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In-microbe formation of nucleotide sugars in engineered *Escherichia coli*

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ABSTRACT

Numerous different nucleotide sugars are used as sugar donors for the biosynthesis of glycans by bacteria, humans, fungi, and plants. However, many of these nucleotide sugars are not available either in their native form or with the sugar portion labeled with a stable or radioactive isotope. Here we demonstrate the use of *Escherichia coli* metabolically engineered to contain genes that encode proteins that convert monosaccharides into their respective monosaccharide-1-phosphates and subsequently into the corresponding nucleotide sugars. In this system, which we designated "in-microbe", reactions occur within 2 to 4 h and can be used to generate nucleotide sugars in amounts ranging from 5 to 12.5 μg/ml cell culture. We show that the *E. coli* can be engineered to produce the seldom observed nucleotide sugars UDP–2-acetamido-2-deoxy-glucuronic acid (UDP–GlcNAcA) and UDP–2-acetamido-2-deoxy-xylose (UDP–XylNAc). Using similar strategies, we also engineered *E. coli* to synthesize UDP–galacturonic acid (UDP–GalA) and UDP–galactose (UDP–Gal). ^{13}C- and ^{15}N-labeled NDP–sugars are formed using [^{13}C] glucose as the carbon source and with [^{15}N]NH_4Cl as the nitrogen source.

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Nucleotide sugars are the sugar donors used for the formation of polysaccharides, glycoproteins, proteoglycans, and glycolipids as well as for the synthesis of glycosylated secondary metabolites [1] and glycosylated antibiotics [2]. At least 70 different nucleotide sugars have been identified in bacteria, and 30 activated sugars have been detected in plants. By contrast, humans and fungi are believed to synthesize 10 and up to 15 activated sugars, respectively. Although virtually all organisms produce UDP–glucose and GDP–mannose, only a small number of organisms are capable of forming ADP–glucose, TDP–glucose, GDP–glucose, or CDP–glucose. Moreover, some nucleotide sugars may be unique to a group of organisms. For example, only land plants have been shown to synthesize UDP–apiose. Different metabolic pathways exist for the formation of nucleotide sugars. Thus, photosynthetic organisms fix CO_2 and use it to generate Frc, which can then enter into different metabolic pathways for the synthesis of activated sugars, including GDP–Man, UDP–Glc, ADP–Glc, and UDP–GlcNAc. On the other hand, bacteria, fungi, and mammals rely on acquired carbon for making precursors that enter into nucleotide sugar metabolic pathways.

We recently identified a set of operons in the gram-negative bacteria *Bacillus cereus* subsp. cytotoxis NVH 391-98, which contain genes encoding proteins that catalyze the formation of the seldom observed sugar nucleotides UDP–2-acetamido-2-deoxy-xylose (UDP–XylNAc)[1] and UDP–2-acetamido-2-deoxy-glucuronic acid (UDP–GlcNAcA) [3]. The bacterium contains other operons that may function to generate UDP–GlcA (and possibly TDP–GlcA) and UDP–GalA (and possibly TDP–GalA). Each of the operons also contains genes annotated as glycosyltransferases that may use these activated sugars for glycan synthesis. To study these putative glycosyltransferases, we require a convenient supply of the appropriate activated sugars. We found that purified recombinant enzymes were not cost-effective for synthesizing UDP–XylNAc and UDP–GlcNAcA in amounts sufficient for such studies. Furthermore,

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[1] *Abbreviations used:* UDP–XylNAc, UDP–2-acetamido-2-deoxy-xylose; UDP–GlcNAcA, UDP–2-acetamido-2-deoxy-glucuronic acid; UGlcNAcDH, UDP–*N*-acetylglucosamine dehydrogenase; UXylNAcS, UDP–*N*-acetylxylose synthase; GalAK, galacturonic acid kinase; GalK, galactose kinase; Sloppy, UDP–sugar pyrophosphorylase; IPTG, isopropyl β-D-thiogalactoside; PBS, sodium phosphate and NaCl; LC–ESI–MS/MS, liquid chromatography–electrospray ionization–tandem mass spectrometry; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; MS, mass spectrometry; TEAA, triethylammonium acetate; AcN, acetonitrile; CID, collision-induced dissociation; 1D, one-dimensional; 2D, two-dimensional; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlated; [^{13}C]GlcN-1-P, [^{13}C]glucosamine-1-P; PRPP, 5-phospho-α-^{d}-[^{13}C]ribose-1-diphosphate; PPi, pyrophosphate.

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generating ^{15}N- or ^{13}C-labeled UDP–XylNAc and UDP–GlcNAcA required the use of additional enzymes to form the appropriate isotopically labeled monosaccharides. Here we report an engineered biological system we named "in-microbe" that uses *Escherichia coli* modified to contain sets of genes encoding proteins that transform a sugar into its corresponding sugar-1-phosphate and subsequently into the desired NDP–sugar. We show that the in-microbe system could be adopted to produce a broad range of rare and common activated sugar metabolites, including UDP–GlcNAcA, UDP–XylNAc, UDP–Gal, and UDP–GalA.

Materials and methods

[^{13}C]Glc and [^{15}N]NH_4Cl compounds were purchased from Cambridge Isotope Laboratories. Glc is uniformly labeled. M9 and CA media were purchased from Difco.

Cloning of NDP–sugar metabolic enzymes was carried out as described in the following sections.

Formation of plasmids harboring UDP–N-acetylglucosamine dehydrogenase and UDP–N-acetylxylose synthase from Bacillus

The gene encoding the *B. cereus* UDP–*N*-acetylglucosamine dehydrogenase (UGlcNAcDH) was amplified using pET28b:BcUGlcNAcDH as the template along with forward (5′-TGCTGCCACCGCTGAGCAAATTAATACGACTCACTATAGGGG-3′) and reverse (5′-CCAAGGGGTTATGCTAGTTATTGCTCAGC-3′) primer sets and Phusion Hot Start DNA Polymerase (New England Biolabs). The primers were designed with a 15-nucleotide perfect homology (underlined) to the region of pET28 flanking the *Blp*I site. Following polymerase chain reaction (PCR) amplification, the DNA was digested with *Dpn*I and purified using a spin column (Qiagen). The DNA was cloned by in-fusion reaction (Clontech, according to the manufacturer's instructions) to a *Blp*I/linearized pET28b-BcUXylNAcS. The resulting plasmid (pET28b:BcUXylNAcS + BcUGlcNAcDH) has two T7 promoters: one for the expression of UDP–*N*-acetylxylose synthase (UXylNAcS) and the other for the expression of UGlcNAcDH.

Formation of plasmids harboring galacturonic acid kinase, galactose kinase, and UDP–sugar pyrophosphorylase

Plasmids with genes encoding *Arabidopsis* galacturonic acid kinase (GalAK, At3g10700), galactose kinase (GalK, At3g06580), and UDP–sugar pyrophosphorylase ("Sloppy", At5g52560) were generated as described previously [4]. The *Nco*I–*Not*I fragment of Sloppy derived from pET28b:At5g52560#a73f/2#2 was subcloned to the pACy-duet-1 vector to create pACy:At5g52560#6. GalK (pET28a:At3g06580) and Sloppy (pACy:At5g52560) plasmids were cotransformed into the BL21(DE3)-derived *E. coli* strain (Novagen) for coexpression, and clones were selected on medium supplemented with 30 μg/ml chloramphenicol and 50 μg/ml kanamycin. Plasmid harboring GalAK (pET28b:At3g10700) and Sloppy (pACy:At5g52560) were cotransformed into the BL21(DE3)-derived *E. coli* strain. An empty vector was used as a control.

Extraction of NDP–sugars

Bacterial strains (3 ml) harboring the different expression plasmids were grown overnight in LB medium (per l: 10 g Bacto tryptone, 5 g Bacto yeast extract, and 10 g NaCl) or M9/CA medium (per l: 6.78 g sodium phosphate dibasic, 3 g potassium phosphate monobasic, 0.5 g NaCl, 1 g ammonium chloride, and 8 g casamino acid) supplemented with chloramphenicol (30 μg/ml) and kanamycin (50 μg/ml) at 37 °C and 250 rpm. Portions of the culture medium were used to inoculate fresh medium (5 ml) and allowed to grow to an OD_{600nm} of 0.4 and 0.6. The medium was then supplemented with an appropriate carbon source (sugar or ^{13}C-labeled sugar at 0.2% [w/v]) or nitrogen source ([^{15}N]NH_4Cl at 0.2% [w/v]). Isopropyl β-D-thiogalactoside (IPTG, 0.5 mM) was added, and the cells were allowed to grow for up to 4 h at the indicated temperature. A portion (3 ml) of the culture was removed and centrifuged (18,000*g*, 1 min, 22 °C). The cell pellet was washed twice with 4 volumes of 10 mM sodium phosphate (pH 7.5) and 150 mM NaCl (PBS) and then suspended in 75 mM NaF. Then, 10 volumes of cold chloroform/methanol (1:1, v/v) was added, and the sample was mixed for 30 min on ice. The suspension was centrifuged (18,000*g*, 4 min, 22 °C), and the upper aqueous phase was collected and recentrifuged. Portions of this aqueous phase were analyzed by high-performance anion exchange chromatography, liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS), and ^1H NMR (nuclear magnetic resonance) spectroscopy.

Analysis of NDP–sugars

The aqueous extracts of the *E. coli* cells were separated on a Q15 anion exchange column (1 mm i.d. × 250 mm, Amersham) using an Agilent series 1100 HPLC (high-performance liquid chromatography) system equipped with an autosampler, a diode array detector, and ChemStation software (version B.04.02). Samples (30 μl) were injected, and the column was washed at 0.2 ml min^{-1} for 3 min with 20 mM ammonium formate (pH 8.4). Nucleotides were then eluted with a linear gradient from 20 to 500 mM ammonium formate over 23 min. Nucleotides were detected by A_{261nm} and quantified using calibration curves generated from standard UDP–sugars. The peaks corresponding to UDP–GlcNAcA (retention time [*Rt*] = 23.2 min), UDP–XylNAc (*Rt* = 16.0 min), UDP–Gal (*Rt* = 16.9 min), and UDP–GalA (*Rt* = 23.8 min) were collected, lyophilized, and analyzed by NMR spectroscopy and ESI–MS/MS. Column fractions (0.4 ml) were collected, and either they were lyophilized and reconstituted in D_2O (150 μl) for ^1H NMR analysis or a portion was analyzed by LC–ESI–MS/MS.

For ESI–MS (mass spectrometry) analysis, each column fraction (47.5 μl of the 0.4 ml) or standard nucleotide sugar (1–100 μM in 47.5 μl) was mixed with 2.5 μl of 0.4 M triethylammonium acetate (TEAA, pH 7.0, Sigma) and 50 μl of acetonitrile (AcN). Samples were analyzed using a linear ion trap mass spectrometer (LTQ-XL, ThermoFisher) with direct infusion at a rate of 3–10 μl/min using a Harvard Apparatus syringe pump. Samples (100 μl) were also introduced via the HPLC autosampler at 25 μl/min with 10 mM TEAA (pH 7.0) in 50% AcN [5]. Negative ion spectra were recorded over the mass range *m*/*z* 100–1000. Prominent ions in the mass spectrum were selected and subjected to collision-induced dissociation (CID) with a helium collision gas pressure of 3×10^{-3} Torr and collision voltage set to 25 V to give an MS/MS product ion spectrum. UDP–Glc (exact theoretical mass of 566.047) gave an $[M-H]^-$ ion at *m*/*z* 565.08, and when fragmented it produced a major product ion at *m*/*z* 323.00 corresponding to a $[UMP-H]^-$ ion (UMP calculated theoretical mass of 324.036). The MS assignments of other NDP–sugars are summarized in Supplemental Table S1 (see Supplementary material).

Analyzes of ^{13}C- or ^{15}N-labeled NDP–sugars by NMR spectroscopy

Overnight cultures of *E. coli* were diluted into M9/CA medium and grown to OD_{600nm} = 0.6. The culture (5 ml) was induced by IPTG (0.5 mM), [^{13}C]Glc or [^{15}N]NH_4Cl was added (0.2%), and the culture was grown up to 4 h. After NDP–sugar extraction, sample was chromatographed and HPLC column fractions (0.4 ml) were collected, lyophilized, and then dissolved in D_2O (150 μl) for

NMR spectroscopic analysis. One-dimensional (1D) ^{1}H- and two-dimensional (2D) ^{13}C HSQC (heteronuclear single quantum coherence) or ^{15}N HMBC (heteronuclear multiple bond correlated) spectra of the HPLC-purified UDP–GlcNAcA or UDP–XylNAc (lyophilized and resuspended in 150 μl of D_2O) were collected at 25 °C using a Varian/Agilent Direct Drive 600- or 900-MHz spectrometer. The HSQC spectra were collected with a carbon spectral width of 170 ppm, centered at 75.1 ppm, and with 128 points of 200 transients each. The HMBC spectra were collected with a nitrogen width of 65.8 ppm, centered at 139.6 ppm, and with 64 points of 400 transients each.

Results and discussion

Engineering E. coli to produce UDP–GlcNAcA and UDP–XylNAc

Cultures of *E. coli* harboring the UDP–GlcNAc dehydrogenase gene were shown to generate a product that had a retention time of 23.2 min on anion exchange chromatography (see Fig. 1 and compare panel A with panel B [control]). The peak was collected and analyzed by LC–ESI–MS/MS (Fig. 1C) and by ^{1}H NMR (see Supplemental Fig. S1 in Supplementary material). The ESI–MS of the product contained an $[M–H]^-$ ion at m/z 620.08, and CID of this ion gave a predominant fragment ion at m/z 403.00 corresponding to deprotonated UDP. The chemical shifts of the product ^{1}H NMR spectra (Supplemental Fig. S1) are consistent with UDP–GlcNAcA [3].

E. coli produced approximately 12.5 μg/ml UDP–GlcNAcA. These data suggest that the introduced gene shunts the endogenously synthesized UDP–GlcNAc to UDP–GlcNAcA in amounts that do not have a discernible effect on the growth of the bacterium. The amounts of UDP–GlcNAc or UDP–GlcNAcA formed were not increased by adding Frc or L-glutamine to the LB culture medium (data not shown). In most experiments, the cell pellet was suspended with NaF because a previous study [6] had suggested that this compound inhibits enzymes that degrade NDP–sugars. However, comparable yields of UDP–GlcNAcA were obtained if the bacterial cells were resuspended in water rather than NaF.

We next determined whether *E. coli* could be engineered to produce UDP–XylNAc. For this purpose, we introduced a single plasmid that independently drives the expression of the *B. cereus* genes encoding UDP–GlcNAc dehydrogenase and UDP–XylNAc synthase. Aqueous extracts of cells harboring both genes (Fig. 1D) but not the control cells (Fig. 1E) gave a distinct peak (see arrow 2) when analyzed by HPLC. The ESI–MS of the collected peak (Fig. 1F) contained an $[M–H]^-$ ion at m/z 576.08 as expected

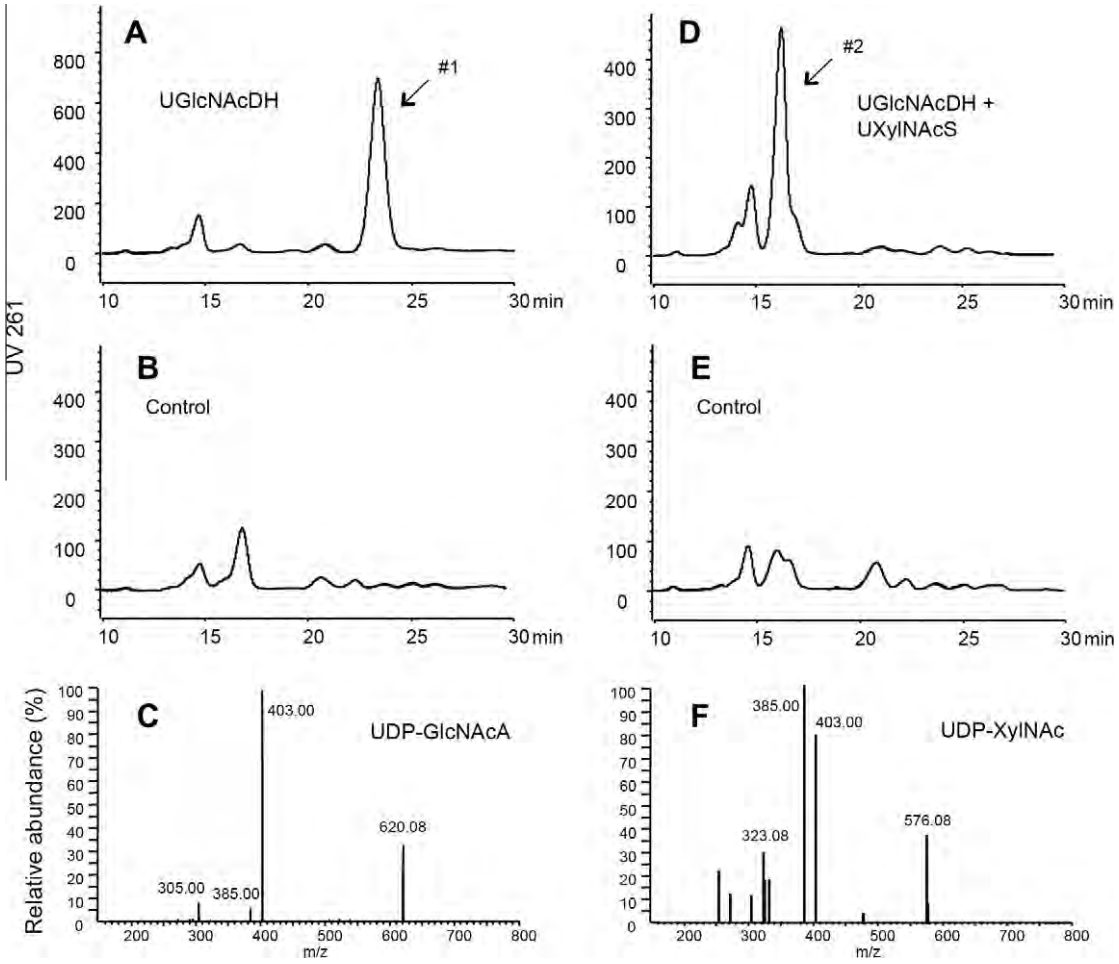

Fig.1. Analyses of UDP–GlcNAcA and UDP–XylNAc produced in-microbe by engineered *E. coli* using anion exchange chromatography and ESI–MS. Cell cultures [expressing UDP–GlcNAc dehydrogenase (A), UDP–GlcNAc dehydrogenase and UDP–XylNAc synthase (D), or plasmid control (B and E)] were extracted 4 h after induction. An aliquot from each sample was separated by Q15-HPLC [25] and analyzed at A_{261nm} (A, B, D and E). The peaks labeled 1 and 2 were collected and analyzed by ESI–MS (C and F). Panel A shows the formation of UDP–GlcNAcA (marked by arrow 1) in engineered cells when compared with control cells (B), and panel D shows the formation of UDP–XylNAc (marked by arrow 2) in engineered cells when compared with control cells (E). The labeled peaks in panels A and D correspond to UDP–GlcNAcA (23.2 min) and UDP–XylNAc (16.0 min), respectively. Panels C and F show the negative ion mode MS/MS analysis of the parent $[M–H]^-$ UDP–sugars and their MS/MS fragments (see details in Supplemental Table S1).

for UDP–XylNAc [3]. Negative ion MS/MS analysis of this ion fragment gave fragment ions at *m/z* 385.00 and 403.00 corresponding to [UDP–H–water]$^-$ and [UDP–H]$^-$, respectively. The ^{1}H NMR spectrum of the product (Supplemental Fig. S2) contained signals with chemical shifts characteristic of UDP–XylNAc [3]. The yield of UDP–XylNAc was 5 μg/ml culture. It is worth noting that the entire UDP–GlcNAcA was shunted to UDP–XylNAc.

Isotope labeling of UDP–GlcNAcA and UDP–XylNAc

We have shown that engineered *E. coli* can rapidly generate and accumulate UDP–GlcNAcA and UDP–XylNAc. Next, we determined whether isotopically labeled UDP–sugars could also be produced. For this purpose, we supplemented the growth medium of *E. coli* harboring UDP–GlcNAc dehydrogenase with [U-^{13}C]glucose. The NDP–sugars were extracted and the ^{13}C signal was determined by NMR spectroscopy. A 2D ^{1}H–^{13}C HSQC experiment (Fig. 2) showed that all six carbons of the GlcNAcA ring of UDP–GlcNAcA had a signal intensity consistent with ^{13}C enrichment. This is likely due to the metabolic conversion of [^{13}C]Glc to [^{13}C]Glc6P and [^{13}C]Frc6P, which give rise to [^{13}C]glucosamine-1-P ([^{13}C]GlcN-1-P), the precursor for UDP–[^{13}C]GlcNAc (see metabolic scheme in Supplemental Fig. S5) [7]. The five carbons of the ribose moiety of UDP–GlcNAcA are also ^{13}C, and this is likely due to [^{13}C]glucose coming from the pentose shunt generating D-[^{13}C]ribose-5-P, the precursor for 5-phospho-α-D-[^{13}C]ribose-1-diphosphate (PRPP). In *E. coli,* PRPP can be coupled with orotic acid to form orotidylate, which gives rise to UMP and UTP (Supplemental Fig. S6). UTP and GlcNAc-1-P form UDP–GlcNAc in *E. coli.* The two carbons on the acetate group (NAc) of GlcNAcA moiety are also ^{13}C. This is likely due to the glycolysis I pathway (see illustrated metabolic pathway in Supplemental Fig. S5), where glucose is converted to pyruvate. The pyruvate carbons can then be incorporated into the acetyl moiety of acetyl-CoA (coenzyme A). Interestingly, the uracil carbons of UDP–GlcNAcA are not labeled. This possibly can be explained by the metabolism leading to pyrimidine synthesis in *E. coli.* The C-2 and N-3 atoms in the pyrimidine ring come from carbamoyl phosphate, whereas the remaining atoms in the pyrimidine ring (N-1, C-6, C-5, and C-4) come from aspartate (see illustration in Supplemental Fig. S7). A 1D ^{1}H spectrum without ^{13}C decoupling of labeled UDP–GlcNAcA showed the relative amount of ^{13}C satellites to the central ^{12}C peak to be in excess of 90%, indicating that more than 90% of UDP–GlcNAcA is labeled with ^{13}C.

The uracil ring in the above short labeling experiments was not labeled. To determine whether that ring can be labeled in the in-microbe system, we examined whether ^{15}N can be incorporated into UDP–GlcNAcA. For this purpose, we fed *E. coli* with [^{15}N]ammonium chloride. A 2D HMBC experiment (Fig. 3) demonstrated that the N-3 of the uracil ring and the nitrogen atom of the NAc group of GlcNAcA moiety of UDP–GlcNAcA are selectively labeled with ^{15}N, whereas N-1 is not labeled. Labeling of N-3 of the uracil ring is explained by the incorporation of [^{15}N]NH_3 into L-glutamine forming the carbamic acid and carbamoyl phosphate. Labeling of the nitrogen of *N*-acetyl-glucosaminuronic acid moiety is explained by incorporation of the [^{15}N]ammonia into L-glutamine (i.e., NH_3 + phosphorylated glutamate) rather than its incorporation with ketoglutarate into glutamate (see metabolic scheme in Supplemental Fig. S7). Interestingly, the N-1 of the uracil ring of UDP–GlcNAcA was not labeled with ^{15}N under the conditions described. To confirm this specific labeling of N-3, we fed the cell with [^{15}N]L-glutamine. As expected, the N-3 of the uracil ring was labeled ^{15}N, as was the nitrogen atom of the NAc group of GlcNAcA moiety of UDP–GlcNAcA. No label was found of the nitrogen N-1 of uracil, as expected. In *E. coli,* the N-1 is derived from the nitrogen group of aspartic acid (Supplemental Fig. S7).

Additional proof that the N-3, but not the N-1, was labeled came from ^{15}N HMBC acquired using a 21.1-Tesla magnet. At this field, the ribose H-1′ and the uracil H-5 are separated, and if N-1 were labeled, one would expect to see a coupling to the ribose H-1′. However, the ^{15}N HMBC data confirmed that the observed ^{15}N signal was coupled only to the uracil H-5 and not to the ribose H-1′. In addition, the coupling between uracil H-5 and H-6 to uracil N-1 is expected to be very similar [8], but we obtained a strong signal coupled to H-5 and a very weak signal coupled to H-6. This is more

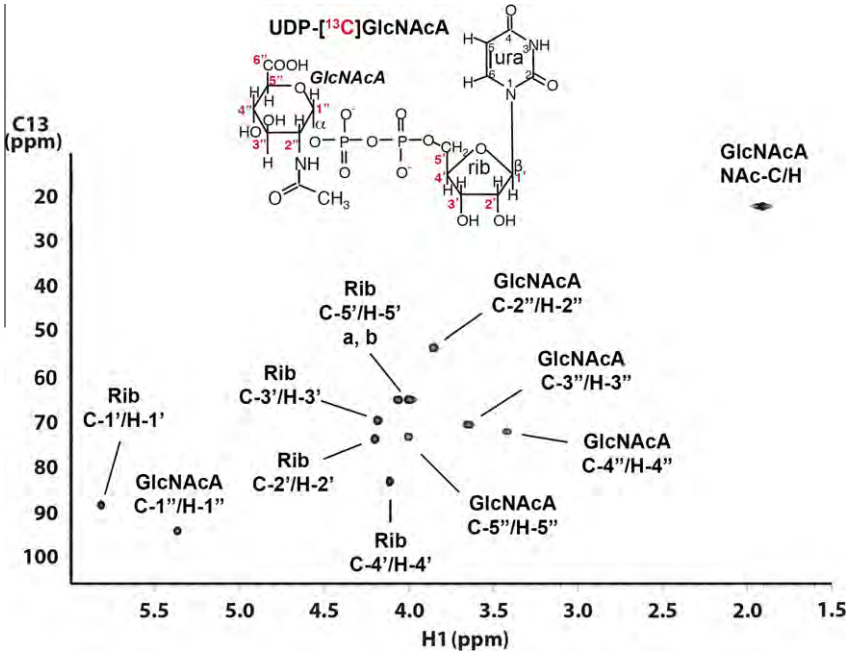

Fig.2. 2D ^{13}C HSQC NMR spectra of the HPLC-purified UDP–[^{13}C]GlcNAcA. Shown are 600-MHz 2D ^{13}C–^{1}H NMR spectra of UDP–GlcNAcA purified from engineered *E. coli* cells (harboring UDP–GlcNAc dehydrogenase) grown in the presence of uniformly labeled [^{13}C]glucose. At this concentration, the unlabeled uracil 5 and 6 C–H signals are not detectable.

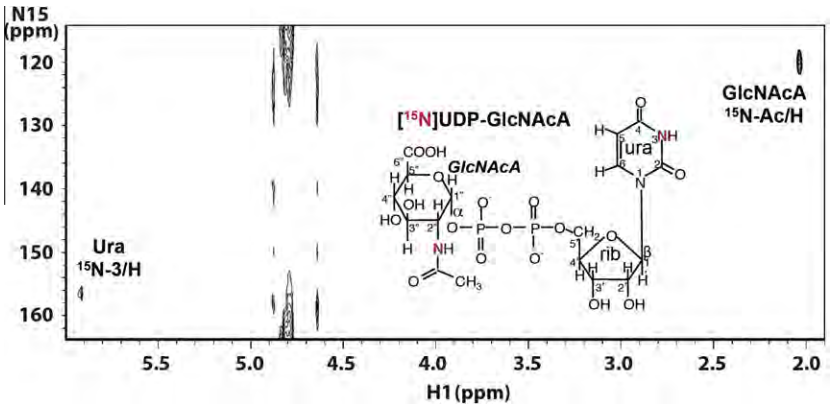

Fig.3. 2D ^{15}N HMBC NMR spectra of the HPLC-purified [^{15}N]UDP–GlcNAcA. Shown are 600-MHz 2D ^{15}N–^{1}H NMR spectra of UDP–GlcNAcA purified from engineered *E. coli* cells (harboring UDP–GlcNAc dehydrogenase) grown for 4 h in the presence of [^{15}N]NH_4Cl. The atoms highlighted in red are ^{15}N-labeled. (For interpretation of the reference to color in this figure legend, the reader is referred to the Web version of this article.).

compatible with the three-bond N-3–H-5 and four-bond N-3–H-6 couplings. Taken together, we confirmed that the ^{15}N signal identified from uracil ring is on N-3.

In a similar experiment, we fed the *E. coli* strain that carries both the UDP–GlcNAc dehydrogenase and UDP–XylNAc synthase genes with either [^{13}C]glucose or [^{15}N]NH_4Cl. 2D HSQC NMR experiments of the NDP–sugars isolated from cells grown in the presence of [U-^{13}C]glucose revealed that ^{13}C is in the carbons of the XylNAc and ribose but is not, as found previously, in the uracil ring of UDP–XylNAc (Fig. 4). From the 1D ^{1}H spectrum of labeled UDP–XylNAc, the relative amount of ^{13}C satellites confirms more than 95% ^{13}C enrichment. Feeding *E. coli* with [^{15}N]ammonia again demonstrated that N-3 of the uracil ring and the nitrogen atom of the NAc group of XylNAc moiety of UDP–XylNAc are ^{15}N, as shown in Fig. 5.

In-microbe system to produce UDP–Gal and UDP–GalA

To determine whether *E. coli* could be engineered to accumulate other NDP–sugars, we introduced genes encoding GalK and UDP–sugar PPase (Sloppy) or GalAK and Sloppy [4]. GalK in the presence of ATP converts α-D-galactose to α-D-Gal-1-P, which in the presence of Sloppy and UTP is converted to UDP–Gal [4]. In vitro, the UDP–sugar PPase more effectively catalyzes the reverse reaction, and unless pyrophosphate (PPi) is depleted, the reaction proceeds toward the formation of Gal-1-P rather than UDP–Gal. In vivo, however, it is believed that PPi derived from synthesis of DNA, RNA, and many other nucleotide metabolic pathways is readily converted to 2Pi by phosphatases and PPiases. Hence, we expected that the *E. coli* phosphatases could deplete PPi and drive the uridylylation reaction toward the formation of UDP–Gal. The engineered bacteria cells harboring GalK and Sloppy genes were supplemented with galactose and accumulated a compound that eluted from the HPLC column with a retention time of 16.9 min (see Fig. 6A, marked by arrow 3). Control cells supplemented with Gal did not accumulate this compound (Fig. 6B). Analysis of the compound by MS and MS/MS (Fig. 6C) showed that the parent ion at m/z 565.08 is fragmented to an ion with m/z 323.00, suggesting that the product is a UDP–hexose and a UMP, respectively. ^{1}H NMR analysis (Supplemental Fig. S3) provides chemical shift values

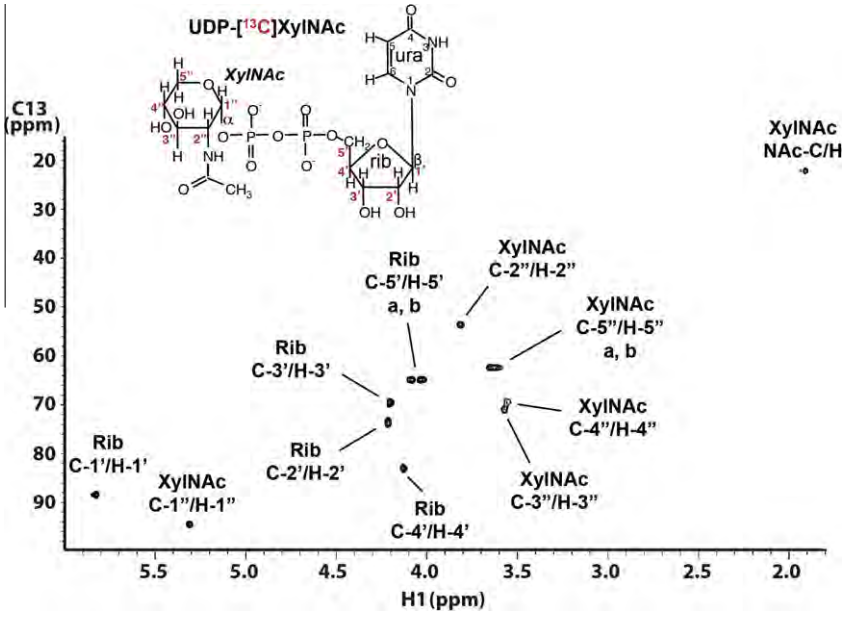

Fig.4. 2D ^{13}C HSQC NMR spectra of the HPLC-purified UDP–[^{13}C]XylNAc. Shown are 600-MHz 2D ^{13}C–^{1}H NMR spectra of UDP–XylNAc purified from engineered *E. coli* cells (harboring UDP–GlcNAc dehydrogenase and UDP–XylNAc synthase) grown for 4 h in the presence of uniformly labeled [^{13}C]glucose. At this concentration, the unlabeled uracil 5 and 6 C–H signals are not detectable.

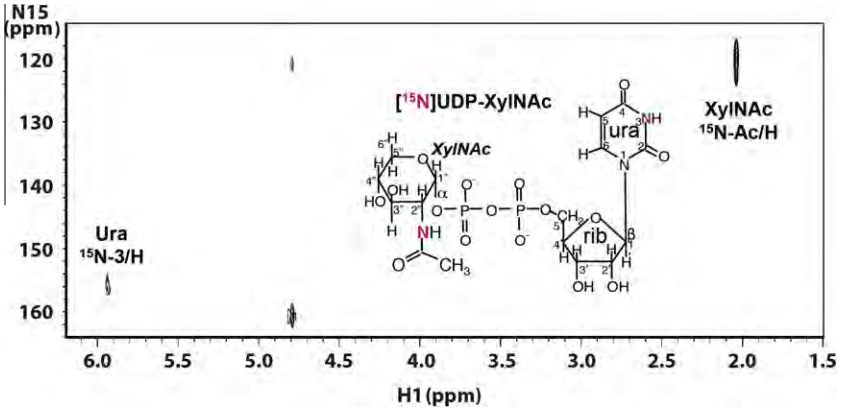

Fig.5. 2D ^{15}N HMBC NMR spectra of the HPLC-purified [^{15}N]UDP–XylNAc. Shown are 600-MHz 2D ^{15}N–^{1}H NMR spectra of UDP–XylNAc purified from engineered *E. coli* cells (harboring UDP–GlcNAc dehydrogenase and UDP–XylNAc synthase) grown in the presence of [^{15}N]NH_4Cl. The atoms highlighted in red are ^{15}N-labeled. (For interpretation of the reference to color in this figure legend, the reader is referred to the Web version of this article.)

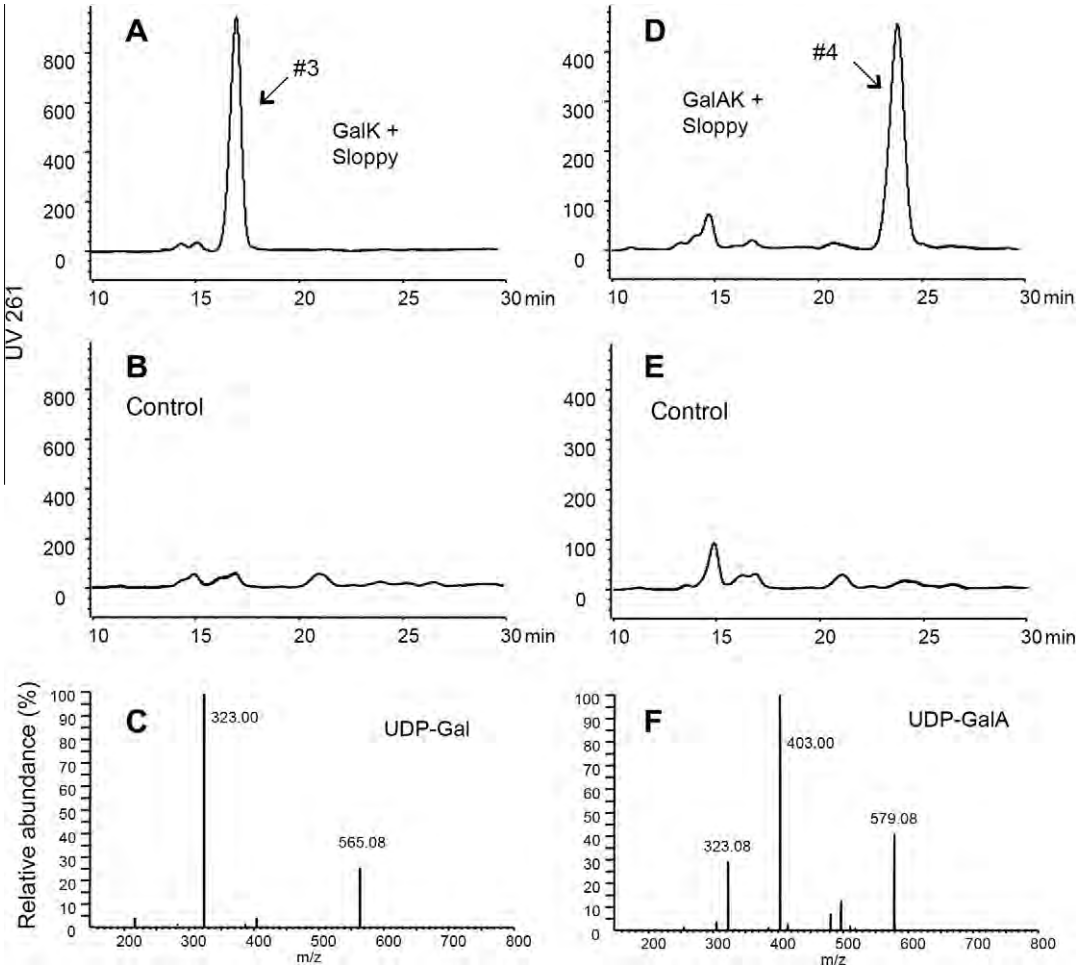

Fig.6. Characterization of UDP–Gal and UDP–GalA produced in-microbe using anion exchange chromatography and ESI–MS. *E. coli* [expressing GalK and Sloppy genes (A), GalAK and Sloppy (D), or plasmid control (B and E)] were grown for 4 h in the presence of the additives as indicated, and the NDP–sugars were then extracted. An aliquot from each sample was separated by Q15-HPLC [25] and monitored at A_{261nm} (A, B, D and E). Panel A shows the formation of UDP–Gal (arrow 3) in engineered cells supplemented with Gal, and panel B shows control cells supplemented with Gal. Panel D shows the formation of UDP–GalA (marked by arrow 4) in engineered cells supplemented with GalA, and panel E shows the control cells supplemented with GalA. The HPLC peaks in panels A and D are UDP–Gal (16.8 min) and UDP–GalA (23.8 min). Panels C and F show the MS/MS analysis performed at the negative mode of the parent UDP–sugars. The parent ions and fragmentations are listed in Supplemental Table S1.

and coupling constants that are consistent with UDP-α-galactose (UDP-Gal). The *E. coli* line is deficient in GalK activity (*galk*). The yield of UDP–Gal in this in-microbe system was 12.4 μg/ml.

The next system we examined was *E. coli* engineered to contain GalAK and Sloppy genes. This cell line, when grown in the presence of galacturonic acid, accumulated a product that eluted from the

anion exchange column with a retention time of 23.8 min (Fig. 6D, arrow 4). No product was formed if no GalA was added. Analysis of the product by MS and MS/MS (Fig. 6F) showed that the parent ion at m/z 579.08 is fragmented into two ions with m/z 403.00 and 323.08, suggesting that the product is a UDP–uronic acid and the ion fragments are $[UDP–H]^-$ and $[UMP–H]^-$, respectively. ^1H NMR analysis (Supplemental Fig. S4) gave chemical shifts and coupling constants that are consistent with UDP–GalA. The yield of UDP–GalA was 6.4 μg/ml. Sloppy, a promiscuous UDP–sugar PPase from plants [4,9], can be replaced by other promiscuous NDP–sugar PPases from various species to convert diverse sugar-1-Ps to their corresponding UDP–sugars. For example, a UDP–sugar PPase from *Trypanosoma cruzi* converts Glc-1-P, Gal-1-P, Xyl-1-P, and GlcA-1-P to their corresponding UDP–sugars [10]. A bacterial RmlA that normally converts Glc-1-P and TTP to dTDP-Glc has been engineered to use multiple sugar-1-Ps as substrates [11]. Similarly, a promiscuous sugar nucleotidyltransferase from archaea has been used to form different UDP–sugars and dTTP–sugars [12].

Toward improving the yields of UDP–sugar

We measured the time course of NDP–sugar production. The cells were grown in 20 ml of LB medium, and an aliquot (3 ml) was removed immediately after induction with IPTG (time 0) and then at hourly intervals for 5 h. The NDP–sugars were extracted and quantified by HPLC and verified by LC–ESI–MS/MS. The results show that within 2 h, the formation of NDP–sugar reached its maximum (Fig. 7). We then compared the amounts of NDP–sugar formed when *E. coli* was grown in flasks or in test tubes. The cells grew faster in the flask, possibly due to increase aeration, and produced between 20% and 30% more NDP–sugars than cells grown in test tubes (data not shown).

We analyzed the requirement of sugars added for the NDP–sugar production. Engineered *E. coli* harboring GalAK and Sloppy requires exogenous GalA to produce UDP–GalA. Engineered *E. coli* harboring GalK and Sloppy produces a small amount of UDP–Gal in the absence of added galactose. This is likely due to the contamination of residue Gal in the rich medium. However, a substantial (3-fold) increase in the production of UDP–Gal could be obtained by adding Gal to the growth medium. *E. coli* harboring UDP–GlcNAcDH alone or the combined activity of UDP–GlcNAcDH and UDP–XylNAc synthase produced the corresponding UDP–GlcNAcA and UDP–XylNAc without adding additional carbon sources to the rich medium. In addition, the yield is comparative to the ones fed with 0.2% Glc, Frc, or L-glutamine in the same medium.

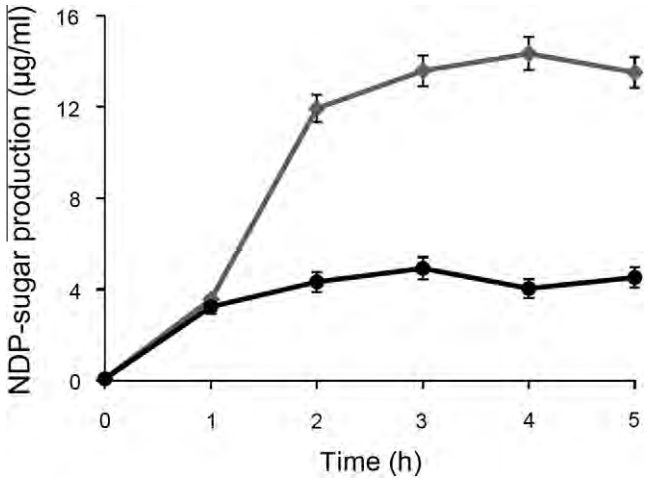

Fig.7. Time course for in-microbe production of NDP–sugars. *E. coli* cells harboring UDP–GlcNAcDH (light line) or UDP–GlcNAcDH and UDP–XylNAcS (dark line) were grown, and an aliquot was removed after the addition of IPTG (time 0) and then at hourly intervals. The amounts of NDP–sugars [UDP–GlcNAcA (diamonds) and UDP–XylNAc (circles)] produced are plotted. Each value (μg/ml) is the mean of triplicate reactions, and the values varied by no more than 5%.

Comparison of in-microbe system and other methodologies for the preparation of NDP–sugars

Several procedures have been described for the biologically based synthesis of nucleotide sugars, including (i) in vitro enzymatic reactions [11,13–15]; (ii) in vitro synthesis of an NDP–sugar coupled with a glycosyltransferase engineered in bacteria, also known as a "one-pot reaction" [16–19]; and (iii) the in-microbe method described in this article. Several groups have successfully generated milligram–gram amounts of UDP–Gal [20–22], UDP–GalNAc [22], and radioactive UDP–GlcNAc [23] using homogeneous enzymes. Azido-radioactive precursor [24] of UDP–GlcA was also produced in vitro using a similar method. We have generated UDP–XylNAc and UDP–GlcNAcA using recombinant proteins [3]. Nonetheless, these processes are time-consuming and require the addition of costly NAD^+ and UDP–GlcNAc. Moreover, an additional five recombinant enzymes (hexokinase, phosphoglucose isomerase, glutamine:Fru-6-P amidotransferase, GlcN phosphate mutase, and glmU) are required to generate ^{15}N- and ^{13}C-labeled UDP–XylNAc and UDP–GlcNAcA using such in vitro methods. Expression plasmids harboring these five genes were not available to us, so we sought to develop an in vivo system that we call "in-microbe". Using our in-microbe methods, we showed that [^{13}C]Glc and [^{15}N]L-glutamine are readily incorporated into UDP–GlcNAcA and UDP–XylNAc (see Figs. 2–5). This in-microbe method relies on endogenous *E. coli* enzymes to carry out the initial enzymatic reactions and, thus, eliminates the need for exogenous hexokinase, phosphoglucose isomerase, glutamine:Fru-6-P amidotransferase, GlcN phosphate mutase, and glmU.

Purified recombinant enzymes often lose activity during storage or may become inactive. Using such partially active enzymes will result in decreased product yield. In contrast, our in-microbe system maintains enzymatic activity, thereby alleviating such potential problems.

Butler and Elling [21] reviewed some of the disadvantages of using in vitro enzymatic conversion. For example, the use of recombinant enzymes might not be successful in large-scale industrial processes. By contrast, our in-microbe methodology provides an efficient and convenient way to produce both normal and labeled NDP–sugars. In addition, it should be readily scalable to obtain NDP–sugars in amounts sufficient for small-scale research and potentially at large-scale industry use.

Currently, we are using the in-microbe system to generate many other nucleotide sugars, including UDP–xylose and UDP–GlcA. The system will enable one to inexpensively generate a sufficient amount of other labeled derivatives such as deuterium-labeled NDP–sugars, radioactive NDP–sugars, and modified NDP–sugars (e.g., azido-sugar nucleotide, deoxy-sugar nucleotide) that are critical for glycobiology research. The system also provides means to evaluate biological transformation (i.e., pathways) of metabolites and determine how precursors enter different metabolic pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.12.028.

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