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Identification by saturation mutagenesis of a single residue involved in the α-galactosidase AgaB regioselectivity

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The α-galactosidase AgaB of *Bacillus stearothermophilus* displays a major 1,6 and a minor 1,3 regioselectivity. The wild-type enzyme was subjected to directed evolution (random mutagenesis and in vitro recombination) using a double screening strategy based on the elimination of the 1,6 regioselectivity and the analysis by TLC of the transglycosylation products. One of the AgaB mutants (E500) exhibited a new 1,2 regioselectivity and a rather high level of transglycosylation. The corresponding gene contains 10 mutations compared to the *agaB* gene and we demonstrated by saturation mutagenesis that the G442R substitution strongly contributes to the emergence of this new regioselectivity. Moreover, other single point mutations at this position led to new mutants displaying other kinds of regioselectivity demonstrating the importance of this position in the subtle kinetic control of transglycosylation.

Keywords: directed evolution, regioselectivity, transglycosylation, saturation mutagenesis

Introduction

The enzymatic synthesis of the saccharides has become a very powerful alternative due to the use of glycosidases [1]. These enzymes usually hydrolyze glycosidic bonds but can also catalyze the formation of glycosidic linkages via their transferase activity. Moreover these enzymes are very attractive due to their stability, high stereoselectivity and low cost [2–4]. Some of them display a high regioselectivity, such as the β-galactosidase from *Bacillus circulans* [5], some thermophilic β-galactosidases [6], the α-galactosidases from green coffee beans [7] or from *Penicillium multicolor* [8]. However most of them present a rather low regioselectivity which often leads to the synthesis of a mixture of regioisomers during transglycosylation [9–11].

In a previous study, we analyzed the potential of the newly cloned α-galactosidase of *Bacillus stearothermophilus* (AgaB) to catalyze the regioselective synthesis of oligosaccharides [12]. This enzyme belongs to the family 36 of glycosylhydrolases and its amino acids chain has a 83 414 molecular weight (Genbank, accession number AY013287.1). The active site consensus sequence is located between amino acids 360 and 369 [13]. The high regioselectivity of the wild-type enzyme led to the synthesis of the major product 4-nitrophenyl α-D-galactopyranosyl-[1 → 6]-α-D-galactopyranoside(α-Gal-[1 → 6]-α-Gal-O-*p*-NP) (53%) and of the minor product 4-nitrophenyl α-D-galactopyranosyl-[1 → 3]-α-D-galactopyranoside (α-Gal-[1 → 3]-α-Gal-O-*p*-NP) (4%). We then undertook the directed evolution of this α-galactosidase in order to improve its 1,3 regioselectivity. With one round of random mutagenesis and one recombination of the best mutants by StEP (Staggered Extension Process, [14]), we found that several evolved enzymes lost most of their activity towards the 1,6 linkage both in hydrolysis and in synthesis. This led to the mutant enzyme E901 exhibiting a preference for the 1,3 linkage [15]. This work constituted the first example of the modification of glycosylhydrolase regioselectivity by directed evolution which does not rely on structural data and rational design.

Herein, we report the further analysis of glycosidase mutants previously obtained by this in vitro evolution process. We reasoned that the initial screening procedure based on the suppression of the 1,6 regioselectivity of AgaB should allow the selection of clones presenting regioselectivity other than 1,3 or 1,6. Among those, we first characterized such a mutant (E500) presenting a high transferase activity with a major 1,2 regioselectivity which was not detectable in the wild-type

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enzyme. By sequence comparison, one of the mutations was identified as being strongly involved in the appearance of this new regioselectivity. Then, saturation mutagenesis at this position emphasized the subtle control of the regioselectivity by this residue. Thus this galactosidase provides a convenient model system to determine to what extent directed evolution is able to change glycosidase regioselectivities.

Materials and methods

Saturation mutagenesis

The DNA template used for the saturation mutagenesis was the plasmid pAMG22 which contains the 2.2 kb wild type α-galactosidase *agaB* gene [16] from *Bacillus stearothermophilus* under the control of the Ptac promoter in the pBTac2 vector [16]. Primers D (5′-CAATTAATCATCGGCTCG) and F (5′-AATCTTCTCTCATCCGCC) flank the gene before the Ptac promoter and after the *Pst*I restriction site. Degenerate synthetic oligonucleotides used for the G442 codon mutagenesis were G442N1 (5′-GCCCGCGTTCGGAANNNCGAAACCAGC TTG) and G442N2 (5′-CAAGCTGGTTTCGNNNTTCCG AACGCGGGC). The upstream gene fragment was amplified by *Pfu* DNA polymerase with D and G442N2 oligonucleotides and downstream fragments were amplified with G442N1 and F oligonucleotides. The amplified fragments were mixed and the full length gene was amplified with D and F oligonucleotides.

Cloning of mutants

Mutagenized *agaB* genes were digested by the *Eco*RI and *Pst*I restriction enzymes, and cloned back into the pBTac2 vector which was previously digested by the same enzymes. The resulting plasmids were transformed into competent XL1 blue cells that were plated on LB agar plates containing 20 μg/ml of tetracycline and 100 μg/ml of ampicillin. After 18 h of culture, colonies were picked up and spotted with sterile toothpicks onto two LB agar plates. One plate was stored and the other one was incubated for 2 h at 55°C. IPTG was not used for the induction since previous work indicated it neither increased significantly the α-galactosidase activity nor the band intensity corresponding to the AgaB subunit on SDS-PAGE (data not shown). The clones retaining a thermostable α-galactosidase activity were identified by pouring an X-α-Gal solution (2 mg/ml) on the plate and 120 blue clones were further characterized. Some mutagenized *agaB* genes were sequenced at the G442 codon level by MWG-Biotech.

Screening of the transglycosylation activity by means of thin layer chromatography (TLC)

Transglycosylation α-galactosidase activities were determined in the crude extracts of selected clones which were prepared as follows: each clone was cultivated in LB medium and 1.5 ml of overnight cultures was centrifuged, resuspended in 0.5 ml of 0.1 M sodium phosphate buffer pH 7.0 and subject to brief sonication. Then the lysate was centrifuged for 15 min at 13,000 rpm and α-galactosidase activity was determined in the supernatant. In an Eppendorf tube, 3 μL of crude extract of the AgaB mutants were added to 25 μL of a 90 mM solution of *p*NPαGal in phosphate buffer (0.1 M, pH 7). The mixture was allowed to react overnight at room temperature (since after 1 to 12 hours the wild-type enzyme and mutant activities are inhibited by pNP) and the conversion of the components was followed by means of TLC (precoated silica gel 60 sheets Merck F254). Using Seymour eluent (60-30-3-5, MeOH-$CHCl_3$-AcOH-H_2O), all the disaccharides synthesized were separated. The Rf values for *p*NPαGal, α-Gal-[1 → 3]-α-Gal-O-*p*-NP, α-Gal-[1 → 2]-α-Gal-O-*p*-NP, α-Gal-[1 → 6]-α-Gal-O-*p*-NP and galactose were respectively 0.59, 0.35, 0.30, 0.26 and 0.14.

Kinetics of transglycosylation reactions by means of *in situ* proton NMR spectroscopy

Kinetics experiments were performed using a 500 MHz Bruker spectrometer according to a procedure previously published in a previous paper [12]. In a tube, 21 mg (70 μmol) of *p*NPαGal and the amount of salts necessary for 0.78 mL of 0.1 M (pH 7.0) phosphate buffer were lyophilized, dissolved in D_2O and lyophilized once more. The buffer reference mixture containing the silylated reference [0.78 mL of a 10 mmol/L of 3-(trimethylsilyl)-propanesulfonic acid sodium salt solution] was prepared as above and adjusted to pD = 7.0. The α-galactosidase preparation (0.48 units) was dried in a desiccator containing P_2O_5 and redissolved in 0.18 mL of the phosphate buffer reference mixture. The solutions were warmed to the temperature desired for the reaction and the enzymatic preparation was added to the reactant solution. The resulting mixture was immediately filtered into an NMR tube and the reaction was allowed to proceed in the magnet of the spectrometer pre-adjusted to the temperature of the reaction. Using this procedure, it was possible to perform the first measurements about 5 min after the introduction of the enzyme in the NMR tube. The yields ρ obtained for the self condensation disaccharides were calculated from the ^{1}H NMR spectra using the integrations of the anomeric protons peaks according to the relationship:

$$\rho\,(\%) = \{2\,x_i/[x_{gal} + x_{p\mathrm{NPgal}} + 2(x_{ij})]\}\,100$$

where x_i, x_{ij}, x_{gal} and $x_{p\mathrm{NPgal}}$ are respectively the molar fractions of a given self condensation disaccharide, the sum of the molar fractions of all the self condensation disaccharides synthesized, the molar fraction of galactose and of the remaining *p*NPgal.

Identification of the self-condensation disaccharides: the structure of the disaccharides has been elucidated by means of proton and carbon NMR spectroscopy using standard bidimensional procedures. The NMR data are given in

Table 1. The chemical shifts (solvent D_2O) are quoted from acetone at 2.04 ppm (^1H) and 29.9 ppm (^{13}C).

Preparation of the His-tagged AgaB mutants

For the determination of kinetic parameters, the carboxy terminal hexahistidine tag was added to native and mutant enzymes: the corresponding genes were amplified with the D and AHISP (5′-ATATATCTGCAGTTAGTGGTGGTGGTG GTGGTGTTGTTGAACAGCTTTCAATCGCC) primers, digested with *Eco*RI and *Pst*I restriction enzymes and cloned in the pBTac2 vector. *E. coli* XL1 blue cells transformed with constructs were grown overnight in LB medium with ampicillin and tetracycline, and lysed by brief sonication in a solution containing NaCl 300 mM, imidazole 20 mM, sodium phosphate 50 mM pH 8.0. Enzymes were purified with nickel-NTA resin (Qiagen) and eluted with the same buffer containing 250 mM of imidazole. Fractions containing the α-galactosidase activity were used immediately for kinetic studies. The concentration of the purified enzymes (typically 0.5–3 mg/ml) was determined by the bicinchoninic acid method [18] using bovine serum albumin as the standard. The purity of the preparation was checked by SDS-polyacrylamide gel electrophoresis. Imidazole was not removed from the eluted enzymes since at 12.5 mM (the highest concentration used for the determination of kinetic parameters), no inhibiting effect was observed with the wild-type or mutant α-galactosidases.

Enzyme kinetics

Kinetic studies with *p*NPαGal were performed at 25°C in microtitre plates by following changes in absorbance at 405 nm using microtitre plates reader (iEMS, Labsystem) or in cuvettes using a Kontron Uvikon 860 spectrophotometer equipped with a cell holder connected to a circulating water bath. In both cases the rates of substrate hydrolysis were determined using a continuous assay. The buffer employed for all kinetic experiments with wild type and mutant α-galactosidases was 100 mM sodium phosphate buffer pH 7.0. The reaction mixtures (100 µL), containing the *p*NPαGal and buffer, were preincubated at 25°C prior to addition of 100 µL of diluted enzymes (with the Kontron spectrophotometer, volumes of 200 µL were used). The initial rates of enzyme-catalyzed hydrolysis were measured at five, six or seven different substrate concentrations ranging from about 0.15 K_m to 5 K_m. Values of K_m and k_{cat} were determined from the initial rates by means of non-linear regression analysis.

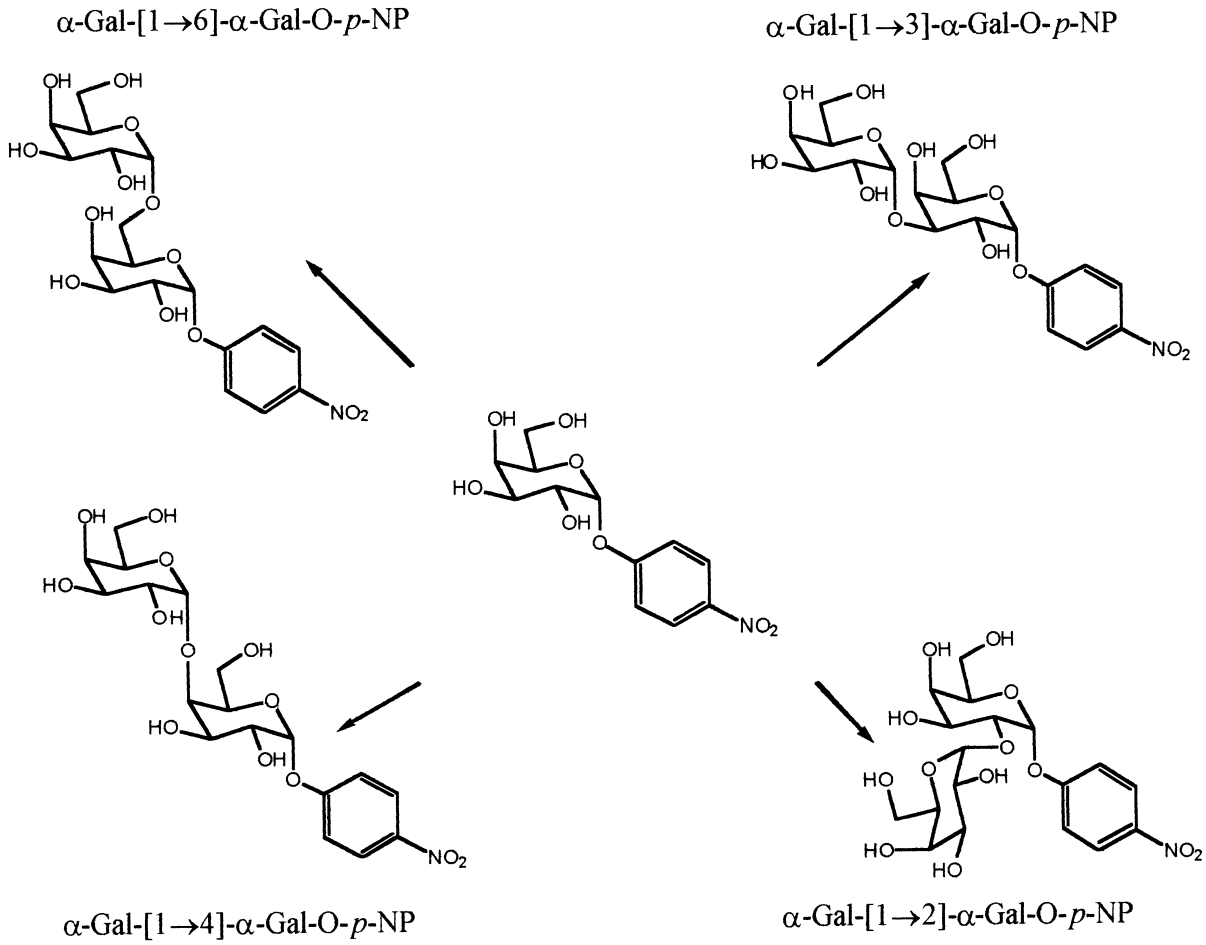
Results and discussion

Characterization of a mutant with a new regioselectivity

In vitro evolution of the *agaB* gene encoding an α-galactosidase of *Bacillus stearothermophilus* has previously been performed in order to improve its 1,3 regioselectivity at the expense of its original 1,6 regioselectivity [15]. In the screening procedure, we selected the mutants displaying a low 1,6 hydrolytic activity on melibiose while keeping a hydrolytic activity on X-gal. Among these mutants, some of them displayed a quite high 1,3 regioselectivity in hydrolysis (E33 and E901). We reasoned that this screening procedure could also lead to mutants with completely new regioselectivities which were not present in the wild type enzyme as illustrated by the Scheme 1. The best 18 mutants having a low hydrolytic activity on melibiose were screened for the transglycosylation reaction by TLC where self-condensation products were detected after incubation of the mutant enzymes with *p*NPαGal. A careful observation of the different TLC patterns revealed that other regioselectivities had emerged for several clones obtained after random mutagenesis and *in vitro* recombination (Figure 1). Some of them produced a regioisomer migrating on the TLC plates between the 1,3 and 1,6 ones which was not produced by the wild type enzyme. In this

Table 1. ^1H and ^{13}C chemical shifts of the self-condensation disaccharides synthesized with the galactosidase mutants

^1H	Gal-α-(1→2)-Gal-α-O-pNP	Gal-α-(1→3)-Gal-α-O-pNP	Gal-α-(1→6)-Gal-α-O-pNP	^{13}C	Gal-α-(1→2)-Gal-α-O-pNP	Gal-α-(1→3)-Gal-α-O-pNP	Gal-α-(1→6)-Gal-α-O-pNP
1	6,09	5,88	5,90	1	93,8	95,2	96,3
2	4,14	4,20	4,04	2	71,4	66,3	69,3
3	3,83	4,25	3,84	3	68,7	74,1	70,7
4	3,96	4,32	4,13	4	68,6	65,4	68,0
5	4,07	4,27	3,80	5	68,9	71,0	75,1
6	3,73/3,73	3,73/3,73	4,11/4,09	6	60,7	60,9	69,0
1′	5,13	5,24	4,80	1′	95,5	96,8	97,6
2′	3,77	3,91	3,29	2′	67,4	68,2	69,3
3′	4,19	4,00	3,64	3′	70,6	69,2	71,8
4′	3,75	4,06	3,68	4′	67,5	69,3	67,7
5′	3,98	3,97	3,61	5′	71,7	72,0	71,7
6′	3,70	3,74	3,79	6′	60,5	61,2	60,5



Scheme 1. Structure of the disaccharides which could be synthesized from the self-condensation of α-*p*-nitrophenylgalactoside catalyzed by an α-galactosidase.

respect, the E500 mutant seemed particularly interesting since it allowed the synthesis of mostly only one new regioisomer. This new oligosaccharide was purified and identified by means of two-dimensional NMR experiments as the autocondensation product α-Gal-[1 → 2]-α-Gal-O-*p*-NP. Then, this mutant was further characterized for its transglycosylation abilities. Figure 2 presents the kinetics of self-condensation of *p*NPαGal followed by NMR, as previously described [19]. The E500 mutant enzyme was able to produce relatively high yields (25%) of the new disaccharide α-Gal-[1 → 2]-α-Gal-O-*p*-NP, while other regioisomers, α-Gal-[1 → 3]-α-Gal-O-*p*-NP and α-Gal-[1 → 6]-α-Gal-O-*p*-NP, represent only a small percentage of the transglycosylation products. Thus, this *in vitro* directed evolution approach has allowed the *agaB* enzyme to switch to a new regioselectivity while keeping a high transferase activity.

The gene sequence of the E500 mutant shows 10 amino acid changes not homogeneously distributed in the enzyme. Six substitutions (A2S, T4A, I34V, E46A, R48A, S50P) were found in the N-terminal part of the protein. Among the four others, three (N117S, A198E, K399E) have already been detected in the E33 and E901 mutants [15] exhibiting a preference for 1,3 regioisomers. It is reasonable to assume that the presence of a highly mutated N-terminal region in this mutant simply reflects that this location is highly permissive and does not have roles in either the structural stability or substrate recognition. Thus, the G442R mutation, original in this mutant, might be involved in the emergence of the 1,2 regioselectivity.

Saturation mutagenesis of the codon 442

To validate the importance of the G442R mutation in the control of the regioselectivity, we carried out a saturation mutagenesis [20] on the wild type *agaB* gene at this position. 120 mutant clones still displaying a thermostable α-galactosidase activity were analyzed for their regioselectivity in transglycosylation with *p*NPαGal as a single substrate. Once the reaction spontaneously stopped (we have already shown in a previous paper [15] that the increasing concentrations of *p*NP, but not the pH variation, are responsible of this effect), reaction products were subjected to TLC analysis (data not shown) and the different regioisomers synthesized by each mutant were

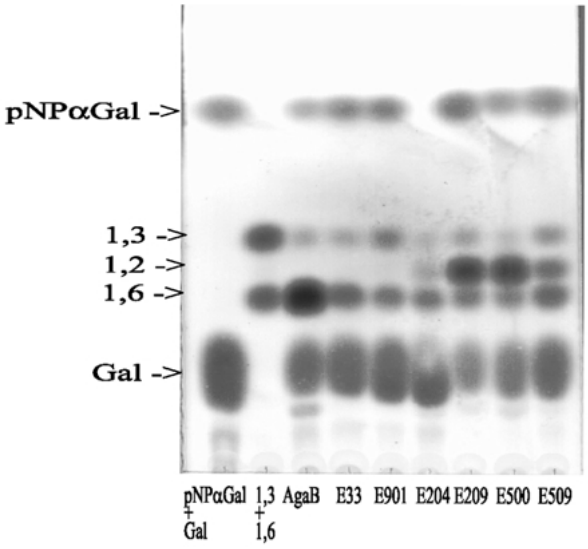


Figure 1. Analysis of transglycosylation products obtained by self-condensation of *p*NPαGal (90 mM) with wild type and evolved α-galactosidases AgaB, on a TLC plate. Locations of regioisomers α-Gal-[1 → 3]-α-Gal-O-*p*-NP, α-Gal-[1 → 6]-α-Gal-O-*p*-NP and α-Gal-[1 → 2]-α-Gal-O-*p*-NP are indicated by the 1,3, 1,6 and 1,2 abbreviations respectively. Gal = Galactose.

identified. An AgaB mutant (L3) producing the highest percentage of 1,2 regioisomer was further characterized. The mutation at the mutagenesis site was identified as being G442R. Thus, the importance of the G442R mutation in the E500 mutant for the 1,2 regioselectivity was confirmed by this single point mutation experiment. The transglycosylation regioselectivity of the L3 mutant was also analyzed by *in situ* proton NMR spectroscopy at 25°C (Figure 2). As shown with E500, this single point mutant was able to produce a significant yield (12%) of the disaccharide α-Gal-[1 → 2]-α-Gal-O-*p*-NP as the major transglycosylation product. The mutations N117S, A198E, K399E previously considered to contribute to the decrease in 1,6 regioselectivity (since they were found in all three E33, E901 and E500 mutants) could finally play a minor role. However, they could still have an effect on the yield and the kinetics of the transglycosylation reaction since the highest level of α-Gal-[1 → 2]-α-Gal-O-*p*-NP produced by the single mutant L3 is two times lower and reached at a 4 times lower reaction rate than with E500.

Two other single point mutants displaying different transglycosylation patterns on TLC (F24 and F1) were also sequenced and characterized for their transferase activity by NMR. The F24 mutant allowed the synthesis of the three regioisomers 1,2, 1,6 and 1,3, in order of decreasing yield respectively (Figure 2). Thus although F24, corresponding to a G442I mutation, presents a slightly higher level of transglycosylation, it is characterized by a lower 1,2 regioselectivity than the L3 mutant. On the other hand, autocondensation of *p*NPαGal with F1 mutant, identified by the G442Y mutation, led to the synthesis of only 1,3 and 1,6 regioisomers in similar yield at a quite low level. These results further emphasize the critical role of the residue in position 442 in controlling the outcome of the transglycosylation reaction.

Kinetic characterization of the mutants

Further analysis of AgaB mutants was carried out by the determination of their kinetic constants for the hydrolysis of *p*NPαGal (Table 2). This substrate was chosen first because it is the only substrate that is accepted by all the mutants thus allowing comparison, and second because the K_m for other substrates like *p*NP-disaccharides are very high (within 30–100 mM range) preventing accurate kinetic parameters. As the determination of the k_{cat} necessitates work on the purified enzyme, forms of the native and mutant enzymes with C-terminal hexahistidine tags were engineered so that efficient purification of all enzymes was achieved on a nickel-NTA resin. We checked that the addition of the His-tag did not modify the kinetic properties of the wild-type and mutant AgaB enzymes by comparing their K_m with the enzymes without the tag. No significant differences were observed for wild-type and mutant enzymes except for the mutant E500 in which the His-tag induced a 4-fold decrease in K_m (K_m = 0.25 mM without His-tag). Mutants E500 and L3 containing the mutation G442R, exhibit a lower K_m than for the wild-type enzyme, while other mutants F1 and F24 showed increased K_m values. One possible explanation is that the Arg mutation introduces a positive charge which could stabilize the aromatic cycle of the *p*NPαGal within the active site by cation-π interaction. However, by considering the former NMR kinetics of self-condensation (Figure 2), it can be stated that high differences of K_m values (50-fold between F24 and E500) do not have large effects on the yields of transglycosylation products. On the other hand, and as generally observed for mutant enzymes, all k_{cat} values were lower than that of native enzyme. This is in agreement with the selection process which was first based on the output of mutants having lost their hydrolytic activity on melibiose. Once again no correlation could be established between the k_{cat} values and the behaviour of enzymes during transglycosylation (Table 2). This emphasizes the importance of our second screening strategy which was based on the direct detection of the synthesis of disaccharides by TLC.

Conclusion

All these results taken together show that an evolution screening strategy based on the decrease of 1,6 regioselectivity allowed the isolation of AgaB mutants displaying both 1,3 and 1,2 regioselectivities; for some unknown reason we have never observed mutants owing the 1,4 regioselectivity. Unlike in our former study [15], we were able to isolate AgaB mutants in which only one mutation entailed dramatic regioselectivity changes. The replacement of glycine at position 442 by three different amino acids gave rise to three AgaB mutants displaying different regioselectivities in synthesis: the G442R

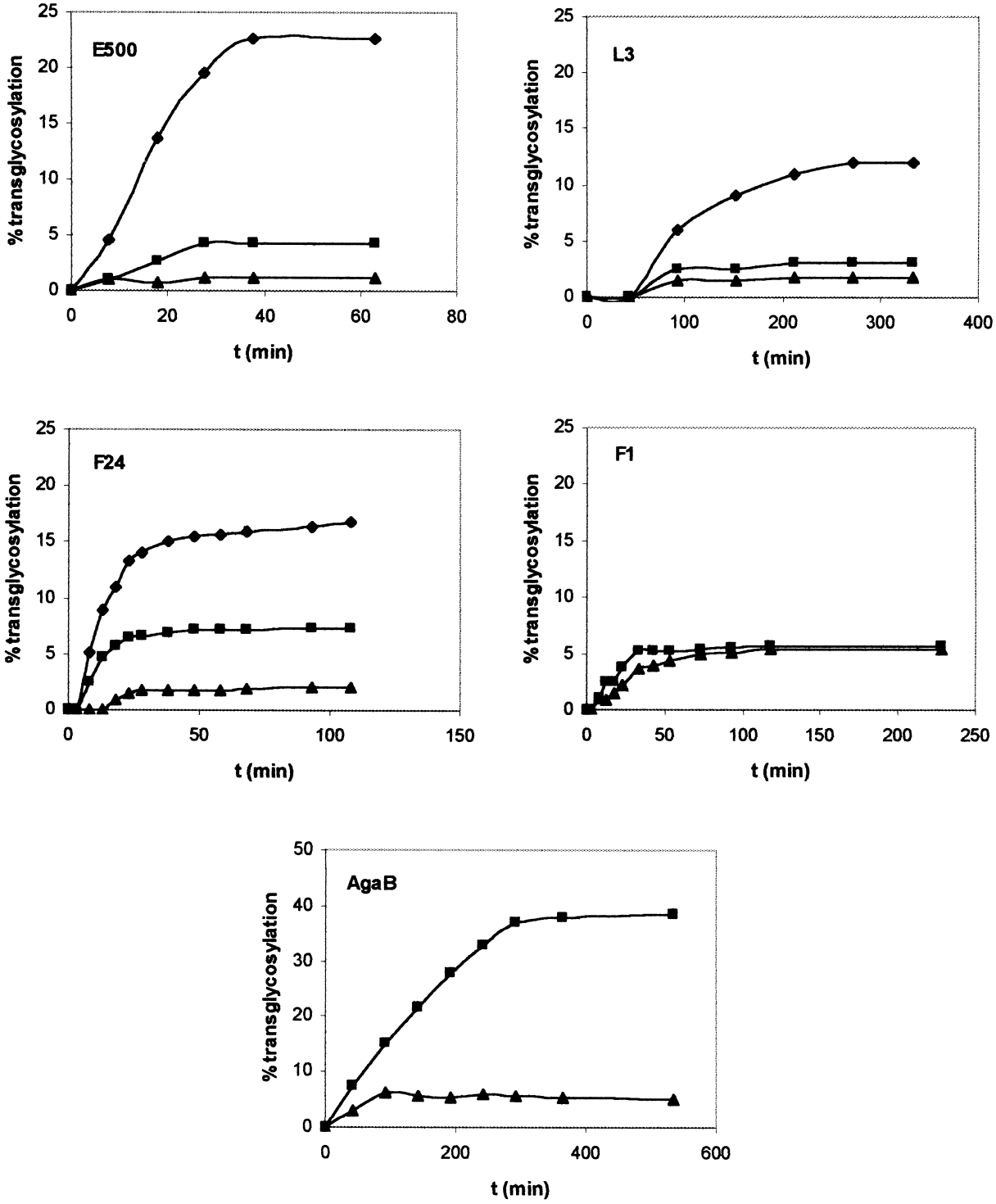


Figure 2. Kinetics of the synthesis of the self-condensation disaccharides α-Gal-[1 → 6]-α-Gal-O-*p*-NP (■), α-Gal-[1 → 3]-α-Gal-O-*p*-NP (▲) and α-Gal-[1 → 2]-α-Gal-O-*p*-NP (◆) catalysed by AgaB and the α-galactosidase mutants E500, L3, F24 and F1 at 25°C. The hydrolysis products of *p*NPαGal are not represented on this figure.

substitution strongly contributes to the emergence of the major 1,2 regioselectivity of the E500 mutant; the G442Y substitution gives a mutant, F1, which exhibits a regioselectivity similar to that of a previously identified mutant, E901 [15] displaying 6 other mutations. This last observation demonstrates that several solutions are possible to reach the same enzyme activity. A study of the three-dimentional structure of AgaB enzyme is currently in progress in order to examine the possible contribution of different substitutions to the modifications of AgaB enzyme behaviour.

Table 2. Kinetic parameters of AgaB and of the mutant α-galactosidases with *p*NPαGal as substrate. The regioselectivity is expressed as the maximum molar percentages of *p*NPαGal which have formed a given disaccharide and thus are also the yields for the formation of this compound

		Regioselectivity (%)					
Enzyme	*Mutation(s)*	*1,6*	*1,2*	*1,3*	k_{cat} (s^{-1})	K_m (*mM*)	k_{cat}/K_m ($s^{-1}\,mM^{-1}$)
AgaB		38.5	0	3.2	26.5 ± 0.25	0.92 ± 0.01	28.7
E500	A2S,T4A, I34V, E46A, R48A, S50P, N117S, A198E, G442R, K399E	4.2	22.6	1.2	0.525 ± 0.025	0.060 ± 0.003	8.55
L3	G442R	3.1	12	1.8	0.695 ± 0.005	0.19 ± 0.01	3.7
F24	G442I	7.3	16.8	2	17.45 ± 0.05	13.3 ± 0.15	1.31
F1	G442Y	5.7	0	5.4	4.05 ± 0.05	6.3 ± 0.1	0.64

Finally, this study illustrates the efficiency of *in vitro* molecular evolution techniques in altering finely-tuned enzyme properties. Yet, transglycosylation catalyzed by glycosidases is a kinetically controlled reaction which depends on subtle variations in the enzyme structure. For these reasons, regioselectivity engineering of glycosidases is beyond the resolution of present structural analysis and thus beyond our ability to predict the result. Here, as one round of mutagenesis and *in vitro* recombination was successful to alter the regioselectivity of AgaB, we expect that, by carrying out several repetitive cycles and screening, the most regioselective mutants could be improved for their transferase activity.

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References

1 Ichikawa Y, Look GC, Wong C-H, Enzyme-catalyzed oligosaccharide synthesis, *Anal Biochem* **202**, 215–38 (1992).
2 Yoon J-H, Ajisaka K, The synthesis of galactopyranosyl derivatives with β-galactosidases of different origins, *Carbohydr Res* **292**, 153–63 (1996).
3 Vetere A, Paoletti S, Complete synthesis of 3′-sialyl-N-acetyllactosamine by regioselective transglycosylation, *FEBS Lett* **399**, 203–6 (1996).
4 Vic G, Scigelova M, Hastings JJ, Howarth OW, Crout DHG, Glycosidase catalysed synthesis of oligosaccharides: trisaccharides with the α-D-Gal-(1 → 3)-D-Gal terminus responsible for the hyperacute rejection response in cross-species transplant rejection from pigs to man, *Chem Commun* 1473–4 (1996).
5 Vic G, Hastings JJ, Crout DHG, Glycosidase-catalysed synthesis of glycosides by an improved procedure for reverse hydrolysis: application to the chemoenzymatic synthesis of galactopyranosyl-(1 → 4)-O-α-galactopyranoside derivatives, *Tetrahedron: Asymm* **7**, 1973–84 (1996).
6 Fang J, Xie W, Li J, Wang PG, Chemical and enzymatic synthesis of glycoconjugate 3: synthesis of lactosamine by thermophilic galactosidase catalysed galactosylation on a multigram scale, *Tetrahedron Lett* **39**, 919–22 (1998).
7 Singh S, Scigelova M, Vic G, Crout DHG, Glycosidase-catalysed oligosaccharide synthesis of di-, tri- and tetra-saccharides using the *N*-acetylhexosaminidase *from Aspergillus oryzae* and the β-galactosidase from *Bacillus circulans*, *J Chem Soc*, *Perkin Trans* **1**, 1921–26 (1996).
8 Weignerova L, Sedmara P, Hunkova Z, Halada P, Kren V, Casali M, Riva S, Enzymatic synthesis of iso-globotriose from partially protected lactose, *Tetrahedron Lett* **40**, 9297–9 (1999).
9 Murata T, Usui T, Preparation of oligosaccharide units library and its utilization, *Biosci Biotech Biochem* **61**, 1059–66 (1997).
10 Chiffoleau-Giraud V, Spangenberg P, Dion M, Rabiller C, Transferase activity the synthesis of β-(1,3)-disaccharides, *Eur J Org Chem* 757–63 (1999).
11 Petzelbauer I, Reiter A, Splechtna B, Kosma P and Nidetzky B, Transgalactosylation by thermostable β-glycosidases from *Pyrococcus furiosus* and *Sulfolobus solfataricus*. Binding interactions of nucleophiles with the galactosylated enzyme intermediate make major contributions to the formation of new β-glycosides during lactose conversion, *Eur J Biochem* **267**, 5055–66 (2000).
12 Spangenberg P, André C, Dion M, Rabiller C, Mattes R, Comparative study of new α-galactosidases in transglycosylation reactions, *Carbohydr Res* **329**, 65–73 (2000).
13 Fridjonsson O, Watzlawick H, Gehweiler A, Rohrhirsch T, Mattes R, Cloning of the gene encoding a novel thermostable α-galactosidase from *Thermus brockianus* ITI360, *Appl Environ Microbiol* **65**, 3955–63 (1999).
14 Zhao H, Giver L, Shao Z, Affholter JA, Arnold FH, Molecular evolution by staggered extension process (StEP) *in vitro* recombination, *Nat Biotechnol* **16**, 258–61 (1998).
15 Dion M, Nisole A, Spangenberg P, André C, Glottin-Fleury A, Mattes R, Tellier C, Rabiller C, Modulation of the regioselectivity of a *Bacillus* α-galactosidase by directed evolution. *Glycoconjugate J* **18**, 215–23 (2001).

16 Ganter C, Böck A, Buckel P, Mattes R, Production of a thermostable, recombinant α-galactosidase suitable for raffinose elimination from sugar beet syrup. *J Biotechnol* **8**, 301–10 (1988).
17 Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JD, Smith JA, Struhl K, *Current Protocols in Molecular Biology*, New York, Wiley, 1994.
18 Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**, 76–85 (1985).
19 Spangenberg P, Chiffoleau-Giraud V, André C, Dion M, Rabiller C, Probing the transferase activity of glycosidases by means of *in situ* NMR spectroscopy, *Tetrahedron: Asymmetry* **10**, 2905–12 (1999).
20 Hayashi N, Welschof M, Zewe M, Braunagel M, Dubel S, Breitling F, Little M, Simultaneous mutagenesis of antibody CDR regions by overlap extension and PCR, *Biotechniques* **17**, 310–15 (1994).

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