Increasing the Transglycosylation Activity of α-Galactosidase From *Bifidobacterium adolescentis* DSM 20083 by Site-Directed Mutagenesis

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Abstract: The α-galactosidase (AGA) from *Bifidobacterium adolescentis* DSM 20083 has a high transglycosylation activity. The optimal conditions for this activity are pH 8, and 37°C. At high melibiose concentration (600 mM), approximately 64% of the enzyme–substrate encounters resulted in transglycosylation. Examination of the acceptor specificity showed that AGA required a hydroxyl group at C-6 for transglycosylation. Pentoses, hexuronic acids, deoxyhexoses, and alditols did not serve as acceptor molecules. Disaccharides were found to be good acceptors. A putative 3D-structure of the catalytic site of AGA was obtained by homology modeling. Based on this structure and amino acid sequence alignments, site-directed mutagenesis was performed to increase the transglycosylation efficiency of the enzyme, which resulted in four positive mutants. The positive single mutations were combined, resulting in six double mutants. The mutant H497M had an increase in transglycosylation of 16%, whereas most of the single mutations showed an increase of 2%–5% compared to the wild-type AGA. The double mutants G382C-Y500L, and H497M-Y500L had an increase in transglycosylation activity of 10%–16%, compared to the wild-type enzyme, whereas the increase for the other double mutants was low (4%–7%). The results show that with a single mutation (H497M) the transglycosylation efficiency can be increased from 64% to 75% of all enzyme–substrate encounters. Combining successful single mutants in double mutations did not necessarily result in an extra increase in transglycosylation efficiency. The donor and acceptor specificity did not change in the mutants, whereas the thermostability of the mutants with G382C decreased drastically.

Keywords: α-galactosidase; transglycosylation; site-directed mutagenesis; homology modeling; substrate specificity; thermostability

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INTRODUCTION

Prebiotic substrates like raffinose and stachyose are easily degraded by α-galactosidases from bifidobacteria (Garro et al., 1999; Leder et al., 1999; Roy et al., 1992; Xiao et al., 2000). In previous research (Leder et al., 1999; van den Broek et al., 1999; Van Laere et al., 1999), an α-galactosidase from *Bifidobacterium adolescentis* (AGA) was isolated, cloned, and expressed in *Escherichia coli* cells. The enzyme was classified in glycoside hydrolase (GH) family 36. AGA possessed transglycosylation activity (van den Broek et al., 1999) and was able to elongate acceptor substrates, like melibiose, raffinose, and stacchyose, with (α1 → 6)-galactosyl residues at their non-reducing end (van den Broek et al., 1999; Van Laere et al., 1999). However, not all enzyme–substrate encounters result in a transglycosylation reaction, since water can compete with the saccharides as acceptor molecule, resulting in a hydrolysis reaction.

Glycosyl transferases synthesizing (α1 → 6) galactosyl oligosaccharides also occur naturally and these enzymes do not seem to have hydrolytic activity. Interestingly, two different types of plant transferases occur in the same GH family as the *B. adolescentis* AGA (GH36) and the closely related family GH27. One of these transferases is stacchyose synthases (GH36), which catalyzes the synthesis of stacchyose by galactosyl transfer from galactinol to raffinose, and of verbascose by galactosyl transfer from galactinol to stacchyose, as well as by self-transfer of the terminal galactosyl residue from one stacchyose to another (Peterbauer et al., 2002). The other transferases are galactan:galactan galactosyltransferases (GGTs) from GH-family 27. These GGTs are involved in the biosynthesis of the long-chain raffinose family oligosaccharides (RFOs) by catalyzing the transfer of an α-galactosyl residue from one RFO molecule to another (Haab and Keller, 2002). The amino acid sequence of these plant α-galactosyl transferases might be used to pinpoint important residues for transglycosylation reaction, which could subsequently be introduced in AGA by site-directed mutagenesis.

Several articles indicate that it is possible to enhance the efficiency to perform transglycosylation reactions by mutagenesis of an enzyme (Hansson et al., 2001; Jorgensen et al., 2001; Matsui et al., 1994; Mayer et al., 2000). From the results of these articles, several possible factors have been suggested to promote the transglycosylation reaction of an enzyme. First, aromatic amino acids are involved in stabilizing the binding of the substrate through non-polar stacking interactions (Matsui et al., 1994); replacement of these residues with non-aromatic residues might change the geometry of binding oligosaccharides, and therewith enhance transglycosylation (factor I). Second, Tyr, Trp, and His residues can form strong H-bonds with water and the substrate, thereby positioning these molecules in a manner that favors hydrolysis reactions (Brzozowski and Davies, 1997) (factor II). Third, amino acids, capable of forming H-bonds, that are located further away from the catalytic site might favor transglycosylation by trapping water molecules, which might therefore not be available for hydrolysis (Hansson and Adlercreutz, 2001) (factor III). Fourth, a high hydrophobicity of the entrance of the catalytic site might reduce the amount of water entering the enzyme and lower the hydrolysis activity (Kuriki et al., 1996; Saab-Rincon et al., 1999) (factor IV). This factor probably only applies to enzymes which contain a pocket-like structure (Davies and Henrissat, 1995), because the water molecule can enter the catalytic site only at one position. Groove-like structures are more open and allow water molecules to enter the catalytic site at many places.

Our goal was to improve the transglycosylation activity of the AGA from *B. adolescentis* DSM20083 and therewith gaining more insight in the structure–function relationships underlying the transglycosylation reaction. By improving the transglycosylation activity, the enzyme might be used more efficiently to obtain longer or rare oligosaccharides for prebiotic use. The transglycosylation activity of the AGA is characterized and amino acids close to the catalytic site are modified by site-directed mutagenesis. The *aga* gene was mutagenized according to the amino acid sequences of both the stacchyose synthases and GGTs and the above mentioned possible factors suggested to be determinants of transglycosylation activity.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

DNA cloning was performed using the *E. coli* strain XL1 blue MRF' (Promega, Madison, WI). The *E. coli* strain was grown in Luria-Bertani (LB) broth or solidified LB medium (15 g agar/L) supplemented with 100 μg/mL ampicillin, when appropriate.

The *E. coli* cells containing the AGA gene (*aga*) and the mutants were grown in LB broth or solidified LB medium supplemented with 100 μg/mL ampicillin and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG).

Chemicals, Substrates, and Enzymes

Chemicals and substrates were purchased from Sigma (St. Louis, MO) unless stated otherwise. Restriction enzymes and other enzymes used for DNA manipulation were obtained from MBI Fermentas (St. Leon Rot, Germany) and were used according to the instructions of the manufacturer.

Cloning of the Wild-Type *aga* Gene

The wild-type AGA gene from *B. adolescentis* DSM 20083, was described by Van den Broek et al. (1999). A polymerase chain reaction (PCR) was carried out with *Pfu*-turbo polymerase (Stratagene, La Jolla, CA) to obtain the *aga* open reading frame of the wild-type gene. Two primers were used for amplification of the *aga* gene (*Xba*I and *Hind*III restriction sites were introduced at the beginning and at the end of the gene, respectively, for cloning purpose). Primers used were (with the restriction sites underlined): AR1GAL (5′-GCGCTCTAGAGCAATGACGCTCATTCA), and AR2 REVGAL (5′-CGCGAAGCTTTACTCAGATGCGGACTA). The forth-coming DNA fragments were ligated into a *Xba*I/*Hind*III digested *p*Bluescript vector (Stratagene) or *p*ETBlue Perfectly Blunt Vector (Novagen, Darmstadt, Germany). The recombinant plasmid was transformed into *E. coli* XL1 blue MRF' cells for expression.

Construction of Mutants

The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to make selected point mutations according to the instructions of the manufacturer. For each single mutant, a sense/antisense primer pair was designed, which included silent mutations encoding restriction sites to verify the inserted mutations (sense primer, introduced mutations in boldface, codon for mutated amino acid italic, restriction sites underlined, and restriction enzymes in parentheses): D380N (5′-GGAACGCTTCGTCGTG***AAC***GATGGCTG-GTTCGG**C**GCCCGCCG-3′) (*Nar*I); D381N (5′-GGA-ACGCTTCGTCGTGGAT***AAC***GGCTGGTTCGG**C**GCC CGCCG-3′) (*Nar*I); G382C (5′-TCGTGGATGAT***TGC***-TGGTTCGG**A**GCCCGCCGAGAC-3′) (*Ban*I); F384L (5′-GTGGATGATGGCTGG***CTC***GG**A**GCCCGCCGAG-AC-3′) (*Ban*I); Y492A (5′-CGGCGAACTCGGCATCG-AT***GC***CATCAAATGGGATCAC-3′) (*Cla*I); Y492G (5′CGGCGAACTCGGCATCGA**T*****GG***CATCAAATGG-ATGGGATCAC-3′) (*Cla*I); W495L (5′-CGGC-ATC-GA**T**TACATCAAA***TTG***GATCACAACAAATACG-TCACCG-3′) (*Cla*I); W495Y (5′-GCTGGTCGGCAT-CGA**T**TACATCAAA***TAC***GATCACAACAAATACG-3′) (*Cla*I); H497M (5′-CGGCATCGA**T**TACATCAAATGG-GAT***ATG***AACAAATACGTCACCG-3′) (*Cla*I); N498C (5′-CGGCATCGA**T**TACATCAAATGGGATCAC***TGC***-AAATACGTCACCG-3′) (*Cla*I); Y500L (5′-GGATC-ACAACAAA***CTC***GTCACCGAAGCGGTGTCG**C**CG-CG**G**ACCGG-3′) (*Sac*II). The wild-type AGA gene (*aga*)

was used as the template DNA. For each double mutant, a sense/antisense primer pair of the single mutants was used, accept for Y492G-H497M (5′-CGAACTCGGCATCGA**T-*GGC***ATCAAATGGGAT***ATG***AACAAAT-3′, template: mutant Y492G), and H497M-Y500L (5′-ATCGACTAC-ATCAAATGGGAT***ATG***AACAAA***CTC***GTCACCGA-3′, template: mutant H497M).

Plasmid DNA was isolated by following the Qiagen plasmid purification method (Qiagen, Hilden, Germany). Positive colonies were selected according to the restriction pattern of the plasmids upon digestion with the specific restriction enzyme for the silent mutation. To verify, if the point mutation was actually introduced, the DNA–nucleotide sequence of the plasmid was determined. After verification, the desired mutant plasmid was transformed to *E. coli* XL1 blue MRF' cells.

Isolation and Characterization of Mutants

Cells from an *E. coli* cell culture (1 L, LB-medium, 37°C, overnight) containing the wild-type or mutated *aga* genes were harvested by centrifugation (10 min; 3,000*g*; 4°C). The supernatant was removed and used for activity measurement; the cells were suspended in 40 mL 20 mM sodium phosphate buffer pH 7 and disrupted by sonic treatment (10 min; amplitude 30%, duty cycle 0.3 s on, 0.7 s off; Digital Sonifier, Branson, Danbury, CT) on ice. Subsequently, the suspension was centrifuged (10 min; 13,000*g*; 4°C), the cell extract was collected, and the pellet suspended in 40 mL 20 mM sodium phosphate buffer pH 7 and a second sonic treatment was performed. The cell extracts were pooled and applied onto a Q-Sepharose Hiload 26/10 (Amersham, Little Chalfont, UK) anion-exchange column. Elution took place with a linear gradient of 0–0.5 M NaCl in 20 mM sodium phosphate pH 7 at a flow rate of 4 mL/min. Fractions with the highest AGA activity were pooled and further purified on a Superdex 200 PG Hiload 16/26 (Amersham) size-exclusion column. Elution was performed with 0.15 M NaCl in 20 mM potassium phosphate buffer pH 7.0 at a flow rate of 1 mL/min. Fractions with the highest AGA activity were pooled and concentrated with anion-exchange chromatography under the same conditions as described above.

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. SDS–PAGE was carried out on the Pharmacia Phastsystem according to the instructions of the supplier (Amersham). Coomassie brilliant blue staining was used for the detection of proteins on PhastGel 8%–25% gradient gels (Amersham).

Enzyme Assays

Side activity of the purified enzymes was measured by determining the hydrolysis of *p*-nitrophenyl-α-D-glycosides (*p*NP-α-L-arabinopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-β-D-xylopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-β-D-fucopyranoside, and *p*NP-α-L-rhamnopyranoside) at 37°C after 10 min incubation. The reaction mixture (125 μL) consisted of 2 mM potassium phosphate buffer pH 6.0, and 0.2 mg/mL *p*NP-glycoside solution. The reaction was stopped by adding an equal volume of 0.5 M glycine/NaOH buffer pH 9.0, containing 2 mM EDTA. The color formation was measured at 400 nm.

α-galactosidase activity was measured by determining the hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside (*p*NP-Gal) under the conditions mentioned above. One unit (U) of activity was defined as the amount of enzyme that liberates 1 μmol of galactose per min under the specified conditions. The molar extinction coefficient of *p*-nitrophenol under these assay conditions was 13,700/Mcm.

Optimal transglycosylation conditions of the wild-type *aga* were measured with 0.04 U AGA and 300 mM melibiose in McIlvain buffer (0.1 M citric acid/0.2 M di-sodium phosphate), in a total volume of 100 μL. The enzyme's optimal transglycosylation pH determined with McIlvain buffers in the range of pH 3–8 and a temperature of 40°C during 4 h. For determination of the optimal transglycosylation temperature, temperatures between 20 and 70°C were used for incubation at pH 8 during 4 h. After incubation, the samples were 10 times diluted with McIlvain buffer pH 5, and were stopped by heating the incubation mixtures for 5 min at 100°C. All reactions were carried out in duplicate. After centrifugation (10 min, 10,000*g*) the supernatant was analyzed by high-performance anion-exchange chromatography (HPAEC).

Acceptor specificity was measured with 300 mM melibiose, 300 mM acceptor substrate, 0.04 U AGA in McIlvain buffer pH 8, in a total volume of 100 μL at 40°C during 7 h. The incubations were diluted, stopped, and analyzed as described before. All reactions were carried out in duplicate.

Transglycosylation activity was measured with 600 mM melibiose, 0.04 U AGA (the amount of units added, was calculated from the hydrolytic activity towards *p*NP-Gal for both wild-type and mutant AGA) in 100 mM Tris-HCl buffer pH 8, in a total volume of 100 μL at 40°C during 7 h. The incubations were diluted, stopped, and analyzed as described before. All reactions were carried out in triplicate.

Analytical Methods

HPAEC was performed using a Thermo-Quest HPLC system equipped with a Dionex CarboPac PA-1 (4 mm ID × 250 mm) column in combination with a CarboPac PA guard column (3 mm ID × 25 mm) and a Dionex ED40 PAD-detector (Dionex Co., Sunnyvale, CA). A flow rate of 1 mL/min was used with a combination of linear gradients of sodium acetate and NaOH: 0–20 min, 16 mM NaOH; 20–25 min, 16–100 mM NaOH; 25–40 min, 0–10 mM NaOAc in 0.1 M NaOH; 40–45 min, 10–100 mM NaOAc in 0.1 M NaOH. Each elution was followed by a washing step of 5 min 1,000 mM sodium acetate in 0.1 M NaOH and an equilibration step of 15 min 16 mM NaOH.

Statistical Analysis

Statistical analysis of the transglycosylation data was performed using General Linear Model in SPSS 10.0.7. *Z*-values >|2| were considered as outliers. Simple contrasts were calculated to determine significant differences between the mutant enzymes with the wild-type AGA as the reference group at $\alpha < 0.05$.

DNA Sequencing and Sequence Analysis

An automated DNA sequencer 373 (Applied Biosystems, Foster City, CA) was used to determine the nucleotide sequence of the gene. The DNA sequence data of *aga* are available at GenBank nucleotide databases under the accession number AF124596. The BLAST2 program ((Altschul et al., 1997), available at http://www.ncbi.nlm.nih.gov/) was used for searching sequence homologies. The alignment of the amino acid sequences is made by use of the clustalW multiple alignment function with a Pam250 matrix and was manually edited. This alignment and the SWISS-MODEL Version 36.0003 program (Guex and Peitsch, 1997; Peitsch, 1995; Schwede et al., 2003) (available from http://www.expasy.org/swissmod/SWISS-MODEL.html) were used for homology modeling.

RESULTS

Transglycosylation Conditions for Wild-Type AGA From *B. adolescentis*

The wild-type AGA from *B. adolescentis* was used to synthesize longer oligosaccharides, using melibiose as a donor substrate (Fig. 1). The transglycosylation products were identified as α-D-Gal*p*-(1 → 6)-α-D-Gal*p*-(1 → 6)-D-Glc*p*, and α-D-Gal*p*-(1 → 6)-α-D-Gal*p*-(1 → 6)-α-D-Gal*p*-(1 → 6)-D-Glc*p* by NMR analysis (data not shown). These results were in agreement with those obtained with the native AGA from *B. adolescentis* (Van Laere et al., 1999). A third transglycosylation product was found at a retention time of 21 min, which is suggested to be α-D-Gal*p*-(1 → 6)-D-Gal*p*, since this peak increased when both melibiose and galactose were incubated with the AGA (determination of acceptor specificity). The percentage of enzyme–substrate encounters resulting in hydrolysis and transglycosylation reactions catalyzed by AGA was determined as follows: the concentration of galactose represented the amount of hydrolysis, whereas the concentration of glucose represented the sum of hydrolysis and transglycosylation. The highest relative amount of transferase activity was found at pH 8 and 37°C (data not shown). At a melibiose concentration of 300 mM, approximately 50 of every 100 enzyme–substrate encounters resulted in a transfer reaction. At higher melibiose concentrations the transglycosylation activity reached a maximum of 69% (Fig. 2).

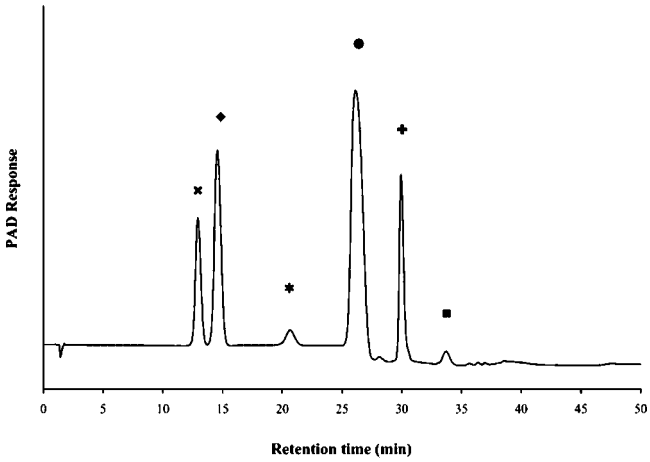

Figure 1. High-performance anion-exchange chromatography (HPAEC) diagram of the incubation of the wild-type α-galactosidase (AGA) from *Bifidobacterium adolescentis* with melibiose. The reaction was carried out during 7 h at pH 8, and 37°C. The initial concentration of melibiose was 300 mM. ✖, galactose; ◆, glucose; ✱, tentative galactosyl dimer (α-D-Gal*p*-(1 → 6)-α-D-Gal*p*); ●, melibiose (α-D-Gal*p*-(1 → 6)-α-D-Glc*p*); ✚, galactosyl–melibiose (α-D-Gal*p*-(1 → 6)-α-D-Gal*p*-(1 → 6)-α-D-Glc*p*); ■, galactosyl–galactosyl–melibiose (α-D-Gal*p*-(1 → 6)-α-D-Gal*p*-(1 → 6)-α-D-Gal*p*- (1 → 6)-α-D-Glc*p*).

Characterization of *aga* Gene and Design of Single Mutants

It was found in the literature that it is possible to enhance the transglycosylation activity of a GH by mutagenesis (Hansson et al., 2001; Jorgensen et al., 2001; Matsui et al., 1994; Mayer et al., 2000). Since AGA already possesses a high transglycosylation activity in comparison with other α-galactosidases (Spangenberg et al., 2000), it seems an interesting enzyme for the synthesis of oligosaccharides. However, for more efficient elongation of oligosaccharides, it is important to enhance the transglycosylation activity and reduce the hydrolytic activity.

The 3D-structure of AGA is not known. It belongs to GH36, of which no 3D-structure is currently available. However, GH36 has a strong relationship with GH27 and consequently these families are grouped in the same clan (GH-D). Four 3D-structures from GH27 are known: α-galactosidase from *Gallus gallus* (Garman et al., 2002), *Oryza sativa* (Fujimoto et al., 2003), *Homo sapiens* (Garman and Garboczi, 2004), and *Trichoderma reesei* (Golubev et al., 2004).

An amino acid alignment was made with AGA and four groups of enzymes, including the four α-galactosidases of which a 3D-structures is known (Fig. 3). Group I contained bacterial GH36 α-galactosidases, group II GH36 plant stacchyose synthases, group III GH27 α-galactosidases, and group IV GH27 GGTs. Based on the 3D-structures and sequence comparisons, the catalytic residues of AGA were suggested to be D496 and D562. The alignment showed that these catalytic residues and the residues D380 and D381 were conserved in all enzymes. Two more amino acids were conserved in all enzymes, i.e., W383 and K494. Figure 3

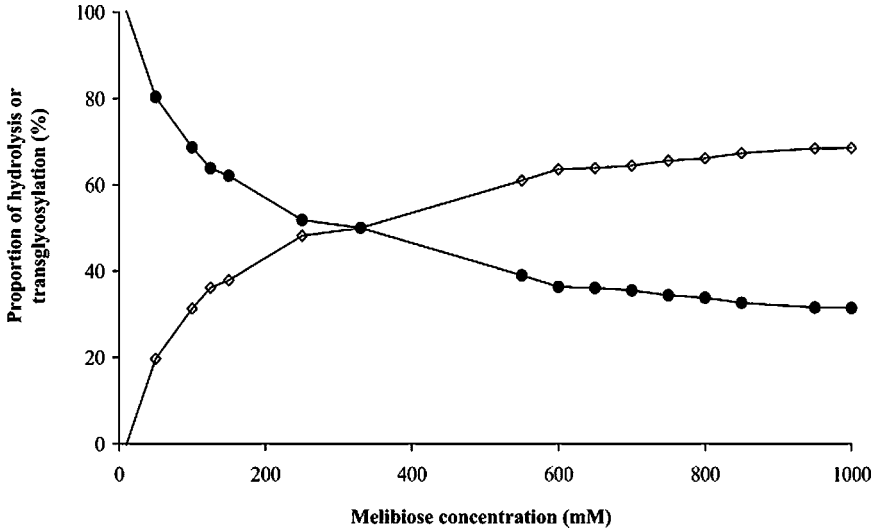

Figure 2. Influence of the melibiose concentration on the proportion of hydrolysis and transglycosylation activity of wild-type AGA. The reaction was carried out during 7 h at pH 8 and 37°C. ●, Hydrolysis; ◇, transglycosylation.

shows three different regions of the alignment. Two regions show the regions around the catalytic amino acids of D496 (B) and D562 (C). The other shows the region around the residues D380 and D381 (A).

A putative 3D-structure of the catalytic site of AGA was constructed by homology modeling, using the structure of *O. sativa* (Fujimoto et al., 2003) as a template (Fig. 4). Of the four enzymes with a known 3D-structure, the one from *O. sativa* had the highest identity (23%) with AGA. Amino acids 371–584 were incorporated in the model. The putative 3D-structure of AGA showed that the residues D496, D562, D380, and D381 were all located in the catalytic site, at a distance of approximately 6–8 Å from each other. This indicated that, besides the catalytic residues D496 and D562, also D380 and D381 might be important for the catalytic activity of the enzyme. To conclude on this, two mutants were made by site-directed mutagenesis, in which the Asp was replaced by an Asn (D380N and D381N). K494 might be involved in substrate binding, as the Lys residue in the α-galactosidase from rice (Fujimoto et al., 2003), but mutagenesis of this residue was not further pursued since it was conserved in all enzymes.

Special attention was devoted to Tyr, Trp, His, and Phe residues close to the catalytic residues, because these amino acids might play an important role in positioning the substrate molecule and catalytic water through stacking interactions and/or H-bonds (factors I and II). Mutation of these residues to non-aromatic amino acids might reduce the hydrolytic activity of AGA, and increase its transglycosylating activity. Combining the amino acid sequence and the putative 3D-structure of AGA, six aromatic residues were found, which were located in or close to the catalytic site (Fig. 4). The putative 3D-structure shows that catalytic residue D496 is located on a strand containing amino acids 491–497 (indicated in red). This strand is schematically represented in Figure 5. The aromatic side chains of Y492, H497, and Y500 are located in the catalytic cleft (Figs. 4 and 5). These amino acids were subjected to site-directed mutagenesis to replace the aromatic side chain by a non-aromatic one, resulting in four mutants Y492G, Y492A, H497M, and Y500L. The Gly residue in mutant Y492G was chosen because it was conserved in three stacchyose synthases from GH36 (Fig. 3). The Met in mutant H497M was chosen because it is hydrophobic, non-aromatic, and present in other bacterial GH36 enzymes (Fig. 3). The Leu and Ala in the mutants Y500L and Y492A, respectively, were chosen because they are hydrophobic and non-aromatic.

The three other aromatic residues F384, W495, and W559 are located near the catalytic site, but point away from the cleft. Therefore, they are less available for positioning the substrate or water. They might, however, be involved in capturing water molecules, and make them less available for hydrolysis (factor III). Two of these residues were modified to a hydrophobic, non-aromatic residue, Leu (F384L and W495L). Since GGTs are synthesizing enzymes, the W495 was replaced by a Tyr, because this Tyr is conserved in GH27 GGTs.

Two more mutants were created, based on the amino acid sequence of these GGTs. The residues G382 and N498 of AGA were both replaced by a Cys residue (Figs. 3 and 4). Cys residues are conserved at the corresponding positions of G382 and N498 in GGTs.

Hydrolysis and Transglycosylation Activity of Mutants

Eleven different single mutations in the *aga* gene were obtained: D380N, D381N, G382C, F384L, Y492A, Y492G, W495L, W495Y, H497M, N498C, and Y500L. These mutant genes were cloned and expressed in *E. coli* XL1 blue MRF' cells. Cell extracts were used for purification of the mutant AGAs by anion-exchange chromatography and size-exclusion chromatography. The mutants were not completely pure after these chromatographic steps, as judged by SDS–PAGE

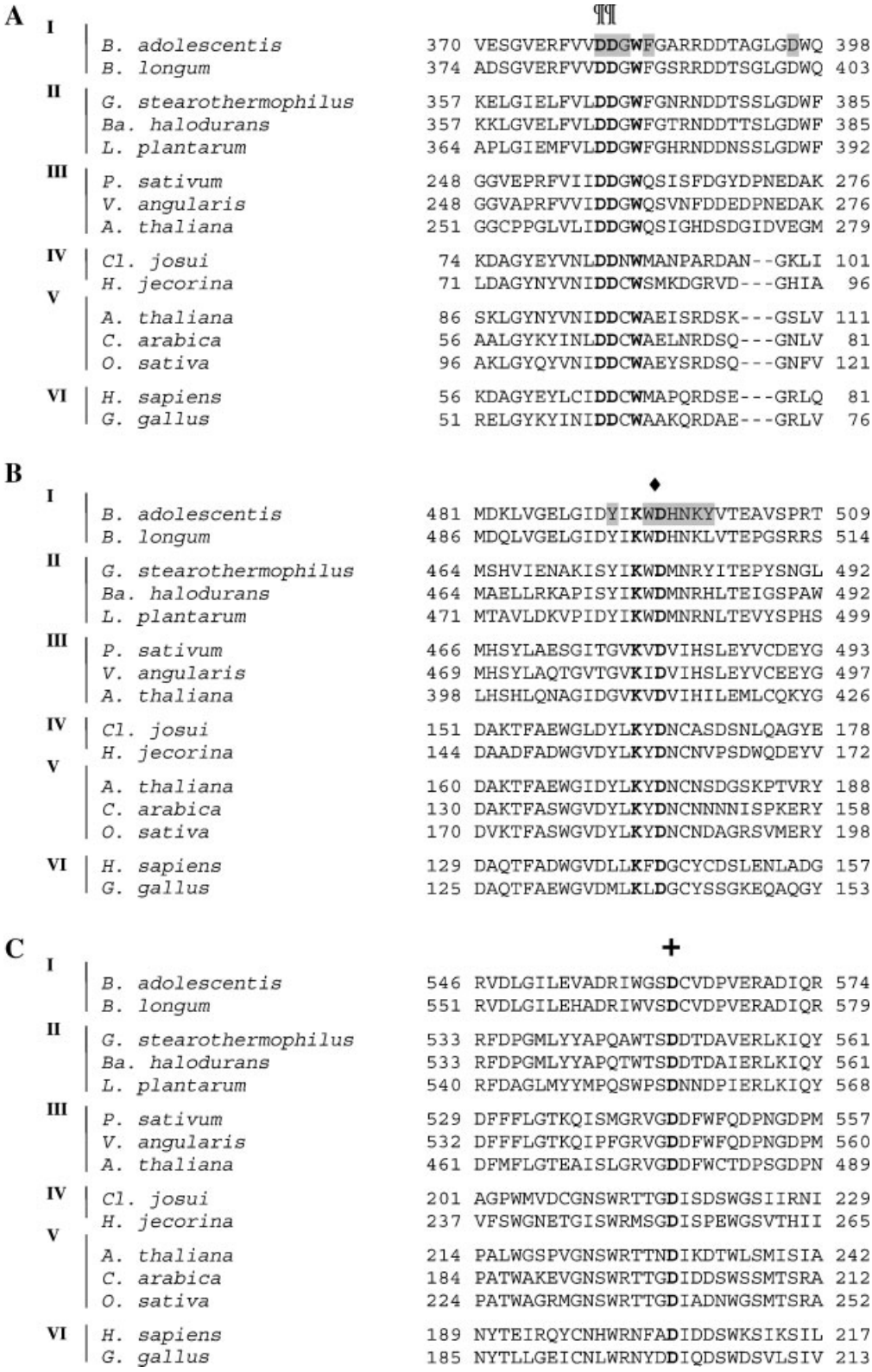

Figure 3. Alignment of the highly conserved regions of *B. adolescentis* AGA and enzymes from GH36 and GH27. A: Region including aspartates involved in catalysis; (B) region including catalytic acid/base, and (C) region including the catalytic nucleophile. I: Bacterial GH36 α-galactosidases (*B. adolescentis* (GenBank accession no. AF124596), *B. longum* (AF160969), *Geobacillus stearothermophilus* (AF038547), *Bacillus halodurans* (NP_243089), and *Lactobacillus plantarum* (AF189765)); (II) plant raffinose or stacchyose synthases GH36 (*Pisum sativum* (AJ4311087), *Vigna angularis* (Y19024), and *Stachys affinis* (AJ344091)); (III) GH27 α-galactosidases (*Clostridium josui* (AB025362), *Trichoderma reesei* (CAA93244), *Homo sapiens* (AAA51676), *Gallus gallus* (AAA16614), *Oryza sativa* (AAM92832)); (IV) GH27 plant galactan:galactan galactosyltransferases (GGTs) (*Arabidopsis thaliana* (NP_196455), *Coffea arabica* (T50781)); conserved amino acids are indicated in bold; *enzymes with a known 3D-structure. For the *B. adolescentis* AGA: amino acids used for site-directed mutagenesis are marked with a gray box; ◆, catalytic residue D496; ✚, catalytic residue D562; ¶, D380 and D381.

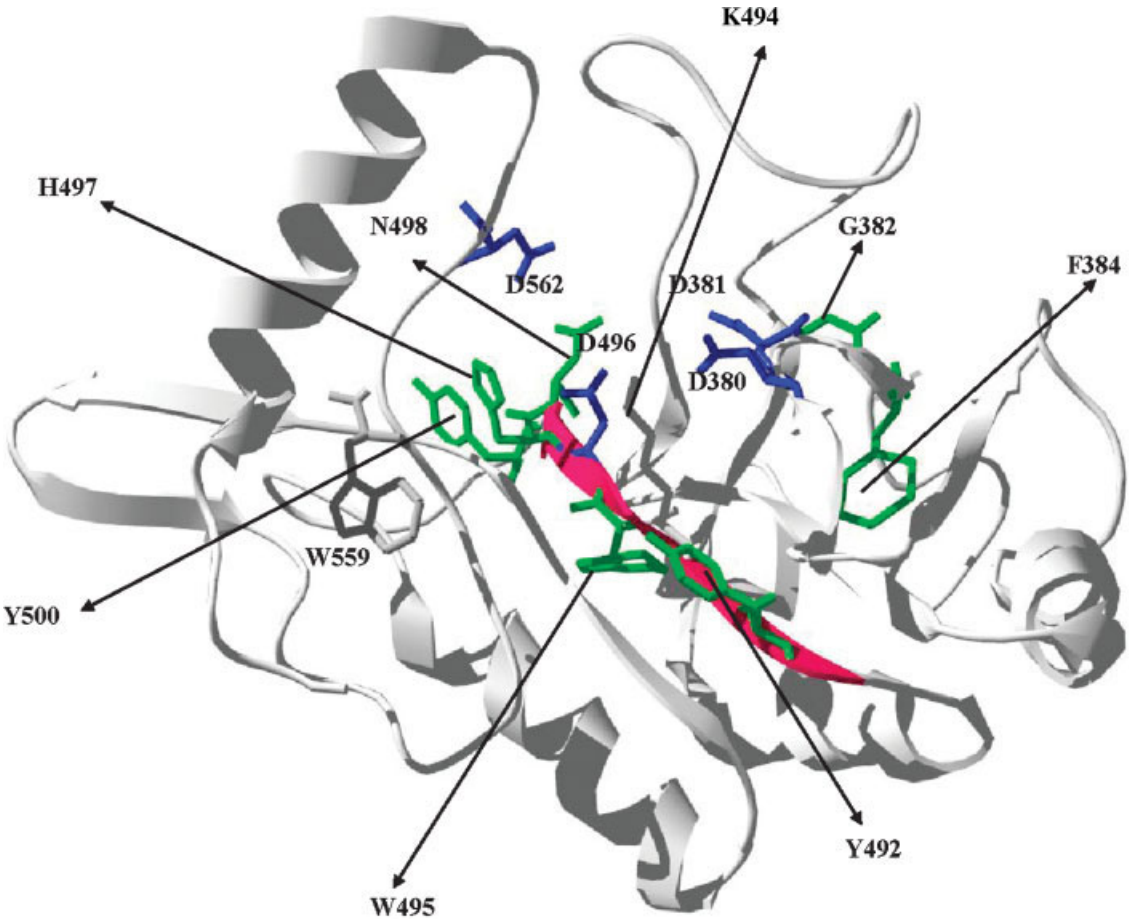

Figure 4. Putative 3D-structure of AGA from *B. adolescentis*, obtained by homology modeling, using the 3D-structure of α-galactosidase from *O. sativa* as a template. The putative catalytic residues, D496 and D562, and the residue involved in substrate binding, D380 and D381 are indicated in blue. Amino acid residues which are subjected to site-directed mutagenesis are shown in green. Residues W559 and K494 are indicated in gray. The strand of amino acids 491–497 was colored red.

and Coomassie brilliant blue staining. However, no activity towards a series of *p*NP-glycosides, other than *p*NP-Gal, was found. The *E. coli* cells itself did not show AGA activity. The AGA hydrolytic activity towards *p*NP-Gal and the transglycosylation activity towards melibiose were determined. These results showed that the hydrolytic activity was lost in the mutants D380N and D381N, suggesting that these residues were indeed involved in the catalytic activity of the enzyme. All other mutant enzymes showed hydrolytic activity towards *p*NP-Gal (data not shown). For the transglycosylation experiments, the amount of enzyme added was based on the hydrolytic activity towards *p*NP-Gal; an equal amount of enzyme activity was added to the incubation mixtures. The mutants G382C, Y492G, H497M, and Y500L showed a higher transglycosylation efficiency than the wild-type enzyme (Fig. 6), for the mutations Y492G, H497M, and Y500L this increase was significant ($P < 0.05$). Only mutant H497M increased by 16% in transglycosylation activity under the conditions tested, whereas that of the other mutants increased by approximately 5%. The percentage of enzyme–substrate encounters resulting in transglycosylation of the mutants was analyzed at optimal transglycosylation conditions for the wild-type AGA. For this enzyme, it was found that this parameter was independent of the incubation time; similar results were obtained for the mutant H497M (data not shown).

To enhance the transglycosylation activity further, double mutants were made. All four single mutations, which gave an increased transglycosylation, were combined to six double mutants: G382C-Y492G, G382C-H497M, G382C-Y500L, Y492G-Y500L, Y492G-H497M, and H497M-Y500L. The double mutants were also cloned and expressed in *E. coli* cells and purified as mentioned before. The hydrolytic activity towards *p*NP-Gal was determined and seemed to be unaltered in the mutant enzymes (data not shown). The transglycosylation activity of the double mutants increased significantly ($P < 0.05$). The mutants G382C-Y500L and H497M-Y500L increased to 10% and 16%, respectively, compared to the wild-type, whereas the other mutant enzymes had an increase of approximately 4%–7% (Fig. 6). This means that the best double mutant had approximately the same transglycosylation activity as single mutant H497M.

Acceptor Specificity and Stability of Mutants

Different acceptor substrates were examined (Table I). The presence of a new product peak in the HPAEC diagrams

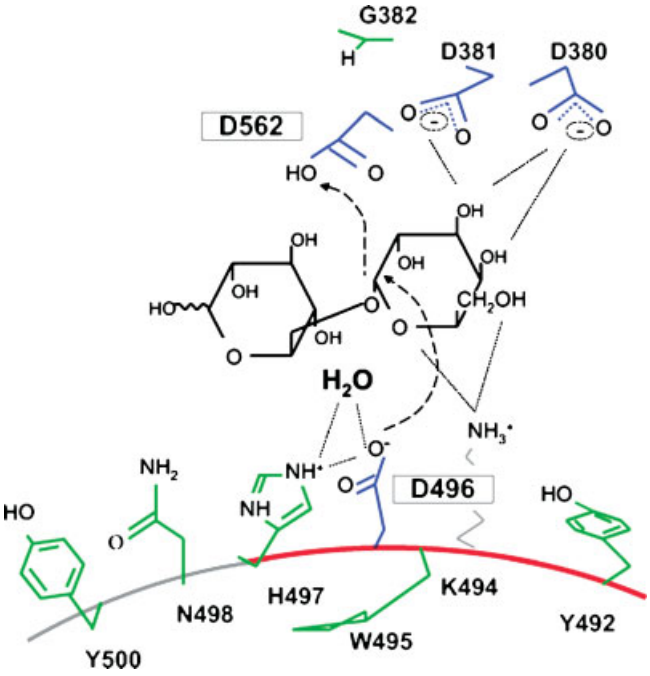

Figure 5. Schematic representation of the catalytic site of AGA from *B. adolescentis* with melibiose as substrate based on the putative 3D-structure. The putative catalytic residues, D496 and D562, and the residues involved in substrate binding, D380 and D381 are indicated in blue. Amino acid residues subjected to site-directed mutagenesis are shown in green. Residue K494 is indicated in gray. The strand of amino acids 491–497 is colored red. The possible H-bonds are indicated with dotted lines. The action of the catalytic residues are indicated by arrows.

indicated the presence of transglycosylation activity towards the acceptor molecule. D-Galactose, D-glucose, L-glucose, D-mannose, lactose, maltose, cellobiose, and sucrose were found to be good acceptors for the wild-type AGA, when melibiose was used as donor. AGA used both the acceptor substrate and melibiose as acceptor. Pentoses (e.g., Xyl), hexuronic acids (e.g., GalA), and deoxyhexoses (e.g., Rha) did not serve as acceptor molecules (Table I). Also alditols did not serve as acceptor molecules. Disaccharides, containing a hexose at the non-reducing terminus, were found to be good acceptors. From these experiments, it was concluded that AGA required a hydroxyl group at C-6 at the non-reducing end of the acceptor substrate to perform transglycosylation. In most cases, one sugar unit was linked to the acceptor molecule; the yield of longer oligosaccharides was very low. The transglycosylation activity of the mutants towards hexose (Glc), pentose (Xyl), deoxyhexose (Rha), and hexuronic acid (GlcA) was unaltered (Table I).

Due to the mutations in the AGA gene, the folding of the enzyme might be changed and therewith the stability of the enzyme. The wild-type AGA was found to be stable for at least 24 h at temperatures below 50°C. The temperature stability of the double mutant enzymes was unaltered, except for the enzymes containing the mutation G382C. These were unstable at temperatures above 37°C.

DISCUSSION

The *B. adolescentis* AGA was subjected to site directed mutagenesis, to unravel the structure–function relation of the enzyme, particularly with respect to transglycosylation. In the putative 3D-structure of AGA four Asp residues were found in the catalytic site of the enzyme. Two residues were identified as the catalytic residues, i.e., D496 and D562. The two other residues, D380 and D381, were subjected to site-directed mutagenesis; mutation from Asp to Asn resulted in an inactive enzyme. Therefore, it was concluded that these

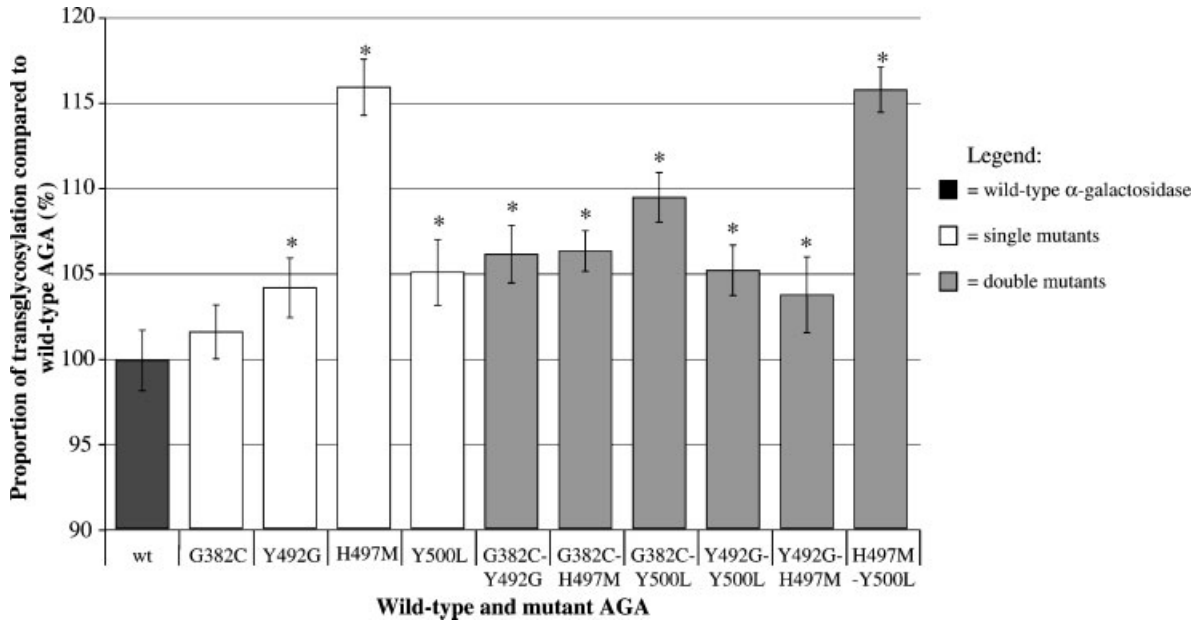

Figure 6. Percentage of transglycosylation of single mutants and double mutants made from *B. adolescentis* AGA. The reaction was carried out during 7 h at pH 8 and 37°C. The initial concentration of melibiose was 600 mM. The amount of enzyme added was based on their hydrolytic activity towards *p*NP-Gal; an equal amount of enzyme activity was added to each incubation. *Indicates a significant difference with the wild-type enzyme ($P < 0.05$).

Table I. Acceptor specificity for the transglycosylation reactions of the wild-type α-galactosidase (AGA) from *Bifidobacterium adolescentis*.

Substrate type	Substrate	Wild-type	Mutants
H	D(+)-Galactose	+	n.d.
	D(+)-Glucose	+	+
	L-Glucose	+	n.d.
	D(+)-Mannose	+	n.d.
	Lactose	+	n.d.
	Maltose	+	n.d.
	D(+)-Cellobiose	+	n.d.
	Sucrose	+	n.d.
P	D(+)-Xylose	−	−
	L(+)-Arabinose	−	n.d.
D	L(+)-Rhamnose	−	−
HA	D-Galacturonic acid	−	n.d.
	D-Glucuronic acid	n.d.	−
A	D(−)-Sorbitol	−	n.d.
	Ethanol	−	n.d.

n.d., not determined.

The reaction was carried out during 7 h at pH 8 and 37°C. The initial concentration of melibiose and that of the acceptor substrate were both 300 mM. The ability of AGA to use a substrate as acceptor is indicated with +. When AGA is unable to use a substrate as acceptor, this is indicated with −. H, hexose at the non-reducing end; P, pentose; D, deoxyhexose; HA, hexuronic acid; A, alditol.

Asp residues were also involved in the catalysis, suggesting that the enzyme has a catalytic tetrad. Such a tetrad has also been described for β-glucanase from barley (Chen et al., 1995). There, the two non-catalytic residues of the catalytic tetrad were suggested to be involved in substrate binding (Chen et al., 1995). Catalytic triads were also found in different types of enzymes (Amaya et al., 2003; Debeche et al., 2002; Schubot et al., 2001; Shaw et al., 2002). The residues D380 and D381 probably play a key role in substrate binding, through H-bonds between both Asp and the substrate (Brzozowski and Davies, 1997; Chen et al., 1995; Debeche et al., 2002; Namchuk and Withers, 1995), and may also play a role in influencing the ionization status of the catalytic residues (Brzozowski and Davies, 1997; Chen et al., 1995; Debeche et al., 2002).

The transglycosylation efficiency was increased significantly in three single and six double mutants. The largest increase was found with the mutants H497M and H497M-Y500L. As shown in Figure 5, H497 was located next to the catalytic D496. It is proposed that this His residue stabilizes the catalytic Asp via H-bonds with the substrate or water in a triangular arrangement, therewith positioning them, and stabilizing the transition state of the Asp and positioning the water molecule for hydrolysis. Replacing the His by a Met residue increased the transglycosylation efficiency of AGA, without altering the absolute activity of the enzyme. Apparently, the Met residue is unable to position the water molecule, thereby increasing the likelihood of other substrates to act as acceptors, which increases transglycosylation activity. These results correspond with factor II, mentioned in the ''Introduction.''

Two other mutations also showed a reasonable increase in transglycosylation activity, i.e., Y500L and Y492G. The Tyr residues were assumed to stabilize substrate binding through stacking interactions (factor I). Based on the putative 3D-structure of AGA, it was found that the residues Y500 and Y492 were not located near the catalytic site (Figs. 4 and 5), which makes stacking interactions with the substrate molecules unlikely. The residue Y492 was, besides a Gly, also mutated to an Ala. The latter mutation decreased the transglycosylation activity. It is unclear what the role of this Tyr residue can be. The stacchyose synthases from GH36 have a Gly residue at the position of Y492. Residue Y500 was located on a coil in a low-similarity region of the putative 3D-model. Since modification of Y500 had a high impact on transglycosylation, compared to Y492, it is suggested that the coil will be folded differently, more close to the catalytic site.

Using GHs to elongated oligosaccharides has an advantage compared to using glycosyl transferases, since low-cost donor substrates can be used. An increase in transglycosylation activity of 16% was reached in this research with only a single mutation. This result will be discussed with results from the literature. For example, β-glucosidase CelB from *Pyrococcus furiosus* showed an increase in transglycosylation activity of approximately 17% after mutation of a Phe to a Tyr residue (Hansson and Adlercreutz, 2001). After mutation, the transglycosylation activity of the enzyme accounted for 69% of all enzyme–substrate encounters. Different mutants of neopullanase from *Bacillus stearothermophilus* showed an increase in transglycosylation between 17% and 21% (Kuriki et al., 1996), which resulted in a maximum of 52% of all enzyme–substrate encounters being a transglycosylation reaction. An α-amylase mutant from *Saccharomycopsis fibuligera*, in which a Tyr was replaced by a Trp residue, produced transglycosylation products from a glucoheptasaccharide up to 25% of the total amount of oligosaccharides in the reaction mixture, whereas no transglycosylation products were formed by the wild-type enzyme (Matsui et al., 1994). Because of the difference in calculation of the transglycosylation activity, it is difficult to compare our results with results found in literature. However, the increase in transglycosylation activity obtained with mutants of AGA seemed to be in the same order of magnitude as found in literature. Wild-type AGA already had a high transglycosylation activity compared to other enzymes, and its transglycosylation activity could be increased to 75% of all enzyme–substrate encounters in mutant H497M.

We also showed that AGA was able to use different acceptor substrates, as long as they contain a hydroxyl group at the C-6 position at the non-reducing end. This property of the enzyme could be used to make rare oligosaccharides. If used as prebiotics, these oligosaccharides might be less easy to ferment and reach the more distal areas of the colon (Voragen, 1998), where the main disorders of the gut occur (Gibson et al., 2000). The transglycosylation products formed in this research were mainly tri- and tetra-saccharides. It will be interesting to elongate oligosaccharides even further, which makes it necessary to improve the transglycosylation even more.

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