Materials and Methods

Chemicals and Reagents
Standard tetracyclines (chlorotetracycline, doxycycline, mectetacycline, minocycline, and rolitetracycline) used in this study were purchased from Tokyo Chemical Industry (Tokyo, Japan). Isopropyl-β-D-thiogalactopyranoside (IPTG), α-D-glucose 1-phosphate, acetyl phosphate, and uridine diphosphate were purchased from GeneChem (Daejeon, South Korea). For analytical purpose, high-performance liquid chromatography (HPLC) grade acetonitrile and water were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). Other chemicals and solvents used in this research were purchased from standard commercial sources made for research purpose with the highest quality.

Plasmids, Microorganisms, and Culture Condition
Three different recombinants, acetate kinase (ack) gene assembled in pET24ma (pET24ma-ACK), α-D-glucose 1-phosphate uridylyltransferase (galU) gene cloned in pET32a (pET32a-GalU) [1], and UDP-glycosyltransferase (yjiC) cloned in pET28a (pET28a-YjiC) [2] were used in this study. *Escherichia coli* BL21(DE3) (Stratagene, USA) was used for recombinant protein production. All recombinant *E. coli* strains were cultured in Luria-Bertani (LB) broth medium. Cultures were incubated at 37°C with shaking (200 rpm). Kanamycin (50 µg/ml, final concentration) and ampicillin (100 µg/ml, final concentration) were used for colony selection and maintenance of recombinant strains.

Protein Expression and Soluble Fraction Preparation
All recombinant proteins were expressed in 50 ml flask containing LB broth medium supplemented with appropriate amount of antibiotics. Prior to expression, 3 ml of seed culture of each recombinant was prepared in LB medium cultured at 37°C overnight with shaking (200 rpm). Then 200 µl of this inoculum was transferred to 50 ml LB medium containing appropriate antibiotic in a sterile flask and incubated at 37°C with shaking (200 rpm). When cell growth reached approximate optical density of 0.5 at 600 nm, IPTG at final concentration of 0.5 mM was added to the culture. Culture flasks were then transferred to 20°C and incubated for 20 h with shaking (200 rpm). After 20 h of culture, cells were harvested for soluble protein extraction by centrifugation at 842 × g for 10 min. Cell pellets were collected and washed twice with 100 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol. Finally, cells were re-suspended in 1 ml of the same buffer containing 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). These cells were then kept at −70°C for later analysis. Cells were thawed and lysed by sonication using a Sonosnasher (Sonicator, Heat Systems, Ultrasonic, Inc.). The cell lysate was collected in microcentrifuge tubes after centrifugation at 13,475 × g for 30 min at 4°C. The supernatant part was transferred to new sterile tube and protein fraction was subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The same crude enzyme was used for single vessel multi-enzyme reactions.

Analytical Procedures
The quenched reaction mixture was filtered through 0.2 µm filter and subjected to ACQUITY ultra-pressure liquid chromatography (UPLC, Waters Corp., Billerica, MA, USA) PDA (LG 500 nm) coupled with high resolution quadrupole time-of-flight electrospray mass spectrometry (HR-QTOF ESI/MS) (SYNAPT G2-S (Waters Corp.)) in positive mode ionization. UPLC-PDA was performed using mobile phases consisting of HPLC grade acetonitrile (ACN) and water containing 0.05% trifluoroacetic acid. UPLC-PDA binary program was set for 12 min. ACN concentrations were as follows: (0-100)% from (0-7 min), 100% from (7-9.50 min), and (100-0)% from (9.51-12 min).

References