ARTICLE

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Selective Retinol Production by Modulating the Composition of Retinoids From Metabolically Engineered *E. coli*

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ABSTRACT: Retinoids can be produced from *E. coli* when introduced with the β-carotene biosynthesis pathway and the BCMO gene. *E. coli* has no inherent metabolic pathways related to retinoids, therefore only retinal should be produced from the cleavage of β-carotene by BCMO. However, retinol and retinyl acetate were also produced in significant amounts, by the non-specific activity of inherent promiscuous enzymes or the antibiotic resistance marker of the retinal-producing plasmids. Retinol was produced by the *ybbO* gene of *E. coli* which encodes oxidoreductase and retinyl acetate was produced by the chloramphenicol resistance gene, called *cat* gene which encodes chloramphenicol acetyltransferase, present within the pS-NA plasmid that also contains the mevalonate pathway. The composition of retinoids could be modulated by manipulating the relevant genes. The composition of retinol, a commercially important retinoid, was significantly increased by the overexpression of *ybbO* gene and the removal of *cat* gene in the recombinant *E. coli*, which suggests the possibility of selective retinoid production in the future.

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KEYWORDS: retinoids; retinol; retinyl acetate; *ybbO*; chloramphenicol resistance gene (*cat* gene)

Introduction

Retinoids, also known as vitamin A, play essential and diverse functions in vision, bone development, regulation of cell proliferation and differentiation, immunity, skin health as antioxidants, and reducing the risk of certain cancers (Blomhoff and Blomhoff 2006; Ross 1993). Retinoids are defined as a class of compounds that consist of four isoprenoid units with a cyclic polar end group, and the variations in the end group create different classes of retinoids (Barua and Furr 1998). The end groups of alcohol, aldehyde, carboxylic acid and ester give retinol, retinal, retinoic acid, and retinyl ester, respectively (Fig. 1).

Retinoid biosynthesis has mostly been studied in animals. Animals are incapable of synthesizing β-carotene themselves, so they must meet their needs through plants in their diet. β-Carotene from plants is cleaved to retinal by β-carotene 15,15′-monooxygenase (BCMO; EC 1.14.99.36), and then further converted into different classes of retinoid (Goodman et al., 1966; Kim and Oh 2009). Retinal is converted to retinol by either alcohol dehydrogenase (EC 1.1.1.1) or retinol dehydrogenase (EC 1.1.1.-), which is then changed to retinyl ester by various *O*-acyltransferases (EC 2.3.1.-) (Hoog and Ostberg 2011; Lee et al., 2009; Shi and Cheng 2009). Retinal can also be oxidized by either aldehyde oxidase (EC 1.2.3.1) or retinal dehydrogenase (EC 1.2.1.36) into retinoic acid for degradation (Garattini et al., 2009).

Retinoid metabolism in bacteria has not been studied in great extent, only mainly about β-carotene 15′,15′-monooxygenase (BCMO) in photosynthetic bacteria (Peck et al., 2001; Sabehi et al., 2005). We have previously achieved retinoid production from *E. coli* introduced with the β-carotene biosynthesis pathway and the BCMO gene (Jang et al., 2011). *E. coli* does not contain an inherent metabolic pathway related to retinoids; therefore only retinal should have been synthesized from β-carotene by BCMO. However, it was found that retinol and retinyl acetate were also produced. It seems that some inherent promiscuous enzymes in *E. coli* exhibit non-specific activity towards retinal. This study was aimed at finding out

Hui-Jeong Jang and Bo-Kyung Ha contributed equally to this work.

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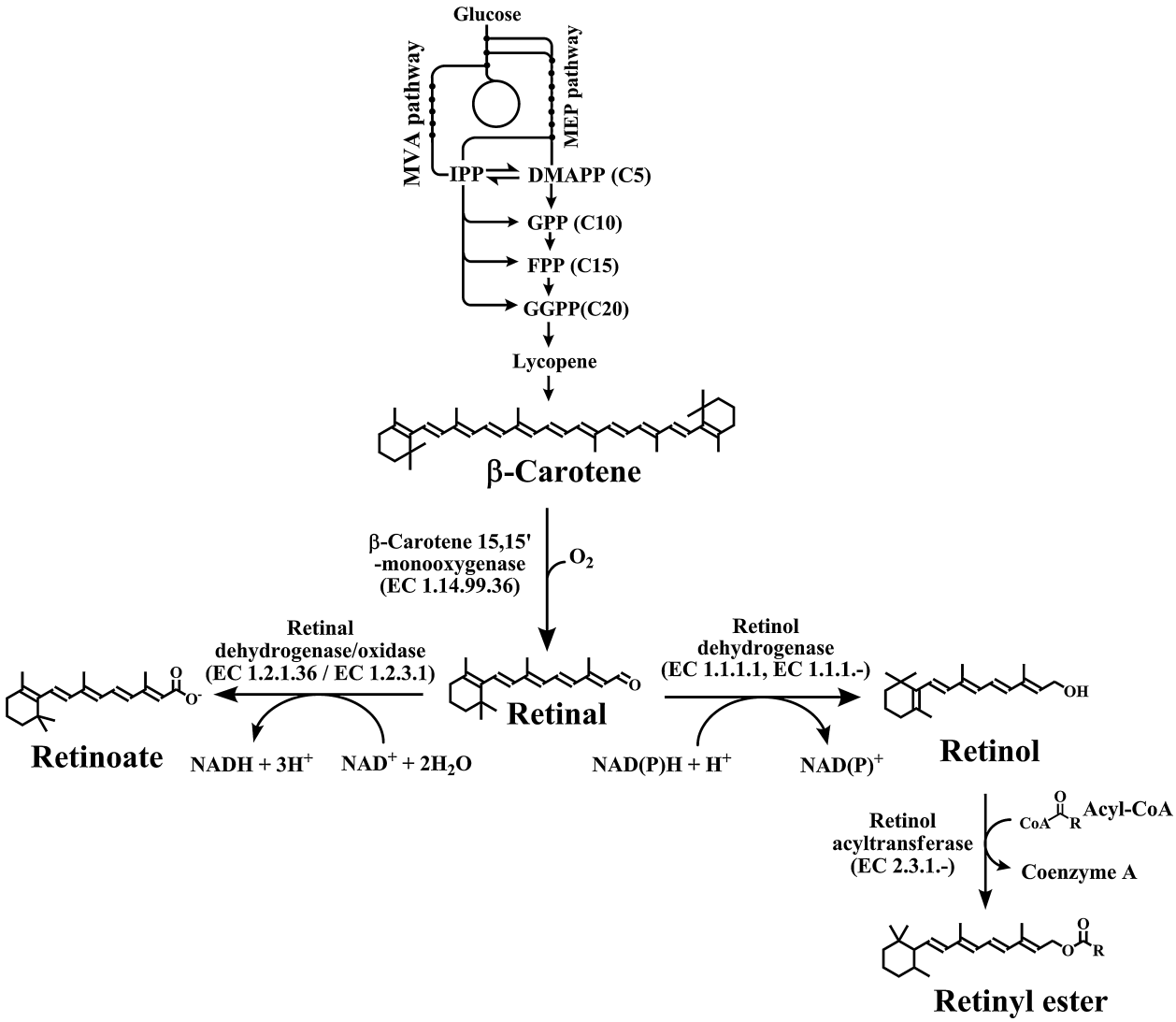

Figure 1. The conversion of β-carotene to different classes of retinoid, including retinal, retinol, retinoic acid, and retinyl esters. Retinoid biosynthesis in *Escherichia coli* using the MEP pathway and the exogenous MVA pathway.

which inherent promiscuous enzymes in *E. coli* could convert retinal into retinol or retinyl acetate, which then opens up new possibilities for the production of a desired class of retinoid, using specific enzymes.

Materials and Methods

Bacterial Strain and Culture Conditions

E. coli DH5α (F^-, ϕ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1 *endA*1, *hsdR*17(r_K^- m_K^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyr*A96, *rel*A1) was used for gene cloning and retinoid production. The culture for retinoid production was carried out in 2 YT medium (16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter) using a shaking incubator at 29°C and 250 rpm. Glycerol and arabinose, as the main and auxiliary carbon sources, were added at concentrations of 2% (w/v) and 0.2% (w/v), respectively. Ampicillin (100 μg/mL), chloramphenicol (50 μg/mL), and kanamycin (50 μg/mL) were added to the culture as required. Cell culture was carried out in a test tube and growth was determined by measuring the optical density at 600 nm (OD_{600}). For the two-phase culture for retinoid production, 5 mL of dodecane (Cat. No. 297879, Sigma, St. Louis, MO, USA) was layered over 5 mL of culture medium.

Gene Cloning and Plasmid Construction

The plasmids and PCR primers used in this study are listed in Table I. Common procedures, including genomic DNA preparation, restriction digests, transformations, and other standard molecular biology techniques, were carried out as described in the literature (Sambrook and Russell 2001). PCR was performed using *pfu* DNA polymerase (Solgent Co., Daejeon Korea) with a standard protocol. The pTrc99A, pSTV28, pSTV28K, pBluescript, and pBBR1MCS2

Table I. Strains, plasmids, and primers used in this study.

Strains, plasmids and primers	Description	Reference or source
Strains		
DH5α *ΔybbO*	DH5α (*ΔybbO*::FRT)	In this study
DH5α *ΔeutE*	DH5α (*ΔeutE*::FRT)	In this study
DH5α *ΔpuuC*	DH5α (*ΔpuuC*::FRT)	In this study
Plasmids		
pBluescript	pUC origin, *lac* promoter, *lacZ*, and *bla*	Stratagene
pBBR1MCS2	Broad-host-range, Km^r lacPOZ mobRP4	
pSTV28	P_{lac} expression vector, pACYC184 origin, *lacZ*, Cm^r	Takara
pTrc99A	P_{trc} expression vector, pBR322 origin, $lacI^q$, Amp^r	Amersham Bioscience
pSTV28K	P_{lac} expression vector, pACYC184 origin, *lacZ*, Km^r	(Zhou et al., 2014)
pKD46	repA101ts, oriR101, *exo, bet,gam,* tL3, P_{BAD}, *araC*, and *bla*	AY048746 (Datsenko and Wanner 2000)
pKD13	Template plasmid, oriR6Kgamma, tL3LAM, FRT, *kan, bla*, and *rgnB*	AY048744 (Datsenko and Wanner, 2000)
pKD3	Template plasmid,oriR6Kgamma, FRT, *cat, bla, rgnB*	AY048742 (Datsenko and Wanner, 2000)
pCP20	cI857λts, FLP, *cat,* and *bla*	(Datsenko and Wanner, 2000)
pT-DHBSR	pTrc99A containing *crtE, crtB*, and *crtI* from *P. agglomerans*, *crtY* from *P. ananatis*, *ipiHP1* from *H. pluvialis, dxs* from *E. coli,* and the codon-optimized *blh* gene (SR) from uncultured marine bacterium 66A03	(Jang et al., 2011)
pS-NA	pSTV28 containing *mvaE* and *mvaS* from *E. faecalis*; *mvaK1*, *mvaK2*, and *mvaD* from *S. pneumoniae*; and *idi* from *E. coli*	(Yoon et al., 2009)
pBBR2-ybbO	pBBR1MCS2 containing *ybbO* from *E. coli*	In this study
pS-NAK	pSTV28K containing *mvaE* and *mvaS* from *E. faecalis*; *mvaK1*, *mvaK2*, and *mvaD* from *S. pneumoniae*; and *idi* from *E. coli*	(Zhou et al., 2014)
pT-DHBSRybbO	pT-DHBSR containing *ybbO* from *E. coli*	In this study
Primers[a]		
ybbO-F	5′-CGGGTACCGAGGAGGTAATAAT**ATG**ACTCATAAAGCAACGGAGATCC-3′	
ybbO-R	5′-CGCTCGAG**TTA**CCCCTGCAATATTTTGTCCATC-3′	
SR-F2	5′-G**ACTAGTGAATTCAGGAGGTAATAAAT**ATG**G**-3′	
SR-R2	5′-CGTTTAATTAA**TTA**GTTTTTGATTTTGATACGGGAAGAG-3′	
ybbO-F2	5′-CGTTTAATTAAAGGAGGTAATAAT**ATG**ACTCATAAAGCAACG-3′	
ybbO-R2	5′-CGTACTAG**TTA**CCCCTGCAATATTTTGTCCATC-3′	
KOybbO-F	5′-GTTTATTGCCGACTGGATGGCGAAGCAGTTGCAGCCTTTAGTAAATCATG**ATTCCGGGGATCCGTCGACC**-3′	
KOybbO-R	5′-GACATGGGGGCTTAAGCGCGCGCTTCAACTCACCCCTGCAATATTTTGTC**TGTAGGCTGGAGCTGCTTCG**-3′	
KOybbOCF-F	5′-**GCCTGCAAACTATGGTCGCC**-3′	
KOybbOCF-R	5′-**CAGCACCGGAGTGGTCATCG**-3′	
KOeutE-F	5′-GTGGTGTCTGGCGGTCAGGTAATTTTCCACAAATAAGGCAGAACATCATG**ATTCCGGGGATCCGTCGACC**-3′	
KOeutE-R	5′-ATTGTTCGTCGTGCGCCATCTGTTACTCCTTAAACAATGCGAAACGCATC**TGTAGGCTGGAGCTGCTTCG**-3′	
KOeutECF-F	5′-**GGTAATCCTGACGGGCAATGC**-3′	
KOeutECF-R	5′-**CGACACCACATCGCAGGTGC**-3′	
KOpuuC-F	5′-ACGTTCACAAACTGCATATATCTGATAGACGTGAAACAGGAGTCATAATG**ATGGGAATTAGCCATGGTCC**-3′	
KOpuuC-R	5′-CGTAGTAACTGCTGGTATGTTCGGTCATTTCAGGCCTCCAGGCTTATCCA**TGTAGGCTGGAGCTGCTTCG**-3′	
KOpuuCCF-F	5′-**CAGAGGCGGCTTGAAGGATG**-3′	
KOpuuCCF-R	5′-**GTGATTGACTCATTCAGCGTGTCG**-3′	

[a]Template binding regions are indicated with bold letters, start and stop codons are underlined, and restriction sites are double underlined.

plasmids were used for gene cloning and gene expression (Table I). The pT-DHBSR plasmid containing the genes for retinoid biosynthesis and the pS-NA and pS-NAK plasmids with the MVA pathway operon were used as described previously (Jang et al., 2011; Yoon et al., 2009; Zhou et al., 2014). The alcohol dehydrogenase gene *ybbO* was amplified using PCR from *E. coli*, and then, the PCR product was digested using *Kpn*I and *Xho*I for insertion into the corresponding sites on pBBR1MCS2, which resulted in pBBR2-ybbO. For pB-SRybbO, SR gene and *ybbO* gene were amplified using the primer sets of SR-F2/SR-R2 and ybbO-F2/ybbO-R2, respectively. PCR products were then digested using *Pac*I to be inserted into the *EcoR*V site on pBluescript. To clone pT-DHBSRybbO, pB-SRybbO was digested using *Spe*I to be inserted into the corresponding site on pT-DHB.

Gene Deletion

Primers used for gene deletion are listed in Table I. Three genes, *ybbO, eutE*, and *puuC*, were knocked out by the one-step inactivation method (Datsenko and Wanner 2000). Linearized DNA flanked by FLP recognition target sites and homologous sequences were amplified by PCR using pKD3 and pKD13 as the template for chloramphenicol and kanamycin resistance, respectively. Insertion sequences of the KOybbO PCR product and the

KOeutE PCR product which contain a kanamycin marker for knock-out of *ybbO* and *eutE*, respectively, were amplified using the primer set of KOybbO-F/KOybbO-R and KOeutE-F/KOeutE-R from the pKD13 plasmid. The KOpuuC PCR product containing a chloramphenicol marker for knock-out of *puuC* was amplified using the primer set of KOpuuC-F/KOpuuC-R from the pKD3 plasmid. Three PCR products were electroporated to *E. coli* DH5α (pKD46) competent cells that express the RED recombinase using 0.2% L-arabinose. Positive clones were selected by relevant antibiotics at 37°C and verified with PCR analysis using primer sets of KOybbOCF-F/KOybbOCF-R, KOeutECF-F/KOeutECF-R, and KOpuuCCF-F/KOpuuCCF-R. Subsequently, the knock-out mutants were transformed with pCP20 plasmid, which expresses the FLP recombinase. The transformants harboring pCP20 were grown at 30°C to remove the marker and then the temperature-sensitive plasmid pCP20 was removed by overnight growth at 43°C. The resulting three knock-out strains were named DH5α *ΔybbO*, DH5α *ΔeutE*, and DH5α *ΔpuuC*.

Analysis of Retinoids

In the two-phase culture system with a dodecane overlay, the upper dodecane phase containing the retinoids was collected and centrifuged for 10 min at 14,000 rpm to remove all cellular particles. The dodecane phase was analyzed by HPLC (LC-20A, Shimadzu, Kyoto, Japan) using the Symmetry C18 (250 mm × 4.6 mm, 5 μm) with Sentry Guard C18 (15 mm × 4.6 mm, 5 μm) HPLC columns (Waters, Milford, USA). A flow rate of 1.5 mL/min and column temperature of 40 °C were applied, and the mobile phase was composed of methanol and acetonitrile in 95:5 ratio. The detection wavelengths were 370 nm for retinal, and 340 nm for retinol and retinyl acetate. Retinal (Cat. No. R2500), retinol (Cat. No. R7632), and retinyl acetate (Cat. No. R4632) were purchased from Sigma (USA), dissolved in acetone, and used as standard compounds. The results are presented in the form of mean ± SD from three independent experiments.

Results and Discussion

Searching for Inherent Enzymes in *E. coli* That Contribute to Retinoid Modification

In animals, retinal is either oxidized to retinoic acid or reduced to retinol. Retinoid metabolism has not been reported in *E .coli*, but retinal, retinol and retinyl acetate were all produced in recombinant *E. coli* harboring the β-carotene synthesis pathway and the BCMO gene in our previous work (Jang et al., 2011). However, retinoic acid was not produced; it is possible that although aldehyde dehydrogenase of *E. coli* non-specifically contributes to the conversion of retinal into retinoic acid, retinoic acid is rapidly degraded following its production due to its instability (Barua and Furr 1998; Perlmann 2002). Thus, investigation was carried out on the possibility of the presence of inherent aldehyde dehydrogenases in *E. coli* that are capable of producing retinoic acid from retinal. If such enzymes do exist, the production of retinoids could be improved by deleting the genes involved in the oxidation of retinal to retinoic acid in *E. coli.* In order to find such enzymes, similar searches were performed at NCBI BLAST (http://blast.ncbi.nlm.nih.gov) between proteins of *E. coli* MG1655 (taxid:511145) and two amino acid sequences that are known to code for retinal dehydrogenase: CBY96723 from *Salmonella enterica* and NP_733798 from *Homo sapiens*. The gene *eutE* showed 97% similarity with CBY96723 whereas the gene *puuC* showed 63% similarity with NP_733798. In *E. coli*, the two genes *eutE* and *puuC* encode predicted aldehyde dehydrogenase/ethanolamine utilization protein and γ-Glu-γ-aminobutyraldehyde dehydrogenase, respectively.

For enzymes that can covert retinal into retinol, a similarity search against the retinol dehydrogenase amino acid sequence of ACC72923 from *H. sapiens* revealed the gene *ybbO* to be of highest similarity at 52%. In *E. coli, ybbO* is annotated as a predicted oxidoreductase. In addition, a recent work by Rodriguez et al (2014) has reported that YbbO has an aldehyde reductase activity and is capable of reducing decanal (C10) to decanol (C10) (Rodriguez and Atsumi 2014).

To search for enzymes responsible for the conversion of retinol into retinyl acetate, similar searches were performed against known amino acid sequences which encode retinol acyltransferase: NP_004735 from *H. sapiens*, YP_001185024 from *Shewanella putrefaciens* CN-32, and YP_723688 from *Trichodesmium erythraeum* IMS 101. No homologue of retinol acyltransferase was identified from the BLASTP analysis. Thus, acyltransferase related genes were searched within the plasmids that were introduced into *E. coli*. It was realized that the chloramphenicol resistance *cat* gene within the pSTV28 vector containing the mevalonate pathway (pSNA) encodes chloramphenicol acetyltransferase. According to recent reports, chloramphenicol acetyltransferase catalyzes the acetylation of substrates other than chloramphenicol (for eg, isobutanol and perillyl alcohol) and produces ester derivatives (Alonso-Gutierrez et al., 2013; Rodriguez et al., 2014).

Three chromosomal genes in total were predicted to be involved in the interconversion of retinoids: *eutE, puuC*, and *ybbO.* These genes were alternately deleted from the *E. coli* genome to investigate their roles in retinoid metabolism.

Retinoid Production by Deletion of *eutE, puuC,* or *ybbO* Gene

If either *eutE* or *puuC* is involved in the oxidation of retinal to retinoic acid, deletion of these genes will prevent the biological degradation of retinoids via retinoic acid, resulting in an enhancement in total retinoid production. In addition, if *ybbO* contributes to the reduction of retinal to retinol, its deletion will increase the retinal:retinol ratio. Therefore, in order to study the effect of these three genes on retinoid production, deletion strains of the three genes were introduced with the retinoid biosynthesis gene and the mevalonate pathway (Fig. 2). The total retinoid production was slightly lower than the wild type (140 mg/L), at 125 mg/L in all of *eutE, puuC*, and *ybbO* deletion mutants, but these amounts are not significantly different. On the other hand, retinal: retinol production was 70:54 mg/L in the wild type, and 80:34 mg/L in the *ybbO* deletion mutant. In proportions, retinal, retinol, and retinyl acetate were produced at 64%, 27%, and 9% for the *ybbO* deletion mutant, and 50%, 40%, and 10% for the wild type. In comparison to the wild type, the *ybbO* deletion mutant showed a

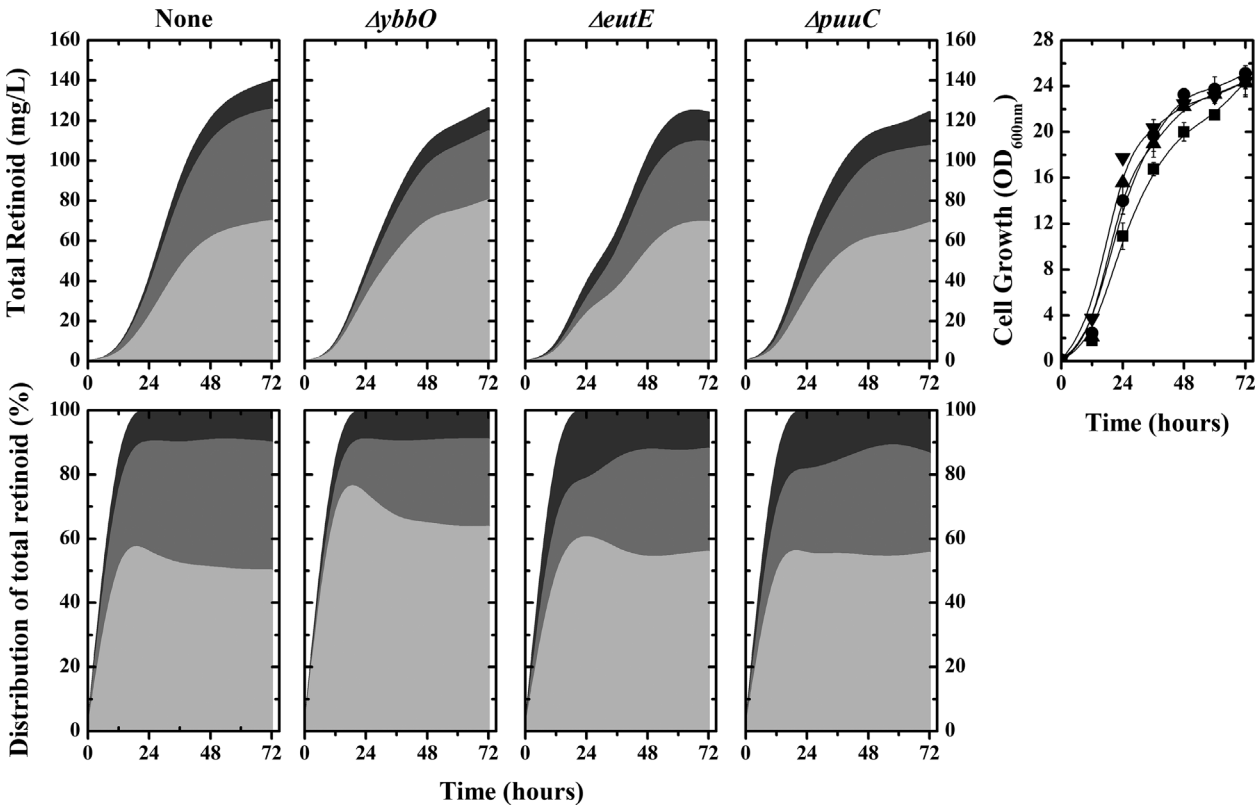

Figure 2. Retinoid production and cell growth of *E. coli* DH5α deletion mutants harboring pT-DHBSR and pS-NA. Deletion mutants were DH5α Δ*ybbO*, DH5α Δ*eutE*, and DH5α Δ*puuC*. The cultures were carried out for 72 h in 2 YT medium containing 2% (w/v) glycerol and 0.2% (w/v) arabinose at 29°C with overlay of 5 mL dodecane. For retinoid production, retinal, retinol, and retinyl acetate are indicated in light gray, gray, and dark gray, respectively. For cell growth, the *E. coli* DH5α deletion mutants are indicated as symbols; DH5α, closed squares (■); DH5α Δ*ybbO*, closed circles (●); DH5α Δ*eutE*, closed triangles (▲); DH5α Δ*puuC*, closed reversed triangles (▼). The proportions of different classes of retinoid are represented as percentages of the total retinoid production. Retinal, retinol, and retinyl acetate are indicated in light gray, dark gray, and black, respectively.

higher proportion of retinal, but a lower proportion of retinol. There was no change in the retinoid composition in *eutE* and *puuC* deletion mutants, compared to the wild type. Therefore, it can be concluded that *eutE* and *puuC* genes are not associated with retinoid metabolism, whereas *ybbO* gene contributes to the conversion of retinal to retinol, as expected. Since retinol production was not completely blocked in *ybbO* deletion mutant, it is expected some gene exists which has similar roles as retinol dehydrogenase. As mentioned above, the deletion of *eutE* and *puuC* did not affect retinoid production nor the proportion of retinoids. Therefore, these two genes were overexpressed in order to further investigate their effect on retinoid production. In the overexpression strains, both the total retinoid production and the proportion of retinoids were shown to be similar to the wild-type, and a lack of retinoic acid production was observed (Data not shown). Therefore, it is suggested that the genes *eutE* and *puuC* are not involved in the production of retinoic acid from retinal.

Retinoid Production by Overexpression of *ybbO* Gene

The *ybbO* gene was overexpressed to study its role in further detail. It is expected that the conversion from retinal to retinol will be increased, thus the proportion of retinol will increase. The *ybbO* gene is reported to have transferred from an eukaryotic retinol dehydrogenase ancestor to the *E. coli* genome in the human intestine via horizontal gene transfer, as shown by sequence comparison and phylogenetic analysis (Baker 1998). Retinoid production from retinoid-producing *E. coli* strain overexpressed with the *ybbO* gene was measured (Fig. 3). Total retinoid production did not differ significantly between the wild type and the *ybbO* overexpression strains. However, retinal and retinol were produced at 21 and 67 mg/L, respectively, which is an increase in retinol production but a decrease in retinal production as compared to the wild type strain. In proportion of the total retinoid production, retinol production was 53% in the overexpression strain, which is a 1.4-fold increase from 38% in the wild type. In the overexpression strain, retinyl acetate was produced at 38 mg/L, which is 30% of total retinoid. It is suggested that the production of retinyl acetate increased due to the concentration of retinol increasing, since retinyl acetate is converted from retinol. In conclusion, the *ybbO* gene was confirmed to have a retinol dehydrogenase activity that is able to convert retinal into retinol, a property that could be used for the selective production of retinol from *E. coli*.

Acetylation of Retinol by *cat* Gene

It was found through Protein BLAST that homologues of retinol acyltransferase do not exist within the *E. coli* genome, thus the

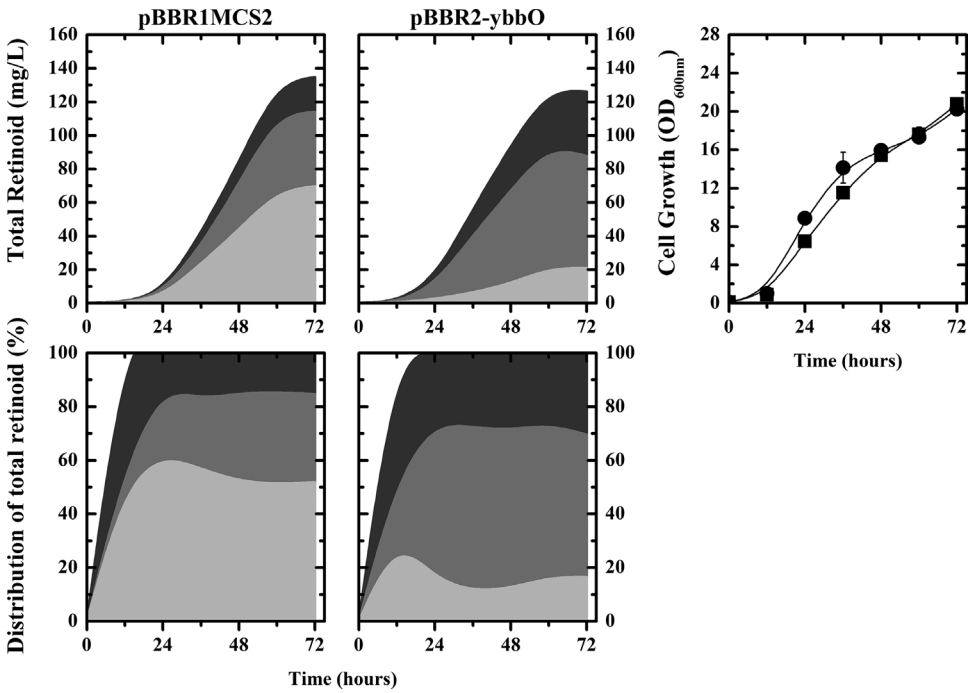

Figure 3. The effect of *ybbO* overexpression on retinoid production and cell growth of recombinant *E. coli* DH5α harboring pBBR2-ybbO, pT-DHBSR, and pS-NA. The cultures were carried out for 72 h in 2 YT medium containing 2% (w/v) glycerol and 0.2% (w/v) arabinose at 29°C with overlay of 5 mL dodecane. For retinoid production, retinal, retinol, and retinyl acetate are indicated in light gray, gray, and dark gray, respectively. For cell growth, *E. coli* DH5α containing the additional plasmids are indicated as symbols; *E. coli* (pT-DHBSR/pSNA/pBBR1MCS2), closed squares (■); *E. coli* (pT-DHBSR/pSNA/pBBR2-ybbO), closed circles (●). The proportions of different classes of retinoid are represented as percentages of the total retinoid production. Retinal, retinol, and retinyl acetate are indicated in light gray, dark gray, and black, respectively.

conversion of retinol to retinyl acetate must be due to a heterologous gene. The only gene used in this study with an *O*-acetyltransferase activity is chloramphenicol *O*-acetyltransferase (EC 2.3.1.28). Chloramphenicol *O*-acetyltransferase is encoded by the *cat* gene and used as a chloramphenicol resistance marker for the pSTV28 plasmid vector; in this study, the *cat* gene was contained within the pS-NA plasmid for the mevalonate pathway. The *cat* gene within the pS-NA plasmid was replaced with kanamycin resistance gene (ABC61923; UQ-NPTII) to see whether chloramphenicol *O*-acetyltransferase is involved in the conversion of retinol to retinyl acetate. Retinoid production in *E. coli* harboring pS-NA that contains the *cat* gene and in *E. coli* harboring pS-NAK that contains the kanamycin resistance gene was compared (Fig. 4). Total retinoid production was lower with pS-NAK at 125 mg/L, compared to 140 mg/L with pS-NA. The reduction in total retinoid production with pS-NAK is due to the lack of production of retinyl acetate from retinol. For this reason, retinol production was expected to be higher with pS-NAK. However, since retinol production did not increase as expected, this requires further investigation. Retinyl acetate production in the pS-NA strain was about 14 mg/L; the pS-NAK strain merely produced 1 mg/L as expected. Retinal and retinol were produced at 70 and 56 mg/L with pS-NA, and at 68 and 55 mg/L with pS-NAK, which showed no significant differences. However, the retinal:retinol:retinyl acetate ratio in the pS-NA strain was 50%:40%:10%, whereas in the pS-NAK strain without the *cat* gene, retinal and retinol were produced at 55% and 45%, respectively. The change in the proportion was not due to changes in retinal or retinol production, but due to the lack of retinyl acetate production.

It can be concluded that chloramphenicol *O*-acetyltransferase contributes to retinoid modification, more specifically, to the conversion of retinol to retinyl acetate in a retinol acetyltransferase-manner. In homology comparison with known retinol acyltransferases, chloramphenicol *O*-acetyltransferase showed 11% similarity with NP_004735 from *H. sapiens*, 12% similarity with YP_001185024 from *Shewanella putrefaciens* CN-32, and 14% similarity with YP_723688 from *Trichodesmium erythraeum* IMS 101. From the low homology, it can be predicted that the *cat* gene is not involved specifically with retinyl acetate production, thus the amount of retinyl acetate was actually low in proportion to total retinoid production. In order to increase the proportion of retinyl acetate for its selective production, the activity of the *cat* gene could be improved via directed evolution, or the genes mentioned above—NP_004735 from *H. sapiens,* YP_001185024 from *Shewanella putrefaciens* CN-32, or YP_723688 from *Trichodesmium erythraeum* IMS 101—could be introduced into retinoid-producing *E. coli*.

Selective Retinol Production

It was shown that the *ybbO* gene plays a role in the conversion of retinal to retinol, and the *cat* gene is involved in the conversion of retinol to retinyl acetate. The *ybbO* gene was overexpressed at the same time as using the pS-NAK plasmid which replaces the *cat* gene,

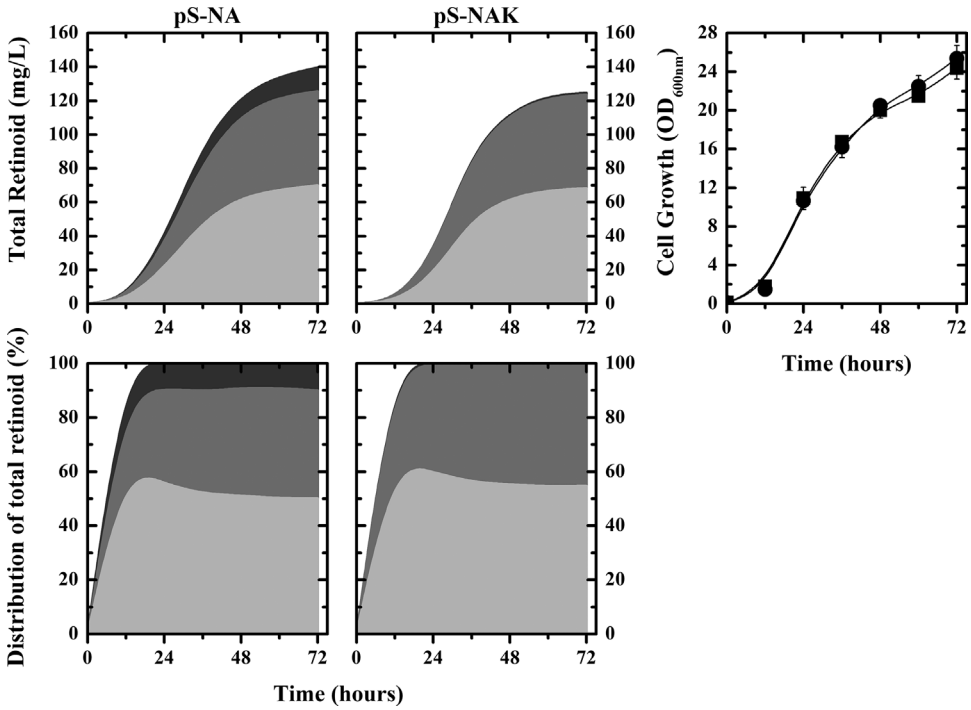

Figure 4. Retinoid production without the formation of retinyl acetate and cell growth of recombinant *E. coli* DH5α harboring pS-NAK and pT-DHBSR. The cultures were carried out for 72 h, in 2 YT medium containing 2% (w/v) glycerol and 0.2% (w/v) arabinose at 29°C with overlay of 5 mL dodecane. For retinoid production, retinal, retinol, and retinyl acetate are indicated in light gray, gray, and dark gray, respectively. For cell growth, the whole mevalonate pathway plasmids are indicated as symbols; pS-NA, closed squares (■); pS-NAK, closed circles (●). The proportions of different classes of retinoid are represented as percentages of the total retinoid production. Retinal, retinol, and retinyl acetate are indicated in light gray, dark gray, and black, respectively.

in order to produce *E. coli* that selectively produces a high proportion of retinol. The *ybbO* gene was placed after the SR gene in the retinoid-producing plasmid pT-DHBSR to produce the pT-DHBSRybbO plasmid. This plasmid was used along with either pS-NA or pS-NAK, both of which contain the mevalonate pathway, to compare retinoid proportion (Fig. 5). *E. coli* (pT-DHBSRybbO/pS-NA) resulted in a lower total retinoid production of 109 mg/L, as compared to 140 mg/L from *E. coli* (pT-DHBSR/pS-NA). In pT-DHBSRybbO strain, since the *ybbO* gene was placed after the SR gene which is located at the front, genes that produce beta-carotene, the precursor of retinal, including *crtE, crtI, crtB, ipi*HP1, and *crtY* are distanced from the promoter. This may have caused the polarity effect of gene expression in the operon, which would influence the production of the precursor. When the amount of carotenoids was measured, both strains had 3 mg/L of beta-carotene left in the cell. On the other hand, there was an absence of lycopene with pT-DHBSR whereas there was a trace of lycopene at 1 mg/L with pT-DHBSRybbO (Data not shown). After 72 h of culture, OD_{600nm} of cell growth was 24 and 21 for pT-DHBSR and pT-DHBSRybbO, respectively. Although pT-DHBSRybbO gave lower total retinoid production, retinal production was 7 mg/L, ten-fold lower than that of 70 mg/L from pT-DHBSR. Retinol and retinyl acetate were produced at 78 and 24 mg/L in pT-DHBSRybbO, and at 56 and 14 mg/L in pT-DHBSR; pT-DHBSRybbO showed both higher retinol and retinyl acetate production. In proportion of total retinoid, retinal production was reduced to 6% whereas both retinol and retinyl acetate production were increased to 72% and 22%, respectively. When *E. coli* (pT-DHBSRybbO/pS-NAK) was used for retinoid production, the amount of retinal and retinol produced was 5 and 76 mg/L, respectively, which is indifferent from *E. coli* (pT-DHBSRybbO/pS-NA). However, retinyl acetate production was reduced to 5 mg/L, which lead to a decrease in total retinoid production from 109 to 87 mg/L. This is a similar result as shown in Figure 4, whereby the total retinoid production decreased due to the reduction in retinyl acetate production. The proportion of retinal: retinol:retinyl acetate was 6%:88%:6%. In comparison to pS-NA, the proportion of retinal is the same, but the proportion of retinol increased by 16% and the proportion of retinyl acetate decreased by 16%. In conclusion, with the pT-DHBSRybbO plasmid, even though the total retinoid production was lower than that with the three vector system which uses the pBBR2-ybbO plasmid (Fig. 3), the yield percentage of retinol reached a high 88%. Thus, we were able to develop retinoid-producing *E. coli* that selectively produces retinol.

Conclusion

Retinol and retinyl acetate were produced along with retinal, even though *E. coli* does not contain metabolic pathways related to retinoids. Thus, we selected genes of inherent *E. coli* enzymes or genes within the retinal-producing plasmid, and investigated their effect on retinoid production. As a result, genes which contribute to the production of retinol and retinyl acetate were identified. In addition, it was shown that selective retinol production is possible

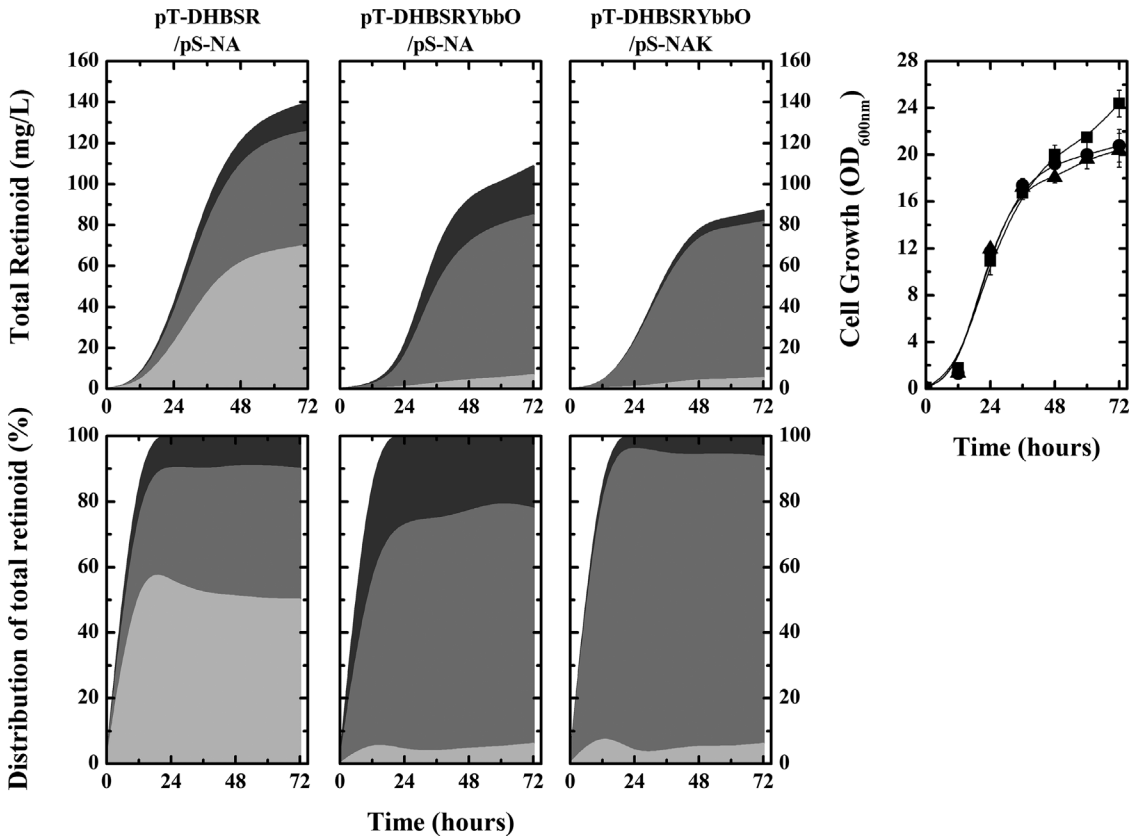

Figure 5. The effect of pT-DHBSRybbO plasmid for selective production of retinol and cell growth of recombinant *E. coli* DH5α harboring pT-DHBSRybbO with pS-NA and pS-NAK. The cultures were carried out for 72 h in 2 YT medium containing 2% (w/v) glycerol and 0.2% (w/v) arabinose at 29°C with overlay of 5 mL dodecane. For retinoid production, retinal, retinol, and retinyl acetate are indicated in light gray, gray, and dark gray, respectively. The cell growth of *E. coli* (pT-DHBSR/pS-NA), (pT-DHBSRybbO/pS-NA), and (pT-DHBSRybbO/pS-NAK) are indicated as symbols of closed squares (■), closed circles (●), and closed triangles (▲), respectively. The proportions of different classes of retinoid are represented as percentages of the total retinoid production. Retinal, retinol, and retinyl acetate are indicated in light gray, dark gray, and black, respectively.

via metabolic engineering, which provides an industrially meaningful method for the production of retinol, an increasingly sought-after cosmetic ingredient.

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