J. Microbiol. Biotechnol. (2010), **20**(12), 1689–1695
doi: 10.4014/jmb.1007.07058
First published online 1 October 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Identification and Characterization of a Pantothenate Kinase (PanK-sp) from *Streptomyces peucetius* ATCC 27952

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Received: July 30, 2010 / Revised: August 31, 2010 / Accepted: September 1, 2010

Pantothenate kinase (PanK) catalyzes the first step in the biosynthesis of the essential and ubiquitous cofactor coenzyme A (CoA) in all organisms. Here, we report the identification, cloning, and characterization of *panK-sp* from *Streptomyces peucetius* ATCC 27952. The gene encoded a protein of 332 amino acids with a calculated molecular mass of 36.8 kDa and high homology with PanK from *S. avermitilis* and *S. coelicolor* A3(2). To elucidate the putative function of PanK-sp, it was cloned into pET32a(+) to construct pPKSP32, and the PanK-sp was then expressed in *E. coli* BL21(DE3) as a His-tag fusion protein and purified by immobilized metal affinity chromatography. The enzyme assay of PanK-sp was carried out as a coupling assay. The gradual decrease in NADH concentration with time clearly indicated the phosphorylating activity of PanK-sp. Furthermore, the ca. 1.4-fold increase of DXR and the ca. 1.5-fold increase of actinorhodin by *in vivo* overexpression of *panK-sp*, constructed in pIBR25 under the control of a strong *erm*E* promoter, established its positive role in secondary metabolite production from *S. peucetius* and *S. coelicolor*, respectively.

Keywords: Coenzyme-A, pantothenate kinase, coupling assay, *Streptomyces peucetius*, *Streptomyces coelicolor*, *Streptomyces clavuligerus*

Coenzyme A (CoA) is a ubiquitous essential cofactor that plays a crucial role in the metabolism of carboxylic acids, including short- and long-chain fatty acids. About 4% of known enzymes utilize CoA as an obligate cofactor [2] and it is the source of 4'-phosphopantetheine, the prosthetic group of carrier proteins of fatty acid, polyketide, and nonribosomal peptide synthases [15, 16]. Starting from pantothenic acid, CoA is biosynthesized in five steps and this pathway is universal in both prokaryotes and eukaryotes [2, 6]. During the biosynthesis, pantothenate (vitamin B5) is first phosphorylated to 4'-phosphopantothenate by pantothenate kinase (PanK or CoaA), and then condensed with cysteine and decarboxylated to form 4'-phosphopantotheine in reactions catalyzed by the 4'-phosphopantothenoylcysteine synthase (CoaB) and 4'-phosphopantothenoylcysteine decarboxylase (CoaC) domains of a bifunctional enzyme in prokaryotes and by two distinct proteins in eukaryotes [16]. The 4'-phosphopantotheine is then converted to dephospho-CoA by phosphopanthetheine adenylyltransferase (CoaD) and finally phosphorylated by dephospho-CoA kinase (CoaE) at the 3'-OH of the ribose to form CoA. PanK is proposed to be the master regulator of CoA biosynthesis in mammalian cells [25].

Several studies on the regulation of primary metabolism related to PanK have been conducted [12]. Furthermore, the phosphorylating activity of PanK from different species has been characterized. First, *Escherichia coli* CoaA (CoaA*Ec*) was cloned as a prokaryotic type I CoaA [30]. Thereafter, eukaryotic PanK isoforms were isolated from *Aspergillus nidulans* (AnPanK), mouse (mPanK), and human (hPanK) [6, 10, 26, 36]. Similarly, PanK from *Bacillus anthracis* has been well characterized [22]. On the other hand, CoaAs insensitive to CoA and acyl-CoAs were recently identified from *Staphylococcus aureus* (CoaA*Sa*), *Pseudomonas aeruginosa* (CoaA*Pa*), and *Helicobacter pylori* (CoaA*Hp*) as prokaryotic types II and III CoaAs [5, 8, 11, 18]. Likewise, genetic analysis of coenzyme A biosynthesis in *Saccharomyces cerevisiae* was also recently performed [23].

Numerous studies have identified the potential of CoA biosynthetic enzymes because CoA compounds, including acetyl-CoA, propionyl-CoA, malonyl-CoA, and methylmalonyl-CoA, are essential precursors for the biosynthesis of

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polyketides and other complex biomolecules. *E. coli* has already been metabolically engineered to increase CoA production [34], in which a 10-fold increase in CoA and a 5-fold increase in acetyl-CoA were seen on overexpression of *panK* and supplementation with pantothenic acid [24]. However, the metabolic engineering of *E. coli* is not easily applied to actinomycetes, which is a much more difficult bacterial phylum to engineer. In this study, identification of *panK-sp* with detailed sequence analysis was carried out (Fig. S1A−S1B); thereafter, we sought to characterize its activity *in vitro* and *in vivo*. PanK in *S. peucetius* is believed to play a key role in increasing the CoA inside the cell (Fig. 1), ultimately leading to increased secondary metabolite production through sequential steps [20].

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Streptomyces peucetius ATCC 27952 was cultured at 28°C in R2YE medium [14] for 2−3 days for the preparation of genomic DNA. *Escherichia coli (E. coli)* strains were grown at 37°C in Luria−Bertani (LB) medium in both liquid and agar plates supplemented with the appropriate amount of antibiotics when necessary (ampicillin 100 µg/ml). The pGEM-T easy vector (Promega, USA) and pET-32a(+) (Novagen, Germany) were used for the cloning of polymerase chain reaction (PCR) products and for the expression of the gene. *E. coli* XL1-Blue (MRF) (Stratagene, USA) was used as a host cell for recombinant plasmid preparation and DNA manipulation, whereas *E. coli* BL21 (DE3) (Stratagene, USA) was used as the host for the expression (Table S1). Reagent-grade chemicals were purchased from Sigma/Aldrich or Merck, UK. In some cloning experiments, isopropyl-β-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were included in the LB agar plates for screening purposes. Plasmids were propagated in JM110 to obtain nonmethylated DNA for transformation into the *S. peucetius* [21], *S. coelicolor* [14], and *S. clavuligerus* strains [13]. For protoplast transformation, individual strains were cultured in R2YE medium (5% sucrose, 0.02% potassium sulfate, 1% magnesium chloride, 1% glucose, 0.5% yeast extract, and 0.01% Difco casamino acid) at 250 rpm and 28°C for 24 h. The growing seed culture was then transferred to 50 ml of R2YE medium at 250 rpm and 28°C for 36 h. *Streptomyces* transformants were supplemented with thiostrepton

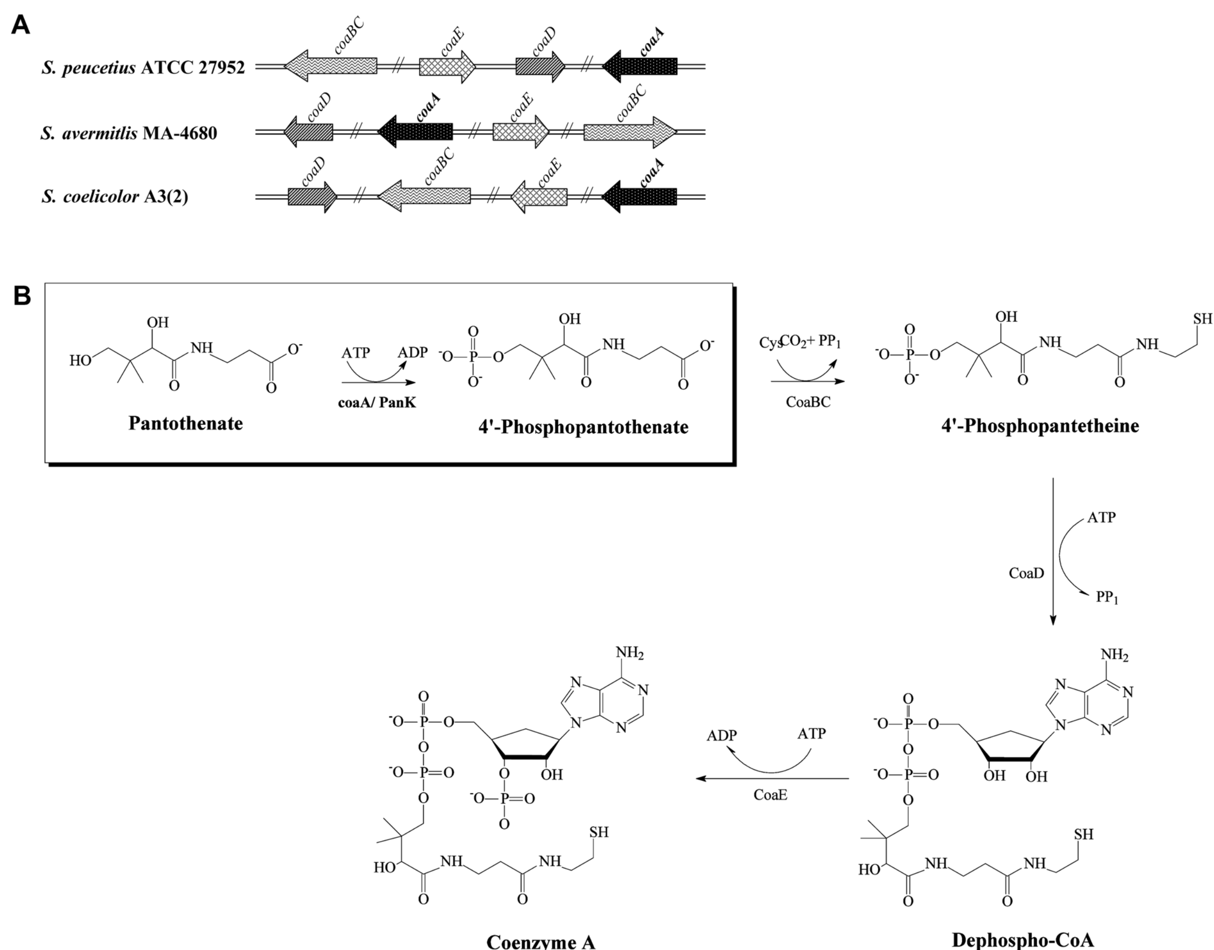

Fig. 1. A. CoA biosynthetic genes organization in *S. peucetius* compared with *S. coelicolor* and *S. avermiltis*. CoaA ORFs are highlighted with a black-filled arrow. **B**. Biosynthetic pathway of coenzyme A.

(12.5 µg/ml, 25 µg/ml, and 5 µg/ml) for secondary metabolite production. All bacterial strains used in this study are listed in the table below (Table S1).

DNA Isolation and Manipulation

The *panK* and neighboring genes were assigned by open reading frame (ORF) finder and by BLAST [1]. Standard methods were used for DNA cloning, plasmid isolation, and restriction enzyme digestion [14, 28]. Plasmids were isolated from *E. coli* by the alkaline lysis method. Electrophoresis was conducted in agarose gels with Tris-EDTA buffer. The DNA sequence was determined on an automated DNA sequence analyzer. The computer-based analysis and comparison of nucleotide and protein sequences were performed with the BLAST, FASTA, CLUSTALW, and GENEDOC programs.

Construction of Recombinant Plasmids

A set of primers, *panK-sp*-F 5'-GAC **GGA TCC** CAT ATG ATC ACT TCG CCG CCA-3' and *panK-sp*-R 5'-CGC C**TC TAG A**TC CTA GAG CTT GCG CAG TGA-3' (the underlined letters indicate the restriction sites for *Bam*HI and *Xba*I), was used to amplify nucleotide sequences of *panK-sp*. The Polymerase chain reaction (PCR) was performed in a thermocycler (Takara, Japan). The amplification conditions for PCR were 94°C for 7 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and finally 72°C for 10 min. DNA amplification was performed in a total volume of 20 µl containing 5 µl of PCR Mix (Genotech, Korea). The PCR product was cloned into pGEM-T (Promega, USA) and sequenced prior to cloning into the expression vector to verify that no mutation had been introduced during PCR amplification. It was then cloned into the *Bam*HI−*Xba*I sites of pET-32(a) (Novagen, USA) to construct pPKSP32. The pPKSP32 was introduced into *E. coli* BL21 (DE3) by heat pulse transformation [28]. Similarly, the amplified PCR product of *panK-sp* was cloned into the *Xba*I−*Eco*RI sites of pIBR25 under the control of the *erm*E* promoter in order to generate the expression recombinant plasmid pA25 to be expressed in *Streptomyces* strains.

Expression and Purification

The recombinant plasmid was transformed into *E. coli* BL21 (DE3). The transformed colony was cultured, and the overnight culture was diluted 1:50 in fresh medium. IPTG (isopropyl-β-D thiogalactopyranoside) was added at a final concentration of 0.4 mM when the culture reached an optical density at 600 nm (OD_{600}) of 0.7, and the induction was carried out for 20 h at 20°C. Following induction, the cells were washed and resuspended in 25 ml of 50 mM Tris-HCl buffer (pH 7.5). The cell pellets were disrupted by ultrasonication, and the crude cell extract was obtained by centrifugation at 12,000 ×*g* for 20 min. The molecular masses of denatured proteins were observed in SDS−PAGE analyses (as described by Laemmli [17]) with standard molecular weight protein markers (Novagen, USA) (Fig. 2) [17]. His-tagged protein in crude cell extracts was purified by immobilized Co^{2+}-affinity chromatography (Talon, USA) according to the manufacturer's instructions. The proteins were eluted with a linear gradient of imidazole (from 10 to 500 mM) in water. The pure fractions were dialyzed against storage buffer (50 mM Tris-HCl buffer, pH 7.5) and the purified proteins were analyzed by 12% SDS−PAGE. Protein concentration was determined by the Bradford assay using BSA as a standard [4].

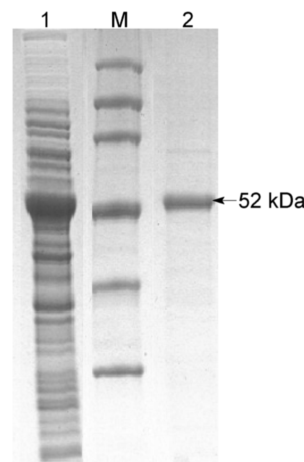

Fig. 2. SDS−PAGE of pPKSP32a expressed in *E. coli* BL21 (DE3).
Lane 1: Soluble PanK-sp; Lane 2: molecular weight marker; Lane 3: purified PanK-sp.

Reconstituted Assay of PanK-sp

The activity of PanK-sp was determined with substrate, panthothenate and ATP. The reaction mixture consisted of 80 U/ml of each pyruvate kinase and lactate dehydrogenase, 200 µM NADH, and 200 µM phosphoenolpyruvate in 50 mM HEPES (pH 7.5) containing 2 mM $MgCl_2$ in a 1-ml cuvette. The concentration of pantothenate was varied from 5 mM to 30 mM at 2.5 mM increments. The reaction mixtures were incubated at 25°C for 5 min, and the reaction was initiated by the addition of PanK-sp at a final concentration of 100 nM in a final assay volume of 1 ml. The progress of the reaction was monitored at 340 nm for 20 min at 2-min intervals. ADP formation was monitored by coupling it to pyruvate kinase and lactate dehydrogenase. The activity was measured as the change in the absorbance at 340 nm while monitoring the depletion of NADH in the UV moniter (Shimadzu, UV-1601PC). This change was observed only in the presence of enzyme and all other components of the reaction mixture, confirming the activity as being the ATP-dependent phosphorylation of pathothenate.

DNA Sequence Accession Number

The nucleotide sequence of *panK-sp* reported in this paper has been deposited in the NCBI nucleotide sequence database under Accession No. GU183626.

Analysis of Secondary Metabolites Production

To study the growth rate, *S. peucetius*, and *S. peucetius* PA25 (harboring plasmid pPA-25); *S. coelicolor*, and *S. coelicolor* PA25; and *S. clavuligerus* and *S. clavuligerus* PA25 were grown in NDYE, YEME, and CA production media (medium composed of 2.0% glycerol, 3% tryptic soy broth, 1% peptone, and 1.05% MOPS at pH 6.5) at 28°C, respectively, after 36 h of incubation in R2YE seed media. The cell pellets were collected at intervals of 12 h by centrifuging 50 ml of culture broth of each strain at 6,000 ×*g*. Cell pellets were washed with distilled water and dried at 72°C in a vacuum oven to constant weight. Dried cell pellets were obtained for growth rate analysis.

Fifty ml of culture broth of each *S. peucetius* strain was centrifuged for 15 min at 6,000 ×*g* to remove cell pellets. The supernatant was

extracted with 2 volumes of $CHCl_3$:CH_3OH (9:1). The extract was dried under reduced pressure using a rotary evaporator and reconstituted with 1.5 ml of methanol. A 15-µl aliquot of the extract was analyzed by HPLC using a reverse-phase C_{18} column with 100% methanol (solvent B) and distilled water (solvent A, pH 2.34 by trifluoroacetic acid) for 71 min, with a flow rate of 1 ml/min by the following method: 0−50 min (0−100% B), 50−60 min (100% B), and 60−70 min (100% A). Peaks were monitored using a UV absorbance detector at 254 nm. Authentic doxorubicin was used as a reference.

The *S. coelicolor* supernatant was extracted with 2 volumes of ethyl acetate. The extract was dried under reduced pressure using a rotary evaporator and reconstituted with 1 ml of methanol. Actinorhodin synthesis was estimated spectrophotometrically by measuring the optical density at 630 nm of cell-free culture supernatants.

To measure clavulanic acid synthesis, a 2-ml sample was taken from each culture every 12 h during the fermentation process (48 h), and the supernatant was used for secondary metabolite production and CA assays [27]. The filtered fermentation supernatant (90 ml) was reacted with 30 ml of 3 M imidazole solution (pH 6.8) at 37°C for 40 min [3]. The imidazole derivative was diluted 15-fold with ddH_2O and its absorbance was measured at 312 nm [19]. CA yields in *S. clavuligerus* strains were determined by comparison with a calibration curve generated from a pure CA standard. CA was also analyzed by high-performance liquid chromatography (HPLC) using a C-18 column.

Results and Discussion

Sequence Analysis of *panK*

Sequence analysis of the whole genome of *S. peucetius* ATCC 27952 revealed the presence of a 0.99-kb open reading frame (ORF), designated as *panK-sp.* The CoA-biosynthetic genes in *S. peucetius* are located from 1,278 kb to 4,625 kb in the chromosome. The *panK-sp* is located between nucleotides 4,624,859 bp and 4,625,935 bp (Fig. 1A). *panK-sp* lacks the DXGGS/T motif characteristic of the type II PanKs, as well as some of the critical homologies to PanK type III. Therefore, the *panK-sp* identified from *S. peucetius* is a type I PanK. Moreover, unlike other type I PanK, the *panK* from *S. peucetius* has 261-Leu instead of 261-Phe based on multiple alignment. Takagi *et al*. [33] recently reported that the presence of a leucine residue instead of phenylalanine hinders the formation of a hydrophobic pocket. This characteristic of pantothenate enzyme makes it non-inhibitory to CoA formation [31].

panK-sp contains an A-type ATP-binding consensus sequence, G*XXXX*GKS [7]. It also contains the well-conserved residue lysine (Leu-115), which is a critical residue for ATP binding [9, 29]. The sequence of *panK-sp* was further compared with that of *panK* from other strains (Fig. S1A). Alignment of *panK-sp* showed 83% identity to *S. avermitilis* (Accession No. NP_826138), 70% identity to *S. coelicolor* (Accession No. NP_628896) and 52% identity to *E. coli* (Accession No. NP_418405). Furthermore, the dendrogram in Fig. S1B shows the evolutionary relationships among the amino acid sequences of some PanK proteins. PanK-sp exhibited a high degree of aa homology with the corresponding enzymes of other *Streptomyces*. The overall comparison of CoA biosynthetic genes further clarifies the relationship among *Streptomyces* strains (Table 1).

Expression and Purification of PanK-sp

The 996-bp *panK-sp* was amplified from genomic DNA of *S. peucetius* by PCR amplification and then cloned into expression vector pET-32a(+) at the *Bam*HI−*Xba*I sites. The recombinant plasmid pPKSP32 was introduced into *E. coli* BL21 (DE3) and was expressed as described in the Materials and Methods section. Expression was easily accomplished in the whole cell by induction with 0.4 mM IPTG for 20 h at 20°C. Since stability is one of the most challenging aspects of the *in vitro* study, we added 10% glycerol along with 1 mM DTT, 1 mM PMSF, and 0.1 mM EDTA in 50 mM Tris-HCl buffer (pH 7.5). The molecular weights of the His-tagged proteins were confirmed by 12%

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strains and plasmid vector	Relevant features	Sources/References
E. coli XL1 blue	General cloning host	Stratagene PBL
E. coli BL21 (DE3)	General cloning host	Stratagene PBL
JM110	*dam dcmsupE44 thi leu rpsL lacYgalT ara tonA thr Tsx*Δ*(lac–proAB) F [traD36proAB*$^+$ *lacZ*Δ*M15]*	Stratagene PBL
S. peucetius ATCC27952	Doxorubicin parental strain	ATCC
S. coelicolor M145	SCP1$^-$ SCP2$^-$	Keiser *et al.* [14]
S. clavuligerus NRRL3585	Wild type, cephamycin and clavulanic acid producer	NRRL
pGEM-T easy	General cloning vector, Amp^r	Promega, USA
pET32a (+)	*E. coli* expression vector, Amp^r	Novagen
pPKSP32a	pET32a(+) with 0.99 kb *Bam*HI−*Eco*RI PanK-sp ORF	This work
pIBR25	*Streptomyces* expression vector, with *erm*E* promoter and Tsr^r	Sthapit *et al.* [32]
pA-25	pIBR25-based recombinant plasmid for expression of *panK-sp*	This work

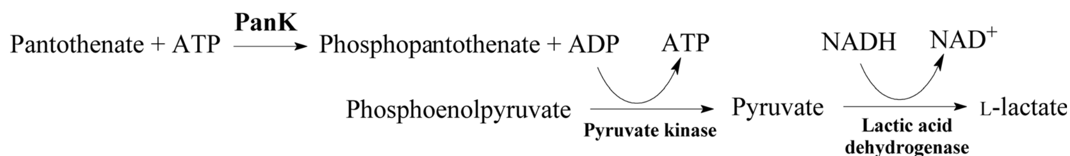

Fig. 3. Scheme for coupling enzyme assay.

SDS−PAGE and were consistent with the predicted size. During the purification process using Co^{2+}-affinity chromatography, the target proteins were eluted at a concentration of 500 mM imidazole in the elution buffer provided by the manufacturer. The pure dialyzed fractions were analyzed by 12% SDS−PAGE (Fig. 2).

Characterization of *S. peucetius* CoaA

Generally, when the enzyme reaction does not result in a change in absorbance, a coupling assay for the enzyme using a spectrophotometer can be applied. Since coupled reactions involve multiple enzymes, these assays present a number of potential problems that are not encountered with direct or indirect assays. In this kind of enzyme assay, the enzymatic reaction of interest is paired with a second enzymatic reaction that can be conveniently measured. In a typical coupled assay, the product of the enzyme reaction of interest is the substrate for the enzyme reaction with which it is coupled for convenient measurement (Fig. 3). Therefore, to test the role of PanK-sp in pantothenate phosphorylation, we carried out a coupling assay. The pH, substrate concentration, and enzyme concentration were each varied. The most suitable pH was 7.5. The optimal pantothenate concentration was 17.5 mM, which utilized 100 nM of purified enzyme. A control experiment was performed to cross-check the role of the enzyme. Denatured protein showed no phosphorylating activity and nor did the protein isolated from vector only, which was also used as a control. To further assess the activity of PanK with pantothenate, additional NADH was used in the reaction mixture to study the NADH consumption during phosphorylation. During this experiment, NADH was gradually consumed in the reaction (Fig. 4). PanK-sp had no activity at temperatures below 15°C.

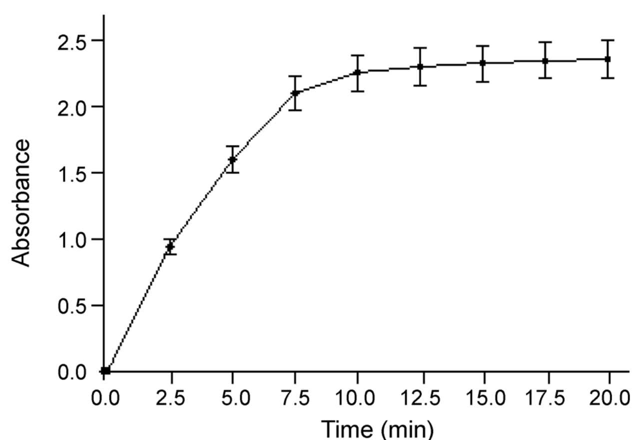

Fig. 4. Saturation plot for absorption of NADH (340 nm) optimized at 17.5 mM concentration of pantothenate to assess the phosphorylating activity of PanK-sp.

Effect of PanK-sp Expression on Antibiotic Producer Strains

The role of pantothenate kinase in secondary metabolite production is of significant interest. In this study, we overexpressed pantothenate kinase in *S. peucetius*, *S. coelicolor*, and *S. clavuligerus* to study its effect on their respective metabolite production.

After transformation of pA25, recombinant strains of *S. peucetius* PA25, *S. coelicolor* PA25, and *S. clavuligerus* PA25 were selected for analysis of DXR, actinorhodin, and clavulanic acid production, respectively.

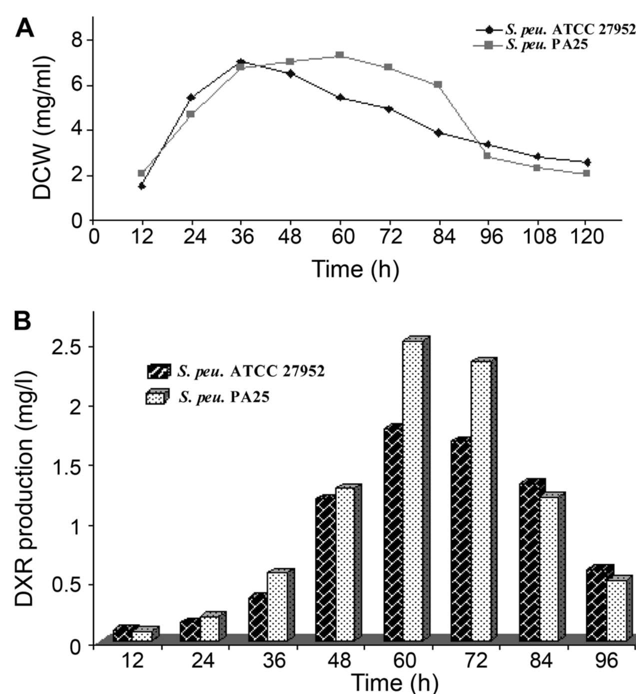

Fig. 5. Cell growth and production of DXR by *S. peucetius* strains.

A. Growth rate profile. Growth curve obtained by introduction of pA25 in *S. peucetius* compared with wide-type *S. peucetius*. **B**. Production of DXR by *S. peucetius* PA25 with respect to *S. peucetius* ATCC 27952.

The biomasses of both wild-type and pA25-containing recombinant strains were higher than those of the parental strains in *S. peucetius* as well as *S. coelicolor*, but in the case of *S. clavuligerus*, the dry cell weight of parental and recombinant strains were comparable. In *S. peucetius*, the cell growth smoothly increased up to 36 h and was then sustained for a longer period than in the parental strain; that is stationary phase for up to 84 h (Fig. 5A). The DXR production was increased by 1.4-fold (2.5 mg/ml) in *S. peucetius* PA25 at 60 h (Fig. 5B).

To study the influence of *panK-sp* on the production of actinorhodin, the *panK-sp* was heterologously expressed in *S. coelicolor*. *S. coelicolor* and *S. coelicolor* PA25 were cultured in YEME medium and actinorhodin was isolated as described in the Materials and Methods section.

In *S. coelicolor,* the cell growth increased smoothly up to six days and was then sustained for a longer period than in the parental strain; that is stationary phase for up to 5 days (Fig. 6A). The amount of actinorhodin produced by *S. coelicolor* PA25 was approximately 1.5 times greater than that produced by the parental strain (Fig. 6B). Our results from these two strains showed that the influence of *panK-sp* increased the biomass. Moreover, expression of *panK-sp* elevated the CoA pool, which eventually led to the product enhancement.

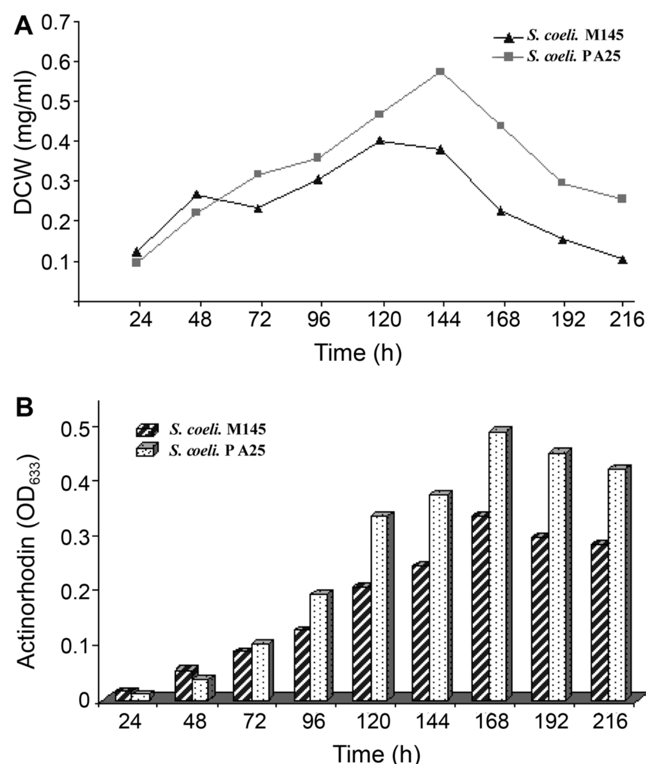

Fig. 6. Cell growth and production of ACT by *S. coelicolor* strains.

A. Growth rate profile. Growth curve obtained by introduction of pA25 in *S. coelicolor* M145 compared with *S. coelicolor* M145. **B**. Production of ACT by *S. coelicolor* PA25, with respect to *S. coelicolor* M145.

To further cross-check the role of *panK-sp* in secondary metabolite production, we heterologously expressed *panK-sp* in *S. clavuligerus* [clavulanic acid (a non-polyketide compound) producer]. From the clavulanic acid production analysis, we found that PanK-sp had negligible effect on non-polyketide-type compound production (data not shown). Since, CoA cannot be produced by PanK-sp in the non-polyketide compound producer, the level of clavulanic acid production remained unchanged.

During primary metabolism in the cell, formation of CoA is regulated by five enzymes. PanK, being one of them, plays a crucial role of phosphorylation of pantothenate. Since polyketide-type compounds are biosynthesized from CoA, the role of *panK* is believed to be very important in secondary metabolite (polyketide-type compounds) formation, which has been clearly demonstrated by our findings. PanK is also proposed to be the master regulator of CoA biosynthesis in mammalian cells [25]. Several kinetic studies on the same enzymes have established a significant regulatory role in CoA biosynthesis [30, 23]. The formation of phosphopantothenate by phosphorylation of pantothenate is the rate determining step in CoA biosynthesis in bacteria [12]. Pantothenate kinase activity in different organisms is also regulated through feedback inhibition by CoA and its thioesters [35]. PanK activity is inhibited *in vivo* and *in vitro* by unacylated CoA and less efficiently by CoA (especially nonesterified CoA) and its thioesters in a competitive manner by binding to the ATP site [31, 34]. Therefore, further research would be focused on inhibition of PanK-sp activity, thereby overcoming the inhibition to increase its catalytic activity.

Acknowledgment

This work was supported by the Agenda Program (No 200905FP014), Rural Development Administration, Republic of Korea.

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