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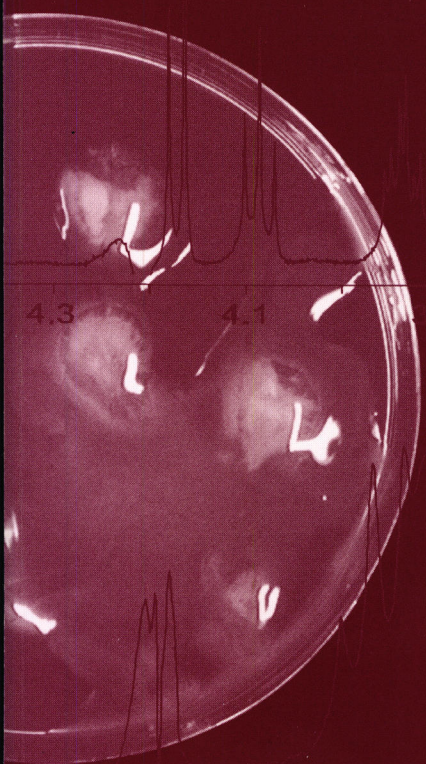
Molecular characterization of a fructosyltransferase and a glucansucrase



Ineke van Geel-Schutten

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RIJKSUNIVERSITEIT GRONINGEN

Exopolysaccharide synthesis by *Lactobacilus reuteri*:

Molecular characterization of a fructosyltransferase and a glucansucrase

Proefschrift

ter verkrijging van het doctoraat in de
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aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. D.F.J. Bosscher
in het openbaar te verdedigen op
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Gerritdina Hendrika van Geel-Schutten
geboren op 10 augustus 1962
te Almelo

Promotores	:	Prof.dr. L. Dijkhuizen Prof.dr. P.H. Pouwels
Beoordelingscommissie	:	Prof.dr. B.W. Dijkstra Prof.dr. W.N. Konings Prof.dr. O.P. Kuipers

Voor Henk,
aan Eva, Lisa en Hanna

Omslag: Glucan en fructan producerende kolonies van *Lactobacillus reuteri* op een MRS-s agar plaat; ^{1}H NMR spectra van het glucan en het fructan geproduceerd door *Lb. reuteri*

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Contents

Voorwoord

Dit voorwoord, dat waarschijnlijk het meest gelezen onderdeel van dit proefschrift wordt, is misschien wel het moeilijkst geweest om te schrijven. Het is ook het gedeelte waarover het langst is nagedacht. Het is bedoeld om iedereen te bedanken die direct of indirect heeft meegewerkt aan het tot stand komen van dit proefschrift, en dat zijn een heleboel mensen. Ik wil, ondanks het risico dat ik iemand oversla, toch een poging wagen iedereen te noemen.

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Lieve Eva, Lisa en Hanna: wat stelt een promotie nou voor vergeleken met het behalen van een zwemdiploma, met het voor het eerst naar school gaan of met het zetten van de eerste stapjes?

Ineke

1

General introduction

Lactic Acid Bacteria and Exopolysaccharide Synthesis

PREFACE

This introduction provides background information about the research described in this thesis. First, some features of the strains used for the experiments, i.e. lactobacilli, are described. This description includes their carbohydrate metabolism, their application, and a few remarks on the limited possibilities of gene cloning in members of this genus. Special attention is given to *Lactobacillus reuteri*, the strain selected for the research described in the chapters 3, 4 and 5 of this thesis. The second part of this chapter describes exopolysaccharide (EPS) synthesis by lactic acid bacteria (LAB) in general. The EPSs produced by LAB can, based on their monosaccharide composition and biosynthetic pathway, be divided into two classes, the homopolysaccharides and the heteropolysaccharides. Examples of both EPS types are given, and the mechanisms of their biosynthesis are described. The *Lb. reuteri* strain described in this thesis synthesizes two types of homopolysaccharide, using sucrase types of enzymes.
General properties of sucrase enzymes are described in more detail in part 3 of this introduction. Finally, an outline of this thesis is given in part 4.

1 LACTOBACILLI

1.1 General description and applications

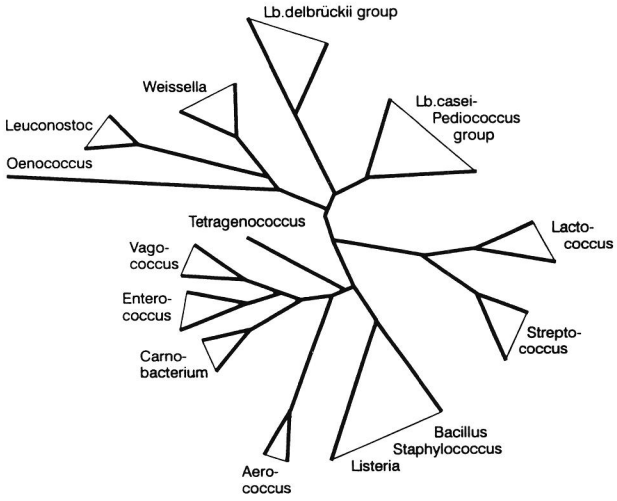
Lactobacilli are facultatively anaerobic Gram-positive microorganisms which produce lactic acid as the major end product during the fermentation of carbohydrates. Lactobacilli are found in habitats where rich, carbohydrate containing substrates are available. Examples of such habitats are mucosal membranes 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 (Wood and Holzapfel 1995). 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 (LAB) (Stiles and Holzapfel 1997). A phylogenetic tree of the LAB is given in Figure 1.

Historically, the genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* form the core of the LAB group (Axelsson 1998). Members of these genera play an essential role in the fermentation of food (for instance cheeses, milks, breads, wines, pickles and meats) and feed. LAB 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. LAB biochemical processes which are of technological importance are carbohydrate and citrate metabolism, proteolysis, and production of antimicrobials and exo-polysaccharides (Daly et al. 1996). There is also an growing interest in LAB because of the assumed health benefits (pro- and prebiotics) they confer and various other medical applications.

1.2 Carbohydrate metabolism

The general metabolism and physiology of LAB 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 necessary solutes. Carbohydrates are metabolized very rapidly via fermentation. This fast fermentation of carbohydrates, coupled to substrate level phosphorylation, is an essential feature of the metabolism of lactobacilli.

(a)



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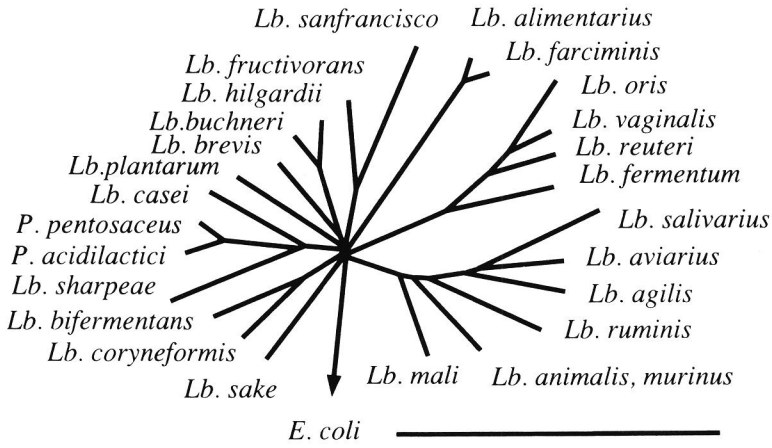

Figure 1 (a) Schematic, unrooted phylogenetic tree of lactic acid bacteria, including some aerobic and facultatively anaerobic Gram-positive bacteria of the low G + C subdivision. A phylogenetic tree of the *Lactobacillus casei-Pediococcus* group to which *Lb. reuteri* is related is given in (b). The bar indicates 10% expected divergence. (a) from Axelsson (1998), (b) from Schleifer and Ludwig (1995).

1.2.1 Transport and metabolism of monosaccharides

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.

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 the products of a heterofermentative fermentation. In general, hexoses are metabolized via the Embden-Meyerhof-Parnas (glycolysis) pathway in homofermentative lactobacilli, whereas the 6-phosphogluconate/phosphoketolase pathway is used in heterofermentative strains (Figure 2).

In the presence of oxygen or other oxidants increased amounts of acetate may be produced at the expense of lactate or ethanol, whereby one additional mole of ATP is gained via the acetate kinase reaction. Thus, variations in the metabolic end products may occur. 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 (Axelsson 1998).

1.2.2 Transport and metabolism of disaccharides

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 cleaved by specific hydrolytic enzymes to monosaccharides, which enter the major pathways described above. When a PTS is involved, specific phosphohydrolases split the phosphorylated disaccharide into a free monosaccharide and a monosaccharide phosphate. 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.

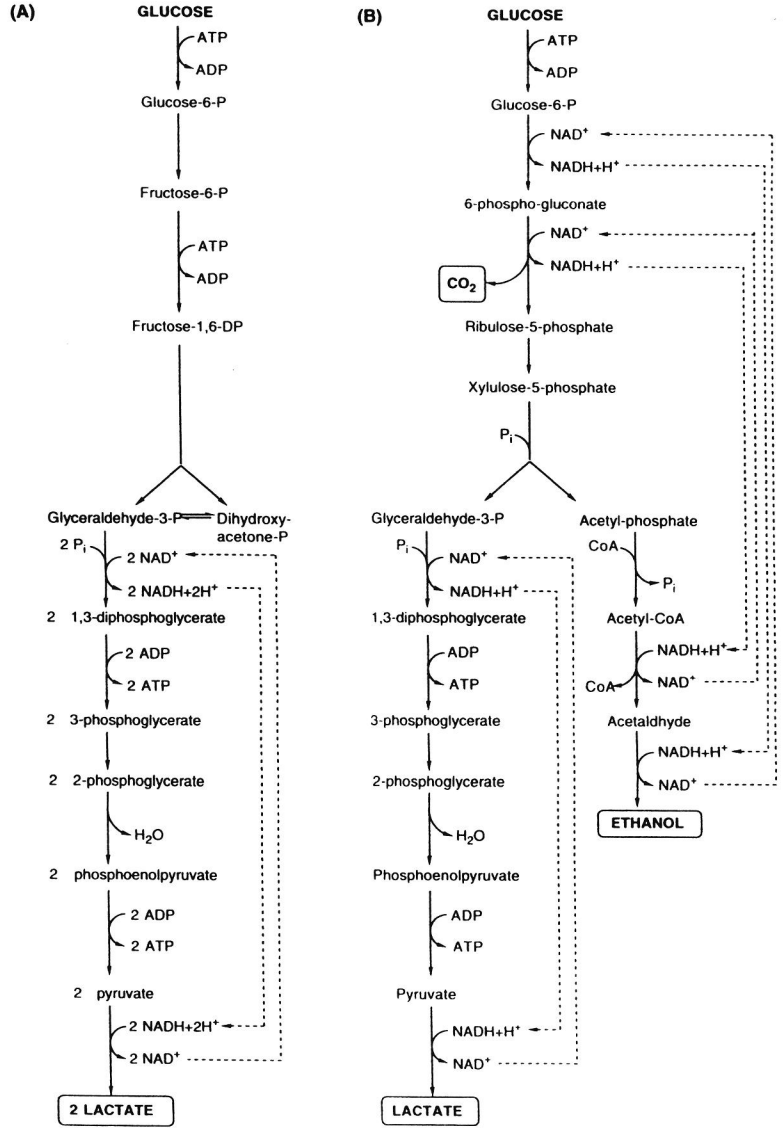

Figure 2 Major pathways of glucose fermentation in lactic acid bacteria.
A. Homolactic fermentation (glycolysis, Embden-Meyerhof-Parnas pathway)
B. Heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway)
From Axelsson (1998)

Sucrose may also act as a donor of monosaccharides for exopolysaccharide (EPS) formation in certain lactic acid bacteria. In dextran production, by *Ln. mesenteroides* for instance, the sucrose is cleaved by a dextransucrase. The glucose moiety is used for dextran synthesis and fructose is fermented in the usual manner (Axelsson 1998). Oral streptococci are also capable of synthesizing glucose containing polymers from sucrose. In some of these streptococci, the fructose moiety of sucrose can be used for polymer synthesis. In this way, levans and inulin-like polymers are formed. The enzymes responsible for the synthesis of these glucose and fructose containing polysaccharides from sucrose are located extracellularly (see paragraph 3).

1.3 Gene cloning in Lactobacilli

Lactobacillus strains generally have been very difficult to access genetically. Therefore, in the past, most molecular biological research on LAB has been performed on lactococci and streptococci. Progress in research on the genetics and molecular biology of lactobacilli has been further complicated by the fact that lactobacilli are a very heterogeneous group of bacteria. Another barrier for genetic studies has been the inefficiency of transformation systems for lactobacilli. This barrier is now mostly overcome since relatively efficient electroporation techniques have become available in recent years. Knowledge about molecular biology of *Lactobacillus* is evolving very rapidly now (Von Wright and Sibakov 1998). However, not all *Lactobacillus* strains are genetically well accessible yet and results on transformation efficiency are strain dependent. Furthermore, cloning of *Lactobacillus* genes in *E. coli* frequently results in structural instability with concomitant loss of parts of the plasmid vectors (Pouwels et al. 1996). Gene disruption is in some cases very complicated to achieve. The use of integration vectors for *Lactobacillus* strains harboring plasmids, like the *Lb. reuteri* strain described in this thesis, often does not result in the desired gene disruption (Leer, R. personal communication).

1.4 *Lactobacillus reuteri*

Lb. reuteri belongs to the obligately heterofermentative lactobacilli. The species was originally isolated from human breast milk (Kandler et al. 1980). Its ecosystem is the gastrointestinal tract of a broad spectrum of hosts, including humans and all mammals and avian hosts examined to date. It is the major component of gut lactobacilli and the only enterolactobacillus known to be indigenous to such a broad spectrum of hosts. Another

interesting characteristic, not noted in other enterolactobacilli, is that *Lb. reuteri* strains isolated from different hosts have distinctive colony morphologies while retaining similar physiological and genetic characteristics. This may indicate an adaptation of its surface properties to meet specific host colonization site requirements during the evolution of symbiotic relationships with various hosts (Casas et al. 1998).

Lb. reuteri is the only enterolactobacillus shown to be capable of producing and secreting the antimicrobial metabolite reuterin (3-hydroxypropanal), that 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 (Stiles and Holzapfel 1997, Casas et al. 1998). 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 the DNA synthesis (Ouwehand 1998).

Because of this reuterin production, *Lb. reuteri* strains are of interest for various applications. For instance, El-Ziney et al. (1996) suggested that reuterin, or *Lb. 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 *Lb. reuteri* is reutericin 6, a peptide of 2.7 kDa. 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 *Lb. delbrueckii* subsp. *bulgaricus* (Kabuki et al. 1997).

In addition to the application of *Lb. reuteri* for its antimicrobial properties, *Lb. reuteri* is considered to be useful as an effective health promotor (probiotic, see below). Application of the strain in feeds is mentioned as an alternative for the addition of antibiotics. Moreover, there are indications that *Lb. reuteri* administration would result in a cost-effective enhancement of productivity in the poultry industry.

1.4.1 *Lactobacillus reuteri* and pre- and probiotics

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 (Havenaar and Huis in 't Veld 1992). Probiotic effects are considered to include inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activity, increase of the immune response and reduction of

cholesterol levels (du Toit et al. 1998). LAB 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. 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.

Prebiotics are defined as enhancers of probiotic strains or of beneficial endogenic strains of the gastrointestinal tract. Certain oligosaccharides and polysaccharides for instance are resistant to digestion by enzymes of the gastro-intestinal tract. They can, however, be fermented by beneficial microorganisms, such as bifidobacteria. Prebiotics therefore, selectively stimulate the growth of these microorganisms (Salminen et al. 1998, Casas et al. 1998). Symbiotics are combinations of pro-and prebiotics.

The efficacy of some strains of *Lb. reuteri* as a probiotic has been demonstrated in poultry, in various other animals, and in humans (Casas et al. 1998). Administration of *Lb. 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 (De Smet et al. 1998, Du Toit et al. 1998). In humans, *Lb. reuteri* is, for example, effective as a therapeutic agent against acute rotavirus diarrhea in children. Administration of *Lb. reuteri* to patients hospitalized with diarrhea resulted in shortening and amelioration of acute diarrhea. These therapeutical benefits were observed within 24 h of the onset of treatment (Shornikova et al. 1997). The effectiveness of *Lb. reuteri* as a probiotic apparently is very broad: Casas et al. (1998) have proposed that strains of *Lb. reuteri* can manifest a probiotic effect on their hosts when these are challenged by either bacterial, viral, or protozoal stressors.

Lb. reuteri's mode of action as a probiotic remains to be determined. Preliminary studies indicate that gut colonization by *Lb. reuteri* may be of importance in 1) immunopotentiating the host's cell-mediated and humoral immune responses and 2) 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 (Casas et al.1998). 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.

Lb. reuteri has already been selected as an additional probiotic culture to supplement the more commonly used *Lactobacillus acidophilus* and *Bifidobacterium* species. Milk to which the three cultures were added, named "BRA" milk, has been introduced on the Swedish market (Speck et al. 1993). A "probiotic enhanced" (symbiotic) yogurt (SymBalance, ToniLait AG, Bern, Switzerland) has recently been introduced in the market. It contains four human probiotic bacteria, including *Lb. reuteri*, and inulin as a prebiotic, which selectively stimulates bifidobacteria growth in the human colon (Casas et al. 1998). *Lb. reuteri* strain SD2112 is commercially exploited as a health promoting strain by BioGaia (Raleigh, North Carolina, USA; Sanders and Huis in 't Veld 1999).

2 EXOPOLYSACCHARIDE PRODUCTION BY LACTIC ACID BACTERIA

Some lactic acid bacteria, including species of *Lactobacillus*, are known to produce extracellular polysaccharides. These EPSs are large molecules with molecular masses varying between 10^1 to 10^4 kDa, i.e. approximately 50 to 50,000 glycosyl units. The physiological role of EPS in these bacteria has not been clearly established, and is probably diverse and complex. It has been suggested that EPS may play a role in protecting the cell against desiccation, phagocytosis and phage attack. EPS may also contribute to the provision of higher oxygen tension and participate in the uptake of metal ions. Furthermore, EPS may function as adhesive agents and facilitate interactions between plants and bacteria. Most EPS do not appear to function as energy sources for growth, since slime-forming LAB are usually not capable of catabolizing the polymer which they synthesize (Cerning 1990). Homopolysaccharides (glucans) formed by oral streptoccocci apparently have a major influence on the formation of 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 (Russell 1990).

EPS can be used as viscosifying, stabilizing, emulsifying, prebiotic, gelling or water binding agents in the food as well as in the non food industry (Whitfield 1988, Roller 1992, Sutherland 1972). Moreover, some polysaccharides may contribute to human health, either as non-digestible food fraction, or because of their antitumoral, antiulcer, immunomodulating or cholesterol lowering activity (De Vuyst and Degeest 1999). Depending on their composition and mechanism of biosynthesis, EPS can be divided in two classes: heteropolysaccharides and homopolysaccharides.

2.1 Heteropolysaccharides produced by lactic acid bacteria

Heteropolysaccharides from LAB are composed of a variety of sugar residues, mostly glucose, galactose, fructose and rhamnose. Sometimes charged groups like acetate, phosphate or glycerolphosphate are also present. Heteropolysaccharides are usually produced in low amounts (below 100 mg/l). In fermented milks and yogurt, for example, they are produced in situ by the starter cultures and are essential for the consistency of the products.

Heteropolysaccharides are produced by for instance *Lb. sake* (Van den Berg et al. 1993, 1994), *Lb. casei* (Kojic et al. 1992), *Lb. delbrueckii* spp *bulgaricus* (Cerning et al. 1986, Lamothe et al. 1999), *Lb. helveticus*, *Lb. paracasei, Lb. acidophilus* (Robijn 1996), *Lb. delbrueckii* (Grobben et al. 1995), *Lb. rhamnosus* (Macedo et al. 1999, Provencher et al. 1999), *Streptococcus salivarius* ssp *thermophilus* (Cerning et al. 1988, Stingele et al. 1996, Griffin et al. 1996, Dunn et al. 1999), and different *Lactococcus* strains (Cerning 1990, Cerning et al. 1992, Kitazawa et al. 1993).

Heteropolysaccharides are synthesized at the cytoplasmic membrane by glycosyltransferases, utilizing precursors formed intracellularly. The biosynthetic pathways of heteropolysaccharides show some similarities with the biosynthesis of cell wall components such as lipopolysaccharides, peptidoglycan and teichoic acid. The isoprenoid glycosyl carrier lipids in the cytoplasmic membrane, which are of importance for the biosynthesis of these components of the bacterial cell wall, also are involved in EPS biosynthesis. They function as acceptors on which the repeating units are assembled and as anchor for the polymerization of the repeating units to the mature polysaccharide. Furthermore, sugar nucleotides serve as precursors in the biosynthesis of different cell wall components as well as in the biosynthesis of heteropolysaccharides (Oba et al. 1999). The sugar nucleotides play an essential role in sugar interconversions as well as sugar activation, which is necessary for monosaccharide polymerization. Several enzymes involved in the biosynthesis of heteropolysaccharides, e.g. those involved in the synthesis of the sugar nucleotides, are not necessarily unique to EPS formation, but also play important roles in other metabolic pathways, such as the metabolism of sugars. On the other hand, EPS producing LAB employ specific glycosyl transferases for the assembly of the repeating units. Figure 3 shows a schematic representation of pathways involved in the heteropolysaccharide synthesis of *Lc. lactis* subsp. NIZO B40.

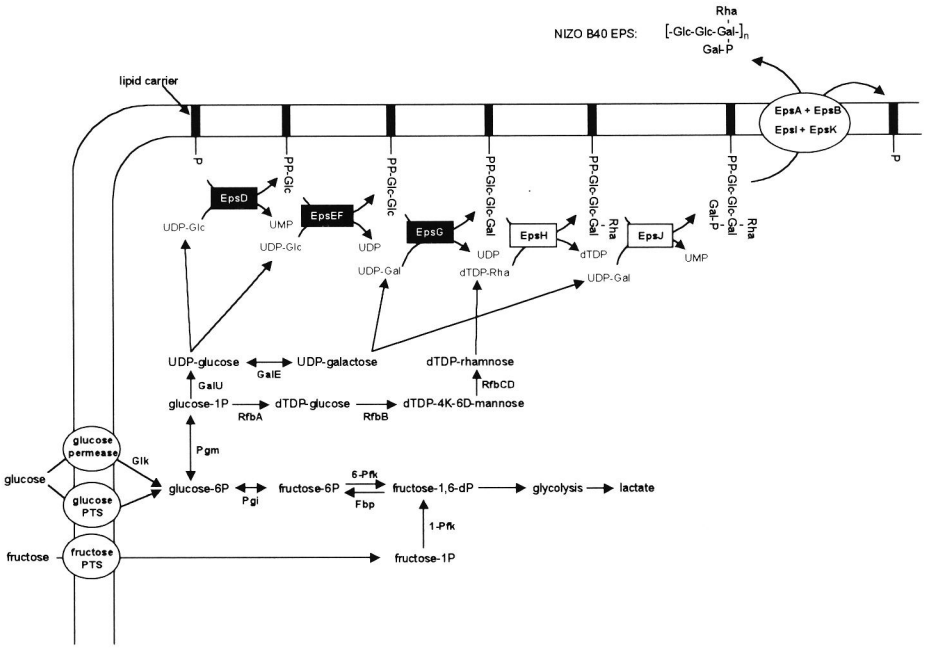

Figure 3 Schematic representation of pathways involved in of *Lc. lactis* subsp. NIZO B40 EPS biosynthesis. Relevant steps in both general metabolism (sugar nucleotide biosynthesis from glucose and fructose, lower left) and EPS biosynthesis (*eps* gene cluster encoded steps of repeating unit synthesis, export and polymerization, upper left) are depicted. Enzymes involved in general metabolism are indicated. The role of specific *eps* gene products has been experimentally determined (black boxes), or is predicted on the basis of homologies (white boxes). Glc, glucose; Gal, galactose, Rha, rhamnose; UDP-Glc, UDP-Gal and dTDP-Rha are sugar nucleotides. From Kleerebezem et al. (1999).

The ability to produce heteropolysaccharides is often an unstable characteristic in LAB. In mesophilic LAB, loss of the slime producing property has been attributed to loss of plasmids. However, there is no evidence for plasmid encoded EPS biosynthetic pathways in some thermophilic LAB strains producing EPS. In these strains the genes required for EPS biosynthesis are apparently located on chromosomal DNA. Here, the genetic instability could be due to mobile genetic elements, or to a generalized genomic instability, including DNA deletions and rearrangements (De Vuyst and Degeest 1999). The genes encoding the enzymes involved in heteropolysaccharide synthesis

and secretion in *S. thermophilus* species (Sfi6 and NCFB 2393), *Lb. delbruckii* subsp. *cremoris* and *Lc. lactis* subsp. *cremoris* are organized in gene clusters. The *eps* gene cluster of *S. thermophilus* Sfi6, for instance, contains 13 genes and is located on a chromosomal DNA fragment with a size of 14.5 kb (Stingele et al. 1996). The other known streptococcal *eps* gene clusters are also located on chromosomal DNA (Almiron-Roig et al. 1999, Griffin et al. 1996). In *Lc. lactis* subsp. *cremoris* NIZO B40 a gene cluster of 12 kb with 14 *eps* genes is located on a 40 kb plasmid. As illustrated in Figure 4, the gene cluster is preceded by an iso-IS 982 insertion sequence element (van Kranenburg et al. 1997, 1999, Kleerebezem et al. 1999).

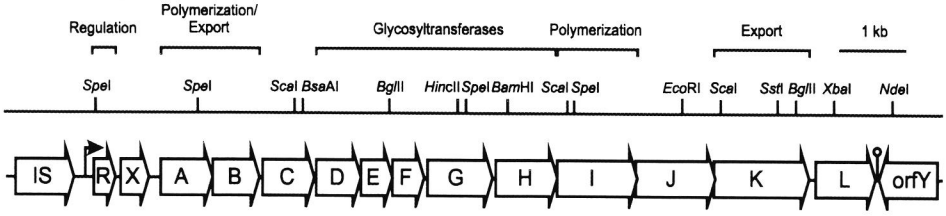

Figure 4 Physical and genetic map of the eps gene cluster of plasmid pNZ4000 of Lc. lactis subsp. NIZO B40. (From Van Kranenburg et al. 1999).

The organization, transcriptional pattern and deduced functions of the genes in the different *eps* gene clusters appear to be highly conserved. The genes are organized in four functional regions: a regulatory region at the 5' end of the gene cluster, a region involved in polymerization and export, a central region with genes showing homology with glycosyltransferases for biosynthesis and, finally, another region involved in polymerization and export (Figure 4) The streptococcal and lactoccoccal eps gene clusters are transcribed as one unit. The first gene of the cluster is preceded by an *E. coli* consensus promotor. More knowledge of the molecular organization and of factors regulating expression will make it possible to enhance EPS production and to increase the possibilities of modifying the structure and thus the properties of the heteropolysaccharides (De Vuyst and Degeest 1999, Boels et al. 1999, Ramos et al. 1999, Kleerebezem et al. 1999).

2.2 Homopolysaccharides

Homopolysaccharides are composed of one type of glycopyranosyl residue. They are synthesized outside the cell by enzymes of the sucrase type in the presence of a donor molecule, sucrose, and an acceptor molecule, e.g. the growing polymer molecule.

Examples of homopolysaccharides produced by lactic acid bacteria are fructans, such as levan (*S. salivarius*) and inulin-like polymers (*S. mutans* strains JC2, JC-1 and BHT and *S. salivarius* SS2) and glucans, such as dextran (*Ln. mesenteroides*, and *Lb. hildegardii*), mutan (various streptococci) and alternan (*Ln. mesenteroides*) (Cerning 1990, Milward and Jacques 1990, Pidoux et al. 1990, Shiroza et al. 1988, Hamada and Slade 1980, Simms et al. 1990, Ebisu et al. 1975, Leathers et al. 1997).

Levans are fructans with 2→6 linked β-fructofuranoside residues. They are either unbranched or branched at the C1 position. Inulin consists of 2→1 linked β-fructofuranoside residues with some branching at the C6 position. Levan production has been frequently studied in *Zymomonas mobilis* and *Bacillus* species. However, limited information is available about levan production by lactic acid bacteria: it has only been described for streptococci. Chapters 2 and 3 provide the first example of fructan synthesis by a *Lactobacillus* species.

Almost all glucans produced by lactic acid bacteria are composed of α-glucopyranosyl moieties; the various types of glucan differ in their linkages. A common feature of all dextrans is the preponderance of α-(1→6) linkages with some branching points at position 2, 3 or 4. Mutans differ from dextrans in containing a high percentage of α-(1→3) linkages; often a water-soluble fraction rich in α-(1→6) linkages and a water-insoluble fraction rich in α-(1→3) linkages are found. Alternan consists of alternating α-(1→6) and α-(1→3) linkages. Recently, the production of a β-(1→3) glucan by a *Lactobacillus* species has been reported (Duenas et al. 1999, Duenas-Chasco et al. 1998).

There is a tremendous variation in glucans due to differences 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. Many factors, including the type of growth media, incubation time, sucrose concentration and the presence of polysaccharide degrading enzymes, probably influence the molecular weight, structure and physical characteristics of these polymers.

2.3 Oligosaccharides

There is a growing interest in homopolysaccharides and oligosaccharides derived from homopolysaccharides, for instance for prebiotic purposes. Inulin, levan, several fructo-oligosaccharides and gluco-oligo saccharides are known to stimulate the growth of bifidobacteria in the human colon. Oligosaccharides can be synthesized by the same sucrases enzymes as the enzymes responsible for the synthesis of polysaccharides. However, the oligosaccharide synthesis reaction, 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 necessary for the acceptor reaction. Depending on for instance the nature of the acceptor molecule and sucrose concentration, different oligosaccharides can be obtained (Monsan and Paul 1995, Casas et al. 1998, Gibson et al. 1994, Quirasco et al. 1995, 1999, Patel et al. 1994, Fegy et al. 1999, Argüello et al. 1999, Alcade et al. 1999).

3 SUCRASES

Sucrases are extracellular enzymes belonging to the glycosyl transferases, which catalyze the transfer of a monosaccharide from an oligosaccharide or high-energy compound to another carbohydrate molecule as acceptor. In the presence of efficient acceptors, low molecular weight oligosaccharides are synthesized instead of the usually synthesized high molecular weight polymers. The enzymes responsible for the synthesis of glucose polymers have been referred to in the literature as glucosyltransferases or glucansucrases. The sucrases responsible for the production of polymers constituted of fructose are only referred to as fructosyltransferases or, in the case of levan synthesis, as levansucrases.

3.1 Fructan synthesizing sucrases

There are two types of polymers with fructose as the constituent sugar, inulin and levan. Inulin is synthesized by inulosucrase (sucrose: 2→1-β-D-fructan 1-β-D fructosyltransferase, E.C. 2.4.1.9).
Levan is synthesized by levansucrase (sucrose: 2→6-β-D-fructan 6-β-D fructosyltransferase, E.C. 2.4.1.10) by the following reaction:

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

All known levansucrases catalyse the fructosyl transfer from sucrose to numerous acceptors other than levan. Examples of possible acceptors are water (hydrolysis of sucrose), short-chain acylalcohols and various mono- and disaccharides. (Chambert and Petit-Glatron 1991). Within the LAB, levansucrase has been found only in *S. salivarius* and *S. mutans* (Giffard et al. 1993, Shiroza and Kuramitsu 1988). The enzyme is also found in other bacteria, e.g. *Zymomonas mobilis, Erwinia amylovora, Acetobacter amylovora, Bacillus polymyxa, Bacillus amyloliquefaciens, Bacillus stearothermophilus* and *Bacillus subtilis* (Song et al. 1995, Kyona et al. 1995, Gunasekaran 1994, Geier et al. 1993, Arrieta et al. 1996, Li 1995, Tang et al. 1990. Steinmetz et al. 1995). All these enzymes have been characterized at the gene level. At the amino acid level, the levansucrases of Gram positive and those of Gram negative bacteria show low similarity (about 20%; S. van Hijum, Chapter 4 of this thesis). The molecular masses of most bacterial levansucrases vary between 50 and 100 kDa (for instance Shiroza et al. 1988, Steinmetz et al. 1985, Sutherland et al. 1993). A value of 140 kDa, however, has been reported for the enzyme of *S. salivarius*. The levansucrase of this organism appears to be bound to the cell wall. Part of the enzyme is released into the culture medium in the presence of sucrose (Milward and Jacques, 1990). Thus far, no fructosyltransferase has been detected in lactobacilli. Most of the research on levansucrases has been performed on enzymes from *Zymomonas, Bacillus* and, to a lesser extent, *Acetobacter* species.

Chapters 2 and 3 of this thesis provide the first description of a levansucrase activity and its levan product in a *Lactobacillus* species. Chapter 4 describes the molecular characterization of a gene coding for this levansucrase.

3.1.1 Mechanism of catalysis and structure/function relationships of fructan synthesizing enzymes

In comparison to glucansucrases (see below), very little is known about fructan synthesizing sucrases. Only a few studies have focussed on structure/function relationships. The 3D structure of the levansucrase of *B. subtilis* has been reported by Lebrun and Rapenbosch (1980). This levansucrase molecule possesses a very elongated ellipsoid structure with overall dimensions of 26 x 32 x 117 Å. However, the resolution of this tertiary structure is low, 3.8 Å. Protein structures with a higher resolutions are necessary for detailed structure/function relationship studies.

The proposed mechanism of catalysis for fructosyltransferases is a two step mechanism involving a bifunctional catalysis in which an acidic group and a nucleophilic group of the protein are important for transfructosylation

(Sinnott 1987). Isolation of a stabilized enzyme-fructosyl complex of the levansucrase of *B. subtilis* and sucrose has been possible; the nucleophilic group binding to the fructosyl group of sucrose has been identified as the β-carboxy group of an Asp residu. In this enzyme fructosyl complex, β-carboxy group of the aspartic acid was found to be covalently linked to the C-2 of the fructosyl moiety ester bond (Chambert and Gonzy-Treboul 1976). Furthermore, site directed mutagenesis experiments with the levansucrase of *B. subtilis* (SACB) showed that the amino acid at position 331 (Arg^{331}) is also of importance for the specificity and efficiency of the transfructosylation process. Arg^{331}His mutants, for instance, showed a lower polymerizing and a higher oligosaccharide forming activity than the wild type enzyme. It has been suggested that the side chain of this Arg residu at position 331 could act as a proton donor in the bifunctional catalysis (Chambert and Petit-Glatron 1991).

Up to now there are no further reports of site directed mutagenesis of levansucrases and the limited information available on levansucrases is insufficient for a full understanding of its mechanism.

3.2 Glucansucrases

Glucansucrases are extracellular enzymes synthesizing various glucans from sucrose. Glucans are produced by numerous lactic acid bacteria, e.g. by various species of *Leuconostoc* and *Streptococcus* and by some species of *Lactobacillus* and *Lactococcus*. One example is dextran, which is synthesized by dextransucrase (sucrose: 1→6-α-D-glucan 6-α-D glucosyltransferase, E.C. 2.4.1.5) according to the following irreversible reaction:

$$\text{sucrose} + (1\rightarrow6\text{-}\alpha\text{-D-glucosyl})_n \rightarrow \text{D-fructose} + (1\rightarrow6\text{-}\alpha\text{-D-glucosyl})_{n+1}$$

Various strains of *Streptococcus* and *Leuconostoc* produce more than one glucansucrase. *S. mutans* 6715 (serotype g) for instance produces 3 kinds of glucansucrase (Shimamura et al. 1983), whereas four distinct glucansucrases have been isolated from *S. sobrinus* (Walker et al. (1990). *Ln. mesenteroides* B-1355 produces three glucansucrases (Smith et al. 1998). The glucansucrases of *Streptococcus* species are produced constitutively, whereas the *Leuconostoc* glucansucrases are produced by sucrose induction, except for some constitutive mutants that have been isolated (Mizutani et al. 1994, Kim and Robyt 1995). Sucrose is the only natural substrate for glucan synthesis. Energy necessary to catalyze the reactions is provided by the

cleavage of the glycosidic bond in sucrose. All known glucansucrases are large enzymes with an average molecular weight of 160,000 Da.

The nomenclature of glucan synthesizing enzymes is confusing. The enzymes from *Leuconostoc* species are commonly called dextransucrase (DSR), whereas the streptococcal enzymes are referred to as glucosyltransferase (GTF). Other glucansucrases are for example alternansucrase (ASR; EC 2.4.1.140), amylosucrase (EC 2.4.1.4) and a streptococcal glucosyltransferase, synthesizing mutan (EC 2.4.1.-).
Also a glucansucrase synthesizing only α-(1→2) linkages has been isolated (Smith et al 1998). Amylosucrase is not found in lactic acid bacteria but only in *Neisseria* strains (Buttcher et al. 1997, De Montalk et al. 1999).

3.2.1 Mechanism of catalysis of glucansucrase

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. A broad spectrum of carbohydrates can be used as acceptors. In addition to the synthesis of glucan and oligosaccharides, the enzymes are 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.

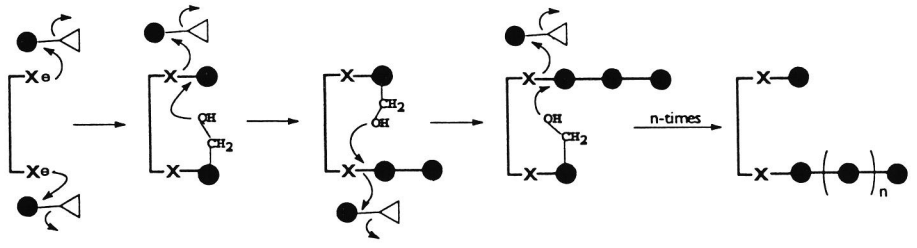

Figure 5 Two-site insertion mechanism for the synthesis of *Ln. mesenteroides* B-512F dextran by dextransucrase. X orients the glucosyl units so that their C6 hydroxyl groups can make an attack onto C1 of the opposed glucosyl unit. X, nucleophylic group; ●, glucosyl unit, ▽, fructosyl unit. From Robyt (1996).

Principally, glucan synthesis occurs via the formation of covalent glucosyl- and glucanyl-enzyme complexes (Mooser and Iwaoka 1989). The glucosyl residue from sucrose is then transferred to the reducing end of the glucan chain in glucan synthesis, whereas it is transferred to the non-reducing end of the acceptor molecule in the acceptor reaction. The formation of the covalent glucosyl-enzyme complex is the only reversible step in the catalytic reaction (Robyt 1996).

Using pulse-chase techniques with ^{14}C-sucrose and immobilized *Ln. mesenteroides* dextransucrase, Robyt et al. (1974) concluded that the glucose is added to the reducing end of the growing chain by a two-site insertion mechanism (Figures 5 and 6). According to this mechanism, the reaction occurs in two steps involving two sucrose binding sites (nucleophiles, presumably carboxylate anions). In the first step the nucleophilic sites attack two sucrose molecules to give two covalent glucosyl-enzyme intermediates. In the second step the C-6 hydroxyl of one of the glucosyl intermediates makes a nucleophilic attack onto C-1 of the other glucosyl intermediate to form an α-(1→6) glucosidic linkage and an isomaltosyl intermediate. The newly released nucleophilic site attacks another sucrose molecule to give a new glucosyl-enzyme intermediate. The C-6 hydroxyl of this new glucose-enzyme intermediate then attacks the C-1 of the isomaltosyl-intermediate to give an α-(1→6) linkage and the formation of an isomaltosyl-enzyme intermediate. This process continues in a similar manner between the two sites, giving the synthesis of an α-(1→6) linked glucan chain by the addition of glucose to the reducing end of the growing chain and the apparent insertion of glucose between the enzyme and the dextran chain. The dextran chain is synthesized de novo without the need for any primer (i.e. a molecule like an oligosaccharide or glucan chain, necessary to start the synthesis reaction) and without the need for the dissociation of the dextran chain from the active site before the next glucose residue is added.

The mechanism for mutan synthesis by *S. mutans* is essentially the same and differs from dextran synthesis only by having the glucosyl-enzyme intermediates oriented in such a way that their C-3 hydroxyl groups are stereochemically placed to perform a nucleophilic attack onto C-1. This would result in the formation of α-(1→3) glucosidic linkages. The synthesis of alternan can likewise be postulated, with the intermediates stereochemically positioned differently.

Branching of the glucans can take place without a separate branching enzyme when a glucan chain acts as an acceptor and attacks the covalent glucosyl- or glucanyl-enzyme complexes. In this way, glucose or a glucan chain can be transferred to the acceptor chain where they are attached through branch linkage.

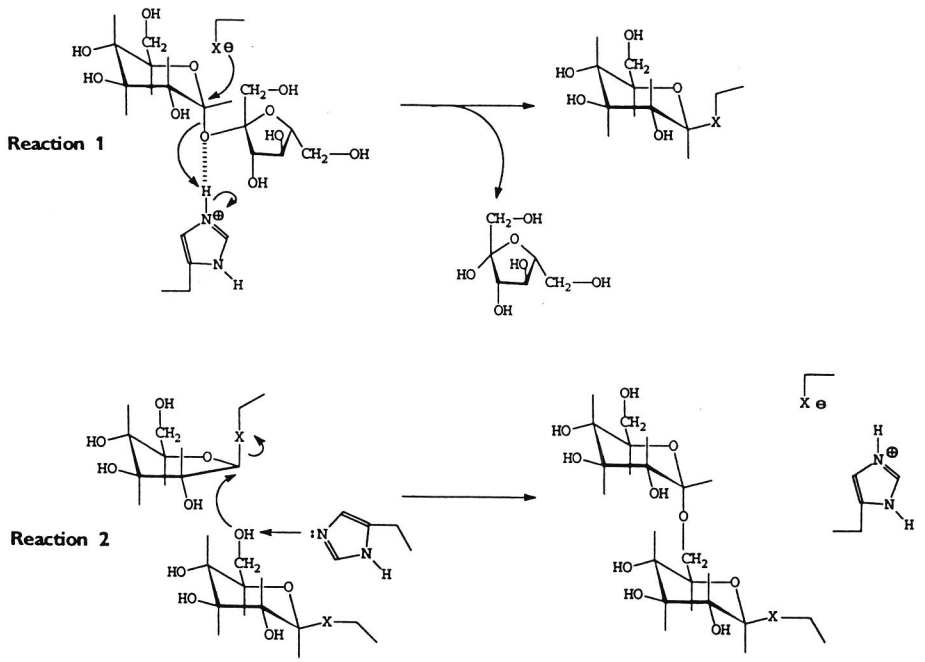

Figure 6 Mechanism for the cleavage of sucrose and the formation of an α-(1→6) glucosidic bond by dextransucrase. Reaction 1: nucleophilic displacement and protonation of the fructose moiety to form a glucosyl-enzyme intermediate. Reaction 2: formation of an α-(1→6) glucosidic bond by attack of a C6 hydroxyl group onto C1 of a glycosyl-enzyme complex: the attack is facilitated by abstraction of a proton from the hydroxyl group by the imidazole group. From Robyt (1996).

For oligosaccharide synthesis in the acceptor reaction, the enzyme transfers glucose to carbohydrate acceptors. In this secondary reaction, the acceptor binds to a separate acceptor binding site. The mechanism of action of the acceptor reaction is one of terminating glucan synthesis by the release of the glucosyl and glucanyl units from the covalent enzyme-intermediate. Using maltose in an equilibrium dialysis experiment, one acceptor binding site was found in *Ln. mesenteroides* B-512FM. Two sucrose binding sites were found by equilibrium dialysis with 6-deoxysucrose, a strong competitive inhibitor for dextransucrase. Thus, the active site of *Ln. mesenteroides* B-512FM dextransucrase contains two sucrose binding sites and one acceptor binding site (Su and Robyt 1994).

The proposed two-site insertion mechanism described above only elucidates part of the catalytic mechanism. However, it is interesting because it explains that elongation of glucan occurs at its reducing end without the presence of an exogenous primer at the beginning of the reaction. Besides the above mentioned study of Su and Robyt (1994), there are only a few other studies suggesting the existence of two sucrose binding sites (Germaine and Schachtele 1976, Koba and Mayer 1991). However, up to now, only one site, capable of making a covalent bond with the glucose moiety originating from the breakdown of sucrose has been clearly identified by Mooser and Iwakoa (1989). They isolated a covalent glucosyl-enzyme complex from a quenched reaction of the glucosyltransferase from *S. sobrinus* with radiolabeled sucrose. The glucosyl-enzyme complex was stabilized by the carboxyl group of Glu or Asp. In subsequent studies an Asp was identified at the catalytic site by isolation of active site peptides of two *S. sobrinus* glucosyltransferases (Mooser et al. 1991).

Thus, despite of many years of research (the enzyme was first described in 1941 by Hehre), the mechanism of glucansucrase catalysis is in the main still unknown.

3.2.2 Structural and functional organization of glucansucrases and their encoding genes

More than ten streptococcal and four *Leuconostoc* glucansucrase encoding genes (*gtf* genes and *dsr* genes respectively) have been cloned and sequenced. Analysis and comparison of the different amino acid sequences of those genes show that these enzymes are closely related and have a common structure (Figure 7).

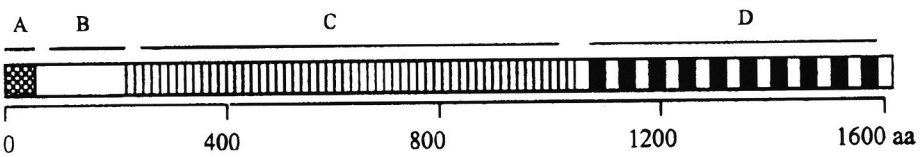

Figure 7 Schematic general structure of glucansucrases, derived from nucleotide sequences of cloned glucansucrase genes. A, signal peptide; B, N-terminal variable region; C, catalytic domain; D, C-terminal glucan binding domain. From Monchois et al. (1999a).

They are composed of four distinct structural domains: their N-terminal end begins with (1) a signal peptide of 32-34 amino acids, followed by (2) a highly variable stretch of 123-129 aa, (3) the highly conserved catalytic or sucrose binding domain of about 1,000 amino acids and (4) the C-terminal end of about 500 amino-acids (i.e. one third of the molecule), composed of a series of tandem repeats. The C-terminal end constitutes the glucan binding domain (Monchois 1999). The presence of both a C-terminal glucan binding and a sucrose binding catalytic domain is necessary to keep a fully active enzyme (Abo et al 1991).

3.2.2.1 The C-terminal glucan binding domain

Ferreti et al. (1987) were the first to determine the complete nucleotide sequence of a streptococcal glucosyltransferase, the gene encoding GTF-I from strain *S. sobrinus* Mfe28. They assigned the location of a glucan-binding site to the carboxy terminal region of the protein.
This glucan binding domain containing multiple repeats (A and C, see below) was subsequently found in the sequences of other glucosyltransferase genes from oral streptococci (Russell 1990) and in the dextransucrase genes from *Leuconostoc* species (Monchois 1999).

The C-terminal domain of all glucansucrases sequenced is composed of a series of repeated units, which have been divided into four classes; A, B, C and D-repeats. These repeat sequences exhibit high similarity to the repeats in the glucan binding protein from *S. mutans* as well as the ligand binding domains in *Clostridium difficile* toxin A and the lysins from *S. pneumoniae* (Giffard at. al 1993). Within these repeats, a common conserved repeat, designated the YG repeat, can be discerned. The YG repeat is, amongst others, defined by the presence of one or more aromatic residues (of which one is usually tyrosine), followed by glycine 3-4 residues downstream, a hydrophobic residue, a neutral polar residue (usually glycine or asparagine) and 1-3 hydrophobic residues (Giffard and Jacques 1994). The number and distribution of the A, B, C and D repeats is specific for each enzyme. A repeats are always present, often in an A-C pattern. However, it appears that D repeats are specific to enzymes produced by *S. salivarius* ATCC 25975. *Ln. mesenteroides* NRRL B-512F dextransucrase contains besides A and C repeats also N repeats, which have not been identified in streptococcal GTF's. These N repeats are not highly conserved but possess the main characteristics of YG repeats (Monchois et al. 1998).

The exact involvement of the different repeats in glucan structure determination has not been elucidated. However, except for GTF-L (*S. salivarius*), enzymes producing an insoluble glucan possess the same YG

repeat pattern, A-A-C-A-C-A-C. The minimum number of these repeated units necessary to ensure glucan binding properties is different for enzymes producing a soluble glucan compared to those producing an insoluble one, the latter enzymes appearing less sensitive to deletions. Lis et al. (1995) showed by constructing deletion derivatives with different amounts of repeats, that the four C-terminal repeats of GTF-S from *S. mutans* GS-5 constituted part of the minimum domain required for glucan binding. Deletion of the three C-terminal A-C units of GTF-G from *S. gordonii* resulted in an enzyme which produced a glucan with only α-(1→6) branches instead of α-(1→6) and α-(1→3) branched glucan produced by the wild type enzyme.

Different conserved amino acids of the C-terminal domain may be involved in glucan binding. The clustered aromatic residues (Tyr, Trp, Phe) may stabilize the binding between glucan and protein by interacting with the carbon ring of the glucopyranosyl residues. The polar residues (Lys, Gly and Phe) or acid (Asp) may allow the creation of hydrogen bonds with hydroxyl residues of sugar, whereas residues like Lys, Gly, Asp or Ser, able to introduce flexibility into the protein structure, may allow the glucosyl residue to be correctly orientated with respect to the binding sites (Monchois 1999).

The C-terminal domain is not the unique determinant in the structure of the glucan produced. This was shown by in frame fusions of the C-terminal domain of *gtfB* (encoding GTF-I, producing an insoluble glucan), with the catalytic domain of *gtfD* (encoding GTF-S, producing a soluble glucan) from *S. mutans*. This resulted in an enzyme producing an insoluble glucan, whereas the inverse in-frame fusion did not result in an enzyme producing a soluble glucan (Monchois, Willemot and Monsan 1999). Moreover, Shimamura et al. (1994) were able to identify six amino acids in GTF-S (producing soluble glucan) and in GTF-I (producing insoluble glucan) of *S. mutans* GS5, not located in the C-terminal glucan binding domain, which influenced the nature of the glucan produced. By site directed mutagenesis of these amino acids they were able to alter the nature of the glucan produced by the enzymes: increased levels of soluble glucan by GTF-I and insoluble glucan by GTF-S.

Besides the role in glucan binding and the possible involvement in the determination of the structure of the synthesized glucan, the presence of the C-terminal glucan binding domain appears to be necessary for glucansucrase activity. However, the precise role of this domain in enzyme catalysis remains largely unknown. The glucan binding domain may be of importance for the polymer chain growth. Some enzymes with deletions at the C-terminal end retain hydrolytic activity but glucan binding and synthesis properties disappear. For instance, the *drsT* gene of *Ln. mesenteroides* lacks 5 nucleotides in the ORF, resulting in a frameshift which causes premature termination of mRNA translation. The encoded glucansucrase showed a 50 kDa deletion at the

C-terminal end and lacked the glucan binding domain. This enzyme still possessed a weak sucrose hydrolyzing activity, but was unable to synthesize glucan. Glucan synthesis and sucrose cleavage could be restored by constructing a new gene (*drsT5*), in which the 5 nucleotides were present, resulting in synthesis of the complete protein of 210 kDa (Funane et al. 1999). Monchois et al. (1998) suggested that the C-terminal domain could also play a facilitating role in the transfer of products from the catalytic site. Truncations in the glucan binding domain of DSR-S from *Ln. mesenteroides* did modify the V_{max} of the dextran synthesis, but the K_m for sucrose, optimum pH and energy of activation of this enzyme were not affected by these C-terminal deletions.

3.2.2.2 The catalytic domain

The catalytic domain of glucansucrases is highly conserved, as shown by protein sequence alignments. However, these sequence alignments have not allowed identification of amino acids essential for particular steps in the mechanism of enzyme catalysis. This shows that the knowledge of primary sequences of glucansucrases is not sufficiently developed for investigating structure/function relationships.

A conserved Asp at the active site of a glucosyltransferase GTF-S of *S. sobrinus* was identified by the isolation of a stabilized glucosyl-enzyme complex in which the carboxyl group of the Asp was a covalently linked with the glucose moiety from sucrose (Mooser and Iwakoa 1989). The importance of an Asp residue in sucrose binding, Asp^{451} was confirmed by Kato et al. (1992) for the GTF-I enzyme from *S. mutans* GS5. Conversion of this residue to Glu, Asn or Thr resulted in mutant proteins without enzymatic activity. Mooser et al. (1991) also confirmed the existence of a catalytic Asp residue in GTF-I and GTF-S of *S. sobrinus* by the isolation and determination of the amino acid sequence of active site peptides. They suggested that at least one other amino acid has to be involved in the catalytic process to facilitate the fructose release by donating a proton. In addition to Asp acid residues, the involvement of His residues has been illustrated by different experiments (Fu and Robyt 1988, Funane et al. 1993, Tsumori et al. 1997).

Up to now, there are no structural data of the glucansucrases. However, in the absence of structural information an alternate approach has been to undertake molecular modeling studies of the enzymes. Secondary structure prediction studies of the catalytic domain of α-(1→3) and α-(1→6) synthesizing glucosyltransferases, like *Leuconostoc* dextransucrases and *S. mutans* glucosyltransferases GTF-I and GTF-S, tend to show that glucansucrases posses an $(\beta/\alpha)_8$ barrel structure like glycosidases (including α-amylase), cyclodextrin glycosyltransferase (CGTase), isoamylase and a

dextran glucosidase of *S. mutans*. This motif is characterized by the presence of 8 β-sheets (E1-E8) located in the core of the protein alternated with 8 α-helices (H1-H8) located at the surface of the protein. The α/β-barrel of the glucosyltransferases is circularly permuted. Whereas the barrel domains in α-amylases and related amylolytic enzymes begins with a β-strand, the α/β barrel in the glucan-synthesizing glucosyltransferases starts with an α helix and ends with a β-strand equivalent to α-helix 3 and β-strand 3, respectively, in the α-amylases (Figure 8; MacGregor et al. 1996).

An alternative alignment of the glucosyltransferase GTF-I from *Streptococcus downei* with different α-amylases also resulted in the classification of this protein as a member of the 4/7 superfamily of the glucohydrolases. This family includes α-amylases and cyclodextrin glycosyltransferases which belong to family 13. An important characteristic of this superfamily is the presence of aspartates/glutamates at the C terminal end of the fourth and seventh strand of the $(\beta/\alpha)_8$ barrel (Devulapalle et al. 1997).

Much information is available about structure function relations of family 13 enzymes. Furthermore, the 3D protein structures of several family 13 enzymes are known (for example Uitdehaag 1999). Therefore, the above mentioned structure predictions allowed the assignment of conserved amino acid residues in GTFs, possibly playing a role in the catalytic mechanism of the glucansucrase enzymes. For GTF-I of *S. mutans* GS-5 for instance, the conserved amino acids Trp^{491}, and His^{561} were demonstrated to be essential for catalysis by site-directed mutagenesis experiments. These residues were selected as targets for mutagenesis by the alignment of GTFs with the α-amylase family proteins. Conversion of Trp^{491} or His^{561} to either Gly or Ala resulted in mutants which had completely lost activity. Asp^{413}, however, was not essential for enzyme activity. Asp^{413}Thr mutants retained 12% of the wild type activity and exhibited a K_m for sucrose which was identical to that of the parental enzyme, suggesting that this Asp^{413} is not part of the sucrose binding site of the enzyme. This residue could function as an essential proton donor in catalysis or in the essential β→α loop structure of the enzyme (Figure 8; Tsumori et al. 1997).

The enzymes of the α-amylase family contain three acidic amino acids with carboxyl groups important for catalysis, at or near the C-terminal β-strands 4, 5 and 7. Equivalent residues occur invariably in glucosyltransferases: Asp^{415}, Glu^{453} and Asp^{526} in GTF-I (*S. mutans*); Asp^{437}, Glu^{475} and Asp^{547} in GTF-S (*S. mutans*); and Asp^{551}, Glu^{589} and Asp^{662} in the *Leuconostoc* dextransucrase DSRS (Figure 8; McGregor et al. 1996). These observations suggest that these residues may be involved in the catalytic mechanism of the glucosyltransferases. Substitutions of these residues in

GTF-I supported this hypothesis, since they resulted in reduction of enzyme activity. The mutated enzymes retained less than 0.01% of the wild type activity (Devulapalle et al. 1997). Furthermore, mutation of Asp^{511} and Asp^{551} in *Ln. mesenteroides* NRRL B-512F glucansucrase, both to Asn, completely suppressed the dextran and oligosaccharide synthesis. Mutation of Asp^{513} and His^{661} to Asn and Arg respectively, resulted in greatly reduced dextransucrase activity. According to sequence alignments with glucosyltransferases, α-amylases and cyclodextrin glucanotransferases, His^{661} may have a hydrogen-bonding function (Monchois et al. 1997).

For GTF-S of *S. mutans* possible roles of other amino acid residues have been assigned. The residues His^{546} and Gln^{920} of GTF-S may stabilize the transition step, whereas Asp^{440}, Asp^{441}, Ala^{476}, Trp^{477} and Ser^{478} may play a role in binding of acceptor molecules and in the transfer of the glucosyl residue to the non reducing end of the acceptor molecules. Furthermore, calcium binding by Asp^{397} and chloride binding by Arg^{436} of GTF-S have been demonstrated. (Figure 8; MacGregor et al. 1996, Monchois et al. 1999).

All the above mentioned (mutagenized) amino acids are located in the putative $(\beta/\alpha)_8$ barrel of the catalytic core of the glucansucrases. Recently, Monchois et al. (1999a) demonstrated, by different truncations upstream of this putative $(\beta/\alpha)_8$ barrel of GTF-I, the importance of the N- terminal region of the catalytic core in catalysis. Site directed mutagenis of amino acid residues in this region, Trp^{344}Leu, Glu^{349}Leu and His^{355}Val, resulted in a severely reduced mutan and oligosaccharide synthesis (respectively 3%, 30% and 15% residual activity). The first mutation had also a strong effect on the k_{cat} value suggesting that Trp^{344} may be involved in the catalytic mechanism of the enzyme. The mutation in His^{355} resulted in reduced oligosaccharide yields with longer acceptors and in a reduced mutan yield, suggesting that this residu is of importance in binding of acceptors and glucan elongation.

Although the above described approaches of alignments and site directed mutagenesis provide information about the role of parts of the glucansucrase enzyme and its mechanism, more research is necessary in the future for a full understanding of the action of the enzyme. Hopefully, crystallization efforts will succeed, making the elucidation of the 3D protein structures of the enzymes, and subsequently of structure/function relationships, possible. Eventually, this may lead to carbohydrate engineering.

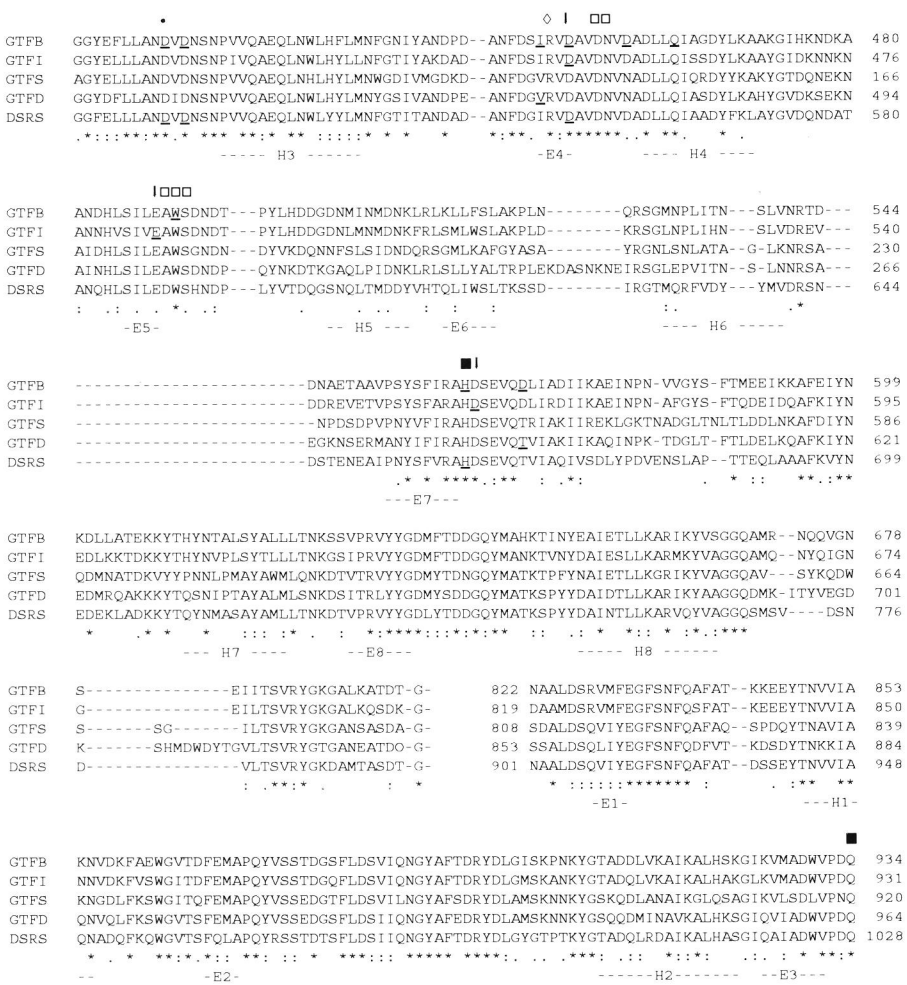

Figure 8 Sequence alignment of a part of the conserved catalytic domain of glucansucrases, GTF-B (GTF-I of *S. mutans*), GTF-D (GTF-S of *S. mutans*), GTF-I *(S. downei*), GTF-S (*S. downei*), DSR-S (*Ln. mesenteroides*). *, identical or conserved residues in al sequences; :, conserved residues; ., semi-conserved residues; --- gap in the sequence, **aa**, amino acids which have been mutated; aa-aa-aa peptide sequences used to generate antibodies; ⇓, putative catalytic residues; •, putative calcium binding sites; ■, putative residues stabilizing the transition state; , residues possibly playing a role in the binding of acceptor molecules and in the transfer of the glucosyl residue; ◇, putative chloride binding sites: **-Ex-**, localization of β-strands; **-Hx-**, localization of a-helices according to MacGregor et al. (1996). Adapted from Monchois et al. (1999a).

4 OUTLINE OF THIS THESIS

Because of their GRAS (Generally Regarded As Safe) status, lactobacilli are of importance in the production of food and feed. Moreover, some strains are known as probiotics. Some lactobacilli possess the ability to produce polysaccharides. The polysaccharides produced by lactobacilli could be useful as viscosifying, stabilizing, emulsifying, gelling or water binding agents in the food as well as in the non food industry, or may contribute to human health, either as prebiotic, or because of their antitumoral, antiulcer, immunomodulating or cholesterol lowering activity. Furthermore, the EPSs may play a role in colonization of the probiotic strains.

The aim of the research described in this thesis was to isolate EPS producing *Lactobacillus* strains and to characterize (1) the polymers produced, (2) the enzymes responsible for the synthesis of these EPS molecules and (3) the genes encoding these enzymes.

In Chapter 2 a new method for the screening of lactobacilli for the production of EPS is presented. This procedure resulted in the isolation of *Lactobacillus* strains producing large amounts of polysaccharides from sucrose, mostly glucans. One of these strains, identified as *Lb. reuteri*, a known probiotic strain, produced a glucan and a fructan during growth on sucrose. This is the first example of a *Lactobacillus* strain producing more than one type of polysaccharide and also of levan synthesis by a *Lactobacillus* strain.

Chapter 3 describes the characterization of the polysaccharides (a linear, unbranched levan and a glucan with a unique structure) produced by *Lb. reuteri* and the identification of the corresponding glucan- and levansucrases. Spontaneous mutants of *Lb. reuteri*, lacking levansucrase activity (e.g. strain 35-5) or lacking both levan- and glucansucrase activity (e.g. strain K-24), were isolated from samples of chemostat cultures of the wild type strain.

Chapter 4 describes the isolation of the first levansucrase gene of a *Lactobacillus* strain. Using degenerate primers based on conserved sequences of known levansucrases, part of a *Lb. reuteri* gene encoding a levansucrase was amplified by PCR. The gene was isolated, characterized, expressed in *Escherichia coli* and the products synthesized with sucrose as substrate were characterized. Based on alignments of the deduced amino acid sequences of other levansucrases and that of the gene of *Lb. reuteri*, a phylogenetic tree of levansucrases was constructed. *Lb. reuteri* levansucrase was found to be most closely related to the levansucrase SacB *S. mutans*.

Chapter 5 describes the isolation and characterization of the first glucansucrase gene of a *Lactobacillus* strain. The deduced amino acid

sequence of the *Lb. reuteri* glucansucrase was compared with glucansucrase enzymes from other LAB. Based on these alignments a phylogenetic tree of glucansucrases was constructed. The results show that *Lb. reuteri* glucansucrase, synthesizing a unique soluble glucan, is closely related to ASR from *Ln. mesenteroides* an enzyme synthesizing alternan. The gene was cloned and expressed in *E. coli* and the properties of the glucan synthesized by the recombinant enzyme were compared with those of the glucan synthesized by *Lb. reuteri*.

Finally, Chapter 6 contains a summary of the research described and concluding remarks on the contents of this thesis.

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2

Screening and characterization of *Lactobacillus* strains producing large amounts of exopolysaccharides

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ABSTRACT

A total of 182 *Lactobacillus* strains were screened for production of extracellular polysaccharides (EPS) using a new method: growth in liquid media with high sugar concentrations. Sixty EPS positive strains were identified; 17 strains produced more than 100 mg/l soluble EPS. Sucrose was an excellent substrate for abundant EPS synthesis. The ability to produce glucans appears to be widespread in the genus *Lactobacillus*.

The monosaccharide composition of EPS produced by *Lb. reuteri* strain LB 121 varied depending on the growth conditions (solid versus liquid medium) and the sugar substrates (sucrose versus raffinose) supplied in the medium. Strain LB 121 produced both a glucan and a fructan on sucrose, but only a fructan on raffinose. This is the first report of fructan production by a *Lactobacillus* species. EPS production increased with increasing sucrose concentrations and involved extracellular sucrase-type of enzymes.

INTRODUCTION

Polysaccharides find numerous industrial applications (Roberts 1995, Sutherland 1993, 1998). The food industry is especially interested in natural thickeners, such as guar gum, locust bean gum, pectin, starch (all from plants), gelatin (animals), alginate, carrageen, agar (all from seaweed), xanthan gum and gellan gum (all from bacteria). Most of these thickeners are additives, however, and increasingly considered less desirable.

Lactic acid bacteria are food-grade organisms, possessing the GRAS (Generally Recognized As Safe) status, and are known to produce extracellular polysaccharides (EPS) which contribute to the texture of fermented milk (Cerning 1990, Roller and Dea 1992). EPS from these bacteria thus may provide a new generation of food grade thickeners. Lactic acid bacteria often also contribute positively to the taste, smell, or preservation of the final product. Lactic acid bacteria produce both homopolysaccharides (Cerning 1990, Dunican and Seeley 1965, Robyt 1995), e.g. glucans (*Ln. mesenteroides*, streptococci) and fructans (streptococci), and heteropolysaccharides (Van den Berg et al. 1995, Cerning 1990, Cerning et al. 1994, Grobben et al. 1995, Gruter et al. 1992, Van Kranenburg et al. 1997, Stingele et al. 1996). The properties of EPS from lactic acid bacteria vary strongly, depending on monosaccharide composition, branching degree, and types of glycosidic linkages present (Cerning 1990, Robyt 1995).

Synthesis of heteropolysaccharides by lactobacilli is currently studied intensively (Van den Berg et al. 1995, Cerning et al. 1994, Grobben et al. 1995, Yamamoto et al. 1994, 1995). Few studies have focussed on synthesis of homopolysaccharides by lactobacilli (Dunican and Seeley 1965, Pidoux et al. 1990, Sharpe et al. 1972). Limited information thus is available about glucan synthesis, and no reports have appeared about fructan synthesis, by lactobacilli. Here we report the results of a new screening procedure for EPS producing lactobacilli, using liquid growth media with high sugar concentrations. In addition, the effects of growth conditions on EPS production by two selected *Lactobacillus* strains were studied.

MATERIALS AND METHODS

Strains, media and batch fermentations

A wide variety of *Lactobacillus* species, originally isolated from a variety of sources and habitats, e.g. (fermented) foods, the gastro-intestinal tract of laboratory animals and human dental plaque, was used (TNO Nutrition

and Food Research culture collection). *Lactobacillus reuteri* strains LB 121 and LB 180 have been deposited with the BCCM/LMG Culture Collection (accession numbers LMG18388 and LMG 18389 respectively).

All strains were grown anaerobically at 37°C in MRS medium (de Man et al. 1960), solidified with 20 g/l agar when appropriate. EPS production was screened in modified MRS media containing 100 g/l of glucose (MRS-g), fructose (MRS-f), maltose (MRS-m), raffinose (MRS-r), sucrose (MRS-s), galactose (MRS-gal) or lactose (MRS-l), instead of the 20 g/l glucose normally present in MRS medium. All media were autoclaved at 121°C for 15 min. In the preparation of modified MRS, the sugars were autoclaved separately.

Infusion flasks (350 ml) equipped with a magnetic stirrer, incubated in an anaerobic glove cupboard, or Applikon (Schiedam, The Netherlands) /Bioflow III (New Brunswick Scientific, Edision, U.S.A) fermentors (2.5 liter), flushed with nitrogen, were used for anaerobic batch fermentations at 37°C. In the latter case, *Lactobacillus* strains were grown at pH 4.8 or 5.8, controlled by automatic addition of 2 M or 4 M NaOH.

Each experiment described was performed at least in triplicate; data presented are averages with a standard deviation of less than 10%.

Screening for EPS synthesis

Modified MRS media (10 ml) were inoculated (1%) with strains pregrown in MRS medium. After 3 days incubation 1 ml culture samples were centrifuged (4 min x 11,000 *g*) followed by EPS isolation from supernatants. Two volumes of cold (4°C) ethanol were added to one volume culture supernatants; the mixtures were stored overnight at 4°C. Precipitates were collected by centrifugation (15 min x 2,000 g) and resuspended in one volume of demineralized water. After precipitation with two volumes of cold ethanol and centrifugation, pellets were dried at 55°C. EPS amounts were determined by measuring dry weight or total carbohydrate content of the precipitates. The EPS sugar composition also was determined (see below). Uninoculated modified MRS media served as controls. EPS slime surrounding colonies growtn on MRS agar plates, was sampled with an inoculum loop and its sugar composition was determined (see below).

Determination of EPS monosaccharide composition

Complete hydrolysis of dried EPS was carried out by incubating samples for 2 h in 1 M H_2SO_4 at 100°C. Monosaccharides were determined by High Performance Anion Exchange Chromatography using a Carbopac PA1 Column (4 x 250 mm, DIONEX) and Pulsed Amperometric Detection (PAD).

Sugars were eluted with a gradient of NaOH (0-90 mM in 25 min). Rhamnose (5 μg/ml), arabinose (50 μg/ml), galactose (30 μg/ml), glucose (105 μg/ml), xylose + mannose (26 + 51 μg/ml) and fructose (138 μg/ml) in 1 M H_2SO_4 were used as references. Using this method the detection limit for arabinose, rhamnose, galactose and glucose is 1 μg/ml, for xylose/mannose and fructose 5 μg/ml. Fructose in fructan of strain LB 121 was determined using the improved resorcinol reagent (Yaphe and Arsenault 1965).

EPS purification and analysis

EPS produced after two days growth on MRS-s or MRS-r was isolated as described above. Instead of drying, the EPS was dialyzed (cellulose dialysis tube, Sigma D-9777, cut off 12 kD) at 4°C against water for 3 days, and then followed by freeze-dried. EPS was redissolved in 0.1 M potassium phosphate (pH 6.7) and filtered over an 0.45 μm filter; 100 μl was used for High Performance Gel Permeation Chromatography (HP-GPC) analysis, at room temperature using an isocratic HPLC pump (Waters model 501). Samples were injected with an automatic injector (Gilson model 231) on a Bio-Gel TSK guard column, followed by Bio-Gel TSK 60 and a Bio-Gel TSK 30 columns. Samples were eluted at a flow rate of 0.6 ml/min with 0.1 M potassium phosphate (pH 6.7) as mobile phase. EPS was detected with an refractive index detector (Erna ERC-7510).

Enzyme localization studies

Lactobacillus strains were grown in MRS medium with 30 g/l sucrose and harvested by centrifugation (15 min x 10,000 *g*) in the late exponential growth phase. Cells were washed twice with saline solution (8.5 g/l NaCl) and resuspended to the original volume in 0.05 M citric acid/0.10 M Na_2HPO_4 buffer, pH 5.5. Culture supernatants were dialyzed (cellulose dialysis tube, Sigma D-9777, cut off 12 kD) for 24 h at 4°C against the same citric acid/Na_2HPO_4 buffer (4 times replaced by fresh buffer). The overall EPS biosynthetic enzyme activity was assayed by incubating washed cell suspensions and dialyzed supernatants with sucrose (final concentration 33 g/l) for 0-24 h at 37°C. EPS production was measured as total carbohydrate content of ethanol precipitable material (see below).

Other assays

Protein was determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin as standard. Cells were first boiled for 20 min in

1 M NaOH. Biomass (dry weight) was estimated by multiplying protein content by a factor 2 (Gottschalk 1986).

D- and L-lactic acid, and sucrose, were determined enzymatically using commercial kits (Boehringer Mannheim).

The total amount of carbohydrate was determined with phenol-sulphuric acid (Dubois et al. 1956) with sucrose as standard.

Lactobacillus strains were identified by polyacrylamide gel electrophoresis of proteins (Van den Berg et al. 1993).

RESULTS

Screening for EPS production

A total of 182 *Lactobacillus* strains were screened for EPS production on MRS-f, MRS-g, MRS-gal, MRS-l, MRS-m, MRS-r and MRS-s. Sucrose was an excellent substrate for abundant EPS synthesis; only a few strains produced EPS on other sugars. Sixty EPS positive strains were identified; 17 strains produced more than 100 mg/l of soluble EPS.

Monosaccharide composition of EPS

The EPS monosaccharide composition of the most productive strains, and of strains producing EPS from more than one sugar, was determined on liquid and solid modified MRS media. The main constituent of most EPS molecules was glucose and, to a lesser extent, fructose (Table 1). Other EPS monomers also were found occasionally, but only in small amounts. Strains LB 181 and LB 182 were exceptional, producing EPS on liquid MRS-s and MRS-l with not only glucose and fructose but also relatively high percentages of other sugars. On MRS-l, strain LB 181 produced EPS with 31.5% mannose, 25.5% glucose, 22% glucuronic acid, 17% galactose, and 4% arabinose. On MRS-s, this strain produced EPS with 30% fructose, 23% mannose, 21% glucose, 17.5% glucuronic acid, 5% galactose, and 3.5% arabinose. On MRS-l, strain LB 182 produced an EPS composed of 28.5% glucose, 27% mannose, 21% galactose, 19.5% glucuronic acid, and 4% arabinose. On MRS-s, this strain produced EPS with 41% fructose, 24% glucose, 16% mannose, 11% glucuronic acid, 5% galactose, and 3% arabinose.

Table 1 Monosaccharide composition of partially purified EPS produced by *Lactobacillus* strains after 3 days incubation at 37 °C on liquid and/or solid modified MRS media with 100 g/l of various sugars. nd, not determined

Strain number	Sugar in medium	Solid/ liquid medium	Glucose in EPS % (w/w)	Fructose in EPS % (w/w)	Other sugars in EPS % (w/w)	Total amount of EPS (mg/l)
23	sucrose	liquid	84	0	16	255
	sucrose	solid	95	5	<1	ND
24	sucrose	liquid	85	0	15	130
33	sucrose	liquid	93	<1	<1	>1000[a]
	sucrose	solid	96	4	4	ND
34	sucrose	liquid	88	0	12	420
	sucrose	solid	98	1	<1	ND
44	sucrose	liquid	86	0	14	285
86	sucrose	liquid	92	2	6	1055
	sucrose	solid	92	5	3	ND
116	sucrose	liquid	95	1	4	1350
121	sucrose	liquid	24	75	1	4800
	sucrose	solid	65	34	1	ND
	raffinose	liquid	1	96	3	2655
180	sucrose	liquid	95	2	3	4050
181	lactose	liquid	25	0	75	160
	sucrose	liquid	21	30	49	145
182	sucrose	liquid	24	41	35	250
	lactose	liquid	29	0	71	110

[a] Separation of strain LB 33 biomass and EPS was inefficient, resulting in underestimation of the amount of EPS formed

Strain LB 121 was unusual in producing EPS with varying monosaccharide composition. The glucose and fructose ratio varied from 1:3 (liquid MRS-s) to 2:1 (solid MRS-s) and 1:96 (liquid MRS-r) (Table 1). These variations either reflect changes in sugar composition of a specific EPS, or synthesis of different EPS molecules (see below).

Growth and EPS production

Strains LB 121 and LB 180 were studied in more detail, in view of the high levels of EPS produced by both and the variations observed in EPS monosaccharide composition with growth conditions (strain LB 121). Both were identified as *Lactobacillus reuteri* strains by polyacrylamide gel electrophoresis of proteins (Van den Berg et al, 1993).

Growth in MRS-s medium with increasing initial sucrose concentrations (0 - 100 g/l) resulted in increased biomass levels up to 20 g/l of sucrose. EPS production by both strains, however, continued to increase up to 100 g/l of sucrose, with strain LB 180 producing more than 20 g/l of EPS (Figure 1). Replacement of sucrose by equal amounts of glucose and fructose, supplied either as a mixture or separately, dramatically affected EPS production. Virtually no EPS was produced on MRS-g or MRS-(g + f). The amount of biomass formed was about the same in MRS-s, MRS-g and MRS-(g + f) (< 0.1 g/l). Relatively little growth (0,15-0.20 g/l biomass) and virtually no EPS production (< 0.1 g/l) occurred in MRS-f medium.

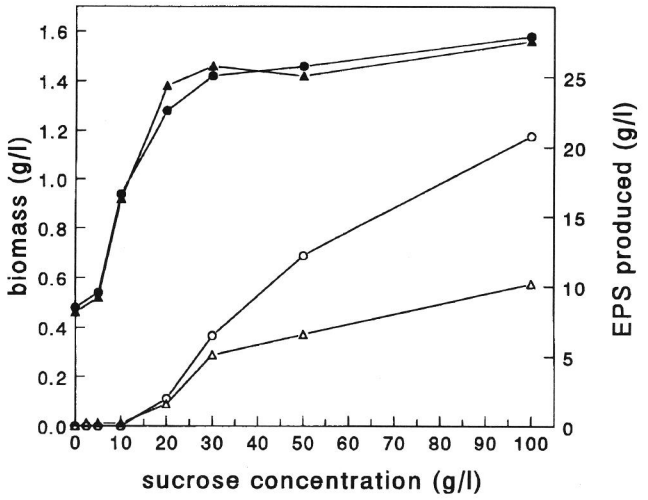

Figure 1 Effects of the initial sucrose concentration on extracellular polysaccharide (EPS; ○ △) and biomass (• ▲) production by strains LB 121 and LB 180 after incubation under anaerobic conditions for 3 days at 37°C in sucrose-containing MRS medium (MRS-s). The amount of EPS formed was quantified by measuring the total carbohydrate content of the ethanol precipitates. ○ • LB 180; △ ▲ LB121

Anaerobic growth in MRS-s medium without pH control resulted in abundant EPS production (i.e. over 10 g/l) by both strains (Figures 2 and 3). Several differences were observed between the two cultures. Strain LB 121 continued to grow and to produce lactate for 50 h. Growth of strain LB 180 (and lactate production) terminated after 20 h, reaching relatively low final lactate levels. Strains LB 121 and LB 180 both produced EPS in the early exponential growth phase, but only strain LB 180 continued to produce EPS in the stationary phase. Strain LB 121 rapidly produced high levels of EPS, reaching a maximum concentration after 15 h. Strain LB 180 produced EPS less rapidly but converted relatively more sucrose into EPS and less into lactate. Growth, EPS synthesis, and sucrose utilization of both cultures, ceased before exhaustion of sucrose from the medium.

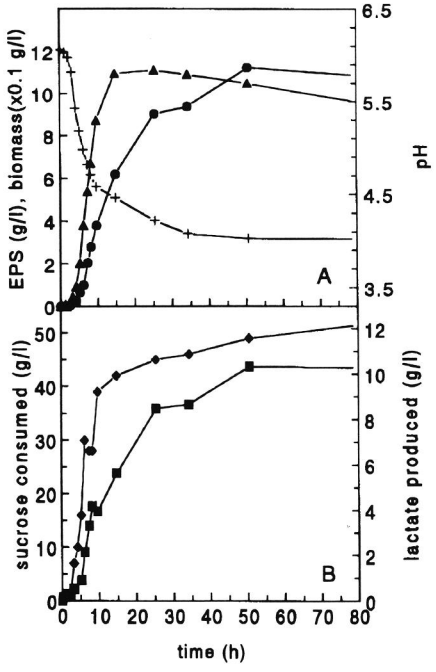

Figure 2A, B Growth and EPS Production of LB 121 under anaerobic conditions at 37°C in MRS-s medium without pH control. A ● Biomass, ▲ EPS production, + pH; B ♦ sucrose consumption, ■ lactate production

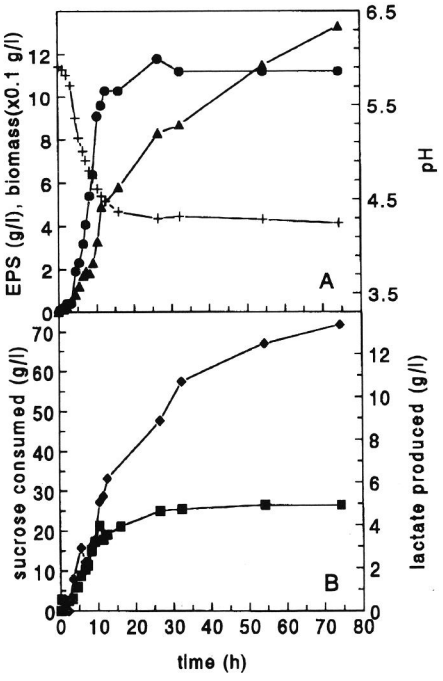

Figure 3A, B Growth and EPS Production of LB 180 under anaerobic conditions at 37°C in MRS-s medium without pH control. A ● Biomass, ▲ EPS production, + pH; B ♦ sucrose consumption, ■ lactate production

Growth of both strains in MRS-s medium was also studied in batch fermenters with automatic pH control. At pH 5.8, both strains completely consumed the sucrose from the medium within 30 h (Figures 4C, 5C), resulting in a much higher lactate production (Figures 4D, 5D) than in the absence of pH control (Figures 2B, 3B). Control of the pH at 4.8 resulted in reduced rates of sucrose consumption and lactate production by both strains (Figures 4C, D and 5C, D). Both strains formed much more biomass at pH 5.8 (Figures 4A, 5A), but the total amount of EPS produced after 30 h of fermentation was almost the same at both pH values for both strains (Figures 4B, 5B).

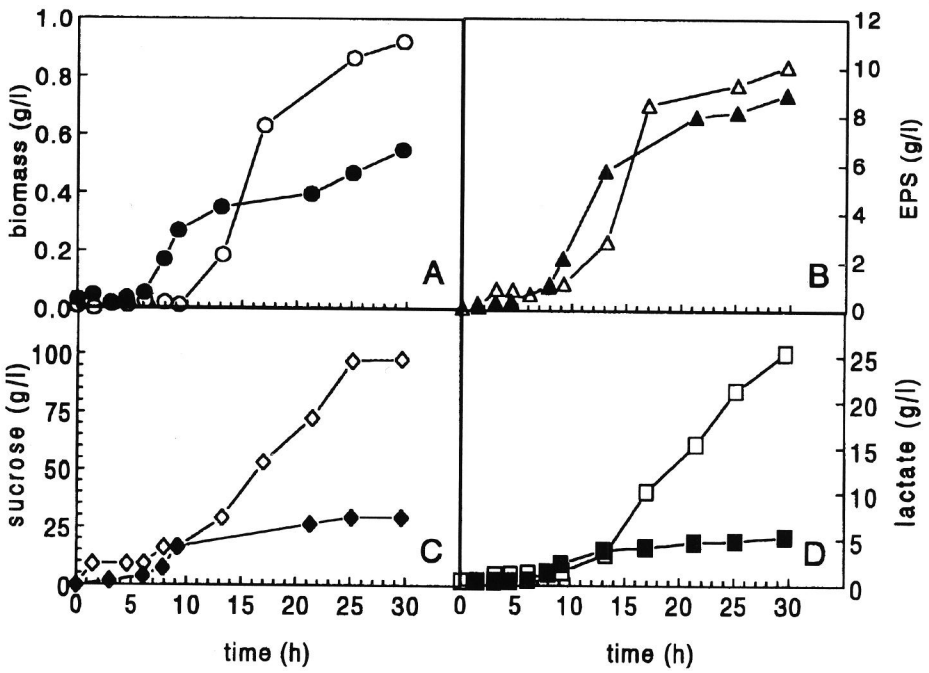

Figure 4A-4D Growth of strain LB121 under anaerobic conditions at 37°C in MRS-s medium in fermentors with automatic pH control at pH 5.8 (○ △ ◇ □) or pH 4.8 (● ▲ ◆ ■). **A** Biomass, **B** EPS production, **C** sucrose consumption, **D** lactate production

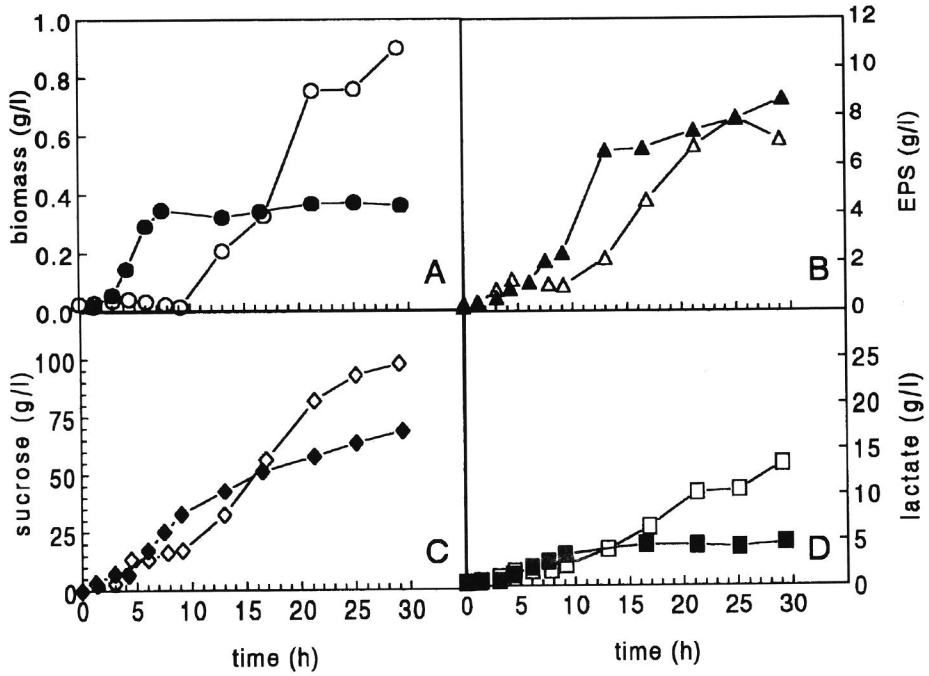

Figure 5A-5D Growth of strain LB180 under anaerobic conditions at 37°C in MRS-s medium in fermentors with automatic pH control at pH 5.8 (○ △ ◇ □) or pH 4.8 (● ▲ ◆ ■). **A** Biomass, **B** EPS production, **C** sucrose consumption, **D** lactate production

Localization of EPS biosynthetic enzymes

Washed cell suspensions and dialyzed culture supernatants of strain LB 121 converted sucrose into EPS (Table 2). The EPS biosynthetic enzyme(s) of LB 121 thus occurred both in a cell-free and a cell-associated form. EPS production by strain LB 180 was much higher in culture supernatants than in cell suspensions. The EPS biosynthetic enzyme(s) of strain LB 180 thus occur(s) largely in a cell-free form. With both strains there were no indications for cell lysis.

Table 2 EPS production by washed cell suspensions and dialysed supernatants of LB 121 and LB 180, obtained from cultures grown in MRS medium with 30 g/l sucrose. Incubations were under anaerobic conditions at 37°C, pH 5.5 with 33 g/l sucrose. EPS was determined by measuring total carbohydrate content of ethanol precipitates.

Incubation time (hours)	EPS produced (g/l)			
	LB 121 cells	LB 121 sup.	LB 180 cells	LB 180 sup.
0	0	0	0	0
0.5	0.38	0.72	< 0.1	0.54
1	0.89	1.1	< 0.1	0.80
2	1.5	1.6	< 0.1	1.1
4	2.4	2.3	0.11	1.1
24	5.2	5.2	0.48	1.6

EPS analysis

HP-GPC analysis of purified EPS of strain LB 121 grown on MRS-s showed two peaks with retention times of 20 and 27 min. EPS of cells grown on MRS-r only showed a peak at 27 min. Also EPS produced by strain LB 180 grown on MRS-s only showed a single peak at 20 min. Strain LB 180 did not produce EPS on MRS-r.

Monosaccharide analysis of the HP-GPC peak fractions revealed that strain LB 121 synthesized two polymers on sucrose, a glucan (retention time 20 min) and a fructan (retention time 27 min); on raffinose only the fructan was produced (retention time 27 min). Strain LB 180 produced only a glucan on MRS-s (retention time 20 min).

DISCUSSION

Screening for EPS synthesis by lactic acid bacteria is usually carried out on agar media with relatively low glucose or lactose concentrations (Van den Berg et al. 1993, Cerning 1990, Vedamuthu and Neville 1986, Vescovo et al. 1989). Following this approach, EPS producers are identified on the basis of colony ropiness. Our data show that growth in liquid MRS medium with relatively high sugar concentrations provides an excellent alternative, yielding a large number of EPS positive strains (60 out of 182 strains tested). In comparison, use of skimmed milk medium with 10 g/l glucose only yielded

30 EPS producing strains out of 607 strains tested (Van den Berg et al. 1993). The new method presented in this paper, using high sugar concentrations in liquid media, is more laborious but allows identification of strains with a high EPS production potential, and does not depend on EPS ropiness. Several (10%) of the strains tested produced over 100 mg/l EPS; 5 strains produced even more than 1 g/l EPS (Table 1), levels that are 10-100 fold higher than those previously reported for lactobacilli (Van den Berg et al. 1995, Grobben et al. 1995).

EPS molecules produced in relatively large amounts (>100 mg/l) contained almost exclusively glucose (and fructose) (Table 1). This was not due to the screening method used: several strains were identified producing relatively low amounts of EPS with other monosaccharides (mannose, arabinose, galactose). Sucrose clearly was the best substrate for EPS synthesis (Table 1) and appears to be the direct substrate for EPS synthesis by extracellular enzymes in strains LB 121 and LB 180 (Table 2).
Synthesis of glucans (and fructans) by strains LB 121 and LB 180, and most of the other high EPS producing strains identified (Table 1), thus may involve sucrase-type of extracellular (Table 2) enzymes (Dols et al. 1997, Monchois et al. 1997, Robyt 1995, Robyt and Walseth 1978).
These glycosyltransferase enzymes catalyze transfer of glucosyl or fructosyl residues from sucrose to a glucan or a fructan polymer respectively. Low EPS producing strains (i.e. LB 181, LB 182) on the other hand synthesize hetero-polysaccharides and may employ intracellular nucleotide-sugar dependent glycosyltransferases with different sugar substrate specificity (Van den Berg et al. 1995, Van Kranenburg et al. 1997, Stingele et al. 1996).

The EPS monosaccharide composition of strain LB 121 varied with the growth conditions (liquid or solid medium) and sugar substrates (sucrose or raffinose) supplied in the medium (Table 1). These differences may be caused by variations in expression or stability of the glucan and fructan biosynthetic enzymes. Moreover, raffinose is known to be a substrate for levansucrase, but not for glucansucrase (Robyt and Walseth 1978). This explains the observed synthesis of only the fructan by LB 121 on raffinose medium (Table 1). Similar observations have been made for *Streptococcus mutans* strain Ingbritt (Russell 1978). During growth on sucrose the water-soluble EPS produced by this bacterium consisted both of a dextran (40%) and a fructan (60%). With raffinose, the only product found was a water-soluble fructan.

Glucan and fructan synthesis by lactic acid bacteria (*Leuconostoc* and *Streptococcus* species) is well documented (Monchois et al. 1997, Robyt 1995, Robyt and Walseth 1978, Russell 1978), but not for the genus *Lactobacillus*. Only a few reports have described glucan synthesis by a few *Lactobacillus* species (Dunican and Seeley 1965, Sharpe et al. 1972). Our data

indicate that the ability to produce glucans is in fact widespread in the genus *Lactobacillus.* This is the first report of fructan production by a *Lactobacillus* species.

Both LB 121 and LB 180 were identified as strains of *Lb. reuteri*, but their pattern of EPS synthesis in MRS-s medium without pH control differed (Figures 2 and 3) from strictly growth-associated (strain LB 121) to continued synthesis after growth had stopped (LB 180). In pH controlled batch fermentors both strains produced approximately the same amount of EPS at pH 4.8 and pH 5.8 (t = 30 h), although at pH 4.8 less biomass and lactate were formed, and less sucrose was consumed (Figures 4 and 5). Not all of the sucrose consumed was recovered in as EPS and lactate (plus equimolar amounts of ethanol and CO_2): The action of sucrase-type of enzymes not only resulted in EPS formation but also in accumulation of glucose and fructose, part of which subsequently will be metabolized again by the cells. Also short EPS fragments (oligosaccharides) may have been produced that were not precipitated with 67% ethanol.

In conclusion, we have used a new method for screening lactic acid bacteria for EPS production that did not depend on EPS ropiness, using high sugar concentrations in liquid growth medium. This allowed identification of *Lactobacillus* strains producing large amounts of EPS. The EPS molecules produced by strains LB 121 and LB 180 possess interesting properties for industrial applications. EPS structures, properties of the sucrase-type of enzymes, and factors determining EPS monosaccharide composition and yields, are currently studied in more detail.

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3

Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by *Lactobacillus reuteri* wild-type and mutant strains

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ABSTRACT

Lactobacillus reuteri LB 121 cells growing on sucrose synthesize large amounts of a glucan (D-glucose) and a fructan (D-fructose) with molecular masses of 3,500 and 150 kDa, respectively. Methylation studies and ^{13}C or ^{1}H nuclear magnetic resonance analysis showed that the glucan has a unique structure consisting of terminal, 4-substituted, 6-substituted and 4,6-disubstituted α-glucose in a molar ratio of 1.1:2.7:1.5:1.0. The fructan was identified as a (2→6)-ß-D-fructofuranan or levan, the first example of levan synthesis by a *Lactobacillus* species.

Strain LB 121 possesses glucansucrase and levansucrase enzymes that occur in a cell-associated and a cell-free state after growth on sucrose or raffinose, but remain cell-associated during growth on glucose or maltose. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sucrose culture supernatants, followed by staining of gels for polysaccharide synthesizing activity with sucrose as a substrate, revealed the presence of a single glucansucrase protein of 146 kDa.

Growth of strain LB 121 in chemostat cultures resulted in rapid accumulation of spontaneous exopolysaccharide-negative mutants that had lost both glucansucrase and levansucrase (e.g., strain K-24). Mutants lacking all levansucrase activity specifically emerged following a pH shift-down (e.g., strain 35-5). Strain 35-5 still possessed glucansucrase and synthesized wild-type glucan.

INTRODUCTION

A variety of high-molecular-weight polysaccharides produced by plants (cellulose, pectin, and starch), seaweeds (alginate and carrageenan), and bacteria (alginate, gellan, and xanthan) find applications as viscosifying, stabilizing, emulsifying, gelling, or water binding agents in the food and nonfood industries (Sutherland 1993, Whitfield 1988). All these poly saccharides are additives, however, and therefore they are considered less desirable in the food industry.

Lactic acid bacteria are food-grade organisms that possess the GRAS (Generally Recognized As Safe) status and are known to produce an abundant variety of exopolysaccharide (EPS) molecules (Cerning 1990, Dunican and Seeley 1965, Roller and Dea 1992), which contribute to the texture of fermented milk. EPS from these bacteria may allow development of a new generation of food grade polysaccharides. Lactic acid bacteria often also contribute positively to the taste, smell, or preservation of the final product.

Synthesis of heteropolysaccharides by lactic acid bacteria, including lactobacilli, is currently studied intensively (Breedveld et al. 1998, Cerning 1990, Garcia-Garibay and Marshall 1991, Grobben et al. 1995, Robijn et al. 1995, Stingele et al. 1996, Van den Berg et al. 1995, Van Kranenburg et al. 1997). Synthesis of homopolysaccharides (e.g., dextran and levan) has been studied mainly in *Ln. mesenteroides* and in streptococci (Cerning 1990, Monchois et al. 1997, 1999, Robyt 1995). Limited information is available about homopolysaccharide biosynthesis in lactobacilli (Dunican and Seeley 1965, Pidoux et al. 1990, Sharpe et al. 1972).

Recently, we have screened a large collection of lactobacilli for strains producing EPS from sucrose. One of these strains, identified as *Lactobacillus reuteri* strain LB 121, synthesized large amounts of water-soluble EPS material with both glucose and fructose as constituents (Van Geel-Schutten et al. 1998). The present study reports the biochemical and mutational identification of the biosynthetic enzymes involved and provides a structural characterization of the glucan and fructan synthesized by strain LB 121.

MATERIALS AND METHODS

Strains, media, and growth conditions

Lb. reuteri strain LB 121 (LMG 18388) and mutants derived from it, strain 35-5 (LMG 18390) and strain K-24 (LMG 18391), were grown anaerobically at 37°C in MRS medium (De Man et al. 1960). Modified MRS media, containing 100 g of raffinose (MRS-r) or sucrose (MRS-s) liter^{-1}, instead of the 20 g glucose liter^{-1} normally present in MRS medium, was used for EPS production under cultivation conditions with or without pH control (Van Geel-Schutten et al. 1998). When appropriate, media were solidified with 20 g of agar liter^{-1}. All media were autoclaved for 15 min at 121°C. Sugars were autoclaved separately.

Chemostat cultivation (Bioflow III fermentors, working volume 1.5 liter) was performed in 0.5 x MRS-s medium, flushed with nitrogen. The pH was kept automatically at 5.5 with 4 M NaOH. After about 5 h of growth, fresh medium was pumped into the fermentor at a dilution rate of 0.05, 0.1, 0.2 or 0.4 h^{-1}.

Identification of spontaneous mutants in EPS biosynthesis

Samples from chemostat cultures were appropriately diluted and spread on MRS agar plates. A number of colonies from each plate were picked randomly and grown anaerobically in culture tubes containing 10 ml MRS-s. After 3 days of growth, EPS was isolated and determined as described below. Mutant strains producing either no EPS or EPS with a different appearance when purified and dried were selected for further studies.

Enzyme assays

Glucansucrase (E.C. 2.4.1.5) and levansucrase (E.C. 2.4.1.10) activities were measured at 37°C by monitoring the release of fructose and glucose, respectively, from sucrose. Reaction mixtures (1 ml) contained $CaCl_2$ (50 mg.liter^{-1}), acetate buffer (200 mM, pH 5.5), sucrose (50 mM), and appropriately diluted enzyme. Samples (100 μl) were withdrawn at regular intervals, and 5 μl 2 M NaOH was added to stop the reactions. Glucose and fructose formed were quantified enzymatically by monitoring the reduction of NADP as described previously (Mayer 1987). Glucose was measured first in a reaction mixture containing Tris-HCl (50 mM, pH 7.6), ATP (2.5 mM), NADP (1 mM), $MgSO_4$ (10 mM), hexokinase (3,000 U.liter^{-1}), and glucose-6-phosphate dehydrogenase (1,500 U.liter^{-1}). Fructose concentrations were

measured in the same reaction mixture but with the addition of phosphoglucoisomerase (7,000 U.liter^{-1}).One glucansucrase or levansucrase activity unit is defined as the amount of enzyme producing 1 μmol of monosaccharide per min. All enzyme assays were performed in triplicate; data presented are averages with a standard deviation of less than 10%.

Activity staining of EPS synthesizing enzymes

MRS, MRS-s and MRS-r (10 ml) media were inoculated with 200 μl of overnight cultures of strains LB 121, 35-5, and K-24 and incubated at 37°C for 8 h. Cells were removed by centrifugation, and proteins in the supernatants were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (see below). After electrophoresis the gels were washed three times with demineralized water and incubated overnight at 37°C in acetate buffer pH 5.5 (50 mM HAc, 1% (vol/vol) Tween-80, 1 mM $CaCl_2$) with 1% (wt/vol) sucrose. Glucansucrase activities were detected by staining gels for polysaccharides by a periodic acid-Schiff (PAS) procedure (Zacharius et al. 1969). Gels loaded with the same supernatants and incubated without sucrose were used as controls.

Enzyme localization studies

Cells of strain LB 121 and of mutant strain 35-5, in the exponential phase of growth on MRS with 30 g glucose, maltose, raffinose or sucrose liter^{-1}, were harvested by centrifugation (25 min x 2,500 *g*). The cells were washed twice with 0.05 M citric acid/0.1 M Na_2HPO_4 (pH 5.5) and resuspended in the same buffer, containing 50 g sucrose liter^{-1} and 50 μg chloramphenicol ml^{-1}. Cell suspensions were incubated anaerobically at 37°C, and sampled at regular intervals to determine the amount and monosaccharide composition (see below) of the EPS produced. Supernatants were filtered through a 0.2 μm pore size filter (Millipore), diluted 1:1 with 0.1 M citric acid/0.2 M Na_2HPO_4 (pH 5.5) with 50 g sucrose liter^{-1}, and treated in the same way as the cell suspensions.

Isolation and purification of EPS

Cells were harvested by centrifugation (10 min x 11,000 *g*). Two volumes of cold ethanol were added to culture supernatants, and the mixtures stored overnight at 4°C. Precipitated material was collected by centrifugation (20 min x 2,500 *g*), resuspended in demineralized water and mixed with two volumes of cold ethanol. Samples were centrifuged (20 min at 2,500 *g*) and

the pellets were dried at 100°C. EPS amounts were determined by measuring final dry weights.

HP-GPC, GLC, and MS

High Performance Gel Permeation Chromatography (HP-GPC) analysis was carried out at room temperature, using a Progel TSK guard column, followed by a Progel TSK G6000 PW column, and a refractive index detector (Erna ERC-7510). Samples were eluted at a flow rate of 0.6 $ml.min^{-1}$ with 0.1 M $NaNO_3$ as mobile phase. Gas-Liquid Chromatography (GLC) measurements were performed on a Chrompack CP9002 gas chromatograph, equipped with a CP-Sil 5CB fused silica capillary column (25 m × 0.32 mm, Chrompack), using a temperature program of 120–240°C at 4°C/min. GLC data were collected and processed with Maestro Chromatography Software. GLC–mass spectrometry (MS) analysis was carried out on a MD800/8060 system (electron energy, 70 eV; Fisons instruments), using a DB-1 fused silica capillary column (30 m by 0.32 mm; J&W Scientific). A temperature program of 140–240°C at 4°C/min was used.

Molecular mass determination

The average molecular mass of the polysaccharides was determined by size exclusion chromatography. To determine the size distribution of the polysaccharides, EPS produced after two days of growth on MRS-s or MRS-r was isolated as described above. Instead of drying, EPS was dialyzed (cellulose dialysis tube. [Sigma D-9777]; cut off 12 kDa) at 4°C against water for 3 days. Lyophilized EPS was dissolved in 0.1 M $NaNO_3$, filtered over a 0.45 μm filter (Millipore), and analyzed on HP-GPC (Van Geel-Schutten et al. 1998).

Monosaccharide analysis

After complete hydrolysis of EPS (2 h in 1 M H_2SO_4 at 100°C), glucose was determined by HPLC (Van Geel-Schutten et al. 1998) and fructose by an improved resorcinol reagent (Yaphe and Arsenault 1965). The absolute configurations of the monosaccharides were determined by GLC analysis of the trimethylsilylated (–)-2-*butyl* glycosides (Gerwig et al. 1978, 1979) on CP-Sil 5CB.

Methylation analysis

Polysaccharides were permethylated using methyl iodide and solid sodium hydroxide in methyl sulfoxide (Ciucanu end Kerek 1984). After hydrolysis with 2 M trifluoroacetic acid (2 h, 120°C), the partially methylated monosaccharides were reduced with $NaBD_4$. After neutralization, removal of boric acid by co-evaporation with methanol, and acetylation with acetic acid anhydride (3 h, 120°C), the mixtures of partially methylated alditol acetates obtained were analyzed by GLC on CP-Sil 43CB and by GLC–MS on DB-1 (Jansson et al. 1976, Kamerling and Vliegenthart 1989).

NMR spectroscopy

Prior to NMR spectroscopic analysis (Bijvoet Center, Department of NMR Spectroscopy) samples were exchanged twice in 99.9 atom% D_2O (Isotec) with intermediate lyophilization and finally dissolved in 99.96 atom% D_2O (Isotec). Proton-decoupled 75.469 MHz ^{13}C NMR spectra were recorded on a Bruker AC-300 spectrometer (probe temperature 80°C). One-dimensional ^{1}H NMR spectra were recorded on a Bruker AMX-500 spectrometer (probe temperature 80°C). The HOD signal was suppressed by applying a WEFT pulse sequence (Hård et al. 1992). Chemical shifts are expressed in ppm by reference to internal acetone ($\delta = 2.225$) for ^{1}H or to external methanol ($\delta = 49.00$) for ^{13}C. Proton spectra were recorded in 16K data sets, with a spectral width of 5,000 Hz. Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation; when necessary, a fourth-order polynomial baseline correction was performed.

Gel electrophoresis

SDS-PAGE was performed according to Laemmli (1970) using the Phast System from Pharmacia with 10-15% polyacrylamide gels. After activity staining the gels were silver stained (Heukeshoven and Dernick 1985). Lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), and phosphorylase b (97,400) were used as molecular mass references.

Other assays

Fermentation patterns of the bacterial strains were established using API CHL 50 tests (bioMérieux, Marcy l'Etoile, France). Protein was determined

according to Lowry et al. (1951) with bovine serum albumin as a standard. Intact cells were first boiled for 20 min in 1 M NaOH.

Chemicals

All biochemicals were obtained from Boehringer, Mannheim.

RESULTS

EPS synthesis by strain LB 121

Strain LB 121 grown in batch cultures, with or without pH control, produced large amounts of non-ropy EPS on both MRS-s and MRS-r media (Table 1). Monosaccharide analysis, including determination of absolute configurations, revealed the presence of both D-glucose and D-fructose (in a 1:2 ratio) in the EPS material synthesized by strain LB 121 grown on MRS-s; on MRS-r a polymer with only D-fructose was produced (Table 1). Repeated subculturing (ca. 350 generations) of strain LB 121 on MRS-s in batch culture did not affect EPS levels.

Table 1 Amount and composition of EPS synthesized by *Lb. reuteri* strains grown on MRS-sucrose and MRS-raffinose at 37°C during 3 days.

Strain	Sugar in medium	EPS conc. ($g.liter^{-1}$)	% D-Glucose in EPS (w/w)	% D-Fructose in EPS (w/w)
LB 121	sucrose	9.8	32	68
	raffinose	7.3	0	100
35-5	sucrose	9.7	100	0
	raffinose	0	0	0
K-24	sucrose	0		
	raffinose	0		

Isolation of spontaneous mutants in EPS biosynthesis

During growth of strain LB 121 in chemostat cultures the amounts of EPS synthesized varied strongly, and no stable and reproducible steady states were obtained. This was due to accumulation of spontaneous mutants in

chemostat cultures. Continuous cultivation of strain LB 121 at pH 5.5 resulted in a rapidly decreasing EPS production in time: EPS concentrations dropped from 10 g.liter^{-1} in batch cultures on MRS-s to 1.5 and 2.5 g.liter^{-1} after 20 generations of growth on 0.5 x MRS-s at dilution rates of 0.05 h^{-1} and 0.2 h^{-1}, respectively. In view of the non-ropy character of strain LB 121 EPS, samples from these cultures were spread first on MRS agar (no EPS production) and individual colonies were checked for the ability to synthesize EPS during growth in batch culture in MRS-s liquid medium. After 20 generations of growth 25 individual colonies were examined for EPS production. Of these colonies, 21 produced no EPS at all (e.g., mutant strain K-24) or less than 1 g.liter^{-1}; only 4 colonies produced the same amount of EPS as strain LB 121 (about 10 g.liter^{-1}). Strain K-24 produced no EPS on either MRS-s or MRS-r (Table 1); no EPS synthesizing revertants were observed during further studies.

Strain LB 121 cells growing in chemostat cultures at pH 5.5 and dilution rates of 0.05 h^{-1} or 0.2 h^{-1} were also subjected to a shiftdown to pH 4.5. Within 10 generations of growth at either dilution rate, numerous mutants (e.g., strain 35-5) were identified producing EPS material that, when dried, had a different appearance from that of strain LB 121 EPS and was composed of D-glucose only. All mutants tested (> 25) in these experiments synthesized EPS material with D-glucose only. Interestingly, strain 35-5 grown in batch culture on MRS-s produced the same amount of EPS as strain LB 121 (about 10 g.liter^{-1}), but this was now composed of glucose only. No EPS was synthesized by strain 35-5 in MRS-r medium (Table 1). Mutant strain 35-5 turned out to be very stable during further studies. Mutants producing EPS composed of fructose only were not detected. Non-producing mutants similar to strain K-24 did not appear in chemostat cultures run at pH 4.5 for prolonged periods of time. After a switch of cultures back to pH 5.5, non-producing mutants similar to K-24 started to accumulate again.

Strains 35-5 and K-24 showed the same fermentation profiles as strain LB 121 in API 50 CHL tests, including the ability to ferment sucrose and raffinose, confirming the identity of the mutant strains as derivatives of strain LB 121. The mutations resulting in loss of EPS synthesizing activity apparently have not affected the ability of these strains to grow on the various sugar (mono- and disaccharides) substrates tested. Strains K-24 and 35-5 were selected for further characterization.

EPS size and monosaccharide analysis

The HP-GPC elution patterns and size distribution analysis of the different EPS species synthesized by strains LB 121 and 35-5 were studied

(Figure 1). On MRS-s, strain LB 121 produced EPS with two size distributions (15.0 and 19.4 min). Strain LB 121 on MRS-r (19.4 min) and strain 35-5 on MRS-s (15.0 min) synthesized EPS with one size distribution. Monosaccharide analysis of HP-GPC fractions revealed that the polymer eluting at 19.4 min consisted solely of fructose (fructan) whereas the polymer eluting at 15.0 min consisted solely of glucose (glucan). Molecular masses of 3,500 and 150 kDa were determined for the glucan and fructan, respectively. Strain LB 121 thus synthesizes both a glucan and a fructan on sucrose. On raffinose, strain LB 121 produces the fructan only. Mutant strain 35-5 synthesizes only the glucan on sucrose and has lost the ability to produce the fructan (Table 1; Figure 1).

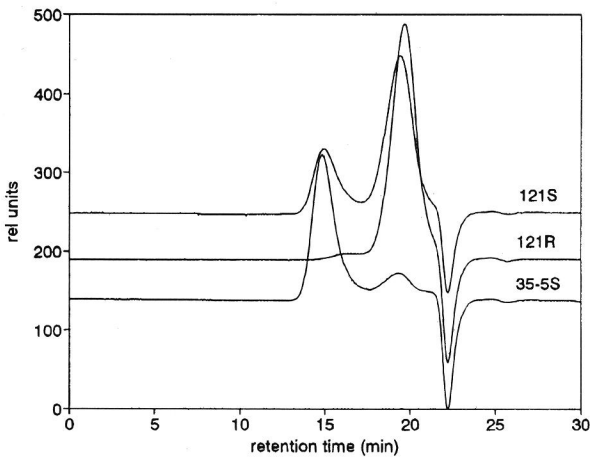

Figure 1 HP-GPC elution patterns of native EPS from, (121S) *Lb. reuteri* strain LB 121 grown on sucrose, (121R) strain LB 121 grown on raffinose, and (35-5S) mutant strain 35-5 grown on sucrose.

Methylation analysis

Methylation analysis showed that the EPS synthesized by strain LB 121 on MRS-s consists of terminal, 4-substituted, 6-substituted and 4,6-disubstituted glucose in a molar ratio of 1.1:2.7:1.5:1.0, together with a large amount of 6-substituted fructose. These results, in combination with data presented above, indicate the presence of a branched glucan and a uniformly linked fructan. Methylation analysis of the EPS synthesized by strain LB 121 on MRS-r revealed the presence of merely 6-substituted fructose, indicating a uniformly linked fructan (levan). Methylation analysis of the EPS synthesized

by mutant strain 35-5 on MRS-s revealed the presence of the same four glucose derivatives, in a comparable molar ratio, as identified in the strain LB 121 EPS produced on MRS-s.

NMR spectroscopy

In the 1D ^{13}C NMR spectrum of the fructan synthesized by strain LB 121 on MRS-r (spectrum not shown) six carbon signals are observed (Table 2). The C-2 resonance (δ = 105.0) indicates the occurrence of ß-fructofuranose. Comparison of the ^{13}C chemical shifts of the fructan with published chemical shifts of Me-ß-D-Fru*f* (1) and *Zymomonas mobilis* levan (2) demonstrates the fructan to be: $[\rightarrow 6)$-ß-D-Fru*f*-$(2\rightarrow]_n$. In the 1D ^{1}H NMR spectrum of the fructan (Figure 2B, Table 2) no signals in the anomeric region (δ = 5.3 - 4.3) were found, confirming the absence of anomeric protons. The observed peak pattern fits the fructofuranose configuration.

Table 2 ^{1}H and ^{13}C NMR chemical shifts[a] of 1 recorded in D_2O at 80°C.

1H and 13 C NMR	Chemical shift[a] (coupling constant [Hz])
Proton	
H-1a	3.750 (12.3)
H-1b	3.704 (12.1)
H-3	4.176 (8.2)
H-4	4.090 (7.9)
H-5	3.941 (3.3)
H-6a	3.891 (10.6)
H-6b	3.631 (7.7)
Carbon	
C-1	61.7
C-2	105.0
C-3	78.1
C-4	76.6
C-5	81.2
C-6	64.3

[a] Given in parts per million relative to the signal of internal acetone (δ = 2.225) for proton and relative to the α-anomeric signal of external glucose (δ = 49.00) for carbon. Coupling constants (Hz) are included in parentheses.

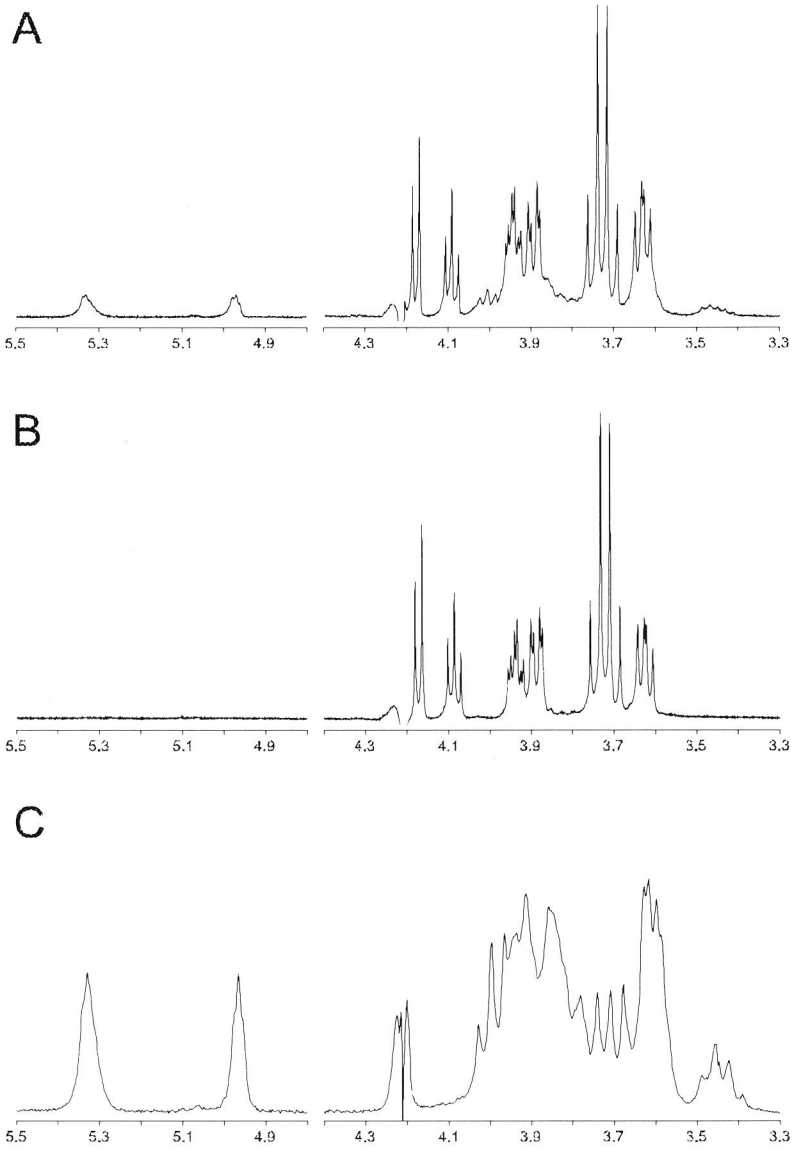

Figure 2 500 MHz ^{1}H NMR spectra of EPS produced by (A) *Lb. reuteri* strain LB 121 grown on sucrose (mixture of glucan and fructan), (B) strain LB 121 grown on raffinose (fructan), and (C) mutant strain 35-5 grown on sucrose (glucan), recorded in D_2O at 80°C.

The 1D ^{1}H NMR spectrum of the glucan synthesized by strain 35-5 grown on MRS-s (Figure 2C) showed two broad signals in the anomeric region (δ = ~ 4.97 and ~ 5.35). Comparison of the spectrum with ^{1}H NMR data of potato starch (16) demonstrates that the glucan consists of (1→4)- and (1→6)-linked α-glucopyranose residues. Due to poor resolution of the spectrum it is not possible to trace the terminal and the (1→4,6)-linked residues as indicated by the methylation analysis. The lineshape of the anomeric signals is characteristic for a glucan with various glucosyl linkages.

Comparison of the 1D ^{1}H NMR spectrum of the polysaccharide material synthesized by strain LB 121 grown on MRS-s (Figure 2A) with the spectra of the fructan and the glucan demonstrates that both the fructan and the glucan are synthesized by strain LB 121 grown on MRS-s.

EPS biosynthetic enzymes

High activities of both glucansucrase (5.7 U.mg of protein^{-1}) and levansucrase (6.9 U.mg of protein^{-1}) were detected in supernatants of strain LB 121 cultures grown on MRS-s. Supernatants of mutant strain 35-5 cultures grown on MRS-s only contained glucansucrase activity (4.4 U.mg of protein^{-1}); strain 35-5 had lost all levansucrase activity. Mutant strain K-24 had completely lost both glucansucrase and levansucrase activities. In strain LB 121 both sucrase enzymes showed maximum activity in the stationary phase of growth. In contrast, glucansucrase of strain 35-5 reached maximum activity during exponential growth and declined drastically at the end of the growth phase, reaching a fairly low activity level (0.6 U.mg of protein^{-1}) in supernatants of overnight cultures.

Activity staining of EPS biosynthetic enzymes

After SDS-PAGE of proteins in supernatants of cultures of strains LB 121, 35-5 and K-24 grown on various sugars, gels were incubated with sucrose. Proteins able to synthesize polysaccharides from sucrose were visualized by PAS staining (Figure 3). Supernatants of strain LB 121 grown on sucrose or on raffinose, and of mutant strain 35-5 grown on sucrose, each showed a single activity band on the gels corresponding to enzymes with a molecular mass of 146 kDa. Supernatants of these strains grown on glucose did not show any activity bands with sucrose (see below). No activity bands were observed with mutant strain K-24. Control gels loaded with supernatant samples, but incubated without sucrose, did not show any bands after PAS staining. Incubation of the SDS-PAGE gels with raffinose followed by PAS staining did not reveal positive bands. Apparently, only glucan(sucrase activity)

and not levan(sucrase activity) can be detected by PAS staining. The data also show that after SDS-PAGE the single glucansucrase enzyme present is free of polysaccharide and has remained active, able to synthesize glucan when incubated with sucrose.

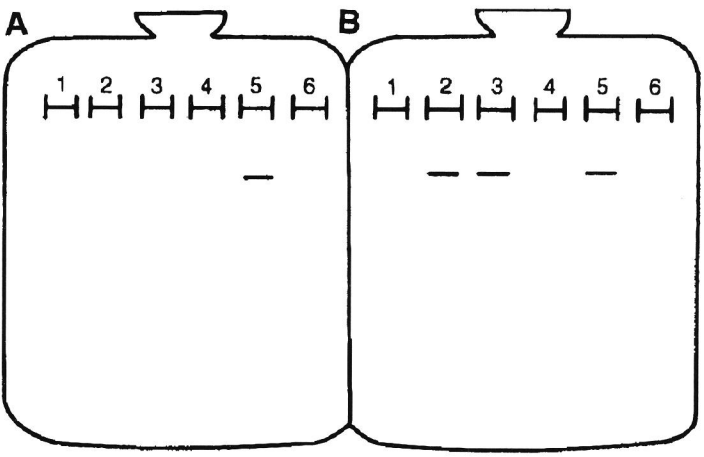

Figure 3 Bands of proteins with glucansucrase activity present in supernatants of *Lb.reuteri* strains grown on MRS, MRS-s or MRS-r, identified in SDS-PAGE gels by polysaccharide staining (PAS) after incubation with sucrose. After activity staining the gels were silver stained (results not shown). Lane 1: strain K-24 grown on MRS-r; lane 2: strain K-24 grown on MRS; lane 3: strain K-24 grown on MRS-s; lane 4:strain 35-5 grown on MRS-r; lane 5: strain LB 121 grown on MRS-s; lane 6: marker proteins. Lane 1: strain 35-5 grown on MRS; lane 2: strain 35-5 grown on MRS-s; lane 3: strain LB 121 grown on MRS-r; lane 4: strain LB 121 grown on MRS; lane 5: strain LB 121 grown on MRS-s; lane 6: marker proteins.

Localization of EPS biosynthetic enzymes

Washed cells and supernatants of strain LB 121 cultures grown on MRS-s synthesized glucan as well as fructan when incubated with sucrose (Table 3). Washed cells and supernatants of strain 35-5 cultures grown on MRS-s synthesized only the glucan from sucrose. Similar observations were made with raffinose and maltose grown cells of strain LB 121 and strain 35-5 when incubated with sucrose (data not shown). The glucansucrase (strain

LB 121 and strain 35-5) and levansucrase (strain LB 121) enzymes thus are synthesized during growth on various sugars; these enzymes occur both in a cell-associated and in a cell-free state following growth on sucrose or raffinose. In contrast, no EPS synthesis was observed with supernatants of glucose or maltose grown cells of strain LB 121 and strain 35-5 when incubated with sucrose. Washed cells of glucose grown cultures, however, clearly synthesized both glucan and fructan (strain LB 121) and glucan only (mutant strain 35-5) from sucrose. The EPS synthesizing enzymes thus remain cell-associated during growth on glucose (Table 3). Also during incubations of washed cells in buffer (pH 5.5) with sucrose no release of EPS synthesizing enzymes was observed.

Table 3 EPS production by supernatants and washed cells of *Lb. reuteri* strain LB 121 and mutant strain 35-5 incubated at 37°C in buffer with sucrose as substrate[a]

Sugar in growth medium	Washed cells/ supernatant	Incubation time (hours)	Glucan produced by strain 35-5 ($g.liter^{-1}$)	Glucan produced by strain LB 121 ($g.liter^{-1}$)	Fructan produced by strain LB 121 ($g.liter^{-1}$)
Sucrose	Washed cells	0	0.28	ND	ND
		2	0.60	0.43	0.41
		10	3.12	1.19	0.92
		22	4.52	1.87	1.57
Sucrose	Supernatant	0	0.36	ND	ND
		2	0.44	0.37	0.55
		10	0.52	0.64	1.06
		22	1.00	0.85	1.70
Glucose	Washed cells	0	0.32	ND	ND
		2	1.24	0.89	0.27
		10	3.76	3.01	0.97
		22	5.72	4.35	1.51
Glucose	Supernatant	0	0.16	ND	ND
		2	0.08	ND	ND
		10	0.04	ND	ND
		22	0.12	ND	ND

[a] Cultures were harvested in the exponential phase of growth and incubated with sucrose plus 50 μg chloramphenicol to block de novo enzyme synthesis.
ND, not detected

DISCUSSION

Lactic acid bacteria, e.g. *Ln. mesenteroides* strains and *Streptococcus* species, synthesize glucans with different structures from sucrose. Examples are dextrans with contiguous α-(1→6)-linked glucose residues, mutans with contiguous α-(1→3)-linked glucose residues, or alternans with alternating α-(1→6)- and α-(1→3)-linked glucose residues, in the main chains. Different dextrans with a varying degree of α-(1→2), α-(1→3), or α-(1→4) branching have been described (Robyt 1995). The present study shows that *Lb. reuteri* strain LB 121 synthesizes a high-molecular-mass branched α-glucan with terminal, 4-substituted, 6-substituted and 4,6-disubstituted a-glucose residues. To the best of our knowledge such a glucan structure has not been described previously. The properties and possible industrial applications of this unique glucan, already produced in large amounts by wild type *Lb. reuteri* strain LB 121 (Van Geel-Schutten et al. 1998 and this study), are currently under investigation.

Lb. reuteri strain LB 121 also synthesizes a low-molecular-mass (2→6)-ß-D-fructofuranan (levan). Levan synthesis in lactic acid bacteria has been reported only for *Streptococcus* species (Milward and Jacques 1990, Shiroza and Kuramitsu 1988)); the present study is the first report of the synthesis of a levan type of polysaccharide in the genus *Lactobacillus*.

The biochemical and mutant data presented here show that strain LB 121 employs glucansucrase and levansucrase enzymes to synthesize the glucan and levan from sucrose. Also, raffinose is a substrate for levan synthesis by the action of the levansucrase; raffinose is not a substrate for glucansucrase (Robyt and Walseth 1978). Both enzymes are synthesized during growth on various sugars and occur in a cell-bound and a cell-free state in sucrose or raffinose cultures, but only in a cell-bound state in glucose or maltose cultures.

The chemostat cultivation technique is a convenient tool to study the effects of various environmental parameters on the physiology of microbial cells (Dijkhuizen 1996, Harder and Dijkhuizen 1983). Attempts to study the physiology of EPS synthesis by strain LB 121 in chemostat cultures failed, mainly because no stable steady state conditions could be established due to the rapid accumulation of mutants. Instability of EPS production in lactic acid bacteria has been observed before during repeated transfer of cells in batch cultures (Cerning 1990, Cerning et al. 1988, Garcia-Garibay and Marshall 1991, Kojic et al. 1992). Repeated subculturing (ca. 350 generations) of strain LB 121 on MRS-s in batch culture, however, did not affect EPS production. It remains unclear why EPS synthesis in strain LB 121 is unstable in chemostat cultures but not in batch cultures. Also, the nature of the (stable) mutations

in strains 35-5 and K-24 remains to be characterized. Interestingly, although mutant strain 35-5 has lost all levansucrase activity, it still synthesizes the same total amount of EPS material as strain LB 121 when grown on sucrose, but this is now composed of the glucan only. Incubation of washed cells harvested from exponential-phase cultures of strains LB 121 and 35-5 with sucrose also resulted in synthesis of similar amounts of EPS material.

Supernatants of sucrose grown cultures of strain LB 121 possess both glucansucrase and levansucrase activities, but PAS staining of SDS-PAGE gels loaded with these supernatants and incubated with sucrose identified only glucan and not the levan. Incubation of these SDS-PAGE gels with raffinose followed by PAS staining did not reveal positive bands. Apparently, levan synthesis cannot be detected by PAS staining, or else the levansucrase enzyme is inactivated during SDS-PAGE. Accordingly, supernatants of strain LB 121 grown on raffinose, possessing both glucansucrase and levansucrase activity and of mutant strain 35-5 grown on sucrose possessing only the glucansucrase, each displayed a single activity band with sucrose at the same 146 kDa position as in strain LB 121. The data thus indicate that the glucansucrase enzyme present in strains LB 121 and 35-5 is a monomeric enzyme with a molecular mass of 146 kDa. Glucosyltransferase proteins of *S. mutans* (Honda et al. 1990, Koga et al. 1983, Shimamura et al. 1982), *Ln. mesenteroides* (Funane et al. 1995), *S. downei* (Gilmore et al. 1990), *S. sobrinus* (Ferreti et al. 1987), and *S. salivarius* (Giffard et al. 1991) have molecular masses of 130 to 180 kDa. The levansucrase protein of strain LB 121 remains to be identified and characterized with respect to its molecular mass and other properties. Fructosyltransferase enzymes studied in various bacteria have molecular masses between 50 and 100 kDa (see, for example, Shiroza and Kuramitsu 1988, Steinmetz et al. 1985 and Sutherland 1993), whereas a 140 kDa enzyme was reported in *Streptococcus salivarius* (Milward and Jacques 1990).

The glucansucrase and levansucrase enzymes of *Lb. reuteri* strain LB 121, as well as the corresponding genes, will be characterized in more detail in further work.

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4

Molecular characterization of a novel fructosyltransferase from *Lactobacillus reuteri* synthesizing a high molecular weight fructan with β-(2→1) linked fructosyl units in *Escherichia coli*

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SUMMARY

A novel *Lactobacillus reuteri* fructosyltransferase gene (*ftfA*) was isolated with PCR techniques. A DNA fragment of approximately 2.6 kb was isolated and characterized, revealing a single open reading frame (ORF) encoding the putative fructosyltransferase (FTFA). Based on the size of the *ftfA* ORF (approximately 2400 bp), the deduced molecular weight of the putative FTFA is 85,598 Da. FTFA showed high similarity with fructosyltransferases from other Gram positive bacteria. Highest similarity (90%) was found with SacB of *Streptococcus mutans*, an enzyme synthesizing a fructan with β-(2→1) linkages only.

FTFA contains a putative N-terminal secretion signal peptide, suggesting that the enzyme would be secreted. The C-terminal amino acid sequence of FTFA contains a cell wall anchoring peptide sequence LPXTG, plus a 20-fold repeat of the motif PXX, where P is proline and X is any other

amino acid. The presence of these PXX repeats has not been reported before in proteins from either prokaryotic or eukaryotic origin.

The *ftfA* gene was successfully expressed in *Escherichia coli*, yielding an active FTFA enzyme. When incubated with sucrose, FTFA produced fructo-oligosaccharides of DP 3-4, plus a high-molecular-weight fructan polymer ($>10^7$ Da) with β-(2→1) linkages only (an inulin). Fructo-oligosaccharides of DP 3-4 were also found in *Lb. reuteri* culture supernatant after cultivation on media containing sucrose. This is the first example of the isolation and molecular characterization of a fructosyltransferase, synthesizing a fructan polymer, in a *Lactobacillus* species.

INTRODUCTION

Lactic acid bacteria (LAB) are food-grade organisms that possess the GRAS (Generally Recognized As Safe) status and are known to produce an abundant variety of homo- and heteropolysaccharides (De Vuyst and DeGeest 1999), which may be of interest for the development of a new generation of food-grade ingredients. LAB polysaccharides can be used as viscosifying, stabilizing, emulsifying, gelling or water binding agents in the food as well as in the non-food industry. Because of their GRAS status, many lactobacilli are of strong importance in the production of food and feed. Moreover, some strains are known as probiotics, beneficially affecting the host by improving the properties of the indigenous population of gastrointestinal microorganisms (Havenaar and Huis in 't Veld 1992).

Homopolysaccharide biosynthesis in lactobacilli has received relatively little attention thus far. The *Lactobacillus* polysaccharides may contribute to human health, either as prebiotics, or because of their antitumoral, antiulcer, immunomodulating or cholesterol lowering activity. The prebiotic effects of fructans (i.e. inulin, levan) and of fructo-oligosaccharides and gluco-oligosaccharides are well-known, stimulating the growth of bifidobacteria in the human colon (Gibson et al. 1994; Casas et al. 1988; Marx et al. 2000).

Limited information is available about fructan synthesis by LAB, with attention mainly focussed on the situation in streptococci. LAB 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. *Streptococcus mutans* JC-2 for instance produces a fructan consisting mainly of ß-(2→1) linked fructosyl units with 5% ß-(2→6) branches (Ebisu et al. 1975, Rosell and Birkhed 1974). Fructan produced by *S. mutans* Ingbritt A contains only ß-(2→1) linked fructosyl units (Baird et al. 1973).

Levans are synthesized from sucrose and raffinose by various bacteria. These polysaccharides are either linear or branched at the C1 position. Their synthesis has been studied in most detail in *Zymomonas mobilis* and in *Bacillus* species. The sizes of the bacterial levans vary strongly, ranging from 20 kDa to several MDa.

The enzymes responsible for synthesis of fructan polymers are generally referred to as fructosyltransferases or, in the case of levan synthesis, as levansucrases. Levansucrase (sucrose: 2,6-β-D-fructan 6-β-D fructosyltransferase, E.C. 2.4.1.10) for instance catalyzes the following reaction:

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

The enzyme has been found in various Gram negative and Gram positive bacteria, e.g. *Z. mobilis, Erwinia amylovora, Acetobacter diazotrophicus, Bacillus polymyxa, Bacillus amyloliquefaciens, Bacillus stearothermophilus* and *Bacillus subtilis* (Song et al. 1995, Kyono et al. 1995, Gunasekaran 1994, Geier et al. 1993, Arrieta et al. 1996, Li 1995, Tang et al. 1990. Steinmetz et al. 1985). Within the LAB, only the *Streptococcus salivarius* and *S. mutans* fructosyltransferases have been studied (Giffard et al. 1993, Shiroza and Kuramitsu 1988). The molecular masses of most bacterial fructosyltransferases vary between 50 and 100 kDa (for instance Shiroza and Kuramitsu 1988, Steinmetz et al. 1985, Sutherland 1993). A value of 140 kDa, however, has been reported for the enzyme of *S. salivarius* (Milward and Jacques 1990). Thus far, no fructosyltransferase has been characterized from lactobacilli.

Previously, we have reported that *Lb. reuteri* strain 121 cultivated on media containing sucrose produces both a glucan and a fructan polymer (Van Geel-Schutten et al. 1999). The fructan polymer is a linear levan, consisting of only β-(2→6) linked fructosyl residues, with an estimated size of 150 kDa (Van Geel-Schutten et al. 1999). The physiological functions of these polysaccharides are unknown.

This paper describes the molecular characterization of a fructosyltransferase from *Lb. reuteri* 121 and the products synthesized from sucrose. This is the first report describing a *Lactobacillus* fructosyltransferase enzyme. Interestingly, it synthesizes both fructo-oligosaccharides and a high molecular weight fructan with β-(2→1) linked fructosyl units only (an inulin).

MATERIALS AND METHODS

Bacterial strains and plasmids

Lb. reuteri strain 121 (LMG 18388) was used (Van Geel-Schutten et al. 1998, 1999). *Escherichia coli* strains JM109 (Phabagen; Hanahan 1983) and Top10 (Invitrogen) were used as hosts for cloning purposes. Plasmid pCR2.1 (Invitrogen) was used for cloning of the Taq amplified PCR fragments. Plasmid pCRbluntII-Topo (Invitrogen) was used for cloning of the Pwo amplified PCR fragments. Plasmid pBAD/myc/his/C (Invitrogen) was used for cloning and expression of the fructosyltransferase gene in *E. coli* Top10 cells.

Media and growth conditions

Lb. reuteri strain 121 was grown at 37°C in MRS medium (Difco, De Man et al. 1960) or in MRS-s medium (i.e. MRS medium with 100 g/l sucrose instead of 20 g/l glucose, Van Geel-Schutten et al. 1998). Because both phosphate and citrate negatively affect detection with Dionex columns, fructo-oligosaccharide production by *Lb. reuteri* was studied with cultures grown in modified MRS-s medium, in which phosphate was omitted and ammonium citrate was replaced by ammonium nitrate. *E. coli* strains were grown aerobically at 37°C in LB medium (Ausubel et al. 1987), where appropriate supplemented with 50 μg/ml ampicillin (for selection of recombinant plasmids) or with 0.02% arabinose (w/v) for induction of the fructosyltransferase gene. Agar plates were made by adding 1.5% agar to the media.

Isolation of DNA

Lb. reuteri DNA was isolated according to Verhasselt et al. (1989) as modified by Nagy et al. (1995).

Plasmid DNA of *E. coli* was isolated using the alkaline lysis method (Birnboim and Doly 1979) or with a Qiagen plasmid kit following the instructions of the supplier (Qiagen GmbH, Germany).

Molecular techniques

General procedures for cloning, DNA manipulations and agarose gel electrophoresis were essentially as described by Sambrook et al. (1989). Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the suppliers (GIBCO BRL, Biolabs, Boehringer

Mannheim). DNA was amplified by PCR techniques (DNA Thermal Cycler 480, Perkin Elmer), using ampliTAQ-DNA polymerase (Perkin Elmer) or Pwo DNA polymerase (Roche biochemicals). DNA fragments were isolated from agarose gels using a Qiagen extraction kit, following the instructions of the supplier (Qiagen GmbH, Germany).

E. coli transformations were performed by electroporation in 0.2 mm cuvettes using the BioRad gene pulser apparatus (BioRad, Veenendaal, The Netherlands) at 2.5 kV, 25 μF and 200 Ω, following the instructions of the manufacturer.

Southern hybridization

DNA was restricted with endonucleases, separated by agarose gel electrophoresis and transferred to a Hybond nylon membrane (Amersham Pharmacia Biotech). For hybridization, probes were labelled using the DIG DNA random primed labelling and detection kit (Boehringer Mannheim, Cat. No. 1093.657), following the manufacturers instructions.

Identification and nucleotide sequence analysis of the fructosyltransferase gene

The fructosyltransferase gene was identified by amplification of chromosomal DNA of *Lb. reuteri* with PCR using degenerated primers (5ftf, 6ftfi and 12ftfi) based on conserved amino acid sequences deduced from different bacterial fructosyltransferase genes (Figure 1, Table 1). Using primers 5ftf and 6ftfi, an amplification product with the predicted size of 234 bp was obtained (Figure 2A). This 234 bp fragment was cloned in *E. coli* JM109 using the pCR2.1 vector and sequenced (Sanger et al. 1977). Analysis of the obtained sequence data confirmed that part of a fructosyltransferase (*ftf*) gene had been isolated. The 234 bp amplified fragment was used to design primers 7ftf and 8ftfi (Table 1). PCR with primers 7ftf and 12ftfi gave a product of the predicted size of 948 bp; Figures 2B); its sequence showed clear similarity with previously characterized *ftf* genes. The 948 bp amplified fragment was used to design primers ftfAC1(i) and ftfAC2(i) (Table 1) for inverse PCR. Using inverse PCR techniques, a 1438 bp fragment of the putative *ftfA* gene was generated, including the 3' end of the putative *ftfA* gene (Figure 2C). The remaining 5' fragment of the putative *ftfA* gene was isolated with a combination of standard and inverse PCR techniques. Briefly, *Lb. reuteri* DNA was restricted with *Xho*I and ligated. PCR with primers 7ftf and 8ftfi, using the ligation product as template, yielded a 290 bp PCR product which was cloned into pCR2.1 and sequenced. This revealed that primer 8ftfi had

annealed aspecifically as well as specifically, yielding the 290 bp product (Figure 2D).

Table 1 Nucleotide sequences of primers used in PCR reactions to identify *ftfA*. Listed from left to right are: primer name (i, reverse primer), location (in bp) in *ftfA* and the sequence from 5' to 3' according to IUB group codes (N = any base; M = A or C; R = A or G; W = A or T; S = C or G; Y = C or T; K = G or T; B = not A; D = not C; H = not G; V = not T). Underneath the primer sequences of ftfA1 and ftfA2i, restriction enzymes used for the forced cloning of the *ftfA* PCR product into pBAD are indicated.

Primer		Nucleotide sequences
ftfAC1	(1176)	CTG-ATA-ATA-ATG-GAA-ATG-TAT-CAC
ftfAC2i	(1243)	CAT-GAT-CAT-AAG-TTT-GGT-AGT-AAT-AG
ftfac1	(1176)	GTG-ATA-CAT-TTC-CAT-TAT-TAT-CAG
ftfAC2	(1243)	CTA-TTA-CTA-CCA-AAC-TTA-TGA-TCA-TG
ftfA1		CCA-TGG-CCA-TGG-TAG-AAC-GCA-AGG-AAC-ATA-AAA-AAA-TG
ftfA2i		*Nco*I *Nco*I
5ftf	(845)	AGA-TCT-AGA-TCT-GTT-AAA-TCG-ACG-TTT-GTT-AAT-TTC-TG
6ftfi	(1052)	*Bgl*II *Bgl*II
7ftf	(1009)	GAY-GTN-TGG-GAY-WSN-TGG-GCC
8ftfi	(864)	GTN-GCN-SWN-CCN-SWC-CAY-TSY-TG
12ftfi	(1934)	GAA-TGT-AGG-TCC-AAT-TTT-TGG-C
19ftf	(1)	CCT-GTC-CGA-ACA-TCT-TGA-ACT-G
20ftfi	(733)	ARR-AAN-SWN-GGN-GCV-MAN-GTN-SW
		TAY-AAY-GGN-GTN-GCN-GAR-GTN-AA
		CCG-ACC-ATC-TTG-TTT-GAT-TAA-C

At this time we obtained the N-terminal amino acid sequence of a fructosyltransferase enzyme purified from *Lb. reuteri* 121 (FTFB; van Hijum et al., in preparation), containing the following 23 amino acids: Q V E S N N Y N G V A E V N T E R Q A N G Q I (Figure 3). The degenerated primer 19ftf was designed on the basis of part of this N-terminal peptide sequence (YNGVAEV), and primer 20ftfi was designed on the 290 bp PCR product. PCR with primers 19ftf and 20ftfi gave a 754 bp PCR product (Figure 2E), which was cloned into pCR2.1 and sequenced.

Both DNA strands of the entire *ftfA* gene were double sequenced. In this way the sequence of a 2.6 kb region of the *Lb. reuteri* DNA, containing the *ftfA* gene was obtained.

```
5ftf
B. amyloliquefaciens SacB        80    GLDVWDSWPLQNAD    93
B. subtilis SacB                 82    GLDVWDSWPLQNAD    95
S. mutans SacB                   243   DLDVWDSWPVQDAK    256
S. salivarius Ftf                282   EIDVWDSWPVQDAK    295
                                       :*******:*:*.
6ftfi
B. amyloliquefaciens SacB        156   QTQEWSGSATFTSDGK  171
B. subtilis SacB                 158   QTQEWSGSATFTSDGK  173
S. mutans SacB                   312   LTQEWSGSATVNEDGS  327
S. salivarius Ftf                351   DDQQWSGSATVNSDGS  366
                                         *:******...**.
12ftfi
B. amyloliquefaciens SacB        440   KATFGPSFLMN       450
B. subtilis SacB                 440   QSTFAPSFLLN       450
S. mutans SacB                   609   NSTWAPSFLIQ       619
S. salivarius Ftf                655   KSTWAPSFLIK       665
                                       ::*:.****::
```

Figure 1 Parts of an alignment of the amino acid sequences of some bacterial fructosyltransferases. Sequences in bold indicate the consensus sequences used to construct the degenerated primers 5ftf, 6ftfi and 12ftfi. (*) indicates a position with a fully conserved amino acid residue. (:) indicates a position with a fully conserved 'strong' group: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW. (.) indicates a position with a fully conserved 'weaker' group: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY. Groups are according to the Pam250 residue weight matrix (Altschul et al. 1990).

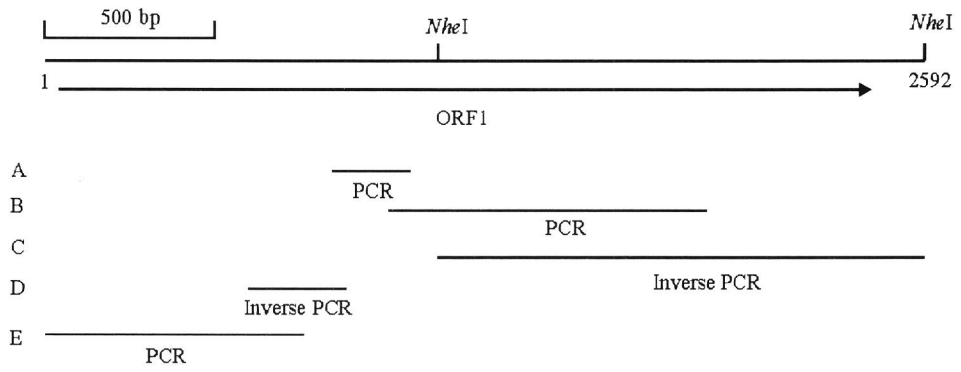

Figure 2 Strategy used for the isolation of the *ftfA* gene from *Lactobacillus reuteri* 121 chromosomal DNA.

Construction of plasmids for expression of the *ftfA* gene in *E. coli* Top10

A 2414 bp fragment, containing the *ftfA* gene starting at the first putative start codon at postion 41, was generated by PCR, using primers ftfA1 and ftfA2i (Table 1). Both primers contained suitable restriction enzyme recognition sites (a *Nco*I site at the 5' end of ftfA1 and a *Bgl*II site at the 3' end of ftfA2i). PCR with *Lb. reuteri* DNA, Pwo DNA polymerase, and primers ftfA1 and ftfA2i, yielded the complete putative *ftfA* gene flanked by *Nco*I and *Bgl*II restriction sites. The PCR product with blunt ends was ligated directly into pCRbluntII-Topo. Using the *Nco*I and *Bgl*II restriction sites, the putative *ftfA* gene was cloned into the expression vector pBAD, downstream of the inducible arabinose promoter and in frame upstream of the Myc epitope and the His tag. The pBAD vector containing the putative *ftfA* gene (pSVH101) was transformed to *E. coli* Top10 and used to study *ftfA* expression. Correct construction of plasmid containing the complete *ftfA* gene was confirmed by restriction enzyme digestion analysis and by sequence analysis, showing an in frame cloning of the *ftfA* gene using the ribosomal binding site provided by the pBAD vector and the first putative start codon (at position 41) of *ftfA* (Figure 3).

Preparation of cell extracts

Cells of *E. coli* Top10 harbouring pSVH101 were grown overnight in LB with 0.02% arabinose. The pellet was washed with 25 mM sodium acetate buffer pH 5.4 and the suspension was centrifuged. Pelleted cells were resuspended in 25 mM sodium acetate buffer pH 5.4. Cells were broken by sonication. Cell debris and intact cells were removed by centrifugation for 30 min at 4°C at 10,000 *g* and the resulting cell free extract (supernatant) was used in the enzyme assays.

Enzyme assays

Fructosyltransferase activities were determined at 37°C in reaction buffer (25 mM sodium acetate pH 5.4, 1 mM $CaCl_2$, 100 g/l sucrose) by monitoring the release of glucose from sucrose, by detecting fructo-oligosaccharides or by determining the amount of fructan polymer produced (Van Geel-Schutten et al. 1999). *E. coli* cell free extracts or *Lb. reuteri* culture supernatants were used as enzyme source.

Sucrose, glucose and fructose were determined enzymatically. Sucrose was converted into glucose and fructose, using invertase (Bio-invert 300) according to the specifications of the manufacturer (Quest Biocon).

Glucose was determined by the hexokinase/glucose-6-phosphate dehydrogenase assay. For determination of fructose, phosphoglucoisomerase was used to convert fructose-6-phosphate into glucose-6-phosphate, followed by the hexokinase/glucose-6-phosphate dehydrogenase assay. The NADPH formed was determined spectrophotometrically at 340 nm. All enzyme assays were performed in duplicate.

Gel electrophoresis

SDS-PAGE was performed according to Laemmli (1970), using the BioRad mini-Protean II slab gel system with 7.5% polyacrylamide gels. The HMW-SDS standard (Pharmacia) was used as molecular mass reference. After electrophoresis gels were stained with Coomassie Brilliant Blue, or an activity staining (periodic acid Schiff, PAS) was carried out as described earlier (Van Geel-Schutten et al. 1999).

Characterization of the fructo-oligosaccharides produced

Fructo-oligosaccharide synthesis was studied in *Lb. reuteri* culture supernatants and *E. coli* (*ftfA*) cell extracts (see above), incubated in reaction buffer (1 mg of protein in 10 ml buffer, incubated overnight at 37°C). Glucose and fructose produced were determined enzymatically as described above. Fructo-oligosaccharides produced were analyzed on a Dionex column. The incubation mixtures were centrifuged for 30 min at 10,000 g and diluted 1:5 in a 100% DMSO solution prior to injection on a Dionex column. A digest of inulin (DP1-20) was used as standard.

Separation of compounds was achieved with anion-exchange chromatography on a CarboPac PA1 column (Dionex) coupled to a CarboPac PA1 guard column (Dionex). Using a Dionex GP50 pump the following gradient was generated: % eluent B = 5% (0 min); 35% (10 min); 45% (20 min); 65% (50 min); 100% (54-60 min); 5% (61-65 min). Eluent A was 0.1 M NaOH and eluent B was 0.6 M NaAc in a 0.1 M NaOH solution. Compounds were detected using a Dionex ED40 Electrochemical Detector with an Au working electrode and a Ag/AgCl reference-electrode with a sensitivity of 300 nC. The pulse program used was: +0.1 Volt (0-0.4 s); +0.7 Volt (0.41-0.60 s); -0.1 Volt (0.61-1.00 s). Data was integrated using a Perkin Elmer Turbochrom data integration system.

A different separation of compounds was done on a cation exchange column in the calcium form (Benson BCX4). As mobile phase Ca-EDTA in water (100 ppm) was used. The elution speed was 0.4 ml/min at a column temperature of 85°C. Detection of compounds was done by a refractive index

(Jasco 830-RI) at 40°C. Quantification of compounds was achieved by using the software program Turbochrom (Perkin Elmer).

Characterization of the fructans produced

Fructan production by *Lb. reuteri* was studied with cells grown in MRS-s medium. Product formation was also studied in *E. coli* (*ftfA*) cell extracts (see above), incubated in reaction buffer (1 mg of protein in 10 ml buffer, incubated overnight at 37°C). Fructans produced were collected by precipitation with ethanol (Van Geel-Schutten et al. 1999). NMR spectroscopy and methylation analysis were performed as described by Van Geel-Schutten et al. (1999). The molecular weight of the fructans was determined by GPC-MALLS as described by Van Geel-Schutten et al. (2000).

Phylogenetic tree constructions

Amino acid sequences from fructosyltransferase enzymes were divided into three groups: from Gram positive bacteria, Gram negative bacteria and plant origin. Each of the individual groups were aligned with ClustalW 1.74 (Thompson et al. 1994), with a gap opening penalty of 30 and a gap extension penalty of 0.5. The three groups were subsequently aligned to each other with ClustalW, using the same parameters. Phylogenetic tree construction was performed using TreeCon 1.3b (no correction for distance estimation, 100 bootstrap samples, Van de Peer and De Wachter 1994).

RESULTS

Isolation and nucleotide sequence analysis of the *Lb. reuteri* fructosyltransferase gene

Using PCR with degenerated primers based on conserved amino acid sequences, found by alignment of a number of fructosyltransferases and chromosomal DNA of *Lb. reuteri* as template, a single amplicon of 234 bp was obtained and sequence analysis confirmed its fructosyltransferase (*ftf*) identity. Southern hybridization with the amplified 234 bp PCR fragment, followed by washing under non-stringent conditions (45°C, 0.5x SSC/ 0.1 SDS), revealed only one hybridizing fragment, suggesting that *Lb. reuteri* chromosomal DNA contains only a single copy of an ftf gene.

Figure 2 shows the cloning strategy (a combination of standard and inverse PCR) used for isolation of the entire *ftf* gene (*ftfA*). In total, a DNA

fragment of 2592 bp was sequenced (Figure 3). It contained an ORF of 2396 bp encoding a putative fructosyltransferase (FTFA). Two putative start codons are present: An ATG codon at position 41, with a putative ribosomal binding site (GGGG) 12 bp upstream, and an ATG codon located at position 68, with a putative ribosomal binding site (AGGA) 14 bp upstream (Figure 3). Starting at position 68, the encoded protein (789 amino acids) has a deduced molecular weight of 85,598 Da and a pI of 4.51.

```
1 tacaatgggg tggcggaggt gaagaaacgg ggttacttct atgctagaac gcaaggaaca 19ftf>
    y  n  g     v  a  e     v  k  k  r     g  y  f     y  a  r     t
    y  n  g     v  a  e     v  n  t  e     r  q  a     n  g  q     i

61 taaaaaaatg tataaaagcg gtaaaaattg ggcagtcgtt acactctcga ctgctgcgct
 1          m  y  k  s     g  k  n     w  a  v  v     t  l  s     t  a  a

121 ggtatttggt gcaacaactg taaatgcatc cgcggacaca aatattgaaa acaatgattc
 18 l  v  f  g     a  t  t     v  n  a     s  a  d  t     n  i  e     n  n  d

181 ttctactgta caagttacaa caggtgataa tgatattgct gttaaaagtg tgacacttgg
 38 s  s  t  v     q  v  t     t  g  d     n  d  i  a     v  k  s     v  t  l

241 tagtggtcaa gttagtgcag ctagtgatac gactattaga acttctgcta atgcaaatag
 58 g  s  g  q     v  s  a     a  s  d     t  t  i  r     t  s  a     n  a  n

301 tgcttcttct gccgctaata cacaaaattc taacagtcaa gtagcaagtt ctgctgcaat
 78 s  a  s  s     a  a  n     t  q  n     s  n  s  q     v  a  s     s  a  a

361 aacatcatct acaagttccg cagcttcatt aaataacaca gatagtaaag cggctcaaga
 98 i  t  s  s     t  s  s     a  a  s     l  n  n  t     d  s  k     a  a  q

421 aaatactaat acagccaaaa atgatgacac gcaaaaagct gcaccagcta acgaatcttc
118 e  n  t  n     t  a  k     n  d  d     t  q  k  a     a  p  a     n  e  s

481 tgaagctaaa aatgaaccag ctgtaaacgt taatgattct tcagctgcaa aaaatgatga
138 s  e  a  k     n  e  p     a  v  n     v  n  d  s     s  a  a     k  n  d

541 tcaacaatcc agtaaaaaga atactaccgc taagttaaac aaggatgctg aaaacgttgt
158 d  q  q  s     s  k  k     n  t  t     a  k  l  n     k  d  a     e  n  v

601 aaaaaaggcg ggaattgatc ctaacagttt aactgatgac cagattaaag cattaaataa
178 v  k  k  a     g  i  d     p  n  s     l  t  d  d     q  i  k     a  l  n

661 gatgaacttc tcgaaagctg caaagtctgg tacacaaatg acttataatg atttccaaaa
198 k  m  n  f     s  k  a     a  k  s     g  t  q  m     t  y  n     d  f  q

721 gattgctgat acgttaatca aacaagatgg tcggtacaca gttccattct ttaaagcaag 20ftfi <
218 k  i  a  d     t  l  i     k  q  d     g  r  y  t     v  p  f     f  k  a
```

```
781 tgaaatcaaa aatatgcctg ccgctacaac taaagatgca caaactaata ctattgaacc
238 s  e  i  k  n  m  p  a  a  t  t  k  d  a  q  t  n  t  i  e

841 tttagatgta tgggattcat ggccagttca agatgttcgg acaggacaag ttgctaattg  5ftf >.
258 p  l  d  v  w  d  s  w  p  v  q  d  v  r  t  g  q  v  a  n     8ftfi <

901 gaatggctat caacttgtca tcgcaatgat gggaattcca aaccaaaatg ataatcatat
278 w  n  g  y  q  l  v  i  a  m  m  g  i  p  n  q  n  d  n  h

961 ctatctctta tataataagt atggtgataa tgaattaagt cattggaaga atgtaggtcc  7ftf >
298 i  y  l  l  y  n  k  y  g  d  n  e  l  s  h  w  k  n  v  g

1021 aatttttggc tataattcta ccgcggtttc acaagaatgg tcaggatcag ctgttttgaa  7ftf >
318 p  i  f  g  y  n  s  t  a  v  s  q  e  w  s  g  s  a  v  l     6ftfi <

1081 cagtgataac tctatccaat tattttatac aagggtagac acgtctgata acaataccaa
338 n  s  d  n  s  i  q  l  f  y  t  r  v  d  t  s  d  n  n  t

1141 tcatcaaaaa attgctagcg ctactcttta tttaactgat aataatggaa atgtatcact  NheI
358 n  h  q  k  i  a  s  a  t  l  y  l  t  d  n  n  g  n  v  s     AC1(i)<>

1201 cgctcaggta cgaaatgact atattgtatt tgaaggtgat ggctattact accaaaactta AC2(i)<>
378 l  a  q  v  r  n  d  y  i  v  f  e  g  d  g  y  y  y  q  t

1261 tgatcaatgg aaagctacta acaaaggtgc cgataatatt gcaatgcgtg atgctcatgt
398 y  d  q  w  k  a  t  n  k  g  a  d  n  i  a  m  r  d  a  h

1321 aattgaagat ggtaatggtg atcggtacct tgtttttgaa gcaagtactg gtttggaaaa
418 v  i  e  d  g  n  g  d  r  y  l  v  f  e  a  s  t  g  l  e

1381 ttatcaaggc gaggaccaaa tttataactg gttaaattat ggcggagatg acgcatttaa
438 n  y  q  g  e  d  q  i  y  n  w  l  n  y  g  g  d  d  a  f

1441 tatcaagagc ttatttagaa ttctttccaa tgatgatatt aagagtcggg caacttgggc
458 n  i  k  s  l  f  r  i  l  s  n  d  d  i  k  s  r  a  t  w

1501 taatgcagct atcggtatcc tcaaactaaa taaggacgaa aagaatccta aggtggcaga
478 a  n  a  a  i  g  i  l  k  l  n  k  d  e  k  n  p  k  v  a

1561 gttatactca ccattaattt ctgcaccaat ggtaagcgat gaaattgagc gaccaaatgt
498 e  l  y  s  p  l  i  s  a  p  m  v  s  d  e  i  e  r  p  n

1621 agttaaatta ggtaataaat attacttatt tgccgctacc cgtttaaatc gaggaagtaa
518 v  v  k  l  g  n  k  y  y  l  f  a  a  t  r  l  n  r  g  s

1681 tgatgatgct tggatgaatg ctaattatgc cgttggtgat aatgttgcaa tggtcggata
538 n  d  d  a  w  m  n  a  n  y  a  v  g  d  n  v  a  m  v  g

1741 tgttgctgat agtctaactg gatcttataa gccattaaat gattctggag tagtcttgac
558 y  v  a  d  s  l  t  g  s  y  k  p  l  n  d  s  g  v  v  l
```

```
1801 tgcttctgtt cctgcaaact ggcggacagc aacttattca tattatgctg tccccgttgc
 578 t  a  s  v  p  a  n  w  r  t  a  t  y  s  y  y  a  v  p  v

1861 cggaaaagat gaccaagtat tagttacttc atatatgact aatagaaatg gagtagcggg
 598 a  g  k  d  d  q  v  l  v  t  s  y  m  t  n  r  n  g  v  a

1921 taaaggaatg gattcaacttgggcaccgagtttcttacta caaattaacc cggataacac 12ftfi <
 618 g  k  g  m  d  s  t  w  a  p  s  f  l  l  q  i  n  p  d  n

1981 aactactgtt ttagctaaaa tgactaatca aggggattgg atttgggatg attcaagcga
 638 t  t  t  v  l  a  k  m  t  n  q  g  d  w  i  w  d  d  s  s

2041 aaatcttgat atgattggtg atttagactc cgctgcttta cctggcgaac gtgataaacc
 658 e  n  l  d  m  i  g  d  l  d  s  a  a  l  p  g  e  r  d  k

2101 tgttgattgg gacttaattg gttatggatt aaaaccgcat gatcctgcta caccaaatga
 678 p  v  d  w  d  l  i  g  y  g  l  k  p  h  d  p  a  t  p  n

2161 tcctgaaacg ccaactacac cagaaacccc tgagacacct aatactccca aaacaccaaa
 698 d  p  e  t  p  t  t  p  e  t  p  e  t  p  n  t  p  k  t  p

2221 gactcctgaa aatcctggga cacctcaaac tcctaataca cctaatactc cggaaattcc
 718 k  t  p  e  n  p  g  t  p  g  t  p  n  t  p  n  t  p  e  i

2281 tttaactcca gaaacgccta agcaacctga aacccaaact aataatcgtt tgccacaaac
 738 p  l  t  p  e  t  p  k  g  p  e  t  q  t  n  n  r  l  p  q

2341 tggaaataat gccaataaag ccatgattgg cctaggtatg ggaacattgc ttagtatgtt
 758 t  g  n  n  a  n  k  a  m  i  g  l  g  m  g  t  l  l  s  m

2401 tggtcttgca gaaattaaca aacgtcgatt taactaaata ctttaaaata aaaccgctaa
 778 f  g  l  a  e  i  n  k  r  r  f  n  -

2461 gccttaaatt cagcttaacg gttttttatt ttaaaagttt ttattgtaaa aaagcgaatt

2521 atcattaata ctaatgcaat tgttgtaaga ccttacgaca gtagtaacaa tgaatttgcc

2581 catctttgtc gg                                                    NheI
```

Figure 3 Nucleotide sequence of the *ftfA* gene and the deduced amino acid sequence of FTFA. The designations and orientation (< for 3' to 5' and > for 5' to 3') of the primers and the restriction enzymes used for (inverse) PCR, are shown at the right hand side of the sequence. Putative start codons (ATG, at positions 41 and 68) and stop codon (TAA, at position 2435) are shown in bold. The positions of the primers used for PCR are shown in bold/underlined. The *Nhe*I restriction sites (at positions 1154 and 2592) used for inverse PCR are underlined. The primers used and their exact positions in the *ftfA* sequence are shown in Table 1. Starting at amino acid 690, the 20 PXX repeats are underlined. At amino acid 755 the LPXTG motif is underlined. The FTFB N-terminal amino acid sequence is indicated in italic and underlined text, the corresponding "FTFA" sequence is in italic.

A hydrophobicity plot (Figure 4) suggests the presence at the N-terminal end of the FTFA protein of 789 amino acids of a putative signal sequence (Von Heijne 1983). A hydrophilic domain comprising amino acid residues 1 to 8 and a hydrophobic domain consisting of residues 9 to 21 (Figure 4). The putative signal peptidase site is located between Gly at position 21 and Ala at position 22 (probability 0.3 according to the PSORT program located on the web at site: http://psort.nibb.ac.jp/). If cleavage occurs at this position, a signal peptide (C-terminally ending with the amino acid residues LVFG) and a mature protein (N-terminally starting with amino acid residues ATTV) will be formed. The putative FTF protein starting from the first Met residue (start codon at position 41) also contains a putative signal sequence, but with a much lower probability (0.017 according to PSORT).

The N-terminal amino acid sequence of the fructosyltransferase purified from *Lb. reuteri* supernatant was found to be QVESNNYNG VAEVNTERQANGQI. This sequence could not be identified in the deduced FTFA sequence. Also the amino acid sequences of three FTFB internal peptide fragments were not present in the putative FTFA sequence (results not shown). Evidently, *ftfA* does not encode the purified fructosyltransferase, which we have tentatively called FTFB.

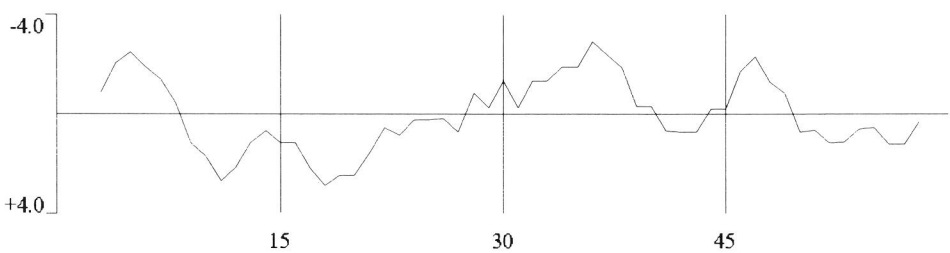

Figure 4 Kyte & Doolittle hydrophobicity plot (Kyte and Doolittle 1982) of the amino acid sequence derived from the putative *ftfA* gene. Amino acid numbers (Figure 3) are indicated below the graph. Kyte & Doolittle values fall within a range of +4 to -4, with hydrophilic residues having a negative score. The most hydrophilic residue has a value of -4.5 (arginine). Values above the axis line are hydrophilic, values below the axis line are hydrophobic.

The C-terminal amino acid sequence of FTFA contains the cell wall anchor amino acid signal LPXTG, plus a 20-fold repeat of the motif PXX (Figure 3), where P is proline and X is any other amino acid. In 15 out of the

20 repeats, however, the motif is PXT. At present there is no explanation for this unique and striking feature; the presence of this motif has not been reported before in proteins from prokaryotic and eukaryotic origin.

Amino acid sequence comparison of *Lb. reuteri* FTFA and other fructosyltransferases

Alignment of the amino acid sequence of FTFA with other fructosyltransferases revealed clear similarities. Highest homology (62% identity, 90% similarity within 539 amino acids) at the amino acid level was found with the *S. mutans* SacB enzyme (Shiroza and Kuramitsu 1988). Based on the alignments with other bacterial fructosyltransferases and site directed mutagenesis studies (Chambert and Petit-Glatron 1991, Batista et al. 1999), putative catalytic residues in *Lb. reuteri* FTFA are Asp^{263}, Glu^{330}, Asp^{415}, Glu^{431}, Asp^{511}, Glu^{514}, Arg^{532} and/or Asp^{551}.

A phylogenetic tree (Figure 5) was constructed on the basis of these alignments. *Lb. reuteri* FTFA shares high sequence similarity with fructosyltransferase enzymes originating from Gram positive bacteria, in particular with *Streptococcus* enzymes. Furthermore, three distinct groups of fructosyltransferases can be identified: from Gram negative- and Gram positive bacteria, and from plants. The fructosyltransferase enzymes of Gram positive- and Gram negative bacteria show low similarity (about 20%).

Expression of the *Lb. reuteri ftfA* gene in *E. coli*

The FTFA encoding gene (nucleotides 41 to 2434; Figure 3) was cloned in an *E. coli* expression vector under the control of an *ara* promoter Fructosyltransferase activity is most conveniently detected by following glucose production from sucrose utilization. Cell extracts of *E. coli* Top10 containing the FTFA encoding ORF (pSVH101) clearly possessed sucrose hydrolyzing activity when incubated in a buffer with sucrose as substrate. The highest sucrose hydrolyzing activity was obtained when *E. coli* (pSVH101) cells were incubated overnight with 0.02% arabinose. Without induction with arabinose no activity was detected. SDS-PAGE of arabinose induced *E. coli* (pSVH101) cell extracts, followed by incubation of gels with sucrose and PAS activity staining, showed a massive band of polysaccharide formation around 80-100 kDa. No sucrose hydrolysis was observed with *E. coli* Top10 extracts containing a vector (pBAD) minus the *ftfA* gene.

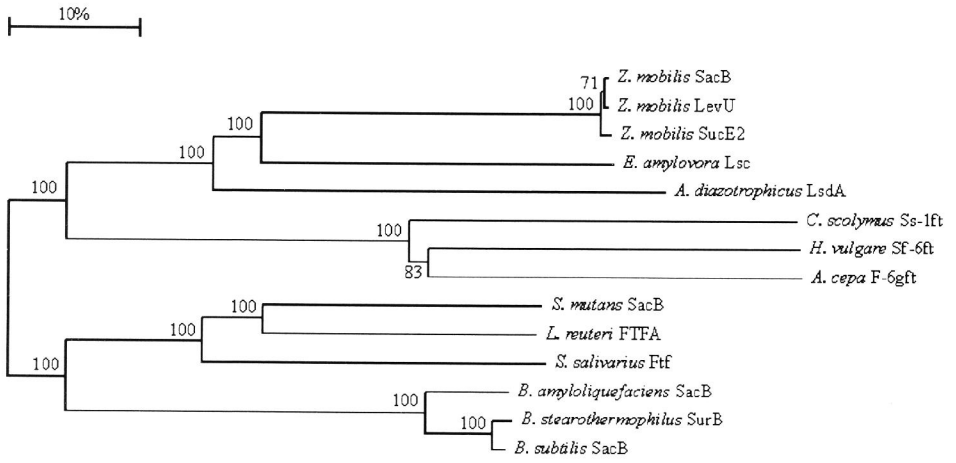

Figure 5 Dendrogram of bacterial and plant fructosyltransferases. The horizontal distances are a measure for the difference at the amino acid sequence level. The length of the upper bar indicates 10% difference. Bootstrap values are given at the root of each branch (in percentages). Fructosyltransferases of Gram positive bacteria are indicated in the lower half of the figure (*B. stearothermophilus* SurB: Li 1995; *B. amyloliquefaciens* SacB: Tang et al. 1990; *B. subtilis* SacB: Steinmetz et al. 1985; *S. mutans* SacB: Shiroza and Kuramitsu 1988; *Lb. reuteri* FTFA: Present study; *S. salivarius* Ftf: Giffard et al. 1993); plant fructosyltransferases are indicated in the middle part of the figure (*Cynara scolymus* Ss-1ft; Hellwege et al. 1997; *Allium cepa* F-6gft: Vijn et al. 1997; and *Hordeum vulgare* Sf-6ft: Sprenger et al. 1995); fructosyltransferases of Gram negative bacteria are shown in the upper part of the figure (*Z. mobilis* LevU: Song et al. 1995; SucE2: Kyono et al. 1995; SacB: Gunasekaran et al. 1994; *E. amylovora* Lcs: Geier and Geider 1993; and *A. diazotrophicus* LsdA: Arrieta et al. 1996).

Analysis of products synthesized from sucrose by *Lb. reuteri* wild type and *E. coli* FTFA

Lb. reuteri culture supernatants incubated with sucrose produced a single fructan (10 g/l) with β-(2→6) linked fructosyl units only (a levan) with an average molecular weight of 150 kDa (Van Geel-Schutten et al. 1999). In the present study we observed that *Lb. reuteri* incubated with sucrose also produces fructo-oligosaccharides of DP 3-4 (approx. 10 g/l), apart from the levan.

Cell extracts of arabinose induced *E. coli* (pSVH101) produced both fructo-oligosaccharides and fructan when incubated in a buffer with sucrose

as substrate. After 17 h of incubation, 16.5 g/l sucrose was consumed and 5.1 g/l fructo-oligosaccharide (mostly DP3 with minor amounts of DP4) and 0.8 g/l fructan was synthesized. Furthermore, 2.6 g/l fructose and 6.0 g/l glucose were released. Methylation analysis of the polymer revealed the presence of ß-(2→1) linked fructosyl units exclusively. The *Lb. reuteri* FTFA thus synthesized a fructan of the inulin type. GPC-MALLS analysis of the inulin polymer produced by the recombinant FTFA indicated that this is a high molecular weight fructan (> 10^7 Da).

DISCUSSION

This paper reports the molecular characterization of the first *Lactobacillus* (*Lb. reuteri*) gene (*ftfA*) encoding a fructosyltransferase (FTFA), producing a high molecular weight inulin with β-(2→1) glycosidic bonds only and fructo-oligosaccharides of DP3-4. Fructo-oligosaccharide synthesis was observed in *Lb. reuteri* culture supernatants, but the inulin type of fructan produced by FTFA in *E. coli* extracts has not been associated with *Lb. reuteri*. Several possibilities can be considered to explain this apparent discrepancy: (1) the *ftfA* gene is silent in *Lb. reuteri*, (2) *ftfA* is not expressed in *Lb reuteri* under the conditions tested, (3) the FTFA enzyme only synthesizes fructo-oligosaccharides in its natural host, and (4) the inulin polymer already has been degraded at the time of harvesting of the cultures. Other possible explanations are that the FTFA enzyme and its polymeric product remain tightly associated with the *Lb. reuteri* cell surface and thus are removed when harvesting culture supernatants, or that the FTFA has other activities in *E. coli* extracts than in *Lb reuteri*.

The N-terminal amino acid sequence of a purified *Lb. reuteri* fructosyltransferase (FTFB; van Hijum et al. in preparation) could not be identified in the deduced FTFA sequence. The primer pair 19ftf, based on this FTFB N-terminal amino acid sequence, and 20ftf had yielded a distinct product comprising the 5' end of *ftfA*. *Apparently, 19ftf* had annealed properly, but upstream of the putative start codons of the *ftfA* gene.

The amino acid sequences of three FTFB internal peptide fragments were not present in the putative FTFA sequence (results not shown). Our data indicate that *ftfA* gene does not encode the purified fructosyltransferase enzyme (FTFB). Preliminary experiments (van Hijum et al., in preparation) suggest the presence of a gene encoding FTFB which in fact synthesizes the 150 kDa levan polymer previously described for *Lb. reuteri* (Van Geel-Schutten et al. 1998, 1999). Hybridization studies under non-stringent conditions with the 234 bp *ftfA* fragment, using *Lb. reuteri* chromosomal DNA as template,

revealed only one hybridizing fragment. The *ftfA* and *ftfB* fructosyltransferase genes in *Lb. reuteri* may share a relatively low sequence similarity only.

Two putative translation start codons are present in the *ftfA* sequence, at positions 41 and 68 (Figure 3). A typical secretion signal peptide according to the Von Heijne rule (Von Heijne 1983) appears to be present in the N-terminus of the putative FTFA protein, most likely initiated from the start codon at position 68 (Figure 3). In order to investigate which of the putative start codons is functional in *E. coli*, the N-terminal amino acid sequence of the *E. coli* FTFA will be determined. The possibility that *ftfA* extends at the 5'-end remains to be determined. To ensure that the complete *ftfA* gene and its surroundings have been identified and characterized, further experiments will involve extending the upstream and downstream sequences of the *ftfA* gene.

Strikingly, a C-terminal repeat of 20 PXX residues is present in the putative FTFA. A slightly larger C-terminal motif (LPXTG), is present in a single copy only (Figure 3), and has been reported for surface proteins of Gram positive bacteria (Rathsam and Jacques 1998, Mesnage et al. 1999), with proteolytic cleavage occurring after the T residue. The function of the *Lb. reuteri* FTFA C-terminal repeat of 20 PXX residues remains to be elucidated. Conceivably, the PXX repeat also plays a role in cell attachment of FTFA.

The *Lb. reuteri* FTFA displays a high degree of amino acid similarity with other streptococcal and *Bacillus* fructosyltransferases. Highest similarity (90% within 539 amino acids) at the amino acid level was found between *Lb. reuteri* FTFA and SacB of *S. mutans* Ingbritt A (Baird et al. 1973, Shiroza and Kuramitsu 1988). This enzyme also synthesizes a fructan polymer containing only β-(2→1) linkages.

At present little is known about the catalytic mechanism of fructosyltransferases. A low resolution 3D structure has been reported for the levansucrase of *B. subtilis* (LeBrun and van Rapenbusch 1980). The proposed mechanism of catalysis for fructosyltransferases is a two-step mechanism involving a bifunctional catalysis in which an acidic group and a nucleophilic group of the protein are important for the transfructosylation reaction (Sinnott 1987). The isolation of a stabilized enzyme-fructosyl complex of the levansucrase of *B. subtilis* and its substrate sucrose has been performed by Chambert and Gonzy-Treboul (1976). The fructosyl moiety of sucrose was covalently linked to the enzyme, probably through a glycosyl ester bond. This bond probably involves the participation of the ß-carboxy group of an Asp residue of the protein and the C-2 of the fructose. Furthermore, site directed mutagenesis experiments with the levansucrase of *B. subtilis* have shown that Arg^{331} (presumably corresponding to Arg^{532} in *Lb. reuteri* FTFA) is also of importance for the specificity and efficiency of the transfructosylation process. *B. subtilis* Arg^{331}His mutants, for instance, showed a lower polymerizing, a

higher hydrolyzing and a higher fructo-oligosaccharide forming activity than the wild type enzyme. It has been suggested that the Arg^{331} side chain acts as a proton donor in the bifunctional catalysis reaction (Chambert and Petit-Glatron 1991). Site directed mutagenesis of the *A. diazotrophicus* levansucrase enzyme (Batista et al. 1999) showed that the $Asp^{309}Asn$ (presumably corresponding to Asp^{415} in *Lb. reuteri* FTFA) strongly affected the sucrose hydrolysis rate of the enzyme, but not its product specificity. The product profiles of the wild type enzyme and mutant $Asp^{309}Asn$ were similar.

A combination of site-directed and random mutagenesis techniques are presently applied to *Lb. reuteri* FTFA to identify structural features determining polymer versus oligomer formation and synthesis of β-(2→6) versus β-(2→1) glycosidic bonds.

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Molecular characterization of a novel glucansucrase from *Lactobacillus reuteri* synthesizing a unique and highly branched glucan with α-(1→4) and α-(1→6) glucosidic bonds

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ABSTRACT

Lactobacillus reuteri produces a unique, highly branched soluble glucan with α-(1→4) and α-(1→6) linked glucosyl units and α-(1-4→6) branching points (Van Geel-Schutten et al. 1999). Based on the nucleotide sequences of conserved regions from known glucosyltransferase genes from lactic acid bacteria, a chromosomal DNA fragment of approximately 5.5 kbp was isolated with PCR techniques. Nucleotide sequencing revealed the presence of ORF1 (*gtfA*) encoding the glucansucrase and part of an upstream ORF2 of unknown function. The size of the *gtfA* ORF was approximately 4100 bp, encoding a protein with a molecular weight of about 140,000 Da. The deduced amino acid sequence of GTFA showed high similarity with other glucansucrases. GTFA consisted, like other glucosyltransferases, of a variable N-terminal domain, a catalytic domain and a C-terminal glucan binding domain. However, an N-terminal secretion signal peptide could not be assigned in GTFA, and its glucan binding domain was shorter than reported for other glucansucrases. In the conserved region downstream of Asp^{494}, 3 out of 9 of

the amino acids conserved in other glucosyltransferases were not found in GTFA. Residues in this region may be involved in binding and stereospecific positioning of the acceptor molecule. Highest similarity (46% identity, 59% similarity) was found with the alternansucrase of *Leuconostoc mesenteroides* NRRL B-1355.

The *gtfA* gene was successfully expressed in *Escherichia coli* DH5α, yielding an active GTFA enzyme. Staining of polyacrylamide gels for GTF activity revealed the presence of four proteins with molecular weights of 165 kDa, 150 kDa, 130 kDa and 125 kDa in *E. coli* extracts, probably resulting from proteolytic activity. Based on NMR spectra, methylation analysis and molecular weight determinations of the polysaccharides, it was concluded that the recombinant GTFA and *Lb. reuteri* produce identical glucans. GTFA is thus responsible for the synthesis of the unique glucan in *Lb. reuteri*. This is the first report describing the molecular characterization of a glucansucrase from a *Lactobacillus* strain.

INTRODUCTION

Many lactic acid bacteria employ large (average Mw of about 160,000 Da) extracellular enzymes , glucansucrases or glucosyltransferases (GTFs, EC 2.4.1.5), for the synthesis of glucans from sucrose:

$$\text{sucrose} + (\text{glucan})_n \rightarrow \text{fructose} + (\text{glucan})_{n+1}$$

The energy released by the hydrolysis of the glycosidic bond in sucrose is used for the synthesis of new glucosidic bonds in the product. The glucosyl moiety from sucrose is transferred to a covalent glucosyl-enzyme intermediate, followed by chain extension at the non-reducing end of the glucan molecule. In addition to the synthesis of high molecular weight glucans, glucansucrases produce low molecular weight oligosaccharides in the presence of suitable acceptor molecules. In this case, the glucosyl residue is transferred to the reducing end of the acceptor molecule (Robyt, 1996).

The glucansucrases studied in most detail are glucosyltransferases from oral streptococci (McCabe and Smith 1973, Abo et al. 1991, Giffard et al. 1993, Robyt and Martin 1983, Musaka et al. 1982, Shimamura et al. 1983, Tagaki et al. 1994, Walker et al. 1990, Tsumori et al. 1997, Kumari et al. 1997, Lis et al. 1995, Kato et al. 1992, Germaine and Schachtele 1976, Russell et al. 1987, Russell 1990) and the dextransucrases from *Ln. mesenteroides* strains B-1355, B-512F and NRRL B-1299 (Miller and Robyt 1984, Robyt and Walseth 1978, Robyt, 1996, Dols et al. 1997a, 1997b,

1997c, Monchois et al. 1996, 1997, 1998a, Goyal and Katiyar 1998, Smith et al. 1998) and *Ln. mesenteroides* ssp. *dextranicum* (Neubauer and Mollet, 1999).

Some glucosyltransferases, such as dextransucrase from *Ln. mesenteroides* strain B-512F, catalyze the formation of linear glucans containing mostly α-(1→6) linkages (dextrans). Other types synthesize dextrans with α-(1→2), α-(1→3) or α-(1→4) branches (Monchois et al. 1999). Alternansucrase from *Ln. mesenteroides* strain NRRL B-1355 synthesizes a glucan with alternating α-(1→6), and α-(1→3) glucosidic bonds (Arguello-Morales et al. 2000). Recently, a glucansucrase of *Ln. mesenteroides* strain B-1355 has been characterized, synthesizing a glucan with only α-(1→2) glucosidic linkages (Smith et al, 1998). There are very few reports, however, about glucan synthesis in lactobacilli (Sidebotham 1974, Hammond 1969, Dunican and Seeley 1963, Van Geel-Schutten et al. 1998, 1999). A biochemical and molecular characterization of the enzyme(s) responsible for glucan synthesis in lactobacilli has not been reported.

The physiological function of the glucosyltransferases in lactobacilli is unknown. The enzymes of oral streptoccci are intensively studied since the glucans produced by these bacteria play an important role in the formation of dental plaque. They are involved in the adherence of the bacteria to each other and to the tooth surface, modulating diffusion of substances through plaque. In addition, they serve as extracellular energy reserves (Loesche 1986, Walker and Jacques 1987).

The genes coding for most of the glucansucrases mentioned above have been cloned and characterized. All the enzymes share a common structure and are composed of four distinct domains: their N-terminal end starts with (1) a signal peptide of 32-34 amino acids, followed by (2) a highly variable stretch of 123-129 amino acids, (3) a highly conserved catalytic or sucrose binding domain of about 1,000 amino acids and (4) a C-terminal end of about 500 amino acids (i.e. one third of the molecule), composed of a series of tandem repeats (Monchois et al. 1999). The C-terminal end constitutes the glucan binding domain. Both the C-terminal and catalytic domains are necessary to keep a fully active enzyme (Abo et al. 1991).

Amino acid sequence comparisons revealed that glucansucrases possess a $(\beta/\alpha)_8$ barrel structure similar to glucoside hydrolases of family 13. This family includes for instance α-amylase and cyclodextrin glycosyltransferase (CGTase). The core of the proteins belonging to this family is constituted of 8 β-sheets alternated with 8 α-helices. In glucansucrases, however, this $(\beta/\alpha)_8$ barrel structure is circularly permuted. Therefore glucansucrases belong to family 70 of the glycoside hydrolases (MacGregor et al. 1996, Devullapalle et al. 1997, Coutinho and Henrissat 1999).

In previous studies we have isolated a strain of *Lactobacillus reuteri*, capable of producing fructan and a highly branched glucan with a unique structure containing α-(1→4) and α-(1→6) linkages. Depending on the carbon source in the culture medium, the sucrase responsible for the synthesis of this polysaccharide material is completely cell-associated or partly released into the culture medium (Van Geel-Schutten et al. 1998, 1999).

Lb. reuteri is a commensal of the gastro-intestinal (GI) tract of a broad spectrum of hosts, including humans and all mammals and avian hosts examined to date. Some strains of *Lb. reuteri* are used as probiotics in poultry, in various other animals, and in humans (Casas et al. 1998). *Lb. reuteri* strain SD2112 for instance, is commercially exploited as a probiotic strain by BioGaia (Raleigh, North Carolina, USA; Sanders and Huis in 't Veld 1999). Although the availability of sucrose in the GI tract is questionable, the exopolysaccharides of probiotic enterolactobacilli such as *Lb. reuteri* could, in addition to other colonizing factors (e.g. adhesins), and analogous to the role of the glucans of oral streptococci, contribute to the colonization of this strain in the GI tract. Furthermore, the polysaccharides produced by the *Lb. reuteri* strain might function as prebiotic substrates.

This paper describes the first molecular characterization of a *Lactobacillus* glucansucrase gene and its expression in *Escherichia coli*. The gene (*gtfA*) encodes a novel type of a glucansucrase from a *Lb. reuteri* species, synthesizing a highly branched glucan with a unique structure containing α-(1→4) and α-(1→6) linkages.

MATERIALS AND METHODS

Bacterial strains and plasmids

Lactobacillus reuteri strain LB 121 (LMG 18388), strain 35-5 (LMG 18390) and strain K-24 (LMG 18391) have been isolated as described previously (Van Geel-Schutten et al. 1998, 1999). *Escherichia coli* DH5α (Phabagen; Hanahan 1983) and *E. coli* JM109 (Promega; Hanahan 1983) were used as hosts for cloning purposes. Plasmids pCR2.1 (Invitrogen) and pEMBL8 (Dente et al.1983) were used for cloning of the glucansucrase gene for sequencing purposes, plasmids pQE60 (Qiagen), pCR2.1-TOPO (Invitrogen), and pBluescriptIISK+ (Stratagene) were used for expression of the *gtfA* gene in *E. coli* DH5α. Plasmids pLP402(uidA)t and pLP503(uidA)t (Pouwels et al. 1996) were used as *E.coli-Lactobacillus* shuttle expression vectors. The different strains and plasmids are listed in Table 1.

Table 1 Strains and plasmids used for cloning and expression of the *gtfA* gene strain or plasmidrelevant characteristics source or reference

Strain or plasmid	Relevant characteristics	Source of reference
Lb. reuteri 121	GTF$^+$, FTF$^+$	Van Geel-Schutten et al. 1999
Lb. reuteri 35-5	GTF$^+$, FTF$^-$	Van Geel-Schutten et al. 1999
Lb. reuteri K-24	GTF$^-$, FTF$^-$	Van Geel-Schutten et al. 1999
E. coli JM109	see reference	Promega; Hanahan 1983
E. coli DH5a	see reference	Phabagen; Hanahan 1983
pCR2.1	*E. coli* vector, 3'T-overhang, Ap^r	Invitrogen
pEMBL-8	*E. coli* vector, containing *LacZ*, Ap^r	Dente and Cortese 1983
pQE60	*E. coli* vector, containing His-tag, Ap^r	Qiagen
pCR2.1-OPO	*E. coli* vector, 3'T-overhang, Ap^r	Invitrogen
pLP503(uitA)t	*E. coli-Lactobacillus* shuttle vector, contains terminator, Ap^r	Pouwels et al. 1996
pLP402(uitA)t	*E. coli-Lactobacillus* shuttle vector, contains terminator, Ap^r	Pouwels et al. 1996
pBluescript SK+	*E. coli* vector, containing *LacZ*, Ap^r	Stratagene
pBXP1500	pBluescriptSKII+ containing *Xba*I/*Pst*I *gtfA* fragment	This study
pBPB2700	pBluescriptSKII+ containing *Pst*I/*Bam*HI *gtfA* fragment	This study
pBGTF	pBluescriptSKII+ containing *gtfA*	This study

FTF, fructosyltransferase, GTF, glucosyltransferase

Media and growth conditions

Lb. reuteri strains 121, 35-5 and K24 were cultivated anaerobically at 37°C in MRS medium (Difco) or in MRS-s medium (i.e. MRS medium with 100 g/l sucrose instead of 20 g/l glucose, Van Geel-Schutten et al. 1998). *E. coli* strains were cultivated aerobically at 37°C in LB medium (Ausubel et al. 1987). *E. coli* strains containing recombinant plasmids were cultivated in LB medium with 100 μg/ml ampicillin. When appropriate, 40 μg/ml X-gal was added. Agar plates were made by adding 1.5% agar to the media.

Isolation of DNA

For the isolation of chromosomal DNA, *Lb. reuteri* was cultivated overnight at 37°C in MRS broth supplemented with 40 mM DL-threonine (Chassy and Giuffrida 1980, Van de Guchte et al. 1989). Cells of 4 ml culture were harvested by centrifugation and resuspended in 10 ml MRS broth supplemented with 40 mM DL-threonine and incubated for 2 h at 37°C. After

centrifugation, cells were resuspended in 400 µl protoplast buffer (10 mM sodium maleate pH 6.1 supplemented with 0.3 M lactose, 10 mM $MgCl_2$, 12% polyethyleneglycol 2000, 0.1 M EDTA (pH 8), 5 mg/ml lysozyme (Sigma, 47.000 U/mg) and 10 U/ml mutanolysine (Sigma)) and incubated for 1 h at 37°C. After centrifugation (1 min, Eppendorf), protoplasts were resuspended in 500 µl 20 mM Tris-HCl, pH 8.0. Subsequently, 100 µl laurylsarcosine and 150 µl 5 M NaCl were added and DNA was extracted as described by Sambrook et al. (1989). Plasmid DNA of *Lb. reuteri* was isolated using a modification of the methods of Anderson and McKay (1983) and Burger and Dicks (1994). Prewarmed (37°C) fresh MRS broth (10 ml) was inoculated with 200 µl of an overnight culture and incubated for 2.5 h at 37°C. Cells were harvested by centrifugation and washed with 2 ml sterile STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8). After centrifugation, the pellet was resuspended in 380 µl solution I (0.5 M sucrose, 50 mM Tris/HCl, 1 mM EDTA, pH 8, containing 2 mg/ml lysozyme (Sigma) and 6.6 U mutanolysin (Sigma)). After 1.5 h of incubation at 37°C, 50 µl of solution II (0.25 M EDTA, 50 mM Tris-HCl, pH 8) and 30 µl of solution III (20% SDS, 50 mM Tris-HCl, 20 mM EDTA, pH 8) were added and the suspension was mixed. NaOH (30 µl of 3 M solution) was added, followed by 50 µl 2 M Tris/HCl and 72 µl 5 N NaCl. After extraction with equal volumes of phenol and chloroform, the DNA was precipitated with ethanol as described by Sambrook et al. (1989).

Plasmid DNA of *E. coli* was isolated using the alkaline lysis method (Birnboim and Doly, 1979) or with a Qiagen plasmid kit following the instructions of the supplier (Qiagen GmbH, Germany).

Molecular techniques

General procedures for cloning, DNA manipulations and agarose gel electrophoresis were essentially as described by Sambrook et al. (1989). Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the suppliers (GIBCO BRL, Biolabs, Boehringer Mannheim). DNA was amplified by PCR techniques (DNA Thermal Cycler 480, Perkin Elmer), using ampliTAQ-DNA polymerase (Perkin Elmer). DNA fragments were isolated from agarose gels using a Qiagen extraction kit, following the instructions of the supplier (Qiagen GmbH, Germany).

E. coli transformations were performed by electroporation in 0.2 mm cuvettes using the BioRad gene pulser apparatus (BioRad, Veenendaal, The Netherlands) at 2.5 kV, 25 µF and 200 W, following the instructions of the manufacturer.

Southern hybridization

DNA was restricted with endonucleases, separated by agarose gel electrophoresis and transferred to a Hybond nylon membrane (Amersham Pharmacia Biotech). For hybridization, probes were labelled with [α-^{32}P]dCTP using a Random Primed DNA labeling kit, following the manufacturer's instruction (Boehringer Mannheim).

Identification and nucleotide sequence analysis of the glucansucrase gene

The glucansucrase gene was identified by amplification with PCR using degenerated primers (GTFpr1, 5'GAYAAKWSIAAKSYIRTIGTISAR GC3' and GTFpr2, 5'GIKCICAIATRATRCCICTRIA3'; Y = T or C, K = G or T, W = A or T, S = C or G, R = A or G, I = A, C, G or T) based on conserved amino acid sequences deduced from different glucosyltransferase genes (*gtfS* of *S. downei*, *gtfC* of *S. mutans*, *gtfI* of *S. downei*, *gtfK* and *gtfM* of *S. salivarius*, and *dsrA* of *Ln. mesenteroides* (Gilmore et al. 1990, Ueda et al., 1988, Ferreti et al. 1987, Giffard et al. 1993, Simpson et al. 1995, Monchois et al. 1996). An amplification product with the predicted size of about 660 bp was obtained (Figure 1a). This 660 bp fragment was cloned in *E. coli* JM109 using the pCR2.1 vector and sequenced (Sanger et al. 1977), confirming that part of a *gft* gene had been isolated. The 660 bp amplified fragment was used to design primers for inverse PCR. Using inverse PCR techniques a 3 kb fragment of the isolated *gtfA* gene was generated (Figure 1b). This 3 kb amplicon was identified by sequencing and probes were designed to isolate the *Eco*RI/*Bgl*II and *Eco*RI/*Hin*dIII fragments from a partial DNA library of *Lb. reuteri* in *E. coli* DH5α (Figure 1c). Positive clones were selected by colony blot hybridization (Datta and MacQuillan 1987) using Hybond-N filters (Amersham Pharmacia Biotech), following the instructions of the supplier, and the cloned fragments were sequenced. Attempts to clone the C-terminal part of the glucansucrase gene in *E. coli* DH5α or JM109 using the partial DNA library strategy with different vectors failed. Therefore, the C-terminal part was isolated by inverse PCR. The remaining fragment, located between the *Eco*RI/*Bgl*II and *Eco*RI/*Hin*dIII fragments, was isolated by PCR techniques (Figure 1d). The amplicons obtained were sequenced directly. To eliminate errors due to the PCR reaction, these fragments were sequenced for at least 4 times, using different clones per PCR reaction. Both DNA strands of the entire glucansucrase gene were sequenced twice. In this way the sequence of a 5.5 kbp region of the *Lb. reuteri* chromosomal DNA, containing the *gtfA* gene and its surroundings, were obtained.

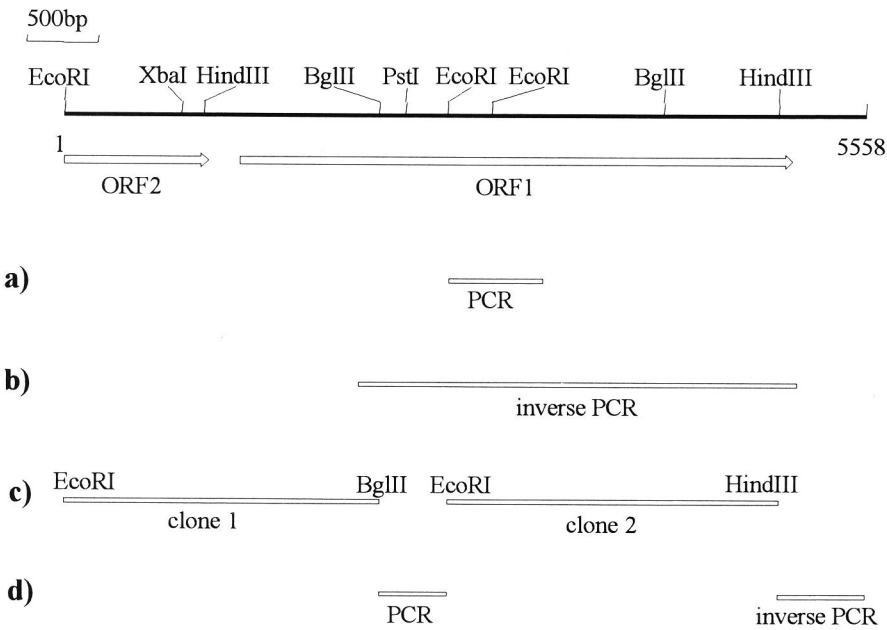

Figure 1 Strategy used for the isolation of the *gtfA* gene from *Lactobacillus reuteri* 121 chromosomal DNA.

Construction of plasmids for expression of the glucansucrase gene in *E. coli* DH5α

A 4.8 kb fragment, containing the entire glucansucrase gene (ORF1), together with part of an upstream open reading frame (ORF2) was generated by PCR, using primers GTFpr3 (5'ACAACCACCATGGAATTAGG TCGCACTGATGTAAC3') and GTFpr4 (5'GCCAGCTGGATCCGTC GACTAG TTTATTTTTGATCAAGCATCTTACC3'). Both primers contained suitable restriction enzyme recognition sites at their 5' ends (*Nco*I in GTFpr3 and *Bam*HI and *Sal*I in GTFpr4). Cloning of this PCR fragment in different vectors failed (data not shown). Therefore, the strategy depicted in Figure 2 was followed. Briefly, the PCR product was restricted with *Xba*I/*Pst*I and *Pst*I/*Bam*HI (Figure 1; *Bam*HI site introduced with GTFpr4). The resulting fragments (1503 bp and 2696 bp, respectively) were cloned separately in pBluescriptIISK+ yielding pBXP1500 and pBPB2700. Ligation of the 2700 bp

*Pst*I/SalI fragment isolated from pBPB2700 in pBXP1500, digested with *Pst*I and *Sal*I, yielded pBGTF (7146 bp) in *E. coli* DH5a.

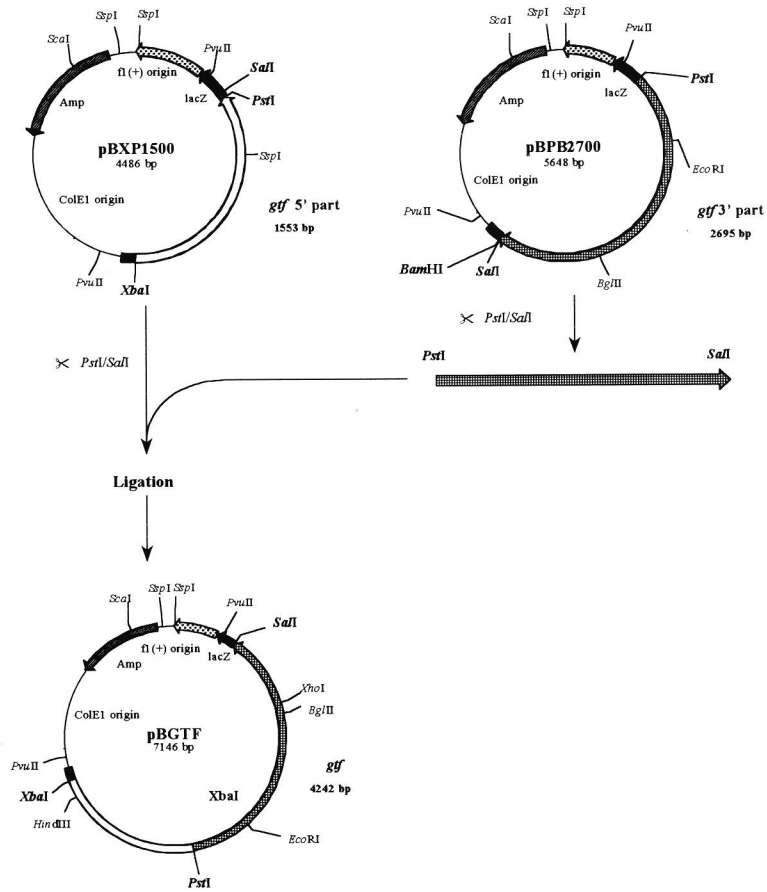

Figure 2 General principle of constructing a recombinant plasmid harbouring the *gtfA* gene. A PCR product containing the *gtfA* gene was digested with *Xba*I and *Pst*I and with *Pst*I and *Bam*HI. The *Xba*I/*Pst*I (depicted in white) was ligated into the multiple cloning site of pBluescriptIISK + in the same direction relatively to the lacZ promotor, resulting in pBXP1500. The *Pst*I/ *Bam*HI part (depicted in grey) was ligated into the multiple cloning site of pBluescriptIISK + in the opposite direction relatively to the lacZ promotor, resulting in pPBP2700. pBXP1500 was used as a vector for subcloning of the 3' end of the *gtfA*. pBPB2700 was digested with PstI and SalI and ligated into pBXP1500, also digested with *Pst*I and *Sal*I. The resulting plasmid pBGTF, contains the entire *gtfA* in the same direction relative to the lacZ promotor. ✂, restriction.

Phylogenetic tree construction

Amino acid sequences were aligned with Clustal W 1.74 (Higgins and Sharp 1988) with a gap opening penalty 30 and a gap extension penalty 0.5. Amino acid sequences were divided in the following groups. Group 1: GTFN (*S. salivarius*), GTFD (*S. mutans*), GTFM (*S. salivarius*), GTFG (*S. gordonii*). Group 2: GTFJ (*S. salivarius*), GTFT (*S. criteci*), GTFS (*S. downei*), GTFK (*S. salivarius*). Group 3: GTFC (*S. mutans*), GTFB (S. mutans), GTFI (*S. downei*). Group 4: GTFA (*Lb. reuteri*), DSRB (*Ln. mesenteroides* NRRL B-1299), DSRA (*Ln. mesenteroides* NRRL B-1299), DSRS (*Ln. mesenteroides* NRRL B-512F), DSRT (*Ln. Mesenteroides* NRRL B-512F), ASR (*Ln. Mesenteroides* NRRL B-1355). First the amino acid sequences were aligned within the groups. The complete alignment was performed by aligning groups 1 to 4 to each other. Tree construction was performed using TreeCon 1.3b (no correction for distance estimation, 100 bootstrap samples, Van de Peer and De Wachter 1994).

Preparation of cell free extracts

Cells of *E. coli* DH5α harbouring pBGTF were harvested by centrifugation after 16 h of growth. The pellet was washed with 50 mM sodium acetate buffer pH 5.5 containing 1 mM CaCl2 and 1% (v/v) Tween 80 and the suspension was centrifuged again. Pelleted cells were resuspended in 50 mM sodium acetate buffer pH 5.5 containing 1 mM $CaCl_2$, 1% (v/v) Tween 80, and 7.2 mM β-mercaptoethanol. Cells were broken by sonication. Cell debris and intact cells were removed by centrifugation for 15 min at 4°C at 14,000 rpm (Eppendorf) and the resulting cell free extract (supernatant) was used in the enzyme assays.

Enzyme assays

Glucansucrase activities were determined at 37°C by monitoring the release of fructose from sucrose, or by measuring the amount of glucan produced (Van Geel-Schutten et al. 1999) using *E. coli* cell free extracts or *Lb. reuteri* culture supernatant in reaction buffer (50 mM sodium acetate, 1 mM $CaCl_2$, 1% Tween 80, 10 g/l sucrose, pH 8). All enzyme assays were performed in duplicate.
Sucrose, glucose and fructose were determined enzymatically. Sucrose was converted into glucose and fructose, using invertase (Bio-invert 300) according to the specifications of the manufacturer (Quest Biocon). Glucose and fructose were determined using commercial kits from Roche Diagnostics, according to

the specifications of the manufacturer. Glucose and fructose were determined with the hexokinase/glucose-6-phosphate dehydrogenase assay, converting fructose-6-phosphate into glucose-6-phosphate with phosphoglucoisomerase. The glucose and fructose assays were performed automaticly using a Cobas Mira Plus autoanalyser (Roche Diagnostics).

Gel electrophoresis

SDS-PAGE was performed according to Laemmli (1970), using the BioRad mini-Protean II slab gel system with 7.5% polyacrylamide gels. The HMW-SDS standard (Pharmacia) was used as molecular mass reference. After electrophoresis gels were stained with Coomassie Brilliant Blue, or an activity staining (periodic acid Schiff, PAS) as described earlier (Van Geel-Schutten et al. 1999).

Characterization of the glucans produced

Glucans were produced using the conditions described under enzyme assays (see above). Glucans produced by *Lb. reuteri* strain 35-5 or with the glucansucrase from the recombinant *E. coli* were collected by precipitation with ethanol (Van Geel-Schutten et al. 1999). NMR spectroscopy and methylation analysis were performed as described by Van Geel-Schutten et al. (1999). The molecular weights of the glucans were determined by high performance size exclusion chromatography (HPSEC) coupled on-line with a multi angle laser light scattering (MALLS) and differential refractive index detection (RI, Schambeck SDF). The HPSEC system consisted of an isocratic pump, an injection valve, a guard column and a set of two SEC columns in series (Shodex SB806MHQ column and TSK gel 6000PW). A Dawn-DSP-F (Wyatt Technology, St. Barbara, CA, USA) laser photometer HeNe (λ = 632.8 nm) equipped with a K5 flow cell, thermostatted by a Peltier heating system was used as MALLS detector. Samples were filtered through a 0.45 μm filter (MILLEX) and the injection volume was 220 μl. Na_2SO_4 (0.1 M) was used as eluent at a flow rate of 0.8 ml/min. Pullulan and dextran samples with Mw ranging from 4.10^4 to 2.10^6 Da were used as standards. Determinations were performed in duplicate.

RESULTS

Isolation and nucleotide sequence analysis of the putative *Lb. reuteri* glucansucrase gene

Based on sequence homology between conserved regions of *gtf* genes, degenerated primers were designed and used for PCR with chromosomal DNA of *Lb. reuteri* 121 as template. A single fragment of 660 bp was obtained and sequence analysis confirmed the *gtf* identity. Southern hybridization of chromosomal DNA of *Lb. reuteri* 121 with the amplified 660 bp PCR fragment, followed by washing under non-stringent conditions (45°C, 0.5x SSC/ 0.1 SDS) revealed only one hybridizing fragment, suggesting that the strain containes only a single copy of a glucansucrase gene. Plasmid DNA of *Lb. reuteri* did not show hybridization.

Figure 1 shows the cloning strategy for isolation of the entire *gtfA* gene. The combined DNA nucleotide sequence (5558 bp) data for the DNA fragments obtained (Figure 1) is shown in Figure 3. It contained an ORF (ORF1) encoding a putative glucansucrase (GTFA) and part of an upstream ORF (ORF2) encoding a polypeptide of 326 amino acids. ORF2 showed no homology to protein sequences in databanks. The glucansucrase gene may encode a protein of 1251 amino acids with a deduced molecular weight of 139,876 Da and a pI of 4.71. A putative ribosome binding site (AGAAG) was located 14 bp upstream from the putative start codon at position 1293. Another potential start codon was located 81 bp upstream with a putative ribosomal binding site (GCAGG) 4 bp upstream of this start codon (Figure 3). Starting at this point, the encoded protein would have a deduced molecular weight of 142,863 Da and a pI of 4.73. In contrast to most other glucansucrase genes, a secretion signal peptide encoding sequence with a cleavage site according to Von Heijne (1983) could not be detected in the 5' end of ORF1.

Inverted repeats were located 62 bp downstream from the termination codon (Figure 3). These repeats could form a stem loop structure (-17.60 kcal/mol), followed by a series of T residues, suggesting the presence of a Rho independent transcription termination signal. Neither putative transcription termination sequences nor unambiguous -10 and -35 consensus promotor sequences could be identified between the termination codon of ORF2 and the putative start codon of *gtfA* (Figure 3).

```
1          TCTACTTCTACACCTGTTTCTGTTTTGCCATCTAATAATACTGAAAAACAAGCTAAAAAT
1   ORF2P  S  T  S  T  P  V  S  V  L  P  S  N  N  T  E  K  Q  A  K  N

61         TATAATGAGCAAGACAAAGGAAACTATGGGAATATTGATACTGCTTACTTTAGCAATAAT
21          Y  N  E  Q  D  K  G  N  Y  G  N  I  D  T  A  Y  F  S  N  N

121        CAATTGCATGTTTCAGGATGGAATGCAACGAACGCATCTCAAGGAACAAACAGTCGACAA
41          Q  L  H  V  S  G  W  N  A  T  N  A  S  Q  G  T  N  S  R  Q

181        ATCATTGTGCGTGATATCACAACCAATAATGAATTAGGTCGCACTGATGTAACAAACAAT
61          I  I  V  R  D  I  T  T  N  N  E  L  G  R  T  D  V  T  N  N

241        GTTGCACGCCCAGACGTTAAGAATGTTCATAATGTTTATAACGCTGATAATTCTGGATTT
81          V  A  R  P  D  V  K  N  V  H  N  V  Y  N  A  D  N  S  G  F

301        GATGTTAATGTCAATATTGACTTTAGCAAGATGAAAGATTATCGGGATTCAATTGAAATT
101         D  V  N  V  N  I  D  F  S  K  M  K  D  Y  R  D  S  I  E  I

361        GTTAGTCGATACAGTGGAAACGGTAAATCTGTTGACTGGTGGTCCCAACCGATCACTTTT
121         V  S  R  Y  S  G  N  G  K  S  V  D  W  W  S  Q  P  I  T  F

421        GACAAAAACAACTATGCTTATCTTGATACATTTGAAGTGAAAAATGGCGAATTACATGCA
141         D  K  N  N  Y  A  Y  L  D  T  F  E  V  K  N  G  E  L  H  A

481        ACCGGATGGAATGCTACTAATAGTGCGATTAACTATAATCACCATTTTGTGATTTTGTTT
161         T  G  W  N  A  T  N  S  A  I  N  Y  N  H  H  F  V  I  L  F

541        GATCAAACGAATGGTAAAGAAGTAGCACGACAAGAAGTTCGTGAAGGTCAATCACGCCCA
181         D  Q  T  N  G  K  E  V  A  R  Q  E  V  R  E  G  Q  S  R  P

601        GATGTTGCTAAGGTATATCCACAAGTAGTTGGTGCTGCCAACTCAGGCTTTAATGTGACA
201         D  V  A  K  V  Y  P  Q  V  V  G  A  A  N  S  G  F  N  V  T

661        TTTAATATCAGTGATTTAGATTATACTCACCAGTACCAAGTTCTTAGTCGTTACAGCAAT
221         F  N  I  S  D  L  D  Y  T  H  Q  Y  Q  V  L  S  R  Y  S  N

721        TCTGATAATGGCGAAGGTGATAACGTTACCTACTGGTTTAATCCACAATCCATTGCTCCT
241         S  D  N  G  E  G  D  N  V  T  Y  W  F  N  P  Q  S  I  A  P

781        GCTAATCAAAGTAACCAGGGTTATCTAGACTCATTTGATATTAGTAAAAATGGTGAAGTA
261         A  N  Q  S  N  Q  G  Y  L  D  S  F  D  I  S  K  N  G  E  V

841        ACAGTAACTGGATGGAACGCTACTGACTTGTCAGAATTACAAAACAACCATTATGTGATT
281         T  V  T  G  W  N  A  T  D  L  S  E  L  Q  N  N  H  Y  V  I

901        CTATTTGATCAGACAGCAGGCAAACAAGTTGCATCTGCTAAAGCTGATTTAATTTCACGT
301         L  F  D  Q  T  A  G  K  Q  V  A  S  A  K  A  D  L  I  S  R

961        CCAGATGTTGCTAAAGCTTATCCACAGTAAAAACAGCTACAAATTCTGGCTTCAAGGTAA
321         P  D  V  A  K  A  Y  P  Q  *

1021       CATTTAAGGTTAATAACTTACAACCGGGTCACCAATACAGCGTTGTAAGTCGTTTCTCTG

1081       CCGATGAAAATGGTAATGGTAATGATAAGCGCCATACAGATTACTGGTTTAGTCCAGTAA

1141       TATTAAACCAGACTGCTTCAAACATTGATACTATTACAATGACATCTAATGGTTTACATA

              rbs
1201       TTGCAGGTTGGATGGCAAGTGATAACTCAATTAATGAAACAACTCCATACGCTATTATCC
              GTFA→  M  A  S  D  N  S  I  N  E  T  T  P  Y  A  I  I

                     rbs
1261       TCAATAATGGAAAAGAAGTTACTCGTCAAAAGATGAGCTTAACCGCCCGTCCAGATGTAG
           L  N  N  G  K  E  V  T  R  Q  K  M  S  L  T  A  R  P  D  V
```

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1321    CAGCAGTATATCCTTCACTTTATAATAGTGCTGTTAGTGGTTTTGACACTACTATTAAAT
10      A  A  V  Y  P  S  L  Y  N  S  A  V  S  G  F  D  T  T  I  K

1381    TGACTAATGATCAATATCAAGCGCTTAATGGCCAATTACAAGTATTGTTACGTTTTTCTA
30      L  T  N  D  Q  Y  Q  A  L  N  G  Q  L  Q  V  L  L  R  F  S

1441    AAGCTGCTGATGGTAATCCAAGTGGTGATAATACTGTAACTGATCAATTTAGTAAAAATT
50      K  A  A  D  G  N  P  S  G  D  N  T  V  T  D  Q  F  S  K  N

1501    ATGCAACTACTGGTGGAAACTTTGACTATGTAAAAGTAAATGGTAATCAAGTTGAATTTA
70      Y  A  T  T  G  G  N  F  D  Y  V  K  V  N  G  N  Q  V  E  F

1561    GTGGTTGGCACGCAACTAACCAATCAAATGATAAAGATTCACAATGGATTATTGTTTTAG
90      S  G  W  H  A  T  N  Q  S  N  D  K  D  S  Q  W  I  I  V  L

1621    TTAATGGTAAGGAAGTAAAGCGTCAATTAGTTAATGATACTAAAGAGGGAGCTGCTGGCT
110     V  N  G  K  E  V  K  R  Q  L  V  N  D  T  K  E  G  A  A  G

1681    TCAACCGAAACGATGTCTACAAAGTAAATCCAGCTATTGAAAACAGTTCTATGTCTGGAT
130     F  N  R  N  D  V  Y  K  V  N  P  A  I  E  N  S  S  M  S  G

1741    TCCAAGGCATTATTACTTTACCTGTGACAGTTAAAAACGAAAATGTCCAACTTGTTCATC
150     F  Q  G  I  I  T  L  P  V  T  V  K  N  E  N  V  Q  L  V  H

1801    GGTTTAGTAACGATGTGAAGACTGGTGAAGGTAACTATGTTGATTTCTGGTCAGAGCTAA
170     R  F  S  N  D  V  K  T  G  E  G  N  Y  V  D  F  W  S  E  L

1861    TGCCTGTTAAGGATAGCTTCCAAAAGGGGAATGGCCCACTTAAGCAATTTGGCTTACAAA
190     M  P  V  K  D  S  F  Q  K  G  N  G  P  L  K  Q  F  G  L  Q

1921    CTATTAACGGTCAACAATATTATATTGACCCAACAACTGGTCAACCACGTAAGAATTTCT
210     T  I  N  G  Q  Q  Y  Y  I  D  P  T  T  G  Q  P  R  K  N  F

1981    TATTACAAAGTGGAAATAATTGGATTTACTTTGATAGTGATACTGGTGTGGGTACTAATG
230     L  L  Q  S  G  N  N  W  I  Y  F  D  S  D  T  G  V  G  T  N

2041    CACTTGAATTACAATTTGCAAAGGGAACTGTTTCATCTAATGAACAATACCGTAACGGTA
250     A  L  E  L  Q  F  A  K  G  T  V  S  S  N  E  Q  Y  R  N  G

2101    ATGCAGCTTACAGTTATGATGACAAGAGTATCGAAAATGTAAATGGTTACTTAACAGCAG
270     N  A  A  Y  S  Y  D  D  K  S  I  E  N  V  N  G  Y  L  T  A

2161    ATACATGGTACCGTCCAAAACAGATCTTAAAGGATGGAACTACCTGGACTGACTCAAAAG
290     D  T  W  Y  R  P  K  Q  I  L  K  D  G  T  T  W  T  D  S  K

2221    AAACAGATATGCGACCAATCTTGATGGTATGGTGGCCTAATACTCTTACCCAAGCATACT
310     E  T  D  M  R  P  I  L  M  V  W  W  P  N  T  L  T  Q  A  Y

2281    ACCTTAATTACATGAAACAACATGGTAATTTATTACCATCTGCTTTACCATTCTTTAATG
330     Y  L  N  Y  M  K  Q  H  G  N  L  L  P  S  A  L  P  F  F  N

2341    CGGATGCTGATCCTGCAGAATTAAATCATTATTCCGAAATTGTGCAACAAAATATTGAAA
350     A  D  A  D  P  A  E  L  N  H  Y  S  E  I  V  Q  Q  N  I  E

2401    AACGAATTAGTGAAACCGGAAATACTGATTGGTTACGTACTTTAATGCACGATTTTGTTA
370     K  R  I  S  E  T  G  N  T  D  W  L  R  T  L  M  H  D  F  V

2461    CTAACAATCCGATGTGGAATAAGGATAGTGAAAATGTTAACTTTAGTGGTATTCAATTCC
390     T  N  N  P  M  W  N  K  D  S  E  N  V  N  F  S  G  I  Q  F

2521    AAGGCGGATTCTTAAAGTATGAAAACTCAGATTTAACGCCTTATGCTAACTCTGATTATC
410     Q  G  G  F  L  K  Y  E  N  S  D  L  T  P  Y  A  N  S  D  Y
```

```
2581    GCTTACTTGGTCGGATGCCAATCAATATTAAGGATCAAACATATCGGGGACAAGAATTCC
430     R  L  L  G  R  M  P  I  N  I  K  D  Q  T  Y  R  G  Q  E  F

2641    TACTTGCTAACGATATTGATAACTCTAATCCTGTTGTTCAAGCAGAACAATTAAACTGGT
450     L  L  A  N  D  I  D  N  S  N  P  V  V  Q  A  E  Q  L  N  W

2701    TATACTATCTCTTGAACTTTGGAACGATCACAGCTAATAATGATCAAGCTAATTTTGATT
470     L  Y  Y  L  L  N  F  G  T  I  T  A  N  N  D  Q  A  N  F  D

2761    CTGTACGGGTAGATGCACCGGATAATATTGATGCCGATCTTATGAATATCGCTCAGGACT
490     S  V  R  V  D  A  P  D  N  I  D  A  D  L  M  N  I  A  Q  D

2821    ACTTTAATGCTGCATATGGTATGGACTCAGATGCTGTCTCAAATAAGCATATTAATATTC
510     Y  F  N  A  A  Y  G  M  D  S  D  A  V  S  N  K  H  I  N  I

2881    TTGAAGACTGGAATCATGCTGATCCGGAATACTTTAATAAGATCGGAAATCCACAATTGA
530     L  E  D  W  N  H  A  D  P  E  Y  F  N  K  I  G  N  P  Q  L

2941    CAATGGATGATACTATTAAGAATTCCCTGAATCATGGGCTTTCAGATGCAACTAATCGTT
550     T  M  D  D  T  I  K  N  S  L  N  H  G  L  S  D  A  T  N  R

3001    GGGGATTAGATGCAATTGTTCATCAGTCATTAGCTGATCGTGAAAATAATTCCACGGAAA
570     W  G  L  D  A  I  V  H  Q  S  L  A  D  R  E  N  N  S  T  E

3061    ATGTTGTAATTCCTAATTACAGTTTCGTTCGGGCTCACGATAATAATTCTCAAGATCAAA
590     N  V  V  I  P  N  Y  S  F  V  R  A  H  D  N  N  S  Q  D  Q

3121    TTCAAAATGCTATTCGTGATGTAACAGGCAAAGATTACCATACTTTCACTTTTGAAGATG
610     I  Q  N  A  I  R  D  V  T  G  K  D  Y  H  T  F  T  F  E  D

3181    AGCAAAAGGGTATTGATGCGTACATTCAAGATCAAAATTCAACAGTGAAGAAATATAACC
630     E  Q  K  G  I  D  A  Y  I  Q  D  Q  N  S  T  V  K  K  Y  N

3241    TTTATAATATTCCGGCTTCATACGCAATTCTTTTAACTAACAAGGATACAATTCCACGTG
650     L  Y  N  I  P  A  S  Y  A  I  L  L  T  N  K  D  T  I  P  R

3301    TATACTATGGTGACTTGTATACTGATGGTGGCCAATACATGGAACATCAAACACGTTACT
670     V  Y  Y  G  D  L  Y  T  D  G  G  Q  Y  M  E  H  Q  T  R  Y

3361    ATGATACTTTAACGAACCTGCTTAAATCACGAGTTAAGTATGTTGCCGGTGGCCAATCAA
690     Y  D  T  L  T  N  L  L  K  S  R  V  K  Y  V  A  G  G  Q  S

3421    TGCAAACAATGAGCGTTGGCGGCAATAATAACATTTTAACTAGTGTTCGTTATGGTAAAG
710     M  Q  T  M  S  V  G  G  N  N  N  I  L  T  S  V  R  Y  G  K

3481    GTGCGATGACAGCTACTGATACTGGTACTGATGAAACCAGAACACAAGGTATTGGGGTTG
730     G  A  M  T  A  T  D  T  G  T  D  E  T  R  T  Q  G  I  G  V

3541    TTGTAAGTAATACGCCAAATCTAAAGCTAGGTGTCAACGATAAAGTAGTTCTTCATATGG
750     V  V  S  N  T  P  N  L  K  L  G  V  N  D  K  V  V  L  H  M

3601    GAGCTGCGCACAAGAACCAACAATATCGGGCAGCCGTGTTGACGACAACTGATGGAGTCA
770     G  A  A  H  K  N  Q  Q  Y  R  A  A  V  L  T  T  T  D  G  V

3661    TTAATTATACTTCTGATCAAGGGGCACCGGTTGCAATGACTGACGAGAACGGTGATCTAT
790     I  N  Y  T  S  D  Q  G  A  P  V  A  M  T  D  E  N  G  D  L

3721    ACTTATCTAGTCATAACCTAGTTGTTAATGGTAAAGAAGAAGCAGATACAGCTGTTCAAG
810     Y  L  S  S  H  N  L  V  V  N  G  K  E  E  A  D  T  A  V  Q

3781    GTTATGCTAACCCTGATGTTTCAGGATATCTTGCTGTATGGGTACCAGTTGGAGCAAGTG
830     G  Y  A  N  P  D  V  S  G  Y  L  A  V  W  V  P  V  G  A  S
```

```
3841    ATAACCAAGATGCTCGAACTGCTCCATCTACTGAAAAGAATAGTGGTAACTCTGCATACA
850     D  N  Q  D  A  R  T  A  P  S  T  E  K  N  S  G  N  S  A  Y

3901    GAACAAATGCTGCTTTTGATTCAAATGTTATTTTTGAAGCCTTTTCTAACTTTGTCTATA
870     R  T  N  A  A  F  D  S  N  V  I  F  E  A  F  S  N  F  V  Y

3961    CACCAACAAAGGAAAGTGAACGTGCTAATGTTCGAATTGCCCAAAATGCTGATTTCTTTG
890     T  P  T  K  E  S  E  R  A  N  V  R  I  A  Q  N  A  D  F  F

4021    CTTCATTAGGTTTTACTTCTTTCGAGATGGCGCCACAATATAATTCAAGTAAAGATCGCA
910     A  S  L  G  F  T  S  F  E  M  A  P  Q  Y  N  S  S  K  D  R

4081    CATTCCTAGATTCAACAATTGATAACGGATATGCGTTTACTGATCGTTATGATCTTGGAA
930     T  F  L  D  S  T  I  D  N  G  Y  A  F  T  D  R  Y  D  L  G

4141    TGAGTGAGCCTAATAAGTACGGAACAGATGAAGATCTACGTAATGCCATTCAAGCGCTCC
950     M  S  E  P  N  K  Y  G  T  D  E  D  L  R  N  A  I  Q  A  L

4201    ATAAAGCTGGCTTACAAGTAATGGCGGATTGGGTTCCTGACCAAATCTATAACCTTCCTG
970     H  K  A  G  L  Q  V  M  A  D  W  V  P  D  Q  I  Y  N  L  P

4261    GAAAAGAAGTTGCTACAGTCACTCGAGTAGATGATCGTGGTAATGTATGGAAAGATGCTA
990     G  K  E  V  A  T  V  T  R  V  D  D  R  G  N  V  W  K  D  A

4321    TCATTAATAATAATCTGTATGTTGTTAATACTATTGGTGGTGGCGAATACCAGAAGAAGT
1010    I  I  N  N  N  L  Y  V  V  N  T  I  G  G  G  E  Y  Q  K  K

4381    ATGGTGGAGCATTCCTCGATAAGTTACAAAAACTTTATCCTGAAATCTTCACAAAGAAGC
1030    Y  G  G  A  F  L  D  K  L  Q  K  L  Y  P  E  I  F  T  K  K

4441    AAGTTTCAACTGGTGTTGCTATTGATCCTTCACAAAAGATAACTGAATGGTCAGCAAAAT
1050    Q  V  S  T  G  V  A  I  D  P  S  Q  K  I  T  E  W  S  A  K

4501    ACTTTAATGGAACAAACATTCTCCATCGTGGTTCTGGTTATGTACTAAAAGCTGATGGTG
1070    Y  F  N  G  T  N  I  L  H  R  G  S  G  Y  V  L  K  A  D  G

4561    GTCAATACTACAACTTAGGTACTACTACAAAGCAATTCTTGCCAATTCAATTAACTGGTG
1090    G  Q  Y  Y  N  L  G  T  T  T  K  Q  F  L  P  I  Q  L  T  G

4621    AAAAGAAACAAGGAAATGAAGGCTTTGTTAAGGGTAATGATGGAAATTACTACTTCTATG
1110    E  K  K  Q  G  N  E  G  F  V  K  G  N  D  G  N  Y  Y  F  Y

4681    ACTTAGCAGGTAATATGGTTAAGAATACCTTTATTGAAGATAGTGTTGGCAACTGGTACT
1130    D  L  A  G  N  M  V  K  N  T  F  I  E  D  S  V  G  N  W  Y

4741    TCTTTGACCAAGATGGTAAGATGGTTGAAAATAAACATTTCGTTGATGTTGATTCTTATG
1150    F  F  D  Q  D  G  K  M  V  E  N  K  H  F  V  D  V  D  S  Y

4801    GTGAAAAAGGTACTTACTTCTTCTTGAAGAATGGTGTATCATTCCGTGGGGGATTAGTGC
1170    G  E  K  G  T  Y  F  F  L  K  N  G  V  S  F  R  G  G  L  V

4861    AAACTGACAATGGTACTTATTACTTTGATAATTATGGAAAGATGGTACGTAATCAAACTA
1190    Q  T  D  N  G  T  Y  Y  F  D  N  Y  G  K  M  V  R  N  Q  T

4921    TTAATGCAGGTGCCATGATTTATACCTTAGATGAAAACGGTAAGCTTATAAAGGCTAGTT
1210    I  N  A  G  A  M  I  Y  T  L  D  E  N  G  K  L  I  K  A  S

4981    ATAATTCAGATGCCGAATATCCAACTTCAACTGATGTTGGTAAGATGCTTGATCAAAATA
1230    Y  N  S  D  A  E  Y  P  T  S  T  D  V  G  K  M  L  D  Q  N

5041    AACTATAATTAGCTGATTTCCGTTTCTTAGAATCGAAAGATTTAATAACTGGGGTTAAAA
1250    K  L  *
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```
5101    CGGCCCTACAAAATCTGATATTGATATAGAGATATTATTTCCTATATCAATATCAGATTT
                ---------------------->          <-------------------

5161    TTGCTTTTTATAAAATTGATTGTGACTAATAAGAATCCGGAAGATAACGTTGTTGTTATA

5221    TCAGTGGATTTAAGCAACATGAATTAATTGAAGATGACGGCAATGATTAAAAGTCGGTCT

5281    GATGATTATTGATGTATTACTAGTATTTGGTTTTTATCATTTATATTTTTACTGTTATTG

5341    GTGTCATATATTCCACAATAACAGTAAAGGTATATATGCTAGTTTATTTTTTAAGTAATT

5401    ATAATATTCTGATTATAATTTGGAAATATTCGCTTTTAGCAAAAAGGTAGTAAACAGATC

5461    AGAATCGTCATTCTGCTTTTCTACTACTAAAAGTCTGTTTTAAATTCTAAACTAAAATAG

5521    GCTAAACACTGATGTTTATCATTTATATTTTTACTGTT
```

Figure 3 Nucleotide sequence of a chromosomal DNA fragment of approximately 5.5 kbp of *Lb. reuteri* and deduced amino acid sequences of of ORF1 (GTFA) and part of the upstream ORF2. Putative start codons are shown in bold. Underlined nucleotides indicate the putative ribosomal binding sites (rbs). Inverted repeats (transcription termination) are depicted with arrows. YG repeats in the C-terminal region are shown in bold italics. *, stop codon.

Amino acid sequence comparison of *Lb. reuteri* GTFA and other glucansucrases

Alignment of the amino acid sequence of GTFA with other glucansucrases (i.e. streptococcal glucosyltransferases and *Leuconostoc* dextransucrases) using BLAST (Altschul et al. 1990), revealed a clear similarity. Also the various domains found in other glucansucrases were present in GTFA. The N-terminal variable region of 210 amino acid was succeeded by a catalytic domain of about 800 amino acids and a C-terminal glucan binding domain of about 260 amino acids.

Highest homology (46% identity, 59% similarity within 1261 amino acids) at the amino acid level was found with alternansucrase (ASR) from *Ln. mesenteroides* NRRL B-1355, encoding a protein of 2057 amino acids (Arguello-Morales et al. 2000). Dextransucrase DSRS of *Ln. mesenteroides* NRRL B-512F also showed high homology (44% identity, 58% similarity within 1270 amino acids) with GTFA (Wilke-Douglas et al. 1989). High similarities were also found between GTFA and GTFD from *S. mutans*, DSRB from *Ln. mesenteroides* and the glucosyltransferases GTFG from *S. gordonii* and GTFN from *S. salivarius*.

Within the central region (putative catalytic domain), high similarity levels (about 50% identity and 60% similarity) were found with other known streptococcal and *Leuconostoc* glucosyltransferases. However, not all of the

conserved amino acids found in the other glucosyltransferases (Monchois et al. 1999) were found in the glucansucrase of *Lb. reuteri*. Particularly in the region directly downstream of Asp^{494}, 3 out of 9 conserved amino acids were not found in GTFA (Figure 4). One of the conserved amino acid substitutions in this region of GTFA, Ile-499, was also found in amylosucrase, a glucosyltransferase from *Neisseria polysaccharea* synthesizing an α(1,4) glucan (Potocki De Montalk et al. 1999).

```
                •                                          ◇ ⇓  ▽▽
GTFD    LLANDIDNSNPVVQAEQLNWLHYLMNYGSIVANDPEANFDGVRVDAVDNVNADLLQIASD  480
DSRS    LLANDVDNSNVVVEAEQLNWLYYLMNFGTITANDADANFDGIRVDAVDNVDADLLQIAAD  576
ASR     LLANDIDNSNPIVQAEQLNWLHYLMNFGSITGNNDNANFDGIRVDAVDNVDADLLKIAGD  650
GTFA    LLANDIDNSNPVVQAEQLNWLYYLLNFGTITANNDQANFDSVRVDAPDNIDADLMNIAQD  509
        *****.****  .*.******* **.*.*.*   *    **** .**** **. ***. ** *
AS      262QWDLN266                                 .290IVRMDAVAFI298
                  ------H3------                        --E4-      -----H4--

                          ⇓▽▽▽
GTFD    YLKAHYGVDKSEKNAINHLSILEAWSDNDPQYNKDTKGAQLPIDNKLRLSLLYALTRPLE  540
DSRS    YFKLAYGVDQNDATANQHLSILEDWSHNDPLYVTDQGSNQLTMDDYVHTQLIWSLTK--S  634
ASR     YFKALYGTDKSDANANKHLSILEDWNGKDPQYVNQQGNAQLTMDYTVTSQFGNSLTHGAN  710
GTFA    YFNAAYGMD-SDAVSNKHINILEDWNHADPEYFNKIGNPQLTMDDTIK----NSLNHGLS  564
        *    ** *   .  .  *. *** *   ** *        ** .*   .       .*..
        ----                -E5--                ---H5----        ---E6--

GTFD    KDASNKNEIRSGLEPVITNSLN-----------------NRSAEGKNSERMANYIFIRA  582
DSRS    ---SD---IRGTMQRFVDYYMV-----------------DRSNDSTENEAIPNYSFVRA  660
ASR     N-RSN----MWYFLDTGYYLNGDLNKKIVDKNRPNSGTLVNRIANSGDTKVIPNYSFVRA  765
GTFA    D-ATN----RWGLDAIVHQS-----------------LADRENNSTENVVIPNYSFVRA  601
           .                                  *          . ** *.**
AS                                                        396FVRS
                       ------------H6-------------                --E7--

        ◆⇓
GTFD    HDSEVQTVIAKIIKAQINPKTDGLTFTLDELKQAFKIYNEDM--RQAKKKYTQSNIPTAY  640
DSRS    HDSEVQTVIAQIVSDLYPDVENSLAPTTEQLAAAFKVYNEDE--KLADKKYTQYNMASAY  718
ASR     HDYDAQDPIRKAMIDHGIIKNMQDTFTFDQLAQGMEFYYKDQENPSGFKKYNDYNLPSAY  825
GTFA    HDNNSQDQIQNAIRDVTGKD--YHTFTFEDEQKGIDAYIQDQ-N-STVKKYNLYNIPASY  657
        **    *  *    .            . * .        *  *        ***.  *. ..*
AS      HD401                                                    ----H7-

GTFD    ALMLSNKDSITRLYYGDMYSDDGQYMATKSPYYDAIDTLLKARIKYAAGGQDMKITYVEG  700
DSRS    AMLLTNKDTVPRVYYGDLYTDDGQYMATKSPYYDAINTLLKARVQYVAGGQ---------  769
ASR     AMLLTNKDTVPRVYYGDMYLEGGQYMEKGTIYNPVISALLKARIKYVSGGQTMATDSSGK  885
GTFA    AILLTNKDTIPRVYYGDLYTDGGQYMEHQTRYYDTLTNLLKSRVKYVAGGQSMQTMSVG-  716
        *..*.***.. *.****.*  . ****    . *    .  ***.*..* .***
AS              488GLPRIYLGD496
        H7-        --E8-                      -------H8------

GTFD    DKSHMDWDYTGVLTSVRYGTGANEATDQGSEATK----TQGMAVITSNNPSLKLNQNDKV  756
DSRS    ---SMSVDSNDVLTSVRYGKDAMTASDTGTSETR----TEGIGVIVSNNAELQLEDGHTV  822
ASR     DL---KDGETDLLTSVRFGKGIMTSDQTTTQDNSQDYKNQGIGVIVGNNPDLKLNNDKTI  942
GTFA    -------GNNNILTSVRYGKGAMTATDTGTDETR----TQGIGVVVSNTPNLKLGVNDKV  765
              . .*****.*      .    .  .      ..*. *.  *.  *.*      .
```

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GTFD    IVNMGAAHKNQEYRPLLLTTKDGLTSYTSDAAAKSLYRKTND----------K-GELVFD  805
DSRS    TLHMGAAHKNQAYRALLSTTADGLAYYDTDENAPVAYTDAN------------GDLIFT  869
ASR     TLHMGKAHKNQLYRALVLSNDSGIDVYDSDDKAPTLRTNDNGDLIFHKTNTFVKQDGTII 1002
GTFA    VLHMGAAHKNQQYRAAVLTTTDGVINYTSDQGAPVAMTDENGDLYLSSHNLVVNGK-EEA  824
          ..** ***** **  . ..  *.  * .*  *        *

GTFD    ASDIQGYLNPQVSGYLAVWVPVGASDNQDVRVAASNKANATG-QVYESSSALDSQLIYEG  864
DSRS    NESIYGVQNPQVSGYLAVWVPVGAQQDQDARTASDTTTNTSD-KVFHSNAALDSQVIYEG  928
ASR     NYEMKGSLNALISGYLGVWVPVGASDSQDARTVATESSSSNDGSVFHSNAALDSNVIYEG 1062
GTFA    DTAVQGYANPDVSGYLAVWVPVGASDNQDARTAPSTEKNSGN-SAYRTNAAFDSNVIFEA  883
           . *  *  .**** *******   ** *         .     . . .* **..*.*
                                                              -E1-

GTFD    FSNFQDFVTKDSDYTNKKIAQNVQLFKSWGVTSFEMAPQYVSSEDG-----SFLDSIIQN  919
DSRS    FSNFQAFATDSSEYTNVVIAQNADQFKQWGVTSFQLAPQYRSSTDT-----SFLDSIIQN  983
ASR     FSNFQAMPTSPEQSTNVVIATKANLFKELGITSFELAPQYRSSGDTNYGGMSFLDSFLNN 1122
GTFA    FSNFVYTPTKESERANVRIAQNADFFASLGFTSFEMAPQYNSSKDR-----TFLDSTIDN  938
        ****    *      .*  **     *    * ***..**** ** *       .**** . *
AS                                  134GLTYLHLMP142
                        ---H1--           --E2-

                                                          ♦
GTFD    GYAFEDRYDLAMSKN--N----KYGSQQDMINAVKALHKSGIQVIADWVPDQ  965
DSRS    GYAFTDRYDLGYGTP--T----KYGTADQLRDAIKALHASGIQAIADWVPDQ 1029
ASR     GYAFTDRYDLGFNKADGNPNPTKYGTDQDLRNAIEALHKNGMQAIADWVPDQ 1174
GTFA    GYAFTDRYDLGMSEP--N----KYGTDEDLRNAIQALHKAGLQVMADWVPDQ  984
        **** *****        .    ***.   .  *. ***  *.* .*******
AS                                                 190DFITNH195
                                  ------H2------   --E3--
```

Figure 4 Alignment of catalytic cores of DSRS of *L. mesenteroides* NRRL B-512F (Wilke-Douglas, 1989), GTFD of *S. mutans* GS5 (Fujiwara et al. 1998), ASR of *L. mesenteroides* NRRL B 1355 (Arguello-Morales et al. 2000), GTFA of *Lb. reuteri* and amylosucrase (AS) of N. polysaccharea (Potocki De Montalk et al. 1999).
*, identical or conserved residues in all sequences; --- gap in the sequence, **AA** amino acids which are conserved in all other glucosyltransferases, but not in GTFA. ⇓, putative catalytic residues; •, putative calcium binding site; ♦, putative residues stabilizing the transition state; ▽, residues possibly playing a role in binding of acceptor molecules and in the transfer of the glucosyl residue; ◇, putative chloride binding sites; **-Ex-**, localization of b-strands; **-Hx-**, localization of α-helices according to MacGregor et al. (1996). Adapted from Monchois et al. (1999).

Phylogenetic tree

As shown by the phylogenetic tree (Figure 5), constructed using the complete amino acid sequences of different glucosyltransferases, GTFA is distinct from the other glucosyltransferases. Again, GTFA is most closely related to the alternansucrase (ASR) of *Ln. mesenteroides* NRRL B-1355.

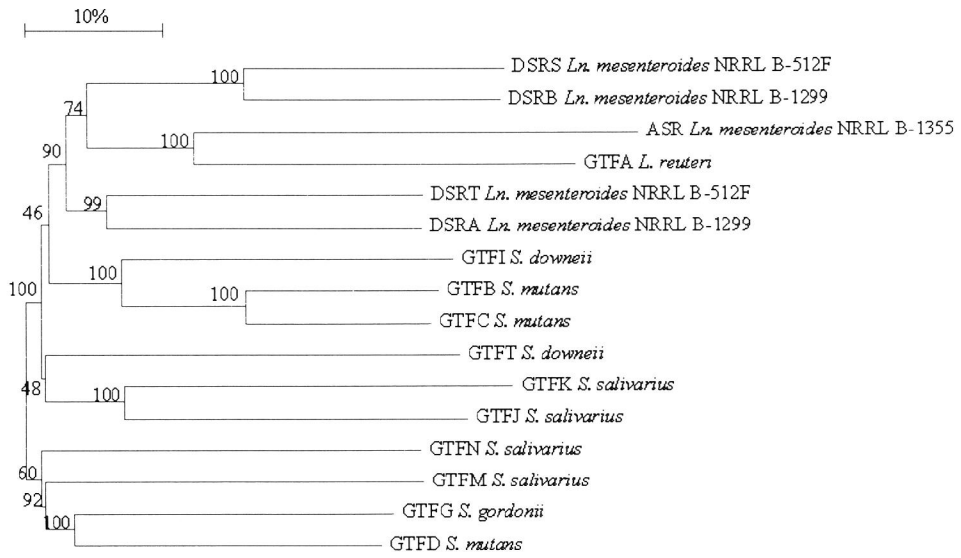

Figure 5 Dendrogram of glucansucrases of lactic acid bacteria, ASR (CAB65910), DSRA (JC5473), DSRB (AAB95453), DSRS (CAB69071), DSRT (BAA90527), GTFB (P08987), GTFC (BAA26110), GTFD (BAA26107), GTFG (AAC 43483), GTFI (BAA0296), GTFJ (CAA77900), GTFK (S22735), GTFM (AAC41413), GTFN (AAC05165), GTFS (P29336), GTFT (AB026123). The horizontal distances are a measure for the differences at the amino acid level. The length of the upper bar indicates 10% difference. Bootstrap values are given at the root of each branch (in percentages).

Expression of *Lb. reuteri gtfA* in *E. coli*

Different methods for constructing plasmids for expression of the *gtfA* gene in *E. coli* failed. Therefore, the approach illustrated in Figure 3 was followed. Transformation of *E. coli* with pBPB2700 resulted in only 13 transformants, of which 3 contained the correct plasmid, whereas transformation of pBXP1500 yielded 864 transformants, of which 294 contained the correct plasmid. Finally, 5 positive clones (out of 18) were found after transformation of *E. coli* with pBGTF. Correct construction of plasmids containing the complete *gtfA* gene was confirmed by digestion analysis of the positive clones with *Bgl*II, *Eco*RI, *Kpn*I, *Xho*I and *Hind*III and by sequence analysis. Sequence analysis also confirmed an in frame cloning of the *gtfA* gene with the start codon of the α-*lac*Z fragment of the β-galactosidase gene.

Analysis of the glucans produced by *Lb. reuteri* and *E. coli* containing pBGTF

Both *E. coli* (*gtfA*) cell free extracts and *Lb. reuteri* sucrose grown culture supernatant produced high molecular weight glucans. The average molecular weight of the glucans produced by GTFA of *Lb. reuteri* strain 35-5 was determined as 4.10^7 Da (±5%), whereas that of the glucans produced by *E. coli* GTFA was 8.10^7 Da (±5%).

As shown in Figure 6, the ^{1}H NMR spectra of the glucans produced by the GTFA present in *E. coli* and the GTFA present in *Lb. reuteri* strain 35-5, producing only the wild type glucan and not the fructan (Van Geel-Schutten et al 1999), were virtually identical. Comparison of both 1H NMR spectra with that of potato starch (Gidley 1985) shows that both glucans consist of α-(1→4) and α-(1→6) linked glucopyranosyl units. The identical nature of the glucans was confirmed by methylation analysis (Table 2).

Table 2 Methylation analysis of the glucans produced by *Lb. reuteri* strains and *E. coli* GTFA

Type of glycosyl units	*Lb. reuteri* 121	*Lb. reuteri* 35-5	*E. coli* GTFA
Glc*p*-(1→	24%	35%	21%
→4)-Glc*p*-(1→	42%	43%	44%
→6)-Glc*p*-(1→	22%	21%	24%
→4,6)-Glc*p*-(1→	12%	11%	11%

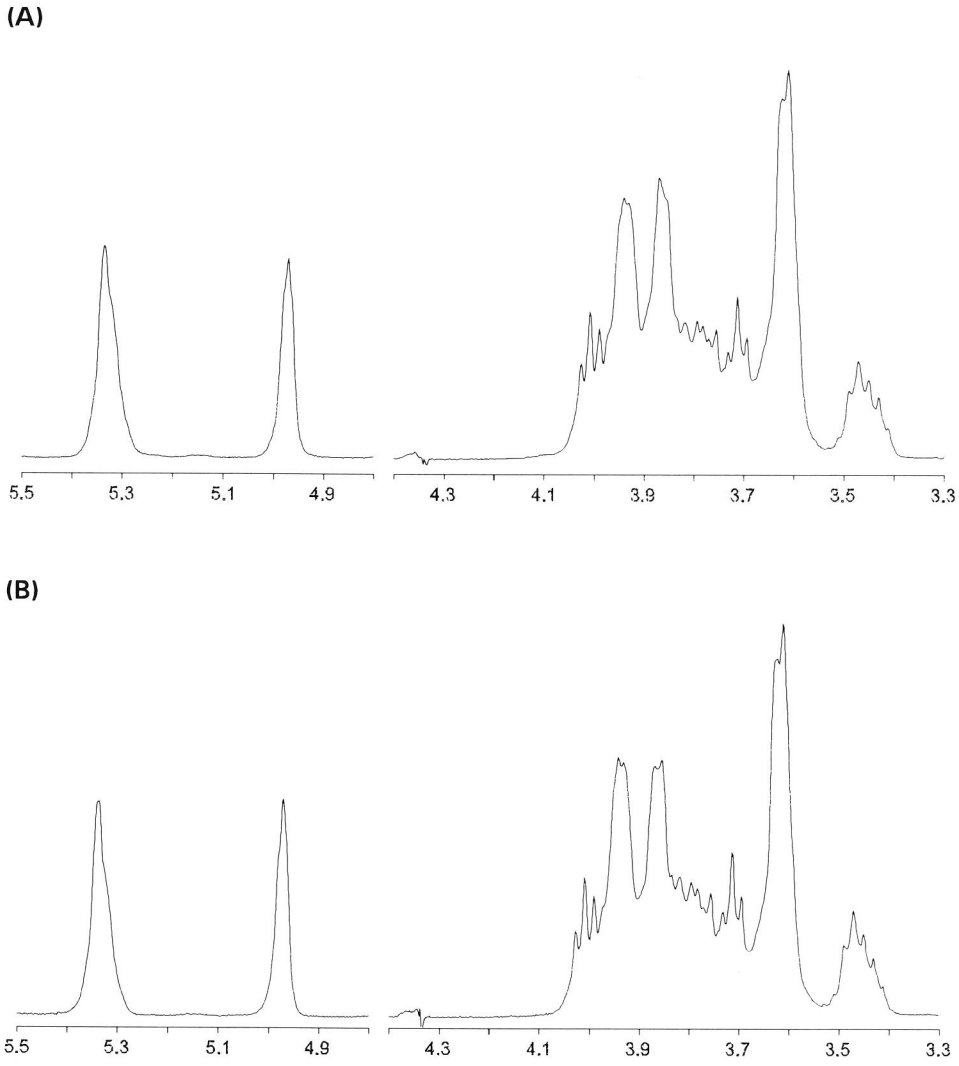

Figure 7 500-MHz ^{1}H-NMR spectra of the glucan produced by *Lb. reuteri* GTFA **(A)** and by *E. coli* **(B)**, recorded in D_2O at 80°C.

DISCUSSION

This paper reports the molecular characterization of the first *Lactobacillus* (*Lb. reuteri*) gene (*gtfA*) encoding a glucansucrase (GTFA), producing a glucan with a unique highly branched structure with α-(1→4) and α-(1→6) bonds. Cloning of *gtfA* in *E. coli* yielded an active glucansucrase, synthesizing the unique glucan. The N-terminal amino acid sequence of the glucansucrase protein is not available; 2 putative translation start positions and ribosomal binding sites (Shine-Dalgarno sequences) could be assigned. The distance between the first putative ribosomal binding site at position 1203 and its putative translation initiation codon is shorter than described for most other genes, whereas the distance between the putative ribosomal binding site at position 1274 and its putative translation initiation codon is larger (Figure 3). Spacers of both lengths, however, have been found previously in *Lactobacillus* strains (Pouwels and Leer 1993, Pouwels, P.H. and Leer, R.J., personal communication).

ORF2, upstream of *gtfA*, showed no significant homology with other sequences available in databases. No promoter sequences or transcription terminating inverted repeats were present between ORF2 and the *gtfA* gene; both genes could thus form part of an operon. Similar observations, an ORF with unknown function upstream from a glucansucrase encoding gene, have been made for *dsrA* in *Ln. mesenteroides* (Monchois et al. 1996). A regulatory role similar to that of the protein encoded by the *rgg* gene located upstream of the gtfG in *S. gordonii* (Sulavik et al. 1992) has been suggested for the product of the ORF upstream of *dsrA*.

The *Lb. reuteri* GTFA showed a high degree of amino acid similarity with other streptococcal and *Leuconostoc* glucosyltransferases. It consisted, like other glucosyltransferases, of a putative N-terminal variable domain, a putative catalytic domain and a putative C-terminal glucan binding domain. In contrast to most other glucosyltransferases, an N-terminal signal peptide could not be detected in GTFR, nor in DSRA from *Ln. mesenteroides* NRRL B-1299 (Monchois et al. 1996). Monchois et al. (1996) concluded that DSRA therefore is an intracellular enzyme. The glucansucrase of *Lb. reuteri*, however, clearly is an extracellular enzyme, or associated with the outside of the cell wall (Van Geel-Schutten et al. 1999). A possible explanation for the lack of an N-terminal signal sequence is that secretion of the glucansucrase protein relies on a signal sequence independent C-terminal secretion signal (Type I or Type III secretion system), as described for Gram-negative bacteria (Salmond and Reeves, 1993).

Based on the alignment with other glucosyltransferases from lactic acid bacteria and site directed mutagenesis studies (Monchois et al 1999a,

Devulapalle et al. 1997, MacGregor et al. 1996), putative catalytic residues in *Lb. reuteri* GTFA are Asp^{494}, Glu^{531} and Asp^{603}. The putative calcium binding site is Asp^{454}, the putative chloride binding site Arg^{492}. His^{602} and Gln^{984} may stabilize the transition state. The residues Asp^{497}, Asn^{498}, Asp^{532} and Trp^{533} may play a role in binding of acceptor molecules and in the transfer of the glucosyl residu. However, a fifth amino acid, Ser, possibly playing a role in acceptor binding or transferring the glucosyl residu, present in other glucosyltransferases (except for DSRA of *Ln. mesenteroides*), was replaced by Asn^{534} in the glucansucrase of *Lb. reuteri* (Figure 4).

The putative C-terminal glucan binding domain of the glucansurase of *Lb. reuteri*, consisting of 262 amino acids, is much shorter than the corresponding domains in streptococcal and *Leuconostoc* glucansucrases (about 500 amino acids). The C-terminal domain of streptococcal and *Leuconostoc* glucosyltransferases consist of a series of tandem repeats which have been divided into four classes: A, B, C and D repeats. These A, B, C and D repeat sequences exhibit high similarity to the repeats in the glucan binding protein from *S. mutans* as well as the ligand binding domains in *Clostridium difficile* toxin A and the lysins from *S. pneumoniae* (Giffard et al.1993). *Ln. mesenteroides* NRRL B-512F dextransucrase contains besides A and C repeats also N repeats, which have not been identified in streptococcal GTF's. These N repeats are not highly conserved (Monchois et al. 1998b). The A, B, C and D repeats present in distinct patterns in the C-terminal domain of other glucansucrases were not found in the glucansucrase of *Lb. reuteri*. However, three repeats, resembling YG repeats defined by Giffard and Jacques (1994) were found: YYFYDLAGNMVKN at position 1126, WYFFDQDGKMVEN at position 1148, and TYYFDNYGKMVRN at position 1195. These repeats could not be assigned as A, B, C, D or N repeats (Figure 3).

Highest overal homology of GTFA at the amino acid level was found with ASR from *Ln. mesenteroides* NRRL B-1355 (Arguello-Morales et al. 2000), responsible for the synthesis of an alternan with 50% α-(1→6) and 50% α-(1→3) linkages, and with DSRS from *Ln. mesenteroides* NRRL B-512F (Wilke-Douglas et al. 1989, Monchois et al. 1997), synthesizing a dextran with 95% α-(1→6) and 5% α-(1→3) linkages. Homology of GTFA with other glucansucrases was highest in the highly conserved putative catalytic domain, which had roughly the same size and structure as the corresponding domains of other glucosyltransferases. However, not all the conserved residues were found in the *Lb. reuteri* GTFA. Relatively many differences with amino acids conserved in other glucosyltransferases were found directly downstream of the putative catalytic Asp^{494} (Figure 4). This region constitutes the α/β barrel 4 of the enzymes of family 13 of glycoside hydrolases (MacGregor et al. 1996). The domain directly downstream of the catalytic Asp contains the

conserved amino acids Asp-Ala-Val-Asp-Asn in other glucansucrases. In CGTase these residues constitute part of the acceptor binding site (residues Asp^{229}-Ala-Val-Lys-His^{233}, in *Bacillus circulans* 251 CGTase), responsible for the stereospecific positioning of the molecule accepting the glucosyl unit. The structure of this acceptor site determines the type of glucosidic bond formed (Uitdehaag et al. 1999). In the corresponding region of GTFA, Pro^{496} is found in a position where a conserved Val is found in other glucosyltransferases (Figure 4). Compared with Val, the presence of Pro causes a more rigid protein structure. This could be of influence on the type of bonds being formed in the glucan synthesized by the enzyme and could therefore be part of the explanation for the unique structure of the glucan with α-(1→4) and α-(1→6) bonds, produced by GTFA. In amylosucrase, a glucosyltransferase synthesizing α-(1→4) bonds, the conserved Val is present. However, immediately downstream of this Val, the conserved Asp-Asn residues are replaced by Ala-Phe (Potocki De Montalk et al.1999). The following amino acid in amylosucrase is Ile, which is also present at that position in GTFA, whereas in other glucosyltransferases a conserved Val is found. This also suggests that the above mentioned region downstream of the catalytic Asp^{494}, may be of influence on the type of bonds being formed. Therefore, Pro^{496} and Ile^{499} of GTFA are likely targets for site directed mutagenesis experiments.

Different recombinant plasmids carrying the *gtfA* gene were constructed. However, attempts to introduce them in *E. coli* failed, in most cases probably due to rearrangements in the host. In order to investigate which part of the *gtfA* gene was responsible for these problems, two fragments of the gene were cloned separately in *E. coli*, using pBluescriptIISK+ (Figure 2). Although both recombinant plasmids were reproduced in the host, transformation of the construct harbouring the 3' end of the *gtfA* gene resulted in only 13 transformants, of which 3 harboured the recombinant plasmid. This, in combination with the cloning problems for sequencing purposes, suggests that the 3' end of *gtfA* contains DNA sequences which are responsible for these problems.

Of course, large pieces of DNA are more difficult to ligate and to clone than small ones, which provide an alternative explanation for the success of the used cloning strategy depicted in Figure 2. With the method described in Figure 2, eventually well defined ligation and transformation mixtures were obtained. This could result in a higher chance of finding the right transformants, compared with a one step cloning of the entire gene.

E. coli clones harbouring the pBGTF plasmid produced an active glucansucrase, as shown by the glucan production and SDS-PAGE followed by PAS activity staining. However, cell free extracts of the *E. coli* strain, harbouring the *gtfA* gene, showed 4 bands of activity on SDS-PAGE gels,

whereas only one band could be detected in culture supernatants of *Lb. reuteri*. The four activity bands in *E. coli* extracts could be the result of proteolytic activity. This was also observed for the *gtfI* gene of *S. downei* cloned in *E. coli* (Ferreti et al., 1987) and for DSRS of *Ln. mesenteroides* NRRL B-512F when expressed in *E. coli* (Monchois et al. 1997). Deletions at the C-terminal part of the GTFA protein do not necessarily result in inactive proteins: deletion of the C-terminal part of other glucosyl-transferases, such as the DSRS of *Ln. mesenteroides*, did result in a decreased initial reaction velocity, but glucan synthesis was still observed, also in SDS-PAGE gels (Monchois et al. 1998b). The protein band of the recombinant GTFA with the lowest mobility corresponded to a molecular weight of 165 kDa, whereas the molecular weight of the enzyme of *Lb. reuteri* is 180 kDa.The molecular weight predicted from the deduced amino acid sequence of the GTFA cloned in *E. coli* was 145 kDa, 20 kDa lower than the largest protein band with glucansucrase activity. The molecular weight of the wild type enzyme was also higher than that predicted from the deduced amino acid sequence of GTFA. This apparently higher molecular weight was not caused by the presence of glucans, since the control gel, incubated with buffer without sucrose, did not show any PAS activity bands (Van Geel-Schutten et al. 1999). During growth on glucose the GTFA was bound to the cell wall, whereas during growth on sucrose part of the enzyme was released into the culture medium. Possibly some kind of membrane anchor is still linked to the enzyme, causing a different mobility in the gel, resulting in an apparently higher molecular weight after SDS-PAGE.

Attempts to confirm by gene disruption that the cloned *gtfA* gene was the gene encoding the active glucansucrase in *Lb. reuteri* failed. Different integration vectors were constructed and used to transform *Lb. reuteri*, but they all integrated in a random manner in the chromosomal DNA of *Lb. reuteri* (data not shown). However, ^{1}H-NMR spectra of the glucans produced by the *Lb. reuteri* and by the *E. coli* GTFA were virtually identical (Figure 6). This, combined with the results of the methylation analysis (Table 2), and the molecular weight determinations of the glucans, shows that the *E. coli* GTFA and the *Lb. reuteri* enzymes synthesize the same glucan with a unique structure: a highly brached glucan with α-(1$\rightarrow$4) and α-(1$\rightarrow$6) bonds. Because of this unique structure it is concluded that the active glucansucrase of *Lb. reuteri* has been characterized.

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Summary

Lactic acid bacteria play important roles in the fermentative production of food and feed. Traditionally, they have been used for the production of for instance wine, beer, bread, cheese and yogurt, and for the preservation of food and feed, e.g. olives, pickles, sausages, sauerkraut and silage. Because of these traditional applications, lactic acid bacteria are food-grade microorganisms that posses the GRAS (Generally Recognized As Safe) status. Due to the various products which are formed during fermentation with lactic acid bacteria, these bacteria contribute positively to the taste, smell and preservation of the final product. In recent years also the health promoting properties of lactic acid bacteria have received much attention.

Lactic acid bacteria are known to produce an abundant variety of exopolysaccharides (EPSs), which contribute to the texture and mouthfeel of dairy products such as yogurt. In addition, it has been suggested that these polysaccharides are bio-active carbohydrates and contribute to human health by acting as prebiotic substrates, nutraceuticals, cholesterol lowering agents or immunomodulants. Because of their GRAS status, EPS of lactic acid bacteria may provide a new generation of food-grade ingredients. Polysaccharides find numerous industrial applications, for instance as viscosifying, stabilizing, emulsifying, gelling, or water binding agents. The polysaccharides presently used for these purposes in the food industry, produced by plants (cellulose, pectin, starch), seaweeds (alginate and carrageenan), and bacteria (alginate, gellan, xanthan), are additives and therefore considered to be less desirable.

The properties, and thus the application potential of EPSs, are dependent on for instance the monosaccharide composition, type of linkages, the degree of branching, the molecular weight, and the presence of charged groups. Based on the monosaccharide composition and their biosynthesis pathways, EPSs produced by lactic acid bacteria can be divided into two groups, heteropolysaccharides and homopolysaccharides.

The first group of EPSs consist of repeating units in which residues of different types of sugars are present. Sometimes charged groups like acetate, phosphate or glycerolphospate are also present.
Heteropolysaccharides are synthesized at the cytoplasmic membrane, utilizing sugar nucleotides as precursor. Different intracellular glycosyltransferases and isoprenoid glycosyl carrier lipids are involved in the biosynthesis. The genes encoding the different enzymes responsible for the heteropolysaccharide synthesis are located on large *eps* gene clusters.

Homopolysaccharides are constituted out of one type of monosaccharide and are often synthesized by a single extracellular enzyme, a sucrase. Homopolysaccharides produced by lactic acid bacteria can roughly be divided into two groups, the fructans, with fructose units, and the glucans, with glucose units. The most commonly found fructans produced by lactic acid bacteria are levan and inulin, synthesized by levansucrase and inulosucrase respectively. In levan, the fructosyl units are linked through β(2→6) bonds, sometimes with branches at the C1 position. The most commonly found glucan in lactic acid bacteria is dextran, in which the backbone consist of α-(1→6) linked glucosyl residues with branching point at the C1, C2 or C4 position. Other examples are alternan (with alternating α-(1→6) and α-(1→3) linkages) and mutan (with α-(1→3) linkages).

The introduction of this thesis (chapter 1) provides an overview of lactic acid bacteria, their carbohydrate metabolism and EPS synthesis, with emphasis on lactobacilli. Special attention is given to the characteristics of *Lactobacillus reuteri*, a probiotic strain with antimicrobial properties, selected for the research described in this thesis (see chapter 2). Examples of heteropolysaccharide synthesis and of homopolysaccharide (mainly fructan and glucan) synthesis by lactic acid bacteria are described. Also the characteristics and common structure of glucansucrase enzymes (glucosyltransferases) of lactic acid bacteria, and a proposed mechanism for glucan synthesis, are described in more detail. Glucansucrases are very versatile enzymes. Sometimes different types of linkages are synthesized by the enzymes: each glucansucrase specifically synthesizes one ore more types of glucosidic bonds. Besides the synthesis of glucans, glucansucrases also are able to hydrolyse sucrose. In addition oligosaccharides are synthesized in the presence of suitable acceptors. Compared to glucansucrases of lactic acid bacteria, little is known of fructan synthesizing sucrases (fructosyltransferases).

Chapter two describes the screening of 182 *Lactobacillus* strains for the production of EPS, using a new method: the strains were grown in liquid media with an excess of different sugars as energy source. EPS produced by the different strains in the different media was collected by ethanol precipitation and the monosaccharide composition was determined. The ability

to produce EPS appeared to be widespread in lactobacilli: sixty EPS producing strains were identified, of which 17 produced more than 100 mg/l. EPS production of two strains, LB 180 and LB 121, identified as *Lb. reuteri* strains, both producing 10-20 g/l of soluble EPS, was studied in more detail. EPS synthesis by both *Lb. reuteri* strains was different. Strain LB 180 produced only a glucan, whereas strain LB 121 produced a fructan as well as a glucan during growth on sucrose. EPS of strain LB 121 was produced almost exclusively during the exponential growth phase, whereas glucan production by strain LB 180 continued in the stationary phase of growth. Almost all the glucan synthesizing enzyme activity of strain LB 180 was found in the culture supernatant during growth on sucrose, whereas in strain LB 121 a substantial part of the EPS synthesizing enzyme activity occurred in a cell-associated form.

Heteropolysaccharide synthesis has been studied intensively in lactobacilli in recent years, with only few studies focussing on the production of homopolysaccharides. Fructan synthesis by strain LB 121 is the first example of fructan synthesis by a *Lactobacillus* species. Therefore, and because of the synthesis of high amounts of two types of homopolysaccharides, strain LB 121 was selected for further research.

Chapter 3 describes the characterization of the glucan and fructan produced by *Lb. reuteri* 121. Methylation and NMR studies identified the fructan as a linear levan with β-(2→6) linkages. The glucan was highly branched with a unique structure consisting of terminal, 4-substituted, 6-substituted, and 4,6-disubstituted α-glucose in a molar ratio of 1.1:2.7:1.5:1.0. The molecular weight of the glucan, estimated as 3,500 kDa, was much higher than that of the fructan (approximately 150 kDa). The enzymes responsible for the synthesis of these polymers occurred in a cell-free and cell-associated form after growth on sucrose or raffinose. However, during growth on glucose or maltose they remained completely cell-associated.

The EPS synthesis of *Lb. reuteri* was not a stable characteristic in continuous cultures. Different spontaneous mutants of *Lb. reuteri* 121 appeared in continuous cultures, such as the EPS-negative mutant strain K-24, lacking both the glucansucrase and levansucrase. Strain 35-5 type of mutants accumulated in continuous cultures following a pH shift down. This mutant still produced the wild type glucan but had lost its ability to produce levan. The mutants obtained from these continuous culture experiments were stable mutants. The glucansucrase enzymes in supernatants of *Lb. reuteri* 121 and 35-5 cultures were at least partially resistant to SDS and incubation at high temperatures and their activity could be detected in SDS-PAGE gels, stained with a PAS (Periodic acid Schiff) activity staining. Levan synthesis, and thus levansucrase activity, could not be detected by this method.

The isolation and characterization of a fructosyltransferase gene of *Lb. reuteri* 121 is described in Chapter 4. The gene (*ftfA*) was located on chromosomal DNA and isolated using PCR techniques. The gene was obtained using PCR and inverse PCR techniques and characterized. It consisted of an ORF of approximately 2400 bp, encoding putative a protein of 798 amino acids with a deduced molecular weight of about 86 kDa. Two putative translation start codons with corresponding ribosomal binding sites could be identified. The presence of a putative signal sequence at the N-terminal end of the protein confirmed the extracellular nature of the enzyme. FTFA contains a putative N-terminal secretion signal peptide, suggesting that the enzyme would be secreted. The C-terminal amino acid sequence of FTFA contains a cell wall anchoring peptide sequence LPXTG, plus a 20-fold repeat of the motif PXX, where P is proline and X is any other amino acid. The presence of these PXX repeats has not been reported before in proteins from either prokaryotic or eukaryotic origin.

The deduced amino acid sequence of FTFA showed high similarity with other bacterial fructosyltransferases. Highest homology was found with the fructosyltransferase SacB of *Streptococcus mutans*. Based on the alignments with other fructosyltransferases a phylogenetic tree was constructed and revealed that three distinctive fructosyltransferase groups could be identified: Gram positive, Gram-negative and plant originating fructosyltransferases.

The *ftfA* gene was successfully expressed in *Escherichia coli* Top10, yielding an active FTFA enzyme, producing an inulin and fructo-oligosaccharides, DP3-4. The deduced N-terminal and internal amino acid sequences of *ftfA* were different from those of a levansucrase (FTFB) purified from supernatants of *Lb. reuteri* 121, synthesizing a levan. It was therefore concluded that *Lb. reuteri* contains at least two fructosyltransferase genes, one encoding the levansucrase purified from supernatants and the one described in Chapter 4 (*ftfA*). This is the first example of the isolation and characterization of a fructosyltransferase gene from a *Lactobacillus* species.

The glucansucrase gene (*gtfA*) of *Lb. reuteri* was isolated in the same way as the *ftfA* gene. Because of severe problems with cloning of parts of the isolated fragment, different approaches were used for the determination of the nucleotide sequence. The sequenced fragment consisted of an ORF encoding a glucansucrase (GTFA) and an upstream ORF with unknown function. The glucansucrase ORF had a size of approximately 4,100 bp, encoding a protein with a molecular weight of about 140,000 Da. The exact size of the gene and of the encoding protein remains to be determined because there were two possible initiation start positions with corresponding ribosomal binding sites. Based on the deduced amino acid sequence, the GTFA showed high homology

with other glucosyltransferase genes from lactic acid bacteria. Like these other glucosyltransferases, it contained a putative N-terminal variable domain, a putative catalytic or sucrose binding domain, and a putative C-terminal glucan binding domain. However, the putative structure of GTFA deviated from the common structure of other glucosyltransferases. First, an N-terminal signal sequence could not be detected, and secondly, the putative glucan binding domain was much shorter than reported for other glucosyltransferases. Highest homology was found with ASR of *Ln. mesenteroides*.

Glucansucrases are closely related to glucosylhydrolase enzymes of family 13. The catalytic domains of glucosyltransferases, containing conserved regions, appear to posses a similar, although circularly permuted, $(\beta/\alpha)_8$ barrel structure, as the glucosylhydrolases enzymes of family 13. Not all the conserved amino acids, however, were found in GTFA. In the regions downstream of the catalytic Asp^{494}, 3 out of 9 of the amino acids, conserved in other glycosyltransferases, were not found in GTFA. Residues in this region may be involved in binding and stereospecific positioning of the acceptor molecule.

Different attempts to clone the *gtfA* gene in *Escherichia coli* DH5α using different vectors failed, probably due to rearrangement reactions of the host. Therefore, a two step cloning strategy was used. Cell free extracts of the recombinant strain produced an active glucansucrase. The recombinant enzyme synthesized the same unique glucan as the glucan produced by *Lb. reuteri*. It can thus be concluded that *gtfA* encodes the active novel glucansucrase of *Lb. reuteri*. This isolation, characterization and cloning of the *gtfA* gene of *Lb. reuteri* is described in Chapter 5. This is the first example of the isolation, characterization and cloning of a *Lactobacillus* glucosyltransferase.

Suggestions for future research

The results obtained by screening a large collection of *Lactobacillus* strains indicate that, although scarcely described, EPS production is widespread in the genus. Probably in the past the conditions used for screening were suboptimal, especially for the identification of homopolysaccharide producing strains. The strain selected for the research described in this thesis is classified as *Lb. reuteri*, an enterolactobacillus, known as an excellent colonizer of the gut. This strain was, together with some other strains, tested because of its good adhering properties to mucosal cells. Although the availability of sucrose in vivo (i.e. in the gastro-intestinal tract) is questionable, it is tempting to speculate that EPS produced by the strain is of influence on the adherence of the bacterial cells. This hypothesis can be easily verified, for instance by testing the adherence of the wild type and the mutant strains of *Lb. reuteri* 121 to mucosal cells. In fact, even the contribution of the produced glucan and levan to the adherence can be evaluated because mutant K-24 is an EPS- mutant, whereas strain 35-5 is only capable of producing the glucan.

Lb. reuteri is not known to produce EPS. However, the two strains selected for the research described in Chapter 2 of this thesis were both identified as *Lb. reuteri* strains. Perhaps EPS production is also widespread amongst the different *Lb. reuteri* strains. This can be tested using the method described in Chapter 2 of this thesis or by amplification of fragments of the glucan and levansucrase encoding genes, using PCR techniques with primers based on conserved regions of the genes.

Lb. reuteri possesses at least two fructosyltransferase genes. Cloning and expression of one of the genes, *ftfA*, in *E. coli* resulted in a recombinant enzyme synthesizing a fructan with β-(2→1) linkages and fructo-oligosaccharides, whereas another protein (FTFB) which is secreted in the culture medium of *Lb. reuteri* produces a high molecular weight levan. To compare both enzymes at the molecular level, cloning and sequencing of the levan synthesizing sucrase are necessary, as well as an extended characterization of the 5' of the *ftfA* gene. Crystallization studies of the purified enzymes, combined with site directed mutagenesis studies, will provide more information about different aspects of the catalysis of the fructan synthesizing enzymes (for instance polymer versus oligosaccharide synthesis, sucrose hydrolysing activity, determination of the linkage type (β-(2→1) versus β-(2→6)) and determination of the chain length of the synthesized fructans). To obtain sufficient enzyme for biochemical characterization and crystallization studies overproduction will be necessary.

The recombinant *E. coli* FTFA protein probably still possesses a signal peptide and is not secreted in the culture medium. To determine the exact position of the cleavage site of the signal sequence, the gene could be cloned in a *Lactobacillus* strain. Strains of this genus probably recognize the signal sequence and export the enzyme to the culture medium. This could also overcome the severe cloning problems observed with cloning of the *ftfA* in *E. coli* Furthermore, isolation of mRNA of *Lb. reuteri* could provide additional information about the expression and physiological roles of the levansucrases. The isolation and analysis of mRNA could also provide valuable information about the glucansucrase of *Lb. reuteri*, such as the exact start position and the regulation of the expression of the gene.

The purification of the glucansucrase of *Lb. reuteri* was, amongst others, hampered by the low amounts of free enzyme in the supernatants of sucrose grown cells. The results of the N-terminal sequence analysis indicated that the very faint band obtained after SDS-PAGE gel electrophoresis was probably contaminated with another protein of unknown function. Concentration of the supernatants containing the enzyme, combined with 2D-gelelectrophoresis might result in sufficient purified enzyme for the determination of the N-terminal amino acid sequence. Translation of this sequence in a nucleotide sequence could result in a correct assignment of the start position of the isolated *gtfA* gene. For characterization studies and for comparison with the recombinant GTFA, purification of larger amounts of the active glucansucrase of *Lb. reuteri* are necessary, as well as purification of the recombinant GTFA.

The precise mechanism of catalysis of glucansucrases is unknown and there are no structural data available. However, structure/function relationships can be studied using mutant enzymes obtained by random mutagenesis or by site-directed mutagenesis. Based on comparison with enzymes from family 13 of the glucosylhydrolases, it can be speculated that the structure of the region downstream the catalytic Asp^{494} is of importance in the determination of the type of bonds being synthesized. Because the glucans synthesized by GTFA differ in type of linkages from other glucosyltransferases, amino acids in this region of GTFA that differ from the conserved amino acids of other glucosyltransferases would be candidates for site-directed mutagenesis experiments.

Compared to homopolysaccharides, heteropolysaccharides are produced in very low amounts by lactic acid bacteria. However, the physical properties, in particular the viscosifying properties, of heteropolysaccharides are often more favourable for different applications. A possibility to obtain large amounts of polysaccharides with improved properties is the (enzymatic) modification of homopolysaccharides. Examples of modification are oxidation,

acetylation or phosphorylation. Furthermore the use of sucrases to obtain hybrid polymers by using different polysaccharides as acceptor and sucrose as fructosyl- or glucosyl donor, creates possibilities for the development of a new class of polysaccharides.

Samenvatting

Melkzuurbacteriën zijn nuttige bacteriën die een belangrijke rol vervullen in de productie van voeding en veevoer. Ze worden van oudsher gebruikt bij de bereiding van bijvoorbeeld wijn, bier, brood, kaas en yoghurt en voor de verlenging van de houdbaarheid van voedingsmiddelen zoals olijven, augurken en zuurkool. Ook bij het inkuilen van gras dragen de melkzuurbacteriën bij aan de conservering van het gras. Vanwege dit traditionele gebruik, worden melkzuurbacteriën beschouwd als veilige GRAS (Generally Recognized As Safe) organismen die in voedingsmiddelen gebruikt mogen worden. Tijdens het fermentatieproces met melkzuurbacteriën ontstaan naast melkzuur, dat een conserverende werking heeft, ook verschillende andere producten. Hierdoor dragen de melkzuurbacteriën vaak ook bij aan de uiteindelijke geur, smaak, en het mondgevoel van het eindproduct.

Melkzuurbacteriën staan de laatste jaren in de belangstelling vanwege hun gezondheidsbevorderende eigenschappen. Zij zijn een onderdeel van de gezonde microflora in de darmen van de mens en van verschillende dieren en helpen zo ziektes te voorkomen. Bepaalde melkzuurbacteriën hebben probiotische eigenschappen, d.w.z. dat wanneer zij, bijvoorbeeld via voedsel, worden toegediend in een levende vorm, bijdragen aan de gezondheid doordat ze de microflora in de darmen verbeteren. Gezondheidsbevorderende eigenschappen die worden toegeschreven aan melkzuurbacteriën zijn bijvoorbeeld het remmen van de groei van ziekte verwekkende micro-organismen, het verhogen van de immuunrespons, het verlagen van het cholesterolgehalte, en het voorkomen van bepaalde vormen van kanker.

Polysachariden zijn lange suikerketens die in de industrie voor verschillende doeleinden worden gebruikt, bijvoorbeeld als verdikkingsmiddelen, stabilisatoren, emulgatoren, geleermiddelen of om water te binden. De polysachariden die thans in de levensmiddelen industrie worden gebruikt, zijn afkomstig van planten (bijvoorbeeld cellulose, pectine en zetmeel), zeewieren (bijvoorbeeld alginaat en carrageen) of bacteriën (bijvoorbeeld alginaat, gellan en xanthaan). Deze polysachariden worden echter beschouwd als additieven en worden daarom door een groot aantal consumenten niet op prijs gesteld. Melkzuurbacteriën zijn in staat om een grote variatie aan exopolysachariden (EPSen) te synthetiseren. EPSen zijn lange suikerketens die door de bacterie aan de buitenkant van de cel gemaakt worden. De EPSen van melkzuurbacteriën kunnen, net als de melkzuurbacterie zelf, voor de mens nuttige eigenschappen hebben. Zo zijn deze EPSen bijvoorbeeld verantwoordelijk voor het mondgevoel en het dik worden van zuivelproducten zoals yoghurt. Daarnaast wordt gesuggereerd dat bepaalde

EPSen bio-actieve eigenschappen hebben en kunnen bijdragen aan de gezondheid door hun prebiotische (d.w.z. het stimuleren van de groei van gezonde bacteriën in de darmflora), cholesterol verlagende of immunomodulerende werking. Vanwege de hierboven genoemde GRAS status van de melkzuurbacteriën zouden de door hen gevormde EPSen toegepast kunnen worden als nieuwe, food-grade (functionele) polysachariden o.a. ter vervanging van de hierboven genoemde polysachariden.

De eigenschappen en toepassingsmogelijkheden van EPSen zijn afhankelijk van bijvoorbeeld de suikersamenstelling, de aanwezige bindingstypen, de mate van vertakking, het molecuulgewicht en de aanwezigheid van geladen groepen. De door melkzuurbacteriën geproduceerde EPSen kunnen op grond van hun suiker samenstelling en hun biosynthetische route worden onderverdeeld in twee groepen, de heteropolysachariden en de homopolysachariden.

Heteropolysachariden bestaan uit repeterende eenheden die zijn opgebouwd uit verschillende suikereenheden. Soms zijn geladen groepen zoals acetaat, fosfaat of glycerolfosfaat aanwezig. Dit type EPS wordt gesynthetiseerd aan de cytoplasma membraan van de cel, waarbij geactiveerde nucleotidesuikers als precursor dienen. Verschillende enzymen, zoals glycosyltransferases, zijn betrokken bij de biosynthese. Daarnaast spelen bepaalde componenten uit de bacteriële cytoplasma membraan, de isoprenoid glycosyl carier lipiden, een rol bij de synthese van heteropolysachariden. Op DNA niveau zijn de genen die coderen voor de enzymen die betrokken zijn bij de biosynthese van heteropolysachariden gegroepeerd als grote *eps* gen clusters. Deze clusters kunnen zowel op het chromosomaal als op het plasmied DNA van de melkzuurbacteriën liggen.

Homopolysachariden zijn opgebouwd uit één type suiker en worden meestal door één enzym, een sucrase, buiten de cel gesynthetiseerd. De homopolysachariden die door melkzuurbacteriën worden gesynthetiseerd, kunnen worden onderverdeeld in twee groepen: de fructanen, opgebouwd uit fructose eenheden, gesynthetiseerd door levan- en inulosucrases (ook wel fructosyltranferases genoemd), en de glucanen, opgebouwd uit glucose eenheden, gesynthetiseerd door glucansucrases (ook wel glucosyltransferases genoemd). Levan, met β-(2→6) bindingen tussen de fructose eenheden, is een voorbeeld van een fructan dat wordt geproduceerd door melkzuurbacteriën. Een ander voorbeeld van een fructan dat door melkzuurbacteriën wordt gevormd is inuline, met β-(2→1) bindingen tussen de fructose eenheden. Het meest voorkomende glucan, geproduceerd door melkzuurbacteriën is dextran, dat bestaat uit een hoofdketen van α-(1→6) gebonden glucosyleenheden met vertakkingen op de C1, C2 of C4 positie van de glucose eenheden in de hoofdketen. Andere glucanen die door melkzuurbacteriën worden gevormd zijn

alternan, opgebouwd uit glucose eenheden die alternerend α-(1→6) en α-(1→3) zijn gebonden en mutan met α-(1→3) gebonden glucosyl eenheden.

In de inleiding van dit proefschrift (hoofdstuk 1) wordt een overzicht gegeven van melkzuurbacteriën, hun suiker metabolisme en hun EPS synthese, waarbij de nadruk wordt gelegd op lactobacillen. Er wordt extra aandacht geschonken aan *Lactobacillus reuteri*, een probiotische stam die in het maagdarmkanaal van alle tot nu toe onderzochte gastheren, inclusief de mens, in grote aantallen voorkomt. Deze stam is geselecteerd voor het onderzoek dat in dit proefschrift wordt beschreven (zie hoofdstuk 2). Er worden voorbeelden gegeven van de synthese van heteropolysachariden en homopolysacharide (met name fructan en glucan) door melkzuurbacteriën. Daarnaast worden de eigenschappen, de gemeenschappelijke structuur van glucansucrase enzymen uit melkzuurbacteriën en een hypothetisch mechanisme voor de synthese van de glucanen behandeld.

Glucansucrases zijn veelzijdige enzymen die uitsluitend sucrose nodig hebben voor de synthese van de glucanen. In sommige gevallen worden verschillende bindingstypen tussen de glucose eenheden gesynthetiseerd; elk glucansucrase is echter specifiek in de synthese van een of meerdere bindingstypen. Naast de synthese van glucanen, zijn de glucansucrases ook in staat om sucrose te hydrolyseren en om oligosachariden (korte suikerketens) te synthetiseren. Deze oligosachariden worden geproduceerd wanneer naast het substraat sucrose, ook geschikte acceptoren voor de glucose eenheden, zoals bijvoorbeeld maltose, aanwezig zijn.

Ook aan de fructosyltransferases, in het bijzonder de levansucrases uit melkzuurbacteriën wordt aandacht besteed in de inleiding; over deze enzymen is echter veel minder bekend dan over de glucansucrases.

In hoofdstuk 2 wordt de screening van 182 *Lactobacillus* stammen met behulp van een nieuwe methode beschreven. Voor deze screening werden de stammen opgekweekt in vloeibare media waaraan een overmaat van verschillende suikers was toegevoegd. EPS productie werd vastgesteld door het gevormde EPS neer te slaan met ethanol, waarna vervolgens de suikersamenstelling van het neergeslagen materiaal werd bepaald. Hoewel EPS productie door *Lactobacillus* stammen nauwelijks beschreven is in de literatuur, bleek deze productie veel voor te komen binnen dit geslacht: er werden 60 positieve stammen geïdentificeerd, waarvan er 17 meer dan 100 mg/l EPS synthetiseerden. De polysachariden die in grote hoeveelheden werden geproduceerd bleken homopolysachariden te zijn, met name glucanen, die werden geproduceerd wanneer sucrose in het groeimedium van de bacteriën aanwezig was.

Twee stammen, gecodeerd als LB 121 en LB 180 werden geselecteerd voor verder onderzoek. Beide stammen werden geïdentificeerd als

Lb. reuteri, maar hun EPS productie was verschillend. Stam LB 121 synthetiseerde alleen EPS tijdens de groei van de bacterie, terwijl bij LB 180 de EPS synthese nog doorging nadat de groei van de bacteriën gestopt was. LB 180 produceerde uitsluitend een glucan, terwijl LB121 zowel een glucan als een fructan synthetiseerde. De sucrase enzymen verantwoordelijk voor de EPS productie van LB 180 werden volledig uitgescheiden in het groeimedium, terwijl bij LB 121 een deel van de enzymen gekoppeld bleef aan de celwand. Vanwege enerzijds de synthese van 2 verschillende polysachariden en anderzijds het feit dat de fructan productie door *Lb. reuteri* 121 het eerste voorbeeld was van fructan synthese binnen het geslacht *Lactobacillus*, is *Lb. reuteri* 121 geselecteerd voor verder onderzoek.

Hoofdstuk 3 beschrijft de chemische karakterisering van het fructan en het glucan dat door *Lb. reuteri* 121 wordt geproduceerd. Het fructan bleek een onvertakt levan met β-(2→6) gebonden fructosyleenheden te zijn met een molecuulgewicht van 150 kDa. Het glucan heeft een unieke, sterk vertakte structuur met α-(1→4) en α-(1→6)gebonden glucose eenheden en een molecuulgewicht van 3,500 kDa. Dit glucan lijkt niet op eerder beschreven glucanen.

De enzymen verantwoordelijk voor de synthese van de polysachariden bleken tijdens de groei van de bacterie op glucose of maltose volledig gebonden te blijven aan de celwand. Tijdens de groei op sucrose of raffinose werd een deel van de glucan en levan synthetiserende enzymen uitgescheiden in het medium. Het glucansucrase bleef na gelelectroforese (SDS-PAGE) nog actief en kon worden aangetoond met een activiteitskleuring op basis van perjoodzuur en het Schiff reagens waarmee gesynthetiseerde suikers roze werden gekleurd. Het levansucrase kon niet worden aangetoond met deze methode.

In continu cultures bleek de polysacharide productie van *Lb. reuteri* geen stabiele eigenschap te zijn: er ontstonden spontaan verschillende mutanten, bijvoorbeeld stam K-24, die noch het glucan, noch het levan konden produceren. Wanneer de pH in de continu culture verlaagd werd, ontstonden mutanten, zoals bijvoorbeeld stam 35-5, die niet meer in staat waren om het levan te produceren, maar nog wel het vermogen om glucan te synthetiseren bezaten. De geïsoleerde mutanten bleken wel stabiel; na herhaaldelijk overenten behielden ze hun eigenschappen.

Hoofdstuk 4 beschrijft de isolatie en de karakterisering van een gen dat codeert voor een fructosyltransferase van *Lb. reuteri*. Het gen ligt op het chromosomale DNA van *Lb. reuteri* en werd geïsoleerd met behulp van PCR technieken. Hiervoor werden gedegenereerde primers afgeleid van geconserveerde aminozuur sequenties van reeds bekende bacteriële fructosyltransferases gebruikt. De nucleotide volgorde van een middels PCR

en inverse PCR geïsoleerd DNA fragment van 2600 bp werd bepaald. Dit geïsoleerde DNA fragment bevatte een open reading frame van ongeveer 2400 bp (*ftfA*) dat voor een eiwit (FTFA) codeerde waarvan de aminozuur volgorde grote gelijkenis vertoonde met de aminozuur volgordes van bekende bacteriële fructosyltransferases. De grootste gelijkenis werd gevonden met het levansucrase (SacB) uit *Streptococcus mutans*. Het molecuulgewicht dat afgeleid kon worden uit de aminozuur volgorde van FTFA was 86 kDa. Het FTFA eiwit bevatte een mogelijk N-terminaal secretie signaal peptide, wat erop wijst dat het eiwit zou worden uitgescheiden door *Lb. reuteri*. Een opvallend kenmerk van het FTFA is, dat in het carboxy terminaal gedeelte een PXT (Pro-willekeurig aminozuur-Thr) motief 15 keer achtereenvolgens voorkomt. Hiervoor kon geen verklaring worden gevonden. Het carboxyterminale gedeelte bevatte tevens een LPXTG sequentie dat verantwoordelijk zou kunnen zijn voor de verankering van het FTFA aan de celwand.

Het *ftfA* gen werd tot expressie gebracht in *Escherichia coli*. Cel vrije extracten van de recombinante *E. coli* bevatten een actief FTFA eiwit dat een fructan met β-(2→1) bindingen en fructose oligosachariden (dp 3-4) synthetiseerde uit sucrose. De afgeleide N-terminale aminozuurvolgorde van het FTFA eiwit is echter duidelijk anders dan die van een levansucrase eiwit gezuiverd uit culture supernatanten van *Lb. reuteri* 121. Het is daarom zeer waarschijnlijk dat in *Lb. reuteri* tenminste twee fructosyltransferases aanwezig zijn. Het *ftfA* gen is het eerste fructosyltransferase gen uit een *Lactobacillus* tam dat geïsoleerd en gekarakteriseerd is.

Het glucansucrase gen (*gtfA*), dat eveneens op het chromosomale DNA van *Lb. reuteri* ligt, werd op dezelfde manier geïsoleerd als het *ftfA*. Het geïsoleerde DNA fragment van 5.5 kb werd gekarakteriseerd door het bepalen van de nucleotide volgorde. Dit fragment bestond uit een open reading frame (ORF) van ongeveer 4100 bp dat codeerde voor het GTFA eiwit met een molecuulgewicht van ongeveer 140 kDa. Aan de 5' kant van het *gtfA* lag een gedeelte van een tweede ORF, waarvan de functie onbekend is.
De afgeleide aminozuurvolgorde van GTFA vertoonde grote gelijkenis met die van glucansucrases/glucosyltransferases uit andere melkzuurbacteriën. Het bestond, net als de andere glucansucrases uit een N-terminaal variabel gedeelte, een katalytisch domein en een C-terminaal glucan bindend domein. Er werd echter geen N-terminaal secretie signaal peptide aangetroffen. Bovendien was het glucan bindende domein korter dan dat van andere glucosyltransferases. De grootste gelijkenis werd gevonden met het alternansucrase ASR uit *Leuconostoc mesenteroides.*

Er zijn geen kristalstructuren beschikbaar van glucansucrases. Ze zijn echter verwant met enzymen uit familie 13 van de glycosylhydrolases. Hiervan is veel bekend over structuur/functie relaties. Het katalytisch domein van de

glucansucrases bevat geconserveerde gebieden die zich in een met enzymen uit familie 13 vergelijkbare $(\beta/\alpha)_8$ structuur bevinden. Niet alle aminozuren die geconserveerd zijn in andere glucansucrases werden ook in GTFA aangetroffen. In het gebied aan de C-terminale kant van een (Asp^{494}) dat mogelijk voor de katalyse belangrijk is, werden zelfs 3 van de 9 aminozuren, geconserveerd in andere glucansucrases, niet aangetroffen in GTFA.

Het *gtfA* gen werd met succes tot expressie gebracht in *E. coli*. Dit resulteerde in een actief recombinant GTFA. De glucanen geproduceerd door het recombinante GTFA en het *Lb. reuteri* GTFA werden met elkaar vergeleken middels NMR, methyleringsanalyses en molecuul gewichts bepalingen. Hieruit bleek dat het recombinant GTFA en het *Lb. reuteri* GTFA identieke glucanen synthetiseerden. De conclusie hieruit was dat GTFA in *Lb. reuteri* verantwoordelijk is voor de synthese van het unieke glucan. Dit is het eerste voorbeeld van de isolatie en moleculaire karakterisering van een glucansucrase uit een *Lactobacillus* stam.

De functie van de glucanen en levanen die door *Lb. reuteri* worden gesynthetiseerd is vooralsnog onbekend. In andere melkzuurbacteriën, de streptococcen, is de functie van deze polysacharides wel bekend. Ze spelen een rol in de vorming van tandplak, waarbij een matrix gevormd wordt van bacteriën en polysachariden op het gebit. De polysachariden zorgen hierbij voor de hechting van de bacteriën aan de tanden. De natuurlijke omgeving van *Lb. reuteri* is het maagdarm kanaal van de mens en van allerlei dieren; de bacterie staat bekend als een uitstekende koloniseerder van de darmen. Dit betekent dat er hechting van de bacterie plaatsvindt in dit ecosysteem. Hoewel niet duidelijk is of en hoeveel sucrose er bijvoorbeeld in de dunne darm beschikbaar is, is het verleidelijk om te speculeren dat, analoog aan de rol van de glucanen en levanen gevormd door streptococcen, de glucanen en levanen geproduceerd door *Lb. reuteri* ook een rol zouden kunnen spelen in de hechting (kolonisatie) van dit organisme.

Stellingen behorend bij het proefschrift

Exopolysaccharide synthesis by *Lactobacilus reuteri*:

Molecular characterization of a fructosyltransferase and a glucansucrase

Ineke van Geel-Schutten
Groningen, 16 juni 2000

1. Het is verwarrend dat de naam glucosyltransferase zowel wordt gebruikt voor glucansucrases als voor enzymen die geactiveerde glucose-eenheden overdragen.

2. Analoog aan de naamgeving van glucansucrase, alternansucrase, dextransucrase en amylosucrase voor enzymen die respectievelijk glucan, alternan, dextran en amylose uit sucrose synthetiseren, zou het enzym dat mutan synthetiseert uit sucrose (E.C. 2.4.1.-) mutansucrase kunnen worden genoemd. Analoog aan de glucansucrases zouden de fructosyltransferases die fructanen synthetiseren uit sucrose, fructansucrases kunnen worden genoemd.

3. De verhoging van de dextransucrase (DSRA) activiteit in *Escherichia coli* DH5α extracten, die werd gevonden na toevoeging van isopropyl β-D-thiogalactoside (IPTG) aan het kweekmedium, kan niet het gevolg zijn geweest van inductie door IPTG.
(Monchois, V. et al. (1996) Gene **182**:23-32)

4. Het valt te betwijfelen of de stelling dat een microbioloog op zijn werk zijn handen beter kan wassen voordat hij naar het toilet gaat dan erna (Proefschrift Peter van der Veen, 1995) ook opgaat voor microbiologen die met lactobacillen werken.
(Dit proefschrift; Reid et al. (1998) Int. Dairy J. **8**:555-562)

5. De betekenis van de muziek van J.S. Bach (1685-1750) voor volksgezondheid en werkgelegenheid kan nauwelijks worden overschat.

6. Dat menselijke individuen die meedoen aan de "rat-race" steeds vaker muisarmen krijgen, is niet het gevolg van genetische manipulatie.

7. Het investeren in muziekonderwijs op basisscholen levert de samenleving uiteindelijk geld op.
(De Volkskrant, 7 april 2000)

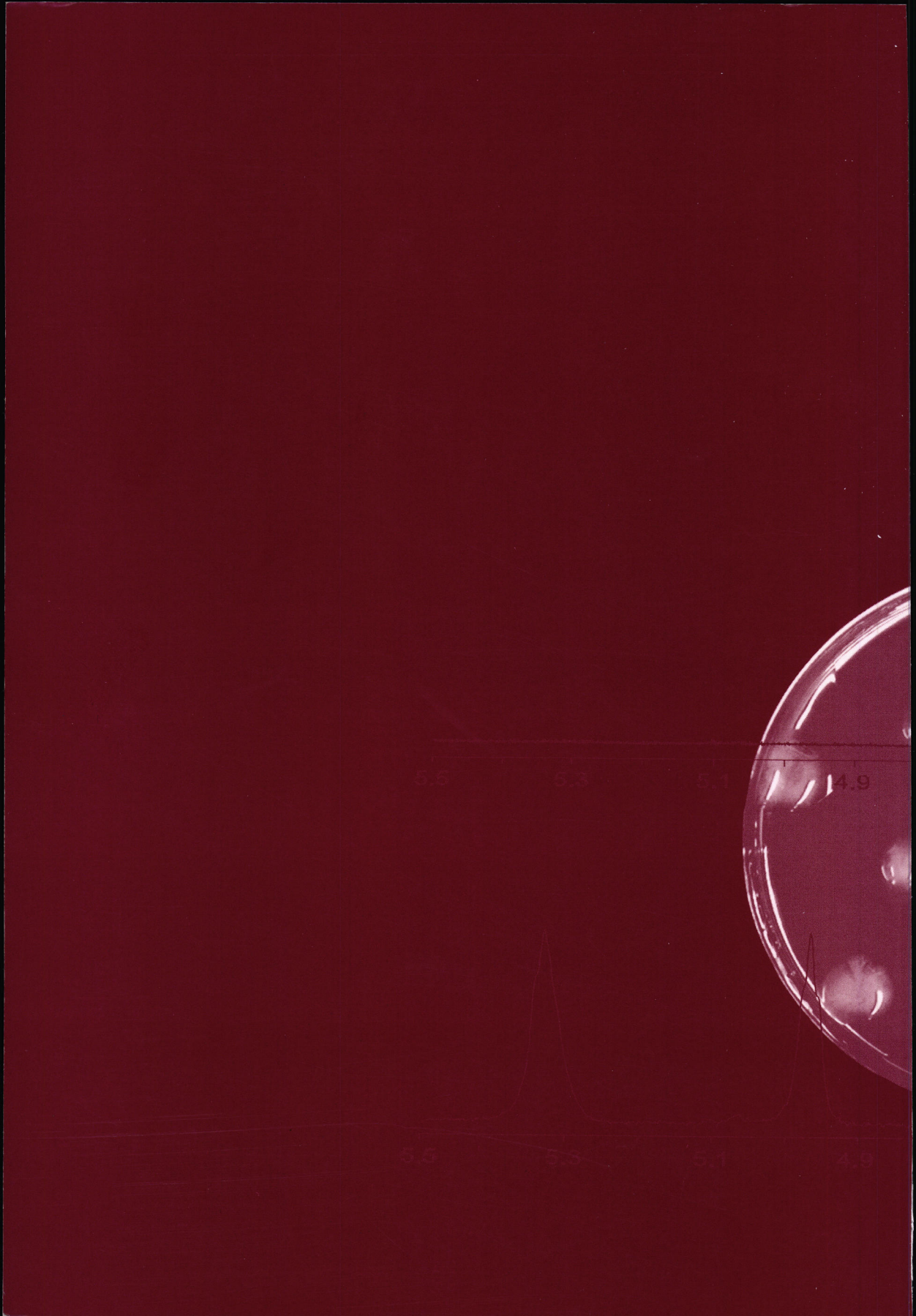
8. De Nederlandse basisscholen zijn onvoldoende ingesteld op en toegerust voor een maatschappij waarin beide ouders van schoolgaande kinderen werken.

9. Een werkgever die alleen kinderopvang voor kinderen van 0 tot 4 jaar subsidieert is kortzichtig; een werkgever die kinderopvang in het geheel niet subsidieert is blind.

10. Het zogenoemde glazen plafond voor vrouwelijke werknemers is mogelijk van spiegelglas.
(Yoo, S.-A. (2000) Management & Bestuur 2:8-9)

11. Een kwaliteitssysteem, zoals dat momenteel wordt opgezet binnen de kinderopvang, geeft ouders geen garanties dat hiermee het belangrijkste kwaliteitskenmerk van de kinderopvang wordt geborgd.

12. Vrouwelijke promovendi die tijdens de periode waarin zij hun promotie-onderzoek uitvoeren zwanger worden, hebben een verhoogde kans op het krijgen van dochters.
(British Medical Journal, 28 augustus 1999; Ineke van Geel)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