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Effects of Increased NADPH Concentration by Metabolic Engineering of the Pentose Phosphate Pathway on Antibiotic Production and Sporulation in *Streptomyces lividans* TK24

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Most of the biosynthetic pathways for secondary metabolites are influenced by carbon metabolism and supply of cytosolic NADPH. We engineered carbon distribution to the pentose phosphate pathway (PPP) and redesigned the host to produce high levels of NADPH and primary intermediates from the PPP. The main enzymes producing NADPH in the PPP, glucose 6-phosphate dehydrogenase (encoded by *zwf*1 and *zwf*2) and 6-phosphogluconate dehydrogenase (encoded by *zwf*3), were overexpressed with *opc* encoding a positive allosteric effector essential for Zwf activity in various combinations in *Streptomyces lividans* TK24. Most *S. lividans* transformants showed better cell growth and higher concentration of cytosolic NADPH than those of the control, and *S. lividans* TK24/pWHM3-Z23O2 containing *zwf*2+*zwf*3+*opc*2 showed the highest NADPH concentration but poor sporulation in R2YE medium. *S. lividans* TK24/pWHM3-Z23O2 in minimal medium showed the maximum growth (6.2 mg/ml) at day 4. Thereafter, a gradual decrease of biomass and a sharp increase of cytosolic NADPH and sedoheptulose 7-phosphate between days 2 and 4 and between days 1 and 3, respectively, were observed. Moreover, *S. lividans* TK24/pWHM3-Z23O2 produced 0.9 times less actinorhodin but 1.8 times more undecylprodigiosin than the control. These results suggested that the increased NADPH concentration and various intermediates from the PPP specifically triggered undecylprodigiosin biosynthesis that required many precursors and NADPH-dependent reduction reaction. This study is the first report on bespoke metabolic engineering of PPP routes especially suitable for producing secondary metabolites that need diverse primary precursors and NADPH, which is useful information for metabolic engineering in *Streptomyces*.

Keywords: Glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, actinorhodin, undecylprodigiosin, *Streptomyces lividans*, pentose phosphate pathway

Introduction

Genus *Streptomyces* is a representative group of industrial microorganisms contributing to the production of various secondary metabolites and hydrolytic enzymes. In addition, it has unique characteristics of morphological differentiation, forming substrates and aerial mycelia, and spores [1]. According to recent analyses of biological systems at the intracellular metabolite level, the balance of redox metabolism generating NADH and consuming NADPH influences the biosynthesis of secondary metabolites (physiological differentiation) and morphological differentiation [2].

Glycolysis and the tricarboxylic acid cycle are the main pathways generating NADH, and the oxidative pentose phosphate pathway (PPP) is the major source of NADPH, which comprises the most central metabolic pathways in

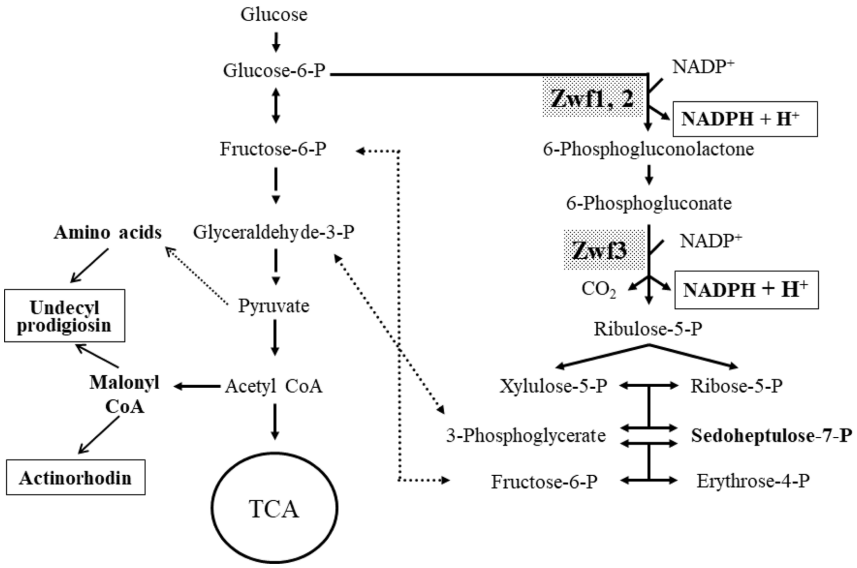

Fig. 1. Overview of central carbon metabolism showing glycolysis, the tricarboxylic acid (TCA) cycle, and the pentose phosphate pathway.

Actinorhodin and undecylprodigiosin biosynthetic pathways are indicated by arrows. The enzymes relevant to this work are shown. Zwf1 and Zwf2, glucose 6-phosphate dehydrogenase; Zwf3, 6-phosphogluconate dehydrogenase; CoA, coenzyme A.

primary metabolism (Fig. 1). NADPH is required for many biosynthetic and detoxification reactions [3]. The flux through the PPP is increased at high NADPH requirements, for example biosynthesis of secondary metabolites such as penicillin [4] and methylenomycin [5], and growth by nitrate reduction [6], but is decreased when the need for NADPH production is reduced [4].

Many primary metabolites in PPP can be used as precursors for primary and secondary metabolite biosynthesis [5]. For example, sedoheptulose 7-phosphate is an important intermediate of primary metabolites as well as of precursors for synthesizing valienamine-containing secondary metabolites, including acarbose [8], validamycin [9], and salbostatin [10]. Therefore, controlling the PPP by systemic metabolic engineering may be a useful tool to improve the secondary metabolite production associated with a strain.

NADPH is produced in the PPP by glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) that convert glucose 6-phosphate to 6-phosphoglucono-D-lactone and 6-phosphogluconate to ribulose 5-phosphate [11], respectively (Fig. 1). In *Streptomyces coelicolor*, two clusters of genes (Accession Nos. AL031107 and AL096839, http://www.sanger.ac.uk/Projects/S_coelicolor/) encoding PPP-related enzymes were identified [12]. G6PDH is encoded by *zwf*1 in the AL031107 cluster and *zwf*2 in the AL096839 cluster, whereas 6PGDH is encoded by *zwf*3 in a separate chromosomal region (Fig. 2A). Genes *zwf*1 and *zwf*2 are located upstream of *opc*, a positive allosteric effector essential for Zwf activity in cyanobacteria [13] and *Corynebacterium glutamicum* [14].

Compared with the primary metabolic pathways, secondary metabolism uses primary metabolites as precursors and has long and complex biosynthetic routes, including oxidation and reduction [15]. Therefore, it might be possible to enhance antibiotic production by increasing the supply of NADPH or precursors by elevating the activity of Zwf. Based on this rationale, the present study aimed to improve biosynthesis of NADPH by engineering metabolic flux through the PPP in *Streptomyces lividans* TK24. We cloned *zwf1* and *zwf*2 encoding G6PDH isozymes, *zwf*3 encoding 6PGDH, and *opc* from *S. coelicolor* A3(2), and expressed them in *S. lividans* TK24 at various combinations. In this article, we report the effects of metabolic engineering through the PPP specifically on production of NADPH, sedoheptulose 7-phosphate, and antibiotics as well as cell growth and morphological differentiation.

Materials and Methods

Bacterial Strains and Culture Media

S. coelicolor A3(2) and *S. lividans* TK24 were acquired from the John Innes Institute (UK). R2-Yeast extract (R2YE) medium [16] was used for maintaining the *Streptomyces* strains, and R2YE plates containing 2% agar were used for regenerating protoplasts

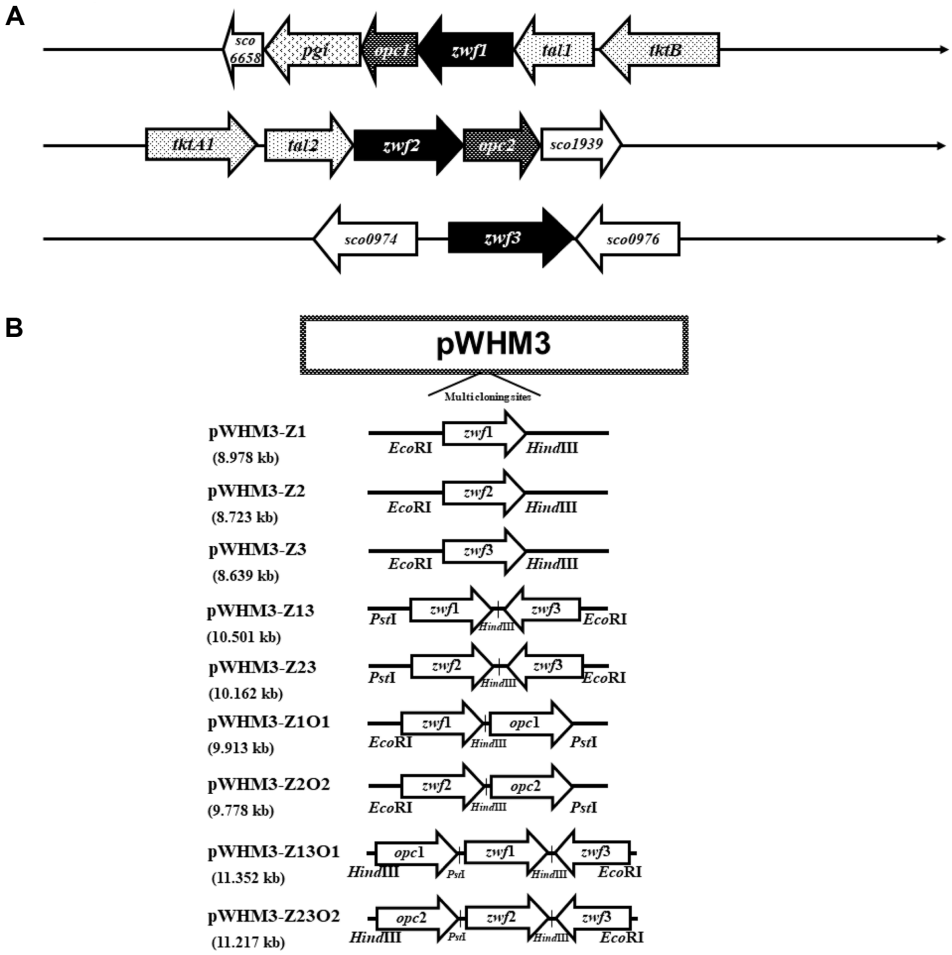

Fig. 2. Gene organization and construction of expression vectors.
(**A**) Gene organization in the chromosomal DNA of *Streptomyces coelicolor* A3(2) containing *zwf*1, *zwf*2, and *zwf*3. Arrows indicate individual open reading frames, with the stop codon marked by the arrowhead. Zwf1 and Zwf2, glucose 6-phosphate dehydrogenase; Zwf3, 6-phosphogluconate dehydrogenase; Tkt, transketolase; Tal, transaldolase; Sco1939, 6-phosphogluconolactonase; Sco6658, 6-phosphogluconate dehydrogenase-like protein; Pgi, glucose-6-phosphate isomerase; Opc; putative allosteric activator for Zwf; others, unknown. (**B**) Construction of expression vectors for *zwf*1, *zwf*2, zwf3, *opc*1, and *opc*2. Primers used for polymerase chain reaction cloning are listed in Table 1, and each restriction enzyme used for subcloning is depicted.

and, after addition of thiostrepton (25 μg/ml), for selecting transformants. The high-copy-number plasmid pWHM3 [17], a *Streptomyces-Escherichia coli* shuttle vector, was used for overexpression in *Streptomyces*. *E. coli* DH5α and ET12567 strains were used as a host for plasmid construction and preparation of the non-methylated DNA for transformation into *Streptomyces*, respectively. The media and growth conditions for *E. coli* were described by Green and Sambrook [18]. Plasmid pMD-T (Takara Bio Inc., Japan) was used for cloning into *E. coli*. *Streptomyces* and *E. coli* were cultivated at 28°C and 37°C, respectively.

Enzymes and Chemicals

DNA-modifying enzymes were purchased from Takara Bio Inc. (Japan). Preparation of plasmid and chromosomal DNA, restriction enzyme digestions, and agarose gel electrophoresis were performed according to standard recombinant DNA techniques described by Green and Sambrook [18]. DNA fragments were extracted from agarose gels with the Gene Extraction kit (DyneBio, Korea). NADPH, antibiotics, and other fine chemicals used for preparing buffers were obtained from Sigma-Aldrich Chemical Co. (USA).

Construction and Transformation of Expression Vectors

DNA fragments containing *zwf1*, *zwf2*, *zwf3*, *opc1*, and *opc2* were cloned by polymerase chain reaction (PCR) using the primers listed in Table 1. The primers were designed from the nucleotide sequence registered in the *Streptomyces* genome project page (http://www.sanger.ac.uk/Projects/S_coelicolor/) to have putative promoter regions including an approximately more than 300-bp

Table 1. Primers used for the polymerase chain reaction.

Primer	Oligonucleotide[a]
Primers for *zwf*1 (glucose 6-phosphate dehydrogenase)	
Zwf1-F_{Eco}	5′-*GAATTC*CCAGCGCCCCCTGTGGGCGT-3′ (EcoRI)
Zwf1-R_{Hind}	5′-*AAGCTT*TCATGGCCTGCGCCAGCTCCG-3′ (HindIII)
Zwf1-F_{Pst}	5′-*CTGCAG*CCAGCGCCCCCTGTGGGCGT-3′ (PstI)
Primers for *zwf*2 (glucose 6-phosphate dehydrogenase)	
Zwf2-F_{Eco}	5′-GTACGAGGAG*GAATTC*GGCACCGA-3′ (EcoRI)
Zwf2-R_{Hind}	5′-GAGAC*AAGCTT*GATGACGACG-3′ (HindIII)
Zwf2-F_{Pst}	5′-GCTGGGCCGCCC*CTGCAG*AAGGCCGGCG-3′ (PstI)
Primers for *zwf*3 (6-phosphogluconate 1-dehydrogenase)	
Zwf3-$F1_{Eco}$	5′-CGG*GAATTC*TGCCGACGAGGGGGAGGGCC-3′ (EcoRI)
Zwf3-$R1_{sal}$	5′-CTG*GTCGAC*CACCACGTCCACGAACGGCTT-3′ (SalI)
Zwf3-$F2_{sal}$	5′-GGACGTGGTG*GTCGAC*CAGGCGGAG-3′ (SalI)
Zwf3-$R2_{Hind}$	5′-*AAGCTT*CTACGCGGTGACCTCGGACCG-3′ (HindIII)
Primers for *opc*1 gene	
Opc1-F_{Hind}	5′-CATGACGGTAAGCTTCGGCTCC-3′ (HindIII)
Opc1-R_{Pst}	5′-CGCGGT*CTGCAG*GACCGCCC-3′ (PstI)
Primers for *opc*2 gene:	
Opc2-F_{Hind}	5′-GTTCC*AAGCTT*CCGGGCACGTCG-3′ (HindIII)
Opc2-R_{Pst}	5′- AA*CTGCAG*GGGCCGTCGGAG-3′ (PstI)

[a]Restriction enzyme sites introduced for subsequent cloning of DNA fragments are shown; the corresponding restriction enzymes are shown in parenthesis.

upstream region of the translational start point. Ex Taq PCR Premix (Takara Bio Inc., Japan) was used in PCR amplification under the following conditions: an initial denaturation step of 5 min at 95°C, 30 cycles of amplification (30 sec at 96°C, 30 sec at 60°C, 1.5 min at 72°C), and a final extension period of 10 min at 72°C. The amplified DNA fragments were digested with the corresponding restriction enzymes presented in Table 1 and cloned into the pMD-T vector with their own promoters. Then, each gene was cloned into the pWHM3 *Streptomyces-E. coli* shuttle vector, generating various combinations of *zwf1*, *zwf2*, *zwf3*, *opc1*, and *opc2* as follows: *zwf*1, *zwf*2, *zwf*3, *zwf*1+*opc*1, *zwf*2+*opc*2, *zwf*1+*zwf*3, *zwf*2+*zwf*3, *zwf*1+ *zwf*3+ *opc*1, and *zwf*2+ *zwf*3+*opc*2 (Fig. 2B). Each resulting plasmid was transformed into *S. lividans* TK24 by the polyethylene glycol-mediated protoplast transformation method [16].

Batch Fermentation of *S. lividans* Transformants in Baffled Erlenmeyer Flasks

A spore suspension (approximately 10^8 spores) stored at −80°C was inoculated into 50 ml of R2YE medium [16] containing thiostrepton (25 μg/ml) in a 500-ml baffled Erlenmeyer flask. The seed culture was grown for 48 h on a rotary shaker (250 rpm), and 10 ml of the seed culture were inoculated into 100 ml of R2YE medium containing thiostrepton (50 μg/ml) in a 500-ml baffled Erlenmeyer flask and cultured for 7 days at 250 rpm.

Batch Fermentation of *S. lividans* Transformants in Fermenter

A spore suspension (approximately 10^8 spores) stored at −80°C was inoculated into 50 ml of R2YE medium containing thiostrepton (25 μg/ml) in a 500-ml baffled Erlenmeyer flask. The seed culture was grown for 48 h on a rotary shaker (250 rpm), and 10 ml of the seed culture was inoculated into 100 ml of Glucose-Yeast extract-Broth medium (33 g glucose and 15 g yeast extract per liter) containing thiostrepton (25 μg/ml) in a 500-ml baffled Erlenmeyer flask. After cultivation for 24 h at 250 rpm, 200 ml of the culture was inoculated into a 3-L fermenter containing 2 L of minimal medium (27.74 g glucose, 1.38 g NaH_2PO_4, 0.75 g KCl, 0.25 g $MgCl_2 \cdot 6H_2O$, 8.5 g $NaNO_3$, 0.28 g Na_2SO_4, 0.59 g citric acid, 0.037 g $CaCl_2$, and 2 ml trace element per liter of distilled water, pH 7.0) containing thiostrepton (50 μg/ml). Glucose, NaH_2PO_4, and other trace elements were sterilized separately and added aseptically. The pH was adjusted to 6.8−7.2 by automatic addition of 1 N HCl or 1 N NaOH. Agitation was fixed at 200 rpm, and aeration was at 1 volume of air per volume of medium per minute.

Preparation of Samples

Three milliliter aliquots of samples were collected aseptically and centrifuged (5,000 ×*g*) at 4°C for 10 min. Mycelium-free supernatant was used for the determination of glucose concentration. Cell pellets were resuspended in 300 μl of phosphate-buffered saline (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, and KH_2PO_4 2 mM, pH 7.4) and disrupted by sonication for 3 min on ice. The cleared cell extract after centrifugation (15,000 ×*g*, 4°C, 30 min) was used for the determination of NADPH and sedoheptulose 7-phosphate.

Analytical Methods

Biomass. After collection by centrifugation, mycelia were washed twice with distilled water and dried at 70°C for 48 h for dry weight determination.

Glucose. The glucose concentration in the culture was determined using the Body Condition Score glucose kit according to the supplier manual (GAGO20-1KT; Sigma-Aldrich Chemical Co., USA).

Quantitation of NADPH and sedoheptulose 7-phosphate. The cell extract (250 µl), 100 µl of Tris-HCl (0.1 M, pH 8.0), 50 µl of $MgCl_2$ (0.01 M), 50 µl of glucose-6-phosphate (0.0006 M), and 50 µl of NADP (0.0002 M) were mixed. After incubation at 28°C for 2 min, the concentration of NADPH was analyzed by high-performance liquid chromatography (HPLC; Waters, USA) equipped with a Supelcosil LC-18-T (25 cm × 4.6 cm ID, 5 µm particles) column. A gradient elution using two mobile phases, 0.1 M KH_2PO_4 (pH 6.0) solution (buffer A) and 0.1 M KH_2PO_4 (pH 6.0) solution containing 10% (v/v) CH_3OH (buffer B), was applied. The gradient for separation started from 100% of buffer A and ended with 100% buffer B in 10 min, with a flow rate of 1.3 ml/min. The peak for NADPH was detected at 254 nm (Photodiode Array 996; Waters), and authentic NADPH was used as the standard. For analysis of sedoheptulose 7-phosphate, 12 mM aqueous ammonium acetate solution was used isocratically at a flow rate of 0.1 ml/min [17]. The peak for sedoheptulose 7-phosphate was detected at 230 nm and compared with authentic reagent.

Quantitation of actinorhodin and undecylprodigiosin. The supernatant of the culture (1 ml) after centrifugation was mixed thoroughly with 0.5 ml of 3 N NaOH. After centrifugation (15,000 ×*g*, 2 min), the actinorhodin concentration was determined by measuring the absorbance of the supernatant at 640 nm in a Genesys 8 spectrophotometer (Fisher Scientific, USA). The cell pellet of the culture (1 ml) after centrifugation was used for extraction of undecylprodigiosin. A volume of acidic methanol (adjusted to pH 1.0 with 1N HCl) equal to the culture aliquot was added to the cell pellet and vortexed thoroughly for 2 min. After removing cell debris by centrifugation (15,000 ×*g*, 2 min), absorbance measurements were obtained at 530 nm. Concentrations of actinorhodin and undecylprodigiosin were calculated using the molar absorptivity ε_{640} = 25,350 M^{-1} cm^{-1} [20] and ε_{530} = 100,150 M^{-1} cm^{-1} [21], respectively.

Results

Construction of Vectors

*zwf*1 and *zwf*2 encoding G6PDH isozymes and *zwf*3 encoding 6PGDH were cloned with their own promoter regions (Fig. 2A). *opc*1 and *opc*2 encoding positive allosteric effectors essential for Zwf activity [13] were also cloned with their own promoters. All the clones were used for subsequent cloning in various combinations, and nine recombinant plasmids containing *zwf*1, *zwf*2, *zwf*3, *zwf*1+*opc*1, *zwf*2+*opc*2, *zwf*1+*zwf*3, *zwf*2+*zwf*3, *zwf*1+*opc*1+*zwf*3, and *zwf*2+*opc*2+*zwf*3 were constructed, generating pWHM3-Z1, pWHM3-Z2, pWHM3-Z3, pWHM3-Z1O1, pWHM3-Z2O2, pWHM3-Z13, pWHM3-Z23, pWHM3-Z13O1, and pWHM3-Z23O2, respectively (Fig. 2B).

Characteristics of *S. lividans* TK24 Transformants in Flask Culture

Cell growth. Each recombinant plasmid was transformed into *S. lividans* TK24 that is frequently used as a *Streptomyces* host for transformation and expression. When the growth of each transformant in R2YE broth in flask batch culture was compared by measuring cell dry weight (CDW), the maximum growth was mainly observed at day 3 (Fig. 3A). All transformants with the nine recombinant plasmids gave higher CDW than that (2.9 mg/ml) of the control (empty vector), and both *S. lividans* TK24/pWHM3-Z1O1 and *S. lividans* TK24/pWHM3-Z1 showed the highest CDW (3.98 mg/ml). According to Avignone Rossa *et al*. [22], the increase in PPP flux could lead to the increase of various intermediates for primary metabolites, resulting in high biomass synthesis. Our data also showed that the recombinant plasmids enhanced biomass synthesis by increasing metabolic flux through the PPP.

NADPH production. The intracellular concentration of NADPH was analyzed by HPLC. The cell-free extract was prepared from the cells collected from 3-ml cultured broth, and the final volume of extract was adjusted to 300 µl. Except for *S. lividans* TK24/pWHM3-Z2, all the transformants with the recombinant plasmids showed 2–5 times higher concentrations of NADPH than that of the control (Fig. 3B). The transformants with pWHM3-Z13, pWHM3-Z23, and pWHM3-Z13O1 showed more than 3 times increased levels of NADPH, and *S. lividans* TK24/pWHM3-Z23O2 especially showed the highest concentration (4.7 times higher than that of the control) of NADPH. This result suggested that the introduction of various recombinant plasmids led to increased metabolic flux through the PPP, generating NADPH at high concentrations. Although all the transformants, including the control, showed maximum growth at day 3, *S. lividans* TK24/pWHM3-Z23O2 showed the maximum growth at day 4 (1.6 times higher than that of control). Considering the growth rate and NADPH production, *S. lividans* TK24/pWHM3-Z23O2 was selected for further fermentation study.

Morphological Characteristics of *S. lividans* TK24 Transformants on R2YE Agar Plate

The in vivo effects of the cloned genes on morphological changes were studied (Fig. 3C). When the transformants of

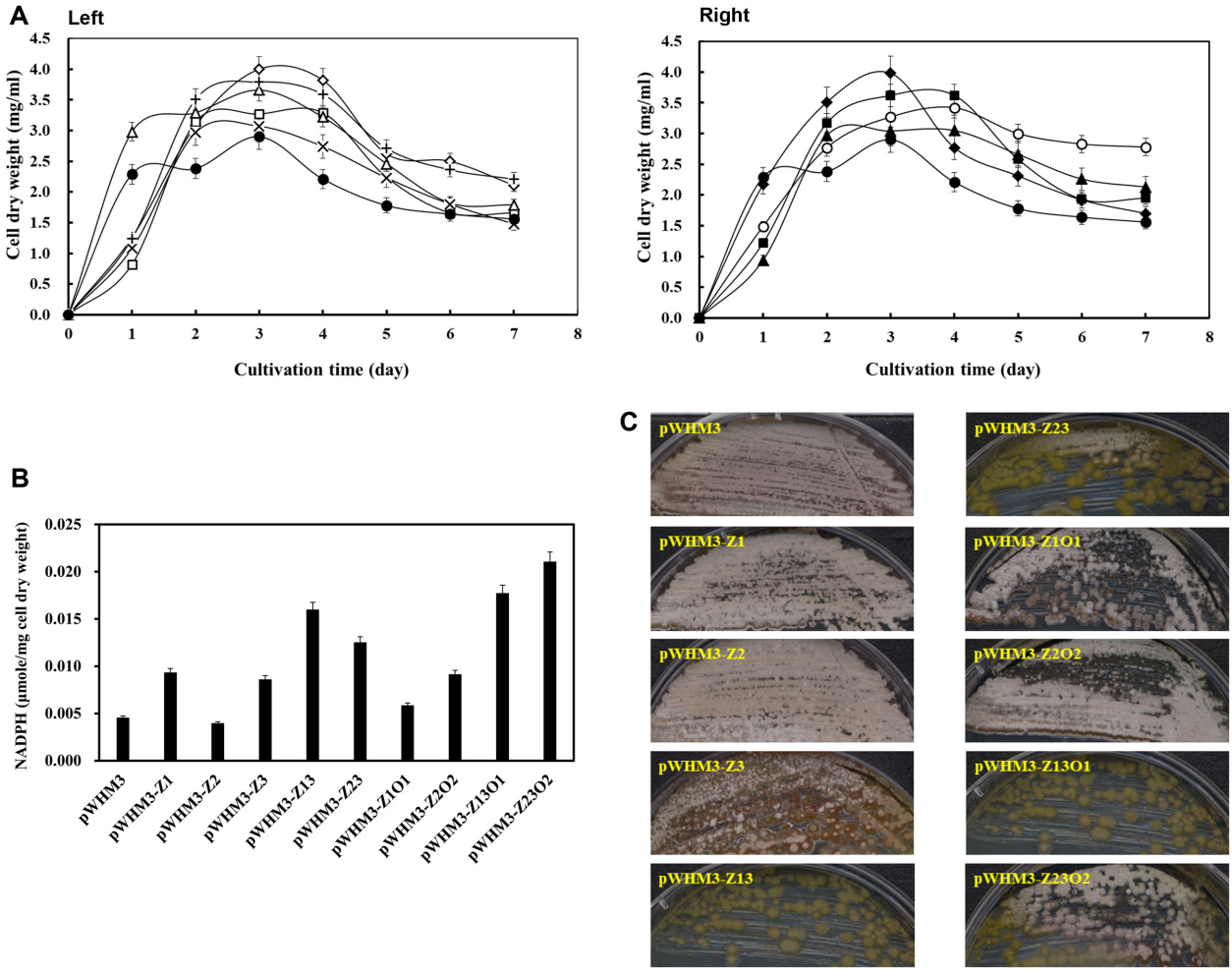

Fig. 3. Cell growth, cytosolic NADPH concentration, and morphological differentiation of *Streptomyces lividans* TK24 transformants.

The transformants were cultured in R2 Yeast Extract (R2YE) broth by shake flask fermentation for analyzing cell growth and cytosolic NADPH concentration. (**A**) Cell dry weight (mg/ml) depending on cultivation time. (Left) ●, *S. lividans* TK24/pWHM3; ◇, *S. lividans* TK24/pWHM3-Z1; □, *S. lividans* TK24/pWHM3-Z2; △, *S. lividans* TK24/pWHM3-Z3; ×, *S. lividans* TK24/pWHM3-Z13; +, *S. lividans* TK24/pWHM3-Z23. (Right) ●, *S. lividans* TK24/pWHM3; ◆, *S. lividans* TK24/pWHM3-Z1O1; ■, *S. lividans* TK24/pWHM3-Z2O2; ▲, *S. lividans* TK24/pWHM3-Z13O1; ○, *S. lividans* TK24/pWHM3-Z23O2. (**B**) The intracellular concentration of NADPH (μmol/mg cell dry weight) at day 3. All the data shown were averaged from the triplicate fermentations. (**C**) Photographs of *S. lividans* TK21 transformants grown on R2YE agar plate for 6 days.

S. lividans TK24 were grown on R2YE agar plate, little difference in colony size was observed. Instead, compared with the control, formation of aerial mycelium and spore was severely inhibited in the strains transformed with pWHM3-Z13, pWHM3-Z23, pWHM3-Z13O1, and pWHM3-Z23O2, which were listed as high cytosolic NADPH producers (Fig. 3C). Moreover, massive production of red pigment (probably undecylprodigiosin), higher than the control, was visually observed on the plate in all the transformants except *S. lividans* TK24/pWHM3-Z13O1 from day 5 (data omitted). These results strongly indicated that engineering metabolic flux through the PPP could significantly affect either typical phenotypic or physiological differentiation in *S. lividans* TK24.

Characteristics of *S. lividans* TK24/pWHM3-Z23O2 in 2-L Fermenter Culture

Cell growth and glucose consumption. The control group showed its maximum growth (5.8 mg/ml) at day 3 and rapid decrease in cell mass after that in minimal medium in 2-L fermenter culture. On the contrary, *S. lividans* TK24/pWHM3-Z23O2 showed maximum growth (6.2 mg/ml) at

day 4, and, thereafter, a gradual decrease of biomass (Fig. 4A). Glucose consumption was similar in both strains during days 1 and 2, but more rapid consumption by the control during days 3 and 5 was observed (Fig. 4A), probably due to more efficient glucose utilization via glycolysis in the control. Instead, *S. lividans* TK24/pWHM3-Z23O2 showed a more efficient use of carbon source for biomaterial synthesis by increased metabolism through PPP flux in spite of slow consumption of glucose. The overall glucose consumption at day 7 was higher in *S. lividans* TK24/pWHM3-Z23O2 than that of the control, probably due to high cell density.

NADPH production. A sharp increase of the intracellular NADPH concentration was observed in *S. lividans* TK24/pWHM3-Z23O2 between days 2 and 3, and in the control between days 3 and 5 (Fig. 4B). The NADPH concentration in *S. lividans* TK24/pWHM3-Z23O2 was remarkably higher than that of the control, but this difference decreased after day 5.

Sedoheptulose 7-phosphate production. Similarly, the intracellular concentration of sedoheptulose 7-phosphate, an intermediate of the PPP, also increased sharply in *S. lividans* TK24/pWHM3-Z23O2 in the early phase of fermentation, but a similar level was detected after day 4, which may be caused by the rapid conversion of sedoheptulose 7-phosphate into other biomaterials (Fig. 4C).

Antibiotic Production by *S. lividans* TK24/pWHM3-Z23O2 in 2-L Fermenter Culture

S. lividans TK24 can produce low amounts of the pigmented antibiotics actinorhodin (blue) and undecylprodigiosin (red) under normal culture condition, but antibiotics production can be stimulated by some environmental or intracellular factors that induce expression of their biosynthetic genes [7, 21]. To confirm the effects of PPP engineering on antibiotics production, we measured the amount of pigments produced by the transformants during fermentation in minimal medium for 10 days. The amount of actinorhodin produced increased depending on the cultivation time; however, *S. lividans* TK24/pWHM3-Z23O2 produced slightly less actinorhodin than that produced by the control (Fig. 5A). Contrastingly, the amount of undecylprodigiosin produced by *S. lividans* TK24/pWHM3-Z23O2 increased continuously and reached its maximum (4.2 mg/l) at day 10 (1.8 times higher than that of the control) (Fig. 5B). All these results strongly indicated that antibiotics production may be influenced in a different way depending on their biosynthetic pathways by engineering metabolic flux through the PPP.

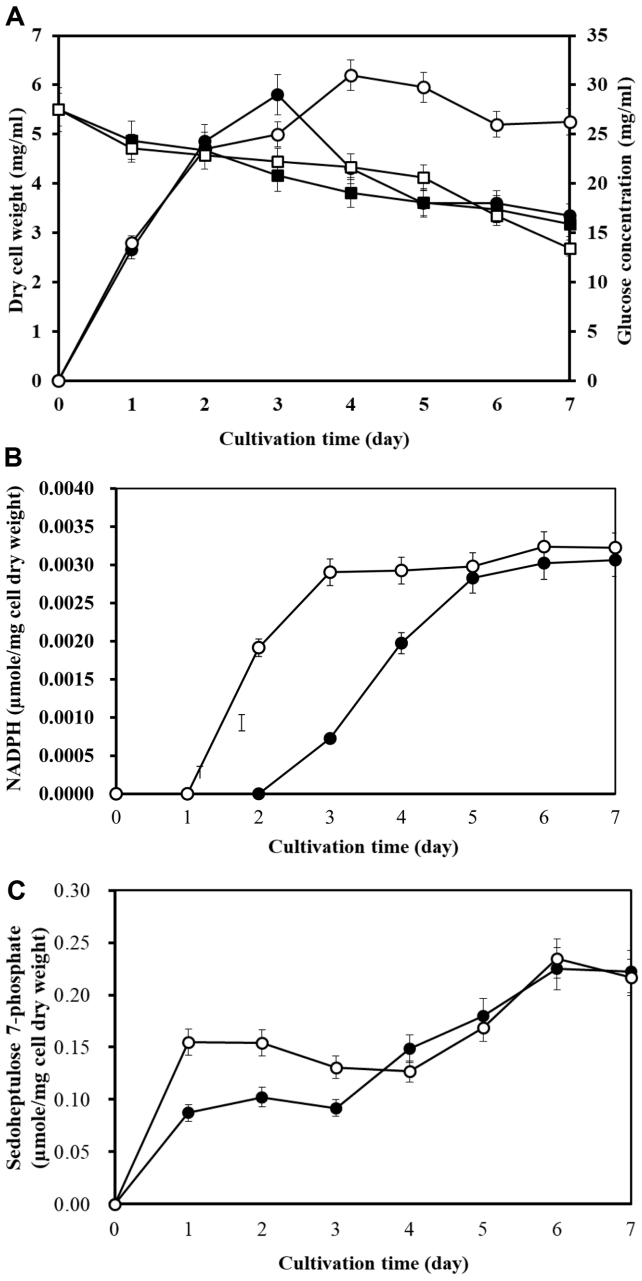

Fig. 4. Cell growth, cytosolic NADPH, and sedoheptulose 7-phosphate concentrations of *Streptomyces lividans* TK24/pWHM3-Z23O2 depending on cultivation time in minimal medium in fermenter culture.

(**A**) Cell dry weight (mg/ml) and the remaining concentration of glucose. (**B**) The cytosolic NADPH concentration (μmol/mg cell dry weight). (**C**) The intracellular concentration of sedoheptulose 7-phosphate (μmol/mg cell dry weight). ●, *S. lividans* TK24/pWHM3; ○, *S. lividans* TK24/pWHM3-Z23O2. ■ and □, glucose concentration in cultures of *S. lividans* TK24/pWHM3 and *S. lividans* TK24/pWHM3-Z23O2, respectively. All the data shown were averaged from the triplicate fermentations.

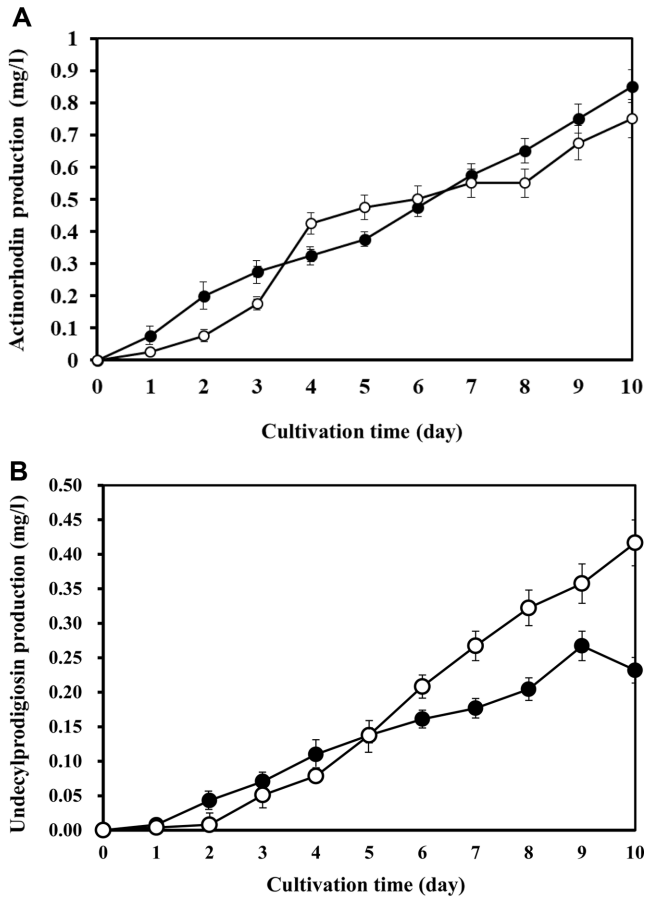

Fig. 5. Actinorhodin (**A**) and undecylprodigiosin (**B**) production by *Streptomyces lividans* TK24/pWHM3-Z23O2 in minimal medium in fermenter culture.

●, *S. lividans* TK24/pWHM3; ○, *S. lividans* TK24/pWHM3-Z23O2. All the data shown were averaged from the triplicate fermentations.

Discussion

Streptomycetes have been studied for the production of a wide range of secondary metabolites and for increasing the production levels of these metabolites. Each secondary metabolite is strain-specifically synthesized and modified by specific tailoring enzymes; however, all the precursors and cofactors are derived from the common primary metabolism. Most of the antibiotic biosynthetic pathways include reductive steps where NADPH is required as a reducing factor. Therefore, engineering the primary metabolism to synthesize precursors or reducing cofactors is a new strategy for developing high-level antibiotic-producing strains [22–24]. Based on this rationale, we demonstrated that engineering the PPP, targeting key enzymes such as G6PDH and 6PGDH, resulted in improved undecylprodigiosin production in *S. lividans* TK24.

Borodina *et al*. [25] reported that deletion of pfkA2 (SCO5426) encoding phosphofructokinase involved in glycolysis resulted in high-level production of pigmented antibiotics (actinorhodin and undecylprodigiosin) because of an increased carbon flux through the PPP in *S. coelicolor* A3(2). It was assumed that the decreased phosphofructokinase activity induced an increase in PPP flux and in flux to pigmented antibiotics and pyruvate, based on genome-scale metabolic model simulations. In addition, G6PDH encoded by *zwf2* played a more significant role than that of the G6PDH encoded by *zwf1* in determining the carbon flux to actinorhodin in *S. coelicolor* [26].

Butler *et al*. [3] demonstrated that the deletion of *zwf1* or *zwf2* resulted in reduced level of Zwf activity to approximately one-half than that observed in the wild-type *S. lividans*. When the pathway-specific transcriptional activator genes for either the actinorhodin (*act*II-ORF4) or the undecylprodigiosin (*red*D) biosynthetic pathway were introduced on multi-copy number plasmids to the mutants, the transformants produced higher levels of antibiotic than those of the wild-type control strains. This result suggested that a lower flux of carbon through the PPP and more efficient glucose utilization via glycolysis resulted in higher levels of antibiotic production, which contradicted the results by Borodina *et al*. [25]. Therefore, other factors as well as the distribution of carbon flux should be considered to develop high producer of antibiotics.

Although all the previous reports [3, 25, 26] described actinorhodin production in a Δ*zwf* background, other metabolic fluxes were not analyzed. Our data clearly demonstrated that Zwf overexpression could lead to increased cell growth, and high intracellular concentrations of NADPH and sedoheptulose 7-phosphate (in early growth phase) in *S. lividans*, probably due to an increased carbon flux through the PPP by enhanced Zwf activity. Interestingly, Zwf overexpression increased undecylprodigiosin production but not actinorhodin production, which is in disagreement with previous reports [3, 25, 26].

Actinorhodin is a blue-pigmented antibiotic produced by *S. coelicolor* A3(2) and has been characterized as one of the best model compounds for studying type II polyketide synthase (PKS) [27]. The type II minimal PKS assembles from eight units of malonate the basic carbon skeleton into octaketide [28], which is further converted to actinorhodin by several ancillary and tailoring enzymes [15].

Prodiginines, including undecylprodigiosin, are a family of tripyrrole red pigments, and they have immunosuppressive and anticancer activities as well as antifungal, antibacterial,

antiprotozoal, and antimalarial effects [29]. Undecylprodigiosin is synthesized from one unit of proline, one unit of glycine, one unit of serine, one unit of acetate, and seven units of malonate via a complex biosynthetic pathway. Although the precise biosynthetic pathways of the two antibiotics are not completely understood, several reductive reactions are necessary for both processes. Specifically, NAPDH-dependent reduction has been observed in undecylprodigiosin biosynthesis [29]. It has been suggested that the biosynthesis of prodigiosins might function as a metabolic sink, consuming the overflow of NADPH [30] or proline from primary metabolism [7]. Thus, the increase of intracellular concentration of NADPH might trigger the biosynthesis of undecylprodigiosin and suppress morphological differentiation.

In *S. lividans*, NADPH was noticeably increased by induction of co-expressed *zwf2+zwf3+opc2*. Concomitantly, the undecylprodigiosin production levels increased by 1.8 times compared with that of the control, in spite of a 10% decrease of actinorhodin production. This tendency was also confirmed in flask cultures using R2YE medium. On day 7 of culture, *S. lividans* TK24/pWHM3-Z23O2 showed a 9% increase in undecylprodigiosin production and 35% reduction in actinorhodin production compare to the control (data not shown). Although previous reports suggested that increased carbon flux into glycolysis (decreased carbon flux through the PPP) might increase primary products for antibiotic biosynthesis, resulting in high production of actinorhodin and undecylprodigiosin in *S. coelicolor* and *S. lividans* [3, 26], our data led us to a different interpretation. In *S. lividans* TK24/pWHM3-Z23O2, the decreased production of actinorhodin was probably due to a small flux into glycolysis, as previously suggested. However, the increased production of undecylprodigiosin was probably because many diverse precursors as well as NADPH-dependent reduction step in its biosynthesis were required. These requirements for undecylprodigiosin production were met by engineering carbon flux through the PPP.

Production of sedoheptulose 7-phosphate, as well as NADPH, increased significantly in the early stages of growth. However, there was a time difference between the production of NADPH/sedoheptulose 7-phosphate and antibiotic production during fermentation. The production of NAPDH and sedoheptulsoe 7-phosphate in *S. lividans* TK24/pWHM3-Z23O2 was significantly higher than in the control (Fig. 4B and 4C) at the early stage of growth, but the production of antibiotics was prolonged at the late stage of growth (Fig. 5). This phenomenon is thought to be due to the marked increase in PPP-mediated primary metabolism by the overexpression of *zwf2* + *zwf3* + *opc2* in *S. lividans*. As a result, the biosynthesis of NADPH and sedoheptulose 7-phosphate increased in the early stage of growth, and thus induced higher cell growth. It was also considered that maintaining a slightly higher level of NADPH than the control until the late growth stage led to a higher production of undecylprodigiosin than the control.

From the metabolic pathway point of view, engineering the PPP in strains producing secondary metabolites using sedoheptulose 7-phosphate as precursor, such as acarbose [8], validamycin [9], and salbostatin [10], may be a valuable tool for industrial application. Thus, enhancing the activity of reduction and various primary precursors may be an effective way for the improved production of certain secondary metabolites in the pharmaceutical industry.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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