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Hybrid reuteransucrase enzymes reveal regions important for glucosidic linkage specificity and the transglucosylation/hydrolysis ratio

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Glucansucrase (GS) (often labelled glycosyltransferase; GTF) enzymes (EC 2.4.1.5) of lactic acid bacteria use sucrose to synthesize a diversity of α-d-glucans with mainly α-(1→4) glucosidic linkages (50% and 70%, respectively), plus α-(1→6) linkages. In the present study, we report a detailed analysis of various hybrid GTFA/O enzymes, resulting in the identification of specific regions in the N-termini of the catalytic domains of these proteins as the main determinants of glucosidic linkage specificity. These regions were divided into three equal parts (A1–3; O1–3), and used to construct six additional GTFA/O hybrids. All hybrid enzymes were able to synthesize α-glucans from sucrose, and oligosaccharides from sucrose plus maltose or isomaltose as acceptor substrates. Interestingly, not only the A2/O2 regions, with the three catalytic residues, affect glucosidic linkage specificity, but also the upstream A1/O1 regions make a strong contribution. Some GTFO derived hybrid/mutant enzymes displayed strongly increased transglucosylation/hydrolysis activity ratios. The reduced sucrose hydrolysis allowed the much improved conversion of sucrose into oligo- and polysaccharide products. Thus, the glucosidic linkage specificity and transglucosylation/hydrolysis ratios of reuteransucrase enzymes can be manipulated in a relatively simple manner. This engineering approach has yielded clear changes in oligosaccharide product profiles, as well as a range of novel reuteran products differing in α-(1→4) and α-(1→6) linkage ratios.

Keywords
glucansucrase; glycosidic linkage; hybrid enzymes; product specificity; reuteransucrase

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The reuteransucrase enzymes of Lactobacillus reuteri strain 121 (GTFA) and L. reuteri strain ATCC 55730 (GTFO) convert sucrose into α-d-glucans (labelled reuterans) with mainly α-(1→4) glucosidic linkages (50% and 70%, respectively), plus α-(1→6) linkages. In the present study, we report a detailed analysis of various hybrid GTFA/O enzymes, resulting in the identification of specific regions in the N-termini of the catalytic domains of these proteins as the main determinants of glucosidic linkage specificity. These regions were divided into three equal parts (A1–3; O1–3), and used to construct six additional GTFA/O hybrids. All hybrid enzymes were able to synthesize α-glucans from sucrose, and oligosaccharides from sucrose plus maltose or isomaltose as acceptor substrates. Interestingly, not only the A2/O2 regions, with the three catalytic residues, affect glucosidic linkage specificity, but also the upstream A1/O1 regions make a strong contribution. Some GTFO derived hybrid/mutant enzymes displayed strongly increased transglucosylation/hydrolysis activity ratios. The reduced sucrose hydrolysis allowed the much improved conversion of sucrose into oligo- and polysaccharide products. Thus, the glucosidic linkage specificity and transglucosylation/hydrolysis ratios of reuteransucrase enzymes can be manipulated in a relatively simple manner. This engineering approach has yielded clear changes in oligosaccharide product profiles, as well as a range of novel reuteran products differing in α-(1→4) and α-(1→6) linkage ratios.

Abbreviations
CGTase, cyclodextrin glucanotransferase; GH, glycoside hydrolase; GS, glucansucrase; GTF, glycosyltransferase; GTFA, reuteransucrase from Lactobacillus reuteri 121; GTFO, reuteransucrase from Lactobacillus reuteri ATCC 55730; RS, restriction site.
domains of GS enzymes possess a ($\beta/\alpha)_8$ barrel structure similar to members of the glycoside hydrolase (GH) family (http://www.cazy.org). The core of proteins belonging to the GH13 family constitute eight $\beta$-sheets alternated with eight $\alpha$-helices. In GTFs, however, this ($\beta/\alpha$) eight-fold structure is circularly permuted [7], as supported by site-directed mutagenesis experiments [8–10] (Fig. 1C). Therefore, GTF enzymes are classified as belonging to the GH70 family [11]. Evolutionary, structurally and mechanistically related families are grouped into ‘clans’. Enzymes from families GH13 (mainly starch modifying enzymes), GH70 and GH77 (4-$\alpha$-glucanotransferases) comprise clan GH-H (also known as the $\alpha$-amylase superfamily) [11].

Recently, several amino acids affecting glucosidic linkage specificity in glucansucrase enzymes have been identified, located close to the catalytic residues [12–15]. However, these residues are identical in the reuteransucrases GTFA and GTFO, both synthesizing $\alpha-(1\rightarrow4)$ plus $\alpha-(1\rightarrow6)$ linkages in their products, but at clearly different ratios. The question remains as to which GTFA/GTFO amino acids determine this difference in the glucosidic linkage ratio. As an initial approach to identify these residues, the regions involved were targeted by characterizing various GTFA/GTFO hybrid proteins, starting out from the N-terminally truncated variants GTFA-dN [6] and GTFO-dN [14]. Their product spectrum on sucrose alone, and with the acceptor substrates maltose and isomaltose, were characterized. The results obtained show that the N-terminal part of the catalytic core (~630 amino acids) of these reuteransucrases, including the three catalytic residues, is the main determinant of glucosidic linkage specificity. A more detailed analysis of this N-terminal part showed that not only the region encompassing the three catalytic residues, but also other regions affect the glucosidic linkage ratio within glucan and oligosaccharide products.

**Results and Discussion**

**The N-termini of the GTFA-dN and GTFO-dN reuteransucrases influence glucosidic linkage specificity**

Deletion of the relatively large N-terminal variable regions in the reuteransucrase proteins (Fig. 1A) had no negative effect on enzyme activity; in addition, their glucosidic linkage specificity was retained [5,6]. Therefore, the much shorter N-terminally truncated variants GTFA-dN and GTFO-dN were used to construct hybrids. To investigate the parts in these reuteransucrase enzymes that control the type of glucosidic linkages...
synthesized in glucan products, the hybrid GTFA-O-dN and GTFO-A-dN proteins were constructed by partial digestion and ligation at a *KpnI* restriction site (Fig. 1B). This *KpnI* site is located between α9 and β1 of the catalytic domain. Parent and both hybrid enzymes were expressed in *Escherichia coli* BL21 DE3 star and purified by Ni-NTA affinity chromatography, followed by anion exchange chromatography (data not shown). GTFA-dN and GTFA-O-dN proteins were produced at comparable and relatively high levels. GTFO-dN and especially GTFO-A-dN were produced at lower levels (data not shown). The GTFO-A-dN variant yielded only very low amounts of soluble protein. By contrast, the GTFA-O-dN hybrid yielded larger amounts of soluble protein than both parents (data not shown). Glucan polymers produced by parent and hybrid enzymes exhibited clear glucansucrase activity with sucrose (data not shown). Glucan polymers produced by parent and hybrid enzymes were subjected to methylation and ¹H-NMR analysis. This revealed that exchange of the C-termini of the catalytic domains plus the glucan binding domains (413 amino acids, 85% identity, 91% similarity) yielded hybrid reuteransucrase enzymes with ratios of glucosidic linkages in their glucans similar to the respective parent proteins (Table 1).

Furthermore, iodine staining of glucan products showed similar results for the GTFO-A-dN and GTFA-O-dN hybrids and their respective GTFO-dN and GTFA-dN parents (Table 1). This indicated that the N-terminal parts of the catalytic domains of both reuteransucrases, including the α3,β4,α4,β5,α5,β6,α6,β7,α7,β8,α8 elements of the permuted (β/α)₈ barrel with the three catalytic residues (Fig. 1C), determine the types and ratios of glucosidic linkages synthesized (Table 1).

### The A1/O1 and A2/O2 regions within the N-termini of the catalytic domains of the GTFA-dN and GTFO-dN reuteransucrases mainly determine glucosidic linkage specificity

To identify regions within the N-termini of the catalytic domains that modulate glucan and oligosaccharide synthesis, six additional hybrid proteins were constructed. The N-terminal parts of the catalytic domains of GTFA-dN and GTFO-dN were divided into three fragments, encompassing: (a) A1/O1, the first part with no structural elements of the (β/α)₈ barrel (243 amino acids; 62% identity, 76% similarity); (b) A2/O2, the middle part including the α3,β4,α4,β5,α5,β6,α6,β7 elements and the three catalytic residues (194 amino acids; 87% identity, 94% similarity); and (c) A3/O3, the third part including the α7,β8,α8 elements (194 amino acids; 86% identity, 93% similarity (Fig. 1). For this purpose, extra restriction sites (RS) were removed or introduced at appropriate places (Fig. 1B). GTFA-dN-RS has two amino acid substitutions, introduced with the extra *SalI* (V985I) and *SacI* (N1179E) sites. GTFO-dN-RS, with a natural *SalI* site, has only one amino acid substitution, introduced with the extra *SacI* site (N1179E). The N-terminally located *KpnI* sites in GTFA-dN-RS and GTFO-dN-RS were removed by introduction of a natural *KpnI* site (Fig. 1B).

### Table 1. Analysis of the glucans produced by purified GTFA-dN and GTFO-dN proteins and derived (hybrid) mutants. Representative data of at least two independent measurements are shown: ≤5% difference.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>I⁰</th>
<th>II Terminal</th>
<th>Methylation (%)</th>
<th>III Chemical shift (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTFA-dN</td>
<td>–</td>
<td>8</td>
<td>44 ± 3</td>
<td>52 ± 3</td>
</tr>
<tr>
<td>GTFO-dN</td>
<td>+</td>
<td>7</td>
<td>72 ± 9</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>GTFA-O-dN</td>
<td>–</td>
<td>11</td>
<td>47 ± 27</td>
<td>57 ± 43</td>
</tr>
<tr>
<td>GTFA-A-dN</td>
<td>+</td>
<td>10</td>
<td>62 ± 15</td>
<td>67 ± 33</td>
</tr>
<tr>
<td>GTFA-dN-RS</td>
<td>–</td>
<td>13</td>
<td>46 ± 25</td>
<td>55 ± 45</td>
</tr>
<tr>
<td>GTFO-dN-RS</td>
<td>+</td>
<td>7</td>
<td>76 ± 7</td>
<td>77 ± 23</td>
</tr>
<tr>
<td>GTFA-O1-dN-RS</td>
<td>–</td>
<td>14</td>
<td>50 ± 18</td>
<td>62 ± 38</td>
</tr>
<tr>
<td>GTFA-O2-dN-RS</td>
<td>–</td>
<td>14</td>
<td>49 ± 19</td>
<td>59 ± 41</td>
</tr>
<tr>
<td>GTFA-O3-dN-RS</td>
<td>–</td>
<td>11</td>
<td>46 ± 26</td>
<td>55 ± 45</td>
</tr>
<tr>
<td>GTFA-A1-dN-RS</td>
<td>–</td>
<td>13</td>
<td>48 ± 22</td>
<td>54 ± 46</td>
</tr>
<tr>
<td>GTFA-A2-dN-RS</td>
<td>–</td>
<td>14</td>
<td>53 ± 15</td>
<td>63 ± 37</td>
</tr>
<tr>
<td>GTFA-A3-dN-RS</td>
<td>+</td>
<td>9</td>
<td>68 ± 13</td>
<td>74 ± 26</td>
</tr>
</tbody>
</table>

a Iodine staining was scored positive when formation of a red complex was observed. b The resolution with NMR was too low to trace the terminal and (α→4,6) linked residues as detected by methylation analysis. Displayed are the anomic signals at 5.0 p.p.m. (α→4,6 linkages) and 5.3 p.p.m. (α→1,4 linkages). 6 Data from three independent batches of GTFA-A3-dN-RS glucan (methylation: 9 ± 2, 68 ± 13, 13 ± 9 and 11 ± 3) NMR (74 ± 10 and 26 ± 10).
silent mutation (Fig. 1B). The GTFA-dN-RS and GTFO-dN-RS enzymes were produced at comparable levels to their parents (data not shown). However, lower amounts of soluble protein were obtained after cell lysis and purification (data not shown). Both these variants displayed glucosucrase activity and their glucan products had a glucosidic linkage distribution similar to their parents (Table 1), indicating that these point mutations had only a minor influence on the ratio of glucosidic linkages synthesized. Subsequently, six different hybrids were successfully constructed using restriction and ligation (GTFA-O1/O2/O3-dN-RS, GTFO-A1/A2/A3-dN-RS; Fig. 1C). These hybrid enzymes were produced at comparable levels. However, for GTFO-A2/A3 and GTFA-O1/O2, only low amounts of soluble proteins were obtained after cell lysis. Again, all six hybrids showed clear glucosucrase activity with sucrose (data not shown) and synthesized glucan products.

Surprisingly, in both reuteransucrases, exchange of the A1/O1 fragments [with no structural elements of the (β/α)6 barrel] had a larger impact on glucosidic linkage distribution than exchange of the A2/O2 fragments [with most structural elements of the (β/α)8 barrel including the three catalytic residues and (putative) acceptor subsites]. GTFA-O1-dN-RS synthesized high amounts of α-(1→4) and low amounts of α-(1→6) glucosidic linkages, differing clearly from the parent GTFA-dN-RS product. The opposite effect was seen for the GTFO-A1-dN-RS glucan product, which was low in α-(1→4) and high in α-(1→6) glucosidic linkages, differing strongly from the parent GTFO-dN-RS product (Table 1). Thus, the A1/O1 fragments determine the ratio of α-(1→4)/α-(1→6) glucosidic linkages synthesized by GTFA and GTFO. A previous study demonstrated that deletions within the A1/O1 region in GTFI led to an inactive enzyme [16]. Removal of a small N-terminal part of this domain led to a slightly less active enzyme. N-terminal deletions heading further towards the C-terminus severely reduced enzyme activity [16]. Further investigations are needed to identify exactly the region and/or amino acids residues of this A1/O1 fragment that determine glucosidic linkage type.

The A2/O2 fragment carries the three catalytic residues: D1024 (nucleophile), E1061 (acid/base catalyst) and D1133 (transition state stabilizer) (Fig. 1C). Amino acid residues upstream and downstream of the nucleophile are virtually identical in both reuteransucrases. The region following the acid/base contains two amino acid residues differences. Previously, these amino acid residues have been mutated (H1065S/A1066N in the A2 region in GTFA, changing GTFA residues into those present in GTFO) [14], with no clear shift in glucosidic linkages present in the polymer products. Amino acid residues in the vicinity of the transition state stabilizer have been shown to affect glycosidic bond type specificity in glucansucrase enzymes [12–15]. However, the residues investigated in those studies are identical in the reuteransucrases GTFA and GTFO. This suggests that amino acid residues further away from the catalytic residues also influence glucosidic bond type specificity. Exchange of the A2/O2 fragments confirmed this, resulting in a similar shift in the α-(1→4)/(1→6) glucosidic linkage ratio, although this is less pronounced than that observed with the exchange of the A1/O1 fragments.

Analysis of the different glucan products showed that exchange of the A3/O3 fragments, containing small sections of the catalytic domains, including the α7,β8,α8 elements, had the least effect on glucosidic linkage type distribution in both reuteransucrases. Exchange of the A3 fragment of GTFA-dN-RS with O3 of GTFO-dN-RS, yielding GTFA-O3-dN-RS, had a minor effect on the type of linkages present in the glucan, resembling the parent GTFA-dN-RS enzyme. The opposite exchange in GTFO-dN-RS showed that the hybrid GTFO-A3-dN-RS is still able to incorporate relatively high amounts of α-(1→4) linked glucose residues in its reuteran, similar to the parent GTFO-dN-RS enzyme (Table 1). Repeated production of this GTFO-A3-dN-RS polymer resulted in determination of an α-(1→4) linkage distribution in the range 65–85% (i.e. more than either of the parent enzymes); each of these polymers stained reddish with iodine (λmax = 525–530). Such large variations were not noticed in different batches of the glucans of the other enzymes studied (differences ≤ 5%). In time, this hybrid GTFO-A3-dN-RS enzyme may be able to further modify its polymer product after maturation (e.g. by a disproportionation type of reaction, as observed for amyllosucrase) [17]. This phenomenon remains to be studied in more detail.

The glucans synthesized by the GTFO-A2-dN-RS and GTFO-A3-dN-RS hybrids both had relatively high amounts of α-(1→4) glucosidic linkages. Nevertheless, the glucan products synthesized by both hybrids appeared to be different. The GTFO-A3-dN-RS glucan product stained red with iodine, similar to the GTFO-dN, GTFO-dN-RS and GTFO-A-dN glucan products (λmax = 520–530), but the GTFO-A2-dN-RS product remained colourless, indicating that no long linear α-(1→4) chains were present (see below) (Table 1). The iodine staining depends on the structure of the α-D-glucan. Linear amyllose, with α-(1→4) linkages only, forms a complex with iodine that results in a blue colour (λmax = 645). Amylopectin, with α-(1→4) linkages plus 1→6 branch points, stains violet
with iodine ($\lambda_{\text{max}} = 545$). The reddish colour observed with the glucan made by GTFO-dN and some of its derivatives ($\lambda_{\text{max}} = 520–530$) indicated that, besides linear $\alpha$-(1→4) glucosidic chains, there was at least some degree of branching by 1→6 linkages (Table 1) [18,19]. This was confirmed by methylation analysis (Table 1). The reason that the reuteran synthesized by GTFA forms no complex with iodine has now become evident. Detailed structural analysis showed that there are no (long) linear $\alpha$-(1→4) glucosidic chains. Instead, the GTFA reuteran contains predominantly alternating $\alpha$-(1→4) and $\alpha$-(1→6) glucosidic linkages [20].

### Products synthesized by parent and hybrid GTFA and GTFO enzymes

Surprisingly, the GTFO-dN-RS enzyme failed to deplete sucrose within 110 h; its hydrolysis decreased two-fold and glucan synthesis (with a ratio of glucosidic linkages similar to GTFO-dN) increased by 30%. This change in GTFO-dN-RS is based on a single point mutation, similar to GTFO-dN. Introduction of the similar mutation in GTFA, suggesting that these two proteins differ in neighbouring amino acid residues in 3D space.

The results obtained in the present study, using hybrid enzyme construction as an initial and relatively crude approach, show that transglucosylation/hydrolysis ratios are relatively easily engineered into the reuteransucrase enzymes (Table 2). The availability of glucansucrase enzymes with a high transglucosylation/hydrolysis ratio, maximizing sucrose use for polymer synthesis, is crucial when aiming for high level production of glucans for (bulk) applications. Previous protein engineering studies of cyclodextrin glucanotransferase (CGTase; GH13 family) [21] and amylo-maltase (GH77 family) [22] enzymes have identified active site residues that are involved in stabilizing the covalent reaction intermediates. Mutagenesis of such residues strongly affects transglucosylation and hydrolysis activity ratios. Application of directed evolution strategies, using random and rational mutagenesis approaches, has allowed conversion of CGTase into an $\alpha$-amylase [23,24]. CGTase protein 3D structural analysis revealed involvement of an induced fit mechanism determining the transglucosylation/hydrolysis ratio [23,25]. A similar mechanism is likely to operate in glucansucrase enzymes (GH70 family), with a fold similar to the GH13 and GH77 proteins (clan GH-H; http://www.cazy.org). Recently, the successful crystallization of a related glucansucrase protein was reported [26]. We are currently exploring the precise molecular mechanisms for transglucosylation and hydrolysis in glucansucrases, aiming to raise the production of $\alpha$-d-glucan polymer synthesis.

### Oligosaccharide synthesis from sucrose and maltose by hybrid enzymes

Interestingly, mutant enzymes GTFO-A2-dN-RS and GTFO-A3-dN-RS synthesized relatively larger amounts of maltotriose [glucose attached via $\alpha$-(1→4) glucosidic linkage to nonreducing end of maltose] and lower amounts of panose [glucose attached via $\alpha$-(1→6) glucosidic linkage to nonreducing end of maltose] than GTFO-dN and GTFO-dN-RS (Table 3) [correction added on 6 November 2008, after first online publication: in the preceding sentence ‘reducing end of maltose’ was corrected to ‘nonreducing end of maltose’ in two places]. Both GTFO-A2-dN-RS and GTFO-A3-dN-RS also synthesized relatively large amounts of $\alpha$-(1→4) glucosidic linkages in their glucan polymers. The opposite effect was observed with mutant GTFO-A1-dN-RS, where slightly lower amounts of maltotriose and slightly higher amounts of panose were synthesized. Thus, the linkage specificity within polymer and oligosaccharide synthesis is conserved, as observed previously for wild-type and mutant glucansucrase enzymes [6,12,14].

### Table 2. Product spectra of purified GTFA-dN and GTFO-dN proteins and derived (hybrid) mutants, incubated with sucrose for 110 h (end-point conversion).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Glucan (%)</th>
<th>Leucrose (%)</th>
<th>Isomaltose (%)</th>
<th>Glucose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTFA-dN</td>
<td>90.5 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td>7.3 ± 0.1</td>
</tr>
<tr>
<td>GTFO-dN</td>
<td>46.9 ± 0.4</td>
<td>5.3 ± 0.3</td>
<td>4.2 ± 0.2</td>
<td>43.5 ± 0.1</td>
</tr>
<tr>
<td>GTFO-O-dN</td>
<td>60.7 ± 1.5</td>
<td>2.5 ± 0.4</td>
<td>3.6 ± 0.2</td>
<td>33.2 ± 0.8</td>
</tr>
<tr>
<td>GTFO-A-dN</td>
<td>62.8 ± 0.2</td>
<td>5.3 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>28.8 ± 0.2</td>
</tr>
<tr>
<td>GTFO-dN-RS</td>
<td>85.5 ± 0.2</td>
<td>1.5 ± 0.3</td>
<td>1.6 ± 0.1</td>
<td>11.3 ± 0.1</td>
</tr>
<tr>
<td>GTFO-dN-RSa</td>
<td>73.9 ± 0.6</td>
<td>2.7 ± 0.9</td>
<td>1.4 ± 0.1</td>
<td>21.9 ± 0.3</td>
</tr>
<tr>
<td>GTFA-O1-dN-RS†</td>
<td>84.3 ± 1.5</td>
<td>1.4 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td>13.6 ± 1.9</td>
</tr>
<tr>
<td>GTFA-O2-dN-RS</td>
<td>85.9 ± 0.3</td>
<td>1.5 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>10.9 ± 0.1</td>
</tr>
<tr>
<td>GTFA-O3-dN-RS</td>
<td>84.0 ± 0.3</td>
<td>2.1 ± 0.2</td>
<td>1.8 ± 0.1</td>
<td>12.1 ± 0.1</td>
</tr>
<tr>
<td>GTFO-A1-dN-RS†</td>
<td>58.9 ± 2.8</td>
<td>1.8 ± 0.1</td>
<td>2.8 ± 0.3</td>
<td>36.5 ± 2.5</td>
</tr>
<tr>
<td>GTFO-A2-dN-RS</td>
<td>51.3 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>3.2 ± 0.2</td>
<td>42.0 ± 0.2</td>
</tr>
<tr>
<td>GTFO-A3-dN-RS</td>
<td>57.7 ± 1.2</td>
<td>2.2 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>37.3 ± 1.1</td>
</tr>
</tbody>
</table>

a Sucrose consumed for 40-60% after 110 h of incubation. b Percentages indicate the relative conversion of sucrose into glucan, oligosaccharides (leucrose and isomaltose) and glucose (hydrolysis). The 100% value is equivalent to the total amount of sucrose consumed after 110 h of incubation.
GTFO-A3-dN-RS synthesized a reuteran with a higher yield than GTFO-dN-RS (Table 4) [correction added on 6 November 2008, after first online publication: in the preceding sentence 'reducing end of isomaltose' was corrected to 'nonreducing end of isomaltose']. Thus, the relatively high amount of \( \alpha-(1\rightarrow4) \) linkages synthesized by this mutant in its polymer was also reflected in oligosaccharide synthesis.

**Conclusions**

The N-termini of the catalytic domains of the GTFA and GTFO reuteransucrases are the main determinants for glucosidic linkage specificity. Within these N-termini, the A1/A2 and O1/O2 parts of the reuteransucrase catalytic domains mainly determine the glucosidic linkages synthesized. Thus, not only the A2/O2 regions containing the catalytic residues, but also the A1/O1 regions make important contributions. Further research is needed to identify more precisely the role of these two different regions and their amino acid residues in glucosidic linkage specificity. The ratio of glucosidic linkages in the oligosaccharide and polymer products of these reuteransucrase enzymes thus could be manipulated in a relatively simple manner, yielding higher yields than GTFO-dN-RS (Table 4) [correction added on 6 November 2008, after first online publication: in the preceding sentence 'reducing end of isomaltose' was corrected to 'nonreducing end of isomaltose']. Thus, the relatively high amount of \( \alpha-(1\rightarrow4) \) linkages synthesized by this mutant in its polymer was also reflected in oligosaccharide synthesis.
clear changes in oligosaccharide distribution and a wide variety of structurally different and novel reuteran products.

Major differences were observed in the transglucosylation/hydrolysis ratios of the parent and derived hybrid enzymes. Whereas GTFO has a high hydrolytic activity with sucrose, hybrid GTFO-A-dN and mutant GTFO-dN-RS had much reduced hydrolysis activities. Interestingly, they maintained the ability of the parent GTFO-dN enzyme to synthesize $\alpha$-(1→4) linkages at a relatively high percentage in their $\alpha$-d-glucan products. Conversion of sucrose in $\alpha$-d-glucans with relatively high amounts of $\alpha$-(1→4) linkages thus has been much improved. This is of great interest because the GTFO/GTFA (hybrid) enzyme synthesizes $\alpha$-d-glucans with both $\alpha$-(1→4) and $\alpha$-(1→6) linkages that are structurally very different from the plant starch (amylose/amylopectin) products with both $\alpha$-(1→4) and $\alpha$-(1→6) linkages [20]. The physicochemical properties of such new $\alpha$-d-glucans remain to be determined, and potentially have new applications with respect to food, cosmetics and pharmaceuticals.

Experimental procedures

Bacterial strains, plasmids, media and growth conditions

_E. coli_ TOP 10 (Invitrogen, Carlsbad, CA, USA) was used as host for cloning purposes. Plasmid pET15b (Novagen, Madison, WI, USA) was used for expression of the different (mutant) _gtf_ genes in _E. coli_ BL21 Star (DE3) (Invitrogen). Plasmids p15-GTFA-dN and p15-GTFO-dN, containing the catalytic and C-terminal glucan binding domains of the _gtfA_ gene (MG-740-1781-His6, 3147 bp, 1049 amino acids) of _Lb. reuteri_ 121 and the _gtfO_ gene (M-746-1781-His6, 3126 bp, 1042 aa) of _Lb. reuteri_ ATCC 55730, respectively, were used as template for mutagenesis [5,6]. _E. coli_ strains were grown aerobically at 37 °C in LB medium [27]. _E. coli_ strains containing recombinant plasmids were cultivated in LB medium with 100 μg·mL$^{-1}$ ampicillin. Agar plates were made by adding 1.5% agar to the LB medium.

Molecular techniques

General procedures for restriction, ligation, cloning, PCR, _E. coli_ transformations, DNA isolation and manipulations, isolation of DNA fragments from gel, and agarose gel electrophoresis were performed as described previously [6]. Primers were obtained from Eurogentec (Seraing, Belgium). Sequencing was performed by GATC Biotech (Konstanz, Germany).

Construction of plasmids for hybrid mutagenesis experiments

Plasmids p15-GTFA-dN and p15-GTFO-dN were partially digested with _BamH_I and _KpnI_ to exchange the _N_ and _C_-termini of the (N-terminally truncated) _gtfA_ and _gtfO_ genes, yielding constructs p15-GTF-AO-dN and p15-GTFOA-dN (Fig. 1).

The QuikChange™ TM site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and the primers AkpnI: 5’-GATACTGGTGATCGTCGAAAC-3’; AsacI: 5’-GGTG AAAGATATGAGCTCTTATATAATCTCGG-3’; and _AsaII_: 5’-GTTGCTAAACGATGTCGACAACTCATAACC-3’ (complementary primers not shown, modified restriction sites are shown underlined, changed bases in bold) were used to sequentially remove the _KpnI_ restriction site and introduce _SacI_ and _SalI_ restriction sites in p15GTFA-dN (Fig. 1B). To remove _KpnI_ and introduce _SacI_ restriction sites in p15GTFO-dN, the primers used were _OkpnI_: 5’-GATACCTGGTGATCGCCGCAAG-3’ and _OasCl_: 5’-GTTAAGAAGTACGAGCTACTACAATATCC-3’ (complementary primers not shown, modified restriction sites are shown underlined, changed bases in bold). Constructs with multiple mutations were made using p15GTFA-dN or p15GTFO-dN containing mutation(s) as template and the appropriate primer pairs.

After successful removal (KpnI, ~ 250 bp) and introduction [SacI (only GTFA, ~ 740 bp) and _SacI_, ~ 1325 bp] of restriction sites (confirmed by DNA nucleotide sequencing), both p15-GTFA-dN-RS and p15-GTFO-dN-RS were digested with _XbaI_ and _SacI_, _SalI_ and _SacI_, and _SacI_ and _KpnI_, and corresponding fragments were exchanged, yielding the six constructs p15-GTFA-O1-dN-RS, p15-GTFA-O2-dN-RS, p15-GTFA-O3-dN-RS, p15-GTFO-A1-dN-RS, p15-GTFO-A2-dN-RS and p15-GTFO-A3-dN-RS (Fig. 1C).

Enzyme activity assays and enzyme purification

Proteins were produced (recombinant _E. coli_ cells were grown for 16 h at 37 °C without induction) and purified by Ni-NTA affinity (Sigma-Aldrich, St Louis, MO, USA) and anion exchange chromatography as described previously [6]. All reactions were performed at 30 °C in 25 mM sodium acetate buffer (pH 4.7), containing 1 mM CaCl$_2$. Glucansucrase activity (U·mL$^{-1}$) was determined as the initial rate by measuring fructose release (enzymatically) from 100 mM sucrose by appropriately diluted GTF enzyme. One unit of enzyme activity is defined as the release of 1 μmol·min$^{-1}$ of fructose [6,28]. Standard incubations were made with 0.1 U·mL$^{-1}$ of purified (mutant) enzyme, except for GTFO-dN-RS (0.014 U·mL$^{-1}$), GTFA-O1-dN-RS (0.009 U·mL$^{-1}$), GTFO-dN-RS (0.005 U·mL$^{-1}$) and GTFO-A3-dN-RS (0.03 U·mL$^{-1}$), for which lower amounts of enzyme were used.
Characterization of the glucans produced

Polymers were produced by incubation of purified (mutant) enzyme preparations with 146 mM sucrose for 7 days, using the conditions described above for the enzyme activity assays, and addition of 1% Tween 80 and 0.02% sodium azide. Glucans produced were isolated by precipitation with ethanol as described previously [28]. All glucans were produced at least twice and analysed by two different methods (see below).

Methylation analysis was performed as described by permethylation of the polysaccharides using methyl iodide and dimethylsulfoxide at room temperature [29].

1D 1H-NMR spectra were recorded on a 500 MHz Varian Inova NMR spectrometer (Varian Inc., Palo Alto, CA, USA) at a probe temperature of 50 °C. Prior to NMR spectroscopy, samples were dissolved in 99.9 atm % D2O (Sigma-Aldrich). Chemical shifts (δ) are expressed in p.p.m. by reference to external acetone (δ 2.225). Proton spectra were recorded in 8 k data sets, with a spectral width of 8000 Hz. Prior to Fourier transformation, the time-domain data were apodized with an exponential function, corresponding to an 0.8 Hz line broadening.

Glucan polymers, amylose type III and amylopectin standards from potato (Sigma-Aldrich) (1% w/v; 15 μL), were stained with 150 μL of iodine solution (1 mg I2 and 10 mg of KI in 10 mL) [18] and visually inspected for the appearance of colour. 1), the concentrations of fructose, glucose, isomaltoolose and leucrose in the reaction medium were determined using anion exchange chromatography (Dionex) as described previously [6]. The percentage of oligosaccharide synthesis from sucrose and acceptor was determined by subtracting the amount of unused acceptor from the initial acceptor concentration.

Analysis of products synthesized from sucrose

After depletion of sucrose (100 mM, 110 h at 30 °C) by GTF (mutant) enzymes (enzyme amount used as indicated above; 0.005–0.1 U·mL⁻¹), the concentrations of fructose, glucose, isomaltoolose and leucrose in the reaction medium were determined using anion exchange chromatography (Dionex, Sunnyvale, CA, USA) as previously described [6]. The amount of fructose released (97.7%), and leucrose (1.7%) and isomaltoolose (0.6%) synthesized from sucrose, corresponds to 100%. Subtracting the free glucose (7.2%; due to hydrolysis) from the free fructose (97.7%) concentration allowed calculation of the yield of reuteran synthesis (90.5%) from sucrose (data of GTFA-dN were used for clarification; Table 2).

Oligosaccharides synthesized from sucrose and (iso)maltose as acceptor substrates

After complete depletion of sucrose (100 mM, 110 h at 30 °C) by GTF (mutant) enzymes (enzyme amount used as indicated above; 0.005–0.1 U·mL⁻¹), incubated with the acceptor substrates maltose or isomaltoolose (100 mM each), the oligosaccharides synthesized were analyzed by anion exchange chromatography (Dionex) as described previously [14]. The percentage of oligosaccharide synthesis from sucrose and acceptor was determined by subtracting the amount of unused acceptor from the initial acceptor concentration.

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References


