LETTER

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Recombinant *E. coli* Prototype Strains for *in Vivo* Glycorandomization

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ABSTRACT *In vitro* glycorandomization is a powerful strategy to alter the glycosylation patterns of natural products and small molecule therapeutics. Yet, such *in vitro* methods are often difficult to scale and can be costly given the requirement to provide various nucleotides and cofactors. Here, we report the construction of several recombinant *E. coli* prototype strains that allow the facile production of a range of small molecule glycosides. This strategy relies on the engineered promiscuity of three key enzymes, an anomeric kinase, a sugar-1-phosphate nucleotidyltransferase, and a glycosyltransferase, as well as the ability of diverse small molecules to freely enter *E. coli*. Subsequently, this work is the first demonstration of "*in vivo* glycorandomization" and offers vast combinatorial potential by simple fermentation.

Glycoconjugation is a powerful tool to enhance the pharmacodymanics and/or pharmacokinetics of small-molecule-based therapeutics, including natural products (*1*). Yet, studies designed to systematically understand or exploit the attachment of carbohydrates in drug discovery remain limited by the availability of practical synthetic and/or biosynthetic tools (*2, 3*). Here, we report the development of two prototype *E. coli* strains for the facile production of small molecule glucosides and glycosides. Through directed evolution, a model promiscuous glycosyltransferase (GT) (OleD-ASP) (*4, 5*) was optimized for use as an *in vivo* glyco-catalyst to provide the OleD variant TDP16. A standard *E. coli* TDP16 overproduction strain, when subsequently grown in the presence of a diverse array of potential acceptors, led to the facile product of corresponding glucosides using endogenous host sugar donors (UDP/dTDP-Glc). Subsequent coexpression of the genes encoding for an engineered promiscuous anomeric kinase (GalK M173L/Y371H) (*6*), engineered promiscuous nucleotidyltransferase (RmlA L89T) (*7*), and TDP16 in *E. coli* led to a prototype strain capable of generating novel glycosides *via* combining unnatural free sugars and aglycons fed to the strain under standard growth conditions. This work stands as the first proof of concept for *in vivo* glycorandomization wherein the demonstrated ability to mix and match non-natural sugars with a range of small molecule acceptors implicates vast combinatorial potential. In addition, prototype strains such as the ones described should open the door for simple large scale fermentation of novel complex glycosides not available *via* conventional biosynthetic methods.

Natural product glycosylation is accomplished by GTs, the donors for which are often exotic nucleotide sugars produced by rather lengthy (5−9 enzymatic transformations) biosynthetic pathways (*2, 8*). Accordingly, the sugar biosynthetic pathways have been manipulated by metabolic engineering to produce novel natural product analogues. The first example of rational glycosyl engineering involved the replacement of an endogenous daunorubicin sugar C-4′ reductase with one of inverting stereospecificity to enable a recombinant *Streptomyces* strain for the anticancer agent epirubicin (*9*). Since this pioneering study roughly a decade ago, efforts have continued toward targeted metabolic glycosyl-engineering of select natural products and such efforts have more recently incorporated "sugar plasmids" harboring entire gene sets encoding for the biosynthesis of specific novel sugar nucleotides (*10–13*). While such studies have enabled the targeted production of

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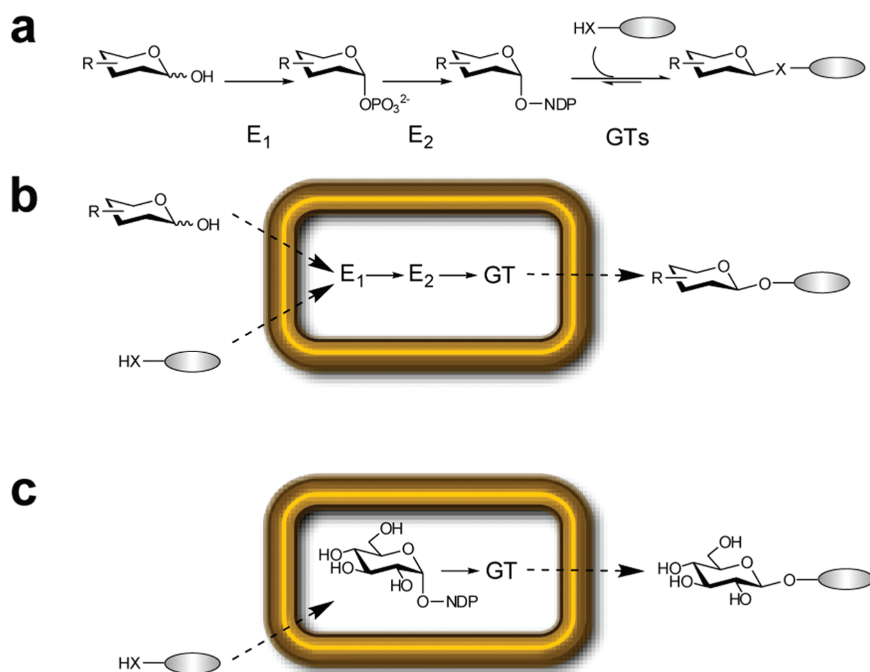

Figure 1. Comparison of methods for glycodiversification of natural products. a) *In vitro* glycorandomization. Reducing sugars are converted to sugar-1-phosphates by E1, a flexible anomeric kinase. E2, a suitably flexible sugar-1-phosphate nucleotidyltransferase, activates each sugar phosphate to the corresponding nucleotide sugar. Large panels of NDP donors are used to probe the specificity of natural product GTs. Gray oval represents diverse natural product or natural product-like aglycons (X = O, S, or NH). b) *In vivo* glycodiversification *via* a "non-natural glycoside host" strain. Reducing sugars and aglycons are fed to a bacterial host engineered to express E1, E2, and a promiscuous GT. The endogenous biosynthetic machinery ensures recycling of necessary cofactors, and aglycons decorated with non-natural sugars are collected from the culture media. c) *In vivo* glucoside host. Aglycons are fed into a bacterial host engineered to express a GT that uses endogenous dTDP/UDPGlc as the glycosyl donor.

non-natural glycosyl analogues of various natural products, the strategy is restricted by the inherent specificity of corresponding sugar nucleotide-forming enzymes and endogenous GTs, and when successful, the corresponding non-natural glycosides are often produced in low yield compared to the parent natural product.

As a response to these limitations, *in vitro* glycorandomization serves to produce diverse sets of sugar nucleotide donors *via* the combination of (i) an engineered promiscuous anomeric kinase (GalK M173L/Y371H), (ii) engineered promiscuous nucleotidyltransferase (RmlA L89T), and (iii) an array of free reducing sugars readily accessible by chemical synthesis or commercial sources (Figure 1, panel a) (*6, 7, 14*). The corresponding availability of these sugar nucleotide sets, in conjunction with the inherent promiscuity of several natural product GTs, enabled the generation of novel natural-product-based glycoside arrays including those based upon aminocoumarins, enediynes, glycopeptides, macrolides, and polyenes (*15–18*). This approach has been further advanced *via* GT-directed evolution to greatly expand donor and acceptor promiscuity and provide, for the first time, variant GTs capable of glycosylating natural products and small molecules for which natural GTs did not exist (*4, 19, 20*). Yet, while *in vitro* glycorandomization has proven to be a useful tool for discovery scale synthesis of novel glycosides, the *in vitro* method requires expensive cofactors, purified proteins, and often optimization of reaction conditions to prevent feedback/forward inhibition by reactants in the coupled system.

Previous studies using model plant GTs have demonstrated that simple aglycons can be taken up by *E. coli* and the resulting glycosides secreted into the culture media (*21–24*). On the basis of this precedent, we envisioned feeding various aglycons and sugars to an *E. coli* strain overproducing the *in vitro* glycorandomization machinery in *E. coli* to provide for the *in vivo* production and utilization of novel sugar nucleotides *en route* to novel glycoside production. Unlike existing *in vivo* approaches that require discrete engineered strains for each different target glycoside to be produced, the approach described herein utilizes a single biocatalytic strain to generate an array of novel glycosides *via* simple alteration of the fermentation input (sugar and aglycon) (Figure 1, panel b). In a simpler version, small molecule glucosides could also be afforded *via* feeding suitable aglycons to a "glucoside" host strain expressing OleD alone (Figure 1, panel c) wherein sufficient endogenous UDP-Glc (1) (Figure 2) is provided by host. Here we report the rapid optimization of OleD to enhance its compatibility with the upstream GalK/RmlA TDP-sugar production pathway and describe the first proof-of-principle demonstration of *in vivo* glycorandomization for the production of diverse natural product glycosides.

Whereas RmlA (i.e., E_2, Figure 1, panel a) is more efficient with TTP than UTP when non-natural sugar-1-phosphates are used as substrates (*7*), our previously described OleD variant "ASP" displays only modest activity toward dTDP-Glc (**2**) (*4*). To maximize the efficiency of OleD for glycosylation within the cytoplasm of *E. coli*, we therefore aimed to improve activity toward **2** by tar-

geted saturation mutagenesis and screening using our recently described 4-methylumbelliferone (**3**, Figure 2) fluorescence-based assay (*19*). The starting point for this mutagenesis was the OleD variant 3-1H12, itself identified from a saturation mutagenesis library (*19*). Variant 3-1H12 differs from the well-characterized ASP variant by a single mutation (A242L) and displays several-fold improvement in activity toward UDP-donor **1** (see Supplementary Table 1 for description of OleD variants used in this study) (*19*) and TDP-donor **2** (see Supplementary Table 2) compared to OleD "ASP". We hypothesized that mutagenesis of active site residues that form the nucleotide binding site would result in the identification of variants with improved activity toward **2**. Analysis of the WT OleD crystal structure revealed 8 residues within the N-terminal domain that were in contact or close to the nucleotide portion of **1** (Supplementary Figure 1). Each of these positions was individually randomized by saturation mutagenesis, affording 8 libraries that were screened using a fluorescence-based assay with **2** as donor, as described in the Supplementary Methods. The variant "TDP16" was identified, and DNA sequencing revealed the novel amino acid substitution Q268 V. The OleD variants "TDP16", "3-1H12", and "ASP" were compared by determining steady state kinetic parameters using either **1** or **2** as donor and the screening target **3** as acceptor (Supplementary Table 2), revealing TDP16 as a superior catalyst for conversion of **2**. Subsequent substrate specificity analysis (Supplementary Table 3) revealed TDP16 to exhibit a donor specificity similar to that of ASP and a marked improvement in activity toward a representative aglycon panel, including 4-methylumbelliferone (**3**), daidzein (**5**), mitoxantrone (**7**), nystatin (**10**), and digitoxigenin (**11**) (Supplementary Table 4; see Figure 2 for structures **3**−**7**).

For the *in vivo* host construction, genes encoding GalK M173L/Y371H and RmlA L89T were cloned into pETDuet1 yielding the vector pDuet-GalK-Ep. The gene encoding TDP16 was cloned into the complementary vector pCDFDuet1, giving pCDF-TDP16. Co-transformation of BL21(DE3) with pDuet-GalK-Ep and pCDF-TDP16 afforded the prototype "non-natural donor" strain (Figure 1, panel b), which expressed soluble GalK, RmlA, and OleD TDP-16 in good yield (~10 mg mL^{-1} culture, data not shown). Similarly, the corresponding universal "glucoside" host (Figure 1, panel c) containing pCDF-TDP16 alone lead to soluble TDP16 production in similar yield.

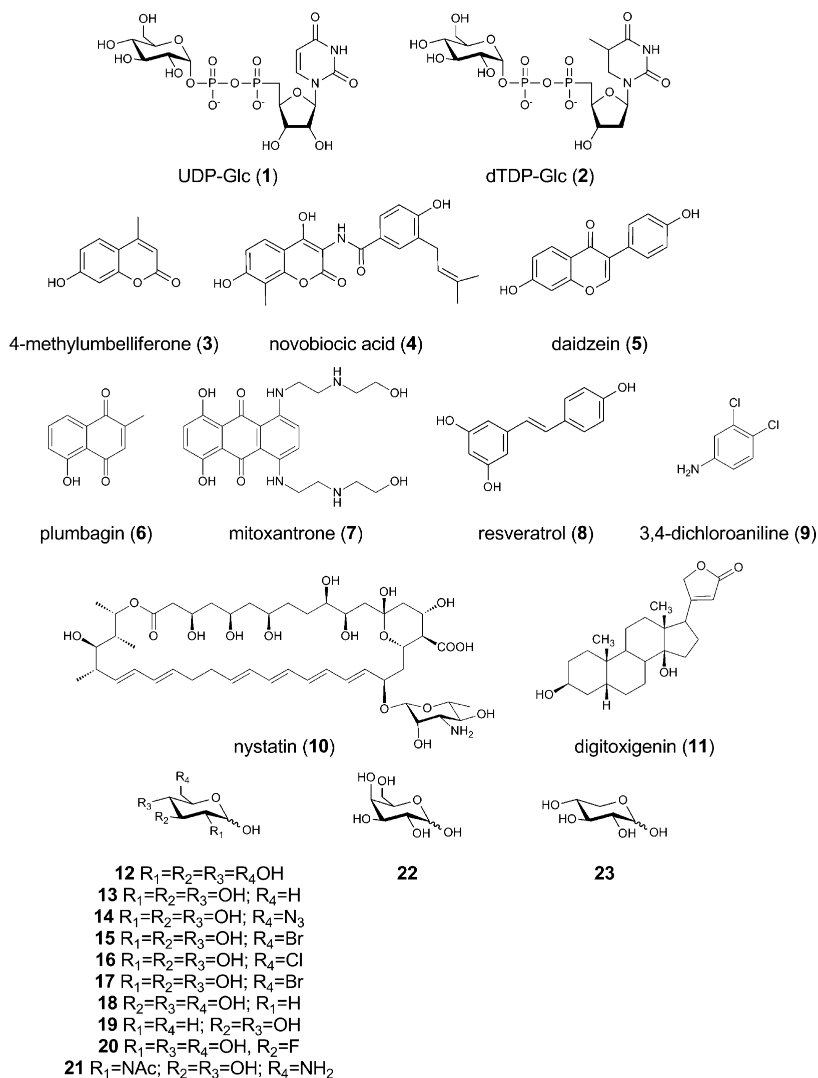

Figure 2. Structures of substrates used in this study.

A panel of known OleD aglycon substrates representing significant structural diversity and a dynamic range of proficiency with OleD (*5*) was chosen to validate our "glucoside" host (**3**−**11**, Figure 2). Following protein expression, each aglycon **3**−**11** was added to a small volume of *E. coli* BL21(DE3) pCDFDuet-TDP16 that had been washed into phosphate-buffered saline (PBS). Aliquots were removed at timely inter-

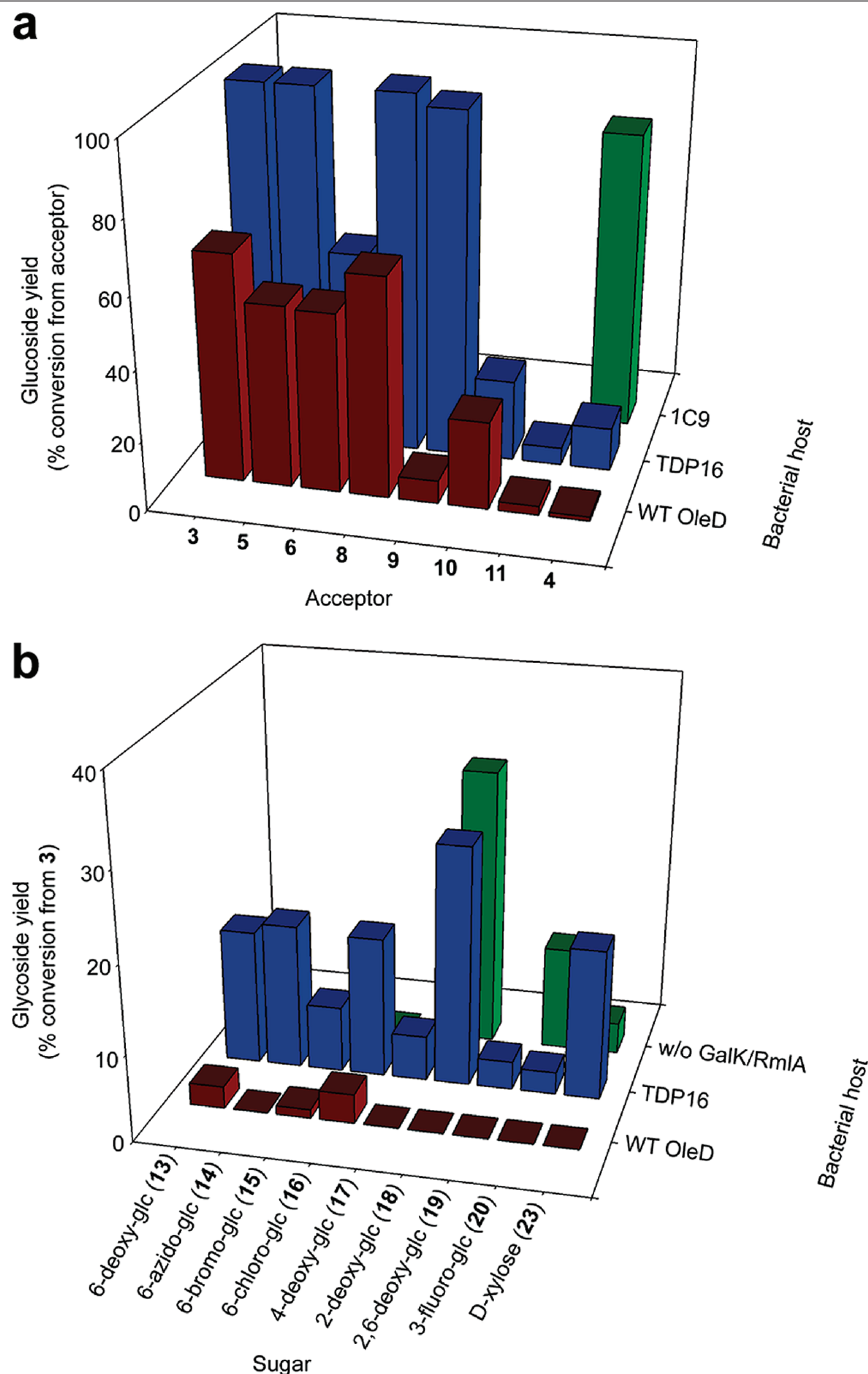

Figure 3. Activity of prototype glycoside producing strains. a) Yields (% conversion from acceptor) of glucosides using the TDP16-, WT-, and 1C9-based glucoside host with a small panel of diverse acceptors. b) Yields (% conversion from 3) of glycosides using the TDP16- and WT-based non-natural glycoside host using acceptor 3 and a panel of free sugars. "w/o GalK/RmlA" refers to the TDP16-based host but lacking the pDuet-GalK/RmlA vector. See Supporting Information for full description of the strains used and details of bioconversion conditions and detection. The standard deviation of the % conversions using data from three independent determinations was less than 20%.

vals, and the culture supernatant was analyzed for glucosides by HPLC (Figure 3, panel a and Supplementary Figure 2). Putative glucoside products were compared to standards prepared by *in vitro* reactions using OleD "ASP" and **1** as donor (*4, 5*) and were also verified by LC−MS analysis (Supplementary Table 5). This analysis revealed that coumarin **3**, aminocoumarin **4**, flavonoid **5**, quinone **6**, polyphenol **8**, amine **9**, and polyene **10** were each converted to the expected glucoside(s) (Figure 3, panel a and Supplementary Figure 2). Curiously, mitoxantrone **7** and digitoxigenin **11** were not converted (evidenced by the absence of product peak and no significant decrease in aglycon peak, as judged by HPLC), even though both are good in *vitro* substrates for TDP16 (Supplementary Table 2) and are likely poorly taken up by *E. coli* or modified within the cell. Expression of WT OleD in place of the variant TDP16 demonstrated that in most cases (except **11**), the TDP16-based strain was a superior host, and bioconversion using a strain that lacked OleD confirmed that glucoside formation in all cases was dependent on OleD (Supplementary Table 5). Product yields varied among these successful biotransformations (Figure 3, panel a) and, with the exception of **7** and **11**, mirrored *in vitro* OleD aglycon specificity. Consistent with this, substitution of TDP16 in the glucoside host with 1C9, an OleD variant previously optimized for activity toward **4** (*20*), led to a 7-fold higher conversion of **4** compared to the TDP16-based host (Figure 3, panel a).

Encouraged by the success of the *in vivo* glucoside host, efforts were next focused upon the prototype host for glycosylation with non-natural sugars (Figure 1, panel b). A panel of sugars was chosen to probe the efficiency and utility of our prototype "non-natural"-donor strain (**12**−**23**, Figure 2). These sugars were chosen to represent diverse levels of proficiency with GalK, RmlA, and OleD. For example, 6-azido-glc (**14**) is a good substrate for the mutant GalK (*14*),

RmlA (as the 1-phosphate) (*25*), and OleD (as the nucleotide) (*4*), whereas D-galactose (**22**) is a relatively good substrate for the GalK (*14*) and RmlA mutants (*26*) (as the 1-phosphate) but a very poor substrate (as the UDP-sugar) for OleD (Supplementary Table 3). Cell suspensions of BL21(DE3) pDuet-GalK-Ep/pCDF-TDP16 were prepared as described in the Supplementary Methods, paying particular attention to wash the cells thoroughly in buffer in order to remove residual sugars from the culture medium. Acceptor **3** (100 μM final concentration) and each sugar (at 4 mM final concentration) were then added to the cell suspension, aliquots were removed at timely intervals, and the culture supernatant was analyzed for glucosides by HPLC (Figure 3, panel b and Supplementary Figure 3). Putative glucoside products were compared to standards prepared by *in vitro* reactions using OleD "ASP" and **3** as donor and were also verified by LC−MS analysis (Supplementary Table 5). In addition to D-Glc (**12**), nine sugars were identified as substrates for the donor strain. Yields varied from 27% conversion for **18** to 2.5% for 3-fluoro-Glc **20**. Notably, these results suggested that 3-fluoro-Glc (**20**) and 2,6-dideoxy-Glc (**19**) NDP-sugars were substrates for the OleD mutant, expanding on the previously established NDP-donor substrate promiscuity. Conversion with D-Gal (**22**) was not detected, likely reflecting the very poor activity of OleD toward UDP-Gal and further suggesting that *in vitro* conversion with UDP-donor needs to be >2% for detectable *in vivo* bioconversion from the free sugar. Substitution of the OleD mutant TDP16 with the WT enzyme resulted in poorer conversions with every sugar tested and no discernible conversion with **14**, **17**, **18**, **19**, **20**, and **23** (Supplementary Table 5). In the absence of added sugar, only the glucoside of acceptor **3** was detected, as expected (at only 26% conversion). In fact, **3**-Glc was detected when any of the sugars **12**−**22** was used, and thus the preferred conversion of glucose to (U/T)DP-Glc by GalK/RmlA competes with conversion of the non-natural sugars. Nonetheless, **3**-Glc is easily separated from the non-natural glycosides by HPLC (Supplementary Figure 3), and total quantities of glycosides produced by the prototype strain are equivalent to 2−9 mg/L of cell suspension, yields that are comparable to other *in vivo* based systems. Additionally, a control strain that did not overexpress the GalK/RmlA mutants displayed conversion only with **18**, **20**, and **23** (Supplementary Table 5), illustrating that these sugars are presumably processed by endogenous nucleotide-sugar biosynthesis machinery.

To assess the impact of host permeability upon conversion efficiency (*e.g.*, in the case of validated *in vitro* substrates **7** and **11**), detergent treatment and physical disruption failed to improve *in vivo* bioconversion (Supplementary Table 5). Subsequent deletion of the *lpp* gene encoding Brauns lipoprotein of BL21(DE3), a mutation previously shown to produce marked improvement of permeability toward diverse small molecules (*27*), led to strains [BL21(DE3)/Δllp/pCDF-TDP16 and BL21(DE3)/Δllp/pDuet-GalK-Ep/pCDF-TDP16] capable of similar or slightly improved bioconversion compared to BL21(DE3)/pCDF-TDP16 and BL21(DE3)/pDuet-GalK-Ep/pCDF-TDP16, respectively (Supplementary Table 4). However, further analysis revealed the Δllp disruption mutants to rapidly lyse even under mild treatment such as washing and/or resuspension in PBS.

In summary, two novel prototype *E. coli* strains for the facile production of small molecule glucosides and glycosides were validated. These strains offer a number of advantages over prior microbial systems for small molecule glycoside production. First, *E. coli* is surprisingly permeable to a range of small molecule acceptors and sugars and is readily amenable to further engineering for strain improvement. Second, OleD mutants can be created that are tailored toward specific aglycon acceptors and/or sugar donors and can easily be substituted for OleD TDP16 within the prototype design described. Third, the *in vivo* glycoside system is amenable to standard large scale fermentation, and in most cases, the corresponding secretion of novel glycoside products greatly simplifies purification of the desired products. Furthermore, this *in vivo* approach circumvents the need for elaborate nucleotide sugar syntheses, cofactor regeneration, and/or enzyme purification required of existing *in vitro* strategies. Cumulatively, the ability to mix and match non-natural sugars with a range of small molecule acceptors offers vast combinatorial potential and also opens the door for similar strategies within important bioactive secondary metabolite-producing bacteria such as drug-producing actinomycetes.

METHODS

For complete materials and methods, including construction of plasmids, mutant library preparation, screening, protein expression and purification, enzyme kinetics, and substrate specificity determinations, see the Supporting Information.

General. Bacterial strain *E. coli* BL21(DE3)pLysS was from Stratagene. NovaBlue was from Novagen. Plasmid pET28/OleD was a generous gift from Prof Hung-Wen Liu (University of Texas-Austin, Austin, TX) and pET28a was from Novagen. All other chemicals were reagent-grade purchased from Fluka, New England Biolabs, or Sigma, unless otherwise stated. Primers were ordered from Integrated DNA Technologies (Coralville, IA). Novobiocic acid (**4**) was prepared as previously described from novobiocin. UDP-Glc (**1**), TDP-glc (**2**), and acceptors **5**−**11** were from Sigma. Sugars **12**, **18**, **20**, **21**, **22**, and **23** were from Sigma; **13**−**17** and **19** were synthesized as previously described. Analytical HPLC was performed on a Rainin Dynamax SD-2/410 system connected to a Rainin Dynamax UV-DII absorbance detector. To eliminate the need to purify acceptors and glucosides from the culture medium, the following optimal wavelengths were used: 254 nm for **3**, **6**, **8**, **9**, and **11**; 325 nm for **4**, 296 nm for **5**, 590 nm for **7**, and 300 nm for **10**. At these wavelengths, the extinction coefficients of acceptor and glucoside were approximately equal. Mass spectra were obtained using electrospray ionization on an Agilent 1100 HPLC-MSD SL quadropole mass spectrometer connected to a UV−vis diode array detector. For LC−MS analysis, quenched reaction mixtures were analyzed by analytical reverse-phase HPLC with a 250 mm × 4.6 mm Gemini 5 μm C18 column (Phenomenex, Torrance, CA) using a gradient

of 10−90% CH_3CN in 0.1% formic acid/H_2O in 20 min at 1 mL min^{-1}, with detection at 254 nm unless otherwise stated.

***In Vivo* Bioconversions.** For *in vivo* glucosylation of acceptors, a starter culture of BL21(DE3) pCDF-TDP16 or other control strain was used to inoculate a suitable volume of LB media containing 50 μg mL^{-1} streptomcyin and grown at 37 °C with shaking. Expression was induced by the addition of 0.1 mM IPTG when the OD_{600} was ~0.6, and the cells were then incubated at 18 °C with shaking for 18 h. Cells were then washed four times with 10× volume PBS at 4 °C. Finally, cells were resuspended in a volume of PBS such that the OD_{600} was 7.0. Acceptor stock solutions (in DMSO) were added to a suitable volume of cells to give 100 μM each of **3**−**9**, 1 mM **10**, and 0.2 mM **11**, and the cell suspensions continued to incubate at 18 °C with rotation. Aliquots (100 μL) were removed at timely intervals. Cells were collected by centrifugation, and the resulting supernatants were analyzed directly by HPLC as described in the Supporting Information and Methods.

For *in vivo* glycosylation of **3** with non-natural sugars, a starter culture of BL21(DE3) pDuet-GalK-Ep pCDF-TDP16 or other control strain was used to inoculate a suitable volume of LB media containing 50 μg mL^{-1} ampicillin and 50 μg mL^{-1} streptomcyin and then grown at 37 °C with shaking. Expression was induced by the addition of 0.1 mM IPTG when the OD_{600} was ~0.6, and the cells were then incubated at 18 °C with shaking for 18 h. Cells were then washed four times with 10× volume PBS at 4 °C. Finally, cells were resuspended in a volume of PBS such that the OD_{600} was 7.0. Acceptor **3** (in DMSO) was added to suitable volumes of cell suspension, and 100 mM stock solutions of each sugar **12**−**23** were added to a final concentration of 4 mM. Aliquots (100 μL) were removed at timely intervals. Cells were collected by centrifugation, and the resulting supernatants were analyzed directly by HPLC as described above.

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Conflict of Interest: J.S.T. is cofounder of Centrose, Madison, WI.

Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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