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Two Trifunctional Leloir Glycosyltransferases as Biocatalysts for Natural Products Glycodiversification

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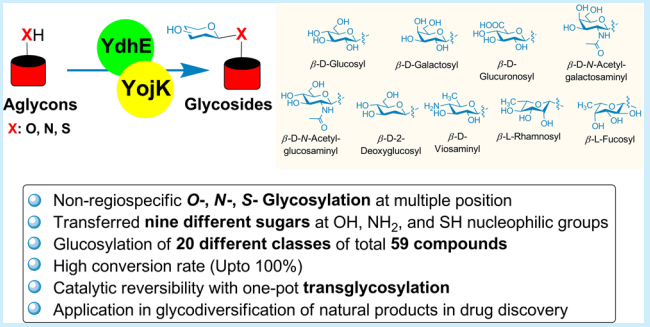
Supporting Information

ABSTRACT: Two promiscuous *Bacillus licheniformis* glycosyltransferases, YdhE and YojK, exhibited prominent stereospecific but nonregiospecific glycosylation activity of 20 different classes of 59 structurally different natural and nonnatural products. Both enzymes transferred various sugars at three nucleophilic groups (OH, NH_2, SH) of diverse compounds to produce *O*-, *N*-, and *S*-glycosides. The enzymes also displayed a catalytic reversibility potential for a one-pot transglycosylation, thus bestowing a cost-effective application in biosynthesis of glycodiversified natural products in drug discovery.



Glycodiversification is developing as one of the most prominent biocatalyst tools to create glycoside libraries of bioactive small molecules.[1] In place of hazardous, time-consuming, and costly chemical synthesis approaches to generating natural product (NP) glycosides, the search for novel glycosyltransferases (GTs) (EC 2.4.1) with wider tolerance of donor and acceptor substrates with permissive catalysis is of enormous importance for producing various glyco-functionalized NPs in an eco-friendly and cost-effective manner.[2]

GTs are the gatekeepers for NPs glycosylation, which is well-known to influence the physical, chemical, and biological properties of the parent molecules.[3] The process of glycosylation is well-characterized in cells, involved in metabolism, cell integrity, molecular recognition, pathogenicity, and post-modification of secondary metabolites during biosynthesis.[4] Since glycans are virtually present in every cells, GTs are ubiquitous in nature and can transfer sugar moieties from activated nucleotide diphosphate sugars (NDP-sugars) to acceptor molecules at nucleophilic OH, NH_2, SH, or CH. According to the recent CAZy classification (http://www.cazy.org/), GTs are classified into 107 different families. Among them, GT1 family proteins are inverting enzymes having a GT-B type 3D structure.[5]

A total of more than 19 000 GT1 family proteins are distributed across five domains of life. However, one-third of these proteins are present in bacteria (see Figure S1A in the Supporting Information). The number of these genes in the database is increasing day by day, because the number of genome sequences of new bacterial strains is rapidly increasing. Based on the genome sequences of 5349 complete bacterial genome sequences available from 2192 unique species in the dbCAN2 meta-server database (http://bcb.unl.edu/dbCAN2/blast.php), a total of 572 269 CAZymes homologues are reported.[6] From the database, we found a total of 3100 GT1 family proteins in the genomes of 1335 unique bacterial strains (see Figure S1B in the Supporting Information). However, few of the GT1 family proteins (∼400) are functionally characterized and a considerably low number of structures have been resolved by crystallography so far. Herein, we focused on GT1 family proteins from the industrially important nonpathogenic *Bacillus licheniformis* DSM 13 strain whose genome sequence (NC_006270.3) has been completed.[7]

According to the CAZy database, *B licheniformis* genome contains 146 CAZyme-related protein encoding genes. Of these, 38 GTs fall into nine different GT families (Figure S1C in the Supporting Information). However, only three GTs (YjiC, YdhE, YojK) belong to the GT1 family of proteins.

Since CAZymes are displayed in modular architecture in genomes with other noncatalytic modules, they often appear in modular fashion. To study the genomic organization of CAZymes, we performed a CAZyme gene clusters (CGC) analysis of the genome sequence of *B. licheniformis*, using the open web server dbCAN2 meta server. The genome contains seven CGCs; however, among 146 CAZymes, most of the enzymes were present out of the CGC clusters, whereas none

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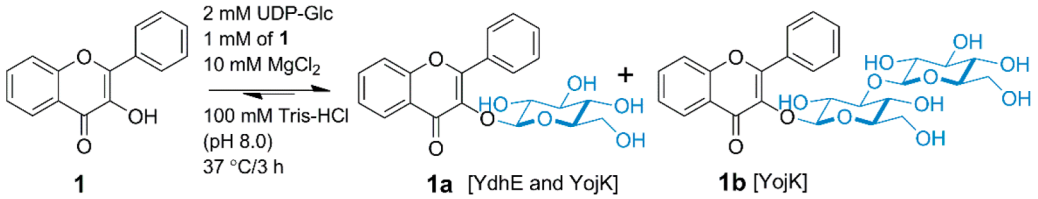
Scheme 1. Phenolic Hydroxyl Glucosylation Reaction by YdhE and YojK



of the GT1 family proteins were present in all of these seven CGCs (see Table S4 in the Supporting Information). A glycogenomics approach has recently been used to characterize several glycosylated natural products (GNPs); the biosynthesis gene clusters including GTs involved in their biosynthesis.[8] To study the possibility of GT1 family proteins involved in secondary metabolites glycosylation in *B. licheniformis*, we analyzed the genome sequence by anti-SMASH bacterial version.[9] However, none of the cluster showed GT1 family proteins involved in their biosynthesis pathways (see Table S5 in the Supporting Information). None of these *in silico* genome analyses provided clues on the involvement of these GTs in NPs glycosylation. However, our previous experiences showed that one of the GT1 family proteins, YjiC, from the same strain exhibited high promiscuity to glycosylate wide range of secondary metabolites from plants and microbes.[10]

The amino-acid sequence alignment of YjiC, YdhE, and YojK showed comparatively low sequence identity and similarity with each other (see Figure S2 and Table S6 in the Supporting Information). Although all three GTs fell into the same clade, they are reasonably divergent from each other in the phylogenetic tree analysis. YjiC is more similar to GTs from *Streptomyces*, whereas YdhE and YojK are more similar to *Bacillus* GTs (see Figures S3 and S4 in the Supporting Information). These two genes were cloned in pET28a(+) vector (see Figure S5 in the Supporting Information) and functionally expressed in *E. coli* BL21 (DE3) for biochemical studies (see Figure S6 in the Supporting Information).

To investigate the glycosylation property of YdhE and YojK, 50 μg/mL—the final concentration—of Ni-affinity purified His_6-tagged fusion pure recombinant enzymes (YdhE 48 kDa and YojK 47 kDa) were reacted with 1 mM 3-hydroxyflavone (3-HF, **1**) in 100 mM Tris-HCl buffer (pH 7.5) containing 2 mM UDP-α-D-glucose (UDP-Glc), and 10 mM $MgCl_2$ (Scheme 1) at 37 °C for 3 h. The high-performance liquid chromatography-photo diode array (HPLC-PDA) showed the conversion of **1** (retention time (t_R = 13 min)) to **1a** (t_R = 9.4 min) by YdhE, but YojK produced an additional product **1b** (t_R = 9 min) in small amounts, both of them showing a similar ultraviolet−visible light (UV-vis) spectrum (Figure S7 in the Supporting Information). High-resolution quadruple time-of-flight electrospray ionization mass spectrometry (HRqTOF-ESI/MS) in positive mode showed **1a** with a proton adduct mass of 401.1221 Da, whereas **1b** displayed a mass of 563.1765 Da, which were exactly 162 and 324 amu greater than the ion peak of **1** (239.0687 Da), respectively, resembling one and two glucose-conjugated derivatives of **1** (see Figure S7). Product **1a** was isolated from YdhE and **1b** was isolated from YojK-catalyzed reactions separately to the purity of 98% using prep-HPLC for NMR studies. The 1D and 2D NMRs of both products identified **1a** as 3-HF-3-*O*-β-D-glucopyranoside, and **1b** as 3-HF 3-*O*-β-D-glucopyranosyl (1−3)-β-D-glucopyranoside. The large coupling constant values (>7.5 Hz) showed conjugation of glucose in β-configuration (see Table S7 in the Supporting Information).

To understand the characteristics of YdhE and YojK, identical reactions were performed with both enzymes using **1** and UDP-Glc at 37 °C. The HPLC profile of the reaction mixture was monitored at different time points, from 5 min to 60 min. Both enzymes achieved maximum conversion of **1** after 30 min (see Figure S8 in the Supporting Information). YojK has ∼50% relative conversion in 5 min incubation, which was 85% with YdhE at the same time. Interestingly, the conversion rate was subtly decreasing in both reactions after 30 min. The optimum activity of the enzymes was found to be observed at 30 °C. Although YojK has relatively low tolerance to temperature variations, YdhE was found to be more stable and active, even below 15 °C and above 37 °C. Similarly, we found the optimal pH of the reaction buffer to be pH 10.0 and 8.8 for YdhE and YojK, respectively. Interestingly, YojK had a higher conversion at a low pH range (pH 3.0−6.5) than YdhE. The divalent metal ion profiling showed that both enzymes are catalytically active, even in the absence of metal ions. However, some metal ions, such as Hg^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+}, decreased the enzymes' activity considerably. The inhibitory effect of some divalent metal ions on enzyme activities of UGTs toward Cu^{2+} and Co^{2+} has been previously reported.[11] The overall results showed that none of the GTs require divalent cations for enzyme activities, but the presence of Mg^{2+}, Mn^{2+}, and Ca^{2+} enhanced the catalytic activity (see Figure S8 in the Supporting Information). The enzyme kinetics of both enzymes using **1** under optimal conditions showed similar *Km* values. But the *Vmax*, *kcat*, and *kcat*/*Km* values of YdhE are higher than those of YojK (see Table S8 in the Supporting Information), revealing a higher catalytic efficiency of YdhE than of YojK.

We then investigated the enzymes for donor substrate flexibility using all five natural nucleotide diphosphate glucoses (NDP-Glcs). The reaction with **1** and the five NDP-Glcs showed that YdhE can accept all five different NDP-Glcs as donor substrates, whereas YojK can accept only ADP-α-D-glucose (ADP-Glc), TDP-α-D-glucose (TDP-Glc), and UDP-Glc (Figure 1). The time profile conversion of **1** to **1a** with different NDP-Glcs showed conversion increment in the order of UDP-Glc > CDP-Glc > TDP-Glc > ADP-Glc > GDP-Glc with YdhE, which was in the order of UDP-Glc > ADP-Glc > TDP-Glc for YojK. The study revealed that both GTs are flexible toward all types of NDP-Glcs as donor substrates, thus increasing the possibility of employing diverse types of sugars as donor substrates for the glycodiversification reactions.

Since GTs are known to synthesize *N*-, *C*-, and *S*-glycosidic linkages,[12] we performed reactions using 3,4-dichlorophenol (**2**), 3,4-dichloroaniline (**3**), and 3,4-dichlorobenzenethiol (**4**) as acceptor substrates and UDP-Glc as the glucose donor substrate (see Scheme 2). As seen in the aforementioned reaction with **1**, both YdhE and YojK catalyzed the conversion

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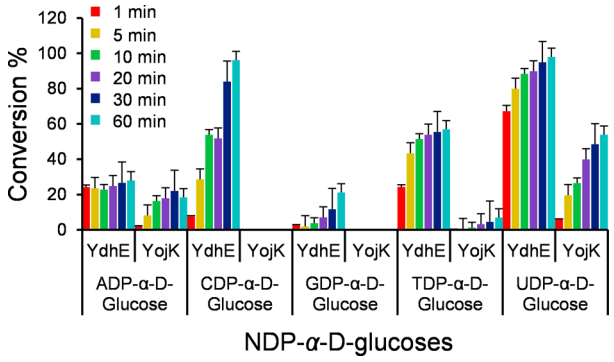
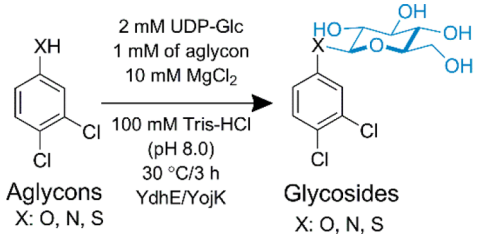


Figure 1. Donor substrate flexibility of YdhE and YojK for the reaction of **1** and NDP-glc at different time intervals.

Scheme 2. *O*-, *N*-, and *S*-Glucosylation Reactions by YdhE and YojK



of **2** (t_R 14.6 min) to its *O*-glucopyranoside derivative (t_R 10.8 min), which we further characterized by UV-vis, HR-qTOF ESI/MS (Figure S9A in the Supporting Information), and 1D NMR analyses, identifying the glucosylated product of **2** as 3,4-dichlorophenyl *O*-*β*-D-glucopyranoside (**2a**). Similarly, the analysis of reaction mixtures of **3** and **4** showed a prominent product peak in all four reaction mixtures. The products (**3a** and **4a**) also had an UV/vis absorbance similar to that of their parent molecules **3** and **4** and HR-qTOF ESI/MS confirmed the conjugation of a glucose moiety to each substrate (see Figures S9B and S9C in the Supporting Information). To further confirm the products by NMR studies, **3a** and **4a** were produced in large volume and purified by prep-HPLC. The NMR analysis confirmed the conjugation of a glucose at the amino functional group of **3** and thiol functional group of **4** in *β*-configuration, thus, producing 3,4-dichlorobenzyl 1-*N*-*β*-D-glucopyranoside (**3a**) and 3,4-dichlorobenzyl 1-*S*-*β*-D-glucopyranoside (**4a**), respectively. Neither of the reaction mixtures showed *C*-glucosylated products in either enzyme reaction.

To expand the possibility of use of YdhE and YojK toward *O*-, *N*-, and *S*-glycosidic linkage formation with various NDP sugars for *O*-, *N*-, and *S*-functionalized natural products, we reacted the same set of three acceptor substrates **2**, **3**, and **4** with eight different NDP-D/L sugars. NDP-D sugars, including UDP-*α*-D-galactose (UDP-Gal), UDP-*α*-D-glucuronic acid (UDP-GlcA), UDP-*α*-D-*N*-acetyl-galactosamine (UDP-GalNAc), UDP-*α*-D-*N*-acetyl-glucosamine (UDP-GluNAc), TDP-*α*-D-2-deoxyglucose (TDP-2dGlc), TDP-*α*-D-viosamine (TDP-Vio), and NDP-L sugars (TDP-*α*-L-rhamnose (TDP-Rhm), GDP-*α*-L-fucose (GDP-Fuc)) were reacted using YdhE and YojK. The HPLC-PDA and HR-qTOF ESI/MS results were fascinating that both YdhE and YojK accepted all three acceptor substrates and most of the donor NDP-D/L sugars and converted them to their respective glycosides (**2a**–**2i**, **3a**–**3i**, **4a**–**4i**) (see Figure 2, as well as Figures S10–S33 in the Supporting Information). Interestingly, both enzymes converted **4** to diverse glycosides with a relatively higher

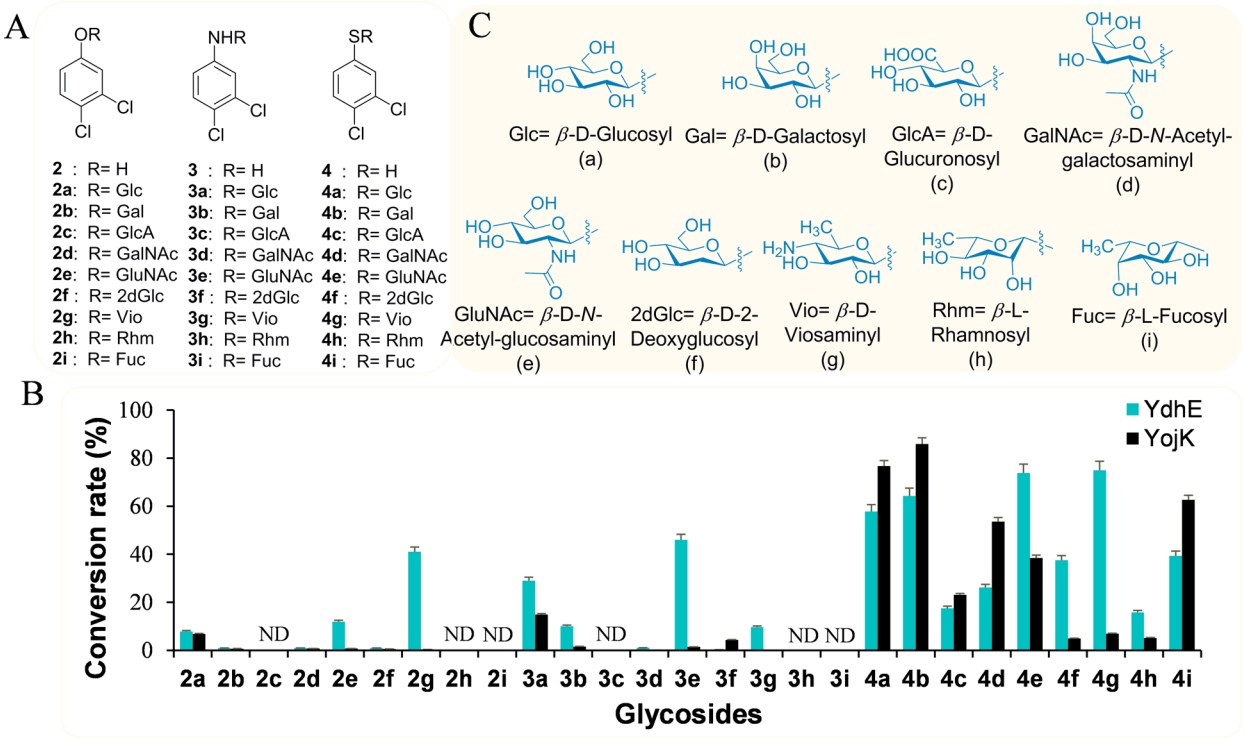


Figure 2. Catalytic promiscuity of YdhE and YojK at the OH, NH_2, and SH functional groups containing compounds with various NDP-D/L sugars. (A) Structures of the *O*-, *N*-, and *S*-glycosides generated. (B) Conversion percentage of each glycosylated molecule by YdhE and YojK. (C) Structures of different sugar moieties conjugated to compounds **2**, **3**, and **4**.

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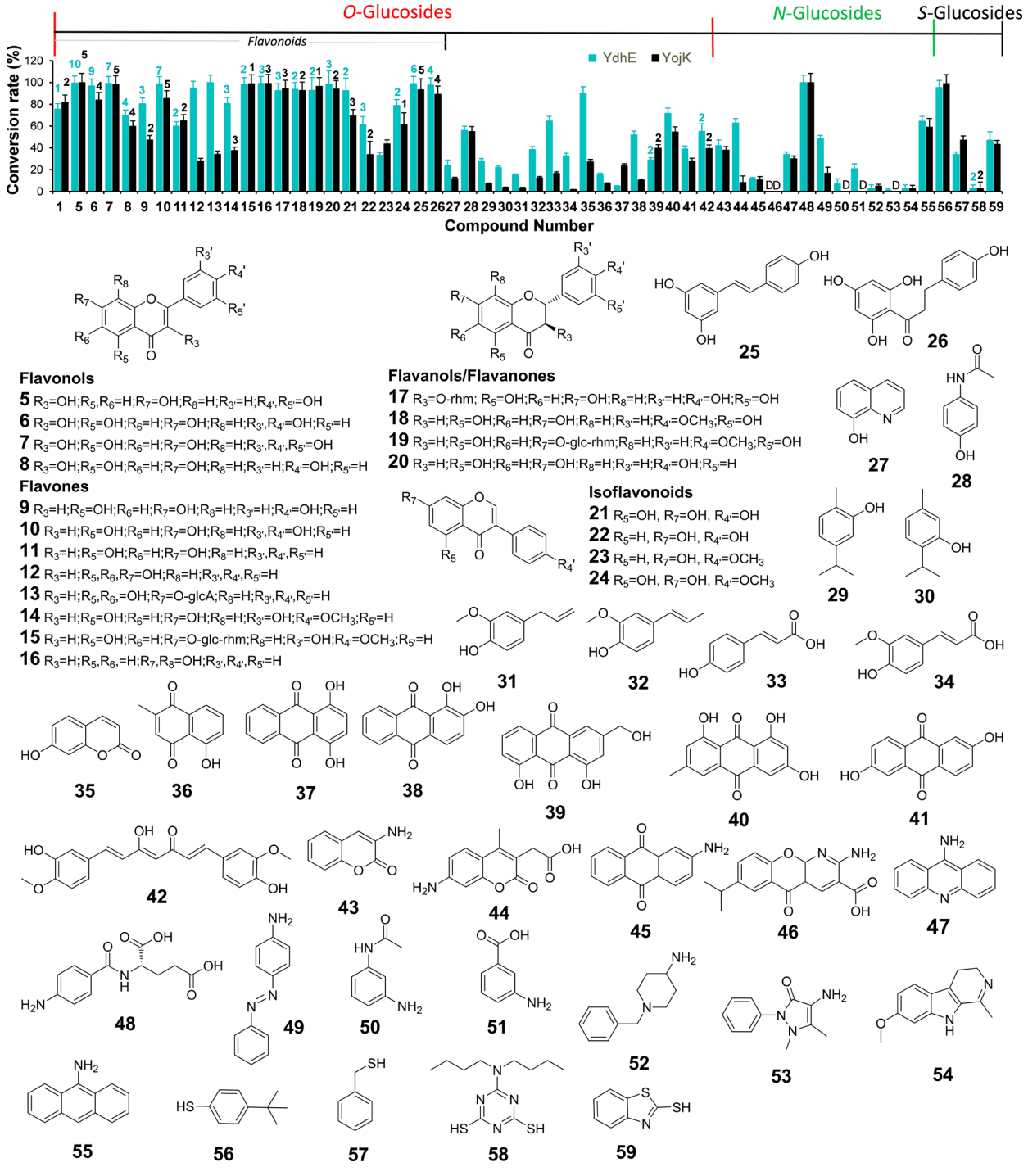

Figure 3. Acceptor substrate promiscuity study of YdhE and YojK with a set of various compounds. The upper panel shows the percent conversion of each compound catalyzed by YdhE and YojK. The number just above the bars represents the number of products generated from the reaction. The numbers of the molecules are listed as shown in the structures of the molecules presented in the lower part of the figure. The symbol "D" indicates that the compound was detected by HR-ESIMS. The compounds used for the reaction, but not accepted by both enzymes, are listed in Figure S90 in the Supporting Information.

conversion percentage than **2** and **3** (see Figure 2). This result showed that the newly identified GTs preferred *S*-glycosylation with flexible activity toward *O*- and *N*-glycosylation. Neither enzymes could conjugate glucuronic acid, rhamnose, or fucose to **2** and **3**.

Inspired by the broad catalytic activity of enzymes with NDP-D/L sugars, we investigated the promiscuity of YdhE and YojK toward 84 different natural and non-natural small molecules. Both enzymes were reacted with 50 hydroxyl groups containing compounds (**1**, **2**, **5**−**42**, **60**−**69**) mostly belonging to plant-originated secondary metabolites; 27 amino functional groups containing metabolites (**3**, **43**−**55**, **70**−**82**), of which most are synthetic compounds and used as human drugs; and seven thiol groups containing synthetic molecules (**4**, **56**−**59**, **83**, **84**) in the Tris-HCl buffer containing $MgCl_2$ and UDP-Glc. The reaction mixtures were analyzed by HPLC-

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PDA followed by HR-qTOF ESI/MS. Both enzymes showed unprecedented substrate promiscuity of *O*-, *N*-, and *S*-glycosylation of various classes of compounds.

Interestingly, the enzymes showed multiple glycosylations at phenolic hydroxyl groups of 23 different structural types of flavonoids, including flavonols (**1**, **5**−**8**), flavones (**9**−**16**), flavanols and flavanones (**17**−**20**), isoflavones (**21**−**24**), *trans*-stilbene (**25**), and dihydrochalcone (**26**) (see Figure 3). The glucosides were identified as *O*-glucosides based on the HRqTOF ESI/MS analysis showing a characteristic mass ion after loss of glucose unit with 162 amu (see Figures S34−S53 in the Supporting Information). For most of the flavonoids compounds, the conversion rate was >75% with both enzymes, except for compounds **8**, **9**, **11**−**14**, **22**, and **23** for only YojK. It is noteworthy that both enzymes exhibited a catalytic potential of >95% with flavonols (**5**−**7**), flavones (**10**, **15**, **16**), flavanols (**17**−**20**), stilbene (**25**), and dihydrochalcone (**26**) (see Figures S54 and S55 in the Supporting Information). Both enzymes accepted hydroxyl groups containing alkaloid 8-hydroxy quinoline (**27**), acetanilide (**28**), monoterpenes (**29**−**32**), phenylpropanoic acids (**33** and **34**), coumarin (**35**), napthoquinone (**36**), anthraquinones (**37**−**41**), and curcumin (**42**) (see Figure 3, as well as Figures S56−S71 in the Supporting Information). In addition, both enzymes demonstrated *N*- and *S*-glycosylation of various classes of compounds. The compounds containing amino-functional groups, including aminocoumarin (**43**, **44**), anthraquinone (**45**), unique synthetic compound (**46**), acridine (**47**), aminobenzene (**48**−**51**), piperidine (**52**), antipyrine (**53**), indole alkaloid (**54**), and anthracene (**55**) were accepted by both enzymes (see Figures S72−S84 in the Supporting Information). The conversion rate of most of the amino functional groups containing molecules tested in this study was <20%, except for aminocoumarin (**43**, **44**), aminobenzene (**48**, **49**), and anthracene (**55**). Similarly, we tested the substrates tolerance capacity of YdhE and YojK with different thiol groups containing molecules, including benzothiol (**4**, **56**), mercaptan (**57**, **83**, **84**), dimercapto triazine (**58**), and benzothiazole (**59**) (see Figure 3, as well as Figures S85−S88 in the Supporting Information). Both enzymes showed robust capabilities to convert **56**−**59** into their respective thiol-glucoside derivatives with the conversion rate of 80% for **56**, followed by conversion rates of >40% with **57** and **59**. Altogether, both enzymes showed broad substrate promiscuity by glycosylating more than 20 different classes of compounds (see Figure 3, as well as Figure S89 in the Supporting Information). However, none of them catalyzed steroid groups of compounds (**60**−**64**), 16-membered macrolactone (**65**), or tetracycline antibiotics (**66**−**69**), five nitrogen bases (**70**−**74**), aliphatic NH_2 group containing compounds (**75**, **76**, **79**), other classes of synthetic molecules with NH_2 (**77**, **78**, **80**−**82**), or compounds with aliphatic SH functional groups into glucopyranosides (see Figure S90 in the Supporting Information).

To elucidate the structure of glucosides, we purified 19 selected glucosides (**1a**, **1b**, **2a**, **3a**, **4a**, **20a**−**20c**, **21a**−**21c**, **29a**, **30a**, **31a**, **39a**, **40a**, **43a**, **49a**, **56a**, **58a**) and used ^{1}H NMR as well as ^{13} NMR. To elucidate the structures of a few selected molecules, various 2D NMRs were performed (see Figures S91−S161 in the Supporting Information). The NMR analysis showed the same beta-stereochemistry of conjugated glucose in all molecules (see Table S7).

To develop an eco-friendly and cost-effective chemo-enzymatic system to synthesize UDP-glc from chemically synthesized *p*-nitro-*β*-D-glucoside (**85**),[12d,13] the catalytic deglycosylation property of YdhE and YojK were exploited in the presence of UDP. The system was eventually coupled with intermolecular transglycosylation reactions with **2**, **3**, and **4** (see Figure 4). The system showed efficient conversion of **2** (>80%), **3** (~40%), and **4** (>50%) into their respective *O*-, *N*-, and *S*-glucosides in the absence of UDP-Glc within 15 min, suggesting a possible application of enzymes for one-pot transglycosylation using various synthetic glucosides without adding UDP-Glc.

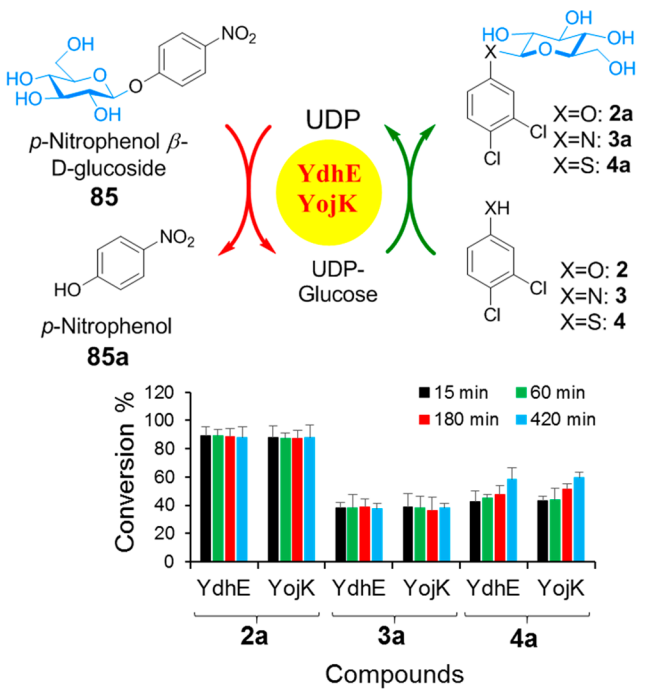

Figure 4. Catalytic reversibility and transglycosylation of compounds **2**, **3**, and **4** by YdhE and YojK. The conversion percent of each substrate is shown at different time intervals.

In summary, we identified two new highly promiscuous GTs, YdhE and YojK, from *B. licheniformis* DSM 13 with unprecedented catalytic efficiency toward different NDP-D/L sugars and above 20 different classes of natural and non-natural compounds to form glycosides. Moreover, both enzymes could synthesize *O*-, *N*-, and *S*-glycosidic linkage with different sugars with a higher activity toward the thiol nucleophilic group than did hydroxyl and amine groups containing compounds. Both GTs found to be superior to previously characterized GTs such as OleD from *Streptomyces antibioticus*,[1g,2a,c] in terms of their wide donor and acceptor substrates promiscuity and catalytic potential to transfer multiple sugars to amine and thiol functional groups without optimizing enzymes' catalytic sites. This study highlights the first *Bacillus* GTs to catalyze numerous druglike compounds to produce *O*-, *N*-, and *S*-glycosides. In addition, the enzymes reacted nonregiospecifically to produce multiple glucosides, along with catalytic reversibility for one-pot transglycosylation reaction at −OH, $-NH_2$, and −SH groups. Cumulatively, these GTs could serve as potential biocatalysts for the glycodiversification of various druglike scaffolds.

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■ ASSOCIATED CONTENT

Supporting Information

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Experimental procedures, HPLC-HRESIMS characterization data, NMR spectra of glucosylated products (PDF)

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R.P.P., P.B., and P.P. performed experiments, analyzed data. R.P.P. wrote the manuscript. T.Y. helped in analyzing NMR data. J.K.S. supervised the work. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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