The Discoidin Domain of Bacillus circulans β-Galactosidase Plays an Essential Role in Repressing Galactooligosaccharide Production

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The recently cloned β-galactosidase from Bacillus circulans ATCC 31382, designated BgaD, contains a multiple domain architecture including a F5/8 type C domain or a discoidin (DS) domain in the C-terminal peptide region. Here we report that the DS domain plays an essential role in repressing the production of galactooligosaccharides (GOSs). We prepared deletion mutants and point-mutated forms of rBgaD-A (deletion of the BgaD signal peptide) to compare their reaction behaviors. The yields of GOSs for all of the point-mutated forms as well as the deletion mutants of rBgaD-A increased as compared to rBgaD-A. In particular, W1540A mutant BgaD-A (rBgaD-A_W1540A) produced much more GOSs than rBgaD-A. Surface plasmon resonance experiments indicated that both the wild-type and the W1540A mutant DS domains showed high affinity for galactosylactose. BgaD-A, which has a wild-type DS domain, showed high hydrolytic activity toward galactosylactose, while the hydrolytic activities of rBgaD-D, without a DS domain, and rBgaD-A_W1540A, with a mutant DS domain were extremely low. The findings obtained in this study indicate that the wild-type DS domain of rBgaD-A has a function that aids galactosylactose molecules to be properly oriented within the active site, so that they can be hydrolyzed efficiently to produce galactose/glucose by inhibiting the accumulation of GOSs.

Key words: β-galactosidase; Bacillus circulans; transgalactosylation; galactooligosaccharides; discoidin domain

β-Galactosidases (β-D-galactoside galactohydrolase, EC 3.2.1.23) producing galactooligosaccharides (GOSs) with various degrees of polymerization has attracted considerable attention, since GOSs are known to be a useful prebiotic.1–4 Hence a number of attempts to isolate β-galactosidases with high transgalactosylation activities from various microorganisms have been reported,5–13 and genes that encode β-galactosidases,13–24 including those of B. circulans ATCC 31382,12,24 have been cloned. However, the lack of a crystal structure as well as a lack of knowledge of the multimeric and monomeric structures of a number of high molecular weight β-galactosidases, except for Escherichia coli β-galactosidase,25 has been a drawback to developing an understanding of the mechanism of transgalactosylation.25–26

We recently have isolated and characterized a β-galactosidase from Bacillus circulans ATCC 31382 and cloned the gene (DDBJ accession no. AB605256).24 Our results indicate that the enzyme has a multiple domain architecture. The polypeptide, designated BgaD, is comprised of a 35-amino acid (a.a.)-long signal peptide, a LacZ domain containing a Glyco_hydro_2_N domain (a sugar-binding domain), a Glyco_hydro_2 domain, and a Glyco_hydro_2,C domain (a TIM barrel domain), a BID_1 domain (a bacterial Ig-like domain, group 1), four repeats of the Big_4 domain (a bacterial Ig-like domain, group 4), and a F5/8 type C domain, known as a discoidin (DS) domain,27 from the amino to the carboxyl terminus, as shown in Fig. 1. We also found, using recombinant enzymes expressed as His-tagged proteins in E. coli cells, that the transgalactosylation activity of rBgaD-A is substantially increased by truncating the C-terminal peptide sequence of 315, 523, or 926 a.a. residues, which includes 1, 2, and 4 repeats of the Big_4 domain, in addition to the DS domain. These findings are consistent with results obtained using isoforms isolated from a commercially available B. circulans ATCC 31382 product (Biolacta®, Daiwa Kasei, Shiga, Japan).12

The DS domain is present in various eukaryotic and prokaryotic proteins, and its various functions have been determined.21–34 It has been reported that the presence of the DS domain enhances the hydrolytic activity of various glycosidases due to high interaction between the substrate and enzyme molecules28–30 but knowledge of the functions of the DS domain in β-galactosidase12,17 is limited. The objective of this study was to elucidate the role of the DS domain in the repression of GOS production. For this purpose, we prepared deletion/
Fig. 1. Domains of B. circulans β-Galactosidase and Its Deletion Mutants.

Sp, signal peptide (1–35 a.a.); a, Glyco_hydro_2,N domain (sugar binding domain) (43–202 a.a.); b, the Glyco_hydro_2 domain (202–320 a.a.); c, the Glyco_hydro_2,C domain (TIM barrel domain) (323–637 a.a.); d, the BID_1 domain (bacterial Ig like domain, group 1) (737–842 a.a.); e, the hydro_4 domain (bacterial Ig like domain, group 4) (858–916, 932–990, 1343–1401, and 1417–1475 a.a.); and f, the F5/8 type C domain (DS domain) (1,506–1,644 a.a.). The domain that showed homology with the LacZ domain of E. coli β-galactosidase is located in the region of 20–842 a.a. residues.

Fig. 2. Amino Acid Sequence Alignment of Several DS Domains.

The peptide sequence (1,506–1,644 a.a. residues) of the DS domain of BgaD isolated in this study was aligned with those of the C2 domain of human coagulation factor VIII (Fa8), a β-mannosidase from Bacteroides thetaiotaomicron (BatM), a chitosanase-glucanase from Paenibacillus fakuiensis (PICG), an endo-β-1,3-glucanase from Paenibacillus sp. CCRC17245 (PspG), and a β-galactosidase from Bifidobacterium bifidum (DSM20215) BIF3 (BIF3). The five conserved amino acid residues are enclosed within a box, and the residues of B. circulans (BgaD) DS domain that were mutated in this study are indicated by black dots. The number on the right-hand side shows the position of the amino acid residue, from the N-terminus.

Materials and Methods

Materials. pCold II vector plasmid and primers were purchased from Takara (Kyoto, Japan) and Life Technologies Japan (Tokyo), respectively. 4'-Galactosylactosylactosylactosylactosylactose as galactosyllactose and o-nitrophenyl β-d-glucopyranoside (o-NPG) were from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of analytical grade unless otherwise noted.

Multiple alignment of the DS domain of B. circulans β-galactosidase. B. circulans β-galactosidase has a multiple domain architecture containing the DS domain described above. The peptide sequence (1,506–1,644 a.a. residues) of the DS domain of BgaD was aligned with those of several eukaryotic and prokaryote DS domains using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Fig. 2). The peptide sequence of the DS domain of BgaD showed homology with those of a β-mannosidase from Bacteroides thetaiotaomicron (GenBank accession no. Q8A3U9) with an identity of 24%,31 a chitosanase-glucanase from Paenibacillus fakuiensis (GenBank accession no. Q93IE7) with an identity of 22%,31 an endo-β-1,3-glucanase from Paenibacillus sp. CCRC17245 (GenBank accession no. ABF15796) with an identity of 30%,32 an endo-β-1,3-glucanase, are intimately involved in stabilizing the DS domain architecture, while the contribution of W1679 is not great. Hence in this study three conserved amino acid residues are generally not high, several aromatic amino acid residues are conserved, as reported by Cheng et al.29 These authors pointed out that W1688 and Y1714, among the conserved amino acid residues in the DS domain of Paenibacillus β-1,3-glucanase, are intimately involved in stabilizing the DS domain architecture, while the contribution of W1679 is not great. Hence in this study three conserved amino acid residues, W1540, W1552, and Y1582 of BgaD, which correspond to W1679, W1688, and Y1714 of Fa8, respectively to W1679, W1688, and Y1714, among the conserved amino acid residues in the DS domain of BgaD, with an identity of 29%,28 an endo-β-1,3-glucanase from Paenibacillus sp. CCRC17245 (GenBank accession no. ABJ15796) with an identity of 30%,32 an endo-β-1,3-glucanase, are intimately involved in stabilizing the DS domain architecture, while the contribution of W1679 is not great. Hence in this study three conserved amino acid residues, 29) These authors pointed out that
transglucosylase activity. In addition, we mutated W1540 to phenylalanine in the preparation of point-mutated $B_{\text{gal}}$D-A.

Construction of expression vectors and transformation. The primers used in this study are shown in Table 1. Deletion mutagenesis was performed as follows: Expression plasmids harboring DNA fragments encoding $B_{\text{gal}}$D-A, deleting 80 aa. residues ($p$ColdII-$b_{\text{gal}}$DA-A80) that correspond to the peptide sequence from the C-terminus to the begin the C-terminal C domain of the enzyme was digested with two cleavage sites corresponding to the C-terminus N-terminus of the domain ($p$ColdII-$b_{\text{gal}}$DA-A80), were constructed, by performing PCR using $p$ColdII-$b_{\text{gal}}$DA-A80 as template and primers 1–3 (Table 1).

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Expression plasmids for the wild-type DS domain in $B_{\text{gal}}$D and its W1540A mutant $p$ColdII-$b_{\text{gal}}$DA-D and the $p$ColdII-$b_{\text{gal}}$DA-D were prepared using a GENEART® Seamless Cloning and Assembly Kit (Invitrogen, Carlsbad, CA). DNA fragments to be assembled were generated by a PCR method with Pcl II vector and $p$ColdII-$b_{\text{gal}}$DA-D and $p$ColdII-$b_{\text{gal}}$DA-D, were assembled, by performing PCR using $p$ColdII-$b_{\text{gal}}$DA-D as template and primers 12–15 (Table 1). DNA fragments were isolated using an Illustra™ GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences, Piscataway, NJ). Seamless cloning and assembly reaction were done following instructions in the manual. DNA fragments encoding 120 amino acid residues (1506–1657 a.a.) of $B_{\text{gal}}$D, harboring the DS domain (1506–1657 a.a.) was inserted into DNA fragment encoding 152 amino acid residues (1506–1657 a.a.) of $B_{\text{gal}}$D-A.

Table 1. Primers Used in This Study

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Recognition site</th>
<th>Sequence (5’→3’)</th>
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<tbody>
<tr>
<td>1</td>
<td>BgaD-A-FW</td>
<td>EcoRI</td>
<td>ACAGAATCTAAGATCCTGCC</td>
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<tr>
<td>2</td>
<td>BgaD-A-Rv1</td>
<td></td>
<td>GAAATTAATGCACTATCATCGGTAATCG</td>
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<td>3</td>
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<td>EcoRI</td>
<td>GAATTC</td>
</tr>
<tr>
<td>4</td>
<td>W1540A Fw</td>
<td>EcoRI</td>
<td>GAATTC</td>
</tr>
<tr>
<td>5</td>
<td>W1540A Rw</td>
<td></td>
<td>GAATTC</td>
</tr>
<tr>
<td>6</td>
<td>W1540F Fw</td>
<td>EcoRI</td>
<td>GAATTC</td>
</tr>
<tr>
<td>7</td>
<td>W1540F Rw</td>
<td></td>
<td>GAATTC</td>
</tr>
<tr>
<td>8</td>
<td>W1552A Fw</td>
<td>EcoRI</td>
<td>GAATTC</td>
</tr>
<tr>
<td>9</td>
<td>W1552A Rw</td>
<td></td>
<td>GAATTC</td>
</tr>
<tr>
<td>10</td>
<td>Y1582A Fw</td>
<td>EcoRI</td>
<td>GAATTC</td>
</tr>
<tr>
<td>11</td>
<td>Y1582A Rw</td>
<td></td>
<td>GAATTC</td>
</tr>
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<td>GENEART_Rv2</td>
<td></td>
<td>GCCTGTCGTCGAAGGAGGACGAATTCATCATCGG</td>
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</table>

*Nucleotides with underlining indicate restriction endonuclease sites, italic letters mean stop codon, and bold letters indicate bases designed for the amino acid mutation.

β-Galactosidase activity measurements. β-Galactosidase activity was assayed using 8 mM o-NPG and 143 mM lactose as substrates in the assay buffer at 40 °C by a method used in a previous study.12,24 For the final enzyme concentration was 2–4 μg/mL, unless otherwise noted. One unit of o-NPG as substrate (Uo, o-NPG) was defined as the amount of protein required to produce 1 μmol of o-NPG per minute at 40 °C at pH 6, and 1 unit with lactose as substrate (Uo, lactose), as the amount of protein required to produce 1 μmol of o-glucose under the same conditions, as reported previously.12,24 Protein concentrations were determined by the BCA method using a BCA Protein Assay Reagent Kit (Pierce Chemical, Rockford, IL) with BSA as standard.

Determination of the maximum yields of the GOSs. The time courses for the β-galactosidase-catalyzed reaction using lactose at a final concentration of 5% (146 mM) as substrate in the assay buffer were determined by carrying out the enzymatic reaction at 40 °C using all of the mutant $B_{\text{gal}}$D-Ax at a final enzyme concentration of about 2 U/l, mL by a method reported previously, 12,24 to determine the maximum yield of each GOS produced. At appropriate time intervals during the reaction, a 100-μL aliquot was withdrawn and boiled for 3 min to terminate the reaction, and the concentrations of substrate and products were determined. The amounts of GOSs produced were quantified by HPLC (LS-20A, Shimadzu, Kyoto, Japan) using a refractive index detector (RID-10A, Shimadzu) equipped with a column (6 mm i.d. × 150 mm YMC-PACK NH2, YMC, Kyoto, Japan). For analyses of lactose and other disaccharides, a 70% CH3CN/H2O solution was used as elution buffer at a flow rate of 0.8 mL/min at 25 °C. For the analysis of GOSs, elution was performed using 60% CH3CN/H2O at a flow rate of 0.8 mL/min at 25 °C. The molecular masses of the tri-, tetra-, and pentasaccharides produced were confirmed by MALDI/TOF MS using Voyager-DETM PRO (Applied Biosystems, Foster, CA), 12 4-Galactosylactose, maltotraose, and maltopentaose (Hayasabara Shoji, Okayama, Japan) were used as standard oligosaccharides for quantification.

Thermal stability and CD measurements. The thermal stability of all of the mutant $B_{\text{gal}}$D-Ax was determined in a temperature range of 20–70 °C in the assay buffer, where the final enzyme concentration was 10 μg/mL. The enzyme activity was assayed at 40 °C and monitored over 2 min. The final concentration of the enzyme was determined by a method described previously. 12,24
0.2–0.4 mg/mL. After incubation of the enzyme solution at each temperature for 1 h, the remaining activity was assayed by carrying out the reaction using α-NPG as substrate, as described above.

Circular dichroism (CD) spectra for β-Gal-A isolated from a commercial product of B. circulans ATCC 31382, BioLacta\(^{10,12}\), rBgaD-A,\(^{23}\) and three point-mutated forms of rBgaD-A, i.e., rBgaD-A\(_{\text{W1540A}}\), rBgaD-A\(_{\text{W1552A}}\), and rBgaD-A\(_{\text{Y1582A}}\) were measured at wavelengths of 190–250 nm at room temperature using a CD spectrometer (J-720W1; Jasco, Tokyo) fitted with a quartz cell with a path length of 1 mm. The enzyme concentration of the samples was approximately 0.2 mg/mL in the assay buffer.

Characterization of rBgaD-A\(_{\text{W1540A}}\). Certain characteristics of rBgaD-A\(_{\text{W1540A}}\) that showed the maximum yield, for each of the GOSs, as described below, were investigated by the same methods and reaction conditions as in previous studies.\(^{12}\) The pH dependencies of the hydrolytic activities for the hydrolysis of α-NPG and lactose were measured at 40°C in 100 mM acetate buffer pH 3.0–5.0, 100 mM phosphate buffer, pH 5.5–8.5, and 100 mM sodium carbonate buffer, pH 9.0–10.5.

To measure the effects of reagents on the stability of rBgaD-A\(_{\text{W1540A}}\), a 20-μL portion of enzyme solution (0.2 μg/mL) was added to 40 μL of 1.0 mM MgCl\(_2\), 1.5 mM PCMB, 0.1 M dithioerythritol, AgNO\(_3\), MgSO\(_4\), 1.10-phenanthroline, 1.5 or 15 mM EDTA-2Na, 15 mM KCl, and 15 mM NaCl dissolved in the assay buffer and pre-incubated for 10 min at 20°C. As a control, 40 μL of the assay buffer was added in place of the reagent solution. The remaining activity was assayed using α-NPG as substrate.

**Biomolecular interaction analysis.** The interaction between rBgaD-D\(_{\text{S}}\) or rBgaD-D\(_{\text{DS}}\) and various saccharides was quantified by measuring surface plasmon resonance (SPR) curves using a BIAcore 1 instrument (BIAcore AB, Tokyo). A ligand (1.8 μg of the wild-type DS domain, rBgaD-D, or the mutant DS domain, rBgaD-D\(_{\text{W1540A}}\), dissolved in 180 μL of 10 mM acetate buffer pH 4.5) was immobilized on a sensor chip CM5 (a surface modified with carboxymethylated dextran) following the manufacturer’s instructions, and the method reported in our previous study.\(^{30}\) Then 180 μL of a solution of an analyte (11–60 nM galactose, glucose, lactose, and galactosylactose), dissolved in the assay buffer was fed at 30 μL/min at room temperature, followed by washing of the chip with the assay buffer, to determine the response curve. For data analysis, BIA evaluation software 3.2 was used, assuming reversible first order kinetics, including mass transfer.

**Enzymatic hydrolyses of galactosylactose.** Hydrolytic activity toward galactosylactose was compared using rBgaD-A, rBgaD-A\(_{\text{W1540A}}\), and rBgaD-D (the rBgaD-A mutant deleting the C-terminal 925 a.a. residues) prepared by a method in our previous paper.\(^{31}\) A 10-μL aliquot of the purified enzyme solution (5.6 U\(_{\text{active}}\)/mL) was added to 400 μL of galactosylactose dissolved in the assay buffer at a final concentration of 5 mM to start the reaction, at 40°C. At appropriate time intervals during the reaction, a 100-μL aliquot of the reaction mixture was withdrawn, and the concentrations of glucose and galactose were determined by previously described methods.\(^{12}\)

**Effects of thermal treatment of rBgaD-A and rBgaD-A\(_{\text{W1540A}}\) on enzymatic activity and GOS yield.** rBgaD-A\(_{\text{W1540A}}\) dissolved in the assay buffer at a final enzyme concentration of about 0.2 mg/mL was pre-incubated for various periods at 60°C, and the remaining activity was assayed using lactose, α-NPG, and galactosylactose as substrates. We also determined the courses of formation of GOSs at 40°C at pH 6.0, using rBgaD-A pre-heated at 60°C and 5% lactose as substrates to determine the maximum yield of each GOS, as described above.

### Results

#### β-Galactosidase activities of various mutant forms of rBgaD-A

All of the mutant rBgaD-As were homogeneous by SDS–PAGE (data not shown). Table 2 summarizes specific activities for the hydrolysis of α-NPG and lactose. The values obtained for β-Gal-A\(^{23}\) isolated from Biolastra\(^{10,4}\) and recombinant β-Gal-A, rBgaD,\(^{24}\) are also shown for comparison. As Table 2 indicates, the specific activities towards the hydrolysis of lactose for all of the mutants decreased as compared to those for rBgaD-A and wild-type β-Gal-A, except for rBgaD-A\(_{\text{C247}}\), in which the entire DS domain was deleted. On the other hand, the specific activities towards the hydrolysis of α-NPG for all the mutants were much lower than those for rBgaD-A and wild-type β-Gal-A, resulting in a considerable decrease in the \(U_{\text{α-NPG}}/U_{\text{lactose}}\) values, as is common for various forms of B. circulans β-galactosidase with high transgalactosylation activity, as reported in our previous studies.\(^{12,13}\) Among the point-mutated forms, rBgaD-A\(_{\text{W1540A}}\) and rBgaD-A\(_{\text{W1540F}}\) showed lower \(U_{\text{α-NPG}}/U_{\text{lactose}}\) ratios.

#### Maximum yields of GOSs for various mutant forms of rBgaD-A

From the time courses of reaction using 5% lactose as substrate, the maximum yields of tri-, tetra-, and pentasaccharides for all of the mutant enzymes were determined in a way similar to that reported in a previous paper.\(^{24}\) then summarized in Table 2. The yields of trisaccharide and tetrasaccharide for all of the mutant forms of rBgaD-A increased as compared to those for β-Gal-A and rBgaD-A, although the yield depended on the type of mutants. rBgaD-A\(_{\text{W1540A}}\) produced tri- and tetrasaccharides at high yields similar to those obtained for the truncated forms of β-galactosidases isolated from Biolastra\(^{10,12}\), while rBgaD-

### Table 2. Specific Activities and Maximum Yields of Galactooligosaccharides Using rBgaD-A Mutants

<table>
<thead>
<tr>
<th>Activity and yield</th>
<th>rBgaD-A(_{\text{(β-Gal-A)}})</th>
<th>rBgaD-A(_{\text{ΔC80}})</th>
<th>rBgaD-A(_{\text{ΔC247}})</th>
<th>rBgaD-A(_{\text{W1540A}})</th>
<th>rBgaD-A(_{\text{W1540F}})</th>
<th>rBgaD-A(_{\text{W1552A}})</th>
<th>rBgaD-A(_{\text{Y1582A}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity ((U_{\text{lactose}}/\text{mg})(^p))</td>
<td>44.6 (44.3)</td>
<td>35.7</td>
<td>48.0</td>
<td>26.3</td>
<td>36.1</td>
<td>18.1</td>
<td>28.7</td>
</tr>
<tr>
<td>Specific activity ((U_{\text{α-NPG}}/\text{mg})(^F))</td>
<td>40.6 (47.2)</td>
<td>11.8</td>
<td>7.7</td>
<td>3.5</td>
<td>4.6</td>
<td>5.6</td>
<td>9.2</td>
</tr>
<tr>
<td>(U_{\text{α-NPG}}/U_{\text{lactose}}) (^d)</td>
<td>0.91 (1.07)</td>
<td>0.33</td>
<td>0.16</td>
<td>0.13</td>
<td>0.13</td>
<td>0.31</td>
<td>0.32</td>
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<tr>
<td>Maximum yield of trisaccharide (%) (^e)</td>
<td>7.0 (7.2)</td>
<td>17.0</td>
<td>20.0</td>
<td>26.0</td>
<td>20.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Maximum yield of tetrasaccharide (%) (^e)</td>
<td>0.0 (0.0)</td>
<td>1.0</td>
<td>10.0</td>
<td>11.6</td>
<td>4.4</td>
<td>0.6</td>
<td>&lt;0.05</td>
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<tr>
<td>Maximum yield of pentasaccharide (%) (^e)</td>
<td>0.0 (0.0)</td>
<td>0.0</td>
<td>0.6</td>
<td>1.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\(^a\)Values in parentheses indicate those obtained for wild-type enzymes purified from a commercial preparation of B. circulans ATCC 31382 (Biolastra\(^{10}\)) obtained previously.

\(^b\)Specific activity toward lactose as substrate.

\(^c\)Specific activity toward α-NPG as substrate.

\(^d\)Ratio of hydrolytic activity toward α-NPG as substrate to that toward lactose as substrate.

\(^e\)Values with respect to the initial amount of lactose.
A_Y1582A and rBgaD-A_W1552A produced much lower yields of trisaccharides with negligible amounts of tetrasaccharide.

Thermal stability and circular dichroism (CD) measurements of various mutant rBgaD-As

The three point-mutated rBgaD-As were stable at temperatures of up to about 50 °C in 1 h of incubation at pH 6.0, while the remaining activity of two deletion mutants, rBgaD-A_A/C80 and rBgaD-A_A/C247, decreased by about 20% (data not shown).

Figure 3 shows the CD spectra of three point-mutated forms, rBgaD-A_W1540A, rBgaD-A_W1552A, and rBgaD-A_W1582A in comparison with those for rBgaD-A and β-Gal-A. As expected, the CD spectra for rBgaD-A and β-Gal-A were similar. The secondary structures of all three mutants were altered by mutating tryptophan to alanine, and the degrees of change in the spectrum were in the order rBgaD-A_A/W1552A > rBgaD-A_A/W1540A ≥ rBgaD-A_A/Y1582A.

Characterization of rBgaD-A_W1540A

Some characteristics of rBgaD-A_A/W1540A, which showed the maximum yield of each GOS were compared to the corresponding values for rBgaD-A or β-Gal-A.

rBgaD-A_A/W1540A showed a pH optimum of 5.5–6.5, similar to rBgaD-A and β-Gal-A, at 40 °C (data not shown). The enzyme was almost completely inhibited by a final 1 mM HgCl₂, and MgSO₄, β-dithiothreitol, PCMB, and 1,10-phenanthroline at a final concentration of 1 mM did not inactivate the enzyme, similarly to β-Gal-A. However, rBgaD-A_A/W1540A was inhibited only slightly by 10 mM EDTA·2Na, in contrast to β-Gal-A, for which the remaining activity was about 50%. rBgaD-A_A/W1540A was stable up to 50 °C, similarly to rBgaD-A.

Biomolecular interaction analysis by SPR

The interaction between rBgaD-DS or rBgaD-DS_W1540A and various saccharides was quantified by determining plasmon resonance response curves using a BIACore J instrument. In every case, the sensor chip was loaded with a non-saturating amount of protein, corresponding to signals of 7675 and 7618 RU (resonance units) for rBgaD-DS and rBgaD-DS_W1540A respectively. When rBgaD-DS or rBgaD-DS_W1540A was immobilized on the sensor chip surface, typical response diagrams were obtained only when galactosyllactose was fed as analyte (Fig. 4). The equilibrium dissociation constants, Kᵤ, determined for rBgaD-DS and rBgaD-DS_W1540A, were very low at 0.85 ± 0.2 × 10⁻⁶ and 1.3 ± 0.4 × 10⁻⁸ M, respectively indicating that rBgaD-DS and rBgaD-DS_W1540A has a high specific affinity to galactosyllactose, although the affinity of the wild-type DS domain protein was slightly higher than that of the mutant DS domain protein. On the other hand, the other saccharides, galactose, glucose, and lactose, showed results indicating rapid association and dissociation rate processes (data not shown), indicating very low affinity to both DS domain proteins.

Differences in hydrolytic activity towards galactosyllactose with rBgaD-A, rBgaD-A_W1540A, and rBgaD-D

Time courses for hydrolysis of 5 mM galactosyllactose were taken using rBgaD-A, rBgaD-D, and rBgaD-A_W1540A, as shown in Fig. 5. rBgaD-A, which contains the DS domain, efficiently hydrolyzed galactosyllactose to galactose and glucose, while rBgaD-D, without the DS domain, hydrolyzed it at a much lower reaction rate, approximately 1/20 of that determined for rBgaD-A, which indicates that the existence of the DS domain is essential to the efficient hydrolysis of galactosyllactose. Furthermore, the hydrolytic reaction rate for rBgaD-A_W1540A, with the point-mutated DS domain was much lower than that obtained for rBgaD-D, indicating the importance of the native structure of the DS domain. The role of the DS domain in the hydrolysis of galactosyllactose is discussed below.

In a reaction mixture obtained using rBgaD-A_W1540A and rBgaD-D as well as rBgaD-A, no GOSs other than galactosyllactose were detected by HPLC. In the hydrolysis of galactosyllactose with the all three enzymes, galactose was produced at a much higher reaction rate than glucose, as shown in Fig. 5, indicating that galactosyllactose was first hydrolyzed to galactose and lactose, followed by the hydrolysis of lactose to glucose and galactose at a much slower rate.
Effects of thermal treatment of rBgaD-A and rBgaD-A_W1540A on enzymatic activity and the GOS yield

Figure 6A and B respectively show semi-logarithmic plots for the time courses of inactivation of rBgaD-A_W1540A and rBgaD-A, in which the remaining activities were evaluated using lactose, o-NPG, and galactosylactose as substrates. The time courses of inactivation determined for rBgaD-A_W1540A were linear, which indicates that the inactivation kinetics followed first-order kinetics in spite of the substrates used in determination of the remaining activity. On the other hand, for rBgaD-A, the time courses of inactivation were different with the substrates used for the remaining activity measurement. The inactivation rates determined with o-NPG and galactosylactose as substrates were lower than that with lactose and deviated slightly from first-order kinetics. The reason for these unusual phenomena is discussed below.

Discussion

The findings reported here indicate that the DS domain of BgaD plays an important role in the repression of GOS production. In particular, the yield of tri- and tetrasaccharides was increased by deletion and by point-mutation of the DS domain. The yield for rBgaD-A_W1540A, in which the entire sequence of the DS domain was deleted, increased to a greater extent than rBgaD-A, consistently with the previously reported findings. On the other hand, the yields of tri- and tetrasaccharides were increased by point-mutating the conserved amino acid residues of the DS domain, although the extent of the increase in yield varied with the amino acid residue mutated.

Jørgensen et al. reported that the transgalactosylation activity of recombinant β-galactosidase from B. bifidum BIF3 was greatly enhanced by the deletion of 580 a.a. residues from its C-terminus, which includes one entire part of the DS domain located near the C-terminus and the greater part of the second one of the two repeats of the DS domain. Although the effect of a point mutation of the DS domain was not investigated by Jørgensen et al., their findings and those of this study suggest that the DS domain of B. bifidum BIF3 and of B. circulans is probably a key factor in controlling transgalactosylation activity, in an inhibitory manner. Jørgensen et al. suggested that one possible reason for the increased yield of GOSs with deletion of the C-terminal peptide region is the more open fragile structure of the deletion mutant, but the results obtained in this study indicate that this is unlikely for rBgaD-A, because the yield of GOSs was markedly increased by a point-mutation of conserved amino acid residues of the rBgaD-A DS domain. In particular, the yields of trisaccharide and tetrasaccharide obtained by rBgaD-A_W1540A were higher than those with rBgaD-A_W1540A, by 30% and 16% respectively.

The CD spectra shown in Fig. 3 were analyzed using the K2D2 program (http://www.ogic.ca/projects/k2d2/) to quantify changes in individual secondary structure. The change of the CD spectrum for rBgaD-A_W1540A, which showed the highest yield of GOSs, was medium among the three mutants: the fractions for the α-helix and the β-sheet determined for rBgaD-A_W1540A were 41.1 and 10.3%, in comparison with 36.2 and 10.5% for rBgaD-A. These findings suggest that an appropriate conformational change or deletion of the DS domain can convert BgaD-A to an enzyme with high transgalactosylation activity. According to interaction studies using the BIAcore J instrument, the wild-type and the W1540A mutant DS domains, BgaD-DS and BgaD-DS_W1540A, showed similar highly specific affinity for the galactosylactose molecule. These results are consistent with the findings of Cheng et al., which indicate that a mutation of the conserved amino acid residues of the Paenibacillus β-1,3-glucanase DS domain affected the stability of the domain more than it did substrate binding affinity. On the other hand, we found in this study that a large difference appeared in the hydrolytic activity toward galactosylactose as between rBgaD-A and rBgaD-D/rBgaD-A_W1540A. The hydrolytic activity of rBgaD-D without the DS domain toward galactosylactose was less than 1/20 that of rBgaD-A. Furthermore, the hydrolytic activity of rBgaD-A_W1540A with the point-mutated DS domain toward galactosylactose was about as low as that obtained for rBgaD-D, despite similar high affinity for galactosyl-
lactose to rBgaD-A, as explained above. These findings suggest that the \textit{B. circulans} DS domain is essential to the efficient hydrolysis of galactosylactose and functions to direct galactosyllactose molecules to a favorable coordinates in the active site for hydrolysis, resulting in the prevention of further transgalactosylation reactions producing GOSs.

BLAST search revealed that \textit{\-galactosidases} from \textit{Bifidobacteria} such as those from \textit{B. bifidum} JCM1254, \textit{B. bifidum} NCIMB41171, and \textit{B. bifidum} DSM20215 in addition to \textit{B. bifidum} BIF3 have a domain architecture conserving a DS domain, which suggests that \textit{Bifidobacteria} \textit{\-galactosidase} can hydrolyze GOSs efficiently yielding glucose or galactose for survival, although further studies are requisite to verify this. We found that \textit{E. coli} \textit{\-galactosidase} (Wako) hardly hydrolyzed galactosyllactose.

It has been reported that the DS domain in some glycoside hydrolases, such as \textit{\textit{\-1,3-glucoamylase}}, \textit{\textit{\-1,3-glucoamylase}}, and \textit{\textit{cellulose}}, functions to aid enzymes in interacting with polysaccharides as substrates, thus increasing catalytic efficiency, but no studies indicating that the DS domain greatly represses the transglycosylation activities of glycoside hydrolases have appeared.

The thermal inactivation rates determined for rBgaD-A at 60°C with o-NPG and galactosyllactose as substrates were appreciably lower than that with lactose as substrate. Such unusual inactivation behavior of the enzyme can be understood by assuming that the thermal stability of the DS domain is lower than that of the region forming the active site of the enzyme. Although the reason is not known, the experimental data obtained so far, including those from this study, indicate that \textit{B. circulans} \textit{\-galactosidases} with the lower U_{o-NPG}/U_{lactose} ratios give higher yields of GOSs. For rBgaD-A, the U_{o-NPG}/U_{lactose} ratio was lowered from 0.91 to 0.21 by incubation at 60°C for 30 min (Fig. 6). This enzyme was used for reaction with 5% lactose as substrate at 40°C to trace the production of GOSs, and the maximum yields of tri- and tetra saccharides were determined to be 22 and 6% respectively. These findings clearly indicate that alteration of the secondary structure of the DS domain decreased the hydrolytic activity of rBgaD-A toward galactosyllactose, which resulted in an accumulation of GOSs.

The findings reported in this study indicate that the DS domain of \textit{B. circulans} \textit{\-galactosidase} plays an important role in the repression of GOS production. However, further studies based on 3D structure are needed to clarify the function of the DS domain in relation to the transgalactosylation and hydrolysis reactions of \textit{B. circulans} \textit{\-galactosidases}.

References