Converting a $\beta$-Glycosidase into a $\beta$-Transglycosidase by Directed Evolution

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Directed evolution was applied to the $\beta$-glycosidase of Thermus thermophilus in order to increase its ability to synthesize oligosaccharide by transglycosylation. Wild-type enzyme was able to transfer the glycosyl residue with a yield of 50% by self-condensation and of about 8% by transglycosylation on disaccharides without nitrophenyl at their reducing end. By using a simple screening procedure, we could produce mutant enzymes possessing a high transferase activity. In one step of random mutagenesis and in vitro recombination, the hydrolysis of substrates and of transglycosylation products was considerably reduced. For certain mutants, synthesis by self-condensation of nitrophophenyl glycosides became nearly quantitative, whereas synthesis by transglycosylation on maltose and on cellobiose could reach 60 and 75%, respectively. Because the most efficient mutations, F401S and N282T, were located just in front of the subsite ($-1$), molecular modeling techniques were used to explain their effects on the synthesis reaction; we can suggest that repositioning of the glycone in the ($-1$) subsite together with a better fit of the acceptor in the ($+1$) subsite might favor the attack of a glycosyl acceptor in the mutant at the expense of water. Thus these new transglycosidases constitute an interesting alternative for the synthesis of oligosaccharides by using stable and accessible donor substrates.

The role of carbohydrates is becoming clearer every day, and the development of oligosaccharides is growing in several medical fields as follows: reduction of inflammatory responses by blocking selectins (1); immunization against cancer (2, 3); adhesion inhibition of pathogenic viruses (4), yeasts (5), and bacteria (6–8); and cell targeting for gene therapy (9). However, the difficulties encountered in synthesizing oligosaccharides by conventional synthetic methods have stimulated the development of enzymatic approaches.

Glycosyltransferases and glycosidases constitute the two major classes of enzymes that can be used in such processes. However, the former are difficult to produce, and their substrates (nucleotide phosphosugars) are not readily available despite the fact that new biological methods seem to circumvent these problems (10). In contrast, glycosidases are robust and soluble enzymes, using cheaper and simpler substrates. They usually catalyze the hydrolysis of glycoside bonds but can also synthesize glycosides, especially through the transglycosylation reaction. Some of them naturally display interesting transfer properties (11, 12). Unfortunately, yields in oligosaccharides remain generally moderate. Moreover, even when high regioselective preferences are exerted by glycosidases, the presence of undesired regioisomers, which make the purification step difficult, has encouraged researchers to improve the properties of glycosidases by different strategies (13).

The first one was the development of glycosyntheses as proposed by Withers and co-workers (14, 15) from exoglycosidases and as proposed by Malet and Planas (16) from endoglycosidases. Several other glycosyntheses have been designed recently, including thermophilic enzymes (17). We constructed the mutant E338S of a thermophilic $\beta$-glycosidase, and we were able to achieve the synthesis of glycosyl-($1\rightarrow3$)-glycosides in yields of up to 90% (18). However, the reactions catalyzed by exoglycosyntheses are somewhat slow relative to wild-type glycosidase activities, requiring large quantities of mutant enzyme and/or extended incubation times (19). This drawback was recently overcome for the glycosynthase Agrobacterium sp. $\beta$-glucosidase E358G, which was submitted to a directed evolution process, resulting in the generation of a mutant 27 times more active (20). Nevertheless, some limitations could still be noticed with this approach as follows. (i) Although aryl glycosides can be used as donors with formate or azide (21, 22), $\alpha$-glycosyl fluorides are more often used because neither self-condensation nor hydrolysis of synthesis products can then be observed. However, these donors are thermolabile, and when the reaction is catalyzed by thermophilic enzymes, high concentrations of enzyme must be used to compete efficiently with the spontaneous hydrolysis of $\alpha$-glycosyl fluorides above 50 °C (18). (ii) The acceptor specificity seems often restricted to sugar such as aryl glycosides because almost no examples of transfer on natural disaccharides could be found with exoglycosyntheses (17, 18). In fact, this property is not derived from the nucleophile mutagenesis of glycosidases but rather from the native enzymes themselves. However, transfer onto natural sugar would be of great interest because the sugar remaining at the reductive end could be functional.

The second strategy is based on directed evolution or directed mutagenesis methods to improve the regioselectivity of the substrate specificity, or the transferase activity exerted by these enzymes. Briefly, in these approaches, the gene encoding for the glycosidase is randomly mutagenized and screened for variants displaying improved properties. Several cycles of in vitro recombination can then be applied between improved variants to accumulate positive mutations (23). We demonstrated previously that directed evolution could produce Bacillus $\alpha$-galactosidase mutants displaying completely new regioselectivities (12, 24), while keeping their transglycosylation abilities. Until now, the improvement by directed evolution of glycosidase transglycosylation activity at the expense of hydrolytic activity has not really been investigated despite the fact that some natural glycosylhydrolyses have furnished evidence that hydrolytic activity can be completely abolished while retaining transglycosidase activity (25). Enhancement of the transglycosylation activities of natural glycosidases has rather been obtained by site-directed mutagenesis of certain exoglycosidases or
endoglycosidases or by protein truncation or fusion; mutations M424K and F426Y significantly improved (18–40%) the synthesis of galacto-oligosaccharides from the *Pyrococcus furiosus* β-glucosidase (26). The transferase activities of α-amylases from *Saccharomyces* or from *Bacillus licheniformis* were increased, respectively, by the substitution of W84L and V286F (27, 28). Jorgensen et al. (29) noticed that a truncated β-galactosidase from *Bifidobacterium* displayed a higher trans-galactosylation/hydrolysis ratio (9:1) than the WT enzyme. 2-Fold higher transferase activity was also obtained by construction of chimera-betw-een-β-glucosidases of *Thermotoga maritima* and of *Cellvibrio gilvus* (30). Finally, six mutations on the *Trypanosoma rangeli* sialidase allowed to increase the trans-sialidase activity to about 10% that of the wild-type *Trypanosoma cruzi* trans-sialidase (31).

Thus, we present here a directed evolution application approach to the β-glycosidase of *Thermus thermophilus* (Tt-β-Gly),2 which we cloned and overexpressed previously (32). This recombinant protein has already been analyzed regarding its ability to catalyse transglycosylation reactions with nitrophenyl β-glycosides as donors and various glycosides as acceptors (11, 33, 34). Its thermostability and β-(1→3) regioslectivity makes this enzyme interesting for the synthesis of oligosaccharides of medical interest. Although a significant level of transglycosylation was obtained with the WT enzyme (up to 50%), the hydrolytic activity is still too high, leading to the cleavage of the transglycosylation products and requiring rigorous kinetic control of the reaction. Here we demonstrate that the transglycosylation activity of this β-glycosidase can be considerably improved by directed evolution, which provided mutants with almost no hydrolytic activity. We also propose a rational three-dimensional explanation for the improved properties of the best mutants based on molecular modeling approaches.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Media**—Selection of ampicillin-resistant (100 μg/ml) *Escherichia coli* was made on LB agar plates. Expression of the *tbgly* gene was performed from the pBtac2 vector in the strain XL1 blue MRF’ of *E. coli*. The expression plasmid containing the WT tbgly gene (GenBank™ accession number Y16753) (1.3 kb) under the control of the Ptac promoter and after the PstI restriction site. 20 pmol of 2-Fold reactions with nitrophenyl β-D-galactoside (9:1) than the WT enzyme. 2-Fold the supernatant was centrifuged for 15 min at 13,000 rpm, and transglycosylation activity of enzyme was determined in the supernatant. For this purpose, protein concentration of crude extracts was estimated by the method of Bradford with lysozyme as the standard protein. Enzyme assays were performed by introducing 5 μl of supernatant in 30 μl of a solution containing 50 mmol/liter of nNP-Gal and 50 mmol/liter of maltose (0.1 mmol/liter in phosphate buffer, pH 7). The mixture was reacted at 55 °C, and conversion of the components was followed by means of TLC ( precoated Silica Gel 60 sheets; Merck F254) using eluent (butanol-1/ethanol/water; 5:3:2, v/v/v), and sheets were revealed with cerium molybdate stain. In order to compare the transglycosylation efficiencies of various enzymes, activity was normalized to the activity using nNP-Gal at 2.5 mmol/liter.

**Purification of WT and Mutant Enzymes**—Recombinant strains expressing the mutant TtβGly genes were grown in 400 ml of LB medium, centrifuged, and resuspended in various volumes (2 or 20 ml) of 100 mmol/liter phosphate buffer, pH 7, depending on the specific activity of enzyme. After sonication and centrifugation, the supernatant was heated 30 min at 70 °C to remove most of the thermolabile proteins and centrifuged again for 10 min at 13,000 rpm. The supernatant was passed through a 0.45-μm filter, and purification of enzyme was achieved by HPLC on a column MonoQ HR 5/5 with a buffer of 20 mmol/liter imidazole, pH 7.4, and a 0 to 0.5 mol/liter NaCl gradient. A sample of purified protein analyzed by capillary electrophoresis (Agilent 2100 Bioanalyser) displayed a single peak thus indicating the homogeneity of the preparation. Protein concentrations were also determined by this method.

**Enzyme Kinetics**—Kinetic studies with purified enzymes on chromogenic substrates (*p*NP-Glc and *p*NP-Gal) were performed in microtitre plates by following changes in absorbance at 405 nm using a microplate reader (iEMS, Labsystem) at 40 °C. The buffer employed for all kinetic experiments with wild-type or mutant Tt-β-Gly was 100 mmol/liter sodium phosphate buffer, pH 7.0. Reaction mixtures (190 μl) containing the substrate and buffer, were preincubated in the plate holder for 10 min prior to addition of 10 μl of enzyme. Transglycosylation activities with nNP-Gal as the only substrate were determined by capillary electrophoresis (Beckman P/ACE System 5000 with an uncoated fused silica capillary, 47 cm). Aliquots (10 μl) of the enzymatic reaction mixture were withdrawn at different times and mixed with 10 μl of *p*NP acetate (2.5 mm), used as internal standard, 20 μl of borax buffer (15 mm) containing SDS (75 mm), and 60 μl of water. Separations were performed at 20 kV with Borax (15 mm) and SDS (75 mm) as running buffer. Products, Gal-Gal-nNP and nNP, were detected and quantified by UV absorbance at 214 nm. Reactions with maltose or cellobiose were

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2 The abbreviations used are: Tt-β-Gly, *T. thermophilus* β-glycosidase; HPLC, high performance liquid chromatography; LB, Luria Bertani; WT, wild-type; DP3, β-1,6-Galp-(1→3)-β-1,3-Galp-(1→4)-β-D-Glc; nNP-Gal, 2-nitrophenyl β-D-galactopyranoside; oNP-Glc, 2-nitrophenyl β-D-glucopyranoside; oNP-Cell, 4-nitrophenyl β-D-celllobiose; pNP-Gal, 4-nitrophenyl β-D-galactopyranoside; pNP-Glc, 4-nitrophenyl β-D-glucopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.
performed with semipurified thermostable enzymes, prepared by heating the cellular extracts for 30 min at 70 °C. We checked that the recombinant proteins represented at least 80% of total protein extract. The latter were introduced into reaction mixtures containing 20 mmol/liter of oN-P-Gal (donor) with 20 mmol/liter of an acceptor (maltose or cellobiose) at 55 °C. Aliquots of 4 μl were withdrawn at different times and mixed with 4 μl of 0.1% arabinose, 26 μl of derivatizing solution of sugars containing 2.85 mg of 8-aminonaphthalene-1,3,6-trisulfonic acid in an H2O/acetic acid reaction mixture (1:7), and 33 μl of sodium cyanoborohydride 1 mol/liter in MeSO. These solutions were warmed for 16 h at 40 °C. Analysis of transglycosylation and hydrolysis products was performed using a Beckman P/ACE System 5000 with an uncoated fused silica capillary. Separations were performed at 20 kV in the presence of 50 mmol/liter sodium phosphate, pH 2.5, at 25 °C. Products were detected and quantified by UV absorbance at 214 nm. Arabinose was used as an internal standard. Between runs, the capillary was washed for 2 min with 100 mmol/liter of NaOH solution followed by water for 2 min and then with 50 mmol/liter sodium phosphate, pH 2.5, for 2 min. 

**NMR Spectroscopy—**NMR spectra were recorded with either a Bruker AX400 or DRX500 at 35 °C. In all cases, chemical shifts in ppm were quoted from the resonance of methyl protons of sodium 3-(trimethylsilyl)-propanesulfonate as an internal reference. Complete analysis of the NMR 1H and 13C resonances and subsequent structure assignment were made using standard two-dimensional sequences (COSY HH and HC).

For mutant A113, enzymatic experiments were performed directly in the NMR tube maintained at 55 °C in the spectrometer. 1H NMR spectra were recorded every hour for 10 h. For the self-condensation reaction, oN-P-Glc (140 μmol) was dissolved in 637 μl of 150 mmol/liter ammonium bicarbonate buffer in D2O (pD 7.8), and 63 μl of the mutant enzyme solution (44 μg of enzyme) prepared as described above was added. For the transglycosylation reaction, oN-P-Gal (donor, 35 μmol) and pNP-Cell (acceptor, 35 μmol) were dissolved in 637 μl of 150 mmol/liter ammonium bicarbonate buffer in D2O (pD 7.8), and 63 μl (44 μg) of the mutant enzyme solution prepared as described above was added.

**Determination of Yields with Mutant F401S—**For the self-condensation reaction, oN-P-Gal (40 μmol) was dissolved in 778 μl of 100 mmol/liter sodium phosphate buffer, pH 7.0, and 22 μl of the mutant enzyme solution (15 μg of enzyme) prepared as described above was added. The reaction was performed at 55 °C for 5 h. Then the solvent was evaporated under reduced pressure, and the residue was dissolved into D2O and analyzed by means of 1H NMR spectroscopy. For transglycosylation reactions, oN-P-Gal (donor, 10 μmol) and acceptor (either maltose or cellobiose, 10 μmol) were dissolved in 499 μl of 100 mmol/liter sodium phosphate buffer in D2O (pD 7.0) and 11 μl (7.7 μg of enzyme) of the mutant enzyme solution prepared as described above was added. The reactions were performed at 55 °C for 23 h. The resulting crude mixtures were then analyzed by means of proton NMR spectroscopic 33855 glycosylation was then analyzed by means of 1H NMR spectroscopy. E3385 glycosylation was used as described previously (18).

**β-D-Galp-(1→3)-β-D-Glcp-(1→4)-β-D-Glcp**—For the β-anomer: 1H NMR (D2O): δ 4.653 (IH-1), 4.543 (IH-1), 4.648 (H-1); 13C NMR (D2O): δ 98.5 (C-1), 81.6 (C-4), 78.3 (C-3; C-5), 77.0 (C-2), 62.9 (C-6) 105.0 (C-1), 87.1 (C-3), 78.7 (C-5), 63.4 (C-6), 106.0 (C-1), 63.7 (C-6). For the α-anomer: 1H NMR (D2O): δ 4.656 (IH-1), 4.546 (H-1), 5.215 (H-1); 13C NMR (D2O): δ 94.5 (C-1), 81.7 (C-4), 78.3 (C-3; C-5), 77.5 (C-2), 62.8 (C-6) 105.0 (C-1), 63.4 (C-6), 106.0 (C-1), 63.7 (C-6). 

**β-D-Galp-(1→6)-β-D-Glcp-(1→4)-β-D-Glcp**—For the β-anomer: 1H NMR (D2O): δ 4.444 (IH-1), 4.533 (IH-1), 4.652 (H-1); 13C NMR (D2O): δ 98.5 (C-1), 80.9 (C-4), 62.8 (C-6), 105.7 (C-1), 71.3 (C-6), 105.1 (C-1), 63.7 (C-6). For the α-anomer: 1H NMR (D2O): δ 4.444 (IH-1), 4.533 (IH-1), 5.216 (H-1); 13C NMR (D2O): δ 95.4 (C-1), 80.9 (C-4), 62.7 (C-6) 106.0 (C-1), 71.3 (C-6), 105.1 (C-1), 63.8 (C-6). 

**β-D-Galp-(1→3)-α-D-Galp-(1→4)-β-D-Glcp**—For the β-anomer: 1H NMR (D2O): δ 5.645 (IH-1), 5.395 (H-1), 5.637 (H-1); 13C NMR (D2O): δ 98.5 (C-1), 79.3 (C-4), 77.2 (C-5), 76.7 (C-2), 63.4 (C-6) 102.1 (C-1), 85.0 (C-3), 75.1 (C-5), 63.2 (C-6), 106.0 (C-1), 63.7 (C-6). For the α-anomer: 1H NMR (D2O): δ 5.645 (H-1), 5.395 (H-1), 5.222 (H-1). 13C NMR (D2O): δ 94.6 (C-1), 79.5 (C-4), 63.3 (C-6) 102.2 (C-1), 85.0 (C-3), 75.1 (C-5), 63.4 (C-6), 106.0 (C-1), 63.7 (C-6). 

**Molecular Modeling—**All molecular modeling calculations were performed with the CFF91 force field (36). For the enzyme, the crystal structure of the native Tt-β-Gly (Protein Data Bank code 1ug6) was used. The modeled ligand is the oligomer of DP3: Gal-β-(1→3)-Gal-β-(1→4)-Glc. For this molecule, the main degrees of freedom are located on the glycosidic linkages; thus, two disaccharide maps (Gal-β-(1→3)-Glc and Glc-β-(1→4)-Glc (not shown here) were generated to evaluate their flexibility according to the semi- relaxed protocol already described (37). The first molecular modeling stage consisted of locating the nonreducing substrate ring (galactopyranose) assumed to fill the most deeply buried subsite (−1). To locate this ring in the catalytic cleft, which is absent in the initial crystal structure, the crystal structure of the covalent intermediate between the β-glucosidase of Bacillus polymyxa and a 2-deoxy-2-fluoroglucose (Protein Data Bank code 1e4i) was used to generate a good starting template. Because of the high similarity of these two enzymes at the catalytic core, the superimposition of the complex with the native Tt-β-Gly gave a good initial orientation of galactose in the modeled Tt-β-Gly complex on the basis of 2-deoxy-2-fluoroglucose ring of the covalent intermediate structure. This complex with monomeric substrate was then refined using an energy optimization (steepest descent algorithm, 10,000 iterations). All sets of hydrogen bond networks were manually built and minimized at this stage. The best solution was kept for subsequent constructions. The Tt-β-Gly complex with β-D-Galp-(1→3)-β-D-Glcp was generated from the previous best energy solution by testing all acceptable glycosidic linkage solutions obtained from the corresponding disaccharide map and minimizing them. Among the starting geometries, only one was kept based on an acceptable distance from the anomeric carbon to the nucleophilic residue Glu-338. Finally, from the best docking solution with β-D-Galp-(1→3)-β-D-Glcp, the same protocol was kept to extend the substrate to DP3 inside the catalytic cleft by testing the four glycosidic solutions of the Glc-β-(1→4)-Glc map.

In a second stage, from the structure of the Tt-β-Gly-DP3 complex, similar complexes involving F401S and N282T mutations were built and performed at 55 °C for 23 h. The resulting crude mixture was then analyzed by means of 1H NMR spectroscopy.
refined. For this series of calculations, all alternative hydrogen bond networks in the vicinity of the mutated residues and all probable hydrogen bond network solutions were manually built and minimized. To compare all these complex structures in terms of transglycosylation preferences, the global binding energy was calculated from these refined docking solutions. For more details, partial binding energies were also estimated for individual subsites in each case. The contribution of each subsite was estimated for substrate monomer and amino acid residues with at least one heavy atom <3.6 Å of a pyranose heavy atom. Hydrogen bonding and stacking terms were discriminated, and a van der Waals term was defined by subtraction from the total energy.

RESULTS

Screening and Characterization of Evolved Tt-β-Gly Mutants—In order to improve the transglycosylation/hydrolysis ratio of the T. thermophilus β-glycosidase (Tt-β-Gly), we first performed a random mutagenesis on the gene tfgly. The screening of variants was carried out in two steps as follows. The first step consisted of isolating mutants displaying low hydrolytic activity and, among them, selecting those having kept a transglycosylation activity and thus displaying a higher transglycosylation/hydrolysis ratio. For the second step, the activities of the best mutants were analyzed quantitatively by capillary electrophoresis and NMR spectroscopy to detect possible regioisomers.

Thereby, the gene tfgly was mutagenized by error-prone PCR, and about 5000 recombinant clones were screened on LB plates containing X-gal (0.1 mmol/liter). At this low substrate concentration, we expected a low level of self-condensation reaction, so that enzyme activity, revealed by the appearance of blue color, was only because of hydrolysis of the substrate (Km of Tt-β-Gly 0.8 mM toward X-Gal). Then the most slightly blue colonies (150) were chosen for further analysis; their enzymatic extracts were used in transglycosylation reactions with oNP-Gal (50 mmol/liter) as donor and maltose (50 mmol/liter) as acceptor. Activity was normalized using oNP-Gal at 2.5 mmol/liter to compare the transglycosylation efficiencies of different enzymes.

By contrast, Phe-401 could be replaced by Asn, Lys, Gly, Pro, or Gln, creating mutants able to catalyze the synthesis of equal amounts of trisaccharides obtained from the transglycosylation of oNP-Gal to disaccharides. Of interest, the A113 enzyme had a remarkable behavior, and its activity was very low because the presence of galactose was hardly detectable. Sequences of mutant N282T/F401S displayed a similar pattern to that of mutant A113. This was justified by the absence of enzymatic activity and, among them, selecting those having kept a transglycosylation activity and thus displaying a higher transglycosylation/hydrolysis ratio. For the second step, the activities of the best mutants were analyzed quantitatively by capillary electrophoresis and NMR spectroscopy to detect possible regioisomers.

Saturating mutagenesis was performed on codons 282, 339, 390, and 401, and the transglycosylation activities of 50–100 recombinant clones were systematically tested using TLC plates in standard conditions (see "Experimental Procedures"). For the first three codons, no enhancement of transglycosylation/hydrolysis ratio was obtained in comparison with mutant F401S. This was justified by the absence of enzymatic activity and, among them, selecting those having kept a transglycosylation activity and thus displaying a higher transglycosylation/hydrolysis ratio. For the second step, the activities of the best mutants were analyzed quantitatively by capillary electrophoresis and NMR spectroscopy to detect possible regioisomers.

Considering the interesting behavior of the A113 enzyme, further mutagenesis was performed to determine the role of each mutation. N339T and F401S displayed a similar pattern to that of WT enzyme, whereas the mutant F401S showed a profile identical to that of mutant A113. This observation confirmed that the substitution F401S was responsible for the strong enhancement of the transglycosylation/hydrolysis ratio. Because the mutant N282T was also particularly efficient (Fig. 1), we built the double mutant N282T/F401S (termed B5) to search for a synergistic effect of mutations.
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**TABLE ONE**

| Mutations found in evolved Tt-β-Gly enzymes |
|--------------------------------------------|
| Enzymes | Mutations |
|---------|-----------|
| WT | Thr-116 Phe-114 Leu-126 Glu-186 Val-262 Asn-282 Pro-333 Ans-339 Ans-390 Phe-401 |
| A55 | Ala |
| A80 | Gln Asp Ala |
| A85 | Thr |
| A86 | Ser |
| A113 | Thr Ser |
| A147 | Leu Thr |

**Kinetic Analysis of the Mutants**—In order to further characterize the reaction kinetics of mutants (N282T, N339T, N339T/F401S, F401S, and N282T/F401S), we purified them in two steps. First, after overexpression in *E. coli*, the crude extracts were heated for 30 min at 70 °C to eliminate most of the thermolabile proteins of *E. coli*. Second, the pure *Tt*-β-Gly mutants (>90%) were obtained after separation by ion exchange HPLC. Kinetics of enzymatic reactions were determined at 40 °C with oNP-Glc and oNP-Gal as substrates. Initial velocities at different substrate concentrations were fitted to the Michaelis-Menten equation in most cases, except for some mutants (N339T/F401S, F401S, and N282T/F401S) where no saturation was reached and where the linear evolution gave only the apparent second order reaction constant, *k*<sub>cat</sub>/<K<sub>m</sub> (Fig. 2A). Apparent kinetic parameters for the wild-type and mutant enzymes are summarized in TABLE TWO. Most of the mutants did not exhibit Michaelis-Menten kinetics, which is expected as the release of *p*NP reflects the overall activity, including both transglycosylation and hydrolysis. To get quantitative information on both transglycosylation and hydrolysis activities, reaction products were measured directly by capillary electrophoresis using *o*NP-Gal as substrate. At low substrate concentrations, the initial hydrolysis rate predominates over the transglycosylation reaction rate. As expected, by increasing the substrate concentration, the transglycosylation/hydrolysis ratio increases (TABLE THREE). Furthermore, WT enzyme exhibits moderate regioselectivity, giving one major *o*NP-β-D-Galp-(1→3)-β-D-Galp transglycosylation product and a minor *o*NP-β-D-Galp-(1→6)-β-D-Galp, as checked by NMR. Most interestingly, after only one round of random mutagenesis, the mutants N282T and N339T/F401S had very low hydrolytic activity even at a low substrate concentration (4 mM), confirming the efficiency of the screening process. At higher substrate concentration (>20 mM), the initial rate of hydrolysis was not detectable for the three N339T/F401S, F401S, N282T/F401S mutants using either capillary electrophoresis, dosage of reducing power, or dosage of free galactose by galactose dehydrogenase. Most surprisingly, the recombinant B5 mutant (N282T/F401S) showed significant hydrolytic activity at low *o*NP-Gal concentration (TABLE THREE). To characterize further the evolution process, the effect of different concentrations of acceptors (cellobiose or maltose) on the activity of *Tt*-β-Gly mutants was tested at a fixed concentration of *p*NP-Gal as substrate (Fig. 2B). The WT enzyme was inhibited by cellobiose because this acceptor was also a substrate for the enzyme and then behaved as a competitor for the *p*NP-Gal substrate. Accordingly, the apparent inhibition constant (K<sub>i</sub>) determined by fitting the inhibition curve was in agreement with the K<sub>i</sub> value (2.4 mM at 60 °C) determined with cellobiose as a substrate. With maltose as acceptor, the WT enzyme was only slightly inhibited. By contrast, with mutant F401S a clear increase in the activity was observed with increasing concentrations of both acceptors, confirming that the deglycosylation step was the rate-limiting step.

**FIGURE 2. Steady-state kinetics of WT and evolved Tt-β-Gly enzymes.** A, typical kinetic data for mutant N339T (●), A113 (▲), and F401S (▲) with pNPGal as substrate. B, effect of cellobiose (filled symbols) and maltose (open symbols) on the rate of substrate cleavage for WT (●) and F401S (▲) mutant. Reactions were performed at 40 °C, pH 7.0, at a fixed concentration of pNP-Gal (5 mM). Apparent K<sub>i</sub> value for cellobiose and WT enzyme is 1.2 ± 0.1 mM. For F401S mutant, apparent K<sub>i</sub> values for the acceptor are 3.8 ± 0.7 mM (cellobiose) and 22 ± 3 mM (maltose).

**Quantification and Identification of Transglycosylation Products**—The transglycosylation products of different reactions were quantified by means of proton NMR spectroscopy and capillary electrophoresis. We first analyzed the kinetics of the self-condensation reaction of mutant N339T/F401S with oNP-Glc as substrate until its full consumption (Fig. 3A). Our results indicated that nearly 100% of oNP-Glc was transformed into the...
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TABLE TWO

Kinetic parameters of WT and mutant Tt-β-Gly enzymes at 40 °C, pH 7.0

Activities were determined by following the release of pNP at 405 nm. ND indicates not determined.

| Mutant          | Substrate | Kcat\textsuperscript{a} \(\text{mm} \) | Km\textsuperscript{b} \(\text{mm} \) | kcat/Km\textsuperscript{c} \(\text{min}^{-1} \text{mm}^{-1} \) | Behavior at high substrate concentration |
|-----------------|-----------|-------------------------------|-----------------|-------------------------------------------------|----------------------------------|
| WT              | pNP-Gal   | 1664 ± 48                     | 5.0 ± 0.4       | 332                                             | Saturation                       |
| WT              | pNP-Glc   | 342 ± 10                      | 0.12 ± 0.01     | 2850 (100)\textsuperscript{d}                   | Substrate inhibition \(K_c = 51 ± 11 \text{ mM} \) |
| N339T           | pNP-Gal   | 544 ± 23                      | 5.9 ± 0.7       | 92.2                                            | Saturation                       |
| N339T           | pNP-Glc   | 395 ± 42                      | 0.31 ± 0.09     | 1274 (45)                                       | Saturation                       |
| A85 (N282T)     | pNP-Gal   | 210 ± 14                      | 32.3 ± 3.6      | 6.5                                             | Saturation                       |
| A85 (N282T)     | pNP-Glc   | 84 ± 3                        | 3.2 ± 0.4       | 26.2 (9)                                        | Saturation                       |
| A113 (N339T/F401S) | pNP-Gal   | ND                            | ND              | 0.06                                            | Linear                           |
| F401S           | pNP-Gal   | ND                            | ND              | 0.6 (0.02)                                     | Linear                           |
| F401S           | pNP-Glc   | ND                            | ND              | 1.2                                             | Linear                           |
| B5 (N282T/F401S)| pNP-Gal   | ND                            | ND              | 4.9 (2)                                        | Linear                           |
| B5 (N282T/F401S)| pNP-Glc   | ND                            | ND              | 0.23 (0.008)                                   | Linear                           |

\textsuperscript{a} Numbers in parentheses indicate the relative overall activity compared with the wild type enzyme.

unique oNP β-D-Glcp-(1→2)-β-D-Glcp regioisomer, whereas only a 50% yield was obtained with the WT enzyme (11). Then we tested the transglycosylation reaction of oNP-Gal with pNP-Cell as acceptor (donor/acceptor molar ratio 1:1); after 11 h of reaction, 78% of the galactosyl unit was transferred to pNP-Cell, with a major (1→3)-regioselectivity. It is interesting to note that the self-condensation β-(1→3)-disaccharide, which is also a product of the transformation, was further used as substrate to synthesize the trisaccharides pNP β-D-Galp-(1→α)-cellobiose (Fig. 38).

By means of capillary electrophoresis, we investigated the ability of these evolved enzymes to transfer a galactose residue to various natural disaccharides (maltose or cellobiose). Samples of selected enzymes (WT, N282T, F401S, N339T/F401S, and N282T/F401S) were introduced into reaction mixtures containing 20 mmol/liter oNP-Gal (donor) with 20 mmol/liter of an acceptor at 55 °C. Aliquots were withdrawn at different times and analyzed with derivatization by 8-aminonaphthalene-1,3,6-trisulfonic acid. Amounts of products were plotted against time for each kind of transglycosylation reaction (Fig. 4).

Concerning the transglycosylation on maltose or cellobiose, evolved enzymes produced a high yield of trisaccharides (50–74% for mutant F401S and N282T/F401S), whereas WT enzyme could not produce more than 8%. Moreover, with mutant N282T/F401S, galactose concentration reached only 13% after 72 h of reaction when using maltose or cellobiose as acceptors (data not shown). For all enzymes, hydrolysis of transglycosylation products started slowly once the maximum transglycosylation yield had been achieved, demonstrating that these mutants had a very low hydrolytic activity on nonactivated substrates. With the most evolved mutant (N282T/F401S), this hydrolysis was not even observed using maltose as an acceptor (Fig. 4A). These results were also confirmed by quantitative analysis of products by NMR (TABLE FOUR). Finally, the structures of reaction products were established by means of standard proton and carbon NMR spectroscopy. Thus, mutant F401S transferred a galactosyl unit to maltose with a major (1→3) regioselectivity (almost 100%), whereas transfer to cellobiose resulted in synthesis of two regioisomers, galactosyl-β-D-(1→3)-cellobiose (66%) and galactosyl-β-D-(1→6)-cellobiose (33%).

Molecular Modeling—In order to understand the effect of positive mutations on transglycosylation activity, the wild-type enzyme-ligand complex was built and compared with similar ones with experimentally found mutations. Because cellobiose was a good acceptor for the transglycosylation from pNP-Gal, the conformation of the trisaccharide β-D-
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Figure 3. Kinetic NMR study of transglycosylation reaction mediated by mutant A113. All assays were conducted at 55 °C using T. thermophilus β-glycosidase mutant A113 in deuterated 150 mmol/liter ammonium bicarbonate buffer (pD 7.8). A, self-condensation reaction with oNP-Glc (200 mmol/liter) as substrate (●) leading only to oNP β-β-x-Glc (●); B, transglycosylation reaction using oNP-Gal (50 mmol/liter) as donor (●) and pHp-Cell (50 mmol/liter) as acceptor leading to the formation of self-condensation disaccharide oNP β-β-x-Galp-(1→3)-β-β-x-Galp (●) and trisaccharides oNP β-β-x-Galp-(1→4)-β-β-x-Galp (●) in the enzyme pocket at subsites (−1), (+1), and (+2) was considered.

Figure 4. Kinetic study of transglycosylation reaction analyzed by capillary electrophoresis with WT (●) and evolved Tt-β-Gly enzymes N282T (●), N339T/F401S (●), F401S (●), and N282T/F401S (●) with oNP-Gal (25 mmol/liter) as donor and with various glycosides as acceptors (25 mmol/liter). A, maltose. B, celllobiose. Percentage of transglycosylation corresponds to the molar yield of galactose transferred to the acceptor.

Galp(1→3)-β-β-x-x-Glc (−1→4)-β-β-x-x-Galp in the enzyme pocket at subsites (−1), (+1), and (+2) was considered.

For the enzyme–ligand complex with the wild type, the first major result is the uniqueness of the docking solution despite the 32 possible conformations: eight possible minima from the Galp-β-(1→3)-Glcp map and four possible minima from the Glcp-β-(1→4)-Glcp map. Fig. 5 shows the modeled substrate deeply buried in the catalytic pocket. Despite the large induced fit of both partners, the trisaccharide still suffers significant deformations at the Galp-β-(1→3)-Glc map (compared with minima calculated for the corresponding disaccharide map) due to the pocket environment (TABLE FIVE).

The docking of the same trisaccharide with F401S and N282T mutants has been studied extensively. For the wild-type, only one docking solution has been found for each mutant. For the Galp-β-(1→3)-Glcp linkage, φ(31-1-O1-C3), ψ(1-C1-01-C3), and τ(01-1-O1-C3) values in F401S (61°, 101°, and 125°, respectively) and N282T (32°, 125°, and 126°, respectively) mutants are significantly closer to the corresponding local minima found in the disaccharide map (67°, 119°, and 120°, respectively) than those of the WT enzyme (17°, 136°, and 128°, respectively). Especially for the F401S mutant, this local (φ,ψ) reorientation led to monomer displacements at subsites (−1) and (+1) as well (Fig. 6).

According to the relative docking energy criteria (0.0, −12.2, and −11.0 kcal mol$^{-1}$ for WT, F401S, and N282T enzymes, respectively), the energy differences are sufficiently significant to allow interpretation. Fig. 6 shows the main structural features of these product–enzyme interactions in the WT enzyme and F401S mutant. In F401S mutant (Fig. 6, white), the trisaccharide has significantly shifted compared with the wild-type docking position (in green) not only in subsite (−1) but also for subsite (+1). This clearly means that F401S mutation affects its neighborhood (Fig. 6, M310 and E392 in pink) for the substrate docking, subsequently yielding a substrate displacement for both monomers involved in the transglycosylation. On the contrary, N282T mutation induces much less rearrangement of vicinal residues Asn-219 and Glu-164 (acid/base residue), and the substrate displacement is not visually significant (not shown in figure) compared with the wild-type substrate position. Nevertheless, the docking energy is just as good as for the
As with the Tt-β-Gly glycosynthases (18), nitrophenyl glucosides were the best acceptors, giving rise to the highest transglycosylation/hydrolysis ratio with our best mutants. Above 20 mmol/liter of substrate, hydrolysis was not detectable with oNP-Gal and synthesis by self-condensation reaction of oNP-Glc became nearly quantitative, whereas with the WT enzyme, maximum yields of 50% were obtained (11). In fact, aryl glucosides are known to bind well to the aglycone site (39) which might be explained by a strong stacking between the Trp-312 (subsite (+2)) and the nitrophenyl group of the acceptor. Even for a transglycosylation reaction using pNP-Cell as acceptor, the percentage of transferred galactosyl units (78%) was similar to that obtained with the alternative glycosynthase strategy (18). Few results have been published concerning the transglycosylation on natural disaccharides (17). However, this possibility would be of great interest because the resulting trisaccharides could be easily modified at the free reducing end. The results presented in TABLE FOUR demonstrate that evolved enzymes exhibit interesting performances in comparison to that of glycosynthase E338S constructed from Tt-β-Gly for the transfer onto cellobiose or onto maltose. With cellobiose as acceptor, the transferase activity of the best mutant was slightly higher (60–74%) than that of glycosynthase (50%). With maltose as acceptor, no trisaccharides were obtained with the glycosynthase, whereas with evolved enzymes, they reached yields of 50–63%. This last result can be explained by the second step of the screening procedure, which used maltose as acceptor to isolate the best transglycosidases. It shows that directed evolution is a convenient method to adapt the enzyme to the desired reaction. By contrast with glycosynthases, in all the reactions catalyzed by the evolved mutants, self-condensation products are synthesized together with the transglycosylation product. However, as self-condensation products are also substrates for the enzymes, a major part is converted into transglycosylation products along the time course of the reaction, so that they finally have little effect on the total yield of oligosaccharide products.

Using nitrophenyl galactosides or nitrophenyl glucosides as acceptors, evolved Tt-β-Gly enzymes and glycosynthase E338S presented the major (1→3)-regioselectivity also observed with WT enzyme. By using maltose as acceptor, the (1→3)-regioselectivity is preserved, but with cellobiose, as with WT enzyme, (1→3) and (1→6) transglycosylation products were observed, demonstrating the importance of the site (+2) in positioning the acceptor for the transfer reaction.

**TABLE FOUR**

**Comparison of percentages of transglycosylation on cellobiose or on maltose obtained with evolved Tt-β-Gly enzymes or with Tt-β-Gly E338S glycosynthase**

The syntheses were performed with oNP-Gal (20 mM) as donor for transglycosylation reactions and α-d-galactopyranosyl fluoride (50 mM) as donor for reactions with glycosynthase. In all reactions, the donor/acceptor molar ratio was fixed at 1. ND indicates not determined.

| Enzyme | Yield | Regioselectivity | Enzyme/donor | Time | Yield | Regioselectivity | Enzyme/donor | Time |
|--------|-------|-----------------|--------------|------|-------|-----------------|--------------|------|
| WT     | 8%    | ND              | 0.07         | 23   | 6%    | ND              | 0.07         | 23   |
| F401S  | 60–74%| 1 → 3 + 1 → 6   | 0.75         | 23   | 50–54%| 1 → 3           | 0.75         | 23   |
| B5 (F401S/N282T) | 73%  | 1 → 3 + 1 → 6   | 18           | 46   | 56–63%| 1 → 3           | 18           | 46   |
| Glycosynthase (E338S) | 50%  | 1 → 3 + 1 → x   | 4.1          | 4.5  | 0%    | 1 → 3           | 4.1          | 15   |

* Milligram of enzyme/mmol donor.
* Ratio obtained by capillary electrophoresis after the indicated incubation time at 55 °C.
* The quantity of glycosynthase used for this synthesis was calculated so that the reaction time at 55 °C was short and thus that the spontaneous hydrolysis of galactosyl fluoride was low.

**DISCUSSION**

**Synthetic Performances of the Evolved Transglycosidases—**This work has essentially shown that directed evolution is able to transform a β-glycosidase into a β-transglycosidase. The drawbacks usually encountered in the transglycosylation approach involve concurrent hydrolysis of substrates and products, which decreases the final oligosaccharide yield. In one step of random mutagenesis and in vitro recombination, the hydrolysis of substrate and transglycosylation products by the Tt-β-Gly enzyme was considerably reduced. Our simple screening procedure proved efficient and could produce mutant enzymes possessing a high transferase activity.

As with the Tt-β-Gly glycosynthases (18), nitrophenyl glucosides were the best acceptors, giving rise to the highest transglycosylation/hydrolysis ratio with our best mutants. Above 20 mmol/liter of substrate, hydrolysis was not detectable with oNP-Gal and synthesis by self-condensation reaction of oNP-Glc became nearly quantitative, whereas with the WT enzyme, maximum yields of 50% were obtained (11). In fact, aryl glucosides are known to bind well to the aglycone site (39) which might be explained by a strong stacking between the Trp-312 (subsite (+2)) and the nitrophenyl group of the acceptor. Even for a transglycosylation reaction using pNP-Cell as acceptor, the percentage of transferred galactosyl units (78%) was similar to that obtained with the alternative glycosynthase strategy (18). Few results have been published concerning the transglycosylation on natural disaccharides (17). However, this possibility would be of great interest because the resulting trisaccharides could be easily modified at the free reducing end. The results presented in TABLE FOUR demonstrate that evolved enzymes exhibit interesting performances in comparison to that of glycosynthase E338S constructed from Tt-β-Gly for the transfer onto cellobiose or onto maltose. With cellobiose as acceptor, the transferase activity of the best mutant was slightly higher (60–74%) than that of glycosynthase (50%). With maltose as acceptor, no trisaccharides were obtained with the glycosynthase, whereas with evolved enzymes, they reached yields of 50–63%. This last result can be explained by the second step of the screening procedure, which used maltose as acceptor to isolate the best transglycosidases. It shows that directed evolution is a convenient method to adapt the enzyme to the desired reaction. By contrast with glycosynthases, in all the reactions catalyzed by the evolved mutants, self-condensation products are synthesized together with the transglycosylation product. However, as self-condensation products are also substrates for the enzymes, a major part is converted into transglycosylation products along the time course of the reaction, so that they finally have little effect on the total yield of oligosaccharide products.

Using nitrophenyl galactosides or nitrophenyl glucosides as acceptors, evolved Tt-β-Gly enzymes and glycosynthase E338S presented the major (1→3)-regioselectivity also observed with WT enzyme. By using maltose as acceptor, the (1→3)-regioselectivity is preserved, but with cellobiose, as with WT enzyme, (1→3) and (1→6) transglycosylation products were observed, demonstrating the importance of the site (+2) in positioning the acceptor for the transfer reaction.

**Structural Basis of the Evolution Process—**After the first step of evolution process, the best evolved enzymes (A113 and A85/A86) bore mutations very close to the active site, but some efficient transglycosidases were also obtained by mutations located far from it (mutants A55, A147). These results illustrate the difficulty of engineering improved
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TABLE FIVE
Partial binding energies for individual subsites

For each subsite, the total partial energy was roughly shared between hydrogen bond (HB), stacking (Stack.), and van der Waals (vdW) terms. Explanations are given under “Experimental Procedures.” For wild type, H-bonds were located only at subsite (−1), O1·OE2(Glu-164), O2·OE2(Glu-338), O3·OE1(Asp-8), O4·OE2(Glu-392). For F401S mutant, a greater H-bond network was present at subsite (−1), O2·OE2(Glu-164), O2·OE2(Glu-338), O3·OE1(Trp-393), O4·NE1(Thr-393), O4·OE1(Glu-392), O6·NE1(Glu-392), and O6·OG(Ser-401). At subsites (+1) and (+2), H-bonds were O6·ND2(Asn-219) and O6·N Ala-285, respectively. For N282T mutant, the H-bond network in subsite (−1) was similar to that of the wild type, plus: O2·OE2(Glu-164) and O3·NE1(Trp-393). At subsites (+1) and (+2), H-bonds were those of F401S mutant.

| Subsite | Tf-β-Gly WT | Mutant F401S | Mutant N282T |
|---------|-------------|--------------|--------------|
|         | Total      | HB | Stack. | vdW | Total | HB | Stack. | vdW | Total | HB | Stack. | vdW |
| (−1)    | −66.4 | −13.6 | −5.6 | −47.2 | −68.1 | −31.9 | −8.7 | −27.5 | −72.9 | −24.7 | −8.2 | −40.0 |
| (+1)    | −14.2 | −14.2 | −19.2 | −2.7 | −16.5 | −16.6 | −3.4 | −13.2 |
| (+2)    | −14.5 | −5.1 | −9.4 | −20.8 | −1.6 | −6.5 | −12.7 | −18.4 | −1.6 | −6.5 | −10.3 |

FIGURE 6. Enzyme/DP3 docking solutions. For wild type, main enzyme residues (in thin sticks) involved in substrate binding are colored in blue. Ball atoms are those forming strong hydrogen bonds; Trp-312 and Trp-385 strongly stack substrate rings in subsites (+2) and (−1), respectively. Corresponding substrate is colored in green. For mutant F401S, residues involved in substrate docking and significantly shifted from wild-type position are colored in purple (Met-310, Ser-401, and Glu-392). (The others have very similar positions and are not represented.) Corresponding substrate is colored in white. For mutant N282T, residues involved in substrate docking and significantly shifted from the wild-type position are colored in yellow (Asn-219, Asn-282, and Glu-164). (The others have very similar positions and are not represented.) Corresponding substrate has a very similar location to that of wild type (not represented).

glycosidases on a rational basis. In the second step of the evolution process, based on the recombination of mutations, the N339T substitution in A113, while located just after the nucleophile residue Glu-338, was finally not responsible for the transglycosylation improvement. However, a cumulative effect of the mutations F401S and N282T was observed in the evolved enzyme B5, which displays the highest potential for the transfer on maltose. Among the β-glycosidases of family 1, amino acid Asn-282 remains very conserved, whereas Phe-401 is sometimes replaced by a tyrosine. Analysis of the three-dimensional structure of Tt-β-Gly showed that the most efficient mutation, F401S, was located just in front of the subsite (−1). This was somewhat surprising because the substitution of this residue did not directly entail a modification of subsites (+1)/(+2). Moreover, saturation mutagenesis at this position revealed that it can be replaced by several different amino acids providing mutants with similar high transglycosylation activity. This result is consistent with the slight improvement of transglycosylation activity observed with the P. furiosus β-glucosidase after mutation of the homologous position F426Y (26). This suggests that this residue, or a homologous position in glycosidases of family 1, is one of the best targets to improve the transglycosidase activity of this enzyme family.

Based on the Tt-β-Gly three-dimensional structure, molecular modeling techniques have been used to give three-dimensional insight into the wild-type enzyme and the best mutants experimentally obtained and explain the efficacy of the directed evolution strategy. The major difficulties for the molecular modeling approach was to explain the kinetic differences between the wild-type and mutated enzyme based only on energy interaction and contact surfaces between the enzyme and its substrates. However, we tried to dissect the possible consequences of individual mutations (F401S and N282T) by docking the final product of the transglycosylation reaction. We reasoned that, as the deglycosylation step is the rate-limiting step in the reaction mechanism, improved transglycosidase activity would rely on an increased affinity of the acceptor for the binding site of the mutant. Consequently, the docking energy of the DP3 product, β-D-Galp-(1→3)-β-D-Glcp-(1→4)-β-D-Glcp, may be a good indicator of the efficiency of this reaction. As illustrated in Fig. 6, mutation F401S induces a significant shift in the position of the DP3 product within the binding site, particularly at the level of the subsites (+1) and (−1), although this mutation is located at the (−1) subsite. This better docking relies on two criteria: the binding energy (TABLE FIVE) and the minimum deformation (dihedrals and valence angles) of the DP3 product within the binding site. According to these calculations, this clearly shows the dispatching of steric constraints all along the substrate by taking into account residues directly in contact but also involves induced medium distance effect. Consequently, repositioning of the glycone in the (−1) subsite together with a better fit of the acceptor in the (+1) subsite might favor the attack of a glycosyl acceptor in the mutant at the expense of water.

These molecular modeling results also agree with the steady-state kinetics. Kinetic analysis of the best mutants (Fig. 2B) has shown a clear activation of the mutant enzymes upon addition of a glycosyl acceptor, cellobiose or maltose, which confirms the faster rate of deglycosylation with the glycoside acceptors than with water. A 2.5-fold rate increase was observed with both acceptors, whereas saturation was observed at 20 mM for cellobiose and 80 mM for maltose. This saturation behavior most likely represents the binding of the acceptors to site (+1) and (+2), with apparent $K_m$ values of 3.8 and 22 mM for cellobiose and maltose, respectively.

In conclusion, these results suggest that directed evolution of the glycosidases in transglycosidases could be an alternative to the glycosynthase strategy with the aim of creating new enzymes for oligosaccharide synthesis. Experiments are in progress in order to still broaden the acceptor specificity so that this enzyme could be used for multiple synthesis reactions.

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