a protein with an LPXTG motif to the cell-wall peptidoglycan layer. The spacer region consists of 50 – 125 amino acids. The C-terminal hydrophobic domain consists of about 30 amino acids. K/R/H are C-terminally located positively charged amino acids.

Currently, five different types of anchoring domains from lactic acid bacteria have been described [100]: (i) transmembrane, (ii) lipoprotein, (iii) AcmA-repeats, (iv) surface-layer-protein, and (v) LPXTG motif. *Transmembrane anchors*: These proteins contain an N-terminal hydrophobic domain, enabling them to reside in the membrane. They are not exposed at the cell surface, because at least 100 amino acids are needed for the protein to cross the peptido glycan layer [100]. *Lipoprotein anchors*: these proteins are coupled covalently to the lipid bilayer by the modification of a cysteine residue located adjacently to the signal peptidase cleavage site [100]. *AcmA-repeats anchor*: these repeats can be located in various regions of proteins. The mode of binding to the cell-wall is unclear, but probably non-covalent [100]. *Surface-layer-protein anchor*: some lactic acid bacteria contain a layer of proteins (S-layer) outside the cell-wall. These S-layer proteins contain a large C-terminal region comprising one-third of the total protein size that is thought to interact with the cell-wall [141].

LPXTG motif cell-wall anchors. Figure 8 shows a schematic representation of the attachment of a protein carrying the LPXTG motif to the cell-wall. The LPXTG motif cell-wall anchor is a C-terminally located domain consisting of (i) a spacer region (of 50 to 125 amino acid residues) rich of Proline/Glycine and / or Threonine/Serine residues [52], (ii) the well-conserved sorting signal LPXTG [52], (iii) a stretch of hydrophobic amino acids (about 30) [122], and (iv) two to three positively charged amino acids (Lysine, Arginine, and Histidine). In this model, 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 region signal sequence attached to the membrane. After proteolytic cleavage by signal peptidase (SPase), the N-terminal part of the protein, spaced by the Proline/Glycine and/or Threonine/Serine rich region, is directed outwards of the cell. Subsequently the LPXTG motif is proteolytically cleaved between the Threonine and the Glycine residues by a sortase enzyme and covalently linked to the peptido glycan layer [121, 122].

None of the five previous mentioned anchoring methods have been described for bacterial FTFs. For the cell-wall associated *S. salivarius* FTF, however, a C-terminal region that resembles the LPXTG cell-wall anchoring motif but lacks the actual LPXTG amino acid motif, was reported [143]. A LPXTG cell-wall anchoring motif has been reported for the exo-fructanase gene from *S. mutans* [20]. In **Chapters 2 and 4** we report details of the first LPXTG-carrying FTFs as present in *L. reuteri* 121.

5. Outline of this thesis

In previous studies [205] a screening of more than 180 lactobacilli for EPS production had been performed. This yielded only one strain producing large amounts of a fructan (and a glucan) polymer, *L. reuteri* 121. This strain was selected for further investigation of the fructan polymer structure, polymer synthesis, and enzymes involved in polymer synthesis. *L. reuteri* is a Generally Regarded As Safe (GRAS) bacterium, inhabiting the mammalian intestine, with well-characterized probiotic properties. Analysis of the fructan polymer produced by strain 121 showed that it contained mainly ß(2→6) linked fructosyl units, thus representing an almost unbranched levan polymer [204].

The aim of this thesis was to gain insight in the molecular and biochemical processes underlying the synthesis of the levan polymer in *L. reuteri* 121. **Chapter 1** reviews current knowledge of the synthesis of fructans and the biochemical properties of bacterial FTFs. At the start of our studies (**Chapter 2**), a molecular approach was chosen to clone the gene encoding the levansucrase enzyme, responsible for levan synthesis. Using degenerate PCR primers based on homologous regions present in bacterial FTFs, and a combination of PCR techniques, an *ftf* gene was obtained. However, following expression of this gene, supposedly encoding the levansucrase, in *Escherichia coli*, incubation with sucrose resulted in production of an inulin polymer and large amounts of fructo-oligosaccharides. Unexpectedly, we had isolated an inulosucrase gene from strain 121. Subsequent analysis of *L. reuteri* 121 cultures for inulosucrase activity yielded negative results. No expression of the inulosucrase gene was detected under the conditions tested. However, large amounts of β(2→1) fructo-oligosaccharides (1-kestose and nystose) were found in culture supernatants of strain 121 grown on sucrose, suggesting that an inulosucrase was active *in vivo*.

Levansucrase activity was clearly present and was purified from *L. reuteri* 121 culture supernatants and characterized (**Chapter 3**). Conclusive evidence is presented that this purified levansucrase is responsible for the levan production by *L. reuteri* 121. In a reverse genetics approach the gene encoding the levansucrase enzyme was subsequently cloned, expressed in *E. coli*, and the enzyme biochemically characterized (**Chapter 4**). This revealed striking similarities, but also some differences, with the strain 121 inulosucrase.

In **Chapter 5** a biochemical and kinetic characterization of the *L. reuteri* 121 inulosucrase is presented. This enzyme shows unusual kinetics not seen before for bacterial FTFs. In **Chapter 6** the results reported in this thesis are summarized and discussed.