Characterization of the First $\alpha$-(1→3) Branching Sucrases of the GH70 Family

Marlène Vuillemin, Marion Claverie, Yoann Brison, Etienne Ševerac, Pauline Bondy, Sandrine Morel, Pierre Monsan, Claire Moulis, and Magali Remaud-Siméon

From the Université de Toulouse, Institut National des Sciences Appliquées (INSA), Université Paul Sabatéï (UPS), Institut National Polytechnique (INP), Laboratoire d'Ingénieries des Systèmes Biologiques et des Procédés (LISBP), 135 Avenue de Rangueil, F-31077 Toulouse, France, CNRS, UMR5504, F-31400 Toulouse, France, and Institut National de la Recherche Agronomique (INRA), UMR792 Ingénierie des Systèmes Biologiques et des Procédés, F-3140 Toulouse, France

Leuconostoc citreum NRRL B-742 has been known for years to produce a highly $\alpha$-(1→3)-branched dextran for which the synthesis had never been elucidated. In this work a gene coding for a putative $\alpha$-transglucosylase of the GH70 family was identified in the reported genome of this bacteria and functionally characterized. From sucrose alone, the corresponding recombinant protein, named BR5, mainly catalyzed sucrose hydrolysis and leucrose synthesis. However, in the presence of sucrose and a dextran acceptor, the enzyme efficiently transferred the glucosyl residue from sucrose to linear $\alpha$-(1→6) dextrins through the specific formation of $\alpha$-(1→3) linkages. To date, BR5 is the first reported $\alpha$-(1→3) branching sucrase. Using a suitable sucrose/dextran ratio, a comb-like dextran with 50% of $\alpha$-(1→3) branching was synthesized, suggesting that BR5 is likely involved in the comb-like dextran produced by L. citreum NRRL B-742. In addition, data mining based on the search for specific sequence motifs allowed the identification of two genes putatively coding for branching sucrases in the genome of L. citreum NRRL B-742. The less soluble polymer contained 75% of $\alpha$-(1→6) linkages and 15% of $\alpha$-(1→4) linkages, whereas the more soluble polymer displayed an uncommon comb-like structure consisting of a linear backbone of $\alpha$-(1→6)-D-glucopyranosyl residues grafted with one $\alpha$-(1→3)-linked glucosyl unit on every glucosyl moiety (5–9). Concomitantly with those findings, dextrans were shown to be synthesized by $\alpha$-transglucosylases, commonly named glucansucrases, and today classified in the GH77 family. The GH70 family belongs to the clan GH-H, together with families GH13 and GH77. To date, 279 $\alpha$-transglucosylase sequences have been reported in the CAZY database, and only 25% has been functionally characterized (11). Furthermore, only four three-dimensional structures of GH70 enzymes have been solved (12–15). The enzymes are organized into five different structural domains, A, B, C, IV, and V. The A, B, and C domains are related to GH13 family enzymes, whereas domains IV and V are GH70-specific domains. The fold of these enzymes is unique, and except for domain C, all domains are formed by non-contiguous fragments of sequences that assemble around a U-turn (15). These enzymes are $\alpha$-retaining enzymes. They mainly catalyze $\alpha$-transglucosylation reactions through a classical double displacement mechanism. The reaction starts with the formation of a $\beta$-D-glucosyl-enzyme intermediate involving a nucleophilic aspartate, an acid-base glutamic acid, and a third aspartate that stabilizes the covalent intermediate. In a second step the intermediate can be intercepted by a water molecule (hydrolysis reaction) or by the hydroxyl group of an acceptor (transgluco-

Numerous lactic acid bacteria from the Leuconostoc genus isolated from different habitats, such as sugar juice, fermenting vegetables, or dairy products, have long been known to produce slimy compounds were termed as dextrans, homopolymers of $\alpha-D$-glucopyranosyl units mainly linked by $\alpha$-(1→6) linkages. Dextrins with a high content of $\alpha$-(1→6) linkages and a low degree of branching found their first industrial applications in the 1940s as a source of synthetic blood volume expanders (3). These findings motivated Jeanes et al. (22) to investigate the diversity of polymers produced during sucrose fermentation by different strains of lactic acid bacteria. A total of 96 strains were screened, and their dextrans were structurally characterized. In this study one strain, Leuconostoc mesenteroides NRRL B-742, also known as L. mesenteroides ATCC 13146 and renamed Leuconostoc citreum NRRL B-742 (4), received particular attention. Indeed, this strain, first isolated from a can of spoiled tomatoes in 1927 (1), was shown to produce two types of dextrans that differed by their structures and degree of solubility from sucrose. The less soluble polymer contained 75% of $\alpha$-(1→6) linkages and 15% of $\alpha$-(1→4) linkages, whereas the more soluble polymer displayed an uncommon comb-like structure consisting of a linear backbone of $\alpha$-(1→6)-D-glucopyranosyl residues grafted with one $\alpha$-(1→3)-linked glucosyl unit on every glucosyl moiety (5–9). Concomitantly with those findings, dextrans were shown to be synthesized by $\alpha$-transglucosylases, commonly named glucansucrases, and today classified in the family 70 of glycoside hydrolases (GH70) (10). The GH70 family belongs to the clan GH-H, together with families GH13 and GH77. To date, 279 $\alpha$-transglucosylase sequences have been reported in the CAZY database, and only 25% has been functionally characterized (11). Furthermore, only four three-dimensional structures of GH70 enzymes have been solved (12–15). The enzymes are organized into five different structural domains, A, B, C, IV, and V. The A, B, and C domains are related to GH13 family enzymes, whereas domains IV and V are GH70-specific domains. The fold of these enzymes is unique, and except for domain C, all domains are formed by non-contiguous fragments of sequences that assemble around a U-turn (15). These enzymes are $\alpha$-retaining enzymes. They mainly catalyze $\alpha$-transglucosylation reactions through a classical double displacement mechanism. The reaction starts with the formation of a $\beta$-D-glucosyl-enzyme intermediate involving a nucleophilic aspartate, an acid-base glutamic acid, and a third aspartate that stabilizes the covalent intermediate. In a second step the intermediate can be intercepted by a water molecule (hydrolysis reaction) or by the hydroxyl group of an acceptor (transgluco-
sylation). The synthesis of the polymer occurs via successive transfers of glucosyl units onto the non-reducing extremity of the growing \(\alpha\)-glucan chain (Scheme 1) (16–18).

Among GH70 sucrose-active enzymes, four subgroups of glucansucrases can be distinguished: the dextransucrases, alternansucrases, mutansucrases, and reuteransucrases, which are all efficient polymerases (10). In addition, one branching sucrase (GBD-CD2) was engineered from \(L.\ citreum\) NRRL B-1299 DSR-E dextransucrase (19). This enzyme displays no polymerase activity but catalyzes \(\alpha-(1\rightarrow3)\) dextran branching very efficiently with single \(\alpha-(1\rightarrow2)\)-linked glucosyl units (Scheme 1) (20). Recently, genome sequencing of \(L.\ citreum\) NRRL B-1299 allowed the identification of the first natural \(\alpha-(1\rightarrow3)\) branching sucrase, BRS-A, that shows the same specificity as GBD-CD2. This enzyme was proposed to be involved in the formation of the native dextrans of \(L.\ citreum\) NRRL B-1299 that contain a high percentage of \(\alpha-(1\rightarrow3)\) linkages (21, 22).

The formation of the regular hyper-branched dextran of \(L.\ citreum\) NRRL B-742 is even more intriguing. Several attempts to isolate the GH70 enzymes of \(L.\ citreum\) NRRL B-742 were reported. However, although the enzyme responsible for the production of dextran with both \(\alpha-(1\rightarrow6)\) and \(\alpha-(1\rightarrow4)\) linkages was isolated, the enzyme contributing to the comb-like structure was never identified (5). Of note, most of the GH70 enzyme activities were found strongly attached to the cell wall, making the enzymes difficult to purify (7). Crude enzyme extracts or sucrose fermentation broths were thus used to produce \(\alpha-(1\rightarrow3)\)-branched glucooligosaccharides from sucrose and maltose (7), and these compounds were shown to possess interesting functional properties. In particular, they stimulated \textit{in vitro} growth of beneficial bacteria such as bifidobacteria or lactobacilli and inhibited the development of pathogens such as \textit{Salmonella} \textit{sp.} or \textit{Escherichia coli} (23, 24). In 2000, Kim and Robyt (25) cloned a gene from the mutant strain \(L.\ citreum\) NRRL B-742CB. The encoded protein, DSR-B-742, shared \(>95\%\) amino acid identity with DSR-B dextransucrase from \(L.\ citreum\) NRRL B-1299 and synthesized a quasilinear dextran with \(>95\%\) of \(\alpha-(1\rightarrow6)\) linkages (26). Thus far the mode of synthesis of the comb-like dextran has remained unknown.

A genomic approach was undertaken to gain insight into the formation of this hyper-branched \(\alpha\)-glucan and to have access to enzymatic tools of interest for functional glucooligosaccharide synthesis. First, the genome of \(L.\ citreum\) NRRL B-742 was sequenced (27) and mined to determine its content in GH70 \(\alpha\)-transglucosylase encoding genes. One putative GH70 gene

\[\alpha-(1\rightarrow6) > 50\%: \text{dextran} \]
\[\alpha-(1\rightarrow3) > 50\%: \text{mutan} \]
\[\text{alternated } \alpha-(1\rightarrow6) \text{ and } \alpha-(1\rightarrow3): \text{alternan} \]
\[\alpha-(1\rightarrow6) \text{ and } \alpha-(1\rightarrow4): \text{reuteran} \]
showing divergence from various consensus sequences of GH70 family enzymes was cloned for recombinant expression in *E. coli*. The corresponding protein, named BRS-B, is an α-(1→3) branching sucrase. A shorter version of BRS-B, named BRS-BΔ1, was rationally designed and characterized. The specificity of BRS-B and its truncated form was investigated and revealed the interest of these enzymes for the production of α-(1→3)-branched dextrans with a controlled degree of branching. In addition, comparison of these enzymes with the recently discovered α-(1→2) branching sucrase (BRS-A) allowed the identification of two enzymes in public databases that were found to catalyze dextran branching and were clustered with previously characterized branching sucrases in the GH70 phylogenetic tree.

**Experimental Procedures**

**Isolation of brsB Gene and Analysis of BRS-B Primary Structure**

The *brsB* gene was identified in the *L. citreum* NRRL B-742 genome (27) (NCBI reference sequence: NZ_CCNG01000013.1) by performing a nucleotide BLAST against a homemade GH70 α-transglucosylase-encoding gene database. The protein sequence of BRS-B is deposited under the GenBankTM accession number CDX65123.1. Protein sequence comparison was performed either using protein Blast (NCBI) against the non-redundant protein sequence database (nr) for local alignments or using Clustal Omega for global alignments with other GH70 enzymes. Protein sequence global alignment with GTF180-N glucansucrase (15) and ΔN122-GBD-CD2 (12) was used to determine length and position of domains A, B, C, IV, and V. The signal peptide cleavage was predicted using SignalP 4.1 server.

**Isolation of Genomic DNA**

*L. citreum* NRRL B-742 was grown on 1.5 ml of MRS medium (Sigma-Aldrich) at 30 °C overnight. Genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega, Madison, WI) according to the supplier’s recommendations for Gram-positive bacteria.

**Cloning of brsB and brsBΔ1 Genes**

The gene *brsB* and a truncated form lacking the signal peptide and the last 575 amino acids at the C terminus of the protein (*brsBΔ1*) were amplified by PCR from *L. citreum* NRRL B-742 genomic DNA template. The primers used are described in Table 1. The addition of the CACC sequence (underlined) to the 5′-forward primers allowed the correct insertion of the genes into the pENTR/D-TOPO vector (Life Technologies). From a positive entry clone, LR recombination (Gateway LR Clonase II enzyme mix, Life Technologies) was performed using the destination vectors described in Table 1. Expression clones were selected on LB agar plates supplemented with 100 μg ml⁻¹ of ampicillin. Plasmids were extracted with the Sigma GenElute HP Plasmid Miniprep kit, verified by restriction analyses and sequencing (GATC Biotech, Constance, Germany).

**Recombinant Production of BRS-B and BRS-BΔ1**

**Starter Culture**—*E. coli* BL21-AI and *E. coli* BL21 star DE3 cells (detailed in Table 1) were freshly transformed by pBAD
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49/brsB and pET-55/brsBΔ1, respectively. Thirty milliliters of LB medium, supplemented with ampicillin (100 μg ml⁻¹), were inoculated with 200 μl of transformation mix and incubated overnight at 37 °C under agitation (200 rpm).

Erlenmeyer Flask Cultures—One liter of modified ZYM5052 medium (28) contained (i) 0% lactose, 0% glucose, 0.5% glycerol and 0.01% L-arabinose for BRS-B production or (ii) 0.1% lactose, 0% glucose and 1% glycerol for BRS-B-Δ1 production. Both were supplemented with ampicillin (100 μg ml⁻¹) and were inoculated with the corresponding starter culture at an A₆0₀nm of 0.05. Cultures were incubated at 21 °C under agitation (150 rpm). After a 26-h incubation, cells were harvested by centrifugation, dispersed in 50 mM sodium acetate buffer, pH 7.5, at a final A₆0₀nm 80 and disrupted by sonication. The recombinant enzymes were recovered in the soluble fraction after centrifugation (15,000 × g, 30 min, 4 °C) of the crude cell extract.

Affinity Chromatography Purification of Enzymes

All purification assays were performed using the ÄKTAxpress (GE Healthcare) at 12 °C. For His₈, tag affinity chromatography, cells were centrifuged and resuspended in binding buffer (20 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, 20 mM imidazole, 2.5% (v/v) glycerol) at a final A₆0₀nm of 200. After disruption by sonication, centrifugation (30,000 × g, 30 min, 4 °C), and filtration through a 0.22-μm cartridge (Sartorius Stedim Biotech, Aubagne, France), lysates were applied onto a 1-ml HisTrap HP® column (GE Healthcare) that had been equilibrated with the binding buffer. The proteins were eluted by an imidazole gradient from 10 to 500 mM over 25 min. Eluate fractions of 3 ml were desalted onto a 10-DG column (Bio-Rad) equilibrated with the binding buffer. The proteins were eluted with 50 mM sodium acetate buffer, pH 5.75, supplemented with 250 μM ml⁻¹ bovine serum albumin, 23 μg ml⁻¹ of purified BRS-B-Δ1 at 30 °C and using initial sucrose concentrations ranging from 3 to 600 mM. All initial velocities were measured by quantifying fructose release at the early stage of reaction (when <5% of the substrate is consumed and when leucrose and other oligosaccharide production can be neglected). At regular time intervals samples were withdrawn and immediately heated at 95 °C for 5 min to stop the reaction. Fructose concentration was determined by HPLC as described above. Kₘ, Kᵢ, and Vₘₐₓ parameters were determined using SigmaPlot Kinetics software and fitted to a non-competitive substrate inhibition model.

α-(1→3) Transglucosylation—Kinetic parameters for α-(1→3) transglucosylation were determined at 30 °C in 50 mM sodium acetate buffer, pH 5.75, supplemented with 250 μg ml⁻¹ BSA, 9.5 mg liter⁻¹ of enzyme, 300 mM sucrose, and dextran of 68,400 g mol⁻¹ at final concentrations ranging from 31 to 617 mM. For the sake of clarity, dextran concentrations will be expressed as molar concentrations of anhydroglucosyl units within the dextran, as determined by dividing dextran mass concentrations by 162 g mol⁻¹. All initial velocities were measured when <5% of the sucrose was consumed. Initial α-(1→3) transglucosylation velocities were calculated by subtracting fructose (representing the total activity) by free glucose (representing hydrolysis activity) production rates. Glucose and fructose concentrations were determined by HPLC using the same conditions as those described previously. Kₘₐₓ, Vₘₐₓ were estimated using SigmaPlot Kinetics software and fitted to a Michaelis-Menten model.

Synthesis of α-(1→3)-branched Dextran Using BRS-B and BRS-B-Δ1

Various α-(1→6)-linked dextrans of average molar masses ranging from 1500 to 2 × 10⁶ g mol⁻¹ (Sigma) were incubated at different concentrations with sucrose (9–438 mM) using 1 unit ml⁻¹ BRS-B (or BRS-B-Δ1) at 30 °C in 50 mM sodium acetate buffer at pH 5.75 for 8–16 h. Reactions were stopped by a 5-min incubation at 95 °C. The various tested sucrose/dextran ratios are reported in supplemental Table S1.

HPAEC-PAD Analysis

To determine production yield and assay sucrose depletion, reaction media were analyzed by HPAEC-PAD (high performance anion exchange chromatography with pulsed ampero-
metric detection) using a CarboPac™ PA100 (4 × 250 mm) analytical column coupled with a CarboPac™ PA-100 guard (4 × 50 mm). Glucose, fructose, leucrose, and sucrose were separated using a 30-min sodium acetate gradient from 6 to 300 mM in 150 mM NaOH. The amount of each sugar was determined using standards of 5, 10, 15, and 20 mg kg⁻¹. Samples were prepared in ultra-pure water and diluted to a final concentration of 10–15 mg kg⁻¹ of total sugars for quantitative analyses and a final concentration of 250 mg kg⁻¹ (total sugars) for qualitative analyses. The percentage of glucosyl units from sucrose incorporated into glucose (%Gglucose) or leucrose (%Gleucrose) was calculated as follows.

\[
\%G_{\text{glucose}} = \frac{\text{Area}_{\text{dextran}}}{\text{Area}_{\text{sucrose}}} \times 342 \times 100 \tag{Eq. 1}
\]

\[
\%G_{\text{leucrose}} = \frac{\text{Area}_{\text{dextran}}}{\text{Area}_{\text{sucrose}}} \times 180 \times 100 \tag{Eq. 2}
\]

**High Performance Size Exclusion Chromatography (HPSEC) Analysis**

HPSEC analyses were carried out using Shodex OH-Pak SB-802.5 and SB-805 columns in series coupled with a Shodex OH-Pak SB-G guard column and placed in an oven at 70 °C. Elution was performed using 0.45 M NaNO₃ and 1% ethylene glycol (v/v) as the eluent using a flow rate of 0.3 ml min⁻¹. The samples were diluted at 10 g kg⁻¹ of total sugars and also in 0.45 M NaNO₃ and 1% ethylene glycol.

The percentage of glucosyl units from sucrose incorporated into α-(1→3)-branched dextran was calculated as,

\[
\%G_{\text{dextran}} = \frac{\text{Area}_{\text{dextran}}}{\text{Area}_{\text{sucrose}}} \times 342 \tag{Eq. 3}
\]

The percentage of glucosyl units from sucrose incorporated into small oligosaccharides was deduced as,

\[
\%G_{\text{small oligosaccharides}} = 100\% - (\%G_{\text{dextran}} + \%G_{\text{glucose}} + \%G_{\text{leucrose}}) \tag{Eq. 4}
\]

Here %Gdextran, %Gglucose, and %Gleucrose have been determined from HPSEC and HPAEC-PAD analyses.

**¹H and ¹³C NMR Analysis of Oligosaccharides and Polymers**

To determine α-(1→3) linkage content, freeze-dried polymer samples were dispersed in deuterated water at a final concentration of 20 mg ml⁻¹. NMR spectra were recorded on an Advance 500 MHz spectrometer (Bruker) operating at 500 MHz for ¹H NMR and 125 MHz for ¹³C using a 5-mm z-gradient TBI probe. The data were processed using TopSpin 3 software. One-dimensional ¹H NMR spectra were acquired by using a zgpr pulse sequence (with water suppression). All measurements were performed at 298 K, and chemical shifts were referenced to an internal reference sodium 2,2,3,3-tetramethylsilanol-3-trimethylsilylpropanoate (¹H = 0 ppm) and acetone (¹³C = 31.08 ppm). The percentages of α(1→3) and α(1→6) linkages in α-glucans were calculated by integrating the corresponding anomeric proton signals (30).

**Model Construction**

Sequence alignment between BRS-B (from residues Ile-220 to Ile-1300) and ΔN123-GBD-CD2 was generated using MUSCLE (31). This alignment was checked, locally corrected in variable loop regions, and submitted to SWISS-MODEL online service for protein structure prediction using the alignment mode (32). The same procedure was applied for the sequence of BRS-A (from residues Gly-223 to Val-1289). BRS-B and ΔN123-GBD-CD2 share 53% sequence identity; BRS-A and ΔN123-GBD-CD2 share 60% sequence identity. All drawings were realized using PyMOL software (DeLano Scientific).

**Identification of New Branching Sucrases**

Blast analysis (blastp and blastn) were performed to identify new putative branching sucrases in NCBI public databases. The putative proteins displaying similarities with characterized branching sucrases (i.e. BRS-A, BRS-B, and/or GBD-CD2) were selected considering that the sequence should display (i) one phenylalanine at subsite +1 (position 675, BRS-B numbering), (ii) a non-aromatic residue at subsite +1 (position 711, BRS-B numbering), (iii) one isoleucine at position 783 (BRS-B numbering), (iv) one histidine at position 785 (BRS-B numbering), (v) one lysine at position 789 (BRS-B numbering), and (vi) one valine at position 795 (BRS-B numbering).

**Cloning of the brsC Gene**

First, a synthetic brsC gene (contig GenBank™ accession number AEIZ01000002, positions 68,715–63,391 on reverse strand) was designed to optimize its expression in *E. coli* (GeneCust, Dudelange, Luxembourg). The gene was then amplified by PCR from pUC57/*brsC* plasmid DNA template using the primers described in Table 1. The PCR product was then inserted into the pENTR/*brsC* plasmid vector (Life Technologies). From a positive entry clone, LR recombination (Gateway LR Clonase II enzyme mix; Life Technologies) was performed with the pET-55-DEST destination vector (Merck Millipore). Expression clones were selected on LB agar plates supplemented with 100 µg ml⁻¹ of ampicillin. Plasmids were then extracted using the GenElute HP Plasmid Miniprep kit (Sigma), verified by restriction analyses, and sequenced (GATC Biotech). *E. coli* TOP10 competent cells (Life Technologies) were used for all cloning experiments.

**Recombinant Production of BRS-C and BRS-D Branching Sucrases**

The construction pET28b/*brD* was provided by Biotomik (Cambridge, ON, Canada) where the *brsD* gene (GenBank™ accession number AZBY01000038.1, locus tag LAKU_38c00010) was designed to optimize the heterologous expression in *E. coli*.

**Starter Culture—*E. coli* BL21** Star DE3 cells were freshly inoculated with 200 ml of LB medium supplemented with ampicillin (100 µg ml⁻¹) were inoculated with 200 µl of transformation mix and incubated overnight at 37 °C under agitation (200 rpm).
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**ORF**

| Protein | 567 | 3606 | 4356 | 789 | 6540 | 849 | 5667 | 5808 |
|---------|-----|------|------|-----|------|-----|------|------|

**Blastn (best blast hit)**

| Protein | L. citreum NRRL B-1299 | - | L. citreum NRRL B-1299 | L. citreum B110-1-2 |
|---------|--------------------------|---|--------------------------|---------------------|
| 99% id dsrA | 99% id dsrE | 76% id brsA L. citreum NRRL B-1299 | 99% id dsrF L. citreum B110-1-2 |

**Specificity**

| Specificity | Dextranucrase (Putative) | Dextranucrase (Characterized) | Dextranucrase (Putative) | Characterized herein | Dextranucrase (Putative) |
|------------|--------------------------|-----------------------------|--------------------------|---------------------|--------------------------|

**Results and Discussion**

**Characterization of a Novel GH70 Branching Sucrase from L. citreum NRRL B-742**—The analysis of L. citreum NRRL B-742 genome (27) revealed the presence of five putative α-transglucosylase encoding genes: (i) the dsrB-742 gene previously isolated by Kim et al. (26), (ii) three additional genes, dsrA-like, dsrE-like, and dsrF-like, that share 99% identity with the characterized dextranucrase genes from L. citreum NRRL B-1299 and B110-1-2 (33–35), and (iii) one gene of 5667 bp, named brsB, putatively encoding a 1888-amino acid protein showing 76% of amino acid identity and 83% of similarity with BRS-A from L. citreum NRRL B-1299 (21) (GenBankTM accession number WP_040177263.1, best Blast hit against the non-redundant (nr) protein database) (Fig. 1).

For BRS-B, a signal peptide with a cleavage site between positions 39 and 40 was predicted using SignalP 4.1 server. Based on sequence alignment, the protein was predicted to belong to the GH70 enzyme family and to adopt the same structure as those displayed by other GH70 enzymes (12–14), which consist of five structural domains A, B, C, IV, and V, of which the limits are located in catalytic domain A. By analogy with other GH70 -D-glucosyl-enzyme intermediate are conserved in the BRS-B sequence and the transition state stabilizer, respectively. Domain V is formed by two fragments, one at the N terminus and...
the other at the C-terminal extremity of the protein, and is predicted to constitute both extremities of the U-shape fold (15). Like most of the domains V of GH70 enzymes, this domain is rich in YG repeats that were previously suggested to play a key role in interactions between glucansucrases and their polymers (36). Eight and two YG repeats that could be involved in α-glucan binding were identified at the N- and C-terminal extremities, respectively (Fig. 2). Additionally, at the C terminus, 6 APY repeats were identified and shared 99% of identity with those found at the C-terminal ends of the alternansucrase (ASR) from L. citreum NRRL B-1355 and the branching sucrase BRS-A from L. citreum NRRL B-1299 (513 out of 518 amino acids) (37, 38). The APY repeats of alternansucrase (ASR) were previously deleted without causing any alteration of enzyme activity or specificity (39).

The brsB gene was cloned into pBAD-DEST49 vectors and expressed in E. coli BL21-AI host strain to characterize the corresponding protein. The level of production reached 700 units/liter of culture. Western blot analyses confirmed that the full-length BRS-B was the most abundant protein, even if slightly degraded forms could also be observed (data not shown).

As the native E. coli BL21-AI does not produce any sucrose-active enzymes, the sonication extract was used to characterize the catalytic activities of BRS-B. The optimal temperature and pH were estimated at 30–33 °C and pH 5.5–6 (data not shown). All subsequent assays were thus performed at 30 °C and at pH 5.75. Enzymatic reactions were first carried out in the presence of 292 mM sucrose, the natural substrate for most of GH70 enzymes. The main reaction products formed were glucose and leucrose (O-α-D-glucopyranosyl-(1→5)-D-fructopyranose). A total of 51% of the glucosyl units from sucrose were incorporated into leucrose, and 33% were transferred onto water (hydrolysis reaction). The remaining part, representing 16% of the transferable glucosyl units, was incorporated into various small glycocoligosaccharides, which were not isolated and characterized due to their very low level of production (supplemental Fig. S1). No trace of high molar mass α-glucans was detected using HPSEC-RI, indicating that BRS-B is not an efficient polymerase.

Acceptor reactions were carried out using 1500 g·mol⁻¹ dextran as the acceptor (66.6 m/s) and sucrose as the donor (292 m/s). As seen on HPAEC-PAD chromatograms, the product profile rapidly changed during the course of the reaction (Fig. 3a). The intensity of the peaks corresponding to linear iso-maltooligosaccharides decreased, whereas new signals were clearly visible. The products synthesized after total sucrose depletion were analyzed by NMR spectroscopy. The 1H NMR spectrum showed two peaks assigned to anomeric resonances at 4.99 ppm and 5.33 ppm, which correspond to the anomeric proton of a glucosyl unit engaged in α-(1→6) or α-(1→3) linkages, respectively. Integration of the anomeric signals showed that the amount of α-(1→3) linkages in dextran reached 39% that of the total osidic linkages (Fig. 3b). These results demonstrate that BRS-B catalyzes the transglucosylation reaction from sucrose onto dextran acceptors through the formation of α-(1→3) linkages. Several dextrans of higher molar mass between 39,100 g·mol⁻¹ and 2 × 10⁶ g·mol⁻¹ were also tested as acceptors. All of them were efficiently branched by BRS-B, as evidenced by the percentage of α-(1→3) linkages reaching a maximum of 50%, indicating that each glucosyl moiety of the α-(1→6) backbone was substituted by an α-(1→3)-linked glucosyl residue (Fig. 4a).

The J-Mod spectrum of the dextran shows two anomic 13C chemical shifts at 98.63 ppm and 100.30 ppm corresponding to the 1H anomeric signals at 4.98 ppm and 5.33 ppm of the α-(1→6) and α-(1→3)-linked glucosyl units, respectively. The presence of α-(1→3)-linked branching was further attested by the typical signal at 81.37 ppm, which was assigned to C-3 of the 3,6-O-disubstituted glucopyranosyl units (9, 40). Only one type of resonance is observed at 100.30 ppm, indicating that the α-(1→3) linkages of the dextran are solely branching linkages. This is confirmed by the presence of only one chemical shift at 61.20 ppm attributed to free C6. At 66.33 ppm, the resonances are attributed to bound C6 of the 3,6-O-disubstituted and 6-O-disubstituted glucosyl units of the main chain (Fig. 4b).

To date, BRS-B is the first reported α-(1→3) branching sucrase. Its ability to branch high molar mass dextran up to 50% suggests that BRS-B is likely involved in the synthesis of L. citreum NRRL B-742 comb-like dextran. Based on sequence alignment, the other putative GH70 enzymes encoded by L. citreum NRRL B-742 are predicted to be dextransucrases synthesizing linear dextrans. Dextransucrase activities were previously isolated in the fermentation broth of L. citreum NRRL B-742 (5, 22, 26). It can thus be assumed that the synthesis of the hyper-branched dextran is due to the synergistic action of at least one dextransucrase and the branching enzyme, BRS-B. The dextransucrase would synthesize the α-(1→6) backbone, and the α-(1→3) branching sucrose would be involved in branching via α-(1→3) osidic bond formation. There are several questions that remain, including understanding which dextransucrases of L. citreum NRRL B-742 work in tandem with BSR-B. Additionally, it is not known whether or not elongation and branching are concomitant. These pending questions require additional experiments, such as RT-quantitative PCR studies, secretome analysis at regular intervals of the culture, and knock-out of either polymerases or branching sucrase encoding genes combined with structural analyses of the produced dextran.

Construction of BRS-BΔ1, a Truncated Form of BRS-B—Despite several attempts, the purification of BRS-B did not give satisfying results due to very weak protein binding onto nickel and cobalt affinity resins. This was attributed to an inadequate exposure of the tags, which could prevent correct interaction with the resin. To facilitate the purification process, a shorter form deleted of the signal peptide and all APY motifs at the C-terminal end, was constructed (Fig. 2). Notably, 8 of the 10 identified YG repeats were conserved. The position of the truncations at the C- and N terminus ends were determined from sequence alignment with the α-(1→2) branching sucrase ΔN₁₂₃ GBG-CD2. The production of the truncated protein BRS-BΔ1 was optimized to a level of 1200 units/liter using pET-55-DEST and BL21 Star DE3 E. coli strain. Western blot analysis showed that the protein was not degraded at the N-terminal.
extremity. The C-terminal His₆ tag was then used to purify the enzyme onto Ni-NTA affinity resin. The purification yield reached 85%, with a corresponding purification factor of 17. BRS-B-specific activity on sucrose (292 mM) was estimated at 24 units/mg of protein. The optimal temperature and pH were determined to be between 33 and 40 °C and 5–5.75, respectively (Fig. 5, a and b) and are in the same range as those observed for the non-purified enzyme BRS-B. A slight improvement of transferase activity (near 6%) was observed with the addition of 1 mM Ca²⁺ (Fig. 5c), as previously described for the branching sucrase ΔN123-GBD-CD2 (increase of 13% (12)). In contrast, Zn²⁺ ions had the most deleterious effect on BRS-B-Δ1 activity, which decreased by 23% in the presence of Zn²⁺ ions (Fig. 5c). From a sucrose substrate, BRS-B-Δ1 showed a slightly higher hydrolytic activity than BRS-B and produced 42% glucose, 48% leucrose, and 10% of small oligosaccharides. The profiles of products obtained with dextran acceptors were similar to those obtained with the full-length protein (data not shown). Of note, the deletion of the APY repeats at the C terminus extremity of the protein did not affect the enzyme specificity and efficiency, as previously observed with the alternan-sucrase and inulosucrase (39, 41).
FIGURE 4. $^1$H NMR (a) and $^{13}$C NMR (b) analyses of the purified branched dextran of $2 \times 10^8$ g mol$^{-1}$ synthesized by BRS-B. $^{13}$C chemical shifts are given using acetone as reference ($^{13}$C = 31.08 ppm).
The kinetic parameters of the recombinant protein BRS-B-H9004 were determined with and without dextran acceptor. Without the acceptor, sucrose hydrolysis was inhibited by 150 mM sucrose, and the kinetic data fitted a non-competitive substrate inhibition modeled by Equation 1 with a $K_m$, $K_i$, and $k_{cat}$ values estimated at 7.0 ± 0.7 mM, 595 ± 88 mM, and at 81.4 s$^{-1}$, respectively. An initial rate of transglucosylation was determined at a fixed sucrose concentration (300 mM) and using dextran acceptor (68,400 g mol$^{-1}$) concentrations varying from 31 to 617 mM (in anhydroglucosyl unit equivalents). A classical Michaelis-Menten profile was obtained with $K_m$ dextran and $k_{cat}$ values of 0.27 ± 0.08 mM and 949 s$^{-1}$, respectively. A 12-fold increase of the catalytic constant was observed compared with the reaction without dextran (Table 2). The formation of $\alpha$-(1→3)-branches onto dextran molecules is very efficient ($k_{cat}/K_m$ dextran = 3514 s$^{-1}$ mM$^{-1}$), and the $k_{cat}$ value is close to that reported for $\alpha$-(1→2) branching catalyzed by the $\alpha$-(1→2) branching sucrase, GBD-CD2 (20). An initial sucrose/dextran ratio higher than three has to be used to obtain a highly branched dextran. By controlling the succrose:acceptor ratio and the size of the dextran acceptor, a large array of products can be produced, opening the possibility of new applications for these kinds of molecules.

The kinetic parameters of the recombinant protein BRS-B-D1 were determined with and without dextran acceptor. Without the acceptor, sucrose hydrolysis was inhibited by >150 mM sucrose, and the kinetic data fitted a non-competitive substrate inhibition modeled by Equation 1 with a $K_m$, $K_i$, and $k_{cat}$ values estimated at 7.0 ± 0.7 mM, 595 ± 88 mM, and at 81.4 s$^{-1}$, respectively. An initial rate of transglucosylation was determined at a fixed sucrose concentration (300 mM) and using dextran acceptor (68,400 g mol$^{-1}$) concentrations varying from 31 to 617 mM (in anhydroglucosyl unit equivalents). A classical Michaelis-Menten profile was obtained with $K_m$ dextran and $k_{cat}$ values of 0.27 ± 0.08 mM and 949 s$^{-1}$, respectively. A 12-fold increase of the catalytic constant was observed compared with the reaction without dextran (Table 2). The formation of $\alpha$-(1→3)-branches onto dextran molecules is very efficient ($k_{cat}/K_m$ dextran = 3514 s$^{-1}$ mM$^{-1}$), and the $k_{cat}$ value is close to that reported for $\alpha$-(1→2) branching catalyzed by the $\alpha$-(1→2) branching sucrase, GBD-CD2 (20). An initial sucrose/dextran ratio higher than three has to be used to obtain a highly branched dextran. By controlling the succrose:acceptor ratio and the size of the dextran acceptor, a large array of products can be produced, opening the possibility of new applications for these kinds of molecules.

**Comparison of BRS-B with Other Branching Sucrases and Glucansucrases**—The four functional sequence signatures (motifs I-IV), usually well conserved in the GH70 family and also found in related the GH13 family (10), were aligned with the corresponding motifs found in the characterized GH70 enzymes from other organisms (Table 3). Interestingly, BRS-B exhibits numerous sequence similarities with GBD-CD2 and BRS-A from *L. citreum* NRRL B-1299, two branching sucrases (19, 21). In particular, Phe-675 of motif II and Ile-783, His-785, Lys-789, and Val-795 of motif IV are only conserved in the branching sucrases. Other amino acids are uniquely found in BRS-B. They include residues 673-IS of motif II, 711-PKGE of motif 796-IH of motif IV, and F1184 of motif I (Table 3). The three-dimensional model of BRS-B was constructed by homology modeling using ΔN$_{123}$-GBD-CD2 branching sucrase as template (Fig. 7). The model was compared with the 3D-structures of ΔN$_{123}$-GBD-CD2 (PDB: 3TTQ) and with the inactive mutant of GTF-180-ΔN in complex with sucrose substrate (PDB: 3HZ3). According to the model, BRS-B also adopts a U-shape fold organized in five domains. As shown in Fig. 7,
First α-(1→3) Branching Sucrases from the GH70 Family

**TABLE 2**
Kinetic parameters of truncated variant BRS-B−Δ1 for hydrolysis reaction (in the presence of sucrose) and for α-(1→3) transglucosylation (in the presence of sucrose and dextran 68.4 kg·mol⁻¹⁻¹)

| Enzyme                | $K_m$ sucrose | $K_m$ dex | $V_{max}$ | $k_{cat}$ |
|-----------------------|---------------|-----------|-----------|-----------|
|                       | mM            | mM of anhydroglucosyl units | μmol mg⁻¹ min⁻¹ | s⁻¹       |
| Sucrose               |               |           |           |           |
| BRS-B−Δ1              | 7.0 ± 0.7     | 0.7       | 34.5 ± 1.1 | 81.4      |
| ΔN₁₃₋₋₋₋₋₋₋₋₋₋−Δ−Δ    | 7.5 ± 1.0     |           | 36.3 ± 0.6 | 76        |
| GBD-CD2               | 10.8 ± 0.8    |           | 34.6 ± 0.5 | 109       |
| Sucrose and dextran (68.4 kg·mol⁻¹⁻¹) | | | | |
| BRS-B−Δ1              | 206 ± 34      | 112 ± 33  | 0.27 ± 0.08 | 949 |
| ΔN₁₃₋₋₋₋₋₋₋₋₋₋−Δ−Δ    | 125 ± 21      | 0.3 ± 0.05 | 462 ± 45  | 947 |
| GBD-CD2               | 42 ± 2        | 75 ± 3    | 0.174      | 970       |

**TABLE 3**
Sequence alignment of the conserved motifs (I–IV) of BRS-C and BBRS-D catalytic core with other characterized GH70 enzymes

| Motif II | Motif III | Motif IV | Motif I | Spe |
|----------|-----------|----------|---------|-----|
| GTF-I [Sd] 2,3 | SIRVDADVNDV | 486 | HVSIVEARNSDN | 559 | FARAHDEQVLIRDA | 931 | ADWPDQ | M |
| GTF-B [Sm] | 1011 | 1048 | HLSILEDNGK | 762 | FVRAHDSDQDIPIK | 1168 | ADWPDQ |
| GTF-A [Lr] | 1020 | 1056 | HINILEDWNHA | 1128 | FVRAHDNNSDQIQA | 1508 | ADWPDQ |
| GTF-O [Lc] | 1020 | 1056 | HINILEDWNSS | 1128 | FVRAHDNNSDQIQA | 1508 | ADWPDQ |
| ASR [Lm] | 631 | 668 | HLSILEDNGK | 762 | FVRAHDSDQDIPIK | 1168 | ADWPDQ |
| GTF-W [Lr] | 748 | 785 | HLSYNEGYHSG | 568 | FVNHQDRQ-KNVINQ | 1216 | EDLVMNQ |
| GTF-ML4 [Lr] | 1012 | 1049 | HLSYNEGYHSG | 1121 | FVNHQDRQ-KNLINR | 1479 | EDLVMNQ |
| GTF-180 [Lr] | 1021 | 1058 | HLSILEDNGW | 1131 | FVRAHSAQIQIRQ | 1503 | ADWPDQ |
| DSR-S [Lm] | 547 | 584 | HLSILEWSHN | 657 | FVRAHSDSEQVIAQ | 1023 | ADWPDQ |
| GBD-CD2 [Lc] | 2206 | 2243 | HLSILEAGLA | 2317 | IIHAHKGQGEVGA | 2688 | ADVVDQ |
| BRS-A [Lc] | 668 | 705 | HLSHVEGLDS | 799 | IIHAHKDQIRGVA | 1151 | ADVANQ |
| BRS-B [Lc] | 667 | 704 | HLSHVEKPG | 783 | IVHAKDQIDTVIH | 1182 | ADVFANQ |
| BRS-C [Lc] | 724 | 771 | HLSHVEADQ | 845 | IVHAKDQIDVSN | 1232 | ADVFANQ |
| BRS-D [LbK] | 520 | 557 | HLSHVEGGVA | 638 | IVHAKDQIEKNGV | 1010 | ADVYNQ |

1 Spe, Specificity. Enzymes are grouped together according to their linkage specificity: M for mutansucrase, R for reuteransucrase, A for alternansucrase, 4,6 GT for α-4,6 glucanotransferase, D for dextranucrase, BS for branching sucrase.
2 The two catalytic residues are indicated in bold red the nucleophile (D) and the acid/base catalyst (E). The transition state stabilizer (D) is represented in bold blue. The residues in bold green and in bold orange correspond to residues of substrate binding subsites −1 and +1, respectively, as originally identified in GTF180ΔN: sucrose complex (PDB entry 3HZ3) (15).
3 [Sd] Streptococcus downei, [Sm], Streptococcus mutans, [Lr], Lactobacillus reuteri, [Lm], L. mesenteroides, [Lc], L. citreum.
the BRS-B model displays loops in the vicinity of the catalytic gorge, which differs in length and amino acid composition in comparison with Domain A of BRS-B and Domain B of BRS-B are represented in blue and green, respectively.

Then, we compared the active site residues delineating the subsites −1 and +1 of BRS-B with those of GTF-180-ΔN and N123-GBD-CD2. As shown in Fig. 8, the residues of subsite −1 are all conserved. In subsite +1, BRS-B and N123-GBD-CD2 only differ by one residue: P711 is replaced by G2250 in N123-GBD-CD2. In contrast, only a few residues constituting subsite +1 of GTF-180-ΔN are conserved in BRS-B (i.e., Q792, D788, and E709, BRS-B numbering). In particular, N1029 and W1065 of N123-GBD-CD2 are not conserved in BRS-B. N1029 is replaced by Phe-675 (Fig. 8a). Notably, one phenylalanine was also found in N123-GBD-CD2, and was shown to be critical for dextran branching (12). Moreover, comparison of BRS-B and GTF-180 backbone, also revealed a rearrangement around residues A710 and P711 that was observed in N123-GBD-CD2. This rearrangement was previously suggested to be responsible for a weaker binding of the fructosyl ring in branching sucrases (12). The identification of the determinants for α-(1→3) branching specificity remains difficult in the absence of three-dimensional structures of BRS-B in apo form or in complex with sucrose or
dextran. However, according to the structural comparison of branching enzymes and polymerases, amino acids can be pointed out that constitute interesting targets for mutation to assess their role in the branching specificity.

**Searching for New Branching Sucrases in Public Databases**—The emergence of natural branching activities through the isolation and characterization of BRS-A (21) and BRS-B in *L. citreum* raised the question of the representation of such type of activity in lactic acid bacteria clade. To shed some light on this issue, a blast search against public sequence databases were carried out to ascertain which sequences contain the residues uniquely conserved in branching enzyme catalytic cores. Two new putative branching sucrases (renamed BRS-C and BRS-D) were identified, one in *Leuconostoc fallax* KCTC3537 and the other in *Lactobacillus kunkeei* EFB6. The percentage of amino acid identity and similarity with BRS-A and BRS-B is detailed in supporting information (supplemental Table S2), and the alignment of the conserved motifs is presented in Table 3. Based on the sequence alignment, the two putative branching sucrases are predicted to possess the catalytic triad DED and the five structural domains, A, B, C, IV, and V, specific to the GH70 family enzymes (supplemental Fig. S2). Similar to the characterized BRS-A (21) and BRS-B, YG repeats (36) were mainly identified at the N-terminal extremity of the protein. All newly identified genes encoding putative branching sucrases are located on the reverse strand of their genome, as previously observed for *brsA* in *L. citreum* NRRL B-1299 genome (21) and *brsB* in *L. citreum* NRRL B-742 genome. All of them are organized in tandem with another putative glucansucrase (supplemental Fig. S3).

**FIGURE 9. Analysis of the products synthesized using BRS-C and BRS-D enzymes from sucrose (292 mM) and dextran 1500 g mol⁻¹ (66.6 mM).**

- **a.** comparison of BRS-B and BRS-C HPAEC-PAD chromatograms. (**1**) Enzymatic reaction at the initial time (t = 0 min), (**2**) enzymatic reaction using BRS-B stopped after 8 h, and (**3**) enzymatic reaction using BRS-C stopped after 8 h. G, peak corresponding to glucose fructose (F), leucrose (L), sucrose (S), and isomaltooligosaccharides with a degree of polymerization (DP) of x. c. ¹H NMR spectrum of the products synthesized by BRS-B (1) and BRS-C (2) at 500 MHz in D₂O leucrose (Leu), α-D-glucose (α-D-Glc), β-D-glucose (β-D-Glc), α-(1→6)-linked glucosyl residues (α-(1→6)), and α-(1→3)-linked glucosyl residues (α-(1→3)). d. ¹H NMR spectrum of the products synthesized by BRS-A (1) and BRS-D (2) at 500 MHz in D₂O. The ¹H NMR spectra of the BRS-A modified dextran displayed all the chemical shift characteristics of α-(1→3)-branched dextran. The anomeric region of the spectrum contained three main resonances corresponding, respectively, to anomeric resonance of α-(1→6)-linked β-Glcp residues of the main linear chain with free carbon 2 (4.98 ppm, peak C), to anomeric resonance from α-(1→3)-linked β-Glcp residues of the main linear chain, with the carbon 2 involved in an α-(1→2) linkage with a branched glucosyl unit (5.18 ppm, peak B), to anomeric resonance from α-(1→2)-linked β-Glcp (5.11 ppm, branching points, peak D).

Acid identity and similarity with BRS-A and BRS-B is detailed in supporting information (supplemental Table S2), and the alignment of the conserved motifs is presented in Table 3. Based on the sequence alignment, the two putative branching sucrases are predicted to possess the catalytic triad DED and the five structural domains, A, B, C, IV, and V, specific to the GH70 family enzymes (supplemental Fig. S2). Similar to the characterized BRS-A (21) and BRS-B, YG repeats (36) were mainly identified at the N-terminal extremity of the protein. All newly identified genes encoding putative branching sucrases are located on the reverse strand of their genome, as previously observed for *brsA* in *L. citreum* NRRL B-1299 genome (21) and *brsB* in *L. citreum* NRRL B-742 genome. All of them are organized in tandem with another putative glucansucrase (supplemental Fig. S3).
Characterization of Two New Putative Branching Sucrases and Phylogenetic Analysis—The genes encoding the putative branching sucrases were expressed in *E. coli*. The production levels of the two new proteins are reported in supplemental Table S3. As already observed for BRS-A, BRS-B, and GBD-CD2, the putative BRS-C and BRS-D mainly produced glucose, leucrose, and a low proportion of small oligosaccharides from sucrose substrate (for detailed production yields, see supplemental Table S4). Acceptor reactions were then performed using sucrose and a small dextran of 1500 g/mol as an acceptor. At the end of the reaction, HPAEC-PAD and NMR analyses demonstrated that dextran was modified by BRS-C and BRS-D (Fig. 9). BRS-C from *L. fallax* KCTC 3537 catalyzed (1→3) transglucosylation from sucrose to dextrans, whereas BRS-D from *L. kunkeei* EFB6 was specific for (1→2) transglucosylation. These findings reveal for the first time that the branching specificity is not restricted to *L. citreum* sp. but is also present in *L. fallax* as well as in *L. kunkeei*. Interestingly, the phylogenetic analyses show that the branching sucrases cluster together in a distinct branch (Fig. 10). In addition, the branching sucrase subtree is divided into two subgroups, one corresponding to enzymes showing an α-(1→2) linkage specificity and the other to enzymes with α-(1→3) linkage specificity.

The discovery of the new branching sucrases expands the natural repertoire of the characterized GH70 α-transglucosylases. For years the GH70 family has been mainly known to house glucansucrases. Recently, a new subgroup was disclosed that contains 4,6-α-glucanotransferases, a type of α-transglucosylases that uses (1→4)-linked glucans as a donor instead of sucrose (42, 43). According to our biochemical and phylogenetic analyses, branching sucrases could also constitute a new GH70 subgroup. Five enzymes of this group have now been characterized, including the engineered α-(1→2) branching sucrase GBD-CD2 (19, 20), BRS-A (21), BRS-B, BRS-C, and BRS-D. The route is now opened to promising structure-function relationship studies that will aim at identifying the structural determinants responsible for polymerization or branching ability and linkage specificity. Despite common structural motifs with GH70 glucansucrases, the branching sucrases also display clear distinctive features. According to sequence analyses, dif-

**FIGURE 10. Phylogenetic tree of GH70 enzymes.** Each enzyme is labeled with its GenBank™ accession number and its origin. The species of microorganism are indicated with *Ln.* for *Leuconostoc*, *Lb.* for *Lactobacillus*, *S.* for *Streptococcus*, and *W.* for *Weissella*. Glucansucrase enzymes are highlighted in green, α-4,6-glucanotransferases are in pink, and branching sucrases are in bold and purple. The scale bar corresponds to a genetic distance of 0.05 substitutions per position.
ferences have been highlighted in the substrate binding subsite +1, which can be used to rapidly sort out branching sucrases from genomic data. Mutational and structural studies will now be necessary to better define the signature of the branching specificity and to provide further insight into the branching mechanism. Finally, a broad variety of branched dextrans varying in terms of size, osidic linkage and branching degree can be easily synthesized using these enzymes, emphasizing the potential of GH70 enzymes to access new structures of biotechnological interest.

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