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Large-scale production of *N*-acetyllactosamine through bacterial coupling

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Abstract

A large-scale production system of *N*-acetyllactosamine, a core structure of various oligosaccharides, was established by a whole-cell reaction through the combination of recombinant *Escherichia coli* strains and *Corynebacterium ammoniagenes*. Two recombinant *E. coli* strains over-expressed the UDP-Gal biosynthetic genes and the β-(1→4)-galactosyltransferase gene of *Neisseria gonorrhoeae*, respectively. *C. ammoniagenes* contributed the production of UTP from orotic acid. *N*-Acetyllactosamine was accumulated at 279 mM (107 g L^{-1}) after a 38 h reaction (2.5 L in volume) starting from orotic acid, D-galactose, and 2-acetamido-2-deoxy-D-glucose.

Keywords: Enzymatic synthesis; Glycosyltransferase; Sugar nucleotide; Oligosaccharide.

1. Introduction

Oligosaccharides have been the focus of much research because of their role in biochemical recognition processes [1,2]. The synthesis of oligosaccharides can be achieved through conventional chemical methods, but multiple protection and deprotection steps are required [3]. An alternative approach to synthesize complex oligosaccharides is the enzymatic method using glycosyltransferases [2], which is especially attractive since these enzymes are highly stereo- and regioselective.

Although glycosyltransferases have not been readily available in the past, a number of genes of glycosyltransferases are now becoming readily available through recombinant DNA technology [4–6]. However, glycosyltransferases require sugar nucleotides, which, by and large, are not commercially available [7]. To solve this drawback, an in situ regeneration system of sugar nucleotides has been proposed [8–10], but the system still requires purified enzyme preparations and sugar phosphates that are expensive and unavailable.

Previously, we reported a novel production system of UDP-galactose (UDP-Gal) and globotriose from D-galactose and orotic acid through the coupling of bacteria [11]. The UDP-Gal production system consisted of a recombinant *Escherichia coli* and *Corynebacterium ammoniagenes*. *E. coli* was engineered to express UDP-Gal biosynthetic genes, galactose-1-phosphate uridyltransferase (*galT*), galactokinase (*galK*), glucose-1-phosphate uridyltransferase (*galU*), and pyrophosphatase (*ppa*). *C. ammoniagenes* was able to convert orotic acid to UTP. For the production of

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globotriose, another recombinant *E. coli* that over-expressed the α-(1→4)-galactosyltransferase gene of *Neisseria gonorrhoeae* was coupled with the UDP-Gal production system. In this system, the addition of sugar nucleotides and sugar phosphates was not required at all, nor was a purified enzyme preparation. To demonstrate the utility of our system, we examined the production of *N*-acetyllactosamine (LacNAc).

LacNAc is an important component of various oligosaccharides such as those found in glycoproteins and sialyl Lewis X, and it is used as the starting material for the synthesis of various oligosaccharides, but it has heretofore not been available in large quantities [12].

Herein we describe the results of a production of LacNAc from D-galactose (Gal), 2-acetamido-2-deoxy-D-glucose (GlcNAc) and orotic acid by coupling the UDP-Gal production system with *E. coli* cells that expressed the β-(1→4)-galactosyltransferase gene of *N. gonorrhoeae* (Fig. 1).

2. Results and discussion

Construction of the LacNAc production system.—The basic system for the production of LacNAc is made up of a UDP-Gal production system and a recombinant *E. coli* that over-expresses the β-(1→4)-galactosyltransferase gene (Fig. 1). The UDP-Gal production system consists of a recombinant *E. coli*, which over-expresses the UDP-Gal biosynthetic genes, *galT*, *galK*, *galU*, and *ppa*, and *C. ammoniagenes*, which contributes the production of UTP from orotic acid [11,13]. The

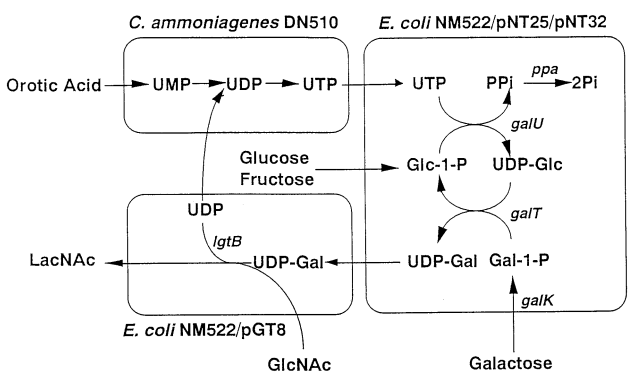

Fig. 1. Scheme of the production system of LacNAc.

β-(1→4)-galactosyltransferase (*lgtB*) gene of *N. gonorrhoeae* F62 (ATCC33084) is cloned by PCR and inserted into pPAC31 under a P_L promoter to form pGT8. The gene is over-expressed through the inactivation of a temperature-sensitive cI857 repressor at 40 °C.

Production of LacNAc.—The reaction was carried out on a 30-mL scale in a 200-mL beaker. *E. coli* cells harboring pGT8 that expressed the β-(1→4)-galactosyltransferase gene of *N. gonorrhoeae* were added to the UDP-Gal production system. GlcNAc, Gal, and orotic acid were also added to the reaction mixture, as well as polyoxyethylene octadecylamin (Nymeen S-215) and xylene to permeabilize the cells. Fructose was added as an energy source. After 27 h, 321 mM (123 g L^{-1}) of LacNAc was produced from 722 mM Gal and 497 mM GlcNAc. Considering the remaining sugars in the reaction mixture, the actual yield of LacNAc was 44% from galactose and 77% from GlcNAc. This result indicates that the coupling of the UDP-Gal production system and recombinant *E. coli* that over-expressed β-(1→4)-galactosyltransferase gene works well. It should be pointed out that the productivity of LacNAc (321 mM) was much higher than that of UDP-Gal itself (72 mM) when compared under the same conditions [11]. The amount of orotic acid added to the reaction mixture was 56 mM, and it was supposed that the compound was recycled via UTP and UDP more than four times [11]. We further carried out the production of LacNAc in a 5-L fermentor to verify the feasibility of large-scale production of LacNAc. As a result of a cellular reaction (2.5 L in volume) for 38 h, 279 mM (107 g L^{-1}) of LacNAc was produced from 578 mM Gal and 290 mM GlcNAc (Fig. 2). Considering the remaining sugars in the reaction mixture, the actual yield of LacNAc was 48% from galactose and 96% from GlcNAc. UDP-Gal was kept at a low level during the reaction (Fig. 2). Almost no peaks other than fructose, GlcNAc and LacNAc were observed after 38 h, as analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) (Fig. 3). The productivity of LacNAc at a 5-L jar fermentor scale was almost the same as that of the

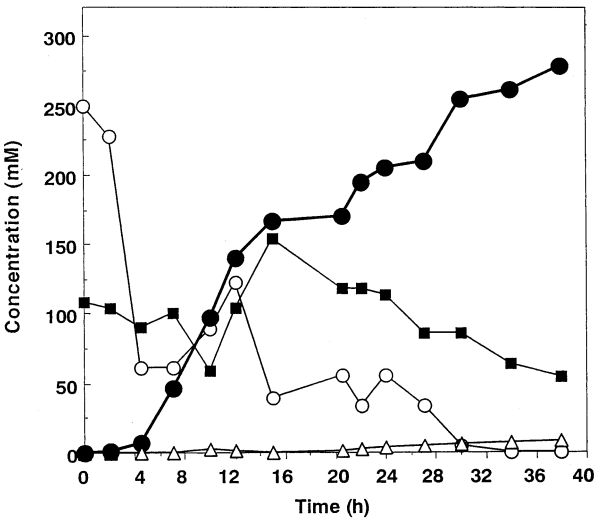

Fig. 2. Time course for LacNAc production on a 2.5-L scale. The amounts of LacNAc (●), GlcNAc (■), Gal (○), and UDP-Gal (△) in the reaction mixture are indicated.

beaker scale. The results clearly showed the high potential of the system through bacterial coupling as a production method for oligosaccharides.

Isolation of LacNAc.—The retention time of the product was identical to that of the authentic LacNAc by HPAEC–PAD analysis (data not shown). The compound (99 g) was isolated through several steps of purification including activated charcoal chromatography from the reaction mixture containing 210 g of LacNAc (see Section 3). In order to secure a pure sample for instrumental analyses, the compound was further purified by gel-filtration chromatography, and the product was freeze-dried. The structure of the purified compound was identified as LacNAc by means of ^{1}H NMR and ^{13}C NMR spectroscopy.

3. Experimental

Materials.—LacNAc was purchased from Oxford GlycoSystems, Inc. (Rosedale, NY). Bio-Gel P-2 was purchased from Bio-Rad Laboratories (Hercules, CA). Orotic acid used for the reaction was the product of Kyowa Hakko Kogyo Co., Ltd. All other chemicals used were commercially available and of analytical grade.

Plasmid construction.—DNA manipulations were performed according to the procedures described by Sambrook et al. [15]. The plasmid pPAC31, which contains the replication origin and ampicillin resistance gene from pBR322, P_L promoter, and temperature-sensitive cI857 repressor from phage lamda, was used for the construction of the expression plasmids [11]. The plasmid pNT25, which contains *galT* and *galK* genes of *E. coli*, and the plasmid pNT32, which contains the *galU* and *ppa* genes of *E. coli*, were described before [11]. The *lgtB* gene for β-(1→4)-galactosyltransferase of *N. gonorrhoeae* (ATCC33084) [4] was amplified by PCR using the primer, 5′-GCGGATCGATTCGGCAGGGAAAGAACAG-3′, to introduce a ClaI site, and 5′-TCTTGGATCCTTTTATTGGAAAGGCACAAT-3′ to introduce a BamHI site. Condi-

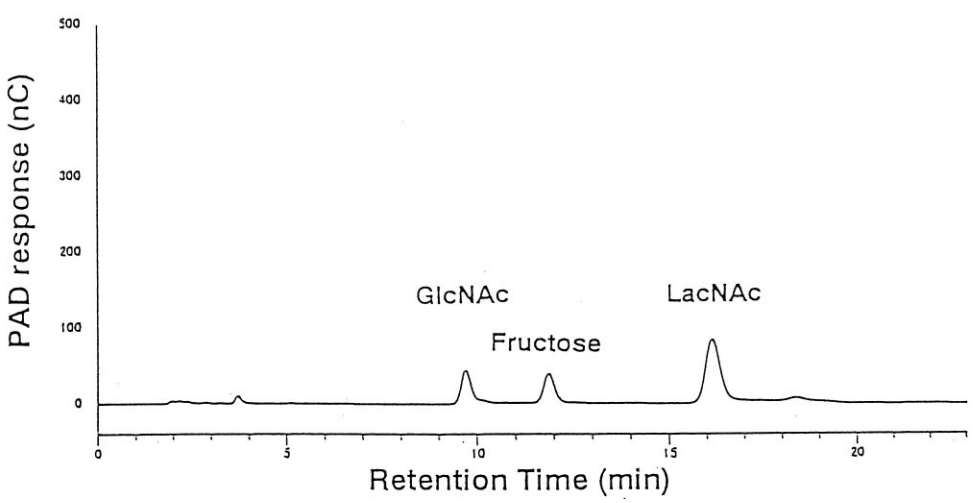

Fig. 3. Carbohydrate analysis of the reaction mixture with HPAEC–PAD.

tions for PCR cycling included denaturation at 94 °C for 1 min, annealing at 37 °C for 2 min and extension at 72 °C for 3 min (30 cycles). The 1.0-kb PCR product was digested with BamHI and ClaI and cloned into the BamHI–ClaI sites in pPAC31 to give pGT8. The DNA sequence was confirmed by the dideoxy sequencing using a model 373A DNA sequencer (Applied Biosystems, Foster City, CA).

Bacterial strains and culture conditions.—*E. coli* strain NM522 was purchased from Stratagene (La Jolla, CA). The expression plasmids were used to transform *E. coli* NM522. *E. coli* strains were cultivated in a 5-L jar fermentor according to the method described previously [11]. *C. ammoniagenes* DN510 cells were cultivated in a 5-L jar fermentor according to the method described previously [16]. Cells were collected by centrifugation and stored at − 20 °C until used.

LacNAc production.—The production of LacNAc was carried out in a 200-mL vessel containing 30 mL of the reaction mixture, i.e., 150 g L^{-1} (wet weight) of *C. ammoniagenes* DN510 cells, 50 g L^{-1} (wet weight) of NM522/pNT25/pNT32 cells, 50 g L^{-1} (wet weight) of NM522/pGT8 cells, 80 g L^{-1} of Fru, 20 g L^{-1} of Gal, 11 g L^{-1} of GlcNAc, 15 g L^{-1} of KH_2PO_4, 5 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 10 g L^{-1} of orotic acid (potassium salt), 4 g L^{-1} of Nymeen S-215 (Nippon Oil and Fats, Tokyo), and 10 mL L^{-1} of xylene. Fru (80 g L^{-1}), Gal (110 g L^{-1}), and GlcNAc (99 g L^{-1}) were added during the reaction. The reaction was done at 32 °C with agitation (900 rpm), and the pH was kept at 7.2 with 4 N NaOH.

The large-scale production of LacNAc was carried out in a 5-L jar fermentor containing 2.5 L of reaction mixture, i.e., 150 g L^{-1} (wet weight) of *C. ammoniagenes* DN510 cells, 50 g L^{-1} (wet weight) of NM522/pNT25/pNT32 cells, 40 g L^{-1} (wet weight) of NM522/pGT8 cells, 50 g L^{-1} of Fru, 50 g L^{-1} of Gal, 30 g L^{-1} of GlcNAc, 20 g L^{-1} of KH_2PO_4, 5 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 10 g L^{-1} of orotic acid (potassium salt), 4 g L^{-1} of Nymeen S-215 (Nippon Oil and Fats, Tokyo), and 10 mL L^{-1} of xylene. Fru (30 g L^{-1}), Gal (80 g L^{-1}), and GlcNAc (60 g L^{-1}) were added during the reaction. The reaction was carried out at 32 °C with agitation (600 rpm) and aeration (1 L min^{-1}), and the pH was kept at 7.2 with 4 N NaOH.

Analyses.—UDP-Gal was analysed according to the method of Lagunas et al. [17]. LacNAc and other saccharides were analysed by means of HPAEC–PAD using a Dionex DX-500 system equipped with a Carbopac PA10 column (Dionex, Sunnyvale, CA) [14]. Nucleotides were measured according to the method described before [18]. Inorganic phosphate was determined with a Determiner IP-S Kit (Kyowa Medex, Tokyo).

Isolation of LacNAc.—The reaction mixture was centrifuged at 7,000 rpm for 10 min at 4 °C to remove the cells. The supernatant containing 210 g of LacNAc was applied to a column of activated charcoal (30 mm × 400 mm) [19]. After the column was washed with 15 bed volumes of H_2O, LacNAc was eluted with five bed volumes of 20% EtOH. Fractions containing LacNAc were collected and freeze-dried, and solid LacNAc (99 g) was obtained. Carbohydrates other than LacNAc were further removed by means of gel filtration with a Bio-Gel P-2 (25 mm × 900 mm). Fractions containing LacNAc were collected and freeze-dried. NMR spectra were recorded in D_2O with a JEOL JNM-A400 instrument using residual HDO as the internal reference.

β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose.—^{1}H NMR (400 MHz, 10 mg/0.6 mL D_2O): α anomer: δ 5.24 (d, 1 H, $J_{1,2}$, 2.5 Hz, H-1), 4.51 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1′), 4.00 (m, 1 H, H-5), 3.96 (d, 1 H, $J_{3',4'}$ 3.4 Hz, H-4′), 3.94 (m, 2 H, H-2, H-3), 3.92 (m, 2 H, H-6a, H-6b), 3.80 (m, 1 H, H-6b′), 3.78 (m, 1 H, H-6a′), 3.77 (m, 2 H, H-4, H-5′), 3.69 (m, 1 H, H-3′), 3.59 (dd, 1 H, $J_{1',2'}$ 7.8 Hz, $J_{2',3'}$ 10.0 Hz, H-2′), 2.08 (s, 3 H, *H*CO); β anomer: δ 4.75 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 4.51 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1′), 4.00 (m, 1 H, H-6b), 3.96 (m, 1 H, $J_{3',4'}$ 3.4 Hz, H-4′), 3.87 (m, 1H, H-6a), 3.80 (m, 1 H, H-6b′), 3.78 (m, 1 H, H-6a′), 3.77 (m, 1 H, H-5′), 3.74 (m, 2 H, H-2, H-4), 3.73 (m, 1 H, H-3), 3.71 (m, 1 H, H-3′), 3.64 (m, 1 H, H-5), 3.57 (dd, 1 H, $J_{1',2'}$ 7.8 Hz, $J_{2',3'}$ 10.0 Hz, H-2′), 2.08 (s, 3 H, *H*CO); ^{13}C NMR (100 MHz, 10 mg/0.6 mL D_2O): α anomer: 175.30 (CH_3CO), 103.79 (C-

1′), 91.38 (C-1), 79.72 (C-4), 76.20 (C-5′), 73.39 (C-3′), 71.83 (C-2′), 71.11 (C-5), 70.13 (C-3), 69.41 (C-4′), 61.86 (C-6′), 60.85 (C-6), 54.57 (C-2), 22.74 (*H*CO); β anomer: 175.56 (CH_3CO), 103.74 (C-1′), 95.71 (C-1), 79.31 (C-4), 76.20 (C-5′), 75.69 (C-5), 73.39 (C-3′), 73.32 (C-3), 71.83 (C-2′), 69.41 (C-4′), 61.86 (C-6′), 60.97 (C-6), 57.08 (C-2), 23.03 (*H*CO).

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