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Converting a β -Glycosidase into a β -Transglycosidase by Directed Evolution*

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Directed evolution was applied to the β -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 nitrophenyl 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 glycosynthases as proposed by Withers and co-workers (14, 15) from exoglycosidases and as proposed by Malet and Planas (16) from endoglycosidases. Several other glycosynthases have been designed recently, including thermophilic enzymes (17). We constructed the mutant E338S of a thermophilic β -glycosidase, and we were able to achieve the synthesis of glycosyl- β -(1 \rightarrow 3)glycosides in yields of up to 90% (18). However, the reactions catalyzed by exoglycosynthases 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. β -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), α -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 α -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 exoglycosynthases (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, 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* α -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 glycosylhydrolases 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

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endoglycosidases or by protein truncation or fusion; mutations M424K and F426Y significantly improved (18–40%) the synthesis of galactooligosaccharides from the *Pyrococcus furiosus* β -glucosidase (26). The transferase activities of α -amylases from *Saccharomycopsis* 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 transgalactosylation/hydrolysis ratio (9:1) than the WT enzyme. 2-Fold higher transferase activity was also obtained by construction of chime-ras between 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 approach applied 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 catalyze transglycosylation reactions with nitrophenyl B-glycosides as donors and various glycosides as acceptors (11, 33, 34). Its thermostability and β -(1 \rightarrow 3) regioselectivity 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 μ g/ml) Escherichia coli was made on LB agar plates. Expression of the $tt\beta gly$ gene was performed from the pBtac2 vector in the strain XL1 blue MRF' of *E. coli*. The expression plasmid containing the WT $tt\beta gly$ gene (GenBankTM accession number Y16753) (1.3 kb) under the control of the Ptac promoter and between the restriction sites EcoRI and PstI was termed pBBGly.

Random and Directed Mutagenesis—Random mutations were introduced by mutagenic PCR (35). Primers D (5'-CAATTAATCATCG-GCTCG) and F (5'-AATCTTCTCTCATCCGCC) flank the gene before the Ptac promoter and after the PstI restriction site. 20 pmol of each primer was mixed with 50 ng of plasmid pBBGly in a 50- μ l PCR. The reaction conditions were as follows: 1× Dynazyme buffer, 1 mmol/ liter of dTTP and dCTP, 0.2 mmol/liter of dATP and dGTP, 7 mmol/ liter MgCl₂, and 2.5 units of polymerase Dynazyme Ext (Finnzymes). The reaction was thermocycled as follows: one cycle at 96 °C, 5 min, then 30 cycles at 96 °C, 30 s; 55 °C, 30 s; 72 °C, 5 min. Mutagenized PCR products were digested by the EcoRI and PstI restriction enzymes and cloned back into the pBTac2 vector digested by the same enzymes.

Mutants N339T and F401S were generated by genetic engineering. The double mutant N282T/F401S was obtained by *in vitro* recombination of the corresponding genes using PCR with overlapping extension. Saturation mutagenesis of codons 282, 339, 390, and 401 were carried out as follows: mutagenic oligonucleotides were designed to present a codon 5'-NNK-3' (or a codon 5'-MNN-3' on the complementary strand) in front of the targeted codon. Amplification of each part of the gene was then reconstituted by overlapping extension and cloned in the same vector. The 1.3-kb DNA fragments encoding for mutant $Tt\beta$ -Gly enzymes were sequenced in both forward and reverse directions.

Screening of Mutants-Random-mutagenized tt BGly genes ligated to the pBtac2 vector were transformed into XL1 blue cells, which were plated on LB agar plates containing 100 μ g/ml ampicillin and 0.1 mmol/ liter X-gal. After 18 h of growth, the slightly blue colonies were grown and analyzed for their activities of transglycosylation. Crude extracts of selected clones were prepared as follows: each clone was grown in LB medium, and 1 ml of overnight cultures was centrifuged, resuspended in 50 μ l of 0.1 mol/liter sodium phosphate buffer, pH 7.0, and subjected to a brief sonication. Isopropyl 1-thio- β -D-galactopyranoside was not used for the induction because, in this system, it did not increase significantly the β -galactosidase activity. Then the lysate was centrifuged for 15 min at 13,000 rpm, and transglycosylation activity 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 μ l of supernatant in 30 μ l of a solution containing 50 mmol/liter of *o*NP-Gal and 50 mmol/liter of maltose (0.1 mmol/liter in phosphate buffer, pH 7). The mixture was allowed to react 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 different enzymes, activity was normalized to the activity using oNP-Gal at 2.5 mmol/liter.

Purification of WT and Mutant Enzymes—Recombinant strains expressing the mutant $tt\beta Gly$ 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 resulting supernatant was passed through a 0.45- μ m 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 (pNP-Glc and pNP-Gal) were performed in microtitration 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 μ l), containing the substrate and buffer, were preincubated in the plate holder for 10 min prior to addition of 10 μ l of enzyme. Transglycosylation activities with oNP-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 μ l) of the enzymatic reaction mixture were withdrawn at different times and mixed with 10 μ l of pNP acetate (2.5 mM), used as internal standard, 20 μ l of borax buffer (15 mM) containing SDS (75 mM), and 60 μ l of water. Separations were performed at 20 kV with Borax (15 mM) and SDS (75 mM) as running buffer. Products, Gal-Gal-oNP and oNP, were detected and quantified by UV absorbance at 214 nm. Reactions with maltose or cellobiose were

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-D-Galp-(1→3)-β-D-Glcp-(1→4)-β-D-Glcp; oNP-Gal, 2-nitrophenyl β-D-galactopyranoside; pNP-Glc, 2-nitrophenyl β-D-glucopyranoside; pNP-Cell, 4-nitrophenyl β-D-glucopyranoside; pNP-Glc, 4-nitrophenyl β-D-glactopyranoside; β-D-g

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 oNP-Gal (donor) with 20 mmol/liter of an acceptor (maltose or cellobiose) at 55 °C. Aliquots of 4 μ l were withdrawn at different times and mixed with 4 μ l of 0.1% arabinose, 26 μ l of derivatizing solution of sugars containing 2.85 mg of 8-aminonaphthalene-1,3,6-trisulfonic acid in an H₂O/acetic acid mixture (17:3), and 30 μ l of sodium cyanoborohydride 1 mol/liter in Me₂SO. 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 55 °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, *o*NP-Glc (140 μ mol) was dissolved in 637 μ l of 150 mmol/liter ammonium bicarbonate buffer in D₂O (pD 7.8), and 63 μ l of the mutant enzyme solution (44 μ g of enzyme) prepared as described above was added. For the transglycosylation reaction, *o*NP-Gal (donor, 35 μ mol) and *p*NP-Cell (acceptor, 35 μ mol) were dissolved in 637 μ l of 150 mmol/liter ammonium bicarbonate buffer in D₂O (pD 7.8), and 63 μ l (44 μ g) of the mutant enzyme solution prepared as described above was added.

Determination of Yields with Mutant F401S—For the self-condensation reaction, oNP-Gal (40 μ mol) was dissolved in 778 μ l of 100 mmol/ liter sodium phosphate buffer, pH 7.0, and 22 μ l of the mutant enzyme solution (15 μ g 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, *o*NP-Gal (donor, 10 μ mol) and acceptor (either maltose or cellobiose, 10 μ mol) were dissolved in 499 μ l of 100 mmol/liter sodium phosphate buffer into D₂O (pD 7.0) and 11 μ l (7.7 μ g 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 spectroscopy.

In addition, galactosyl- β -(1 \rightarrow 3)-maltoside, galactosyl- β -(1 \rightarrow 3)-cellobioside, and galactosyl- β -(1 \rightarrow 6)-cellobioside were obtained by using a multigram scale version of NMR experiments. The crude products were purified by silica gel column chromatography (butanol-1/ethanol/water, 10:3:2, v/v/v) which were performed on Silica Gel Si 60, 40–63 μ m (Merck), using appropriate solvent systems.

Determination of Yields with Mutant B5—For transglycosylation reactions, oNP-Gal (donor, 10 μ mol) and maltose (acceptor, 10 μ mol) were dissolved in 400 μ l of 100 mmol/liter sodium phosphate buffer in D₂O (pD 7.0), and 120 μ l (181 μ g of enzyme) of the mutant enzyme solution prepared as described above was added. The reaction was per-

formed at 55 °C for 23 h. The resulting crude mixture was then analyzed by means of 1 H NMR spectroscopy. E338S glycosynthase was used as described previously (18).

 $\begin{array}{l} \beta\mbox{-}D\mbox{-}Galp\mbox{-}(1\mbox{-}3)\mbox{-}\beta\mbox{-}D\mbox{-}Glcp\mbox{-}(1\mbox{-}4)\mbox{-}D\mbox{-}Glcp\mbox{-}For the $\beta\mbox{-}anomer: ^{1}H NMR (D_{2}O): δ 4.653 ($^{11}H\mbox{-}1), 4.543 ($^{11}H\mbox{-}1), 4.648 ($^{1}H\mbox{-}1); ^{13}C NMR (D_{2}O): δ 98.5 ($^{1}C\mbox{-}1), 81.6 ($^{1}C\mbox{-}4), 78.3 ($^{1}C\mbox{-}5), 77.0 ($^{1}C\mbox{-}2), 62.9 ($^{1}C\mbox{-}6) ($^{11}C\mbox{-}1), 87.1 ($^{11}C\mbox{-}3), 78.7 ($^{11}C\mbox{-}5), 63.4 ($^{11}C\mbox{-}6), 106.0 ($^{11}C\mbox{-}1), 63.7 ($^{11}C\mbox{-}6), For the $\alpha\mbox{-}anomer: ^{1}H NMR (D_{2}O): δ 4.656 ($^{11}H\mbox{-}1), 4.546 ($^{11}H\mbox{-}1), 5.215 ($^{1}H\mbox{-}1); ^{13}C NMR (D_{2}O): δ 94.5 ($^{1}C\mbox{-}1), 81.7 ($^{1}C\mbox{-}4), 78.3 ($^{1}C\mbox{-}3; $^{1}C\mbox{-}5), 77.5 ($^{1}C\mbox{-}2), 62.8 ($^{1}C\mbox{-}6) 105.0 ($^{11}C\mbox{-}1), 63.4 ($^{11}C\mbox{-}6), 106.0 ($^{11}C\mbox{-}1), 63.7 ($^{11}C\mbox{-}6). \end{array}$

 $\begin{array}{l} \beta\mbox{-}D\mbox{-}Galp\mbox{-}(1\mbox{-}\delta)\mbox{-}D\mbox{-}Glcp\mbox{-}(1\mbox{-}4)\mbox{-}D\mbox{-}Glcp\mbox{-}For the $\beta\mbox{-}anomer: 1H NMR (D_2O): δ 4.444 ($^{111}\mbox{H}\mbox{-}1), 4.533 ($^{11}\mbox{H}\mbox{-}1), 4.652 ($^{11}\mbox{H}\mbox{-}1), 71.3 ($^{11}\mbox{C}\mbox{-}6), 105.7 ($^{11}\mbox{C}\mbox{-}1), 80.9 ($^{11}\mbox{C}\mbox{-}1), 80.9 ($^{11}\mbox{C}\mbox{-}1), 80.9 ($^{11}\mbox{C}\mbox{-}1), 80.9 ($^{11}\mbox{C}\mbox{-}1), 80.9 ($^{11}\mbox{C}\mbox{-}1), 80.9 ($^{11}\mbox{C}\mbox{-}1), 71.3 ($^{11}\mbox{C}\mbox{-}0), 105.1 ($^{111}\mbox{C}\mbox{-}1), 80.8 ($^{11}\mbox{C}\mbox{-}1), 80.8 ($^{11}\mbox{-}1), 80.8 ($^$

 $\begin{array}{l} \beta_{\text{-}D\text{-}Galp\text{-}(1\rightarrow3)\text{-}\alpha\text{-}D\text{-}Glcp\text{-}(1\rightarrow4)\text{-}D\text{-}Glcp\text{-}\text{-}\text{For the }\beta\text{-}\text{anomer: }^{1}\text{H}\\ \text{NMR }(\text{D}_{2}\text{O})\text{: }\delta\text{ 5.645 }(^{\text{III}}\text{H}\text{-}1)\text{, }5.395 }(^{\text{II}}\text{H}\text{-}1)\text{, }5.637 }(^{1}\text{H}\text{-}1)\text{; }^{13}\text{C} \text{ NMR}\\ (\text{D}_{2}\text{O})\text{: }\delta\text{ 98.5 }(^{1}\text{C}\text{-}1)\text{, }79.3 }(^{1}\text{C}\text{-}4)\text{, }77.2 }(^{1}\text{C}\text{-}5)\text{, }76.7 }(^{1}\text{C}\text{-}2)\text{, }63.4 }(^{1}\text{C}\text{-}6)\text{ }102.1 }(^{11}\text{C}\text{-}1)\text{, }85.0 }(^{11}\text{C}\text{-}3)\text{, }75.1 }(^{11}\text{C}\text{-}5)\text{, }63.2 }(^{11}\text{C}\text{-}6)\text{, }106.0 }(^{111}\text{C}\text{-}1)\text{, }63.7 }(^{111}\text{C}\text{-}6)\text{.}\\ \text{For the }\alpha\text{-}\text{anomer: }^{1}\text{H} \text{ NMR }(\text{D}_{2}\text{O})\text{: }\delta\text{ }5.645 }(^{111}\text{H}\text{-}1)\text{, }5.395 }(^{11}\text{H}\text{-}1)\text{, }5.222 }(^{1}\text{H}\text{-}1)\text{, }1^{3}\text{C} \text{ NMR }(\text{D}_{2}\text{O})\text{: }\delta\text{ }94.6 }(^{1}\text{C}\text{-}1)\text{, }79.5 }(^{1}\text{C}\text{-}4)\text{, }63.3 }(^{1}\text{C}\text{-}6)\text{ }102.2 }(^{11}\text{C}\text{-}1)\text{, }85.0 }(^{11}\text{C}\text{-}3)\text{, }75.1 }(^{11}\text{C}\text{-}5)\text{, }63.4 }(^{11}\text{C}\text{-}6)\text{, }106.0 }(^{111}\text{C}\text{-}1)\text{, }63.7 }(^{111}\text{C}\text{-}6)\text{.}\\ \text{For } \end{tabular}^{13}\text{C} \text{ NMR }(\text{D}_{2}\text{O}) = 102.6 }(^{1}\text{C}\text{-}1\text{ }and \end{tabular}^{11}\text{C}\text{-}1)\text{, }98.0 }(^{111}\text{C}\text{-}1)\text{.} \text{ For } \\ \text{C}_{19}\text{H}_{34}\text{O}_{16} (518)\text{: } \text{ calculated}, \text{C} 44.02 \text{ }and \text{ }H \text{ }6.56\text{; }found, \text{ C} 43.93 \text{ }H \text{ }6.59\text{.} \end{array}$

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 \rightarrow 3)-Glc- β - $(1\rightarrow 4)$ -Glc. For this molecule, the main degrees of freedom are located on the glycosidic linkages; thus, two disaccharide maps (Gal- β -(1 \rightarrow 3)-Glc and Glc- β -(1 \rightarrow 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-2fluoroglucose 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 \rightarrow 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 \rightarrow 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 Glcp- β -(1 \rightarrow 4)-Glcp 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





SCHEME 1. Test of transgalactosylation reaction used to screen the mutant enzymes.

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 $tt\beta gly$. 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 $tt\beta gly$ 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 (K_m 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 *o*NP-Gal (50 mmol/liter) as donor and maltose (50 mmol/liter) as acceptor at 55 °C (Scheme 1).

The selection of mutants able to catalyze the transglycosylation reaction on an acceptor rather than the self-condensation one was justified by synthetic applications because the two reactions are often in competition (11). Analysis of products by TLC revealed that six clones (A55, A80, A85, A86, A113, and A147) catalyzed the formation of more transglycosylation products than did the WT enzyme (Fig. 1). Moreover, with mutant A113, the hydrolytic activity was very low because the presence of galactose was hardly detectable. Sequences of mutant enzymes (see TABLE ONE) revealed the presence of 1–4 mutations in the different genes. The residue Asn-282 was found to be substituted by a Thr or by a Ser in the two independent mutants A85 and A86, respectively. In addition, the residue Asn-390 was found to be changed into an Ile and into a Thr for the mutants A80 and A147, respectively. Finally, it can be pointed out that the N339T substitution in mutant A113 is localized just after the nucleophile residue Glu-338.



FIGURE 1. Analysis of transglycosylation products of random mutagenized mutants of Tt β -Gly. Enzymatic extracts of different clones were added to 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. After incubation for 24 h at 55 °C, 1 μ l of each reaction mixture was placed on a TLC plate, migrated with eluent (butanol-1/ethanol/water, 5:3:2, v/v/v), and revealed by cerium molybdate stain. Controls: *lane 1*, oNP-Gal with galactose and maltotetraose.

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 to the mutants A85/A86, A113, and A80, respectively. Meanwhile, some clones corresponding to the mutagenesis of the Asn-339 residue gave rise to mutant enzymes with altered regioselectivities (data not shown). 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 *o*NP-Gal to disaccharides as did mutant F401S.

Considering the interesting behavior of the A113 enzyme, further mutagenesis was performed to determine the role of each mutation N339T and F401S. Single mutants were built and tested for their transglycosylation activity under the same conditions. As expected, mutant N339T 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 synergic effect of mutations.

TABLE ONE												
Mutations found in evolved Tt- eta -Gly enzymes												
Amino acids in boldface type are located just around the enzyme pocket.												
Enzymes	Enzymes Mutations											
WT	Thr-116	Phe-114	Leu-126	Glu-186	Val-262	Asn-282	Pro-333	Ans-339	Asn-390	Phe-401		
A55	Ala		Gln	Asp	Ala							
A80									Ile			
A85						Thr						
A86						Ser						
A113								Thr		Ser		
A147		Tyr					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, $k_{\rm cat}/K_m$ (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 oNP-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 $oNP \beta$ -D-Galp-(1 \rightarrow 3)- β -D-Galptransglycosylation product and a minor oNP β -D-Galp-(1 \rightarrow 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 oNP-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-B-Gly mutants was tested at a fixed concentration of pNP-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_i = 1.2 mM) determined by fitting the inhibition curve was in agreement with the K_m 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 *p*NPGal as substrate. *B*, effect of cellobiose (*filled symbols*) and maltose (*open symbols*) on the rate of substrate cleavage for WT (**●**, \bigcirc) and F401S (**■**, \bigcirc) mutant. Reactions were performed at 40 °C, pH 7.0, at a fixed concentration of *p*NP-Gal (5 mM). Apparent *K*_i value for cellobiose and WT enzyme is 1.2 ± 0.1 mM. For F401S mutant, apparent *K*_m 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

Mutant	Substrate	k _{cat}	K _m	$k_{\rm cat}/K_m$	Behavior at high substrate concentration		
		min ⁻¹	тм	$min^{-1} mm^{-1}$			
WT	<i>p</i> NP-Gal	1664 ± 48	5.0 ± 0.4	332	Saturation		
	pNP-Glc	342 ± 10	0.12 ± 0.01	2850 (100) ^a	Substrate inhibition $K_i = 51 \pm 11 \text{ mM}$		
N339T	<i>p</i> NP-Gal	544 ± 23	5.9 ± 0.7	92.2	Saturation		
	pNP-Glc	395 ± 42	0.31 ± 0.09	1274 (45)	Saturation		
A85 (N282T)	<i>p</i> NP-Gal	210 ± 14	32.3 ± 3.6	6.5	Saturation		
	pNP-Glc	84 ± 3	3.2 ± 0.4	26.2 (9)	Saturation		
A113 (N339T/F401S)	<i>p</i> NP-Gal	ND	ND	0.06	Linear		
	pNP-Glc	ND	ND	0.6 (0.02)	Linear		
F401S	<i>p</i> NP-Gal	ND	ND	1.2	Linear		
	pNP-Glc	ND	ND	4.9 (2)	Linear		
B5 (N282T/F401S)	pNP-Glc	ND	ND	0.23 (0.008)	Linear		

TABLE THREE

Transglycosylation and hydrolysis activities of WT and mutant ${\rm Tt}\beta{\rm -Gly}$ enzymes

The activities were measured at 4	40 °C using purified	enzymes and <i>o</i> NP-Gal as	substrate.			
Mutant	oNP-Gal	Transglyo	cosylation ^a	Hydrolysis ^b	Overall activity ^c	
		oNP-Gal(1→3)Ga	l øNPGal(1→6)Gal			
	тм	µmol/(min•mg)	µmol/(min•mg)	µmol/(min•mg)	µmol/(min•mg)	
WT	4	$393 \pm 38 (25)^d$	52 ± 3(3)	1128 ± 81 (72)	1567 ± 113	
	10	1156 ± 54 (43)	128 ± 3(5)	1425 ± 120 (52)	2716 ± 176	
A85 (N282T)	4	119 ± 7 (<i>100</i>)	Undetectable	е	121 ± 17	
	10	229 ± 19 (<i>100</i>)	Undetectable	е	227 ± 43	
A113 (N339T, F401S)	4	1.6 ± 0.1 (80)	Undetectable	0.4 ± 0.1 (20)	2.0 ± 0.1	
	10	3.2 ± 0.1 (78)	Undetectable	0.9 ± 0.1 (22)	4.1 ± 0.3	
F401S	4	65 ± 6 (89)	Undetectable	8 ± 5 (11)	73 ± 7	
	10	73 ± 8 (92)	Undetectable	6 ± 3 (8)	79 ± 8	
B5 (N282T, F401S)	4	2.2 ± 0.1 (56)	Undetectable	1.7 ± 0.3 (44)	3.9 ± 0.4	
	10	4.4 ± 0.6 (<i>60</i>)	Undetectable	2.9 ± 0.4 (40)	7.2 ± 0.3	
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^{*a*} Activity is expressed as the amount of transglycosylation product detected by capillary electrophoresis.

^b Hydrolytic activity was calculated by subtracting the transglycosylation activity from the overall activity. The values are the mean ± S.D. of three independent measurements. ^c The overall activity was determined by the amount of *o*NP released from *o*NP-Gal.

^d Numbers in parentheses indicate the relative amount of transglycosylation and hydrolysis activity.

^e The hydrolysis activity was too low to be accurately determined.

unique *o*NP β -D-Glc*p*-(1 \rightarrow 3)- β -D-Glc*p* regioisomer, whereas only a 50% yield was obtained with the WT enzyme (11). Then we tested the transgly-cosylation reaction of *o*NP-Gal with *p*NP-Cell as acceptor (donor/acceptor molar ratio 1:1); after 11 h of reaction, 78% of the galactosyl unit was transferred to *p*NP-Cell, with a major (1 \rightarrow 3)-regioselectivity. It is interesting to note that the self-condensation β -(1 \rightarrow 3)-disaccharide, which is also a product of the transformation, was further used as substrate to synthesize the trisaccharides *p*NP β -D-Gal*p*-(1 \rightarrow *x*)-cellobioside (Fig. 3*B*).

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 $(1\rightarrow 3)$ regioselectivity (almost 100%), whereas transfer to cellobiose resulted in synthesis of two regioisomers, galactosyl-β-D-(1 \rightarrow 3)-cellobioside (66%) and galactosyl- β -D-(1 \rightarrow 6)-cellobioside (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 *p*NP-Gal, the conformation of the trisaccharide β -D-





Galp-(1 \rightarrow 3)- β -D-Glcp-(1 \rightarrow 4)- β -D-Glcp 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 \rightarrow 3)-Glcp map and four possible minima from the Glcp- β -(1 \rightarrow 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 \rightarrow 3)-Glcp linkage (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. As for the wild-type, only one docking solution has been found for each mutant. For the Gal*p*- β -(1 \rightarrow 3)-Glc*p* linkage, $\phi_{(O5-C1-O1-C3')}$, $\psi_{(C1-O1-C3'-C4')}$, and $\tau_{(C1-O1-C3')}$ values in F401S (61°, 101°, and 125°, respectively) and N282T (32°, 125°, and 126°, respectively) mutants are significantly closer to the corresponding



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

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



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 *o*NP-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.

		Cellobi	ose	Maltose						
Enzyme	Yield	Regioselectivity	Enzyme/donor ^a	Time	Yield	Regioselectivity	Enzyme/donor ^a	Time		
				h				h		
WT	8% ^b	ND	0.07	23	6% ^a	ND	0.07	23		
F401S	60 ^b -74% ^c	$1 \rightarrow 3 + 1 \rightarrow 6$	0.75	23	50 ^b -54% ^c	$1 \rightarrow 3$	0.75	46 ^b -23 ^c		
B5 (F401S/N282T)	73% ^a	$1 \rightarrow 3 + 1 \rightarrow 6$	18	46	56 ^b -63% ^c	$1 \rightarrow 3$	18	$46^{b} - 23^{c}$		
Glycosynthase (E338S)	50%	$1 \rightarrow 3 + 1 \rightarrow x$	4.1^{d}	4.5	0%		4.1	15		
⁴ Milligram of anzumo/mmol donor										

" Milligram of enzyme/mmol donor

^b Ratio obtained by capillary electrophoresis after the indicated incubation time at 55 °C.

^c Ratio obtained by NMR spectroscopy after 23 h of incubation at 55 °C.

^d 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.



FIGURE 5. **Topography of the catalytic pocket.** Longitudinal section of the catalytic pocket of the wild-type enzyme with the inner surface is shown in *blue*. The *pink* surface corresponds to the catalytic residue Glu-164.

F401S mutant; corroborating experimental results show more transglycosylation than with WT enzyme.

Partial binding energy contributions for individual subsites are reported in TABLE FIVE (38) to provide more details for the interpretation of the structural transglycosylation factors. These partial energy calculations only consider short and medium distance energy terms. Therefore, their sum is not exactly the total docking energy. However, their relative differences are suitable for comparisons. Satisfactorily, the Gal*p* ring is much more bound at subsite (-1) than the other Glc*p* rings, and the most important binding term is the one for van der Waals. In all cases, Gal*p* at subsite (-1) and Glc*p* at subsite (+2) are basically stacked with Trp-385 and Trp-312, respectively (Fig. 6). Most surprisingly, in the F401S mutant, Ser-401, directly involved in subsite (-1) definition, lowers the binding energy of subsite (+1) (difference of 5.0 kcal mol⁻¹). more than that of subsite (-1) (difference of 1.7 kcal mol⁻¹). The reverse phenomenon is observed for the N282T mutant (difference of 6.5 and 2.4 kcal mol⁻¹ for subsites (-1) and (+1), respectively).

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 glycosides 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 glycosides 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 \rightarrow 3)-regioselectivity also observed with WT enzyme. By using maltose as acceptor, the (1 \rightarrow 3)-regioselectivity is preserved, but with cellobiose, as with WT enzyme, (1 \rightarrow 3) and (1 \rightarrow 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



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(Asp-8), O4...OE1(Glu-392), O4...OE1(Glu-392), oad...NE1(Trp-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	Tt-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 F4015, 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 *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 *purple* (Met-310, Ser-401, 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 \rightarrow 3)- β -D-Glcp- $(1\rightarrow 4)$ - 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. 2*B*) 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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REFERENCES

- 1. Varki, A. (1997) J. Clin. Investig. 99, 158-162
- Gilewski, T., Ragupathi, G., Bhuta, S., Williams, L. J., Musselli, C., Zhang, X. F., Bornmann, W. G., Spassova, M., Bencsath, K. P., Panageas, K. S., Chin, J., Hudis, C. A.,



Norton, L., Houghton, A. N., Livingston, P. O., and Danishefsky, S. J. (2001) *Proc. Natl.* Acad. Sci. U. S. A. **98**, 3270–3275

- Kudryashov, V., Glunz, P. W., Williams, L. J., Hintermann, S., Danishefsky, S. J., and Lloyd, K. O. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3264–3269
- 4. Le Pendu, J. (2004) in *Protecting Infants through Human Milk* (Pickering, L. K., ed) pp. 135–143, Kluwer Academic/Plenum Publishing Corp., New York
- 5. Brassart, D., Woltz, A., Golliard, M., and Neeser, J. R. (1991) Infect. Immun. 59, 1605–1613
- 6. Krivan, H. C., Roberts, D. D., and Ginsburg, V. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 6157–6161
- Ruiz-Palacios, G. M., Cervantes, L. E., Ramos, P., Chavez-Munguia, B., and Newburg, D. S. (2003) *J. Biol. Chem.* 278, 14112–14120
- Simon, P. M., Goode, P. L., Mobasseri, A., and Zopf, D. (1997) Infect. Immun. 65, 750–757
- Gao, S., Chen, J., Xu, X., Ding, Z., Yang, Y. H., Hua, Z., and Zhang, J. (2003) Int. J. Pharm. (Amst.) 255, 57–68
- 10. Sears, P., and Wong, C. H. (2001) Science 291, 2344-2350
- Chiffoleau-Giraud, V., Spanenberg, P., Dion, M., and Rabiller, C. (1999) Eur. J. Org. Chem. 4, 757–763
- Dion, M., Nisole, A., Spangenberg, P., Andre, C., Glottin-Fleury, A., Mattes, R., Tellier, C., and Rabiller, C. (2001) *Glycoconj. J.* 18, 215–223
- Daines, A. M., Maltman, B. A., and Flitsch, S. L. (2004) Curr. Opin. Chem. Biol. 8, 106–113
- 14. Mackensie, L. F., Qingping, W., Warren, R. A. J., and Withers, S. G. (1998) J. Am. Chem. Soc. 120, 5583–5584
- 15. Prade, H., Mackensie, L. F., and Withers, S. G. (1998) Carbohydr. Res. 305, 371-381
- 16. Malet, C., and Planas, A. (1998) FEBS Lett. 440, 208-212
- Perugino, G., Trincone, A., Rossi, M., and Moracci, M. (2004) Trends Biotechnol. 22, 31–37
- Drone, J., Feng, H. Y., Tellier, C., Hoffmann, L., Tran, V., Rabiller, C., and Dion, M. (2005) *Eur. J. Org. Chem.* **10**, 1977–1983
- 19. Mayer, C., Zechel, D. L., Reid, S. P., Warren, R. A., and Withers, S. G. (2000) *FEBS Lett.* **466**, 40 44
- 20. Kim, Y. W., Lee, S. S., Warren, R. A., and Withers, S. G. (2004) J. Biol. Chem. 279,

42787-42793

- 21. Ly, H. D., and Withers, S. G. (1999) Annu. Rev. Biochem. 68, 487-522
- Perugino, G., Trincone, A., Giordano, A., van der Oost, J., Kaper, T., Rossi, M., and Moracci, M. (2003) *Biochemistry* 42, 8484–8493
- 23. Farinas, E. T., Bulter, T., and Arnold, F. H. (2001) Curr. Opin. Biotechnol. 12, 545-551
- Dion, M., Osanjo, G., Andre, C., Spangenberg, P., Rabiller, C., and Tellier, C. (2001) Glycoconj. J. 18, 457–464
- Albenne, C., Skov, L. K., Mirza, O., Gajhede, M., Feller, G., D'Amico, S., Andre, G., Potocki-Veronese, G., van der Veen, B. A., Monsan, P., and Remaud-Simeon, M. (2004) J. Biol. Chem. 279, 726–734
- Hansson, T., Kaper, T., van Der Oost, J., de Vos, W. M., and Adlercreutz, P. (2001) Biotechnol. Bioeng. 73, 203–210
- Matsui, I., Ishikawa, K., Miyairi, S., Fukui, S., and Honda, K. (1991) *Biochim. Biophys. Acta* 1077, 416–419
- Rivera, M. H., Lopez-Munguia, A., Soberon, X., and Saab-Rincon, G. (2003) Protein Eng. 16, 505–514
- Jorgensen, F., Hansen, O. C., and Stougaard, P. (2001) Appl. Microbiol. Biotechnol. 57, 647–652
- Goyal, K., Jo Kim, B., Kim, J. D., Kim, Y. K., Kitaoka, M., and Hayashi, K. (2002) Arch. Biochem. Biophys. 407, 125–134
- Paris, G., Ratier, L., Amaya, M. F., Nguyen, T., Alzari, P. M., and Frasch, A. C. (2005) J. Mol. Biol. 345, 923–934
- 32. Dion, M., Fourage, L., Hallet, J. N., and Colas, B. (1999) Glycoconj. J. 16, 27-37
- 33. Fourage, L., Dion, M., and Colas, B. (2000) Glycoconj. J. 17, 377-383
- 34. Fourage, L., and Colas, B. (2001) Appl. Microbiol. Biotechnol. 56, 406-410
- 35. Leung, D. W., Chen, E., and Goeddel, D. V. (1989) Techniques (Phila.) 1, 11-15
- 36. Dinur, U., and Hagler, A. T. (1991) in Reviews in Computational Chemistry (Lipkow-
- itz, K. B., and Boyd, D. B., eds) Vol. 2, pp. 99–164, VCH Publishers, New York
 Andre, G., Buleon, A., Vallee, F., Juy, M., Haser, R., and Tran, V. (1996) *Biopolymers* 39, 737–751
- Bak-Jensen, K. S., Andre, G., Gottschalk, T. E., Paes, G., Tran, V., and Svensson, B. (2004) *I. Biol. Chem.* 279, 10093–10102
- 39. Kempton, J. B., and Withers, S. G. (1992) Biochemistry 31, 9961-9969

