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Modulation of the regioselectivity of a *Bacillus* α-galactosidase by directed evolution

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α-galactosidase AgaB of *Bacillus stearothermophilus* was subjected to directed evolution in an effort to modify its regioselectivity. The wild-type enzyme displays a major 1,6 and minor 1,3 regioselectivity. We used random mutagenesis and staggered extension process (StEP) to obtain mutant enzymes displaying modified regioselectivity. We developed a screening procedure allowing first the elimination of AgaB mutants bearing the 1,6 regioselectivity and secondly the selection of those retaining a 1,3 regioselectivity. Our results show that, among the evolved enzymes that have lost most of their activity towards the 1,6 linkage both in hydrolysis and in synthesis, one (E901) has retained its 1,3 activity. However the transglycosylation level reached by this mutant is quite low versus that of the native enzyme. This work constitutes the first example of modification of glycosylhydrolase regioselectivity by directed evolution.

Keywords: directed evolution, regioselectivity, transglycosylation, glycosylhydrolase

Introduction

Oligosaccharide components of glycoproteins and glycolipids are information-rich molecules that guide many biological processes [1]. The understanding of their action in biomolecular recognition led to a rapid development of the methodologies for their synthesis [2,3]. However, carbohydrate molecules are recognized as particularly challenging targets for regioselective glycosylation by either chemical or enzymatic methods because of their structure, bearing multiple hydroxyl groups. Chemical methods used for the synthesis of oligosaccharide units have been extensively developed, but they involve various procedures of protection, glycosylation and deprotection [4].

The enzymatic synthesis of the saccharides has become a very powerful alternative [5] by using either glycosyltransferases or glycosidases. Glycosyltransferases add glycosyl residues on oligosaccharides in a very selective way but use exclusively the nucleotide derivative as a glycoside donor in the reaction [6,7]. Glycosidases can catalyse the formation of glycosidic linkages via their transferase activity. Moreover these enzymes are very attractive due to their stability, high stereoselectivity and low cost [8–10]. Some of them display a high regioselectivity, such as the β-galactosidase from *Bacillus circulans* [11], some thermophilic β-galactosidases [12], the α-galactosidases of green coffee beans [13] or from *Penicillium multicolor* [14]. However most of them present a rather low regioselectivity which often leads to the synthesis of a mixture of regioisomers during the transglycosylation [15,16]. The difficulty of separating the product from the reaction mixture which contains similar compounds hampers their application. Thus various experimental conditions have been studied in order to improve glycosidase regioselectivity such as the addition of organic solvent [17] or the use of different kinds of acceptors [10,11,13,18]. The ratio between the different regioisomers has been modified but none of these experimental conditions was able to provide the formation of a single regioisomer.

The aim of this work is to propose another approach by modifying the properties of enzymes in order to improve their regioselectivity. Directed molecular evolution seems to be the best strategy for this purpose when no information is available about the enzyme structure or the amino acids involved in biocatalysis. Several methods of evolution can be used such as point mutagenesis by error-prone PCR [19] or DNA shuffling [20] as well as the staggered extension process (StEP) [21]. *In vitro* recombination allows the rapid accumulation of beneficial mutations identified in separate genes and their screening often produces multiple improved sequences. A review by

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Petrounia and Arnold [22] lists a number of examples of the *in vitro* evolution of enzymes showing that great improvements can be achieved for several properties: better stability, stronger activity in natural and artificial environments, modified substrate specificity. For example, directed evolution of the *β*-galactosidase of *Escherichia coli* led to a 10- to 20-fold increase in k_{cat}/K_m for the fucose substrates compared to the native enzyme [23].

More recently May *et al.* [24] could invert the enantioselectivity of an hydantoïnase by a few rounds of mutagenesis. By contrast, no experiments have been undertaken to improve the enzyme regioselectivity apart from some work concerning the cytochrome P450 monooxygenase for which the directed evolution led to altered regioselective hydroxylations [25].

In a previous study, we analysed the potential of a newly cloned α-galactosidase to catalyse the regioselective synthesis of oligosaccharides [26]. The AgaB enzyme from the strain KVE39 of *Bacillus stearothermophilus* [27] displayed interesting features since it was easily overexpressed in *E. coli* and its transferase activity was quite high (more than 50% of donor was transformed into autocondensation products). Its high regioselectivity 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) (93%) and of the minor product 4-nitrophenyl α-D-galactopyranosyl-[1 → 3]-α-D-galactopyranoside (α-Gal-[1 → 3]-α-Gal-O-*p*-NP) (7%) (Scheme 1). As this regioselectivity is not particularly useful for oligosaccharide synthesis of biological or medical interest, we decided to try to evolve the activity towards the 1,3 regioselectivity. Indeed this latter could be applied to the synthesis of xenograft antigen α-Gal-[1 → 3]-*β*-Gal-[1 → 4]-GlcNAc [2]. More generally this glycosidase constitutes a model system to determine whether directed evolution is a method able to change and/or to elicit enzymatic regioselectivities which would be of great importance in numerous chemo-enzymatic synthetic processes. In the present study, we have established an original screening procedure using the reversion of a bacterial phenotype and self-synthesized substrates leading to the selection of the desired enzymatic regioselectivity during the hydrolysis and the transglycosylation reactions.

Materials and methods

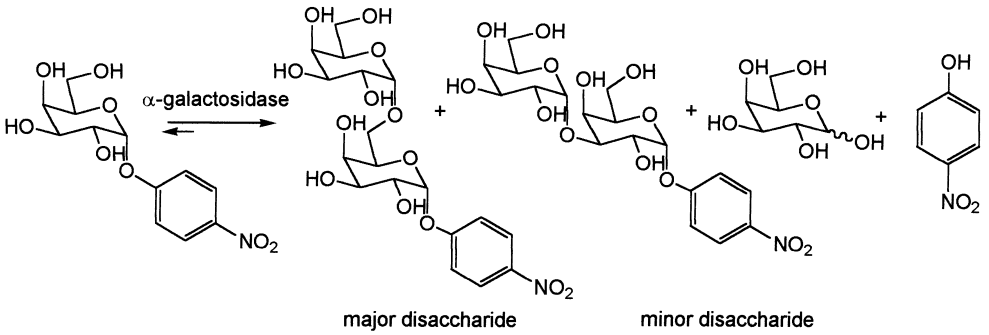
Random mutagenesis and *in vitro* recombination

Plasmid pAMG22 consists of the 2.2 kb wild type α-galactosidase *agaB* gene [28] from *Bacillus stearothermophilus* under the control of the Ptac promoter in the pBTac2 vector [29]. For the first round of evolution, random mutations were introduced by mutagenic PCR [19]. Primers D (5′-CAATTAATCATCGGCTCG) and F (5′-AATCTTCTCT-CATCCGCC) flank the gene before the Ptac promoter and after the *Pst*I restriction site. 100 ng of each primer was mixed with 50 ng of the expression plasmid in a 50-*μ*l PCR. The reaction conditions were: 1 × *Taq* buffer, 5% DMSO, 1 mM of each dNTP, 7 mM $MgCl_2$ and 2.5 U of Goldstar *Taq* polymerase (Eurogentec). The reaction was thermocycled as follows: one cycle at 94°C, 5 min, then 30 cycles at 94°C, 1 min; 50°C, 1 min; 74°C, 3 min and finally one extension step of 5 min at 74°C. Amplification of the DNA fragment was checked by running a small aliquot of the reaction on an agarose gel.

In vitro recombination of five mutant genes was carried out by the StEP method [21] with the following protocol: 100 ng of each primer was mixed with 50 ng of each of the different plasmids in a 50 µl PCR , 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 5% DMSO and 2.5 U of Goldstar *Taq* polymerase. The reaction was thermocycled for 5 min at 95°C, then for 80 cycles at 94°C, 30 sec; 60°C, 15 sec and finally for an extension step of 10 min at 74°C.

Screening of mutant libraries

Mutagenised PCR or StEP reaction products were digested by the *Eco*RI and *Pst*I restriction enzymes before purification using the QIAquick Gel Extraction Kit and cloned back into the pBTac2 vector which was digested by the same enzymes and also purified by the same method. The resulting plasmids were transformed into electrocompetent XL1 blue cells, which were plated on LB agar plates containing 20 µg/ml of tetracycline and 100 µg/ml of ampicillin. After 18 h of growth, colonies were picked and spotted with sterile toothpicks onto TCM agar plates (10 g/l Tryptone, 0.1% sodium



Scheme 1. Transglycosylation reaction catalysed by the α-galactosidase AgaB: the autocondensation reaction with *p*NPαGal.

citrate, 1% melibiose (Mel), 0.5% NaCl, 0.2% KH_2PO_4, 0.1% Ferric ammoniacal citrate, 0.003% neutral red). In this condition the MelA enzyme of XL1 blue cells is not active enough to induce a red colour. The whitest colonies (Mel-) were then spotted on two LB agar plates. One plate was stored and the other one was incubated for 2 h at 55°C. The clones retaining a thermostable α-galactosidase activity were identified by pouring an X-α-Gal solution (2 mg/ml) on the plate and all the blue clones were further characterized.

The 2.2 kb DNA fragments encoding the evolved AgaB enzymes were sequenced in both forward and reverse directions by MWG-Biotech.

Analysis of mutant enzymes for activity and regioselectivity

All 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 subjected to a brief sonication. IPTG was not used for the induction since in this system it neither increased significantly the α-galactosidase activity nor the band intensity corresponding to the AgaB subunit on SDS-PAGE (data not shown). Then the lysate was centrifuged for 15 min at 13,000 rpm and α-galactosidase activitity was determined in the supernatant. Enzyme assays were performed by introducing 1 to 10 µl of supernatant in 150 µl of a 2.5 mM substrate solution (pH 7.0) at 25°C. Once a slight coloration appeared, 850 µl of 0.5 M Na_2CO_3 was added and the absorbance was measured at 405 nm ($\varepsilon_{p\text{-NP}} = 18.500\ \text{l}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$). One unit of activity is defined as the amount of enzyme which liberates 1 µmole of *p*-NP per min under the given assay conditions. Each measurement was repeated at least 3 times and the presented results correspond to the mean values. The protein concentration of crude extracts was estimated by the method of Bradford [30] with lysozyme as a standard. Total activity was obtained using *p*NPαGal as a substrate and regioselectivity was checked using either α-Gal-[1 → 3]-α-Gal-O-*p*-NP or α-Gal-[1 → 6]-α-Gal-O-*p*-NP as substrates, synthesized according to a published procedure [26].

Enzyme kinetics

All kinetic studies with chromogenic substrates were performed in microtitration plates by following changes in absorbance using a microplate reader (iEMS, Labsystem) at 25°C. The buffer employed for all kinetic experiments with wild type and mutant α-galactosidase AgaB was 100 mM sodium phosphate buffer pH 7.0. The reaction mixtures (190 µl), containing the substrate and buffer, were preincubated in the plate holder for 10 min prior to addition of enzyme (10 µl). We checked onto SDS-PAGE that the production level of AgaB, E33 and E901 enzymes were identical (data not shown), so that the ratio of recombinant proteins to the total amount of protein can be considered as constant. Therefore, the differences in activities are clearly related to the catalytic properties of mutant enzymes. The protein concentration in the reaction mixture was 0.6–15 µg/ml and 6–150 µg/ml for the wild-type and mutants, respectively, depending on the substrate. The initial rates of enzyme-catalyzed hydrolysis were measured during 10 min for *p*NPαGal and 2 h for *p*NP-disaccharides, at five to seven different substrate concentrations ranging from about 0.15 K_m to 5-times K_m, when possible. In the case of limited substrate solubility or high K_m, only V_m/K_m constants were determined by measuring initial rates at four low substrate concentrations. Values of K_m and V_m were determined from the initial rates by means of non-linear regression analysis (EnzymeKinetics, Trinity Software).

The measurement of the enzyme-catalyzed hydrolysis of melibiose was carried out in a discontinuous assay in two steps. First, melibiose hydrolysis was initiated by adding 100 µl of enzyme solution to 900 µl of reaction mixture containing melibiose (10–30 mM) in 100 mM sodium phosphate buffer pH 7.0. At various times, aliquots of 100 µl were taken and quenched by heating at 100°C for 5 min. In a second step, the galactose released from melibiose was determined by oxidation with galactose dehydrogenase in the presence of NAD^+ and measuring the increase in NADH at 340 nm using the microplate reader. The reaction mixture containing 150 µl of 0.4 M sodium phosphate buffer, 4 mM $MgSO_4$ pH 7.5, 30 µl of NAD (14 mM),10 µl of the aliquot and 10 µl of a galactose dehydrogenase solution (0.5 mg/ml) was incubated for 1 hour at room temperature and the resulting absorbance was read at 340 nm.

Screening of the transglycosylation activity by means of TLC

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 at room temperature and 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 → 6]-α-Gal-O-*p*-NP and galactose were respectively 0.59, 0.35, 0.26 and 0.14.

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

Kinetic experiments were performed using a 500 MHz Bruker spectrometer according to a procedure published in a previous paper [31]. 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 (2.45 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 into the NMR tube.

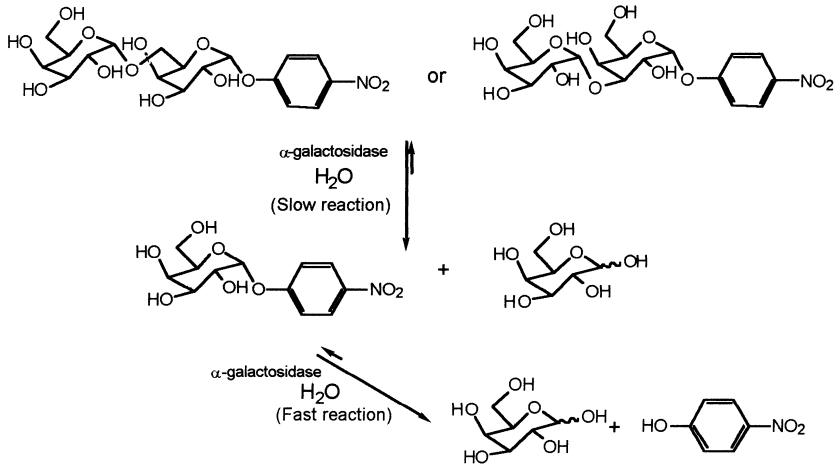
Results

Strategy for evolving α-galactosidase AgaB

The transferase activity of α-galactosidase AgaB of *Bacillus stearothermophilus* analysed by *in situ* proton NMR spectroscopy [26] exhibited a rather low 1,3 regioselectivity and a high 1,6 regioselectivity (Scheme 1). In order to improve the 1,3 regioselectivity, *agaB* gene was submitted to random mutagenesis. The strategy of evolution based only on the selection of a stronger 1,3 activity did not seem appropriate to produce highly regioselective variants because the 1,6 regioselectivity could also remain. Consequently we decided to develop a screening procedure allowing first the elimination of AgaB mutants bearing the 1,6 regioselectivity and secondly the selection of those retaining a 1,3 regioselectivity. Although our objective was to obtain α-galactosidases able to catalyse regioselective synthesis, for the sake of simplicity we decided to screen the regioselectivity of the hydrolytic reaction. Indeed, the screening of the regioselectivity of the transglycosylation reaction is far more difficult to carry out and several researchers have already shown that the regioselectivities of the hydrolysis and of the transglycosylation are quite similar [8,16]. Melibiose (α-Gal-[1 → 6]-Glc), being very close to the α-Gal-[1 → 6]-Gal substrate, was first used to test the ability of mutants to hydrolyse the α-1,6 linkage of galactosides. The clones expressing mutant enzymes which were unable to ferment the melibiose on agar plates, but that kept some α-galactosidase activity could be considered as good candidates for displaying another regioselectivity. In a second step, these mutant enzymes were assayed with the chromogenic substrates α-Gal-[1 → 6]-α-Gal-O-*p*-NP and α-Gal-[1 → 3]-α-Gal-O-*p*-NP. In these reactions, the α-galactosidase activity first releases a galactose residue and 4-nitrophenyl α-D-galactoside (*p*NPαGal), which is then used by the same enzyme as a substrate to finally produce the chromogenic *p*-nitrophenol (*p*-NP) (Scheme 2). Any differences in regioselectivity are then detected by measuring the absorbance at 405 nm. The genes encoding mutant enzymes displaying improved 1,3 regioselectivity were then recombined *in vitro* and the corresponding enzymes were screened by the same protocol. Finally the best candidates were tested for their regioselectivity in transglycosylation which constituted the last screening step.

Screening for improved 1,3 regioselectivity

The 2.2 kb DNA fragment of pAMG22 containing the *agaB* gene [28] was isolated and random mutagenized by error prone PCR to obtain a mutation rate of 0.4% [19]. The PCR product was digested with restriction enzymes *Eco*RI and *Pst*I, ligated back into the pBTac2 vector and introduced into the XL1 blue strain. About one thousand recombinant clones were obtained and spotted on TCM (Tryptone-Citrate-Melibiose) medium giving mostly red colonies (Mel+) but also some white colonies (Mel−) which were then spotted on two LB agar plates. One of them was incubated at 55°C to inactivate the thermolabile α-galactosidase MelA of *E. coli* and the clones Mel- retaining a thermostable α-galactosidase activity were identified by pouring X-α-Gal solution over them. Twenty two blue clones were obtained and their crude extracts were



Scheme 2. Strategy used for the screening of the α-galactosidases produced by the AgaB mutants: the production of *p*NP followed by OD405 reveals the regioselectivity of the enzyme since the hydrolysis of the *p*NPαGal is much faster than that of the disaccharides.

then prepared to test the regioselectivity of the modified enzymes. We first checked that the MelA enzyme of *E. coli*, which requires NAD^+ and Mn(II) ions as cofactors [32], was no longer active in these extracts. Second, each of them were incubated with α-Gal-[1 → 3]-α-Gal-O-*p*-NP or with α-Gal-[1 → 6]-α-Gal-O-*p*-NP at 25°C. The relative 1,3 and 1,6 hydrolytic activities were measured by following the variation of the absorption at 405 nm (although the optimal temperature of AgaB enzyme is 50°C the test was carried out at 25°C because of the thermolability of some mutant enzymes; data not shown). Figure 1A shows the ratio of the 1,3 to the 1,6 hydrolytic activity (*R*H) of each mutant. The enzymes E19, E28, E29, E33, E41 display a higher ratio than that of the native enzyme (0.34) indicating that the AgaB regioselectivity has already evolved after this first round of mutagenesis. In order to further increase the *R*H ratio of α-galactosidase activity the five corresponding genes were recombined *in vitro*. An equimolar mixture of plasmid DNAs containing the genes *agaB19, 28, 29, 33, 41* were subjected to StEP recombination [21]. Screening of 800 clones from this random library by the former protocol resulted in several clones displaying a much higher *R*H ratio, varying between 2.4 and 9 for the best (Figure 1B).

Kinetic characterisation of mutants

In order to understand the possible origin of this regioselectivity evolution, the kinetics of the hydrolytic reaction for some of the best mutants after each round of evolution were compared. The kinetic constants of AgaB, E33 and E901 enzymes were determined on the substrates, α-Gal-[1 → 3]-α-Gal-O-*p*-NP, α-Gal-[1 → 6]-α-Gal-O-*p*-NP and melibiose (Table 1).

The K_m value for *p*NPαGal increases dramatically during the evolution process with a concomitant decrease in the specificity constant V_m/K_m. Though the K_m could not be accurately determined for the evolved α-galactosidase (E901) due to a strong inhibition occurring at the high substrate concentrations, comparison between the K_m increase (>40-fold) and the V_m/K_m decrease (>40-fold) suggests that the catalytic efficiency of the mutant (k_{cat}) is not affected by the evolution process.

The same experiments were carried out with the α-Gal-[1 → 3]-α-Gal-O-*p*-NP, α-Gal-[1 → 6]-α-Gal-O-*p*-NP and melibiose substrates in order to study the regioselectivity of the hydrolytic reaction. The V_m/K_m values of the wild-type AgaB and the selected mutants towards these substrates are much lower than the value for *p*NPαGal and confirm the high K_m towards melibiose (130 mM) previously determined for AgaB [28]. We have checked by NMR kinetic measurements that no Gal-Gal product has been detected by incubation of the *p*-NP-disaccharides substrates with the various mutant enzymes demonstrating that the rate determining step for the chromophore release is the hydrolysis of the Gal-Gal bond of the *p*-nitrophenyl disaccharides. For the α-Gal-[1 → 3]-α-Gal-O-*p*-NP, the V_m/K_m of the evolved enzymes decreased in the same proportions as for *p*NPαGal as shown by the stability of the selectivity ratio (*R*1,3/*p*NPαGal). By contrast, the V_m/K_m of E33 and E901 enzymes towards α-Gal-[1 → 6]-α-Gal-O-*p*-NP decreased much more than towards *p*NPαGal leading to a dramatic modification of the regioselectivity during hydrolysis. This trend was also confirmed with melibiose since this substrate also presents the same structural feature (α-Gal-[1 → 6]-glycoside) at its non-reducing

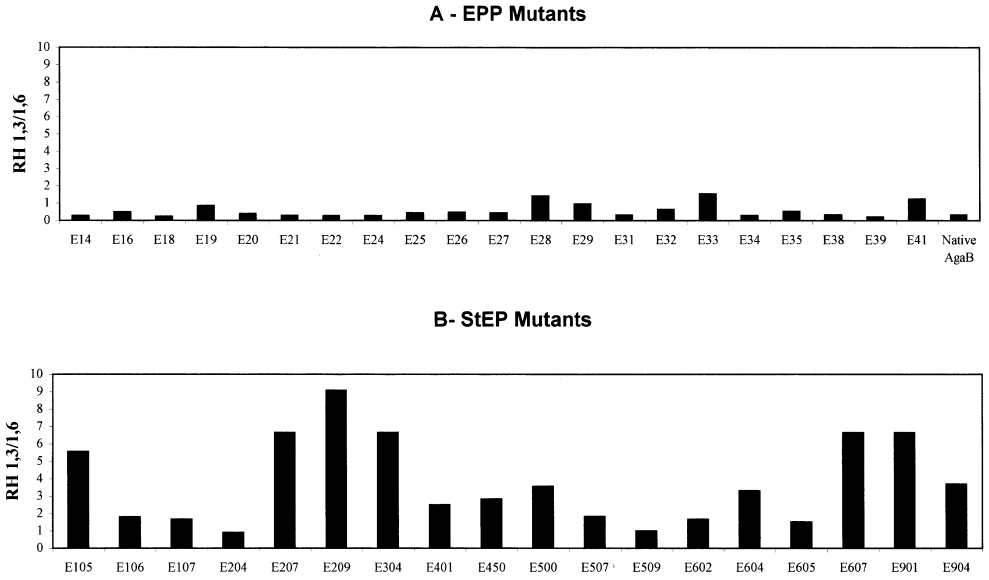


Figure 1. Regioselectivity of evolved α-galactosidases AgaB during the hydrolysis reaction. Ratio of the 1,3 to the 1,6 hydrolytic activity (*R*H) at 25°C determined respectively with 2.5 mM of the α-Gal-[1 → 3]-α-Gal-O-*p*-NP and the α-Gal-[1 → 6]-α-Gal-O-*p*-NP on (**A**) evolved α-galactosidases obtained by error-prone PCR (**B**) evolved α-galactosidases obtained by StEP with the genes *agaB19, 28, 29, 33, 41*.

Table 1. Kinetic parameters of AgaB and of the evolved α-galactosidases E33 and E901 (a) The K_m could not be accurately determined because a strong inhibition occurred at the high substrate concentration. (b) $R_{1,3}/_{pNP\alpha Gal}$ or $R_{1,6}/_{pNP\alpha Gal}$ correspond to the ratio of V_m/K_m towards the 1,3 or 1,6 regioisomers respectively to the V_m/K_m towards *p*NPαGal

	Substrate pNPαGal			*α-Gal-[1 → 3]-α-Gal-O-p-NP*		*α-Gal-[1 → 6]-α-Gal-O-p-NP*			*Melibiose*
Enzyme	K_m *(mM)*	V_m/K_m *(U/mg/M)*	V_m *(U/mg)*	V_m/K_m *(U/mg/M)*	$R_{1,3/pNP\alpha Gal}$[b]	V_m/K_m *(U/mg/M)*	$R_{1,6/pNP\alpha Gal}$[b]	$R_{1,3/1,6}$	V_m/K_m *(U/mg/M)*
Aga B	0.8	1.0×10^4	8	43	4.3×10^{-3}	161	1.6×10^{-2}	0.3	114
E33	3	2.0×10^3	6	12	6.0×10^{-3}	8.3	4.2×10^{-3}	1.4	3.6
E901	>30[a]	2.3×10^2	–	1.1	4.8×10^{-3}	0.16	6.9×10^{-4}	6.9	0.4

extremity. Whereas the native enzyme displays a strong regioselectivity for the 1,6 linkage as exemplified by the low selectivity ratio *R*H1,3/1,6, this ratio is strongly increased for the evolved enzymes E33 and E901. For the most evolved enzyme (E901) a complete swich of the regioselectivity is observed since the selectivity ratio (*R*H1,3/1,6) for the α-1,3 linkage is about 7 compared to the selectivity ratio (*R*H1,6/1,3 = 3.3) for the 1,6 linkage of the wild-type enzyme. Independent determination of the kinetic parameters, K_m and V_m was not possible due to the limited solubility of the disaccharides α-Gal-[1 → 3]-α-Gal-O-*p*-NP and of its 1,6 regioisomer at high concentrations.

Transglycosylation regioselectivity of evolved α-galactosidases

A screening for the transglycosylation reaction was first carried out by TLC where autocondensation products were detected after incubation of the mutant enzymes with *p*NPαGal. We wanted to check whether the increased 1,3 regioselectivity of disaccharide hydrolysis also occurred during the transglycosylation reaction and whether another regioselectivity has emerged. We observed that the best clones in hydrolysis were not systematically the best in transglycosylation: for example, E209 enzyme displaying a high 1,3 regioselectivity during hydrolysis presented a 1,3 transglycosylation regioselectivity below that of E901 or E605 mutants (data not shown). Consequently only the more interesting mutant enzymes from the first (E29, E33) and the second (E901) round of evolution were further analysed: autocondensation of *p*NPαGal was followed by *in situ* proton NMR spectroscopy at 25°C according to a procedure previously described [31]. Figure 2 indicates the molar percentages of each molecular species appearing in the mixture during the transglycosylation reaction (yields were calculated from the integrations of the NMR signals of the various anomeric protons and represent the proportions of *p*NPαGal which have reacted to produce a molecular species). As expected the highly evolved enzyme E901 led to the synthesis of more α-Gal-[1 → 3]-α-Gal-O-*p*-NP than the corresponding 1,6 regioisomer since the ratio (1,3/1,6) of the transglycosylation molecular species (*R*T) varies between 1.2 and 4.25 (and is 1.3 at the time at which the maximum yield of α-Gal-[1 → 3]-α-Gal-O-*p*-NP was obtained). The mutants E29 and E33 displaying lower regioselectivity in hydrolysis are also less regioselective for the saccharide synthesis although the *R*H and *R*T ratios are not always strictly correlated depending on the time of incubation. However, it can be noticed that the yield of the transglycosylation reaction for 1,6 disaccharides is dramatically reduced while the amount of 1,3 disaccharides synthesized (5%) remains constant during the evolution process. Therefore, the apparent increase in the 1,3 regioselectivity is mainly due to decrease of the 1,6 regioselectivity, according to the selection process, but not to improvement of the 1,3 regioselectivity. Moreover, after 1–2 hours at 25°C the mutant enzymes are inhibited while the native enzyme AgaB is still active at the end of the reaction (5 hours). Indeed, we have found an inhibitory effect exerted by the *p*-NP at high concentration (about 50 mM). The use of 2-nitrophenyl α-galactoside (*o*-NPGal) as substrate gave rise to a complete consumption of this substrate. Since the *o*-NP is less soluble than the *p*-NP, it precipitates before reaching inhibitory concentration. However we could not obtain an improvement of the transglycosylation to hydrolysis ratio (data not shown).

In an attempt to improve the 1,3 transferase activity we carried out a backcrossing between AgaB and E901 enzyme. After the StEP with both genes, we obtained five hundred mutants which were analysed for their transglycosylation potential by TLC. None of them displayed either a strong 1,3 regioselectivity or a higher transglycosylation level.

Amino acid substitutions in regioselective α-galactosidases

The genes encoding both enzymes E33 and E901 exhibiting high regioselectivity at each round of *in vitro* evolution were sequenced. Each mutant presented 4 and 6 amino acid changes evenly distributed in the translated E33 and E901 sequences respectively (Table 2). Unfortunately as no X-ray data for this α-galactosidase family is available it is difficult to provisionally assign the effect of each mutation. However we may state that the D376A and K399E mutations are close to the active site since the comparison of the α-galactosidase sequences of the

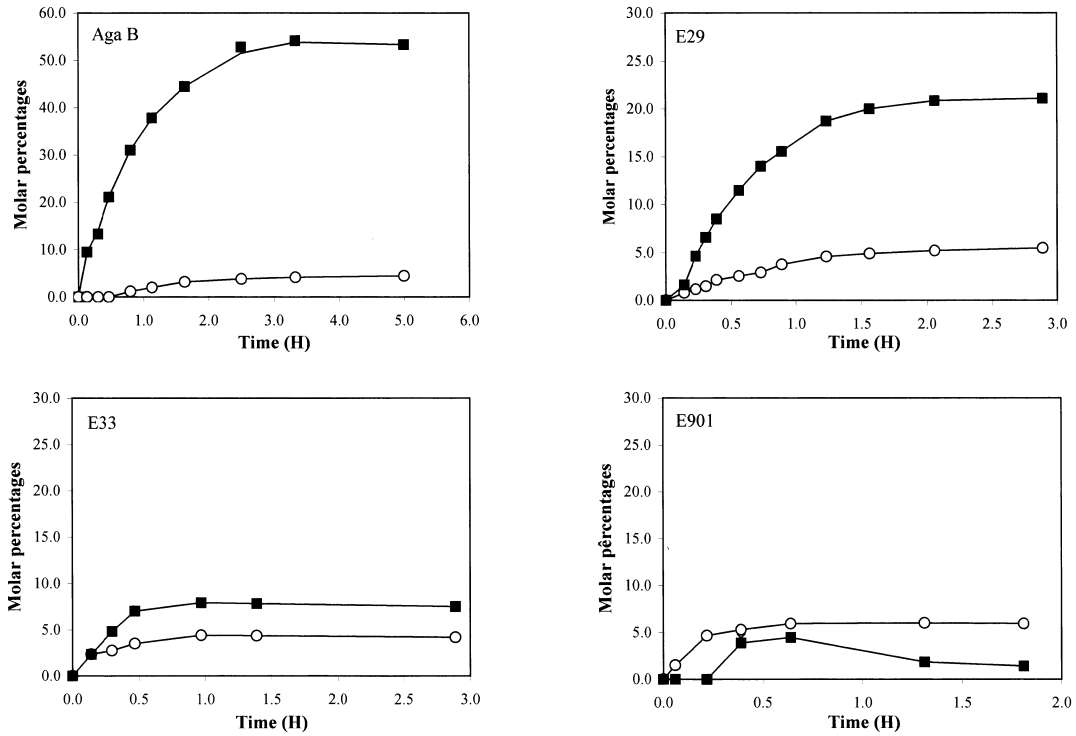


Figure 2. Kinetics of the synthesis of the autocondensation disaccharides α-Gal-[1 → 6]-α-Gal-O-*p*-NP (■) and α-Gal-[1 → 3]-α-Gal-O-*p*-NP (○) catalysed by AgaB and the α-galactosidase mutants E29, E33 and E901 at 25°C. The molar percentages represent the proportions of *p*NPαGal which have reacted to form a given disaccharide and thus are also the yields for the formation of this compound. For each enzyme the same starting concentration of *p*NPαGal was used (90 mM).

Table 2. Amino acid substitutions in the evolved α-galactosidases E33 and E901 displaying a modified regioselectivity

Residue n°	*117*	*198*	*376*	*399*	*461*	*514*	*591*
AgaB	N	A	D	K	E	M	A
E33	S	E	D	E	G	M	A
E901	S	E	A	E	E	V	T

families 36 and 27 revealed a highly conserved region, named α-galactosidase consensus sequence [33], located between amino acids 360 and 369 in AgaB. Moreover the N117S, A198E and K399E mutations may be involved in the decrease of 1,6 regioselectivity since they were found in both mutants. Finally E901 mutations seem to induce at one and the same time higher 1,3 regioselectivity and low transglycosylation level. Indeed as E901 contains 6 substitutions compared with AgaB enzyme, the *in vitro* recombination during the back-crossing experiments should provide 64 different combinations which must have been represented in our library of 500 mutants.

Discussion

We have modified the regioselectivity of the α-galactosidase AgaB *of B. stearothermophilus* by directed evolution in an attempt to improve the regioselective activity for the synthesis of a new linkage by transglycosylation. Our results demonstrate that this method can rapidly change the glycosidase regioselectivity. With one generation of random mutagenesis and one recombination of the best mutants, we found that the most evolved E901 enzyme loses most of its activity towards the 1,6 linkage both in hydrolysis and synthesis. This leads to mutant enzymes exhibiting a preference for the 1,3 linkage. This is consistent with the initial screening of the mutant which relies on the inability of the mutant strains to grow on melibiose (α 1,6 regioisomer). However, the yield of 1,3 disaccharides synthesis for all the selected mutants was not significantly improved compared to the wild type. This leads to some observations about the followed screening strategy: Our selection process was based on the regioselectivity of the hydrolysis reaction assuming that the regioselectivity for the transglycosylation would be identical. This assumption is valid for initial rate measurements of hydrolysis but no longer applies if one compares the initial velocity of hydrolysis and the maximum yield of transglycosylation products which depends on many kinetic constants. Moreover, transglycosylation reactions can occur either by an autocondensation process of the glycoside donor or by the condensation of the donor to another glycoside acceptor. It has been shown that the regioselectivity of these

two processes can be different and dependent on the stereochemistry and the nature of the anomeric substituant of the acceptor [18]. In this respect, an ideal selection process should rely on a positive screening strategy based on the synthesis of the disaccharides. However, no such screening processes are available *in vivo* and classical analysis of product formation by TLC on enzyme extracts is limited to the screening of a library of small size.

The reason for the low transglycosylation level of most of our mutants is still not clear. Since no improvement could be obtained with *o*-NPαGal substrate we cannot invoke the inhibition effect by the *p*-NP. It cannot be assigned to an increase of hydrolysis of transglycosylation products since we checked that with E901 this activity was well below that of AgaB enzyme for both regioisomers at the quite high concentration of 20 mM (data not shown). It could be rather due to a low transferase activity: the mutant enzyme might present less affinity for the acceptor molecules which can therefore entail more *p*NPαGal hydrolysis than condensation reactions.

The interesting outcome of this evolution process is that it is possible to eliminate most of the enzyme activity towards one regioisomer (1,6) while keeping an equivalent transglycosylation activity in terms of yield for the other regioisomer (1,3). This suggests that glycosidase regioselectivity is controlled by several amino-acid residues and that further improvement in the 1,3 transglycosylation activity should be obtainable by screening a mutant library of larger size. Finally using this methodology which does not rely on structural data and rational design, new glycosidases bearing both high transglycosylation and new regioselective activities should be elicited.

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