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Point of VIEW

Fermenting Next Generation Glycosylated Therapeutics

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ABSTRACT Mutants of glycosyltransferases and related sugar nucleotide biosynthetic enzymes have been essential for *in vitro* glycorandomization to create libraries of novel glycosylated natural products and derivatives. These diverse glycorandomized compounds can now be produced *in vivo* economically by fermenting engineered *Escherichia coli* cells that express enzyme mutants.

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Carbohydrate-containing structures play important roles in biological and physiological processes. For example, many natural products have carbohydrate moieties that contribute to their bioactivities. In addition, glycosylation of natural products can generate novel products with enhanced or altered pharmacological and pharmacokinetic properties. Mutants of natural product glycosyltransferases and related sugar-nucleotide biosynthetic enzymes with promiscuous substrate specificities have been generated and used for creating diverse glycosylated natural products by *in vitro* glycorandomization and neoglycorandomization. A recent paper by Thorson *et al.* (*1*) reveals that *in vitro* natural product glycorandomization systems can be readily transformed into *in vivo* systems for economical production of novel glycosylated natural products by fermenting engineered *Escherichia coli* cells. This opens the door for facile access to diverse novel glycosides as potential therapeutics with altered or improved activities.

Glycosyltransferases (GlyTs) are a class of important enzymes involved in the formation of glycosidic bonds in nature. They catalyze the transfer of monosaccharides from activated sugar nucleotides (glycosyltransferase donors) to suitable glycosyltransferase acceptors (ROH), which can be glycans, lipids, proteins, glycoconjugates, or natural products. Applying glycosyltransferases in the enzymatic or chemoenzymatic synthesis of complex carbohydrates and glycoconjugates including glycosylated natural products is limited by the lack of highly active glycosyltransferases that can tolerate diverse donor and acceptor substrates. A number of glycosyltransferases, mainly those from bacterial origin, have been successfully expressed in common *E. coli* expression hosts as soluble active recombinant proteins. Nevertheless, the tolerance of substrate modification by wild-type glycosyltransferases has limits. Therefore, it is necessary to create glycosyltransferase mutants to satisfy the preparative and large-scale enzymatic and chemoenzymatic syntheses of diverse carbohydrate-containing molecules, especially those with natural and non-natural modifications on the carbohydrate units.

Different strategies have been developed for generating and identifying glycosyltransferase mutants with improved substrate promiscuity, including protein X-ray crystal structure based rational design, directed evolution with or without saturation mutagenesis, and both. Unlike hydrolyzing enzymes whose activity can be readily assessed by high-throughput screening assay methods based on the enhanced color or fluorescent signal of the product generated from positive clones, it is usually more difficult to screen and identify improved glycosyltransferase activities of a large amount of mutants generated by directed evolution. Fluorescent compounds that can be used as glycosyltransferase acceptor substrates and whose fluorescent signal decreases after glycosylation, such as 4-methylumbelliferone, have been used to screen

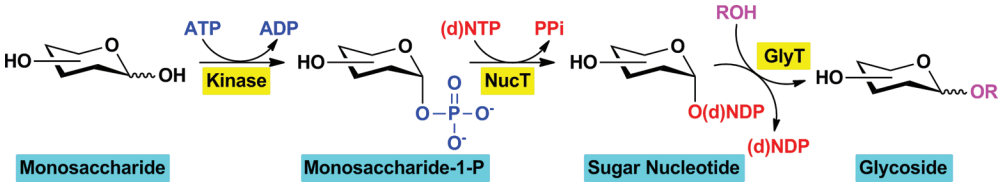

Figure 1. A simple process for the formation of common glycosides catalyzed by glycosyltransferases and related sugar nucleotide biosynthetic enzymes. Abbreviations: NucT, nucleotidyltransferase; GlyT, glycosyltransferase; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; monosaccharide-1-P, monosaccharide-1-phosphate; (d)NTP, nucleoside 5′-triphosphate or deoxynucleoside 5′-triphosphate; PPi, pyrophosphate; (d)NDP, nucleoside 5′-diphosphate or deoxynucleoside 5′-diphosphate. ROH is a glycosyltransferase acceptor that can be a glycan, a glycoconjugate, a peptide/protein, a lipid, or a natural product.

for improved promiscuity of a natural product oleandomycin glycosyltransferase (OleD) from mutants generated by directed evolution. A mutant (OleD-ASP or OleD P67T/S132F/A242V triple mutant) that can tolerate diverse UDP-monosaccharides and various aglycons belonging to or resembling diverse natural product structures (*2*) has been identified. An ELISA-based high-throughput screening method that relies on the use of immobilized acceptor substrates for glycosyltransferase-like enzymes (glycosynthases) and the detection of glycosylated products by carbohydrate-binding proteins after reaction was also developed and used successfully to identify mutants generated by directed evolution for efficient synthesis of glycosphingolipids (*3*). Alternatively, fluorescent-labeled simple glycans that can be freely transported in and out of cells but will be trapped inside cells after glycosylation catalyzed by cytoplasmic glycosyltransferase mutants have been successfully used to identify mutants with improved glycosyltransferase activity by fluorescence-activated cell sorting (FACS) (*4*). Among these methods, the ELISA-based strategy is a general approach that can be quickly adapted by common laboratories and can be applied to most if not all glycosyltransferases or glycosyltransferase-like enzymes as long as carbohydrate-binding proteins that can specifically bind to the glycan products but not the acceptors are available. The acceptors can be tagged with molecules other than lipids, such as biotin, for immobilization. Also, the immobilization of the tagged acceptors and products can be carried out after the enzymatic reactions. The ELISA-based method, however, is less high-throughput compared to FACS-based screening, although this drawback can be overcome with the assistance of automated pipetting, washing, and colony picking systems.

Other than obtaining powerful glycosyltransferases for efficient formation of glycosidic bonds between diverse monosaccharides or derivatives (from donor substrates) and acceptors, it is also necessary to acquire highly active sugar nucleotide biosynthetic enzymes to provide sugar nucleotide donors necessary for glycosyltransferase-catalyzed reactions. Substrate promiscuous sugar nucleotide biosynthetic enzymes are essential if diverse monosaccharides or monosaccharide derivatives are to be transferred to acceptors. For most reactions catalyzed by glycosyltransferases that transfer neutral five- or six-carbon monosaccharides, sugar nucleotides can be generated *in vitro* with a minimum of two enzymes or a bifunctional enzyme containing a kinase activity to produce monosaccharide-1-phosphate from free monosaccharide and a nucleotidyltransferase activity for the synthesis of sugar nucleotide (Figure 1), although *in vivo de novo* biosynthetic pathways may involve many more enzymes. Glycosyltransferases and related sugar nucleotide biosynthetic enzymes for *in vitro* synthesis of diverse glycosylated compounds can come from different species. Usually wild-type enzymes have some flexibility in tolerating substrate modifications, the scope of usable substrates can be expanded further by enzyme mutants.

A galactokinase has been engineered (GalK M173L/Y371H) by the combination of directed evolution and structure-based design (*5*) to transfer a phosphate from ATP to the anomeric carbon of 28 different monosaccharides and derivatives. A promiscuous nucleotidyltransferase mutant (RmlA L89T) that can use both TTP and UTP as nucleotide substrates and diverse α-D-hexopyranosyl phosphates as monosaccharide-1-phosphate substrates has also been identified by structure-based design (*6*). The combined activities of these sugar nucleotide biosynthetic enzymes allow the access to a diverse library of sugar nucleotides for *in vitro* natural product glycorandomization.

In the recent paper by Thorson *et al.* (*1*), an *in vitro* natural product glycorandomization system has been successfully transformed into an *in vivo* system. *E. coli* cells harboring a plasmid encoding a promiscuous *Streptomyces antibioticus* natural product oleandomycin glycosyltransferase (OleD) mutant TDP16 were successfully used to ferment glucosylated diverse natural products or derivatives from aglycons added to the cell culture medium and endogenous UDP-glucose (Figure 2a). Furthermore, *E. coli* cells harboring the TDP16 plasmid and a complementary plasmid encoding engineered promiscuous kinase mutant (GalK M173L/Y371H) and nucleotidyltransferase mutant (RmlA L89T) can ferment novel glycosides from aglycons and unnatural free monosaccharides added to the cell culture medium (Figure 2b). The *in vivo* glycorandomization system takes advantage of the endogenous nucleotides and sugar nucleotides to allow the eco-

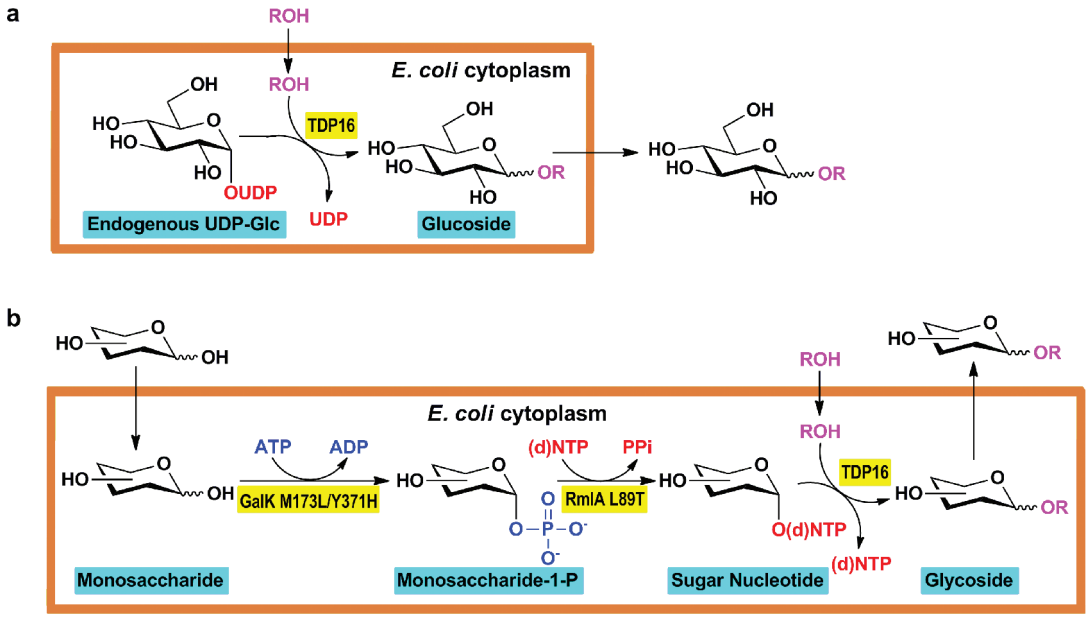

Figure 2. Fermenting diverse glycosides by in vivo glycorandomization. (a) Glucosylation of diverse exogenous aglycons using endogenous UDP-glucose by fermenting *E. coli* cells harboring a plasmid encoding an oleandomycin glycosyltransferase (OleD) mutant TDP16. (b) Glycorandomization of diverse exogenous aglycons using exogenous natural and non-natural free monosaccharides by fermenting *E. coli* cells harboring plasmids encoding an oleandomycin glycosyltransferase (OleD) mutant TDP16, a galactokinase mutant GalK M173L/Y371H, and a nucleotidyltransferase mutant RmlA L89T. Abbreviations: Glc, glucose.

nomic production of novel glycosylated natural products with simple input of aglycons with or without unnatural monosaccharides to generate a large library of diverse glycosides. The process is readily scaled up by simple fermentation. *E. coli* strains harboring novel glycosyltransferase mutants with or without mutants of sugar nucleotide biosynthetic enzymes are important new tools for facile access to novel glycosides.

Thorson and co-workers point out (*1*) that the success for *in vivo* glycorandomization system relies on efficient uptake of aglycons and unnatural monosaccharides by *E. coli* cells. The secretion of the glycosylated product can also help the product purification process. While the *in vivo* glycorandomization system has been proven to be suitable for most substrates that worked for the *in vitro* system, some of the aglycons tested were not compatible with the *in vivo* system. Introducing suitable transporters to the host strains may be explored to solve the problem. Therefore, the *in vitro* system is better suited for initial discovery stage, whereas the *in vivo* system is superior for scaled-up economic production of diverse glycosides.

In the overall glycosyltransferase-catalyzed glycosylation scheme (Figure 1), the addition of the nucleotide to monosaccharides is a transit activation step and the nucleotide does not present in the final glycosylated product (*7*). Nevertheless, the nucleotide added to the monosaccharide-1-phosphate by nucleotidyltransferase has to be compatible with the nucleotide in the sugar nucleotides required by the glycosyltransferases. Therefore, in their report (*1*), Thorson and co-workers generated a new glycosyltransferase OleD mutant TDP16 with improved activity toward dTDP-monosaccharide donors (the preferred product of nucleotidyltransferase mutant RmlA L89T) and diverse aglycons to facilitate the *in vivo* glycorandomization.

In vivo glycorandomization definitely has room for further improvement. One complication is that endogenous monosaccharides and sugar nucleotides can compete with the glycosylation process using intended unnatural glycans. This complication can be overcome by metabolic engineering of the sugar nucleotide biosynthetic pathway of the host strains (*8*).

Combining with combinatorial biosynthesis of natural product aglycons and together with natural product glycosylation pathway engineering (*9*, *10*), the *in vivo* glycorandomization using mutated promiscuous glycosylation enzymes is ready to take the lead on fermenting next generation glycosylated therapeutics.

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