Glucansucrase (GS) (or glucosyltransferase [GTF]) enzymes (EC 2.4.1.5) of lactic acid bacteria (LAB) use sucrose to synthesize a diversity of α-glucans with α1→6 (dextran; found mainly in *Leuconostoc*), α1→3 (mutan; found mainly in *Streptococcus*), alternating α1→3 and α1→6 (alternan; reported only in *Leuconostoc mesenteroides*), and α1→4 (reuteran; synthesized by GTFA and GTFO from *Lactobacillus reuteri* strains) glycosidic bonds (1, 14, 16, 23, 34).

The first glucoside hydrolase 70 (GH70) family 3-dimensional (3D) structures, recently elucidated (9, 38), showed that the catalytic domains of GS enzymes possess a (β/α)₆ barrel structure similar to that of members of the GH13 family, confirming earlier secondary-structure predictions (4, 21). The core of the proteins belonging to the GH13 family comprises 8 β-sheets alternated with 8 α-helices. In GS enzymes, however, this (β/α)₆ barrel structure is circularly permuted (21). Also, the four conserved regions (regions I to IV) identified in members of the α-amylase family GH13 (31) are present in glucansucrases. However, as a consequence of the circular permutation, region I occurs C-terminally to regions II to IV in glucansucrase enzymes.

Upstream of the *gff* gene from *L. reuteri* 121 we identified a second glucansucrase-like gene (*gffB*) (15, 16). However, after cloning and expression in *Escherichia coli*, this GTFB enzyme was inactive with sucrose (15). In parallel investigations, we identified various genes encoding putative GTFB homologs in other *Lactobacillus* strains. This emergence of more GTFB homologs, indicating that they occur more widely, prompted us to investigate the activity, reaction specificity, and product specificity of the *L. reuteri* 121 GTFB enzyme in more detail.

Here we show that the GTFB enzyme uses maltooligosaccharides (MOS), e.g., maltoheptaose (with α1→6 glycosidic linkages only), as substrates to synthesize oligosaccharides with both α1→6 and α1→4 glycosidic linkages into linear oligosaccharide products. We conclude that GTFB represents a novel evolutionary intermediate between the GH13 and GH70 enzyme families, and we speculate about its origin.
glycosidic linkages. Finally, the biochemical characterization of GTFB as a 4,6-α-glucanotransferase enzyme instead of a glucansucrase is discussed.

MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and growth conditions.** Escherichia coli TOP10 (Invitrogen, Carlsbad, CA) was used as the host for cloning purposes. Plasmid pET15b (Novagen, Madison, WI) was used for expression of the fragment (nt) gene in E. coli BL21 Star (DE3) (Invitrogen, Tastraup, Denmark). E. coli strains were grown aerobically at 37°C in Luria-Bertani (LB) medium (2). E. coli strains containing recombinant plasmids were cultivated in LB medium with 100 μg/ml ampicillin. Agar plates were made by adding 1.5% agar to the LB medium.

**Amino acid sequence alignment of GTFB from L. reuteri and phylogenetic tree construction.** Multiple amino acid sequence alignments of GTFB and known glucansucrases and putative 4,6-α-glucanotransferases from lactic acid bacteria were made with the ClustalW interface in MEGA, version 4 (www.megasoftware.net), with gap-opening and extension penalties of 10 and 0.2, respectively. The same program was used to construct a phylogenetic tree of Lactobacillus, Lactococcus, and Streptococcus glucansucrases and 4,6-α-glucanotransferases. Amino acid sequences were acquired from the CAzy (Carbohydrate-Active Enzymes) database (www.cazy.org). A bootstrap test of phylogeny was performed by the neighbor-joining method using 500 replicates.

**Construction of plasmids.** Appropriate primer pairs and template DNA were used to create two different expression constructs with C-terminal His tag: one for the complete GTFB protein (1,587 amino acids), constructed in three separate PCR using the method previously described for L. reuteri 121 GTFA (see below) (16), and one for a variant lacking the N-terminal variable region of GTFB, yielding pET15b-GTFB-D1015N.

**Expression and purification of GTFB.** An overnight culture of E. coli BL21 Star (DE3) harboring the (mutant) GTFB (15) was diluted 1:100. Cells were grown to an optical density at 600 nm (OD₆₀₀) of 0.4 and were induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG); after 4 h of growth, cells were harvested by centrifugation (10 min at 4°C and 10,000 × g). Cell extracts were prepared by sonication, and (mutant) GTFB proteins were purified by Ni²⁺-nitrilotriacetic acid (NTA) and anion-exchange chromatography, as described previously for L. reuteri 121 GTFA (reuteransucrase) (18), with the following modifications: for anion-exchange chromatography, a 1-mL Hitrap Q HP column was used (GE Healthcare, Uppsala, Sweden).

**pH and temperature optima.** The GTFB pH and temperature optima were determined by measuring qualitatively on thin-layer chromatography (TLC) plates (see below) the amounts of saccharides synthesized from 25 mM maltotetraose after overnight incubation (data not shown). Incubation of MOS and other saccharide substrates with GTFB. GTFB (90 nM) was incubated separately overnight with 25 mM sucrose (Acros), raffinose (Sigma), turanose (Sigma), palatinose (Sigma), panose (Sigma), isomaltose (Sigma), MOS with different degrees of polymerization (G2 to G7), or 0.25% amylose-V (Avebe, Foxhol, The Netherlands) in 25 mM sodium acetate buffer, pH 4.7, containing 1 mM CaCl₂, at 37°C.

**Donor and acceptor substrate studies.** Purified GTFB (90 nM in 25 mM sodium acetate buffer, pH 4.7, containing 1 mM CaCl₂) was incubated overnight at 37°C with the donor substrate (0.25% amylose-V) and 25 mM acceptor substrate (glucose [G1] or maltose [G2]). TLC and high-performance anion-exchange chromatography (HPAEC). For TLC analysis of saccharide product mixtures, 1 to 3 μl of the sample was applied to a silica gel 60 F254 plate (Merck, Darmstadt, Germany), and after drying, the plate was run for 6 h in butanol-ethanol-H₂O (5:5:3 [vol/vol/vol]). Then the plate was dried, sprayed with 50% H₂SO₄ in methanol, and left to develop for 10 min at 110°C.

**HPAEC analysis.** Appropriate dilutions of enzyme reaction mixtures were dissolved in 90% dimethyl sulfoxide. A commercial mixture of MOS (DP1 to DP7) was thoroughly washed in methanol, and the mixture was dissolved in 90% dimethyl sulfoxide. A commercial mixture of MOS (DP1 to DP7) was thoroughly washed in methanol, and the mixture was dissolved in 90% dimethyl sulfoxide. A commercial mixture of MOS (DP1 to DP7) was thoroughly washed in methanol, and the mixture was dissolved in 90% dimethyl sulfoxide.
yalized alditols were peracetylated with acetic anhydride-pyridine, 1:1 (vol/vol) (3 h, 120°C), yielding mixtures of partially methylated alditol acetates, which were analyzed by gas-liquid chromatography–electron impact mass spectrometry (GLC-El-MS).

(ii) NMR spectroscopy. One-dimensional 1H nuclear magnetic resonance (NMR) spectra of the incubation sample and fraction P2 were recorded on a Bruker DRX 300 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at a probe temperature of 300 K. Samples were exchanged once with 99.9 atom% D2O, lyophilized, and dissolved in 650 μL D2O. 1H chemical shifts (δ) are expressed in parts per million by reference to internal acetone (δ 2.225). The 1H NMR spectra were recorded with a spectral width of 5 kHz in 16 kHz complex data sets and were zero filled to 32 kHz. A water-eliminated Fourier transform (WEFT) pulse sequence was applied to suppress the HOD signal (7). When necessary, a fifth-order polynomial baseline correction was applied. NMR data were processed using software originally developed by J. A. van Kuik (Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University).

(iii) MS. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) experiments with the incubation sample and fractions O1 and P2 were performed on a Voyager DE Pro mass spectrometer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands) equipped with a nitrogen laser (wavelength, 337 nm; pulse width, 3 ns) (Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University). Positive-ion-mode spectra were recorded in the reflector mode at an accelerating voltage of 24 kV. The mass acquisition range was 500 to 5,000 Da. Samples were prepared by mixing on the target 1 μL of aqueous saccharide solutions with 1 μL 2,5-dihydroxybenzoic acid (10 mg/ml in 40% aqueous acetoni-trile as the matrix solution. GLC-El-MS was performed on a Fisons Instruments GC 8060/MD 800 system (Interscience BV, Breda, The Netherlands) equipped with an AT-1 column (30 m by 0.25 mm; Alltech, Uden, The Netherlands) by using a temperature gradient of 140 to 240°C at 4°C/min (11) (Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University).

RESULTS

Dendrogram and alignment of GTFB. L. reuteri 121 GTFB is the first representative of a group of homologous enzymes identified in different lactic acid bacteria. Genes encoding putative GTFB homologs are present in several Lactobacillus strains, e.g., in L. reuteri ML1, where fragments of a GTFB homolog, gtfML4, were identified upstream of gtfML1, encoding a mutansucrase (15). A GTFB homolog could also be identified in the recently elucidated genome sequence of L. reuteri DSM 20016 (73% identity and 85% similarity in 883 amino acids). Finally, L. reuteri TMW1.106 also contains GTFB (GTF106B) and GTFO (GTF106A) homologs. The GTF106B enzyme showed 92% identity and 95% similarity in 1,383 amino acids with L. reuteri 121 GTFB. However, in contrast to GTFB, GTF106B hydrolyzed sucrose slowly (apparent after 27 h of incubation) (10). Screening of samples from Indonesia also revealed the catalytic part of a putative GTFB homolog in Weissella confusa MBF 8.1 (22). Phylogenetically, these GTFB enzymes cluster together but are also closely related to glucansucrases acting on sucrose as a substrate (Fig. 1) (5, 34). Nevertheless, GTFB has no detectable activity with sucrose (15). In the present study, it is shown (see below) that GTFB uses maltoligosaccharides (MOS) instead as donor and acceptor substrates and that on the basis of the analytical findings, GTFB has to be designated a (1→4)-(α,β)-glucan: (1→4), (1→6)-α,β-glucan β-glucanotransferase (in short, a 4,6-α-glucanotransferase).

Alignments of members of this novel group of GTFB-like enzymes with typical glucansucrases showed similarities (for GTFB and GTFO of L. reuteri 121, 46% identity and 61% similarity in 1,683 amino acids) but also clear and characteristic differences (Fig. 2). The three catalytic residues present (D1024, E1061, and D1125 in L. reuteri 121 GTFB [this numbering is used throughout unless indicated otherwise]) in glucansucrases are also present in the group of 4,6-α-glucanotransferases (D1015, E1053, and D1125) (L. reuteri 121 GTFB numbering). Nevertheless, a large number of amino acid residues conserved in regions I, II, III, and IV of glucansucrases are absent in the 4,6-α-glucanotransferase group of enzymes (Fig. 2). In region II (encompassing the putative nucleophilic residue), the conserved residue V1026 (Pro in GTFA and GTFO) is replaced by Ala in the 4,6-α-glucanotransferases. Region III, the region downstream of the putative acid/base catalyst E1061, is completely different in glucansucrases versus 4,6-α-glucanotransferases. The conserved residue W1063 is replaced by a Tyr residue in the 4,6-α-glucanotransferases (Fig. 2).

Cloning and expression of the gfbB gene. Full-length GTFB, the N-terminally truncated version, and the putative nucleophilic mutant D1015N GTFB were constructed and expressed successfully in E. coli. Following their purification, both the full-length enzyme (Fig. 3) and the N-terminally truncated variant (GTFB-ΔN) (data not shown) showed clear activity on MOS G4 to G7. The GTFB-ΔN variant was not expressed as efficiently as full-length GTFB, and therefore, all further experiments were performed using full-length GTFB. No background activity was detected in E. coli itself, as was evident from a control experiment with an empty pET15b plasmid, which demonstrated that after the His tag purification step, no activity on MOS (maltose to maltoheptaose [G2 to G7]) was detected in the samples obtained (data not shown). The purified full-length D1015N (putative) nucleophilic GTFB mutant also showed no activity on MOS G2 to G7 (data not shown).

GTFB enzyme characteristics. The optimal temperature and pH for GTFB activity with maltotetraose (G4) as a substrate were 30 to 37°C and pH 4 to 5, respectively (data not shown). Assays with various combinations of temperatures and buffers yielded the highest activity levels at 37°C and pH 4.7; these values were used in all subsequent assays.

GTFB donor substrates. GTFB was unable to use sucrose as a donor substrate (Fig. 3, TLC analysis) (15) and was also inactive with the sucrose analogs turanose and palatinose, with raffinose, with the DP5 and DP6 isomaltotriooligosaccharides (IMO), with panose, and with an oligosaccharide mixture obtained by hydrolysis of a partially purified reuteran (produced by the L. reuteri 121 GTFB enzyme) (data not shown). Clear activity, however, was observed after relatively short incubation times with MOS; i.e., G4 and larger MOS yielded a range of different oligosaccharide products (Fig. 3). With substrates of DP6 or larger, polymeric material as well as oligosaccharides started to accumulate (Fig. 3). Under the incubation conditions tested, virtually no activity was observed on maltose.
or maltotriose (Fig. 3). Only low activity was observed with amylose-V, resulting mainly in G1 and G2 release (see below).

**GTFB products from G6 and G7 accumulating in time.** By HPAEC analysis, the first clear reaction products detectable after 1 h with G6 (slightly contaminated with G5) as the substrate were G1 (glucose) and G5 (maltopentaose) (Fig. 4A). Similarly, with G7 (slightly contaminated with oligosaccharides of shorter retention times) as the substrate, the first saccharide products released were G1 (glucose) and G6 (maltohexaose) (Fig. 4B). Later on, incubations with G6 or G7 yielded peaks at retention times of MOS with lower DPs than that of the starting donor, but also peaks that did not fit with the MOS retention times, especially products with longer retention times than that of the starting donor.

**GTFB donor and acceptor substrate studies.** As indicated by HPAEC analysis, incubation of amylose-V with GTFB yielded some glucose (G1) and maltose (G2) (compare Fig. 5A and B). When amylose-V as a donor substrate was incubated with GTFB and glucose as an acceptor substrate, a range of oligosaccharides was synthesized (Fig. 5); also, larger amounts of maltose were detected than for the incubation with amylose-V alone (Fig. 5). Incubation of amylose-V with GTFB and maltose as the acceptor substrate yielded, among others, HPAEC peaks at the positions of panose [Glc-(α1→6)-maltose] and maltotriose (G3) (Fig. 5).

**Characterization of the GTFB products with maltoheptaose (G7).** MALDI-TOF MS analysis of the 150 mM G7 incubation sample showed visible [M + Na]+ peaks in the region of DP3 (m/z 527) up to DP10 (m/z 1,661), with DP5 as the major peak (100%), followed by DP6 (48%) (Table 1). It should be noted that m/z values below 500 were not recorded due to matrix-related noise in that area. The 1-dimensional 1H NMR spectrum (Fig. 6A) revealed, besides the anomeric signals at δ 5.42 to 5.37 (indicative of α1→4 linkages, in the absence of α1→3 linkages), δ 5.225 [reducing -(1→4)-β-Glc] unit and α-anomer of free glucose, δ 4.653 [reducing -(1→4)-β-Glc] unit; H-2 at δ 3.271, and δ 4.634 (β-anomer of free glucose; H-2 at δ 3.273), an additional broad anomeric peak at δ 5.00 to 4.95, indicative of α1→6 linkages (35, 37). The molar ratio of the α1→4-linked, α1→6-linked, and reducing unit glucose residues was 60:18:22. Ethanol treatment of the G7 incubation sample yielded a supernatant fraction (O1) and a precipitate fraction (P2).

**MALDI-TOF MS analysis of fraction O1 showed evidence of the same DP range as that in the incubation sample, with DP5 as the major component(s) (100%), followed by DP6**.
Table 1. Methylation (linkage) analysis of fraction O1 showed the presence of 4-substituted, 6-substituted, and terminal glucose residues at a molar ratio of 63, 17, and 20%, indicating the presence of linear products only (no branched glucose residues).

MALDI-TOF MS analysis of fraction P2 showed visible [M + Na]+ peaks in the region of DP3 (m/z 527) up to DP16 (m/z 2633), with DP5 as the major peak (100%), followed by DP6 (77%). Compared with those in the incubation mixture and fraction O1, the peak intensities of DP7 to DP11 glucosyloligosaccharides were significantly increased. Methylation (linkage) analysis of fraction P2 showed 4-substituted, 6-substituted, and terminal glucose residues at a molar ratio of 52, 35, and 13%, indicating the presence of linear products only. In the 1-dimensional 1H NMR spectrum of fraction P2, the areas of the anomeric signals at δ 5.42 to 5.37 (α1→3,4 linkages [36, 37]), δ 5.226 and 4.655 (α1→3 and α1→2 configuration, respectively, of the reducing glucose unit [37]), and δ 5.00 to 4.95 (α1→6 linkages [35, 37]) occurred at a molar ratio of 55, 33, and 12%, indicating a significant increase in the percentage of α1→6-linked glucose residues over those in the incubation sample. This suggests that the α1→6 linkages are specifically included in the higher-DP linear chains.

DISCUSSION

This paper reports the identification and characterization of a new reaction specificity within the GH70 family, namely, the disproportionation of maltooligosaccharides (MOS), also introducing α1→6 glucosidic linkages into the products formed. *L. reuteri* 121 GTFB is the first member of this group of enzymes that has been characterized in detail. Surprisingly, GTFB has virtually no activity with sucrose. Incubation of *L. reuteri* 121 GTFB with MOS of different DPs produced saccharides with α1→4 glucosidic linkages alone and saccharides that also contained α1→6 glucosidic linkages. Incubation of GTFB with amylose as a donor substrate and glucose or maltose as an acceptor substrate confirmed the ability of the enzyme to synthesize α1→4 glucosidic linkages also. In particular, with maltose as an acceptor,
FIG. 4. HPAEC analysis of the reaction products of 90 nM GTFB incubated for 0 to 8 h in 50 mM sodium acetate buffer, pH 4.7, containing 1 mM CaCl₂ with 25 mM maltohexaose (A) or 25 mM maltotetraose (B).
among other unidentified products, panose [glucosyl-(α1→6)-maltose] and G3 [glucosyl-(α1→4)-maltose] were detected. Preliminary investigation of the products generated when GTFB was incubated with maltoheptaose (DP7) by methylation (linkage) analysis (GLC-EI-MS), 1H NMR analysis, and MALDI-TOF MS revealed the presence of only linear oligosaccharides containing α1→4 and α1→6 glycosidic linkages. There was a range of glucosyloligosaccharides from DP3 to DP16, with an overall content of around 18% α1→6 glycosidic bonds, and the percentage of α1→6 glycosidic linkages was higher in the higher-DP products (33% in

FIG. 5. HPAEC analysis of samples with 0.25% amylose-V (AMV) alone (donor substrate) or amylose-V with 25 mM glucose or 25 mM maltose (acceptor substrates), either without the GTFB enzyme (A) or with GTFB (90 nM) (B) incubated overnight at 37°C in 25 mM sodium acetate buffer, pH 4.7, containing 1 mM CaCl2. Pa, panose.
the ethanol-precipitated fraction). Based on these findings, we have designated the GTFB enzymes (1→4)-α-d-glucan: (1→4)(1→6)-α-d-glucan α-glucanotransferases (in short, 4,6-α-glucanotransferases).

Phylogenetic analysis shows that the 4,6-α-glucanotransferases form a separate group within the glucansucrases (Fig. 1). The three amino acids crucial for catalysis in glucansucrase enzymes (D1024, E1061, and D1133 [L. reuteri 121 GTFA numbering]) (18) are also conserved in the 4,6-α-glucanotransferase group of enzymes (D1015, E1053, and D1125 [L. reuteri 121 GTFB numbering]) (Fig. 2). The recent elucidation of the first 3D structure of the N-terminally truncated glucanase from L. reuteri 180 showed that glucanase enzymes contain 5 domains (A, B, C, IV, and V) and that the peptide chain follows a “U” path. The A, B, and C domains are similar and can be superimposed on the A, B, and C domains present in Bacillus licheniformis α-amylase. Domains IV and V are unique to glucanase enzymes and are also found in the 4,6-α-glucanotransferases. These 4,6-α-glucanotransferase enzymes thus represent structural and functional evolutionary intermediates between family GH13 and GH70 enzymes.

Mutation of the GTFB putative nucleophile amino acid residue (D1015N) resulted in an inactive enzyme, confirming the crucial importance of this residue in catalysis. The other six conserved residues in GH70 enzymes, present in regions I, II, III, and IV, are also conserved in 4,6-α-glucanotransferase enzymes (Fig. 2). Interestingly, a large number of amino acid residues conserved in regions II, III, IV, and I in the glucanase type of enzymes are absent or differ in the 4,6-α-glucanotransferase group of enzymes (Fig. 2). Amino acid residues in regions I to IV contribute to the donor/acceptor substrate binding subsites and therefore are important in determining substrate specificity (38). Differences in amino acid residues contributing to these donor and acceptor substrate binding subsites may explain the differences in substrate specificity between glucanases and 4,6-α-glucanotransferases. The same applies to differences in glycosidic bond specificity between the products synthesized (8, 19).

Although the active sites of glucanases and 4,6-α-glucanotransferases show clear similarities, there are some differences. Questions arise about the active-site organization in

![FIG. 6. Five hundred-megahertz 1-dimensional 1H NMR analysis of maltoheptaose (DP7) incubated with 90 nM GTFB for 7 days in 50 mM sodium acetate buffer, pH 4.7, containing 1 mM CaCl2. (A) Incubation sample; (B) ethanol-precipitated fraction (P2) from the incubation sample. Red., reducing glucose unit.]

TABLE 1. DP distribution of the glucooligomers, as detected by MALDI-TOF MS analysis, in the incubation mixture of maltoheptaose with GTFB, its ethanol supernatant fraction O1, and its ethanol precipitate fraction P2

<table>
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<th>DP</th>
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* ND, not detected under the conditions used.
4,6-α-glucanotransferases, in particular the number of donor substrate binding subsites present. Only one donor subsite is present in glucansucrase enzymes, whereas other amyloytic enzymes, such as amylases and amylomaltases, have more than one donor subsite. In order to address this question, GTFB enzyme incubations with maltohexaose (G6) or maltotetraose (G7) as the substrate were followed over time using HPAEC. The first reaction products detectable with G6 were G1 (glucose) and G5 (maltopentaose) (Fig. 4A). Similarly, with G7, the first products released were G1 (glucose) and G6 (maltohexaose) (Fig. 4B). With both G6 and G7, other MOS of lower order were also present. The data thus suggest that 4,6-α-glucanotransferases have an active-site architecture similar to that of glucansucrases, both containing only a single donor substrate binding subsite (−1).

The enzymes from glycoside hydrolase families GH13, GH70, and GH77 together form clan GH-H (www.cazy.org). All GH-H members employ similar catalytic mechanisms involving a covalent glucosyl-enzyme intermediate and retention of the α-anomeric configuration of the product upon hydrolysis (13, 32). Amylomaltases (also known as 4-α-glucanotransferases) from GH77 are capable of synthesizing large cyclic glucans and disproportionating MOS. Notably, amylomaltases almost exclusively catalyze transglycosylation reactions and only cleave and synthesize α1→4 glycosidic linkages (3). The GH13 enzyme family, acting mainly on starch-like substrates and displaying a large variety of different reaction specificities, is the largest of the GH families at present, organized in different subfamilies in the CAZy database (30). Amylosucrase is the only enzyme from family GH13 that uses sucrose as a substrate to synthesize an amylase type of α-glucan polymer. Amylosucrase enzymes thus have the GH13 type of domain architecture and the GH70 type of glucansucrase activity (24, 29).

All α-amylases have similar domain organizations (see above), even those of archaean origin (20). Glucansucrases are found only in Bacteria, suggesting that the precursor amylase enzyme from which GH70 glucansucrases and present-day amylases have evolved had a similar domain organization. In addition, it is most likely that amyloytic activity, the main activity of α-amylases, emerged first, and that the α-glucanotransferase and sucrose types of activities evolved from the precursor amylase. It appears less likely that a family GH77 enzyme is an intermediate, since these proteins possess various additional domains (B1, partly present in α-amylase members; B2 and B3, unique to amyloyalases); in addition, they lack the C domain (26). Furthermore, GH77 enzymes do not possess α1→6 specificity, in contrast to GH13 and GH70 family members. The GTFB enzyme clearly is a glucansucrase type of protein with regard to its amino acid sequence and domain organization but lacks the ability to act on sucrose. Surprisingly, GTFB acts on MOS, substrates used by various GH13 (and GH77) family members, and is able to cleave α1→4 glycosidic linkages and to synthesize α1→4 and α1→6 glycosidic linkages. GTFB from the GH70 family therefore provides a link between the GH13 α-amylase and GH70 glucansucrase families. The latter use sucrose as a substrate to synthesize linear as well as branched α-glucan polymers, differing in the type of glycosidic linkages (reuteran, α1→4; dextran, α1→6; alternan, α1→3/α1→6; mutan, α1→3), the degree and type of branching, the length of the glucan chains, molecular mass, and the conformation of the polymers (34).

Glucansucrase enzymes also use MOS as acceptor substrates with sucrose as donor substrate. This results in the synthesis of a range of oligosaccharides, e.g., a maltose extended with a series of glucose units bound via α1→6 linkages in the case of dextran transglucosidase. In this case, however, the α1→4 linkages in MOS substrates are not cleaved; MOs are used only as acceptor substrates (6, 12). This is a major difference from the GTFB enzyme, which fails to act on sucrose and instead uses MOS as donor and acceptor substrates, cleaving α1→4 linkages and introducing new α1→4 and α1→6 linkages. GTFB thus appears to be an evolutionary precursor for the glucansucrase type of enzymes with the GH70 domain architecture and the GH13 amyloytic activity (38).

Although 4,6-α-glucanotransferases and glucansucrase enzymes are commonly arranged in tandem on the genome (10, 15), the exact in vivo role of GTFB-like enzymes remains unknown. They may scavenge and modify the oligosaccharides formed by glucansucrase enzymes as substrates for the synthesis of larger saccharides, which are inaccessible to other microbes. They may also play a role in the modification of the glucan synthesized by the glucansucrase enzyme of the microorganism. Interestingly, the genome of L. reuteri DSM 20016 contains only a GTFB-like enzyme and no glucansucrase enzyme. This Lactobacillus strain may be the key to answering questions about the in vivo role of 4,6-α-glucanotransferase enzymes.

Conclusions. Based on the enzymatic activity of GTFB, we propose to give this group of enzymes a separate EC number, different from EC 2.4.1.5 (for common glucansucrase enzymes) or EC 2.4.1.140 (for alternansucrase). We propose to name these enzymes (1→4)-α-β-glucan:(1→4),(1→6)-α-β-glucan α-glucanotransferase enzymes (in short 4,6-α-glucanotransferases). We also propose to divide GH70 family enzymes into two subfamilies in the CAZY database, as has been done for GH13 enzymes (30), namely, glucansucrase enzymes acting on sucrose or on MOS.

The precise in vivo reaction and the physiological function of the GTFB type of enzymes remain to be determined. Clearly, this new group of α-glucanotransferase enzymes provides a valuable asset in the toolbox for saccharide synthesis. The linear oligosaccharides synthesized by GTFB containing α1→4 and α1→6 glycosidic linkages may have interesting physico-chemical properties, which remain to be determined, and may have potentially new applications in the food, cosmetics, and/or pharmaceutical industries. In the future, we will characterize the saccharides synthesized by GTFB in more detail, as well as the characteristic properties of other members of this enzyme group.

ACKNOWLEDGMENTS

We thank Peter Sanders (TNO Quality of Life) for anion-exchange (Dionex) analysis and Rolf Boelens (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University, Utrecht, The Netherlands)
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