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Published in:
Biocatalysis and Biotransformation

DOI:
10.1080/10242420310001618519

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

Publication date:
2003

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Efficient Screening Methods for Glucosyltransferase Genes in Lactobacillus Strains

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(Received for publication 26 May 2003; Revised manuscript accepted 18 August 2003)

Limited information is available about homopolysaccharide synthesis in the genus Lactobacillus. Using efficient screening techniques, extracellular glucosyltransferase (GTF) enzyme activity, resulting in α-glucan synthesis from sucrose, was detected in various lactobacilli. PCR with degenerate primers based on homologous boxes of known glucosyltransferase (gtf) genes of lactic acid bacteria strains allowed cloning of fragments of 10 putative gtf genes from eight different glucan producing Lactobacillus strains (five Lactobacillus reuteri strains, one Lactobacillus fermentum strain, one Lactobacillus sake strain and one Lactobacillus parabuchneri strain). Sequence analysis revealed that these lactobacilli possess a large variation of (putative) gtf genes, similar to what has been observed for Leuconostoc and Streptococcus strains. Homologs of GTFA of Lb. reuteri 121 (synthesizing reuteran, a unique glucan with α-(1→4) and α-(1→6) glycosidic bonds) (Kralj et al., 2002) were found in three of the four other Lb. reuteri strains tested. The other Lactobacillus GTF fragments showed the highest similarity with GTF enzymes of Leuconostoc spp.

Keywords: Glucosyltransferase; Lactobacillus; Glucansucrase; Sucrose; Glucan; Polysaccharide

INTRODUCTION

Various lactic acid bacteria employ large extracellular enzymes, glucosyltransferases (GTFs, EC 2.4.1.5, commonly named glucansucrases), for the synthesis of high molecular weight α-glucans from sucrose. The GTF enzymes of oral streptococci and the dextran- and alternansucrases from Leuconostoc mesenteroides strains have been studied in most detail. All GTFs from lactic acid bacteria share a common structure and are composed of four distinct domains (Fig. 1): their N-terminal end starts with (i) a signal peptide of, followed by (ii) a highly variable stretch, (iii) a highly conserved catalytic or sucrose binding domain of about 1,000 amino acids (Fig. 2), and (iv) a C-terminal glucan binding domain, composed of a series of tandem repeats (Monchois et al., 1999).

There are only a few reports about α-glucan synthesis in lactobacilli (Dunican et al., 1963; Hammond 1969; Sidebotham, 1974; Van Geel-Schutten et al., 1998, 1999, 2001, 2002a,b, 2003). Only the GTFA enzyme responsible for reuteran (a glucan with α-(1→4) and α-(1→6) glycosidic bonds) synthesis in Lactobacillus reuteri strain 121 has been subjected to biochemical and molecular characterization (Kralj et al., 2002).

This paper describes the cloning, identification and characterization of (parts of) 10 gtf genes from various lactobacilli. The data show that a diversity of gtf genes is present in the genus Lactobacillus, as is the case in other genera of lactic acid bacteria (Leuconostoc and Streptococcus).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media and Growth Conditions

Strains previously identified as homopolysaccharide or heteropolysaccharide producers (van Geel-Schutten et al., 1998; unpublished information), Lactobacillus reuteri 121 (LB 121), Lactobacillus reuteri 180 (LB 180), Lactobacillus reuteri ML1 (LB ML1), Lactobacillus reuteri 104R (LB 104R), Lactobacillus fermentum Kg3 (LB Kg3), Lactobacillus sake Kg15 (LB Kg15), Lactoba-
*cillus parabuchneri* 33 (LB 33), *Leuconostoc citreum* 86 (LN 86), *Lactobacillus* sp. 181 (LB 181), *Lactobacillus* sp. 182 (LB 182), were obtained from the culture collection of TNO Nutrition and Food Research, Zeist, The Netherlands. The taxonomic position of the various glucan producing strains was identified by 16sRNA analysis (Gendika, Veendam, The Netherlands).

*Lactobacillus plantarum* WCFS1 (LB WCFS1), with a recently completed genome sequence (Kleerebezem et al., 2003), was obtained from the Wageningen Centre for Food Sciences (WCFS, Wageningen, The Netherlands). The *Lactobacillus reuteri* type strain DSM 20016 (LB DSM) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

**FIGURE 1** Degenerate primer sequences and their annealing sites in the catalytic domain of GTFA of *Lb. reuteri* 121. The four different domains shown are: i, N-terminal signal sequence; ii, variable region; iii, catalytic domain; iv, C-terminal (putative) glucan binding domain.

### Bacterial Strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>Main α-linkages in glucan polymer</th>
<th>Enzymes</th>
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<th>II</th>
<th>III</th>
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**FIGURE 2** Amino acid sequence alignment of highly conserved stretches (I, II, III) in catalytic domains of glucosyltransferases of lactic acid bacteria (see also Bozont et al., 2002; Monchoiz et al., 1999). A: Published sequences of dextran-, mutan- and alternansucrases of *Leuconostoc* and *Streptococcus* strains: GTFB, *S. mutans* G55 (Shiroza et al., 1987); GTFD, *S. mutans* G5 (Honda et al., 1990); GTFI, *S. donovii* Mfe28 (Ferretti et al., 1987); GTFJ, *S. salivarius* ATCC 25975 (Gillard et al., 1991); GTFK, *S. salivarius* ATCC 25975 (Gillard et al., 1993); GTF5, *S. donovii* Mfe28 (Gillmore et al., 1990); DSIR, *L. mesenteroides* NRRL B-1299 (Monchoiz et al., 1997); ASR, *L. mesenteroides* NRRL B-1299 (Monchoiz et al., 1998); DSRE CD1 and CD2, *L. mesenteroides* NRRL-B1299 (Bozont et al., 2002); B: Sequences of *Lactobacillus* glucosyltransferases, previously published or determined in this study: GTFA, *Lb. reuteri* 121; GTF104R, *Lb. reuteri* 104R; GTF180, *Lb. reuteri* 180; GTFML1, *Lb. reuteri* ML1; GTF33, *L. parabuchneri* 33; GTFKg15, *Lb. sake* Kg15; GTFKg3, *L. fermentum* Kg3; GTFML4, *Lb. reuteri* ML1; C: Sequences of *Leuconostoc citreum* 86 glucosyltransferases determined in this study: GTF86-1, GTF86-5, GTF86-8. - sequence gap; ⊕, putative catalytic residue; V, residue possibly playing a role in binding of acceptor molecules and in the transfer of the glucosyl residue; •, putative residue stabilizing the transition state; ND, not determined.
strains were cultivated anaerobically at 37°C in MRS medium (Difco, Franklin Lakes, NJ) (De Man et al., 1960) or in MRS-s medium (MRS-medium with 100 g l⁻¹ sucrose instead of 20 g l⁻¹ glucose). *Escherichia coli* DH5α (Phabagen, Utrecht, The Netherlands) (Hanahan 1983), and *E. coli* TOP 10 (Invitrogen, Carlsbad, USA) were used as hosts for cloning purposes. Plasmid PCR-XL-TOPO (Invitrogen) was used for cloning purposes. *E. coli* strains were grown aerobically at 37°C in LB medium (Ausbel et al., 1987). *E. coli* strains containing recombinant plasmids were cultivated in LB medium with the appropriate antibiotic (50 μg ml⁻¹ kanamycin). Agar plates were made by adding 1.5% agar to the LB medium.

**Isolation of DNA**

Total DNA was isolated according to Nagy et al. (1995), from MRS grown cells. Plasmid DNA of *E. coli* was isolated using a Wizard Plus SV plasmid extraction kit, according to the instructions of the manufacturer (Promega, Madison, WI).

**Molecular Techniques**

General procedures for cloning, *E. coli* DNA transformation, DNA manipulations, and agarose gel electrophoresis were as described (Sambrook et al., 1989). Restriction endonuclease digestions were performed as recommended by the enzyme suppliers (New England Biolabs, Beverly, MA; Roche Biochemicals, Basel, Switzerland). Primers were obtained from Eurogentec (Seraing, Belgium). Sequencing was performed by GATC (Konstanz, Germany). DNA was amplified by PCR on a DNA Thermal Cycler PTC-200 (MJ Research, Waltham, Massachusetts) using Expand High Fidelity DNA polymerase (Roche Biochemicals). Fragments were isolated from agarose gels using a Qiagen gel extraction kit (Qiagen, Hilden, Germany) following the instructions of the supplier.

**Identification and Nucleotide Sequence Analysis of the Glucosyltransferase (gtf) Genes**

The *Lactobacillus* gtf genes were isolated by PCR amplification of chromosomal DNA from the different strains, using degenerate primers (DegFor 5’-GAYAYWSSNAAYCCNYNGTNC-3’ and DegRev 5’-ADRTCNCCRTANAVNYYKNG-3’; Y = Tor C, K = G or T, W = A or T, S = C or G, R = A or G, N = inosine), based on conserved amino acid sequences present in the catalytic core (Fig. 1), deduced from the gtf genes of *Lb. reuteri* (gtfA), *Streptococcus downei* (gtfS), *Streptococcus mutans* (gtfC), *Streptococcus downei* (gtfI), *Streptococcus sali-

**Dendrogram Construction**

Amino acid sequences (~200 amino acids of the catalytic core) were aligned with Clustal W 1.74 (Higgins et al., 1988) with a gap opening penalty of 30 and a gap extension penalty of 0.5. Amino acid sequences were obtained from GenBank: DSRB of *Ln. mesenteroides* NRRL B-1299 (AAB95453), DSRs of *Ln. mesenteroides* NRRL B-512F (AAA53749), DSRE of *Ln. mesenteroides* NRRL B-1299 (AJ430204), GTFA of *Lb. reuteri* strain 121 (AX306822), ASR of *Ln. mesenteroides* NRRL B-1355 (CA865910), GTFB of *S. mutans* G55 (AAA88588), GTF5 of *S. downei* Mfe28 (AA26898), GTFK of *S. salivarius* ATCC 25975 (CAA77898), GTFI of *S. downei* Mfe28 (BAA0296), GTIF of *S. salivarius* ATCC 25975 (CAA77900). The other sequences used were obtained during this study. Tree construction was performed using TreeCon 1.3b (no correction for distance estimation, 100 bootstrap samples, using the neighbor joining algorithm; Van de Peer et al., 1994).

**Activity Staining of Lactobacillus GTF Enzymes**

Aliquots of MRS-sucrose media (10 ml) were inoculated with 200 μl of overnight cultures of *Lactobacillus* strains (LB DSM, LB 121, LB 104R, LB ML1, LB 180, LB 33, LB Kg3, LB Kg15, LB 181, LB 182, LB WCF51) or *Ln. citreum* 86, and incubated at 37°C for 8 h. Cells were removed by centrifugation 10,000 ×
g, and proteins in the supernatants were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (see below). After SDS-PAGE the gel was washed three times (1 min) with demineralized water and incubated overnight at 37°C in a 50 mM sodium acetate buffer, pH 5.5, containing 1% sucrose, 1% Tween 80 and 1 mM CaCl₂. Glucosyltransferase activity was detected by staining the gels for glucans produced with periodate Schiff stain (PAS) as previously described (Van Geel-Schutten et al., 1999).

**Gel Electrophoresis**

SDS-PAGE was performed according to Laemmli (1970) using the Mini-PROTEAN II system (Biorad, Veenendaal, The Netherlands), with 7.5% polyacrylamide gels. After GTF activity staining, gels were stained for proteins with Coomassie BioSafe (Biorad). A High Molecular Weight marker was used as standard (Amersham Pharmacia Biotech, Piscataway, NJ).

**RESULTS AND DISCUSSION**

**Screening for GTF Positive Lactobacillus Strains**

Supernatants from 11 different *Lactobacillus* strains, LB DSM, LB 121, LB ML1, LB 104R, LB 180, LB 33, LB Kg3, LB Kg15, LB 181, LB 182 and LB WCFS1, plus a single *Leuconostoc* strain (LN 86), were loaded on SDS-PAGE. After incubation in sucrose buffer, GTF protein activity bands were identified by PAS staining of the glucans produced (Van Geel-Schutten et al., 1999). With the exception of LB WCFS1 (of which the genome sequence does not encode glucansucrase genes (Kleerebezem et al., 2003)), and LB 181 and LB 182 (previously identified as heteropolysaccharide producers (Van Geel-Schutten et al., 1998)), all strains were positive, showing one or more activity bands at approximately 180 KDa (Fig. 3).

**Isolation and Nucleotide Sequence Analysis of Parts of the Putative Lactobacillus gtf Genes**

Based on sequence similarity between conserved regions, located in the catalytic core of different *gtf* genes of lactic acid bacteria, degenerate primers were designed and used for PCR with chromosomal DNA of the 12 different strains as template. Except for strains LB 181, LB 182 and LB WCFS1, fragments of approximately 660 bp were obtained with all strains tested (Fig. 4). PCR products obtained with LB Kg3 and LB Kg15 chromosomal DNA as template were sequenced directly, using the same degenerate PCR primers. Sequencing of these two PCR products showed in both cases the presence of only one product. The PCR products from the other seven positive strains (LB DSM, LB 121, LB 104R, LB ML1, LB 180, LB 33, and LN 86) were first ligated in pCR-XL-TOPO (Invitrogen). Subsequently, the seven different ligation mixtures were transformed to *E. coli* TOP10. Plasmid DNA was isolated from ten random clones from each transformation. Several *Streptococ-
cis and Leuconostoc species have been shown to contain more than one gtf gene (Funane et al., 2000; Simpson et al., 1995a). Therefore, restriction of the different plasmids (70 in total), with NsiI and EcoRI, was performed as a first screening to identify differences between the plasmids. Based on the restriction analysis, the inserts of five plasmids of each transformation were sequenced. Sequence analysis of five plasmids with chromosomal DNA inserts of LN 86 showed the presence of (parts of) three different (putative) gtf genes (gtf86-1, gtf86-5 and gtf86-8). Strains LB DSM, LB 104R, LB 180, and LB 33 most likely contain one gtf gene each. Strain 121 (gtfA, gtfB) (Kralj et al., 2002; this study) as well as strain ML1 contained at least two gtf genes (gtfML1, gtfML4) (Fig. 2B, C).

The methods used thus allowed efficient identification of GTF positive Lactobacillus (and Leuconostoc) strains, and detection of one or more (putative) gtf genes per Lactobacillus (and Leuconostoc) strain.

### GTF (Fragment) Sequence Comparisons

The amplified products from LB DSM, LB 121, LB 104R, LB ML1, LB 180, LB 33, LB Kg3, LB Kg15, and LN 86, were all identified as partial sequences of (the catalytic domains of putative) gtf genes (Fig. 2B, C). The fragments isolated from LB 104R, LB ML1 (GTFML1) and LB 180 showed the highest similarity at the amino acid level to GTFα of Lb. reuteri 121 (Table 1). The deduced amino acid sequences of the gtf180 and gtf104R encoded (putative) proteins showed very high homology to each other (99% similarity and 99% identity within 206 amino acids). A second (putative) GTF fragment was found in the Lb. reuteri strains ML1 (GTFML4) and 121 (GTFB). These fragments showed homology to each other and to the fragment isolated from Lb. reuteri DSM 20016 (GTFDSM) (~80% identity and ~90% similarity). GTF33 showed the highest similarity with the first catalytic domain (CD1) encoded by dsrE of Ln. mesenteroides NRRL-B1299. GTFKg3 showed the highest homology with DSRB from Ln. mesenteroides NRRL-B1299. GTFKg15 showed the highest homology with DSRS of Ln. mesenteroides NRRL-B512F (Table 1).

### Dendrogram

Construction of a dendrogram (Fig. 5), based on the partial amino acid sequences (approximately. 200 amino acids of the catalytic domains) of GTF enzymes of different lactic acid bacteria, revealed that the fragments of the following putative GTF enzymes (GTF180, GTF104R and GTFML1) isolated from different Lb. reuteri strains cluster closely together with GTFα of Lb. reuteri 121. Three other putative GTF fragments from Lb. reuteri strains (GTFDSM, GTFML4 and GTFB) formed a separate group. The GTF enzymes isolated from the various other lactobacilli cluster with Leuconostoc GTF enzymes.

### CONCLUSIONS

SDS-PAGE activity staining for α-glucan synthesis from sucrose, and PCR based cloning of gtf gene fragments (catalytic domains), allowed fairly rapid identification of putative gtf genes in several Lactobacillus strains. Sequence analysis of the different gtf fragments confirmed their identity. Homologs of GTFα of Lb. reuteri 121 (Kralj et al., 2002) were detected in three Lb. reuteri strains tested. Three other putative GTF fragments from Lb. reuteri strains (GTFDSM, GTFML4 and GTFB) formed a separate group. The other partial GTF sequences showed the highest similarity to glucosyltransferases from Leuconostoc sp. These results show that the large variation of glucosyltransferases previously reported for Leuconostoc and Streptococcus sp. also occurs within the lactobacilli. Conceivably, also such a range of different glycosidic bonds may be present in the glucans synthesized by the various GTF enzymes from lactobacilli. Currently, we are cloning and characterizing the full-length gtf gene sequences.

### TABLE 1

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>(Putative) protein</th>
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<th>Identity (%)</th>
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of the various lactobacilli. Many questions still remain to be answered, e.g. about expression, activity, and glucan synthesis of these (putative) GTF enzymes in their individual hosts, and about the number and nature of glucans (the type of glycosidic bonds present) produced by the various strains.

Acknowledgements

We gratefully acknowledge the contributions of Monica Dondorff, Trifa Omer and Peter Punt (INNO Nutrition and Food Research) to this work. We thank Bart Pieterse for providing Lactobacillus plantarum WCFS1.

References


