

# Digital screening methodology for the directed evolution of transglycosidases

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**Engineering of glycosidases with efficient transglycosidases activity is an alternative to glycosyltransferases or glycosynthases for the synthesis of oligosaccharides and glycoconjugates. However, the engineering of transglycosidases by directed evolution methodologies is hampered by the lack of efficient screening systems for sugar-transfer activity. We report here the development of digital imaging-based high-throughput screening methodology for the directed evolution of glycosidases into transgalactosidases. Using this methodology, we detected transglycosidase mutants in intact *Escherichia coli* cells by digital imaging monitoring of the activation of non- or low-hydrolytic mutants by an acceptor substrate. We screened several libraries of mutants of  $\beta$ -glycosidase from *Thermus thermophilus* using this methodology and found variants with up to a 70-fold overall increase in the transglycosidase/hydrolysis activity ratio. Using natural disaccharide acceptors, these transglycosidase mutants were able to synthesise trisaccharides, as a mixture of two regioisomers, with up to 76% yield.**

**Keywords:** digital imaging/directed evolution/high-throughput screening/transglycosidases

## Introduction

Complex carbohydrates occur in a wide range of contexts in biology, including polysaccharides, proteoglycans, glycoproteins and glycolipids. They have important roles in a number of functions, including cell growth (Kalovidouris *et al.*, 2005), cell–cell interactions (Crocker and Feizi, 1996), immune defence (Rudd *et al.*, 2001), inflammation (Lowe, 2003), as well as viral and parasitic infections (Le Pendu, 2004). In contrast with the situation for peptides and oligonucleotides, the chemical synthesis of complex carbohydrates is an extremely challenging and labour-intensive process, which cannot generally be achieved in an automated fashion or on a large scale. New approaches to complex carbohydrate synthesis and modifications remain an urgent need in biotechnology to facilitate the development of potential applications in food or health industry (Hanson *et al.*, 2004).

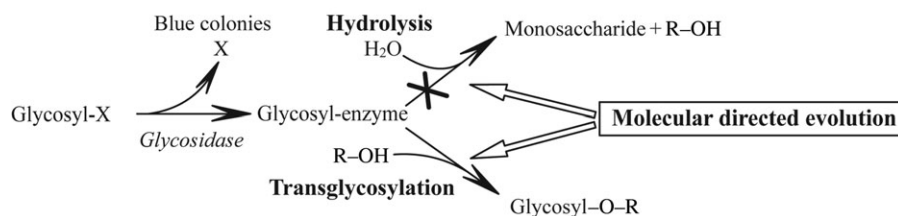
In the past few years directed evolution approaches for protein engineering have proved to be highly useful in improving the stability of enzymes (Eijsink *et al.*, 2005) and for altering their substrate specificities (Dion *et al.*, 2001; Bloom *et al.*, 2005). One of the crucial steps in any directed evolution experiment is the development of a screening assay

to facilitate the screening of large libraries. However, in contrast to the high-throughput screening (HTS) of hydrolases (glycosidases, proteases, lipases, etc), assaying for transfer activity is extremely challenging as no obvious change in fluorescence or absorbance is associated with bond formation. Thus, it is highly desirable to develop HTS methodologies to facilitate the screening of large libraries of mutant enzymes with sugar-transfer activities (Lin *et al.*, 2004; Aharoni *et al.*, 2006; Ben-David *et al.*, 2008).

Most high-throughput catalyst screening processes are based on monitoring spectroscopically the progress of a chemical reaction. There are many fluorogenic or chromogenic substrates that can be used to monitor cleavage reactions such as hydrolysis (Goddard and Reymond, 2004). These substrates often possess masked fluorophores or chromophores with a cleavable moiety, so that the cleavage reactions restore the fluorescence or the absorbance of the chromophores by removing that masking moiety. Such substrates are not available for the reverse reaction, i.e. the bond forming reaction in solution (Konarzycka-Bessler and Bornscheuer, 2003). Furthermore, by using any HTS on these fluorogenic or chromogenic substrates, there are risks of isolating catalysts that are not capable of reacting with a less reactive substrate of synthetic interest (Aharoni *et al.*, 2006). It is thus particularly important that any HTS process include substrates and reactions that are as close as possible to the desired ones.

Herein, we present a new method for HTS of transglycosidase enzyme. This HTS methodology allows the detection, in intact *Escherichia coli* (*E. coli*) cells, of hydrolase mutants that have lost most of their hydrolytic activities while keeping their transferase one. This new screening strategy is illustrated on the glycosylhydrolases family 1  $\beta$ -glycosidase from *Thermus thermophilus* (Tt $\beta$ gly), which was recently evolved in transglycosidases by directed evolution (Feng *et al.*, 2005). This method is based on the digital imaging monitoring (Joo *et al.*, 1999; Delagrave *et al.*, 2001) of reactivation of non- or low-hydrolytic mutants by an acceptor substrate that does not need to be a coloured or a fluorescent compound. Using the chromogenic donor substrate X-glycosyl, mutants with low hydrolytic activity gives light coloured colonies in absence of acceptor. However, in the presence of acceptor, mutants displaying high transferase activity exhibit a higher turnover resulting in an increase in the colony coloration. The formation of X blue product is thus indirectly correlated to the efficiency of the transglycosylation step by an acceptor that differs from water (Scheme 1). Clones exhibiting higher transglycosidase activity are then easily located by digital imaging without the need to lyse the cells and perform many other manipulations otherwise necessary for screening large mutant libraries.

Such screening strategy was implemented to screen mutant libraries of Tt $\beta$ gly glycosidase and allowed us to isolate improved transglycosidase activities on cellobiose substrate acceptors.



**Scheme 1.** Principle of the screening strategy applied to retaining glycosidases. As the mechanism involves a covalent glycosyl-enzyme intermediate, the development of blue colour in colonies is dependent on the reactivation of non- or low-hydrolytic mutants by an acceptor substrate.

## Materials and methods

### Materials

Plasmids pBβGly or pBβGly<sub>F401S</sub> containing the Ttβgly gene and F401S mutant gene were previously constructed from the plasmid pBTac2 (Feng *et al.*, 2005). Oligonucleotides were synthesised by Sigma genosys (Grenoble, France). Kits from Qiagen (QIAprep<sup>®</sup> spin Miniprep Kit) were used for plasmid preparation and extraction. DH5αF'IQ<sup>TM</sup> cells were obtained from Invitrogen.

### Random mutagenesis

Random mutations were introduced by mutagenic PCR (polymerase chain reaction) (Leung *et al.*, 1989). Primer D (5'-CAATTAATCATCGGCTCG) and F (5' AATCTTCTCTCATCCGCC) flank the gene before the tac promoter and after the *Pst*I restriction site. 20 pmol of each primer was mixed with 10 ng of plasmid pBβGly or pBβGly<sub>F401S</sub> in a 50 μl PCR. The reaction conditions were as follows: 1× Dynazyme buffer, 10 mmol dATP and dGTP, 50 mmol dTTP and dCTP, 350 μmol MgCl<sub>2</sub>, 5 mmol MnCl<sub>2</sub> and 1 unit 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; 50°C, 30 s; 72°C, 2 min 30 s; 70°C, 5 min. Mutagenised PCR products were digested by *Eco*RI and *Hind*III restriction enzymes and cloned back into the pBTac2 vector digested by the same enzymes. The plasmids EPP pBβGly and EPP pBβGly<sub>F401S</sub> carrying mutagenised Ttβgly genes was used to transform *E. coli* DH5αF'IQ<sup>TM</sup>.

### Screening of mutants

Transformed cells (400 μl) were plated on nitrocellulose membrane (0.45 μm, 21 × 21 cm<sup>2</sup>) lying on Luria Bertani (LB) agar supplemented with 100 μg/ml ampicillin and were grown at 37°C overnight. The nitrocellulose membrane, on which small colonies (Ø < 0.5 mm) were grown, was transferred on minimal medium (MM) agar (sodium phosphate buffer 100 mM pH 7, agar 15 g/l) plate containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (0.1 mM) and isopropylthiogalactoside (IPTG) (0.05 mM) and incubated 1 h at 30°C. Finally, the membrane was transferred onto fresh MM agar plate containing X-Gal (0.1 mM) and the acceptor sugar (cellobiose 20 or 40 mM) and the plates incubated at 30°C were subjected to digital image analysis.

Two scanned images (Epson 4490, 16 bits grey levels, 600 dpi) were acquired immediately after the transfer onto the acceptor plate ( $T_0$ ) and after an incubation of 2–3 h at 30°C ( $T_f$ ). Digital images were processed using VISILOG 6.3 image analysis software (Noesis, France). These images

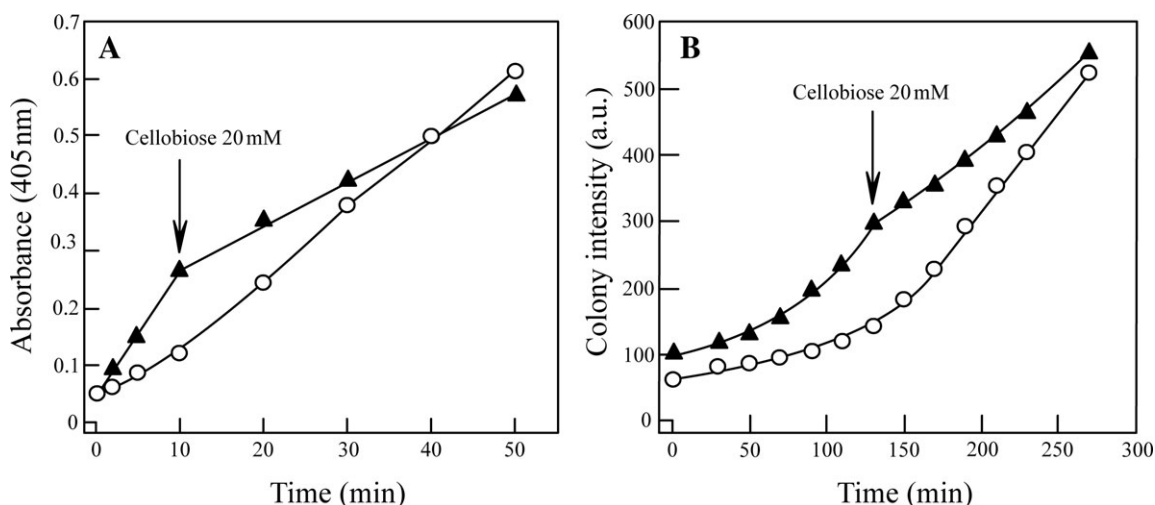
were first aligned using the warp algorithm, which allowed each colony to be exactly superimposed on the two images. Coloration intensities were calculated based on a 65 000 grey scale and automatic colony detection was performed after thresholding to eliminate white colonies. Computer-determined colony boundaries were used to calculate individual colony size measurement and their mean intensity area on each image  $T_0$  and  $T_f$ . The ratio of the mean intensity on each colony [ $R = (T_f - T_0)/T_0$ ] was calculated and used to rank the colony in descending order.

### Kinetic analysis of the mutants

*Whole-cell microtiter plate assay for acceptor activation* Selected colonies were picked and transferred to 250 μl LB (+100 μg/ml ampicillin) in a 96-well microplate and incubated overnight at 37°C. Then IPTG (0.05 mM) was added in each well and enzyme over-expression was allowed for 2 h at 37°C. Next, 50 μl of each culture was transferred to two wells containing 150 μl reaction mixture made of *o*NPGal (2.5 mM), phosphate buffer (100 mM, pH 7) with or without cellobiose (20 mM). The kinetics of *o*NP release was followed for 1 h at 405 nm and the slopes were compared between these assays.

*Enzyme extraction and transglycosylation assay* Five millilitre of an overnight LB medium culture of the selected clones was centrifuged and the cell pellet suspended in 500 μl of lysis buffer (sodium phosphate buffer 50 mM, NaCl 300 mM, pH 8) with lysozyme (1 mg/ml). After sonication, the cell lysate was centrifuged and supernatant was treated 20 min at 70°C to precipitate *E. coli* proteins. After centrifugation (10 000 g), enzyme extracts contained partially purified Ttβgly glycosidase (70–90%) as revealed by capillary electrophoresis (Agilent 2100 bioanalyser). The supernatants were tested without further purification for transglycosylation activity by thin layer chromatography (TLC). Enzyme extracts (5 μl) was introduced into a reaction mixture containing *o*NPGal (25 mM) with cellobiose or maltose (25 mM) in 100 mM phosphate buffer, pH 7 and incubated at 50°C. Aliquots of 2 μl were regularly withdrawn and monitored by TLC (butanol/ethanol/water, 5:3:2).

*Capillary electrophoresis* Prior to capillary electrophoresis analysis, a derivatisation reaction of carbohydrates was performed using 8-aminonaphtalene-1,3,6-trisulphonic acid (ANTS): 4 μl of the released products of the transglycosylation reaction were mixed with 4 μl xylose 10 mM, 6 μl ANTS (0.2 M), 20 μl mixture acetic acid–water (3:17, v/v) and 30 μl NaCNBH<sub>3</sub> (1 M). The ANTS–carbohydrate solution was incubated at 40°C for 15 h.



**Fig. 1.** *In vivo* kinetics on *E. coli* cells expressing WT Ttβgly (▲) and F401S mutant (○). (A) Changes in absorbance at 405 nm of cell culture in presence of *o*NPGal as a donor substrate. (B) Development of the colony coloration on a plate measured by digital imaging in presence of X-Gal (0.1 mM) as a donor substrate. Each point is the mean value of 80 colony intensities. In both experiment, cellobiose was added as an acceptor for the transglycosylation reaction.

The evolution of substrate and product concentrations was monitored by capillary electrophoresis using a Beckman–Coulter P/Ace MDQ system with an uncoated fused-silica capillary (57 cm total length, 47 cm effective length, 75 μm ID). Samples were introduced by pressure (0.5 psi) at the cathode end. Separations were performed at 20 kV in the presence of 50 mM sodium phosphate buffer (pH 2.5) at 25°C. The products were detected and quantified by UV-absorbance at 214 nm. Xylose was used as an internal standard. Between runs, the capillary was rinsed for 3 min with water, followed by 100 mM NaOH for 2 min, water for 2 min then 50 mM sodium phosphate buffer (pH 2.5) for 2 min.

## Results and discussion

### Detection of improved transglycosidase activities in *E. coli* cells

In previous work, we demonstrated that Ttβgly glycosidase mutants with improved transglycosidase activity were activated by increasing concentration of acceptors such as cellobiose or maltose, while the wild-type (WT) enzyme was inhibited in presence of these acceptors (Feng *et al.*, 2005). This kinetic property, which is expected for enzymes with transferase activity using a ping-pong mechanism, was exploited to develop the screening strategy.

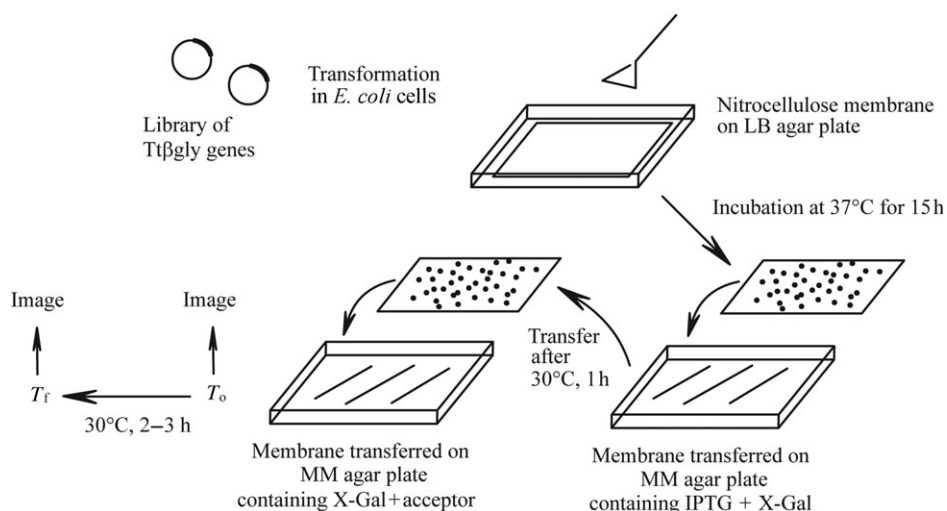
To test the feasibility of this approach, we separately incubated cells expressing either an improved transglycosidase mutant of Ttβgly (F401S) or the WT enzyme in presence of *o*NPGal as a substrate. Then, after an incubation time of 30–60 min, cellobiose acceptor was added to the medium. Both compounds are likely internalised by the lactose permease in the cell (Olsen and Brooker, 1989), since other known cellobiose permease gene, CelB/C and BglS, are not normally expressed (Schnetzer *et al.*, 1987; Reizer *et al.*, 1990). The absorbance at 405 nm was followed continuously before and after the addition of cellobiose acceptor. As shown in Fig. 1A, kinetic curve from cells expressing WT enzyme presented a slight slope reduction after addition of cellobiose

due to the inhibition by this acceptor that can compete with the *o*NP-donor substrate. In contrast, kinetic of *o*NP release with the transglycosidase mutant (F401S) was very slow in presence of donor substrate only, according to the low hydrolytic activity of this mutant, and exhibited a significant increase after addition of acceptor.

Next, we checked that similar kinetic behaviour could be detected by digital imaging directly on colonies growing on agar plates. The procedure used for solid-phase screening is outlined in Scheme 2. *Escherichia coli* culture containing either WT Ttβgly gene or the F401S mutant gene was spread onto nitrocellulose membrane put on agar plates and grown overnight at 37°C. Then, the membrane, on which uniform and small size colonies had grown (<0.5 mm), was transferred onto a fresh agar plate containing X-Gal (0.1 mM) and IPTG (0.05 mM) for 1 h at 30°C. This step allowed the enzyme, which is slightly toxic for *E. coli* growth, to be over-expressed and subsequently to hydrolyse chromogenic X-Gal substrate. Images of the plate were taken at regular intervals and the nitrocellulose membrane was finally transferred on a new plate containing the donor substrate X-Gal and the cellobiose acceptor. Then, digital imaging kinetics were registered during 2–3 h at 30°C. As these kinetic measurements were done on minimal media, no change in colony size was observed during the time course of the experiment. Figure 1B reveals that the kinetics of the colony coloration followed by image quantification had a similar profile to that obtained with liquid cultures. This result demonstrates that the image analysis of the solid phase assay has sufficient sensitivity to detect small kinetic variation in enzyme expressed inside *E. coli* cells. It also confirms that this assay is not dependent on the nature of the chromogenic donor substrate (*o*NPGal or X-Gal) and relies on the efficiency of the deglycosylation of glycosyl intermediate by acceptor substrates.

### Validation of the screening procedures on *E. coli* cell mixtures expressing WT and transglycosidase mutant

To validate this screening strategy, we used it to detect colonies that expressed improved transglycosidase mutant



**Scheme 2.** Procedure for screening a mutant glycosidase library by digital imaging.

(F401S) from a mixture of cells that have been transformed with different ratios of plasmids containing either the WT gene or the F401S mutant gene. Cell mixtures were plated on nitrocellulose membrane and processed as depicted in Scheme 2.

For HTS purpose, only two images were acquired: one, referred as  $T_0$ , immediately after the membrane transfer on the agar plate containing the X-Gal donor substrate and the cellobiose acceptor; and the other, referred as  $T_f$ , after incubation 2–3 h at 30°C on the same plate. Colony intensity at  $T_0$  is then related to only the hydrolytic activity of the expressed enzymes, since coloration developed only in the presence of X-Gal donor, while colony intensity at  $T_f$  was due to hydrolytic and transglycosylation reaction. From image analysis, we calculated the ratio  $R = (T_f - T_0)/T_0$  for each colony, which is directly correlated to the transglycosylation/hydrolysis ( $T/H$ ) activity of the mutants.

During image processing, scanned images proved to be better than images acquired with a digital camera since they benefit from more homogeneous illumination. Furthermore, scanning allows a wider dynamic range (16 bits) and can provide large plate image ( $22 \times 22 \text{ cm}^2$ ) with enough resolution ( $\sim 300$  pixels per colony). Scanned images were processed by careful superposition of  $T_0$  and  $T_f$  images, overall thresholding, geometry recognition of colonies, and local segmentation to obtain individual colony coloration and intensity quantification. The first step of the image processing was to get an exact superposition of the two successive images ( $T_0$  and  $T_f$ ) so that evolution of the mean intensity could be measured accurately on the same colony at different times. This fine tuning of the images was done through a warping algorithm (Visilog 6.3, NOESIS) (Fig. 2).

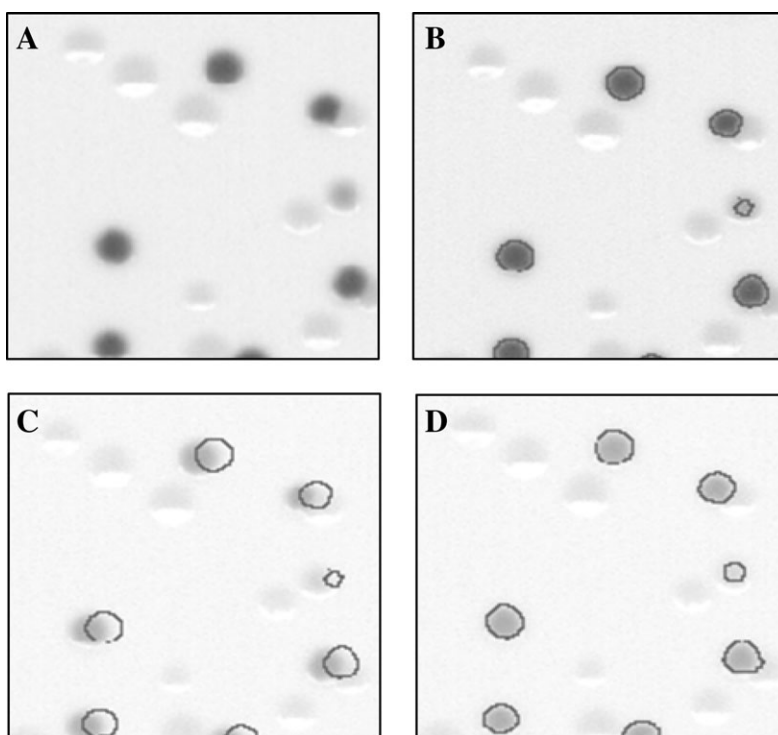
Colonies were screened according to their individual  $R$  value. Table I presents the screening results on mixtures of *E. coli* cells transformed with different ratio of F401S over WT plasmids (5, 1 and 0.1%). Each positive F401S colony that was selected from image analysis was subsequently checked for cellobiose activation in cell suspension with *o*NPGal as substrate. The transferase activity of galactose on cellobiose was also assayed *in vitro* on cell extracts by TLC. For each dilution, our screening process was able to detect

unambiguously the colonies expressing the mutant F401S, which displays high transglycosylation properties and low hydrolytic activity (Feng *et al.*, 2005). The discriminating  $R$  factor was significantly different from colonies expressing WT enzyme and F401S mutant, which validated the choice of this parameter from rapid screening.

#### Identification of improved mutants of *Ttβgly* for transglycosylation

After validating the digital screening on mixtures composed of two components, we used this screening process to probe real libraries of *Ttβgly* mutants. First, we analysed an error prone PCR library that had previously been screened by a low throughput two-step procedure (colony plating and TLC analysis of transglycosylation activities of enzyme extracts) (Feng *et al.*, 2005). From this library, mutants such as A113 (N339T, F401S) were originally selected, from which the transglycosylase F401S mutant was constructed. The extracted plasmid DNA library was transformed in DH5 $\alpha$  strain, which was propagated and plated to give 7796 individual colonies. According to the described screening strategy, 14 were selected for their high  $R$  factor with cellobiose (20 mM) as acceptor. Among the selected colonies, only one clone was a false positive corresponding to the WT enzyme. For one mutant (C5781), we observed a significant increase in transglycosidase activity of the crude cell lysate as judged by TLC analysis and quantified by capillary electrophoresis (Table II). This mutant was sequenced and found to contain a single mutation N390I, which had previously been identified by the low throughput screening procedure (Feng *et al.*, 2005). This result confirms the ability of this HTS strategy to isolate in only one-step mutants that have improved transglycosylation activity.

Next, an error prone library was constructed from the F401S gene in order to improve the transglycosylation activity of these first generation mutants. This library was screened against the natural acceptor disaccharide, cellobiose, on which the F401S mutant transfers galactose at a yield of 60% (Table II). Two plates each containing 6000–10 000 colonies were screened by digital imaging and three positive variants (C2142, C2842, C3460) were selected



**Fig. 2.** Image analysis: (A) scanned image before processing; (B) automated contour detection on the  $T_f$  image. Contour image of colonies from the  $T_f$  image applied to the  $T_0$  image; (C) without image superposition routine and (D) after image superposition with warping routine (Visilog 6.3).

**Table I.** Digital imaging screening on *E. coli* cells transformed with different ratios of F401S over WT plasmids

Plasmid mixture	Number of screened clones	Theoretical F401S clones	Selected clones <sup>a</sup>	Positive F401S clones <sup>b</sup>
95% WT – 5% F401S	1366	68	79	76
99% WT – 1% F401S	1832	18	28	12
99.9% WT – 0.1% F401S	4866	5	4	3

<sup>a</sup>Positives clones selected on the basis of a high  $R$  value (for the experiment at 5% F401S:  $R_{F401S} = 0.34 \pm 0.06$  and  $R_{WT} = 0.09 \pm 0.03$ ).

<sup>b</sup>Selected positive clones were checked by kinetics on colonies in suspension.

(Fig. 3) and checked for their improved transglycosylation efficiency by TLC. Sequence analysis revealed that these mutants contained one or two mutations additional to the F401S mutation (Table II). Interestingly, none of these mutations was in direct contact with the substrates and some (V12G, T161A and A205T) were very far from the active site (Fig. 4). Thus, the C2842 mutant contains two mutations (F401S and N339T), which were previously identified in mutant A113 (Feng *et al.*, 2005). In mutant A113, the mutation N339T was shown to decrease the transglycosylation yield compared with F401S mutant. We can conclude that the additional mutation V12G in mutant C2842, despite its location far from the active site (distance  $>12$  Å), significantly affects the kinetic properties of this mutant towards sugar acceptor (discussed later).

#### Kinetic characterisation of the *Ttβgly* mutants

Next, these mutants were over-expressed and partially purified for further characterisation.

We first checked that they exhibited improved kinetic activation in the presence of the acceptor sugar. When the initial rates of *o*NP released were measured at a fixed concentration of donor (*o*NPGal 5 mM) and an increasing concentration of acceptor (cellobiose), a strong activation of the kinetic was observed with the mutants selected from the screening process (Fig. 5A). Starting from an activation factor of 2.9 with the F401S mutant, this activation factor reached 10.2 and 11.8 for the best mutants (C2842, C3460). The fact that for the variants, we observed an activation of the rate of the ONP or X group release in the presence of high concentration of acceptor is consistent with deglycosylation step being rate determining, at least partially for these substrates. The addition of a competing acceptor would not be expected to have any effect upon the rate of *o*NP or X group release. Even if the glycosylation step is rate determining with *o*NP substrates for WT enzyme, the strong reduction of the hydrolysis rate (Table II) of the variants would be sufficient to change the rate determining step in absence of a competing acceptor. Whatever the effect of the mutations on the glycosylation step, the results of activation presented in Fig. 5 may be interpreted by an increase in deglycosylation rate upon the addition of acceptor, until it reaches the glycosylation rate. However, detailed determination of the kinetic constants is precluded since most mutants did not exhibit Michaelis-Menten kinetics.

To get quantitative information about both transglycosylation and hydrolysis, the kinetics of these reactions were followed directly by capillary electrophoresis using *o*NPGal as donor (25 or 50 mM) and cellobiose or maltose as acceptor (25 or 50 mM) (Table II). The mutant N390I obtained after the first step of random mutagenesis on WT enzyme had a  $T/H$  rate ratio  $\sim 18$ -fold higher than the WT enzyme, which

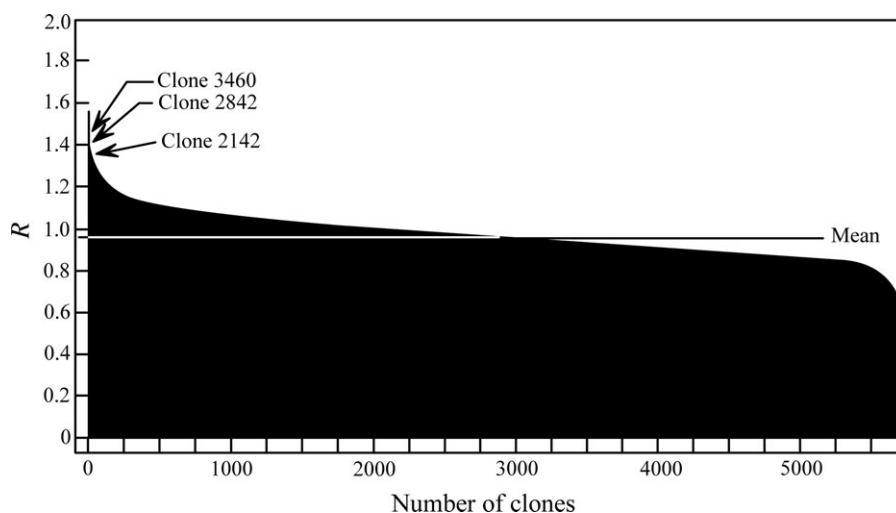
**Table II.** Transglycosylation activities of WT and mutant Tt $\beta$ gly enzymes in the presence of *o*NPGal as donor and cellobiose or maltose as acceptors in 1/1 molar ratio

Mutant	Mutations	Acceptor	[Acceptor] (mM)	Transglycosylation <sup>a</sup> rate [mmol/(min.mg)]	Hydrolysis <sup>a</sup> rate [mmol/(min.mg)]	Overall activity [mmol/(min.mg)]	$V_{trans}/V_{hyd}$	Maximum <sup>b</sup> yield (%)
WT		Cellobiose	25	0.28	1.4	1.68	0.20	12
		Maltose	25	nd <sup>c</sup>	nd	nd	0.16	15
F401S	F401S	Cellobiose	25	0.74	0.07	0.81	10.9	nd
			50	1.49	0.06	1.55	24.8	60
		Maltose	25	nd	nd	nd	1.7	49
C 5781	N390I	Cellobiose	25	nd	nd	nd	3.6	53
C 2142	F401S, T161A	Cellobiose	50	0.34	0.01	0.35	34	65
C 2842	F401S, V12G, N339T	Cellobiose	50	0.06	0.002	0.06	30	70
C 3460	F401S, A205T, H365Y	Cellobiose	50	0.19	0.008	0.194	23	76
		Maltose	50				3.2	46

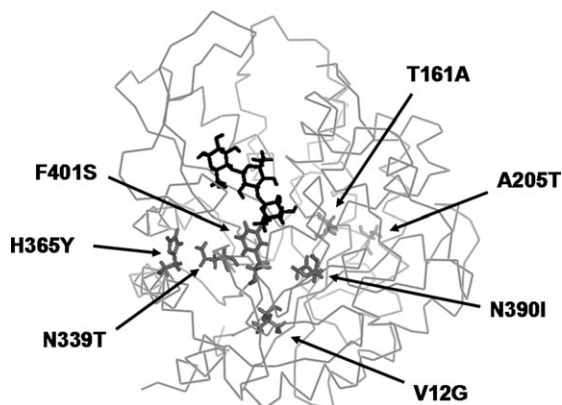
<sup>a</sup>Transglycosylation and hydrolysis activity was determined by separation and quantification of products by capillary electrophoresis.  $V_{trans}/V_{hyd}$  corresponds to the ratio of the initial velocities of transglycosylation products over galactose formation at two different concentrations of donor and acceptor.

<sup>b</sup>Maximum yield corresponds to the transient maximum amount of transglycosylation product obtained after long term incubation.

<sup>c</sup>Not determined.



**Fig. 3.** Results of screening of *E. coli* library expressing mutants of Tt $\beta$ gly on cellobiose acceptor using digital imaging. Activities are expressed as  $R = (T_f - T_0)/T_0$ , where  $T_0$  and  $T_f$  are the colony intensities at the beginning of membrane incubation on X-Gal and acceptor and after 2 h of incubation, respectively.

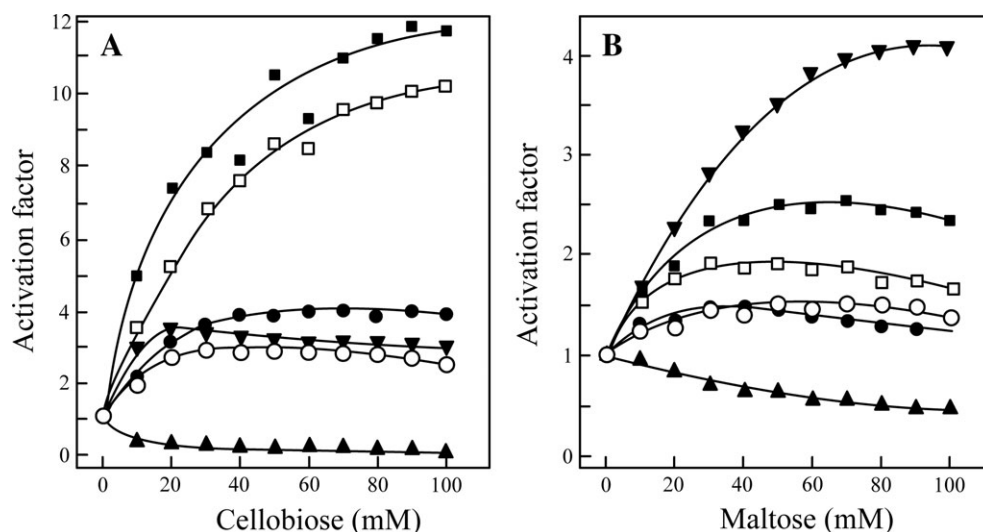


**Fig. 4.** Location of the mutations in the Tt $\beta$ Gly structure. The trisaccharides product (in black) has been docked in the active site.

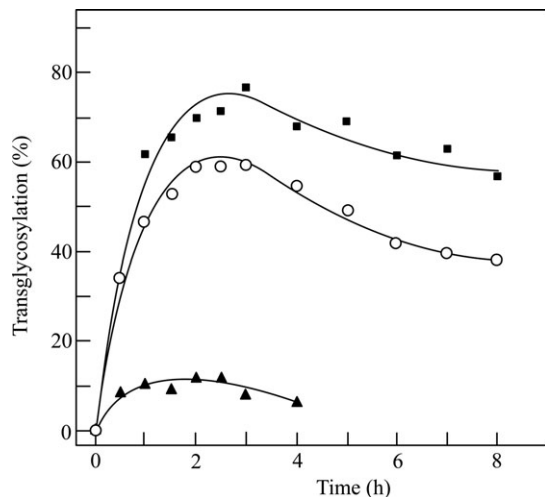
demonstrated the validity and the efficiency of the screening process designed to select for mutants with improved transglycosylation activity. An additional improvement (1.4-fold

for the C2142 mutant) of the  $T/H$  rate ratio was also observed in the second step of random mutagenesis starting from the F401S mutant, which resulted in up to a 70-fold overall increase in the  $T/H$  ratio for some mutants. This increase was mainly due to a marked reduction ( $>175$ -fold) of the hydrolytic performance of the mutant compared with the WT enzyme. An improvement of the absolute transglycosylation rate was only observed for the F401S mutant, while a slight decrease of this rate is observed for mutants obtained after the second step of random mutagenesis (Table II). Nevertheless, the best mutant, C3460, had an overall activity that was  $<10$  times lower than the WT enzyme, making this mutant still valuable for synthetic applications.

To check the specificity of the evolution process toward the cellobiose acceptor used in the screening process, the kinetic of mutants were also followed in presence of maltose, which was an alternative acceptor for the F401S mutant (Feng *et al.*, 2005). The initial rate activation by maltose of the best mutants (2.5-fold increase for C3460 and 1.9 for



**Fig. 5.** Activation of the steady-state kinetics of WT and evolved Ttβgly mutants by cellobiose (A) or maltose (B) with a fixed concentration of *o*NPGal (5 mM). WT Ttβgly (▲), F401S (○), A113 (▼), C2142 (●), C2842 (□) and C3460 (■).



**Fig. 6.** Kinetic study transglycosylation reaction analysed by capillary electrophoresis with WT (▲) and evolved Ttβgly enzymes F401S (○) and C3460 (■) with *o*NPGal as donor and with cellobiose as acceptor. The percentage of transglycosylation corresponds to the molar yield of galactose transferred to the acceptor giving to two trisaccharides ( $\beta$ -D-Galp-(1→3)-cellobioside and  $\beta$ -D-Galp-(1→6)-cellobioside).

C2842) was lower than for cellobiose (Fig. 5B). Consistently, no significant improvement of the *T/H* rate ratio was observed with maltose for the evolved mutant (C3460) compared with F401S mutant. Interestingly, mutant A113 (F401S, N339T) exhibited higher activation by maltose than mutant C2842, suggesting that mutation V12G has an indirect effect on the acceptor selectivity. Both results confirm that the screening process has sorted mutants that had reduced hydrolysis activity but also had a preference for the cellobiose acceptor.

An improvement in the initial *T/H* activity ratio of glycosidases is a prerequisite for obtaining mutants with good synthetic capability. However, because the transglycosylation products were kinetic products, the transglycosylation yield was also affected by the rate of hydrolysis of the product, which should be as low as possible to allow accumulation of

the transglycosylation products. Thus, the progress of the transglycosylation products synthesised by evolved mutants was followed by capillary electrophoresis until nearly complete consumption of substrate (Fig. 6). With WT Ttβgly, a rapid hydrolysis of the transglycosylation products was observed preventing their accumulation and a maximum yield of 12% was calculated for them (Table II). In contrast, evolved mutant like C3460 exhibited slower substrate (*o*NPGal) and products [ $\beta$ -D Galp (1→*x*)- $\beta$ -D-Glcp-(1→4)-D-Glcp] hydrolysis, allowing the latter to reach a maximum transient yield of 76%. These results clearly showed the advantage of the evolved mutants compared with the WT enzyme in relation to their synthetic ability. However, for the cellobiose acceptor, this increase in the transglycosylation yield was not combined with an increase in the regioselectivity of the enzyme since, in all evolved mutants, a mixture (~50/50) of trisaccharides was obtained (1→3 and 1→6 regioisomers). The structures of the transglycosylation products have been determined by NMR and are consistent with the data that we had previously published on these trisaccharides (Feng *et al.*, 2005). This lack of regioselectivity depends on the acceptor since, with maltose as acceptor, we obtained only one regioisomer [ $\beta$ -D Galp (1→3)- $\alpha$ -D-Glcp-(1→4)-D-Glcp].

## Conclusion

We have developed a digital imaging screening strategy for the detection of transglycosidase activity directly on *E. coli* colonies. This technique allowed us to screen >10 000 mutants in few hours with minimum handling and low cost material. Parallel selection could be performed on different acceptor sugars (or acceptors) without the need to synthesis labelled substrates. No such technologies have previously been developed for transglycosidase reactions. The only screening systems developed for sugar-transfer reaction were developed for glycosynthases (Mayer *et al.*, 2001; Kim *et al.*, 2004; Lin *et al.*, 2004; Ben-David *et al.*, 2008) and glycosyltransferases (Aharoni *et al.*, 2006). However, some of these methods were based on a two-step procedure and were not

able to distinguish between synthesis and hydrolysis (Ben-David *et al.*, 2008). Others were based on the use of acceptors modified with either a chromophore (Kim *et al.*, 2004), a fluorophore (Aharoni *et al.*, 2006) or a complex reporter molecule (Lin *et al.*, 2004). As a result, screening with non-natural sugar as acceptor can lead in the selection of mutants that are efficient only on these modified acceptors. Our present screening strategy has a much larger applicability on natural sugars and could be easily adapted to very HTS technology, such as double water-in-oil-in-water emulsions (Aharoni *et al.*, 2005).

We also believe that our methodology could be extended to other transferase reactions, transpeptidation and acetylation, which proceed by a hydrolytic enzymatic mechanism involving two steps via a covalent intermediate. This general digital imaging HTS method should therefore enable the evolution of enzymes with new catalytic activities, providing new synthetic routes for complex carbohydrates and glycoconjugates.

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