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Transferring a Biosynthetic Cycle into a Productive *Escherichia coli* Strain: Large-Scale Synthesis of Galactosides

Xi Chen, Jianbo Zhang, Przemek Kowal, Ziye Liu, Peter R. Andreana, Yuquan Lu, and Peng George Wang*

Department of Chemistry, Wayne State University Detroit, Michigan 48202

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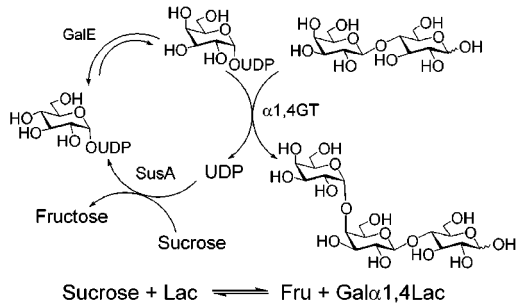
Discoveries concerning the important roles of glycoconjugates in a variety of biological processes have led to an increased demand for efficient synthetic methodologies adaptable for their large-scale production.[1] Compared to chemical synthesis, an enzymatic approach utilizing glycosyltransferases has been proven more efficient in the production of complex carbohydrates.[2] Despite the increasing availability of various glycosyltransferases, the high cost and inadequate availability of sugar nucleotides required by these enzymes have severely limited their applications.[3] Since sugar nucleotides only serve as cofactors in the overall glycosylation in biological system, the best way is mimicking nature to recycle them in situ.[4] Among the existing uridine 5′-diphosphoglucose (UDP-Glc) and uridine 5′-diphosphogalactose (UDP-Gal) recycling schemes, the most elegant one is based on a sucrose synthase (SusA, EC 2.4.1.13).[5] The synthesis and cleavage of sucrose catalyzed by sucrose synthase (sucrose + UDP ⇔ UDP-Glc + fructose) is the only readily reversible transglycosylation reaction involving a sugar nucleotide[6] (Scheme 1). On the basis of this in vitro biosynthetic cycle, our objective has been to create a bacterial strain transformed with a single plasmid which contains the three enzymes along the biosynthetic cycle. The bacterial cells will then be used as a galactoside-producing factory, in which UDP is recycled back to UDP-Gal, providing the donor for galactosyltransferases for large-scale production of custom carbohydrates.

The construction of such a productive *Escherichia coli* strain involved several steps. In addition to the bovine α1,3GT (α1,3galactosyltransferase),[7] GalE (UDP-galactose 4-epimerase) from *E. coli* K-12,[8] α1,4GT (LgtC, α1,4galactosyltransferase) from *Neisseria meningitidis*,[9] and SusA from *Anabaena* sp. PCC 7119 (ATCC no. 29151) were cloned into pET15b vectors separately. To confirm individual enzyme expression, the His-tagged proteins were produced in *E. coli* BL21 (DE3) by a 3-h isopropyl-1-thio-β-D-galactopyranoside (IPTG) induction at 37 °C. The proteins were obtained in soluble, active form and readily purified to approximately 95% homogeneity by an immobilized nickel (Ni^{2+}) affinity column. Then, the three genes comprising a galactosyltransferase gene, *gal*E gene, and *sus*A gene, were assembled into an artificial gene cluster in a temperature-sensitive pLDR20 vector using the pre-constructed pET15b plasmids as templates for the polymerase chain reactions. The choice of pLDR20 vector enabled convenient temperature induction and eliminated the need for inducer, IPTG, required by the pET15b vector system. Furthermore, it allowed for the use of a β-galactosidase negative strain, thus preventing the degradation of lactose (the transferase acceptor) by the host strain. Repeated experiments indicated that it was necessary to add a ribosomal binding sequence (rbs) upstream of each component gene in the construction of the gene cluster to ensure adequate translation of all the enzymes. Townsend et al. reported a similar strategy for coexpression of multiple enzymes.[10] After transformation of the plasmid containing the gene cluster into *E. coli* host, all of the enzymes were simultaneously expressed as soluble proteins, upon induction, by increasing the temperature to 40 °C.

Scheme 1. Biosynthetic Pathway for Galactosides Using Sucrose Synthase



Two examples are reported herein for the synthesis of biomedically important trisaccharides Galα1,4Lac (globotriose, Gb_3) and Galα1,3Lac (α-Gal epitope), respectively. Globotriose is presented in the lipooligosaccharides of the bacterial pathogens *N. meningitidis* immunotype L1 and *N. gonorrhoeae.* It is believed to participate in the process of invasion of human cells by enabling cell−cell adhesion.[11] Additionally, globotriose, found on the surface of mammalian cells, serves as a ligand for the *E. coli*-derived verotoxin, the causative agent of dysentery.[12] α-Gal is the major antigen responsible for the hyperacute rejection in pig-to-human xenotransplantation.[13]

The plasmid maps of pLDR20-CES and pLDR20-αES for the synthesis of Gb_3 and α-Gal, respectively, are shown in Figure 1. Both plasmids were transformed into DH5α and NM522 competent cells subsequently. SDS-PAGE results indicated that cells carrying either plasmid expressed the encoded enzymes in approximately equal amounts.

* To whom correspondence should be addressed. Telephone: 313-993-6759. Fax: 313-577-2554. E-mail: pwang@chem.wayne.edu.

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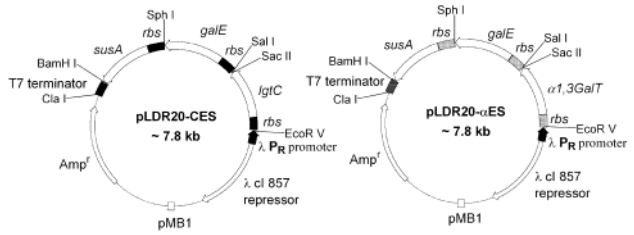

Figure 1. Plasmid maps for pLDR20-CES and pLDR20-αES.

Our whole-cell catalyzed production of oligosaccharides is a two-step process. The first step involves the growth of the recombinant *E. coli* NM522 cells and the expression of the recombinant enzymes along the biosynthetic pathway inside the cell. In the second step the harvested cells are permeabilized by repeated freeze/thaw and employed as catalysts in the reaction. The simple permeabilization allows the transport of substrates and products into/out of the cells.[14] The permeabilized cells, although not healthy and much less viable, can still carry out certain metabolism (e.g., glycolysis) to provide the necessary bioenergetics to drive the enzymatic reactions inside the cells. Importantly, this two-step process allows the use of high cell concentration in the second enzymatic transformation step to achieve a high-yielding fed-batch process.

Small-scale (1 mL) reactions were carried out at room temperature for 36 h to search for the optimum conditions. The formation of the trisaccharides was monitored by HPLC. The prokaryotic sucrose synthase cloned from *Anabaena* sp. has a K_m of 303 mM for sucrose;[15] therefore, a high concentration of sucrose was necessary to force the reversible reaction toward the UDP-Glc-forming direction. Addition of catalytic amounts of UDP was found to affect the efficiency of the glycosylation. In general, increasing amounts of UDP promoted the sucrose-cleaving reaction; however, UDP concentrations above 10 mM caused a decrease of the yield. The effect is due to the reported inhibitory effect of UDP on the glycosyltransferase. Replacement of UDP with catalytic UDP-Glc alleviated the inhibitory effects, and the yield increased dramatically. MES buffer was found to be a better buffer for both Gb_3 and α-Gal-producing cells compared to HEPES buffer commonly used in sucrose synthase studies. The addition of theophylline, a phosphatase inhibitor, led to an increase in yield. Additionally, DTT (dithiothreitol), a thiol-containing reducing agent, enhanced the formation of Galα1,4Lac but had no effect on the yield of the α-Gal epitope. This observation is consistent with the reported thiol requirement for LgtC activity.[16] A careful balance between the product yield and the overall costs indicated that the reaction mixture containing 200 g/L of cells, sucrose (0.5 M), Lac (200 mM), UDP (2 mM), $MgCl_2$ (10 mM), DTT (2 mM), and theophylline (3 mM) was optimal. Repeated control experiments showed that the whole cells serve as multiple biocatalysts with all of the enzymes trapped inside the cells without leaking.

The productive *E. coli* strains were used for large-scale synthesis of Gb_3 and α-Gal. Reactions were performed in a 500 mL volume using 100 g of cells (wet weight) obtained from 20 L of fermentation broth, 86 g of sucrose, 34 g of Lac, 0.40 g of UDP, 0.48 g of $MgCl_2$, 0.15 g of DTT, 0.27 g of theophylline. The accumulation of product was monitored by thin-layer chromatographic (TLC) analysis and quantified by HPLC with RI detection. Time course studies, shown in Figure 2, indicated that the reactions reached saturation after 36 h. Galα1,4Lac accumulated to 44 mM (22 g/L), and Galα1,3Lac, to 36 mM (18 g/L) in the reaction mixture. Notably, HPLC results indicated lack of fructose upon reaction completion. Presumably, it was consumed by the host as an energy source. The reaction was terminated by heating in a boiling water bath for 10 min. The cells were removed by centrifugation. Remaining sucrose and lactose were hydrolyzed to monosaccharides by incubating the supernatant with invertase (0.05 g, 25000 unit, Sigma) and *β*-galactosidase (0.125 g, 1000 unit, Sigma) for 10 h at room temperature. The mixture was then poured into a column packed with graphitized carbon (15 × 5 cm, Supelco, Inc.) and washed with water, and the trisaccharides were eluted with water containing 10% (v/v) acetonitrile. The isolated yields are 22% (11 g) and 18% (9 g) for Gb_3 and α-Gal, respectively. On the basis of the yields, the designed biosynthetic cycle turned over 22 times in the CES cells and 18 times in the αES cells. The products were characterized by HPLC, NMR, and MS spectrometry. Thus, on the basis of commercial prices for small-quantity reagents (most from Sigma, Inc.), the reported 500-mL-scale reaction produced Gb_3 at $8/g and α-Gal at $10/g. Larger-scale production with bulk chemicals will certainly drive the cost much lower.

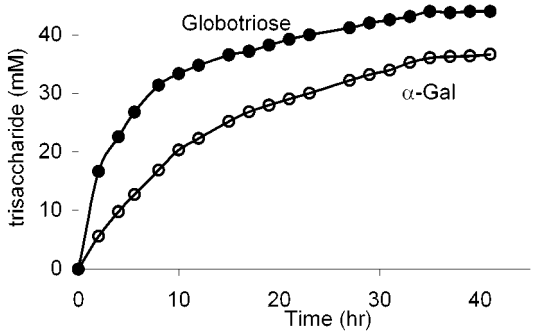

Figure 2. Product accumulation in large-scale synthesis of Gb_3 and α-Gal.

In conclusion, we described herein a simple and efficient system for the large-scale production of galactosides achieved by introducing an artificial gene cluster into a single *E. coli* strain. This artificial cluster, consisting of only three genes, is sufficient for the synthesis of galactosides with concurrent recycling of UDP-Gal. The simplicity of the synthetic cycle, and thus that of the plasmid, allows for further modification, such as the addition or replacement of genes. Work is in progress to incorporate a *β*1,4-galactosyltransferase gene into the pLDR20-CES or pLDR20-αES to form plasmids consisting of four genes for the synthesis of Galα1,4Gal*β*1,4GlcNAc and Galα1,3Gal-*β*1,4GlcNAc starting from *N*-acetylglucosamine. The whole-cell system eliminates the need to purify and preserve the enzymes involved. Moreover, the use of a single bacterial strain instead of multiple strains avoids transport of reaction intermediates between strains and the complication of maintaining the growth of different strains.[3] The strategy was validated by the synthesis of biomedically important globotriose and α-Gal trisaccharides. Since the process uses only inexpensive mono- and disaccharides and catalytic amount of UDP (1%), the production can be economically carried out on industrial scale. Thus, assembling sucrose synthase into an artificial biosynthetic gene cluster offers a simple, versatile, and economical solution to recycle UDP-Glc and UDP-Gal during large-scale production of carbohydrates.

Supporting Information Available: Experimental procedures for cloning and expression of individual enzymes; construction of the plasmid with a galactosides-producing gene cluster and synthetic procedures for globotriose and α-Gal (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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