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Glucose 1-Phosphate Thymidylyltransferase in the Synthesis of Uridine 5′-Diphosphate Galactose and its Application in the Synthesis of *N*-Acetyllactosamine

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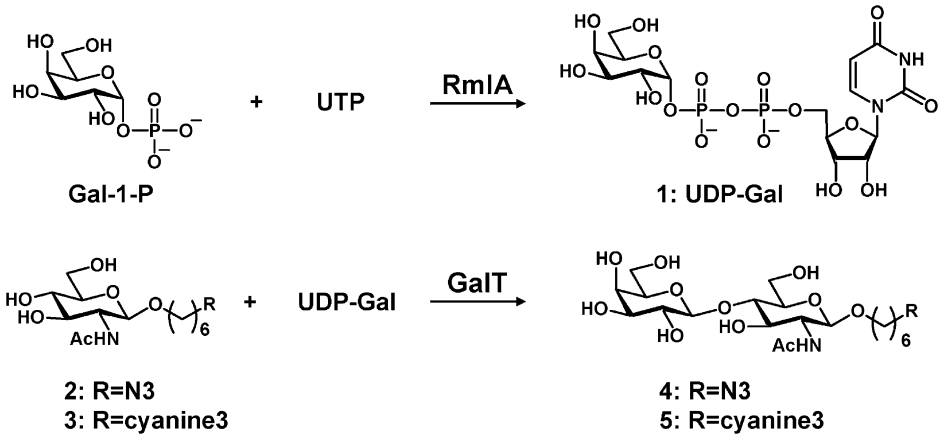
Abstract: In this study, we describe the direct synthesis of uridine 5′-diphosphate galactose (UDP-Gal) by a wild-type bacterial thymidylyltransferase (RmlA), which is used to synthesize thymidine 5′-diphosphate glucose (TDP-glucose) in nature. By using magnesium (Mg^{2+}) as a cofactor and a reaction temperature of 55 °C, a one hundred milligram-scale synthesis of UDP-Gal was achieved by RmlA. In addition, RmlA was site-specifically and covalently immobilized on magnetic nanoparticles (MNPa) The resulting RmlA-MNP complex retained almost 95% of its activity after reuse in ten consecutive enzyme assays. Furthermore, β-1,4-galactosyltransferase (GalT) from *Neisseria meningitides* was successfully overexpressed and purified by using an intein-mediated protein expression system. GalT was relatively stable at 25 °C, and its activity was enhanced in the presence of DTT and BSA. Thus, it was feasible to synthesize *N*-acetyllactosamine (LacNAc) using RmlA and GalT in a sequential addition of enzyme and adjustment of thereaction temperature. These results demonstrate the potential applications of bacterial RmlA in carbohydrate synthesis.

Keywords: *N*-acetyllactosamine; biosynthesis; carbohydrates; enzymes; galactosyltransferase; immobilization; uridine 5′-diphosphate galactose (UDP-Gal); uridylyltransferase

Introduction

Carbohydrates on the cell surface play crucial roles in cell-cell communication, viral invasion, and many biological recognition pathways.[1] The availability of these molecules facilitates their study, on a molecular level, of carbohydrate-protein interactions and helps elucidate the molecular mechanisms of carbohydrate-mediated biological processes. Progress in this vein of research depends on the acquisition of enough material and the purity of the desired carbohydrates.[2] Although many chemical methods have been developed to synthesize complex carbohydrates, most of them are labor-intensive and involve time-consuming synthetic routes[3] that significantly hamper the progress of glycobiology. In contrast, enzymatic synthesis may circumvent the drawbacks of oligosaccharide synthesis, wherein most of the enzymes operate at room temperature, under neutral aqueous conditions, and without the need of substrate functional group protection. The chiral nature of enzymes results in the regio- and stereoselective formation of the product at a remarkably accelerated rate.[4]

Glycosyltransferases have been extensively used in the enzymatic synthesis of oligosaccharides.[4a,5] Glycosyltransferases from microbial sources may be rather flexible due to their ability to synthesize a wide range of oligosaccharide analogues at relatively high yields.[6] These enzymes use sugar nucleotides as donors to incorporate a monosaccharide at the non-reducing end of a growing carbohydrate chain.[1] Many chemical methods have been developed to synthesize NDP-sugars and require multiple steps that result in low overall yields.[7] In contrast, the enzymatic synthesis of sugar-nucleotides is more economical and efficient. Most of the methods therein are based on multienzymatic reactions that begin from inexpensive nucleoside monophosphate (NMP) substrates;[8]



Scheme 1. The reactions catalyzed by RmlA and GalT.

however, the reaction parameters of multienzymatic reactions are complicated and may lead to a low overall conversion yield. Thus, there is an urgent need to develop simple and efficient methods to synthesize sugar-nucleotide donors.

UDP-Gal occurs in living organisms as an intermediate in the catabolic metabolism of D-galactose (Gal). It is also one of the nucleotide sugars that are most relevant in the biosynthesis of glycoconjugates in mammals. Mammals utilize the Leloir pathway to produce UDP-Gal.[9] Previously, Whitesides and co-workers used commercial enzymes to create a regeneration cycle for the synthesis of UDP-Gal based on the Leloir pathway.[10] Moreover, Ozaki and co-workers established an *in vivo* production system of UDP-Gal by combining *Escherichia coli* and *Corynebacterium ammoniagenes*.[11] Wang and co-workers developed a non-covalently immobilized multi-enzyme system, called superbeads, for the *in vitro* synthesis of UDP-Gal using an intermediate regeneration system.[12] Because all of the enzymes that were used in this system are immobilized on solid supports, they are easily recovered and product purification is simple.

Synthetic strategies using enzymes are often limited by the stability of the enzymes and their tolerance to non-natural substrates. Recently, the enzymatic synthesis of sugar nucleotides by bacterial hexose 1-phosphate nucleotidyltransferases was identified as an attractive approach.[13] Due to their broad substrate flexibility, these enzymes have been used in the syntheses of various sugar nucleotides and their analogues. For example, α-D-glucopyranosyl 1-phosphate thymidylyltransferase from *Salmonella enterica* LT2 has been utilized in the synthesis of UDP- and TDP-sugar nucleotides by using a series of hexopyranosyl phosphates.[13a] Lee and co-workers reported the synthesis of various UDP-sugars and NDP-glucoses by the recombinant UDP-sugar pyrophosphorylase of *Thermuscaldophilus* GK24.[13b] Pohl and co-workers reported the first expression of a sugar nucleotidyltransferase from an archaeal source, hyperthermophile *Pyrococcusfuriosus* DSM 3638. They demonstrated its unusual tolerance to heat, pH, and sugar substrates using an electrospray ionization mass spectrometry (ESI-MS)-based assay;[13c,d] however, these approaches were only demonstrated on an analytical scale and the decomposition of UDP-Gal at high temperatures was not discussed.

RmlA, which is a bacterial thymidylyltransferase, is involved in the synthetic pathway of TDP-rhamnose in Gram-negative bacteria.[14] This enzyme exhibits glucose 1-phosphate thymidylyltransferase activity and synthesizes TDP-Glc. Previously, Jakeman showed the capability of RmlA to use both dTTP and UTP nucleotides with six sugar 1-phosphates to efficiently produce both dTDP- and UDP-sugars, respectively.[13e] Herein, we describe the synthesis of UDP-Gal using the wild-type RmlA from *Aneurinibacillus thermoaerophilus* DSM 10155 (Scheme 1). RmlA was easily purified by a single chromatographic step at a good production yield. Recombinant RmlA required a divalent cation for its UDP-Gal synthesis activity. The optimal conditions therein included 10 mM Mg^{2+} in a 50 mM HEPES buffer at a pH 7.5. In a hundred milligram-scale synthesis of UDP-Gal, the yield was 95%.

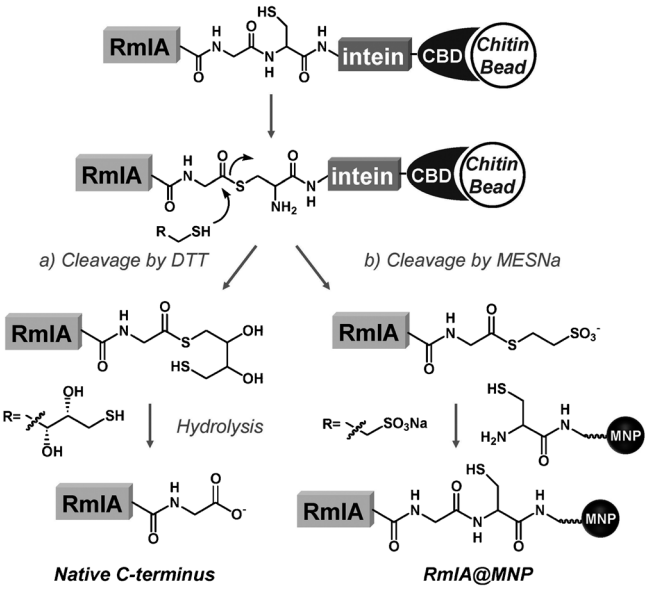
UDP-Gal participates in the synthesis of *N*-acetyllactosamine (LacNAc), which is a well-known and biologically important disaccharide core structure of lactosaminoglycans, tumor-associated antigenic carbohydrates, glycoproteins, and glycolipids. Sialylated and fucosylated derivatives of LacNAc have been characterized as specific ligands for lectins, such as selectins and galectins.[15] Due to their involvement in many biological roles, the number of biological studies investigating LacNAc-based oligosaccharides has increased. Due to the accessibility of UDP-Gal for β-1,4-galactosyltransferase, enzymatic reactions synthesizing LacNAc can be performed as sequential reac-

tions in a one-pot reaction. In a sequential one-pot reaction, there is no need to purify the intermediate, and therefore, it should be a more efficient approach to organic synthesis. Additionally, in comparison to mammalian enzymes, bacterial enzymes have the advantage of easy production in *E. coli*, a broader substrate tolerance, and a superior *in vitro* synthetic efficiency. In combination with β-1,4-galactosyltransferase (GalT) from *Neisseria meningitidis*, a hundred milligram-scale synthesis of LacNAc was obtained from a one-pot synthesis reaction (Scheme 1).

Results and Discussion

Expression and Purification of the Recombinant Enzyme RmlA

As shown in Scheme 2, an intein-mediated protein purification system was used to produce and purify the target enzyme RmlA.[16] The *rmlA* gene fragment was amplified from pRmlA3 and then inserted into the expression vector, pTXB1.[13e,14] It was previously reported that the C-terminal amino acid residue of the fused protein at the intein cleavage site greatly affects the cleavage efficiency of the intein.[17] Thus, a glycine was inserted upstream of the intein cleavage site. The production of enzyme was initiated by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG). The SDS-PAGE analysis of the whole-cell lysate showed a major band at 65 kDa, which was the



Scheme 2. Illustration of the protein isolation method *via* an intein-mediated protein purification system. The intein-mediated protein purification system and native chemical ligation were combined for site-specific covalent immobilization of the target proteins.

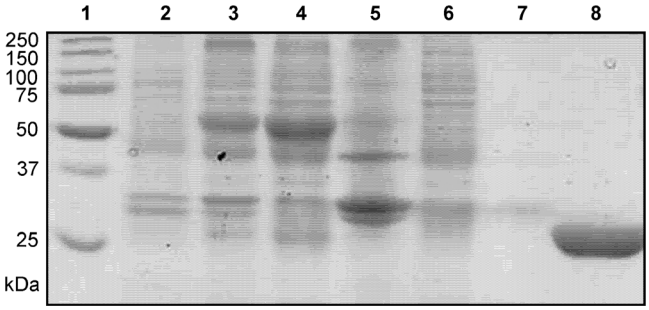


Figure 1. Electrophoretic analysis, 12% SDS-PAGE, of the recombinant RmlA protein expression in *E. coli*. Protein bands were stained with Comassie blue. Lane 1: protein standards; lane 2: crude extract from uninduced cells; lane 3: crude extract from cells induced at 25 °C for 24 h; lane 4: clarified lysate; lane 5: pellet; lane 6: flow through from chitin column; lane 7: wash; lane 8: elution of RmlA after stopping column flow and inducing the cleavage reaction at 4 °C for 16 h.

expected molecular weight of RmlA that had been fused with an intein and a chitin binding domain (RmlA-intein-CBD) (Figure 1). According to the SDS-PAGE, neither the C-terminal fusion protein (RmlA-intein-CBD) nor the cleaved proteins (RmlA and intein-CBD) were observed in the pellet (Figure 1, lane 5). Thus, RmlA was expressed as a soluble protein.

Evaluation of the Activity of RmlA

The activity of RmlA to synthesize UDP-Gal was first tested using galactose 1-phosphate (Gal-1-P) and UTP as substrates in the presence of inorganic pyrophosphatase. Inorganic pyrophosphatase was used to degrade the released pyrophosphate and, thus, drive the UDP-Gal production equilibrium forward. In addition, it was reported that the presence of a divalent metal ion catalyzes the decomposition of the nucleotide diphosphate sugars.[18] Thus, during the assay, UDP-Gal may have decomposed into the 1,2-cyclic phosphate derivative of the sugar and UMP. Based on our observations, as the temperature and incubation time increased, the production of UDP increased due to the decomposition of UTP. Furthermore, commercial UTP is commonly contaminated with up to 3% of UDP, which could interfere with the assay results. To address the aforementioned effects, the impact of temperature on the activity of the purified enzyme was studied using RP-HPLC to monitor the consumption of UTP and the formation of UDP-Gal, UDP, and UMP.

Because the recombinant protein was originally cloned from a thermophilic bacterial strain, *A. thermoaerophilus*, which usually grows at 55–75 °C, the temperature effect on the enzymatic activity was ex-

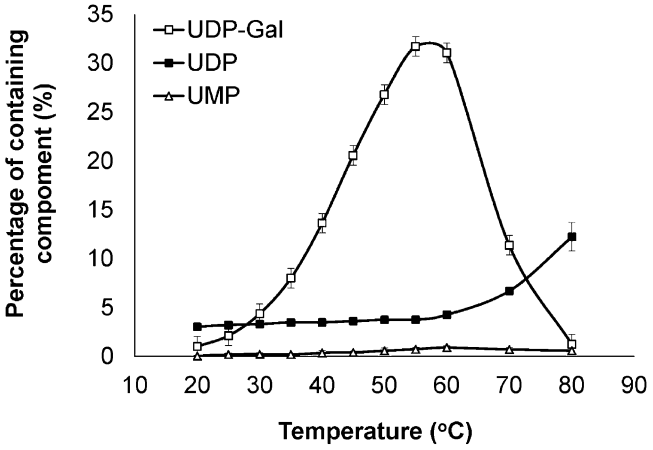


Figure 2. The effect of temperature on the activity of thymidylyltransferase (RmlA). To check activity at various temperatures, RmlA was incubated with standard assay components for 10 min.

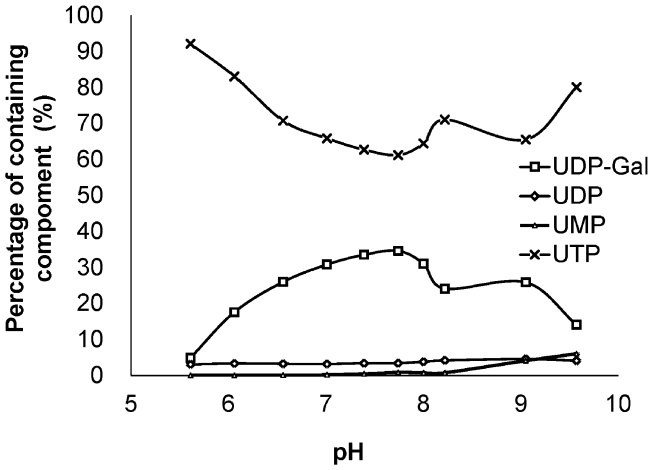


Figure 3. The pH profile of thymidylyltransferase (RmlA) activity. The activities were measured in triplicate at the indicated pH values at 55 °C for 10 min. The buffers (50 mM) that were used included: MES, pH 5.5–6.5; HEPES, pH 7.0–8.2 and CAPSO, pH 9.0–9.5.

amined from 20 to 90 °C (Figure 2). A HEPES-NaOH buffer (pH 7.5) that contained Mg^{2+} as a cofactor was chosen for the initial reaction conditions. The reaction was initiated by adding Gal-1-P to the reaction mixture. The results revealed that the accelerated formation of UDP was observed when the reaction temperature was higher than 60 °C. A similar phenomenon was also observed without the addition of Gal-1-P, indicating that the formation of UDP is due to the decomposition of UTP. Additionally, the divalent metal ion-induced hydrolysis of UDP-Gal was also observed (Supporting Information, Figure S2). The formation of UMP was observed as the amount of UDP-Gal increased due to divalent metal ion-induced hydrolysis. To further confirm this observation, pure UDP-Gal was incubated with the divalent metal ion, and it was subsequently observed that higher concentrations of UDP-Gal at higher temperatures generated more UMP (Supporting Information, Figure S2). To suppress the decomposition of UDP-Gal, the optimal temperature of the RmlA reaction was identified and set to 55 °C. Notably, no product formation was observed in the absence of RmlA, Gal-1-P, Mg^{2+}, or UTP.

As shown in Figure 3, based on observing the UDP formation, the reaction pH was observed to not influence the UTP hydrolysis. Although the UTP consumption rate was high at a pH above 8.5, an accelerated formation of UDP-Gal was not observed. In contrast, the accelerated formation of UMP was observed due to the decomposition of UDP-Gal at high pH (> 8.0). Thus, the optimal pH for the RmlA-catalyzed UDP-Gal production reaction was approximately 7.5.

It has been known that Mg^{2+} can bind to the β- and γ-phosphate of ATP so as to enhance the enzymatic reaction rate of AMP transference.[19] However, the presence of a divalent metal ion may also catalyze the decomposition of the nucleotide diphosphate sugar to produce UMP. To further enhance the production of UDP-Gal and suppress its hydrolysis, the effect of the divalent metal cation was then evaluated. Many divalent metal cations, such as Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, were tested [Figure 4, (**a**)]. In the presence of Mn^{2+}, RmlA exhibited an enhancement of the enzyme activity as indicated by the rapid consumption of UTP. However, the Mn^{2+} ion also accelerated the formation of UMP that is produced by the hydrolysis of UDP-Gal. Decreasing the reaction temperature to 25 °C could have reduced the rate of hydrolysis. Based on observations of the UDP-Gal production [Figure 4, (**a**)], Ni^{2+}, Fe^{2+}, and Mg^{2+} were good cofactors for RmlA; however, during the experiment, a white precipitate appeared when Ni^{2+} or Fe^{2+} was used. Thus, the stability of RmlA in the presence of these specific cations was investigated. The presence of Ni^{2+} and Fe^{2+} decreased the enzyme activity by 50% and 80%, respectively [Figure 4, (**b**)]. In consideration of the highest UDP-Gal production yield and the enzyme stability, Mg^{2+} was the best cofactor for the RmlA-catalyzed reaction.

Because the presence of divalent metal cations decomposed UDP-Gal, its concentration in the reaction buffer may also affect the rate of UDP-Gal hydrolysis (Supporting Information, Figure S2). As shown in Figure 5, the production yield of UDP-Gal was correlated with the Mg^{2+} concentration; however, at higher cation concentrations (e.g., 40 and 50 mM), UDP-Gal decomposition was observed as indicated by the formation of UMP. In consideration of the UDP-Gal stability and to reduce its hydrolysis, the optimal Mg^{2+} concentration was determined to be 10 mM. It is im-

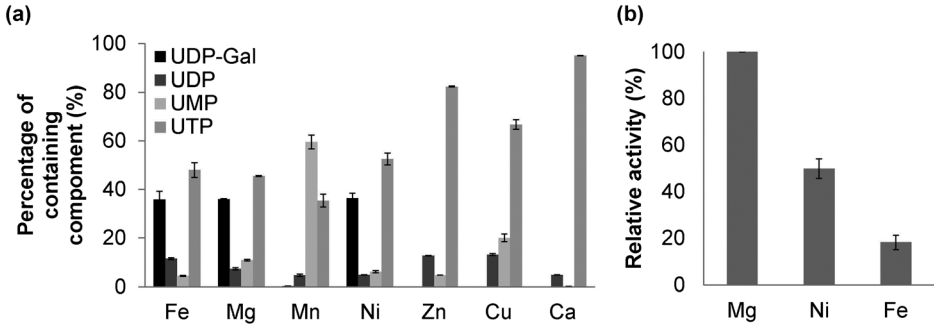


Figure 4. (**a**) The effect of the divalent metal cation on the activity of thymidylyltransferase (RmlA) at 55 °C. (**b**) The stability of RmlA that had been incubated with various metal ions at 10 mM for 10 min at 55 °C. The standard assay mixtures were added at a pH of 7.5 for 10 min at 55 °C.

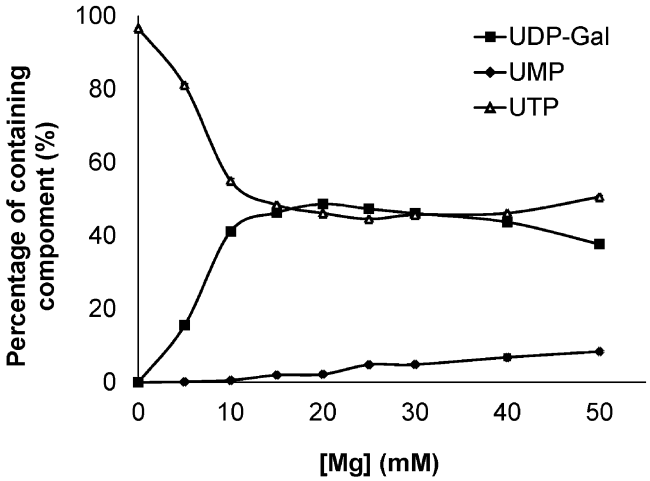


Figure 5. The concentration effect of Mg^{2+} on the activity of thymidylyltransferase (RmlA). The purified RmlA was incubated under standard assay mixtures at a pH of 7.5 with increasing concentrations of Mg^{2+} ions for 10 min at 55 °C.

portant to note that although RmlA is a thermophilic bacterial enzyme, under maximum activity conditions, the purified RmlA lost little of its activity at 55 °C after three hours of incubation. Nevertheless, the enzyme is stable at 25 °C for at least 16 days (Supporting Information, Figure S3).

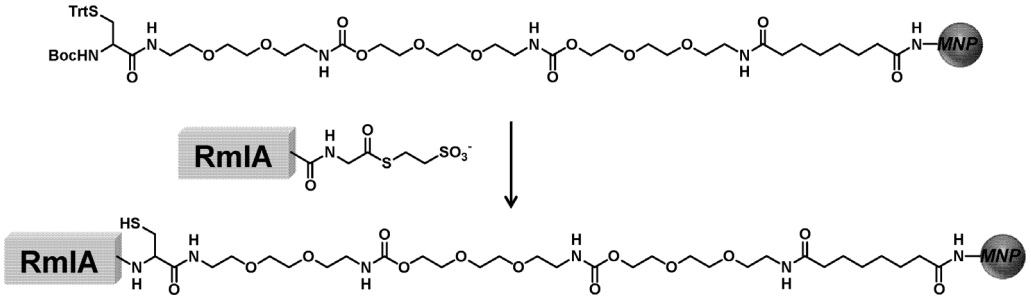
Production of UDP-Galactose

Although Gal-1-P was not a natural substrate of RmlA, it was thought that using optimal reaction conditions and high concentrations of the enzyme should switch the reaction equilibrium to product formation. To investigate the feasibility of using RmlA in practical organic synthesis, a preparative scale (100 mg) synthesis of UDP-Gal was performed using RmlA, Gal-1-P, and UTP with 10 mM Mg^{2+} in a 50 mM HEPES buffer (pH 7.5) at 55 °C for 1.5 h. This reaction resulted in a 90% yield.

Immobilization of RmlA on MNP

Many reports have shown that enzymes that are encapsulated or entrapped on a solid support can retain their activities. In some cases, the encapsulated enzymes are even more stable than those in solution.[20] Previously, we demonstrated that a nano-sized particle was a better support than a micro-sized particle to retain enzyme activity after immobilization.[16] Thus, we immobilized RmlA on a magnetic nanoparticles (MNPs) in a site-specific manner. We used the intein-mediated protein purification system and native chemical ligation (NCL) to generate RmlA-MNP (Scheme 2, pathway b). The advantage of this method was that the enzyme could be directly separated from the reaction mixture by a magnet and reused, and with site-specific immobilization, the highly oriented enzyme should provide a higher activity than one that was randomly immobilized.[21]

It has been suggested that the strong physical adsorption of polyethylene glycol to the hydrophobic core of the protein along with surface adsorption results in the stability of the protein.[22] To reduce the steric hindrance between the enzyme and MNP, a long linker with nine repeated units of ethylene glycol was used. The use of a polyethylene glycol linker should also enhance the solubility of the enzyme-MNP complex. As shown in Scheme 3, the enzyme was immobilized on a PEGylated MNP by NCL. The enzyme concentration on the MNP was determined to be 38.76 μg/RmlA-MNP mg by a BCA protein assay. The recycling ability of RmlA-MNP was then evaluated by assessing the RmlA activity of the same RmlA-MNP in repeated reactions. After being mixed with reagents and allowing the reaction to proceed at 55 °C for 10 min, the RmlA-MNP was separated from the reaction solution with a magnet. As shown in Figure 6, the activity of the RmlA-MNP maintained 95% of its original activity after ten reactions.



Scheme 3. Illustration of the protein isolation method *via* an intein-mediated protein purification system. The intein-mediated protein purification system and native chemical ligation were combined for site-specific covalent immobilization of the target proteins

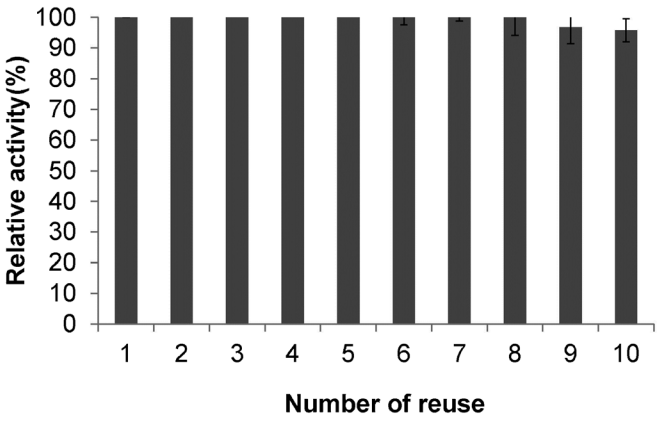


Figure 6. The enzymatic activity of an RmlA-MNP that was repetitively used in an enzyme assay.

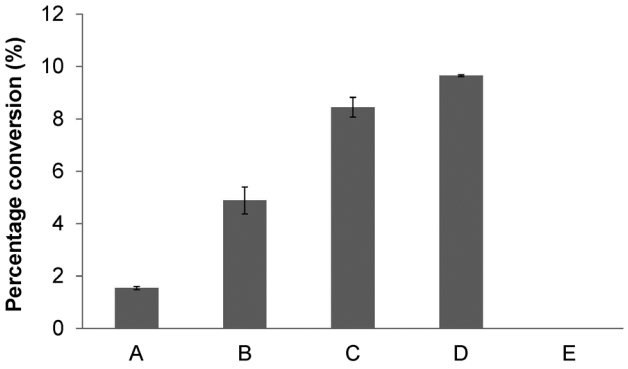


Figure 7. The effects of metal ions, EDTA, and DTT on the activity of GalT. The activity was determined in triplicate enzymatic reactions at 25 °C for 3 min. Different adducts, BSA, DTT, and EDTA, were added to the 50 μL reaction mixture. (A) Normal, (B) 0.2 mM DTT, (C) 1 mg mL^{-1} BSA, (D) 0.2 mM DTT and 1 mg mL^{-1} BSA, and (E) 5 mM EDTA. The purified GalT was incubated with standard assay mixtures at a pH of 7.5 with 10 mM Mn^{2+} ions. The substrates that were used for GalT were GlcNAc-cy3 and UDP-Gal. The Y-axis is the conversion of GlcNAc-cy3 into LacNAc-cy3.

Expression and Purification of the Recombinant Enzyme (GalT)

With UDP-Gal in hand, we wanted to combine it with GalT to synthesize LacNAc. Many β-1,4-GalTs from different sources have been identified.[23] The expression of mammalian GalTs has been achieved using eukaryotic hosts; however, they require expensive tissue culture media and have only produced moderate yields of enzymes. Although the expression of mammalian GalTs by *E. coli* has been reported, these attempts have mainly produced insoluble forms of the enzymes that were difficult to reactivate in large amounts; however, in comparison to mammalian β-1,4-GalTs, bacterial GalTs have the advantage of easy production in *E. coli* and better tolerance to substrate modification.[24]

Previous studies have shown that β-1,4-GalT from *Neisseria meningitides* loses its activity when a histidine tag is fused to either its N- or C- terminus.[25] To facilitate the purification step of the GalT in this study, the β-1,4-GalT gene (*lgtB*) was cloned and then overexpressed in *E. coli* by an intein-mediated protein purification system. Similar to the purification of RmlA, 15 mg of GalT was obtained as a soluble protein from 1 L of *E. coli* culture. To estimate the activity of GalT, GlcNAc-cy3 (compound **3** in Scheme 1) was chosen as a substrate. The specific activity of the purified GalT enzyme was determined to be 2 U at a pH 7.5 and 25 °C.

Optimization of GalT Activity

To improve the reactivity of the GalT, 0.2 mM DTT and 1 mg/mL BSA were added to the solution.[26] As shown in Figure 7, the results reveal that the presence of 0.2 mM DTT and 1 mg/mL BSA enhanced the GalT activity by 3-fold and 5.5-fold, respectively (Figure 7, columns B and C). The combination of both DTT and BSA further increased the Ga1T activi-

ty by 6.3-fold (Figure 7, column D). In contrast, the presence of 5 mM EDTA depleted GalT activity (Figure 7, column E), indicating the requirement of the metal ion for enzymatic activity.

Production of LacNAc-N_3

To demonstrate the use of RmlA in carbohydrate synthesis, LacNAc was selected as a target and GlcNAc-linker-N_3 (**2**) was used as the acceptor. The hydrophobic character of the linker, 6-azidohexan-1-ol, facilitated chromatographic purification of the product. The azido group can easily be reduced to an amine or be linked to another molecule by click chemistry. The optimal reaction temperatures of RmlA and GalT are not the same. Thus, to implement the use of these two enzymes in the synthesis of LacNAc, the reaction was initially set at 55 °C without the addition of GalT. The production of UDP-Gal was monitored by HPLC, which reached a 90% yield in 90 min. Next, the reaction mixture was cooled to 25 °C. GalT and GlcNAc-N_3 acceptor were added to the cooled reaction solution to final concentrations of 2 μM and 10 mM, respectively. The reaction mixture was stirred for 2 h, resulting in an 80% yield of LacNAc-linker-N_3 (**4**) on a 100-mg scale. Notably, RmlA coupled with GalT in one-pot at 25 °C only provides a low yield of LacNAc due to the decomposition of GalT after incubation for 10 h.

Conclusions

Although the general biochemical properties of RmlA are similar to other uridylyltransferases that are found in prokaryotic and eukaryotic cells, RmlA from *A. thermoaerophilus* exhibited an unusual tolerance to high temperatures that facilitates its long-term storage. Due to its broad substrate specificity and reasonably efficient turnover rate of non-native substrates, RmlA served as a useful biocatalyst in the synthesis of UDP-Gal. Using Mg^{2+} as a cofactor, a 100-mg scale synthesis of UDP-Gal was achieved using RmlA. Furthermore, RmlA was site-specifically and covalently immobilized on an MNP using a combination method of intein-mediated protein expression and NCL. RmlA-MNP retains almost 95% of its activity after reuse following ten consecutive enzyme assays. Additionally, by using the intein-mediated protein expression system, we successfully overexpressed and purified β-1,4-GalT. This protein was previously known to exhibit a low activity when a histidine tag was fused to either its N- or C- terminus. GalT was relatively stable at 25 °C, and its activity was enhanced in the presence of DTT and BSA. Thus, the use of RmlA and GalT to synthesize LacNAc was feasible by sequentially adding of enzyme and adjusting the reaction temperature. These results reveal the potential applications of RmlA in carbohydrate synthesis.

Experimental Section

Materials

The enzymes and reagents that were used for the molecular biology procedures, in addition to the DNA ladders and deoxynucleotide triphosphates (dNTPs), were purchased from Violet BioScience or New England Biolabs. The oligonucleotides that were used for DNA amplification were synthesized by Mission Biotech. The vector that encoded the rmlA gene was a kind gift from Joseph Lam (Department of Molecular and Cellular Biology, University of Guelph). The *S. cerevisiae*-derived thermostable inorganic pyrophosphatase was purchased from Sigma as a 1,000 U/mg lyophilized powder. Protein molecular weight standards were obtained from BioRad. The QIAquick gel extraction kit was obtained from Qiagen, and the pGEM-T Easy Vector System I was purchased from Promega. All other chemicals were obtained from Sigma Chemical Company unless otherwise stated.

General Methods

The procedures for standard DNA manipulation including plasmid DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation, and *E. coli* transformation were performed as suggested by the manufacturer. The PCR reactions were performed in a My CyclerTM Thermal Cycler (Bio-Rad). Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Tris-HCl 10–20% gradients, Bio-Rad Laboratories, Hercules, CA). The gels were stained with Coomassie brilliant blue. Protein concentrations were determined from a BCA protein assay kit (Pierce) using bovine serum albumin as the standard.

PCR and Molecular Cloning

DNA amplification was performed in a solution (50 μL) that contained 50 ng of template DNA, 0.4 μM of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, and 2.5 U Taq polymerase. The conditions for PCR were as follows: denaturation at 95 °C for 30 sec, annealing at 55–65 °C for 30 sec, and extension at 72 °C for 1 min. The amplified fragment was cloned into a pGEM-T Easy vector. The insert was further confirmed by a blue/white screen and sequence analysis. The target gene was digested from the constructed T vector and inserted into an NdeI/SapI-treated pTXB1 vector (New England Biolabs). The ligation mixture was then transformed into DH5α and BL21 (DE3) strains of *E. coli*. DNA sequencing was used to confirm in-frame cloning of the full-length target gene. The genes for the enzymes were isolated by PCR amplification using the following primers:
rmlA-F 5′-GGG GGG CAT ATG AAA GGA ATT ATT TTA TCC GGT G GA AGC-3′,
rmlA-R 5′-GCT CTT CAG CAT CCC TCC ACT GTC ACA AGA TCT

TTG TC-3′,
lgtB-F 5′-CCC CAT ATG CAA AAC CAC GTT ATC AGC-3′,
lgtB-R 5′-GCT CTT CAG CAT CCT TGG AAA GGC ACA ATG AAC-3′,
rmlA-h-R 5′-GGG GGG GCT AGC GCC GCC CTC CAC TGT CAC AA GATC TTT GCT-3′, and
lgtB-h-F 5′-GGG GGG GCT AGC GTG CAA AAC CAC GTT ATC AGC TTA GC-3′.

Expression and Purification of the Enzymes

E. coli BL21 (DE3) that was carrying the above recombinant plasmids was cultured in a Luria-Bertani (LB) medium (10 g L^{-1} peptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl; 5 mL total) with ampicillin (100 μg mL^{-1}) overnight at 37 °C in an incubator (Firstek S300R) with vigorous shaking at 200 rpm. They were then transferred into fresh LB medium (500 mL) with ampicillin for another 2 h at 37 °C. When an optical density $OD_{600} = 0.6–0.8$ was reached, the culture was induced with 0.5 mM IPTG for 16–24 h at 16–25 °C. To avoid the cleavage of the thioester of the RmlA-intein fusion protein, the RmlA expression conditions were performed at 25 °C for 24 h with vigorous shaking (200 rpm).The bacterial cells were harvested by centrifugation at 5,000 × *g* at 4 °C for 15 min in a centrifuge (Eppendorff centrifuge 5810R). The cell pellets were re-suspended at 25 mL per liter cell culture in a column buffer (pH 8.0, a 20 mM HEPES buffer that contained 0.1% Triton X-100, 500 mM NaCl, and 0.1 mM EDTA). The cells were disrupted by sonication on ice for 2 min 50 sec in 10 s intervals and then the debris were removed by centrifugation for 60 min at 24,000 × *g* at 4 °C. The cell lysate was collected and applied to a 6 mL chitin bead column that was pre-equilibrated with column buffer. After sample loading onto the chitin column and incubation with the chitin affinity beads for 30 min at 4 °C, the column was washed with 20 column volumes of the same buffer. The resin was then quickly washed with 1 column volume of the same buffer that contained 80 mM DTT, and the effluent was reloaded. The column was clamped at both ends, and on-column cleavage of the intein tag from the fusion protein was performed by incubating the column at 4 °C for 16 h. The purified protein was sequentially eluted by 3 mL of the same buffer that contained 0.75 M, 1.0 M, and 1.5 M NaCl without DTT. According to a BCA protein assay, up to 60 mg of soluble RmlA protein were obtained from 1 L of *E. coli* culture. The effluent was concentrated by centrifugation using a centrifugal filter device (Amicon Ultra, Millpore), divided into aliquots, and stored at −30 °C.

Quantification of Purified Protein

Protein concentration was determined using the BCA™ Protein Assay Kit, and bovine serum albumin (BSA) was used as the standard. The absorbance of each sample was measured at 562 nm in a UV detector. To quantify the protein amount, a protein sample was reacted with BCA reagent at 60 °C for 30 min. The absorbance of the BCA reagent alone was measured at 562 nm. A standard curve was prepared by plotting the 562 nm measurement for each BSA standard *vs.* its concentration in μg/mL. The standard curve was used to determine the protein concentration of each sample.

Enzyme Activity Assay

In general, all RmlA-catalyzed reactions were performed in a 50 mM HEPES buffer (a pH of 7.5) that contained 10 mM $MgCl_2$. After UTP and an equimolar amount of Gal-1-P had been added to the reaction buffer, the pH of the solution was adjusted to 7.5 with 2 M NaOH. The reaction was initiated by the addition of Gal-1-P and allowed to incubate at 55 °C. These samples were then kept on ice until a 25 μL aliquot was injected and analyzed by an RP-HPLC system that was equipped with a membrane on-line degasser, temperature control unit, and UV detector. For the synthesis of UDP-Gal, the reaction was monitored by RP-HPLC analysis. For the pH profiling, the optimal pH for the enzyme activity was investigated by performing the enzymatic reactions in triplicate in a total volume of 50 μL of buffer with a pH that varied from 5.5 to 9.5 (5.5–6.5 MES, 7.0–8.2 HEPES, and 9.0–9.5 CAPSO). Reactions were allowed to proceed for 10 min at 55 °C and then quenched *via* the addition of 10% (v/v) SDS, to a final SDS concentration of 1%. For the recycling ability of RmlA-MNP, the activity was evaluated as described in the analysis of RmlA activity in the above. RmlA-MNP was mixed with reagents at 55 °C for 10 min and separated from the reaction solution with a magnet. The remaining solution was quenched with 1% SDS. The recovered RmlA-MNP was washed with a HEPES buffer (pH 8.0) and then directly used for the next identical reaction. For the activity of RmlA with different metal ions, RmlA was first incubated with Ni^{2+}, Mg^{2+}, or Fe^{2+} in a HEPES buffer at 55 °C for 10 min. Next, a mixture of UTP and Gal-1-P was added and the reaction was left to incubate for 10 min and then quenched via the addition of 10% (v/v) SDS, to a final SDS concentration of 1%. All the reactions were analyzed by RP-HPLC to determine the formation of UDP-Gal.

Hydrolysis of UDP-Galactose

Hydrolysis reactions of UDP-Gal were performed in a 50 mM HEPES buffer (a pH of 7.5) that contained 5–40 mM of the divalent metal ion. After the addition of UDP-Gal to the reaction buffer, the reactions were allowed to shake at 25 °C for 5 h. The reaction was monitored by HPLC analysis.

Reversed-Phase, High-Performance Liquid Chromatography (RP-HPLC) analysis

RP-HPLC analysis was performed using a reverse-phase C18 column (Vydac® 218TP54, GRACE) that was protected with a C18 guard column system. This system also featured a temperature control unit (maintained at 25 °C throughout the experiment) and UV-vis detector, and the system was controlled *via* chromatography software. For RmlA, the reaction was monitored at 270 nm. Buffer A (a 0.1 M potassium phosphate buffer that had been supplemented with 8 mM tetrabutylammonium hydrogen sulfate, pH 5.3) and buffer B (70% buffer A plus 30% methanol, pH 5.9) were prepared and used in two gradient conditions to separate the different compounds. The linear gradient consisted of 100% buffer A for 2.5 min, 0–40% buffer B for 7.5 min, 40–100% buffer B for 1 min, 100% buffer B for 4 min, and 100–0% buffer B for 1 min, followed by an equi-

libration phase of 100% buffer A for 4 min. The flow-rate was maintained at 1 mL min^{-1} for all of the separations. The retention times of UDP-galactose and UTP were 5.7 and 11.6 min, respectively.

The GalT reactions were monitored at 550 nm. Deionized water with 0.1% TFA and acetonitrile was used in two gradient conditions to separate the different compounds. The gradient conditions included 0–36% acetonitrile for 5 min, 36–47% acetonitrile for 15 min, and 47–50% acetonitrile for 2 min, followed by an equilibration phase of deionized water with 0.1% TFA for 5 min. The flow-rate was maintained at 1 mL min^{-1} for all of the separations. The retention times of LacNAc-linker-cy3 and GlcNAc-linker-Cy3 were 17.9 and 18.7 min, respectively.

Thermal Stability of RmlA and GalT

The enzymes were incubated at 25, 37 or 55 °C. Aliquots were taken at various time points for an activity assay. For RmlA, the activity assay was performed in a buffer that contained 50 mM HEPES at a pH of 7.5, 10 mM $MgCl_2$, 5 mM UTP, 5 mM Gal-1-P, and 0.5 EU inorganic pyrophosphatase. For GalT, the activity assay was performed in a buffer that contained 50 mM HEPES at a pH of 7.5, 10 mM $MgCl_2$, 0.25 mM GlcNAc-linker-Cy3, and 0.30 mM UDP-Gal. All of the reactions were monitored over time by RP-HPLC.

Synthesis of UDP-Gal

The synthesis of UDP-Gal was performed in 20 mL of reaction buffer that contained 50 mM HEPES at a pH of 7.5, 10 mM $MgCl_2$, 10 mM UTP, 10 mM Gal-1-P, 10 EU inorganic pyrophosphatase, and 25 mg RmlA. The reaction was first performed at 55 °C for 90 min and monitored by RP-HPLC coupled to a UV detector. To isolate UDP-Gal, the tube was centrifuged for 5 min at 16,200 × *g* at 4 °C, and the clear supernatant was collected. A diluted solution was applied to an ion-exchange column of DEAE-cellulose and was eluted with a 0 to 0.5 M gradient of NaCl. UDP-Gal was desalted with water on a Bio-gel P2 column under gravity flow, and the purified product was lyophilized to give 109 mg (90% yield). NMR spectra were recorded in D_2O and are in agreement with reported data.[10] Electrospray ionization mass spectrometric (ESI-MS) analysis identified a peak at $m/z =$ 565.0471.

Synthesis of LacNAc-N_3

The synthesis of LacNAc-linker-N_3 was performed in 20 mL of reaction buffer that contained 50 mM HEPES at a pH of 7.5, 10 mM $MgCl_2$, 10 mM GlcNAc-linker-N_3, 10 mM UTP, 10 mM Gal-1-P, and 10 EU inorganic pyrophosphatase. The reaction was first performed at 55 °C for 90 min and monitored by RP-HPLC coupled to a UV detector (to measure UDP-Gal). Next, β-1,4-GalT was added. The resulting mixture was incubated at 25 °C for 2 h, and the reaction was monitored by silica thin-layer chromatography (TLC: MeOH/CH_2Cl_2 = 1/2) and RP-HPLC coupled to a UV detector (to measure UDP). The product was purified by silica gel column chromatography, desalted using BioRad P-2 resin, and lyophilized. The reaction resulted in an 80% yield (80 mg). ^{1}H NMR (600 MHz, CD_3OD): $\delta = 1.21$–1.25 (m, 4H), 1.42–1.48 (m, 4H), 1.90 (s, 3H), 3.19 (t, $J = 6.9$ Hz, 2H), 3.41 (t, $J = 8.8$ Hz, 1H), 3.44–3.49 (m, 2H), 3.54 (dd, $J = 3.3$, 10.0 Hz, 1H), 3.57–3.65 (m, 6H), 3.69 (dd, $J = 5.2$, 12.3 Hz, 1H), 3.75–3.79 (m, 2H), 3.85 (dd, $J = 1.9$, 12.3 Hz, 1H), 4.34 (d, $J = 7.8$ Hz, 1H), 4.39 (d, $J = 7.8$ Hz, 1H); ^{13}C NMR (150 MHz, D_2O): $\delta = 22.02$, 24.47, 25.40, 27.81, 28.25, 50.97, 54.96, 59.92, 60.85, 68.38, 70.22, 70.80, 72.29, 72.34, 74.58, 75.18, 78.31, 100.88, 102.71, 174.22; HR-MS-(ESI-TOF): $m/z = 531.2271$, calcd. for $C_{20}H_{36}N_4O_{11}Na$ $[M+Na]^+$: 531.2278.

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