

Review

Engineering the functional fitness of transglycosidases and glycosynthases by directed evolution

George Oyamo Osanjo^{1,2,3*}, Wallace Dimbuson Bulimo⁴ and Francis J. Mulaa^{2,4}

¹Department of Pharmacology and Pharmacognosy, University of Nairobi, PO Box 19676-00202 KNH, Nairobi, Kenya.

²Centre for Biotechnology and Bioinformatics, University of Nairobi, PO Box 30197-00100, Nairobi, Kenya.

³Africa Institute of Biomedical Science & Technology (AiBST-Nairobi), PO Box 19676-00202 KNH, Nairobi, Kenya.

⁴Department of Biochemistry, University of Nairobi, P.O. Box 30197 – 00100, Nairobi, Kenya.

Accepted 10th February 2011

The artificial implementation of the Darwinian theory of evolution to create new variants of functional proteins, a process referred to as directed evolution, has acquired many applications in biochemical engineering. Directed evolution is a handy tool in the nascent science of glycobiology, where it is used in the conversion of glycosyl hydrolases into transglycosidases or for improving the transglycosylation behaviour of glycosynthases. This review focuses on recent applications of the directed evolution approach to harness the transglycosidase potential of glycosidases and to enhance the functional fitness of glycosynthases.

Key words: Directed evolution, transglycosidases, glycosidases, glycosynthases, oligosaccharides.

INTRODUCTION

The important role of glycans as key molecules in bio-molecular communication is well established (Dwek, 1996; Varki, 1993; Gabius, 2008). Essential biological recognition information encoded by oligosaccharides includes the human ABO blood group determinants which mediate transfusion rejection reactions (Hakomori, 1999; Takasaki et al., 1978), promotion of neurogenesis (Murrey and Hsieh-Wilson, 2008), mediation of the inflammatory response (Lowe, 2003; Dube and Bertozzi, 2005), cancer metastases (Lau and Dennis, 2008), infection by some species of bacteria (Ruiz-Palacios et al., 2003), viruses (Marionneau et al., 2002) and involvement in the spermatocyte-ovum fusion during fertilization (Rubinstein et al., 2006).

Oligosaccharides consequently have enormous potential as therapeutic or diagnostic agents. Access to oligosaccharides is however, still hampered by challenges in their syntheses. Chemical synthesis of oligosaccharides is a delicate process that requires many steps of protection and de-protection and often results in low yields (Seeberger and Werz, 2007).

Enzymatic approaches to oligosaccharide syntheses are an excellent alternative. In nature, oligosaccharides are synthesized by the glycosyltransferases (GTs). But the laboratory syntheses using GTs are hindered by their poor stability and expensive substrates. Recent advances such as substrate recycling have yielded moderate improvement in costs (Ruffing and Chen, 2006).

Glycosidases (GHs) catalyze the hydrolysis of glycans *in vivo*. Under suitable *in vitro* conditions glycosidases can also synthesize glycosylated compounds through the transfer of glycosyl groups from a suitable donor to an acceptor substrate (Bucke, 1996). Such reactions are termed transglycosylation. The drawback for transglycosylation reactions is that, the product in turn becomes a substrate for the glycosidase and is thus, hydrolysed. Considerable efforts have been exerted by researchers to improve the transglycosylation reaction of glycosidases, while limiting hydrolysis of product. The fruits of the enzyme engineering efforts so far undertaken include, (1) the development of transglycosidases, which are glycosidases that overwhelmingly catalyze the transfer of glycosyl groups at the expense of the hydrolytic reaction (Feng et al., 2005; Osanjo et al., 2007) and (2) the development of glycosynthases, which are glycosidases whose catalytic nucleophile has been replaced by a non acidic amino acid residue. Glycosynthases are therefore,

*Corresponding author. E-mail: gosanjo@uonbi.ac.ke. Tel: +254 721 794 666.

unable to hydrolyse their catalysis products. The first glycosynthases were developed in 1998 (MacKenzie et al., 1998; Malet and Planas, 1998) and the technology has since been extended to a variety of glycosidases (Trincone et al., 2004; Bojarová and Křen, 2009).

This review focuses on the successes and potential of directed evolution as tool for converting glycosidases into transglycosidases and for improving the transglycosylation activity of glycosynthases.

EXPERIMENTAL STRATEGY FOR DIRECTED EVOLUTION OF GLYCOSIDASES AND GLYCOSYNTHASES

Directed evolution can be viewed as an approach for an accelerated modification of protein scaffolds or properties by mimicking Darwinian evolution under laboratory conditions. Like Darwinian evolution, directed evolution requires the generation of diversity in enzyme genotype followed by selecting the genes that express the desired function, that is, those exhibiting functional fitness. Since the approach is random, a large and diverse gene sequence library must be constructed to maximise the chance of obtaining targeted property. Since its first description as a tool for artificially improving protein function (Stemmer, 1994; Arnold, 1998), directed evolution methodology has become a powerful instrument for engineering properties of enzymes.

Whether the experimental goal is to convert glycosidases into transglycosidases or to improve the yield of transglycosylation for glycosynthases, the strategy for directed evolution is largely similar. The experiments are conducted in three phases that comprise; (1) generation of sequence diversity; (2) library screening or selection; (3) a detailed analysis of the new function or property (Figure 1).

Generation of sequence diversity

The initial task for all directed evolution experiments is to generate a diverse genetic sequence of the protein of interest (Arnold et al., 2001). Sufficient genetic diversity in turn translates into a wide protein sequence space and functional variation. Many methods for generation of sequence diversity are available to the bioengineer (Wong et al., 2006). For glycosidases, the most popular method is error prone polymerase chain reaction (epPCR). Creation of the genetic diversity by epPCR relies on the inherent characteristic of *Taq* polymerase to make errors during DNA synthesis. *Taq* polymerase lacks 3'→5' DNA sequence proof reading capability of other DNA polymerases and therefore, cannot rectify misincorporated nucleotides (Lawyer et al., 1993). The frequency of *Taq* polymerase mediated errors is 1 for every 9000 nucleotides and can be adjusted to the desired rate by

altering the reaction conditions (Tindall and Kunkel, 1988). One way of increasing the frequency of *Taq* polymerase generated errors is using imbalanced dNTPs concentration. Another approach is to replace Mg^{2+} , the natural cofactor for DNA polymerases, with Mn^{2+} or by combination of the two approaches. An overzealous use of these methods may result in achieving high number of misincorporated DNA bases. It should be noted that, a high error rate or indeed a high degree of codon randomization is not always desirable. An optimally randomized genetic library is of course required to achieve a large protein sequence space, but maximal codon randomization may diminish rather than heighten the efficiency of gene randomization due to the degenerate nature of the genetic code. For example, to achieve full codon randomization in which the target codon is replaced by NNN, where N is any of the four bases, 4³ or 64 possible codons are available. Three of these are stop codons, while the rest encode the 20 amino acids. Since some amino acids have more codons than others (for example serine has 6 codons to tryptophan's one), higher randomization may result in codon bias. The representation of the amino acid residues in the library can be equilibrated somewhat by using techniques other than epPCR. A useful alternative is saturation mutagenesis, which consists of replacing the amino acid residue at the site of interest with all the different 20 amino acids. Obviously, the disadvantage of saturation mutagenesis is that, it cannot be done over the whole gene as that would be quite labor intensive.

Fortunately, alternative mutation techniques based on oligonucleotide randomization are now well established and accessible so that randomized oligonucleotides carrying the desired codons can be relatively easily obtained from commercial suppliers. As an example to overcome the codon bias discussed earlier, Hughes et al. (2003) developed a randomization procedure based on oligonucleotides, in which a set of primers containing 20 maximum efficiency (MAX) codons are hybridised on the DNA template and PCR is then used for primer extension.

When homologous sequences are available, genetic diversity can further be generated by a recombination technique. Very many recombination techniques have been developed for protein engineering. The more popular among these methods for enzyme engineering are DNA shuffling, staggered extension process (StEP), the incremental truncation for the creation of hybrid enzyme (ITCHY) and SCRATCHY (see further).

DNA shuffling was the earliest gene recombination technique developed for directed evolution of enzymes (Stemmer, 1994). The method involves the creation of a library of chimeric genes from homologous DNA template sequences (Figure 2). The resultant progeny combines beneficial mutations from several parental genes hence; there is gain in the desired quality. Staggered extension process (StEP) on the other hand, is a PCR-based *in vitro* recombination of entire genes in which PCR is

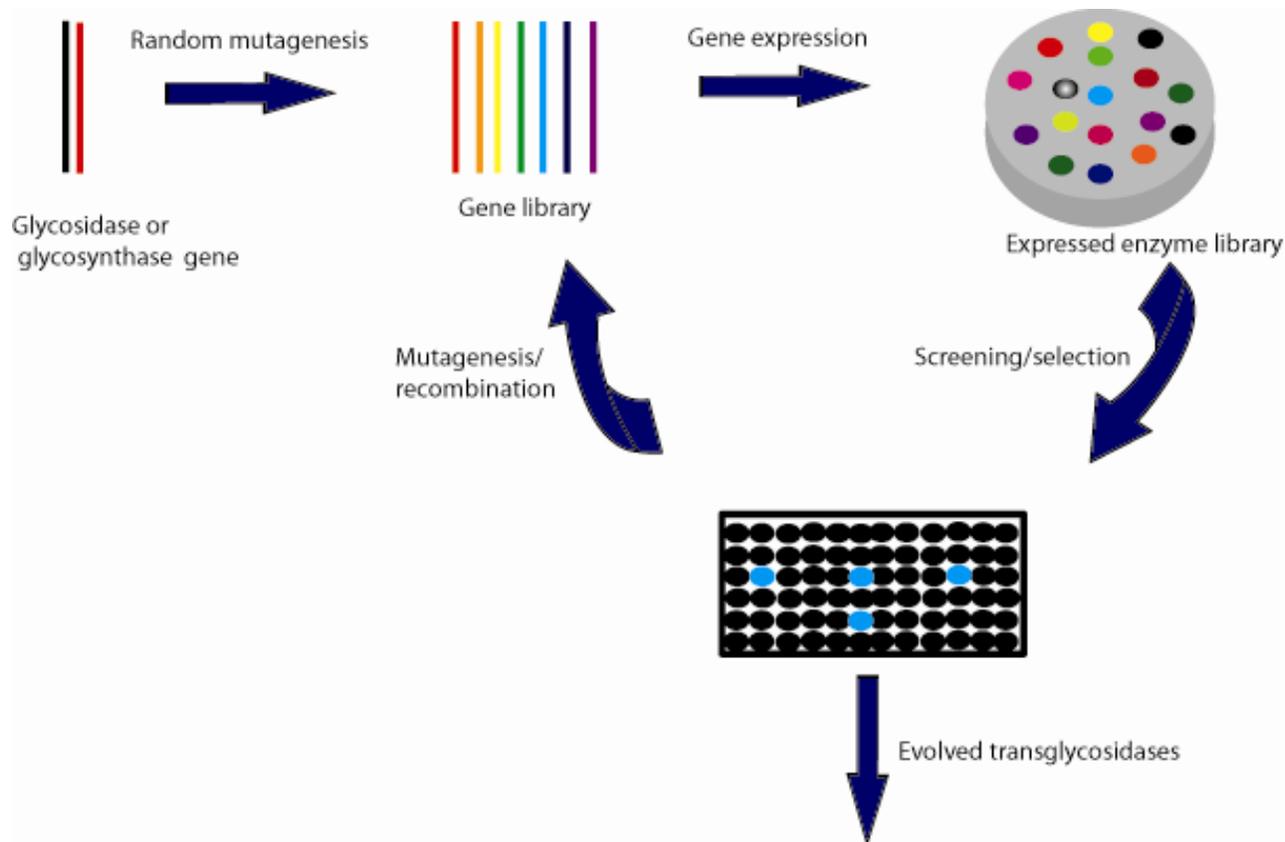


Figure 1. Experimental strategy for directed evolution of transglycosidases. Directed evolution of transglycosidases begins with mutagenesis of glycosidase or glycosynthase genes of interest. The resulting library of mutant genes is expressed to give mutant enzymes which are subsequently screened or selected for the targeted transglycosidase activity. Mutant genes linked to improved activity can be used for the next evolutionary cycle.

performed with very brief renaturation – extension phases (Zhao et al., 1998). The denaturation stage is followed by random annealing of newly synthesized DNA fragments with the template. These fragments are then elongated by partial extension. The process is repeated several times until full-length DNA strands are obtained (Figure 2).

When the goal is to confer a new structural feature on a protein then homology –independent recombination techniques are powerful alternatives. Ostermeier et al. (1999) developed the incremental truncation for the creation of hybrid enzyme (ITCHY) method where libraries are constructed by cloning DNA fragments, from diverse sources, in tandem in a cloning vector (Figure 3). The cloning vector is subsequently linearized and the insert DNA is fragmented by exonuclease digestion. The resultant DNA fragments are used to generate chimeric sequences which are re-ligated into the vector. ITCHY was improved further by adding further recombination steps to develop SCRATCHY (Lutz et al., 2001). SCRATCHY libraries are constructed such that crossover positions are randomly distributed to enhance high recombination frequency.

Screening and selection

Once a library of genetically diverse sequences expressing glycosidase or glycosynthase variants has been constructed, the next phase is to explore the sequence space for proteins fit enough to perform transglycosylation at the targeted level.

This exploration for functional fitness is a key process and is analogous to natural selection in Darwinian evolution (Darwin, 1859). Having generated a library made up of thousands of members, the variant possessing the desired activity must now be found from among largely similar genotypes. For transglycosidases and glycosynthases, this task is often not trivial and is sometimes akin to looking for a needle in a haystack. The task is not rendered easy as the products of transglycosylation are oligosaccharides and as such are neither coloured nor fluorescent and so do not make for easy detection by traditional screening techniques.

Despite the obstacles, the directed evolution process need not perish at selection stage. Various strategies have been devised to surmount the challenges involved in finding the targeted protein variants. They can be

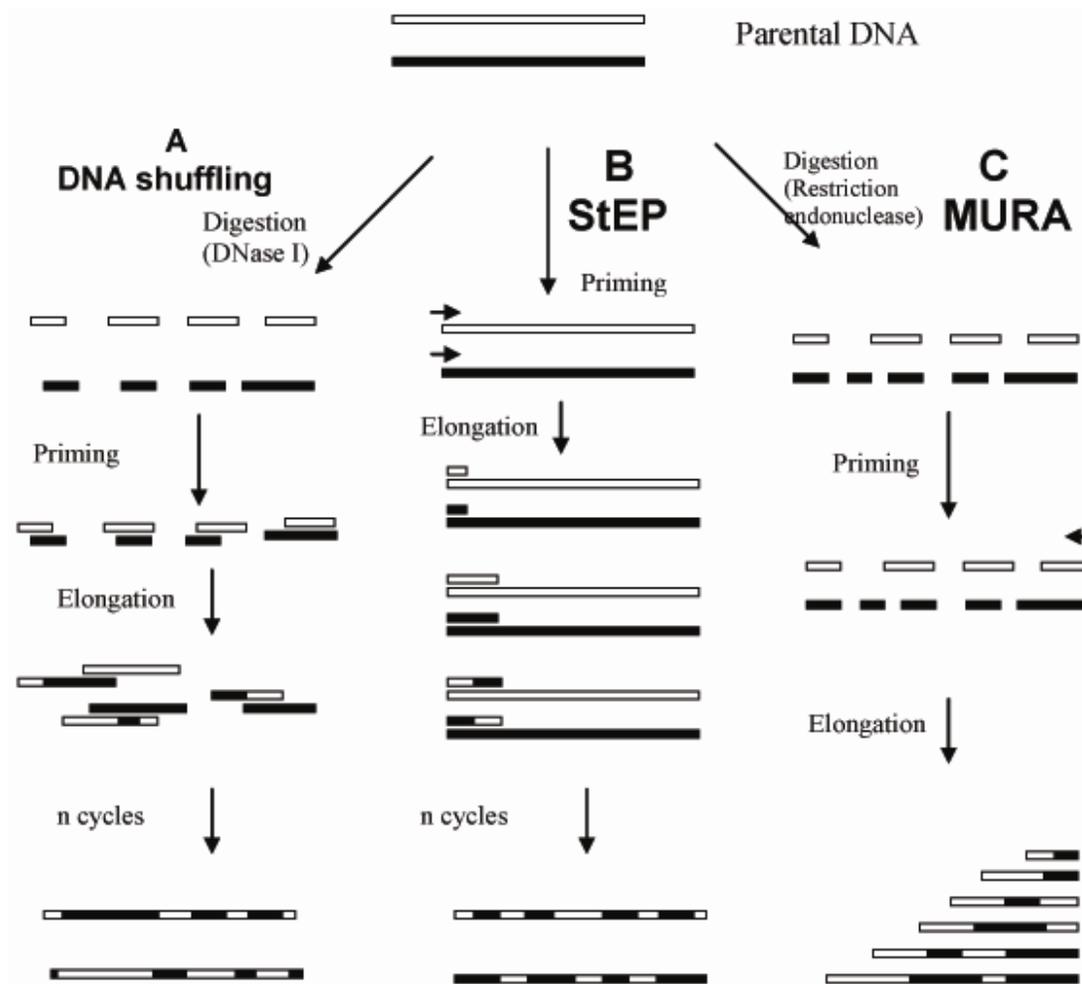


Figure 2. Recombination of DNA sequences by (A) DNA shuffling: DNA fragments are generated from a pool of homologous genes by DNase I then reassembled by PCR to give full-length genes with frequent template switching; (B) StEP: A pool of homologous genes is primed and short fragments produced by brief primer extension enabling template switching; (C) MURA: random DNA fragments are obtained by restriction digestion or PCR then reassembled in the presence of unidirectional primers that contain a restriction site.

grouped into (1) screening methods, that directly detect enzymatic activity or (2) methods for selection, which promote growth of a living cell only when targeted enzymatic activity or functional fitness is present.

An elegant screening methodology has been developed by Kone et al. (2009) who worked on a digital imaging strategy for high-throughput screening of evolved transglycosidase activity. The strategy consists of detecting activation of mutant glycosidases expressed in *Escherichia coli* cells by acceptor substrates. The transglycosylation donor is X-glycoside substrates, where X is a chromogenic aglycone, that turns blue when cleaved to release a free X, while the glycosyl group is transferred to the acceptor. The group X can also be released via hydrolysis by glycosidases. The screen can discriminate enzyme variants possessing both low hydrolytic activity and high transglycosylation activity because in the absence

of an acceptor substrate (at time T_0) transglycosidases with negligible hydrolytic activity fail to hydrolyse the X-glycoside and remain white. On the addition of an acceptor (at time T_1), the donor substrate will be cleaved and the *E. coli* colonies expressing variants with high transglycosidase activity will turn blue at a faster rate than those with lower transferase activity. By determining, the transferase and hydrolysis (T/H) rate ratio for each colony, the authors were able to rapidly screen a library of 10, 000 *Thermus thermophilus* β -glycosidase mutants.

In contrast, Ben-David et al. (2008) developed a high-throughput screening methodology for β -glycosynthases that depends on the detection of hydrofluoric acid, a by-product of β -glycosynthase catalyzed reactions. The procedure was carried out over two steps: In a primary assay about 600 colonies were lifted onto a filter membrane which was then soaked in glycosyl fluoride

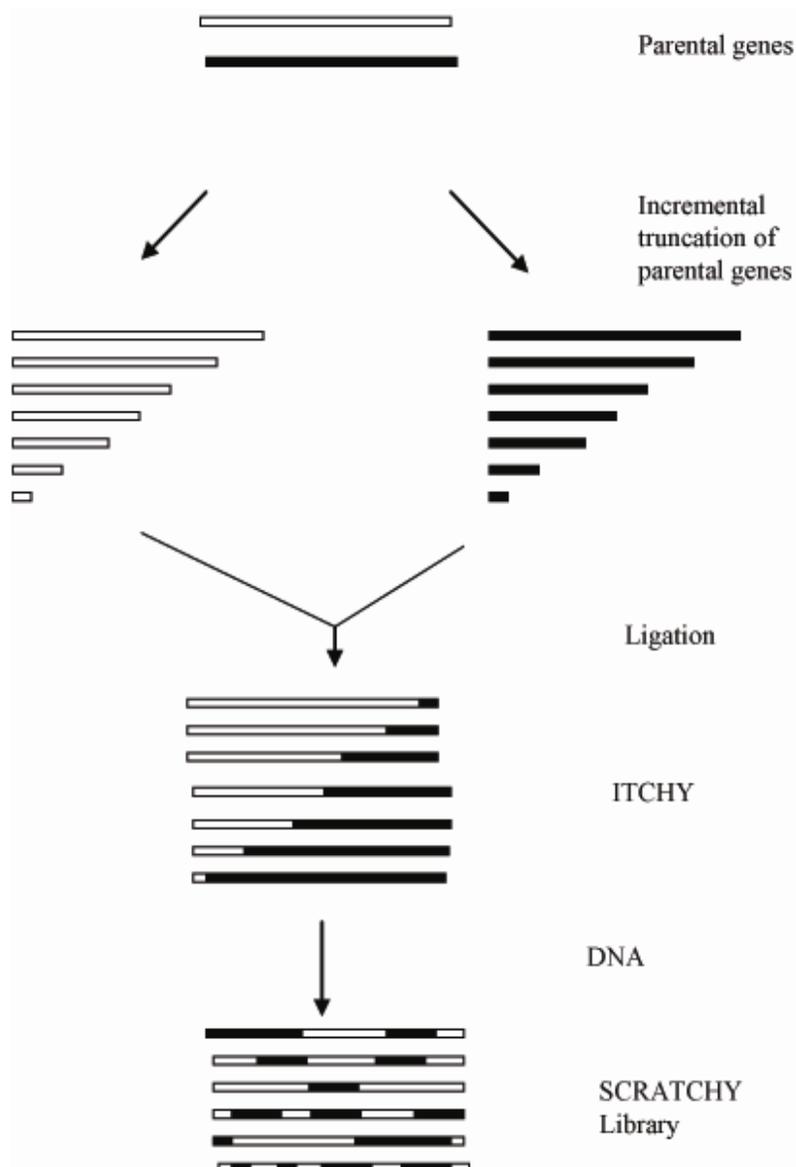


Figure 3. ITCHY and SCRATCHY combinatorial protein engineering. Parental genes are digested by exonuclease III and small aliquots are removed at intervals. The DNA fragments are ligated to form a library of gene fusions. DNA shuffling of ITCHY library gives SCRATCHY library.

substrate and methyl red indicator. Active colonies that turned red were subjected to a secondary quantitative assay that monitored absorbance of protonated methyl red over time in microtitre plates. Mutants associated with high absorption rates were further analyzed. As many as 10,000 mutants of *Geobacillus stearothermophilus* β -xylosynthase could be processed by the authors in a day using the primary assay.

Other screening methodologies have required modifying the acceptor substrate itself to carry a fluorogenic or chromogenic moiety. For example, Withers and colleagues (Mayer et al., 2001; Kim et al., 2004) developed an agar-based screen, in which the cleavage

of a fluorophore from the product of a glycosynthase reaction by another enzyme co-expressed with the mutant glycosynthase in *E. coli* gave fluorescent colonies.

Selection methods obviate the use of the chromogenic or fluorogenic substrates by linking the active enzyme variant to the growth of the cell expressing it. Thus, Lin et al. (2004) developed a chemical complementation technology for rapid selection of Cel7B glycosynthase from *Humicola insolens*. The method utilized a yeast three hybrid strategy in which the acceptor for the enzyme was conjugated to dexamethasone, while the glycosyl donor was coupled to methotrexate. Glycosynthase activity

creates a glycosyl bridge between methotrexate and dexamethasone thus, triggering the transcriptional activation of a LEU2 reporter gene. In the absence of leucine, cells expressing glycosynthase activity have a growth advantage and are selected for.

Examples of functionally fit transglycosidases and glycosynthases developed through directed evolution

Transgalactosidases

Among the early reports on the improvement of the transglycosylation activity of glycosidases by directed evolution was the work by Tellier and colleagues (Dion et al., 2001a,b). The researchers were interested in modifying the regioselectivity of the *Bacillus stearothermophilus* thermostable α -galactosidase AgaB. Native AgaB catalyses the synthesis of galactosides with α (1 \rightarrow 6) linkages as the major product. Following one round of epPCR and StEP, mutants and recombinants were selected based on elimination of the α (1 \rightarrow 6) regioselectivity. The workers developed an enzyme variant that possessed a novel α (1 \rightarrow 2) regioselectivity, commonly found in glycotopes that mediate acute kidney transplant rejection. The gain in transferase activity by the evolved enzyme was 12%.

Amylosucrases and glucansucrases

The amylosucrases and glucansucrases are transglycosidases that form amylose like polymers from sucrose. The group of Remaud-Simeon has studied amylosucrases from different microorganisms with the goal of increasing their transglycosylation yield and thermostability. Accordingly the researchers subjected the *Neisseria polysaccharea* amylosucrase (AS) to directed evolution using epPCR and DNA shuffling, with gene fragmentation accomplished by restriction enzymes. A variant library of 50,000 clones was obtained (van der Veen et al., 2004, 2006). Clones expressing active amylosucrase were identified, selected and screened to determine transglycosylation efficiency using iodine staining of the amylose formed. Variant enzymes with 60% higher activity than wildtype (Asn387Asp mutant) and improved oligosaccharide polymerization (Glu227Gly mutant) were generated. To increase its thermostability, the enzyme was subjected to epPCR and *in vivo* selection with sucrose as the sole carbon source. 4500 colonies were selected from an original epPCR library of 30,000 transformants. Subsequent screening retrieved an evolved enzyme (R20C/A451T double mutant) that had not only 10 times higher thermostability, but also showed better yield for malto-oligosaccharides and amylose at high concentrations of sucrose compared with the wild-type enzyme (Edmond et al., 2008).

Cyclodextrin glucanotransferases (CGTases)

Cyclodextrin glucanotransferases are transglycosidases that convert starch into α (1 \rightarrow 4) linked-circular oligosaccharides (cyclodextrins). Industrially, important cyclodextrins (CDs) include α -, β - and γ -CDs composed of 6, 7 and 8 glycosyl units, respectively. The CDs are used in the pharmaceutical industry as drug delivery vehicles; in the perfume industry as slow releasers of deodorants; in the chemical separation industry for resolution of enantiomers or purification of cholesterol compounds among other applications (Martin Del Valle, 2009).

Use of CGTases is sometimes compromised by other side reactions. Notably, apart from cyclodextrin formation, CGTases can: (1) Cleave starch into short oligosaccharides through amylase activity, (2) form linear oligosaccharides through disproportionation activity and (3) cleave CDs and join them to other oligosaccharides to yield linear products through coupling activity. To increase the transglycosylation/hydrolysis ratio of a CGTase, Kelly et al. (2008) sought to reduce the amylase activity of Tabium *Thermoanaerobacterium thermosulfurigenes* cyclodextrin glucanotransferase (Tabium CGTase) by directed evolution. The researchers first generated sequence diversity of the *cgt* gene by epPCR. A library of 12,000 members was screened for loss of hydrolysis in a microtitre plate assay. CD production was then, detected by addition of phenolphthalein. The process enabled the researchers to isolate a Tabium CGTase S77P mutant with 15-fold reduction in hydrolysis activity, while cyclization activity was retained.

Transfucosidases

Fucosylated oligosaccharides are components of the human ABO blood group antigens, besides playing other roles in innate immunity and neurogenesis (Murrey and Hsieh-Wilson, 2008). At our laboratory, we converted the *Thermotoga maritima* α -L-fucosidase (Tm α -fuc) into an α -L-transfucosidase. Native Tm α -fuc can transfer fucoside units to pNP-galactoside acceptors to form the disaccharide fucosyl galactoside (Fuc- α -(1 \rightarrow 2)- pNP-Gal) at a yield of 7.0%. Following a cycle of epPCR and two recombination steps, an enzyme variant that synthesised the disaccharide Fuc - α -(1 \rightarrow 2)- pNP-Gal at 60% yield was obtained. Furthermore the transferase/hydrolysis ratio of the evolved enzyme was 30 times higher than the native enzyme (Osanjo et al., 2007). Remarkably, only three mutations (T264A, Y267F and L322P) were found to be responsible for this drastic change of activity.

Transglycosidases

Feng et al (2005) developed a β - transglycosidase from the *Thermus thermophilus* β -glycosidase (Tt β gly) for synthesis of β -glycans. Wildtype Tt β gly can transfer β -glycoside

donors to disaccharide acceptors such as cellobiose and maltose with 8% yield. Following one cycle of random mutagenesis, 5000 mutants were first subjected to an agar based screen containing X- β -Gal to detect recombinants with a loss of hydrolytic activity. In a second screening step, enzyme extracts were used in condensation reactions with *O*-nitrophenyl galactoside (*o*NP-Gal) as donor and maltose as acceptor and the reactions products were analysed by TLC and capillary electrophoresis; followed by recombination of the best mutants. The evolved enzymes, carrying F401S and N282T mutations, produced yields of up to 60 and 75% for glycosyl maltosides or cellobiosides, respectively.

Glycosynthases

The development of glycosynthases was a milestone in the synthesis of oligosaccharides using glycosidases. The applications of glycosynthases, nonetheless still face some challenges with substrate specificity, regioselectivity and optimal yields. Directed evolution has been applied to obtain improved variants.

The *Agrobacterium* sp. Abg β glycosynthase, the first glycosynthase to be developed (MacKenzie et al., 1998), had low activity and narrow substrate range with notably low transglycosylation rates when using xylosyl fluoride donor. One round of random mutagenesis with epPCR of the *Abg* glycosynthase gene followed by screening of 10,000 clones yielded an enzyme variant 1D12, carrying the mutation A19T with 3.5 fold increase in activity. This mutant was subjected to a new round of mutagenesis which yielded a new variant, 2F6, with two additional mutations (Q248R and M407V) having an overall 27 fold increase in transfer of xylosyl units relative to the starting glycosynthase (Kim et al., 2004).

Improvement of xylosynthase transfer through directed evolution was also recently undertaken by Ben-David et al. (2008). Screening over 10,000 clones, after the first round of random mutagenesis of the *G. stearothermophilus* xylosynthase XynB2(E335G) using new screening assay based on detection of the release of hydrofluoric acid on agar (*vide supra*) yielded a xylosynthase variant, 27I, that carried four amino acid substitutions: F206L, I211T, C253R and N342K, with 8.6 fold higher transxylosidation activity compared with the parental enzyme. The 27I mutant was further mutagenized in a second round of epPCR and after a screen of 11,000 clones, a new mutant, 29II, carrying six additional amino acid substitutions (V123E, A282T, R291H, T343P, D470G and D597G) having 35 fold increment in activity over the original xylosynthase, was obtained.

In another example, Lin et al. (2004) used oligonucleotide directed mutagenesis to generate a library of *H. insolens* endoglucanase glycosynthase (Cel7B). Cel7B is an endo-acting glycosidase that catalyzes hydrolysis of $\beta(1\rightarrow4)$ glycosidic linkages in cellulose with retention of

anomeric configuration. The authors selected the position Glu197 for saturation mutagenesis, obtaining more than 106 transformants per μ g of DNA. The transformants expressing active enzymes were selected through chemical complementation as already described earlier, resulting in an E197S Cel7B variant with a 5-fold improvement in activity.

In a different strategy, the *endo*- β -*N*-acetylglucosaminidase glycosynthase from *Mucor hiemalis* (Endo-M), a family 85 glycoside hydrolase, was subjected to saturating mutagenesis at the key asparagine 175 position. All possible amino acid residues, except proline, were tested yielding an N175Q mutant whose transglycosylation activity was significantly higher than the original glycosynthase and wild type enzyme (Umekawa et al., 2008; Umekawa et al., 2010).

As apparent from the earlier discussion, the glycosynthase technology has been predominantly used with the retaining β -glycosidases. So far, the glycosynthase approach has only been extended to five α -glycosidases (Okuyama et al., 2002; Honda and Kitaoka, 2006; Wada et al., 2008; Cobucci-Ponzano et al., 2009). Directed evolution has not yet been used to extend the performance of these α -glycosynthases but it could be a complimentary approach to increase the transglycosylation yields and thus, extend the limits of the α -glycosynthase technology.

Finally, through an elegant combination of the glycosynthase technology, directed evolution and rational design, biocatalysts were tailor made for synthesis of glycosphingolipids (Hancock et al., 2009). The *endo*-glycoceramidase II glycosynthase from *Rhodococcus* sp. (EGC glycosynthase), was initially generated by replacing E351 catalytic nucleophile with a seryl residue (Vaughan et al., 2006). The EGC glycosynthase however, possessed low activity and narrow acceptor specificity. Two epPCR libraries of the EGC glycosynthase gene were generated and screened via ELISA to detect sphingolipid synthesis. 32 mutants (from 10,000 colonies) were shown to have increased glycosphingolipid synthesis from a GM1-oligosaccharyl α -fluoride donor and microplate-bound lipid acceptor substrates. Furthermore, the D314Y mutant had much higher affinity for some substrates and broader substrate range than the parent glycosynthase, thus, showing the power of directed evolution.

CONCLUSIONS

The glycosyl hydrolases are evolvable into transglycosidases. Moreover, the desired specificity, stability and regioselectivity characteristics of the enzyme can be achieved by having a sufficiently high molecular diversity library and applying an efficient screening strategy. A big challenge that remains to be overcome is the elimination of hydrolysis of the product. Rational design engineering approaches have overcome this limitation by providing

the glycosynthases, which lack the catalytic nucleophile. Nevertheless, rational design approach by itself does not still provide optimally robust transglycosidases. The reason for this is the immense size of the protein sequence space, which even with the currently considerable computation power remain a challenge to fully explore.

The use of a combination of directed evolution and rational design is therefore, an important strategy to support the development of the transglycosidases, whose uses are rapidly expanding with the growth of the agricultural, food, chemical and pharmaceutical industries.

Acknowledgements

F.J.M. and G.O.O. acknowledge support from the European Union through the LipoYeasts project grant (No. 213068).

REFERENCES

- Arnold FH (1998). Design by directed evolution. *Accounts Chem. Res.* 31(3): 125-131.
- Arnold FH, Wintrode PL, Miyazaki K, Gershenson A (2001). How enzymes adapt: lessons from directed evolution. *Trends Biochem. Sci.* 26: 100-106.
- Ben-David A, Shoham G, Shoham Y (2008). A universal screening assay for glycosynthases: directed evolution of glycosynthase XynB2(E335G) suggests a general path to enhance activity. *Chem. Biol.* 15: 546-551.
- Bojarová P, Křen V (2009). Glycosidases: a key to tailored carbohydrates. *Trends Biotechnol.* 27: 199-209.
- Bucke C (1996). Oligosaccharide Synthesis Using Glycosidases. *J. Chem. Tech. Biotechnol.* 67: 217-220.
- Cobucci-Ponzano B, Conte F, Bedini E, Corsaro MM, Parrilli M, Sulzenbacher G, Lipski A, Dal Piaz F, Lepore L, Rossi M, Moracci M (2009). β -Glycosyl Azides as Substrates for α -Glycosynthases: Preparation of Efficient α -L-Fucosynthases *Chem. Biol.* 16: 1097-1108.
- Darwin C (1859). *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life.* John Murray. London.
- Dion M, Nisole A, Spangenberg P, André C, Glottin-Fleury A, Mattes R, Tellier C, Rabiller C (2001a). Modification of the regioselectivity of a *Bacillus* α -galactosidase by directed evolution. *Glycoconjugate J.* 18: 215-223.
- Dion M, Osanjo G, Andre C, Spangenberg C, Rabiller C, Tellier C (2001b). Identification by saturation mutagenesis of a single residue involved in the α -galactosidase AgaB regioselectivity. *Glycoconjugate J.* 18: 457-64.
- Dube DH, Bertozzi CR (2005). Glycans in cancer and inflammation-potential for therapeutics and diagnostics. *Nat. Rev. Drug Disc.* 4: 477-488.
- Dwek RA (1996). Glycobiology: toward understanding the function of sugars. *Chem. Rev.* 96: 683-720.
- Edmond S, André I, Jaziri K, Potocki-Véronèse G, Mondon P, Bouayadi K, Kharrat H, Monsan P, Remaud-Simeon M (2008). Combinatorial engineering to enhance thermostability of amylosucrase. *Prot. Sc.* 17: 967-976.
- Feng HY, Drone J, Hoffmann L, Tran V, Tellier C, Rabiller C, Dion M (2005). Converting a β -glycosidase into a β -transglycosidase by directed evolution. *J. Biol. Chem.* 280(44): 37088-37097.
- Gabius HJ (2008). Glycans: bioactive signals decoded by lectins. *Biochem. Soc. Trans.* 36: 1491-1496.
- Hakomori S (1999). Antigen structure and genetic basis of histo-blood groups A, B and O: their changes associated with human cancer. *Bioch. Biophys. Acta.* 1473: 247-266.
- Hancock SM, Rich JR, Caines MEC, Strynadka NCJ, Withers SG (2009). Designer enzymes for glycosphingolipid synthesis by directed evolution. *Nat. Chem. Biol.* 5(7): 508-514.
- Honda Y, Kitaoka M (2006). The first glycosynthase derived from an inverting glycoside hydrolase. *J. Biol. Chem.* 281: 1426-1431.
- Hughes DM, Nagel DA, Santos AF, Sutherland AJ, Hine AV (2003). Removing the redundancy from randomised gene libraries. *J. Mol. Biol.* 331: 973-979.
- Kelly RM, Leemhuis H, Rozeboom HJ, van Oosterwijk N, Dijkstra BW, Dijkhuizen L (2008). Elimination of competing hydrolysis and coupling side reactions of a cyclodextrin glucanotransferase by directed evolution. *Biochem. J.* 413: 517-525.
- Kim YW, Lee SS, Warren RA, Withers SG (2004). Directed evolution of a glycosynthase from *Agrobacterium* sp. increases its catalytic activity dramatically and expands its substrate repertoire. *J. Biol. Chem.* 279: 42787-42793.
- Kone FM, Le Behec M, Sine JP, Dion M, Tellier C (2009). Digital screening methodology for the directed evolution of transglycosidases. *Protein Eng. Des. Sel.* 22: 37-44.
- Lau KS, Dennis JW (2008). N-Glycans in cancer progression. *Glycobiology.* 18: 750-760.
- Lawyer FC, Stoffel S, Saiki RK, Chang SY, Landre PA, Abramson RD, Gelfand DH (1993). High-level expression, purification, and enzymatic characterization of full-length *Thermus aquaticus* DNA polymerase and a truncated form deficient in 5' to 3' exonuclease activity. *PCR Methods Appl.* 2(4): 275-287.
- Lin H, Tao H, Cornish VW (2004). Directed evolution of a glycosynthase via chemical complementation. *J. Am. Chem. Soc.* 126: 15051-15059.
- Lowe JB (2003). Glycan-dependent leukocyte adhesion and recruitment in inflammation. *Curr. Opin. Cell. Biol.* 15(5): 531-538.
- Lutz S, Ostermeier M, Moore GL, Maranas CD, Benkovic SJ (2001). Creating multiple-crossover DNA libraries independent of sequence identity. *Proc. Natl. Acad. Sci. USA.* 98(20): 11248-11253.
- MacKenzie LF, Wang QP, Warren RAJ, Withers SG (1998). Glycosynthases: mutant glycosidases for oligosaccharide synthesis. *J. Am. Chem. Soc.* 120: 5583-5584.
- Malet C, Planas A (1998). From β -glucanase to β -glucansynthase: glycosyl transfer to α -glycosyl fluorides catalyzed by a mutant endoglucanase lacking its catalytic nucleophile. *FEBS Lett.* 440: 208-212.
- Marionneau S, Ruvoën N, Le Moullac-Vaidye B, Clement M, Cailleau-Thomas A, Ruiz-Palacios G, Huang P, Jiang X, Le Pendu J (2002). Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals. *Gastroenterology.* 122(7): 1967-1977.
- Martin Del Valle EMM (2009). Cyclodextrins and their uses: a review. *Process Biochem.* 39: 1033-1046.
- Mayer C, Jakeman DL, Mah M, Karjala G, Gal L, Warren RA, Withers SG (2001). Directed evolution of new glycosynthases from *Agrobacterium* beta-glucosidase: a general screen to detect enzymes for oligosaccharide synthesis. *Chem. Biol.* 8(5): 437-443.
- Murrey HE, Hsieh-Wilson LC (2008). The chemical neurobiology of carbohydrates. *Chem. Rev.* 108: 1708-1731.
- Okuyama M, Mori H, Watanabe K, Kimura A, Chiba S (2002). α -glucosidase mutant catalyzes α -glycosynthase-type reaction. *Biosci. Biotechnol. Biochem.* 66: 928-933.
- Osanjo G, Drone J, Solleux C, Tran V, Rabiller C, Dion M, Tellier C (2007). Directed evolution of the α -L-fucosidase into an α -L-transfucosidase for synthesis of oligosaccharides. *Biochemistry.* 46: 1022-1033.
- Ostermeier M, Nixon AE, Shim JH, Benkovic SJ (1999). Combinatorial protein engineering by incremental truncation. *Proc. Natl. Acad. Sci. USA.* 96(7): 3562-3567.
- Rubinstein E, Ziyat A, Wolf JP, Le Naour F, Boucheix C (2006). The molecular players of sperm-egg fusion in mammals. *Semin. Cell. Dev. Biol.* 17: 254-263.
- Ruffing A, Chen RR (2006). Metabolic engineering of microbes for oligosaccharide and polysaccharide synthesis. *Microb. Cell Fact.* 5: p. 25.
- Ruiz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B,

- Newburg DS (2003). Campylobacter jejuni Binds Intestinal H(O) Antigen (Fuc1,2Gal1,4GlcNAc), and Fucosyloligosaccharides of Human Milk Inhibit Its Binding and Infection. *J. Biol. Chem.* 278: 14112-14120.
- Seeberger PH, Werz DB (2007). Synthesis and medical applications of oligosaccharides. *Nature*, 446: 1046-1051.
- Stemmer WPC (1994). DNA Shuffling by random fragmentation and reassembly: *in vitro* recombination for molecular evolution. *Proc. Natl. Acad. Sci. USA*, 9(22): 10747-10751.
- Takasaki S, Yameshita K, Kobata A (1978). The sugar chain structures of ABO blood group active glycoproteins obtained from human erythrocyte membranes. *J. Biol. Chem.* 253: 6086-6089.
- Tindall KR, Kunkel TA (1988). Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry*, 27(16): 6008-6013.
- Trincone A, Rossi M, Moracci M (2004). Oligosaccharide synthesis by glycosynthases. *Trends Biotechnol.* 22: 31-37.
- Umekawa M, Huang W, Li B, Fujita K, Ashida H, Wang LX, Yamamoto K (2008). Mutants of *Mucor hiemalis* endo-beta-N-acetylglucosaminidase show enhanced transglycosylation and glycosynthase-like activities. *J. Biol. Chem.* 283: 4469-4479.
- Umekawa M, Li C, Higashiyama T, Huang W, Ashida H, Yamamoto K, Wang L-X (2010). Efficient glycosynthase mutant derived from *Mucor hiemalis* endo-beta-N-acetylglucosaminidase capable of transferring oligosaccharide from both sugar oxazoline and natural N-glycan. *J. Biol. Chem.* 285: 511-521.
- Varki A (1993). Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology*, 3: 97-130.
- Vaughan MD, Johnson K, DeFrees S, Tang X, Warren RA, Withers SG (2006). Glycosynthase-mediated synthesis of glycosphingolipids. *J. Am. Chem. Soc.* 128: 6300-6301.
- van der Veen BA, Potocki-Véronèse G, Albenne C, Joucla G, Monsan P, Remaud-Simeon M (2004). Combinatorial engineering to enhance amylosucrase performance: Construction, selection, and screening of variant libraries for increased activity. *FEBS Lett.* 560: 91-97.
- van der Veen BA, Skov LK, Potocki-Véronèse G, Gajhede M, Monsan P, Remaud-Simeon M (2006). Increased amylosucrase activity and specificity and identification of regions important for activity, specificity and stability through molecular evolution. *FEBS J.* 273: 673-681.
- Wada J, Honda Y, Nagae M, Kato R, Wakatsuki S, Katayama T, Taniguchi H, Kumagai H, Kitaoka M, Yamamoto K (2008). 1,2- α -L-Fucosynthase: a glycosynthase derived from an inverting alpha-glycosidase with an unusual reaction mechanism. *FEBS Lett.* 582: 3739-3743.
- Wong TS, Zhurina D, Schwaneberg U (2006). The Diversity Challenge in Directed Protein Evolution. *Comb. Chem. High T. Scr.* 9: 271-288.
- Zhao H, Giver L, Shao Z, Affholter JA, Arnold FH (1998). Molecular evolution by staggered extension process (StEP) *in vitro* recombination. *Nat. Biotechnol.* 16(3): 258-61.