Glucansucrases of lactobacilli
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Chapter 6

Efficient screening methods for glucosyltransferase genes in Lactobacillus strains

S. Kralj, G.H. van Geel-Schutten, M.J.E.C. van der Maarel and L. Dijkhuizen

SUMMARY

Limited information is available about homopolysaccharide synthesis in the genus *Lactobacillus*. 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 *Leuconostoc* and *Streptococcus* strains allowed cloning of fragments of 10 putative gtf genes from 8 different glucan producing *Lactobacillus* strains (5 *Lactobacillus reuteri* strains, 1 *Lactobacillus fermentum* strain, 1 *Lactobacillus sakei* strain, and 1 *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) glucosidic 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.

INTRODUCTION

Various lactic acid bacteria employ large extracellular enzymes, glucosyltransferases (EC 2.4.1.5, commonly named glucansucrases, GTFs), 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, 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., 1999d).

![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.](image-url)
Screening for glucosyltransferases in Lactobacillus

There are only few reports about \( \alpha \)-glucan synthesis in lactobacilli (Dunican & Seeley, Jr., 1963, Hammond, 1969, Sidebotham, 1974, van Geel-Schutten et al., 1998, van Geel-Schutten et al., 1999). Only the GTFA enzyme responsible for reuteran (a glucan with \( \alpha \)-(1→4) and \( \alpha \)-(1→6) glucosidic bonds) synthesis in *Lactobacillus reuteri* strain 121 has been subjected to biochemical and molecular characterization (Kralj et al., 2002).

![Table](image)

**Figure 2.** Amino acid sequence alignment of highly conserved stretches (A, B, C) in catalytic domains of glucosyltransferases of lactic acid bacteria (also see (Monchois et al., 1999d) (Bozonnet et al., 2002, Monchois et al., 1999d). I: Published sequences of dextran-, mutan- and alternansucrases of *Leuconostoc* and *Streptococcus* strains. GTFB, *S. mutans* GS5 (Shiroza et al., 1987); GTFD, *S. mutans* GS5 (Honda et al., 1990); GTFI, *S. downei* Mfe28 (Ferretti et al., 1987); GTFJ, *S. salivarius* ATCC 25975 (Giffard et al., 1991); GTFK, *S. salivarius* ATCC 25975 (Giffard et al., 1993); GTFSA, *S. downei* Mfe28 (Gilmore et al., 1990); DSRB, *L. mesenteroides* NRRL B-1299 (Monchois et al., 1997); ASR, *L. mesenteroides* NRRL B-1299 (Arguello-Morales et al., 2000); DSRE CD1 and CD2, *L. mesenteroides* NRRL B-1299 (Bozonnet et al., 2002); II: Sequences of *Lactobacillus* glucosyltransferases, previously published or determined in this study. GTFB, *L. reuteri* 121 (Kralj et al., 2002); GTFB, *L. reuteri* 121; GTF104R, *L. reuteri* 104R; GTF180, *L. reuteri* 180; GTFL1, *L. paracasei* ML1; GTF33, *L. salivarius* 33; GTFL5, *L. salivarius* 5; GTFKg15, *L. sakei* Kg15; GTFKg3, *L. fermentum* Kg3; GTFML4, *L. reuteri* ML4; GTFML1, *L. reuteri* ML1; GTFDSM 20016, *L. paracasei* Cl. 86 (Monchois et al., 1997); III: Sequences of *Lactobacillus* glucosyltransferases previously published or determined in this study. GTFB, *L. reuteri* 121 (Kralj et al., 2002); GTFB, *L. reuteri* 121; GTF104R, *L. reuteri* 104R; GTF180, *L. reuteri* 180; GTFL1, *L. reuteri* ML1; GTF33, *L. paracasei* ML3; GTFKg15, *L. sakei* Kg15; GTFKg3, *L. fermentum* Kg3; GTFML4, *L. reuteri* ML4; I: *L. mesenteroides* NRRL B-1299 (Monchois et al., 1997); ASR, *L. mesenteroides* NRRL B-1355 (Arguello-Morales et al., 2000); DSRE CD1 and CD2, *L. mesenteroides* NRRL B-1299 (Bozonnet et al., 2002); II: Sequences of *Lactobacillus* glucosyltransferases, previously published or determined in this study. GTFB, *L. reuteri* 121 (Kralj et al., 2002); GTFB, *L. reuteri* 121; GTF104R, *L. reuteri* 104R; GTF180, *L. reuteri* 180; GTFL1, *L. reuteri* ML1; GTF33, *L. paracasei* ML3; GTFKg15, *L. sakei* Kg15; GTFKg3, *L. fermentum* Kg3; GTFML4, *L. reuteri* ML4; II: Sequences of *Lactobacillus* glucosyltransferases, previously published or determined in this study. GTFB, *L. reuteri* 121 (Kralj et al., 2002); GTFB, *L. reuteri* 121; GTF104R, *L. reuteri* 104R; GTF180, *L. reuteri* 180; GTFL1, *L. reuteri* ML1; GTF33, *L. paracasei* ML3; GTFKg15, *L. sakei* Kg15; GTFKg3, *L. fermentum* Kg3; GTFML4, *L. reuteri* ML4; III: Sequences of *Lactobacillus* citreum 86 glucosyltransferases determined in this study: GTF86-1; GTF86-5; GTF86-8. - , sequence gap; * , identical residue; : , highly conserved residue; , conserved residue; $\ddagger$ , putative catalytic residue; $\nabla$ , 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.
This paper describes the cloning, identification and characterization of (parts of) 10 \textit{gtf} genes from various lactobacilli. The data show that a diversity of \textit{gtf} genes is present in the genus \textit{Lactobacillus}, as is the case in other genera of lactic acid bacteria (\textit{Leuconostoc} and \textit{Streptococcus}).

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, media and growth conditions**

Strains previously identified as homopolysaccharide or heteropolysaccharide producers (van Geel et al. 1998; unpublished information), \textit{Lactobacillus reuteri} 121 (LB 121), \textit{Lactobacillus reuteri} 180 (LB 180), \textit{Lactobacillus reuteri} ML1 (LB ML1), \textit{Lactobacillus reuteri} 104R (LB104R), \textit{Lactobacillus fermentum} Kg3 (LB Kg3), \textit{Lactobacillus sakei} Kg15 (LB Kg15), \textit{Lactobacillus parabuchneri} 33 (LB 33), \textit{Leuconostoc citreum} 86 (LN 86), \textit{Lactobacillus} sp. 181 (LB 181), \textit{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, The Netherlands). \textit{Lactobacillus plantarum} WCFS1 (LB WCFS1), of which recently the complete genome sequence became available, (Kleerebezem et al., 2003) was obtained from the Wageningen Centre for Food Sciences (WCFS, The Netherlands). The \textit{Lactobacillus reuteri} type strain DSM 20016 (LB DSM) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). All strains were cultivated anaerobically at 37 °C in MRS medium (Difco) (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). \textit{Escherichia coli} DH5\(\alpha\) (Phabagen) (Hanahan, 1983), and \textit{E. coli} TOP 10 (Invitrogen) were used as hosts for cloning purposes. Plasmid PCR-XL-TOPO (Invitrogen) was used for cloning purposes. \textit{E. coli} strains were grown aerobically at 37 °C in LB medium (Ausubel et al., 1987). \textit{E. coli} strains containing recombinant plasmids were cultivated in LB medium with the appropriate antibiotic (50 \(\mu\)g 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 \textit{E. coli} was isolated using a Wizard Plus SV plasmid extraction kit, according to the instructions of the manufacturer (Promega).
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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; Roche Biochemicals). Primers were obtained from Eurogentec. Sequencing was performed by GATC (Germany). DNA was amplified by PCR on a DNA Thermal Cycler PTC-200 (MJ Research) using Expand High Fidelity DNA polymerase (Roche Biochemicals). Fragments were isolated from agarose gels using a Qiagen gel extraction kit (Qiagen) 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’-GAYAAYWSNAAYCCNRYNGTNC-3’ and DegRev 5’-ADRTCNCCRTARTARVTNYKNG -3’; Y=T or 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 salivarius* (*gtfK* and *gtfM*), and *dsrA* of *Ln. mesenteroides* (Kralj *et al.*, 2002, Gilmore *et al.*, 1990, Ueda *et al.*, 1988, Ferretti *et al.*, 1987, Giffard *et al.*, 1993, Simpson *et al.*, 1995b, Monchois *et al.*, 1996).

The PCR conditions for the amplification of the glucosyltransferase genes from the different bacterial strains were as follows: about 100 ng purified DNA as template, 125 pmol of each primer, 2mM dNTP, 10 × reaction buffer, 4.5 mM MgCl₂, 0.7 U Expand High Fidelity DNA polymerase (Roche Biochemicals), were used in the final reaction of 25 μl. The PCR reaction started with a denaturation step (95°C, 5 min). The cycle steps for a total of 35 cycles were as follows: denaturation (95°C, 30 sec), annealing (42 °C, 45 sec) and elongation (72 °C, 1 min).

Amplification products of *Lactobacillus* DNA with the expected size of about 660 bp were either i) directly sequenced, or ii) ligated into pCR-XL-TOPO (Invitrogen) and transformed to *E. coli* TOP 10. From ten random clones, plasmid DNA was isolated and analyzed by restriction using *EcoRI* and *NsiI*. Subsequently five of the ten clones (from each transformation) were sequenced. Determination of the different nucleotide sequences (GATC, Germany) and analysis confirmed *gtf* gene identities.

Dendrogram construction

Amino acid sequences (∼ 200 aa of the catalytic core) were aligned with Clustal W 1.74 (Higgins & Sharp, 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
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(AAA53749), DSRE of *Ln. mesenteroides* NRRL B-1299 (AJ430204), GTFA of *Lb. reuteri* strain 121 (AX306822), ASR of *Ln. mesenteroides* NRRL B-1355 (CAB65910), GTFB of *S. mutans* GS5 (AAA88588), GTFS of *S. downei* Mfe28 (AAA26898), GTFK of *S. salivarius* ATCC 25975 (CAA77898), GTFI of *S. downei* Mfe28 (BAA0296), GTFJ 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 & De Wachter, 1994).

**Activity staining of Lactobacillus GTF enzymes**

MRS-sucrose media (10 ml) were inoculated with 200 µl overnight culture of *Lactobacillus* strains (LB DSM, LB 121, LB 104R, LB ML1, LB 180, LB 33, LB Kg3, LB Kg15, LB 181, LB 182, LB WCFS1) 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 ° 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 (Laemmli, 1970) using the Mini-PROTEAN II system (Bio-Rad), with 7.5% polyacrylamide gels. After GTF activity staining, gels were stained for proteins with coomassie BioSafe (Bio-Rad). The High Molecular Weight marker was used as standard (Amersham Pharmacia Biotech).

**RESULTS AND DISCUSSION**

**Screening for GTF positive Lactobacillus strains**

Supernatants from the twelve different strains: LB DSM, LB 121, LB ML1, LB 104R, LB 180, LB 33, LB Kg3, LB Kg15, LN 86, LB 181, LB 182 and LB WCFS1 were loaded on SDS-PAGE. After incubation in sucrose buffer, GTF 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 contain genes encoding glucansucrases), (Kleerebezem et al., 2003) LB 181 and LB 182 (heteropolysaccharide producers), (van Geel-Schutten et al., 1998) all strains were positive, showing single 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 homology 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 nine different strains as template. In each case, except for LB 181, LB 182 and LB WCFS1 fragments of approximately 660 bp were obtained (Fig. 4). PCR products obtained when using chromosomal DNA of LB Kg3 and LB Kg15 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 six different ligation mixtures were transformed to *E. coli* TOP10. Plasmid DNA was isolated from ten random clones from each transformation. Several *Streptococcal* and *Leuconostoc* species have been shown to contain more than one *gtf* gene (Simpson et al., 1995a, Funane et al., 2000). Therefore, restriction of the different plasmids (60 in total), with *Nsi*I and *Eco*RI, was performed as a first screening to identify differences between the plasmids. Based on the restriction analysis, the inserts of five plasmids 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 (86-1, 86-5 and 86-8). Strain, 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 method used thus allowed detection of several putative *gtf* genes in a single 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 (putative) gtf genes (Fig. 2B,C). The fragments isolated from LB 104R, ML1 (GTFML1) and LB 180 showed highest homology at the amino acid level with GTFA from *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 high homology to each other and to the fragment isolated from *Lb. reuteri* DSM 20016 (GTFDSM) (~80% identity and ~90% similarity). GTF33 showed the highest homology 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).
Screening for glucosyltransferases in Lactobacillus

Table 1. Similarities and identities of sequences of the newly isolated GTF fragments to known GTF sequences shown in Fig. 5.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>(Putative) Protein</th>
<th>Homology to</th>
<th>Genbank</th>
<th>Similarity (%)</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lb. reuteri DSM 20016</td>
<td>GTFDSM</td>
<td>CD2 DSRE of Ls. mesenteroides NRRL B-1299</td>
<td>A3430204</td>
<td>49</td>
<td>29</td>
</tr>
<tr>
<td>Lb. reuteri 104R</td>
<td>GTF104R</td>
<td>GTFA of Lb. reuteri 121</td>
<td>A306822</td>
<td>83</td>
<td>63</td>
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<tr>
<td>Lb. reuteri ML1</td>
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<td>GTFA of Lb. reuteri 121</td>
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<td>58</td>
</tr>
<tr>
<td>Lb. reuteri 180</td>
<td>GTF180</td>
<td>GTFA of Lb. reuteri 121</td>
<td>A306822</td>
<td>83</td>
<td>63</td>
</tr>
<tr>
<td>Lb. parabuchneri 33</td>
<td>GTF33</td>
<td>CD1 DSRE of Ls. mesenteroides NRRL B-1299</td>
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<td>79</td>
</tr>
<tr>
<td>Lb. fermentum Kg3</td>
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<tr>
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<td>72</td>
</tr>
<tr>
<td>Lb. reuteri 121</td>
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<td>CD2 DSRE of Ls. mesenteroides NRRL B-1299</td>
<td>A3430204</td>
<td>49</td>
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<td>Ls. citreum 86</td>
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<td>DSRB of Ls. mesenteroides NRRL B-1299</td>
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<td>98</td>
<td>98</td>
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<tr>
<td>Ls. citreum 86</td>
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<td>ARS of Ls. mesenteroides NRRL B-1355</td>
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</tr>
<tr>
<td>Ls. citreum 86</td>
<td>GTFR6-8</td>
<td>CD1 DSRE of Ls. mesenteroides NRRL B-1299</td>
<td>A3430204</td>
<td>99</td>
<td>99</td>
</tr>
</tbody>
</table>

Dendrogram

Construction of a dendrogram (Fig. 5), based on the partial amino acid sequences (approx. 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 GTFA of Lb. reuteri 121. Three other putative GTF fragments from Lb. reuteri strains (GTFS, GTFL4 and GTFB) formed a separate group. The GTF enzymes isolated from the various other lactobacilli cluster with Leuconostoc GTF enzymes.

Figure 5. Dendrogram of glucosyltransferases of lactic acid bacteria. 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). Sequences of glucosyltransferases determined in this study are indicated with a bold line and are underlined.
Conclusion

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

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