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Research Paper

# Directed evolution of new glycosynthases from *Agrobacterium* $\beta$ -glucosidase: a general screen to detect enzymes for oligosaccharide synthesis

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## Abstract

**Background:** Oligosaccharide synthesis is becoming increasingly important to industry as diverse therapeutic roles for these molecules are discovered. The chemical synthesis of oligosaccharides on an industrial scale is often prohibitively complex and costly. An alternative, that of enzymatic synthesis, is limited by the difficulty of obtaining an appropriate enzyme. A general screen for enzymes that catalyze the synthesis of the glycosidic bond would enable the identification and engineering of new or improved enzymes.

**Results:** Glycosynthases are nucleophile mutants of retaining glycosidases that efficiently catalyze the synthesis of the glycosidic

linkage by condensing an activated glycosyl fluoride donor with a suitable acceptor sugar. A novel agar plate-based coupled-enzyme screen was developed (using a two-plasmid system) and used to select an improved glycosynthase from a library of mutants.

**Conclusions:** Plate-based coupled-enzyme screens of this type are extremely valuable for identification of functional synthetic enzymes and can be applied to the evolution of a range of glycosyl transferases. © 2001 Published by Elsevier Science Ltd.

**Keywords:** Directed evolution; Enzymatic oligosaccharide synthesis; Glycosynthase; Glycosyl transfer

## 1. Introduction

Oligosaccharides play a range of important roles in biological systems, not least of which is their role in cellular communication, and therefore have great potential as therapeutics [1–3]. This potential has not been realized because the chemical syntheses of oligosaccharides are often laborious [4] and prohibitively expensive to scale up for industrial manufacture. An alternative approach is enzymatic synthesis with glycosyl transferases, but on a large scale this can be hampered by the availability and stability of the requisite enzymes, and by the high cost of the nucleoside diphosphosugar donors [5,6]. Although the number of available transferases is increasing rapidly, and the costs of

donors can be reduced by elegant in situ regeneration protocols [7], there remains a need for other enzymatic approaches, particularly to increase the range of accessible products.

One such approach is to use glycoside hydrolases. Approximately 2500 glycoside hydrolases are known and have been classified into some 80 families by sequence similarity [8–11]. All enzymes in a family cleave the glycosidic bond with the same stereochemical outcome, either retaining or inverting; all have the same structural fold; but they may have different specificities [12,13]. The active sites of retaining glycosidases contain two carboxylic acid residues, one acting as a nucleophile, the other as an acid/base catalyst (Fig. 1A), with the reaction proceeding via a covalent glycosyl-enzyme intermediate [14–18]. Retaining glycosidases can also transglycosylate by transferring the glycone of the glycosyl-enzyme to an acceptor sugar rather than to water (Fig. 1B). In this way they can be used to synthesize glycosides, but the yields are typically low be-

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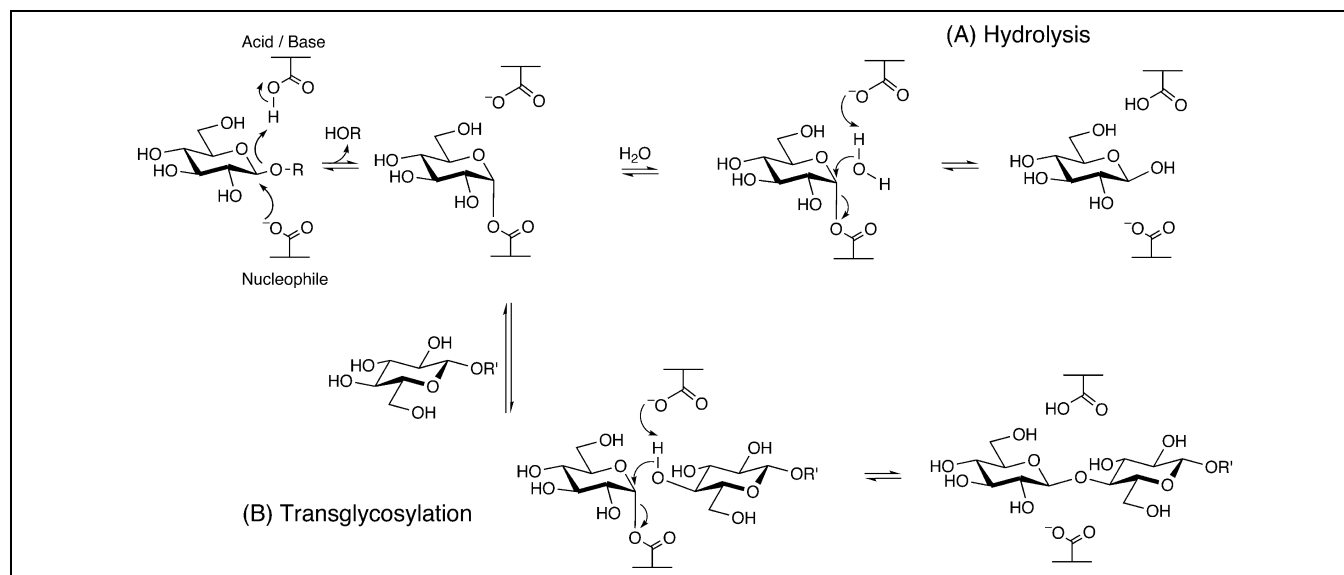


Fig. 1. Mechanisms of retaining glycosidases. (A) A double-displacement hydrolytic mechanism. (B) A transglycosylation mechanism.

cause the product is a substrate for the enzyme and undergoes hydrolysis. Although yields can be improved by the use of non-aqueous solvents or by removal of the product during the reaction, a high-yielding procedure was not generally available until the recent development of specifically mutated glycosidases known as glycosynthases [19]. Mutation of the catalytic nucleophile renders a glycosidase hydrolytically inactive, but the mutant enzyme can transfer an activated donor sugar, in the form of a glycosyl fluoride of the *opposite* anomeric configuration to that of the normal substrate, to a suitable acceptor sugar (Fig. 2). By having the same anomeric configuration as the glycosyl-enzyme intermediate, the glycosyl fluoride mimics the product of the first step of the normal reaction mechanism [19].

Glycosynthases obtained from four enzymes synthesize a range of oligosaccharides in high yields using inexpensive and readily synthesized glycosyl fluorides as glycosyl donors [19–23]. New glycosynthases may be obtained by identifying and mutating promising candidate enzymes, or by directed evolution of existing glycosidases [24–27], but this requires rapid, direct screening methods. Chromogenic and fluorogenic substrates can be used to screen for mutants with altered hydrolytic specificity after DNA shuffling [28] but this is straightforward compared to screening

for the synthesis of a glycosidic linkage because no chromogenic or fluorogenic aglycone is released in the latter case. The screen for glycosynthases described here uses a fluorogenic substrate in conjunction with a second enzyme that releases the fluorophore only from the glycosynthase-derived product (Fig. 3). The method is optimized for *in vivo* screening by using two plasmids: one encoding the screening enzyme, the other the potential glycosynthase gene of interest. The screen was used to detect glycosynthase activity in a library of random mutants of the catalytic nucleophile in *Agrobacterium* sp.  $\beta$ -glucosidase (Abg; EC 3.2.1.21).

## 2. Results and discussion

The screen presented herein depends upon the identification of a coupling enzyme that will cleave only the product of the glycosynthase reaction to release a chromogenic product, but will not cleave the acceptor substrate for the glycosynthase. Fortunately, such enzymes are relatively commonplace for the cleavage of oligosaccharide linkages, in the form of *endo*-glycosidases. For our purposes we needed an *endo*-cellulase, for which we chose Cel5A from *Cellulomonas fimi* (EC 3.2.1.4) which has been shown

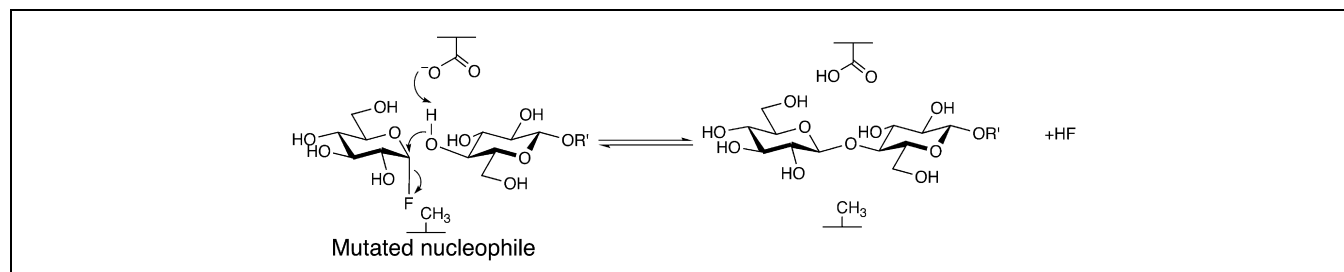


Fig. 2. Proposed mechanism of glycosynthase AbgE358A.

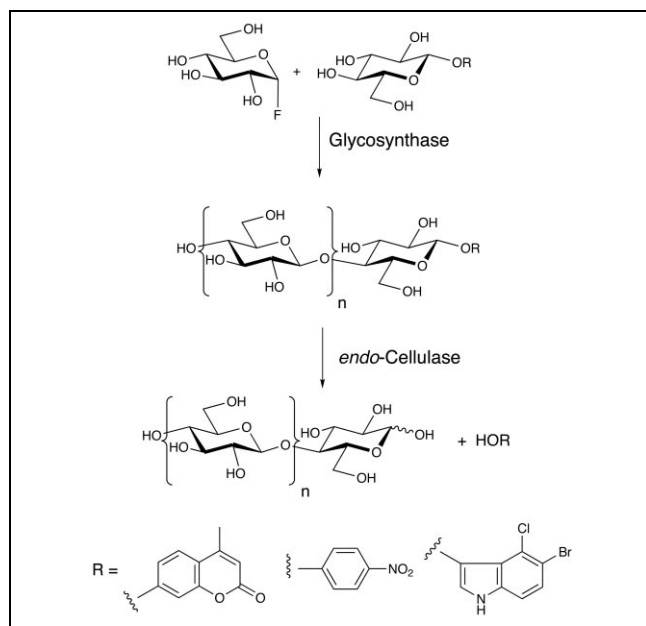


Fig. 3. The chemistry involved in the glycosynthase screen. In this paper, the screen involves the Abg glycosynthase, Cel5A cellulase and  $n=1$  or 2.

previously [29] to have the desired specificity. Thus, our screening project began by sub-cloning the catalytic domain of Cel5A into a protein expression vector to afford large quantities of soluble enzyme.

The Cel5A was first used to analyze *in vitro* Abg glycosynthase reactions performed in the presence of  $\alpha$ -D-glucopyranosyl fluoride (GlcF) and 4-nitrophenyl  $\beta$ -D-glucopyranoside (pNPGlc). TLC analysis of such reaction mixtures revealed an initial build-up of *p*NP-celotrioxide, a product formed by the Abg glycosynthase coupling of two GlcF donor sugars onto pNPGlc. The *p*NP-celotrioxide was then cleaved by Cel5A to release colored *para*-nitrophenolate. The transfer of two donor sugars onto the acceptor sugar prior to the action of the screening enzyme Cel5A is not a prerequisite for a successful screen, but is a consequence of both more rapid catalysis by Abg glycosynthase after addition of one donor sugar and the improved rates of hydrolysis of *p*NP-celotrioxides over *p*NP-cellobiosides by Cel5A (Fig. 3).

Having shown that the assay would work in defined reaction mixtures, it was then necessary to prove its efficacy on plates by lysing bacterial colonies expressing AbgE358S and adding Cel5A, pNPGlc and GlcF. Unfortunately, we were unable to observe glycosynthase activity because of the similar color of LB medium and 4-nitrophenolate. The use of 4-methylumbelliferyl  $\beta$ -D-glucopyranoside (MUGlc) as acceptor, however, was much more successful. Fig. 4A shows a portion of a LB agar plate with four lysed bacterial clones each expressing a known Abg mutant, in the presence of GlcF, MUGlc and purified Cel5A. Within 1 min fluorescence was observed upon addition of Cel5A to a glycosynthase reaction

containing AbgE358S, MUGlc and GlcF. Within a period of 30 min fluorescence was also observed in a similar reaction catalyzed by AbgE358A. Longer incubation times of E358C and E358R with substrates did not reveal any significant sign of glycosynthase activity.

In order to improve the efficiency of the screening process we opted to develop a two-plasmid system whereby the glycosynthase and the coupled enzyme, Cel5A, were co-expressed in the same cell. The two-plasmid screen would be advantageous because it would circumvent the necessity of purifying the Cel5A and of lysing colonies and applying the coupling enzyme. The plasmid pGSVIICel5A was constructed in such a way that both pGSVIICel5A and pET29Abg plasmids could be maintained in the same cell because the vectors' replicons belong to different compatibility groups, and the vectors encode different antibiotic resistances. Plasmid pGSVII was synthesized by restriction digestion of pET29b and pACYC184 and ligation of the expression cassette from the pET vector with the origin of replication and chloramphenicol antibiotic resistance gene of pACYC184.

The Cel5A gene was subsequently sub-cloned into pGSVII to give pGSVIICel5A. The selection and growth of BL21(DE3) cells hosting both plasmids resulted in colonies that expressed both enzymes (data not shown). Initial attempts to repeat the screen on the agar plate gave colonies that were fluorescent but the contrast was very low. The contrast was improved by replica-plating the colonies onto filter paper and then applying GlcF and MUGlc to the colonies. Fig. 4B shows the fluorescence observed from bacterial colonies replica-plated onto filter paper to which substrates were applied. The E358S and E358A expressing colonies once again gave strong fluorescence, and once more no fluorescence was observed on the E358R mutant. However, this time the E358C mutant gave weak fluorescence. TLC analysis of reaction mixtures containing GlcF, pNPGlc and AbgE358C indeed revealed very low glycosynthase activity, below that of the original glycosynthase AbgE358A.

With the two-plasmid screening technology in hand it

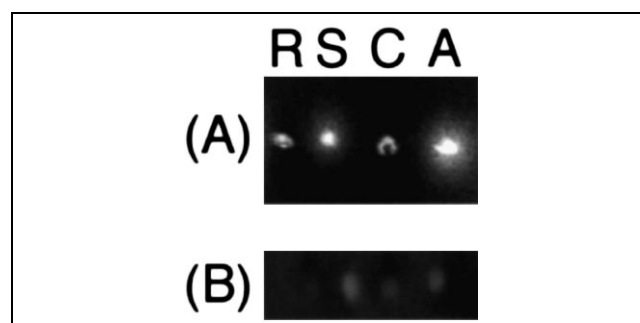


Fig. 4. The screening of defined mutants of Abg for glycosynthase activity. (A) Addition of GlcF, MUGlc and Cel5A purified enzyme to the lysed colonies (after 1 h). (B) Addition of GlcF and MUGlc to colonies expressing known Abg mutants and Cel5A replica-plated onto filter paper (after 1 h).

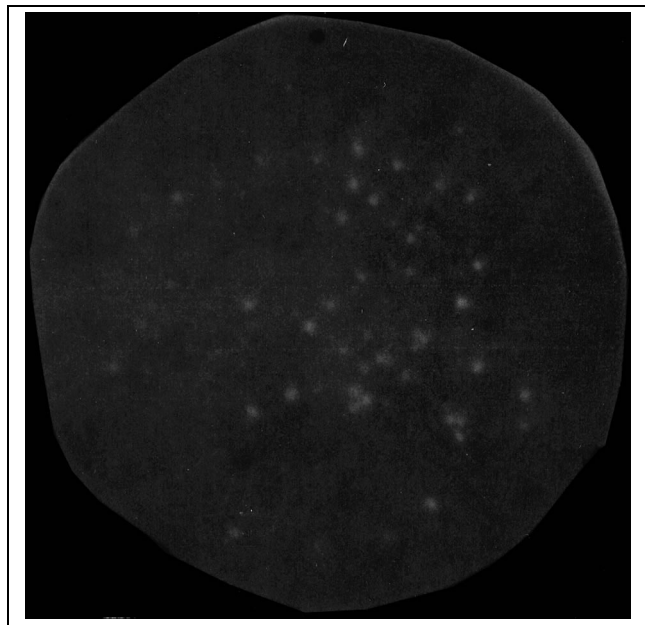


Fig. 5. The screening of a bacterial plate containing colonies expressing the nucleophile library of Abg and Cel5A (pAbgE358X and pGSVIlCe-15A). The colonies were replica-plated onto filter paper and only those colonies not active on X-Glc were incubated with GlcF and MUGlc.

was necessary to validate the approach on a real random library. In order to ensure that mutants of different activity were obtained within a reasonably sized library, saturation mutagenesis was performed at only one position – that of the catalytic nucleophile. This was achieved using a four-primer strategy in which degenerate nucleotides were incorporated at the positions corresponding to the codon for the catalytic nucleophile. The library was then screened for glycosynthase activity. To ensure that the plate was not swamped with fluorescence from wild-type Abg hydrolytic activity, colonies were first plated upon 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucopyranoside (X-Glc). Wild-type Abg hydrolyzes X-Glc, releasing a blue indigo dye. The colonies were then transferred to filter paper and those that did not release the indigo dye were spotted with GlcF and MUGlc. Fluorescence was observed on a number of these non-wild-type colonies (Fig. 5). An initial library of 136 colonies was screened and plasmids purified that encoded the active mutant enzymes. Eight of these plasmids were sequenced and the codons at the nucleophile were TCC $\times$ 3 and TCG (Ser), GCT and GCC $\times$ 2 (Ala), and GGC (Gly). A second library of 200 colonies was screened and plasmids purified that encoded the active mutant enzymes. Ten of these plasmids were sequenced and the codons at the nucleophile were TGC, TGT, TGC and TGT (Cys), GCT $\times$ 3 (Ala), and TCT, TCG, and TCA (Ser). From the rates of fluorescence release observed by screening, E358G appeared to have comparable activity to E358S.

The E358G and E358C enzymes were then over-expressed in BL21(DE3) cells and purified on His-bind resin.

The rates of glycosylation of pNPGlc by  $\alpha$ -D-galactopyranosyl fluoride (GalF) catalyzed by E358G and E358C were determined using a fluoride ion-selective electrode to monitor fluoride release. An acceptor sugar with an axial 4-hydroxyl group does not act as a substrate for glycosynthase reactions catalyzed by Abg mutants and, therefore, the use of GalF as a donor sugar for kinetic studies ensured that the observed release of fluoride corresponded to a single galactosyl transfer. Upon varying GalF concentration at a fixed concentration of pNPGlc (20 mM) standard saturation kinetic behavior was observed for E358G. The apparent kinetic parameters were  $K_m = 118 \pm 23$  mM,  $k_{cat} = 216 \pm 16$  min $^{-1}$  and  $k_{cat}/K_m = 1.8$  min $^{-1}$  mM $^{-1}$ . By contrast, at a fixed concentration of pNPGlc (22 mM), and using E358S as catalyst, the kinetic parameters were  $K_m = 220 \pm 12$  mM,  $k_{cat} = 177 \pm 4$  min $^{-1}$  and  $k_{cat}/K_m = 0.80$  min $^{-1}$  mM $^{-1}$  [20]. The glycine mutant therefore acts as a two-fold better enzyme than the serine mutant, and 50-fold better than the original alanine mutant  $k_{cat}/K_m = 0.034$  min $^{-1}$  mM $^{-1}$ . By contrast, saturation with GalF was not observed with E358C, a value for  $k_{cat}/K_m = 0.018$  min $^{-1}$  mM $^{-1}$  being obtained. Therefore, the second-order rate constant for transglycosylation catalyzed by E358C was two-fold lower than that of the original glycosynthase AbgE358A.

The screen has identified two new glycosynthases from Abg, the E358G and E358C mutant enzymes. In conjunction with our previous work [19,20] there are now four mutants of Abg that function as glycosynthases. These mutant enzymes E358G, E358S, E358A and E358C have relative second-order rate constants of 102:44:2:1 as determined by measurements with pNPGlc and GalF. The discovery of two new glycosynthases, namely E358G and E358C, demonstrates the validity of the *in vivo* screen for detecting new and improved glycosynthases from a library of randomly mutated clones. The mutant E358G is a two-fold improvement on the best glycosynthase mutant, E358S, developed previously from Abg. The broad range of activities that have been detected by the screen shows the sensitivity of the method for enzyme discovery, even though the agar plate assays are not fully quantitative. Quantitation can be readily achieved by picking the most interesting colonies into a 96-well plate and monitoring glycosynthase reactions with a fluorescent plate reader. The further application of the screen to the discovery and evolution of enzymes with new and improved catalytic activities is being actively pursued in our laboratory.

### 3. Significance

Recombinant DNA methodologies for the generation of novel, or improved, enzyme activity rely upon a successful screen to permit selection of improved variants. Conceptually, the screening of enzymes that catalyze hydrolytic reactions is facile because one of the released products is



generally chromogenic. By contrast, the selection of improved enzyme activity for the synthesis of a glycosidic linkage is particularly difficult because no chromophore is released upon enzyme catalysis. The results described here demonstrate the first general screening method for enzymes that catalyze glycosyl transfer. Use of the screen has uncovered an improved glycosynthase E358G from *Agrobacterium*  $\beta$ -glucosidase, which efficiently catalyzes the formation of the glycosidic linkage between two sugars. General screens of this type for glycosidic linkage synthesis will enable facile screening for new glycosyl transfer catalysts that, in turn, will expedite the synthesis of oligosaccharide molecules for new therapeutic applications.

#### 4. Materials and methods

Plasmid-containing strains were grown in Luria–Bertani (LB) broth containing 50  $\mu\text{g ml}^{-1}$  kanamycin (LB<sub>kan</sub>), or kanamycin and 35  $\mu\text{g ml}^{-1}$  chloramphenicol (LB<sub>kan/cam</sub>). All cells were grown at 37°C unless otherwise stated. Expand and *Pwo* DNA polymerases and deoxynucleoside triphosphates were from Roche Boehringer Mannheim. Restriction endonucleases, T4 ligase and pACYC184 plasmid were from New England Biolabs. Electrocompetent *Escherichia coli* Top10 cells and pZero/pCRBlunt cloning kits were from Invitrogen. XL1-Blue cells were from Stratagene. pET expression vector, electrocompetent *E. coli* BL21(DE3) cells and His-bind metal chelation resin were from Novagen. Preparation of oligonucleotide primers and DNA sequencing was performed at the Nucleic Acids and Peptide Service Unit, University of British Columbia. MUGlc and pNPGlc were obtained from Sigma Chemical Company Ltd. GalF and GlcF were synthesized as described previously [19].

##### 4.1. Cloning and purification of *C. fimi* cellulase D (*Cel5A*)

The catalytic domain of cellulase D from *C. fimi* was subcloned into pET29b(+) [30]. The signal sequence and the cellulose binding module were removed in order to produce a cytoplasmic soluble His<sub>6</sub>-tagged fusion protein within *E. coli*.

The following primers were used: 5'-G GAA TTC CAT ATG ACC GGC GAC GAC TGG CTG CAC (forward primer) and 5'-CCC AAG CTT GAC CTG CGA GAT CGA CGT GCC (reverse primer). Underlined are the restriction sites *Nde*I and *Hind*III introduced by the two primers. A 1224-bp fragment was obtained by PCR using 10  $\mu\text{M}$  oligonucleotide primers, 1 mM concentrations of the four deoxynucleoside triphosphates, 5% dimethylsulfoxide and 25 ng of the plasmid pDAM 2-1 [30] in 100  $\mu\text{l}$  DNA polymerase buffer. After heating the mixture to 95°C, the reaction was started by addition of Expand DNA polymerase (5 U). Twenty-five PCR cycles (45 s at 94°C, 30 s at 65°C and 80 s at 72°C) were performed in a thermal cycler (Perkin Elmer, GeneAmp PCR System 2400). The PCR fragment was subcloned into pZero2.0 digested with *Eco*RV and subsequently cloned into pET29b(+) using *Nde*I and *Hind*III restriction sites to

yield pETCel5A. To minimize necessary sequencing, a 698-bp *Bsi*WI/*Sac*II fragment from pDAM 2-1 was swapped into pETCel5A. The sequence of the PCR-amplified gene was confirmed and pETCel5A was further used for production of purified Cel5A protein. An overnight culture of freshly transformed BL21(DE3) cells (10 ml) was used to inoculate 1 l of LB<sub>kan</sub> medium. This culture was grown to an OD<sub>600</sub> of 2 and then cooled to 30°C, induced by adding 50  $\mu\text{M}$  IPTG, and grown overnight. The cell pellet was harvested and resuspended in 30 mM Tris–HCl pH 8.0, containing 5 mM imidazole. The cell suspension was passed twice through a French press at 5°C, centrifuged at 10000  $\times g$  for 30 min and the soluble extract purified by Ni<sup>2+</sup> chelation chromatography. The protein was then dialyzed against 30 mM Tris–HCl pH 8.0 and stored at 4°C.

##### 4.2. Saturation mutagenesis of *Abg*

The DNA encoding the catalytic nucleophile (E358) was mutated by a modified four-primer method to give a PCR product that was ligated into pZero. The pZero plasmid was digested with *Eco*RI and *Xho*I and a 600-bp fragment was purified by Qiaex II gel extraction. The plasmid pET29AbgHis6 [20] was digested and purified by an identical procedure. The two DNA fragments were ligated together and transformed into XL1-Blue cells to give pAbgE358X. The primers used were (A) 5'-GGT CTT CAA GGG CGA ATA TCC, (B) 5'-GAG AAC CTC GAG CCC CTT GGC AAC CCC ATG GTT CC (underlined is the restriction site *Xho*I introduced by the primer), (C) GAC GCC CAT ATT GTA GCA GGC ACC GTT NNN GGT GAT GTA GCA CTC CGG CAG, and (D) AAC GGT GCC TGC TAC AAT ATG GGC, (N = A, C, G, T). A silent mutation (bold) was introduced which removes a *Kas*I/*Nar*I site near the random mutation site (NNN). Primers A and C were used in one PCR while B and D were used in another. The reactions used 8  $\mu\text{M}$  oligonucleotide primers, 1 mM concentrations of the four deoxynucleoside triphosphates, and 30 ng of the plasmid pET29AbgHis6 in 100  $\mu\text{l}$  DNA polymerase buffer with *Pwo* DNA polymerase (5 U). Twenty-five PCR cycles (30 s at 94°C, 30 s at 55°C and 60 s at 72°C) were performed in a thermal cycler. PCR products were purified by Qiaex II gel purification (Qiagen) on a 1% agarose gel stained with ethidium bromide. 2 ng of each quantitated PCR product was combined for a second PCR that used 1 mM concentrations of the four deoxynucleoside triphosphates, in 100  $\mu\text{l}$  DNA polymerase buffer with *Pwo* DNA polymerase (5 U). After five cycles (30 s at 94°C, 30 s at 55°C and 60 s at 72°C) primers A and B were added to a final concentration of 4  $\mu\text{M}$  and the PCR continued for a further 25 cycles.

##### 4.3. Construction of plasmid pGSVII*Cel5A*

The plasmid pACYC (New England Biolabs) was cut with *Psh*AI and *Cla*I. A 2560-bp fragment was purified by Qiaex II gel purification. Plasmid pET29b(+) was cut with *Cla*I and *Sma*I and a 1470-bp fragment purified. The two fragments, *Psh*AI–*Cla*I and *Cla*I–*Sma*I, were ligated together. A plasmid of 4000 bp was isolated from transformed *E. coli* XL1-Blue cells grown on LB<sub>cam</sub>

and named pGSVII. The catalytic domain of Cel5A was cloned into this plasmid using the *Nde*I and *Hind*III restriction sites.

#### 4.4. Preparation of BL21(DE3) pGSVII Cel5A electrocompetent cells

BL21(DE3) cells were transformed with pGSVII Cel5A. A colony was chosen and grown overnight in 50 ml of LB<sub>cam</sub>. An aliquot (5 ml) was transferred to 500 ml fresh LB<sub>cam</sub> until an OD<sub>600</sub> of 0.6. The cells were spun down in a JA10 rotor (Beckman) at 5000 rpm for 10 min at 4°C. The supernatant was discarded and the cells resuspended in autoclaved, ice-cooled water (500 ml). The centrifugation and resuspension was repeated and the cells resuspended in 10% sterile glycerol (10 ml). Aliquots (50 µl) were stored at –78°C until used.

#### 4.5. In vitro screening with purified Cel5A on defined Abg mutants

*E. coli* BL21(DE3) cells with one of the Abg plasmids pET29AbgE538A, C, S, R [20] were grown on LB<sub>kan</sub> agar plates containing IPTG (80 µM). The cells were lysed over chloroform (2 min). Each colony was then incubated with equal volumes of GlcF (1 M) and MUGlc (6 mM) and Cel5A enzyme (3 mg ml<sup>–1</sup>). Fluorescence was detected using a UV transilluminator emitting at 312 nm. Wild-type activity was observed within 1 min and after 20 min fluorescence was observed on glycosynthase-active colonies.

#### 4.6. In vivo screening with pGSVII Cel5A on defined Abg mutants

*E. coli* BL21(DE3) pGSVII Cel5A cells were transformed with one of the Abg plasmids pET29AbgE538A, C, S, R [20] and subsequently grown on LB<sub>kan/cam</sub> agar plates containing IPTG (80 µM). The induced colonies were transferred onto filter paper and incubated with equal volumes of GlcF (1 M) and MUGlc (6 mM).

#### 4.7. In vivo screening with pGSVII Cel5A on pAbgE358X

*E. coli* BL21(DE3) pGSVII Cel5A cells were transformed with pAbg358X and grown overnight on LB<sub>kan/cam</sub> plates. The library was replicated onto LB<sub>kan/cam</sub> plates containing 80 µM IPTG. The induced colonies were grown for 24 h, transferred onto filter paper and incubated with equal volumes of GlcF (1 M) and MUGlc (6 mM).

#### 4.8. Purification of Abg mutant enzymes and transglycosylation kinetics

All enzyme purification and transglycosylation rate determinations were performed as described in [20].

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