Exopolysaccharide synthesis by Lactobacilus reuteri

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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.