Exploring the Nucleophilic *N*- and *S*-Glycosylation Capacity of *Bacillus licheniformis* YjiC Enzyme

Puspalata Bashyal¹, Samir Bahadur Thapa¹, Tae-Su Kim¹, Ramesh Prasad Pandey^{1,2*}, and Jae Kyung Sohng^{1*}

¹Department of Life Science and Biochemical Engineering, Sun Moon University, Asan 31460, Republic of Korea

²Department of Department of Pharmaceutical Engineering and Biotechnology, Sun Moon University, Asan 31460, Republic of Korea

YjiC, a glycosyltransferase from *Bacillus licheniformis*, is a well-known versatile enzyme for glycosylation of diverse substrates. Although a number of *O*-glycosylated products have been produced using YjiC, no report has been updated for nucleophilic *N*-, *S*-, and *C*- glycosylation. Here, we report the additional functional capacity of YjiC for nucleophilic *N*- and *S*- glycosylation using a broad substrate spectrum including UDP- α -D-glucose, UDP-*N*-acetyl glucosamine, UDP-*N*-acetyl-galactosamine, UDP- α -D-glucuronic acid, TDP- α -L-rhamnose, TDP- α -D-viosamine, and GDP- α -L-fucose as donor and various amine and thiol groups containing natural products as acceptor substrates. The results revealed YjiC as a promiscuous enzyme for conjugating diverse sugars at amine and thiol functional groups of small molecules applicable for generating glycofunctionalized chemical diversity libraries. The glycosylated products were analyzed using HPLC and LC/MS and compared with previous reports.

Keywords: *Bacillus licheniformis*, natural product, glycosylation, diversification

Glycosylation is a well-characterized glycosyltransferase (GT) enzyme-catalyzed reaction, in cells, involved in metabolism, cell integrity, molecular recognition and pathogenicity, and post-modification of secondary metabolites during biosynthesis [1–4]. GTs are ubiquitous in nature and transfer sugar moieties from activated nucleotide diphosphate sugars (NDP-D/L-sugars) to acceptor molecules. Leloir GTs are NDP-sugar dependent and transfer sugar units to lipid, nucleic acid, natural products, and other small molecules at nucleophilic oxygen (*O*-), nitrogen (*N*-), sulfur (*S*-), or carbon (*C*-). According to the recent CAZy classification (<http://www.cazy.org/>), GTs are classified into 110 different families. Among them, GT1 family proteins are inverting enzymes having GT-B type 3D structure transferring diverse sugars to small molecules [5, 6]. The glycosylation of natural products (NPs) influences the physical, chemical and biological properties of the parent molecules. Especially, the sugar conjugation to therapeutically important NPs alters the pharmacological and pharmacokinetic properties including water solubility, stability, specificity, as well as biological actions of the compounds [7, 8].

Due to the emerging resistance to the different therapeutics, recent research has been focused on designing/developing bioactive molecules by modification of previously known compounds using various approaches such as by applying microbial enzymes and cells as biocatalysts. Glycosylation is one of the most prominent tools to create glycoside libraries of bioactive small molecules as glycosylation results in an alteration in the pharmacokinetic properties of the parent compounds [9]. In this context, the search for novel glycosyltransferases with tolerance to diverse sets of donor substrates and acceptor compounds is expanding in importance. GTs from various organisms have been used to glucosylate diverse sets of plant natural products, specifically flavonoids [10, 11].

In this study, we have investigated the application of a GT, YjiC from non-pathogenic *Bacillus licheniformis* DSM 13 strain for glycosylation of various industrially important amino (NH₂) and thiol (SH) functional group-containing acceptor substrates. YjiC has been extensively studied for its donor and acceptor substrate promiscuity towards nucleophilic *O*-glycosylation of diverse sets of natural products using NDP-D/L-sugars as donor substrates [12, 13]. Nucleophilic *N*-, *S*-, *C*-glycosylation is regarded as rare in comparison to *O*-glycosylation of natural products. This puts the emphasis on research with those GTs capable of not only *O*-glycosylation but also able to generate other natural products with uncommon glycosidic linkages. GTs able to form C-C glycosidic linkages are gaining attention because of the stability of C-C bonds, and the resulting activity of both glycosyl and aglycone parts [14]. Likewise, *N*- and *S*-linked glycosidic linkages are also equally important for developing novel natural products with potential biological activity. In this study, we explored promiscuous YjiC enzyme for *N*- and

Received: January 16, 2020
 Accepted: March 15, 2020

First published online:
 March 20, 2020

*Corresponding authors

J.K.S.
 Phone: +82-41-530-2246
 Fax: +82-41-544-2919
 E-mail: sohng@sunmoon.ac.kr
 R.P.P.
 E-mail: ramesh.pandey25@gmail.com

Supplementary data for this paper are available on-line only at <http://jmb.or.kr>.

pISSN 1017-7825
 eISSN 1738-8872

Copyright© 2020 by
 The Korean Society for
 Microbiology and
 Biotechnology

Fig. 1. O, N, and S-glycosylation reactions catalyzed by YjiC. Reaction ingredients were as follows: 4 mM UDP-Glu 2 mM of aglycon, 10 mM MgCl_2 , 100 mM Tris-HCl (pH 8.0), 37 °C/3 h, and YjiC enzyme.

S-glycosylation of different thiol and amino group-containing compounds as acceptor substrates and various NDP-D/L-sugars as donor substrates.

Previously constructed pET28-YjiC [15, 16] was used to produce enzyme in *E. coli* BL21(DE3). The His₆-fused YjiC enzyme purified using Ni^{++} affinity chromatography (Fig. S1) was used for in vitro enzyme reactions. The O-, N- and S- trifunctional property of pure YjiC was investigated by reacting the enzyme with 2 mM of 3-dichlorophenol (1), 3-dichloroaniline (2) and 3-dichlorobenzenethiol (3), 4 mM UDP- α -D-glucose (UDP-Glu), in 100 mM Tris-

Fig. 2. HPLC-PDA and HR-QTOF ESI/MS analysis. (A) 3,4-dichlorophenol (1) (ii) and UDP- α -D-glucose reaction with YjiC (i). UV/VIS analysis of product 1a (iii) and standard 1 (iv). ESI/MS analysis of the product, 1a in positive mode (v). (B) 3,4-Dichloroaniline (2) (ii) and UDP- α -D-glucose reaction with YjiC. (i) UV/VIS analysis of product 2a (iii) standard 2 (iv). ESI/MS analysis of the product, 2a in positive mode (v). (C) 3,4-Dichlorobenzenethiol (3) (ii) and UDP- α -D-glucose reaction with YjiC. (i) UV/VIS analysis of product 3a (iii) and standard 3 (iv). ESI/MS analysis of the product 3a in positive mode (v).

Fig. 3. Catalytic promiscuity of YjiC at OH, NH₂, and SH functional group-containing compounds with diverse NDP-D/L-sugars. (A) Structures of the O-, N-, and S- glycosides generated. (B) Conversion percentage of each glycosylated molecules by YjiC. (C) Structures of different sugar moieties conjugated to compounds 1, 2, and 3. ND: not detected.

HCl buffer containing 10 mM MgCl₂ at 37°C for 3 h (Fig. 1). Reactions were analyzed using high-performance liquid chromatogram (HPLC) which showed the conversion of **1** (retention time (t_R 14.5 min) to **1a** (t_R 10.7 min) (Fig. 2Ai-2Aiv), conversion of **2** (retention time (t_R 13.6 min) to **2a** (t_R 10.5 min) (Fig. 2Bi-2Biv), and **3** (retention time (t_R 17.9 min) to **3a** (t_R 11.3 min) (Fig. 2Ci-2Civ). Analyzing all three samples by high-resolution quadrupole time-of-flight electrospray ionization mass spectrometry (HR-QTOF ESI/MS) in positive ion mode showed the masses of m/z^+ 347.0056 Da, m/z^+ 324.0398 Da, and m/z^+ 362.9830 Da, which exactly resembled the glucose-conjugated mass of **1**, **2** and **3** respectively (Fig. 2). After analyzing the conversion rate of these newly derived products (**1a**, **2a**, and **3a**), interestingly, conversion of **3a** was maximum, which was 43.8% compared to **1a** (11.3%) and **2a** (25.2%) (Fig. 3B). We have compared these products (**1a**, **2a**, and **3a**) with those generated after glycosylation of **1**, **2**, and **3** using YdhE and YojK [17]. Since the HPLC chromatogram, UV-VIS and ESI/MS spectra of products produced by YjiC aligned exactly with the products of YdhE and YojK (data not shown), we confirmed conjugation of glucose in β -configuration.

Furthermore, the substrates **1**, **2** and **3** were reacted with eight different types of NDP-D/L-sugar donors 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), and GDP- α -L-fucose (GDP-fuc)) (Figs. 3C, S2-S25). When the reaction was carried out using **1** as an acceptor substrate and with eight different sugar donors, HPLC-PDA and HR-QTOFESI/MS analysis confirmed only two products, **1e** and **1h** (Figs. S5 and S8). No product was detected with other NDP-sugar donors (Figs. S2-S7 and S9). When identical reactions were carried out with **2**, distinct conversion of **2** was seen to **2b**, **2e**, and **2h** (Figs. S10-S16) while with the rest of the other sugar donors, products (**2c**, **2d**, **2f**, **2g**, **2i**) were detected in ESI/MS (Figs. S11-S17). Similarly, in the identical reactions with **3**, the glycosylated products were produced with all eight sugar donors (Figs. 3B and S18-S25). These results revealed that YjiC enzyme was also able to form diverse sugar-conjugated N- and S-glycosidic linkages. Interestingly, the conversion to glycosides was higher towards the thiol functional

Amine functional group containing compounds **Thiol functional group containing compounds**

Fig. 4. Amine and thiol functional group-containing compounds used as acceptor substrates in this study.

group rather than the amine and hydroxyl functional groups (Fig. 3B).

After exploring the potential of YjiC to tolerate diverse NDP-D/L-sugars and transfer them to amine and thiol functional groups, we carried out enzyme reactions with purified YjiC with a set of aglycone compounds containing amine and thiol functional groups (Fig. 4) under the same set of reaction conditions using UDP-Glu as sugar donor. The HPLC-PDA and HR-QTOF ESI/MS analysis revealed glycosylated products of the substrates 4, 6, 7, 8, 10, 11, 12, 14, 16, 17, 19, and 20 belonging to the different classes of natural products (Figs. S26-S37). However, with the substrates 5, 9, 13, 15, and 18, the products were not detected as expected (Figs. S38-S41).

Since conjugation of bulky sugar groups to natural products dramatically changes polarity, water solubility, and binding affinity of parent compounds, glycosylation has been developed as a tool to engineer natural products with different sugars [17]. Importantly, conjugation of different sugars in the same parent molecule helps to study the effect of glycodiversification in altering biological activities, which eventually helps in studying the structure activity relationship of newly developed compounds to various targets. Additionally, glycans are directly involved in cell-cell interactions, binding to molecules, and recognition in cells. Many microbial natural product glycosides contain deoxysugars and are known to play crucial roles in biological activity, stability and binding affinity of drugs to target sites [18]. Thus, conjugation of sugars to drug scaffolds helps to gain new or enhanced binding affinity. Likewise, conjugation with amino deoxysugars to natural products changes the basicity of the molecules [19] while thiol-linked and amine-linked glycosides also bring changes in physical, chemical, and biological properties [20].

In summary, different experimental results demonstrated that YjiC from *B. licheniformis* has very versatile, functional potential to transfer diverse sugars to not only nucleophilic O- but also towards N- and S- groups, thus expanding possible use of YjiC for the chemical library of N- and S-glycosides. Additionally, the enzyme has more activity with thiol glycosylation in comparison to hydroxyl and amine group-containing compounds. Cumulatively, YjiC could serve as a potential biocatalyst for the glycodiversification of various drug scaffolds, like YdhE and YojK [21].

Acknowledgments

This research was supported by a grant from the Next-Generation BioGreen 21 Program (SSAC, Grant#: PJ013137).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Kilcoyne M, Joshi L. 2007. Carbohydrates in therapeutics. *Cardiovasc. Hematol. Agents Med. Chem.* **5**: 1876-1897.
2. Kren V, Martinková L. 2001. Glycosides in medicine: "The role of glycosidic residue in biological activity". *Curr. Med. Chem.* **8**: 1303-1328.
3. Křen V, Řezanka T. 2008. Sweet antibiotics - The role of glycosidic residues in antibiotic and antitumor activity and their randomization. *FEMS Microbiol. Rev.* **32**: 858-889.
4. Wrodnigg TM, Sprenger F. 2004. Bioactive carbohydrates and recently discovered analogues as chemotherapeutics. *Mini Rev. Med. Chem.* **4**: 437-459.
5. Campbell JA, Davies GJ, Bulone V, Henrissat B. 1997. A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *Biochem. J.* **326**: 929-939.
6. Coutinho PM, Deleury E, Davies GJ, Henrissat B. 2003. An evolving hierarchical family classification for glycosyltransferases. *J. Mol. Biol.* **328**: 307-317.
7. Weymouth-Wilson AC. 1997. The role of carbohydrates in biologically active natural products. *Nat. Prod. Rep.* **14**: 99-110.
8. Butler MS. 2004. The role of natural product chemistry in drug discovery. *J. Nat. Prod.* **67**: 2141-2153.
9. Xiao J, Muzashvili TS, Georgiev MI. 2014. Advances in the biotechnological glycosylation of valuable flavonoids. *Biotechnol. Adv.* **32**: 1145-1156.
10. Ko JH, Kim BG, Ahn JH. 2006. Glycosylation of flavonoids with a glycosyltransferase from *Bacillus cereus*. *FEMS Microbiol. Lett.* **258**: 263-268.
11. Ahn BC, Kim BG, Jeon YM, Lee EJ, Lim Y, Ahn JH. 2009. Formation of flavone di-O-glucosides using a glycosyltransferase from *Bacillus cereus*. *J. Microbiol. Biotechnol.* **19**: 387-390.
12. Bashyal P, Pandey RP, Thapa SB, Kang MK, Kim CJ, Sohng JK. 2019. Biocatalytic synthesis of non-natural monoterpene O-glycosides exhibiting superior antibacterial and antinematodal properties. *ACS Omega.* **4**: 9367-9375.
13. Thapa SB, Pandey RP, Bashyal P, Tokutaro Y, Sohng JK. 2019. Cascade biocatalysis systems for bioactive naringenin glucosides and quercetin rhamnoside production from sucrose. *Appl. Microbiol. Biotechnol.* **103**: 8281-8281.
14. Hultin P. 2005. Bioactive C-glycosides from bacterial secondary metabolism. *Curr. Top. Med. Chem.* **5**: 1299-1331.
15. Pandey RP, Parajuli P, Koirala N, Park JW, Sohng JK. 2013. Probing 3-hydroxyflavone for in vitro glycorandomization of flavonols by YjiC. *Appl. Environ. Microbiol.* **79**: 6833-6838.
16. Pandey RP, Parajuli P, Shin JY, Lee J, Lee S, Hong YS, et al. 2014. Enzymatic biosynthesis of novel resveratrol glucoside and glycoside derivatives. *Appl. Environ. Microbiol.* **80**: 7235-7243.
17. Gantt RW, Peltier-Pain P, Thorson JS. 2011. Enzymatic methods for glyco(diversification/randomization) of drugs and small molecules. *Nat. Prod. Rep.* **28**: 1811-1853.
18. Elshahawi SI, Shaaban KA, Kharel MK, Thorson JS. 2015. A comprehensive review of glycosylated bacterial natural products. *Chem. Soc. Rev.* **44**: 7591-7697.
19. Zhang J, Singh S, Hughes RR, Zhou M, Sunkara M, Morris AJ, et al. 2014. A simple strategy for glycosyltransferase-catalyzed aminosugar nucleotide synthesis. *Chembiochem* **15**: 647-652.
20. Ati J, Lafite P, Daniellou, R. 2017. Enzymatic synthesis of glycosides: from natural O- and N-glycosides to rare C- and S-glycosides. *Beilstein J. Org. Chem.* **13**: 1857-1865.
21. Pandey RP, Bashyal P, Parajuli P, Yamaguchi T, Sohng JK. 2019. Two trifunctional lelior glycosyltransferases as biocatalysts for natural products glycodiversification. *Org. Lett.* **21**: 8058-8064.