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Characterization of Dephosphocoenzyme A Kinase from *Streptomyces peucetius* ATCC27952, and Its Application for Doxorubicin Overproduction S

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Dephosphocoenzyme A (CoaE) catalyzes the last step in the biosynthesis of the cofactor coenzyme A. In this study, we report the identification and application of CoaE from *Stretomyces peucetius* ATCC27952. After expression of *coaE*, the protein was found to have a molecular mass of 28.6 kDa. Purification of the His-tagged fused CoaE protein was done by immobilized metal-affinity chromatography, and then *in vitro* enzymatic coupling assay was performed. The increasing NADH consumption with time shed light on the phosphorylating activity of CoaE. Furthermore, the overexpression of *coaA* and *coaE* independently under the *ermE** promoter in the doxorubicin -producing wild type strain, resulted in 1.4- and 1.5-fold enhancements in doxorubicin production, respectively. In addition, the overexpression of both genes together showed a 2.1-fold increase in doxorubicin production. These results established a positive role for secondary metabolite production from *Streptomyces peucetius*.

Keywords: Coenzyme A, dephosphocoenzyme A kinase, doxorubicin, heterologous overexpression, *Streptomyces peucetius*

Introduction

Coenzyme A (CoA) and its thioester derivative acetyl-CoA play vital roles in different reactions of microorganisms, during the biosynthesis of secondary metabolites. CoA acts as an acyl group carrier and carbonyl activating group in various cellular metabolic reactions, and also helps in the synthesis of fatty acids, polyketides, and nonribosomal peptides. CoA is generally synthesized in a five-step process that requires four molecules of ATP, by using pantothenate and cysteine [8, 9, 19] (Fig. 1).

In the CoA biosynthesis pathway, dephospho-CoA kinase which is designated as CoaE, catalyzes the final step of phosphorylating the 3'-hydroxy group of the ribose sugar moiety [12]. The conformation of dephospho-CoA kinase was based on the enzyme coupling assay and identification of the reaction product, using *Escherichia coli* protein. The gene coding for CoaE is present in almost all kingdoms, except for plants [5, 10]. Various research studies have been performed on the regulation of the primary metabolism that is associated with CoaA [6], and the phosphorylating activity of CoaA from different species has been further characterized [18]. Various studies show the importance of CoA biosynthetic enzymes, because CoA compounds, including acetyl-CoA, propionyl-CoA, malonyl-CoA, and methylmalonyl-CoA, are needed for the biosynthesis of secondary metabolites. *E. coli* has already been engineered to increase metabolites. As for CoA production [21] during an overexpression of CoaA, there is a 10-fold increase in CoA and a 5-fold increase in acetyl-CoA, which were supplemented with pantothenic acid [14]. However, it is difficult to perform the same metabolic engineering technique to actinomycetes, because it is a complex bacterial phylum to engineer in *E. coli* [11].

Interestingly, the reverse transcription-polymerase chain reaction (RT-PCR) analysis of CoaA, CoaBC, CoaD, and CoaE from *Streptomyces peucetius* ATCC27952 showed a relatively low expression level of CoaE in comparison with the other genes. In this study, we characterized CoaE in *S. peucetius* ATCC27952, through gene overexpression in

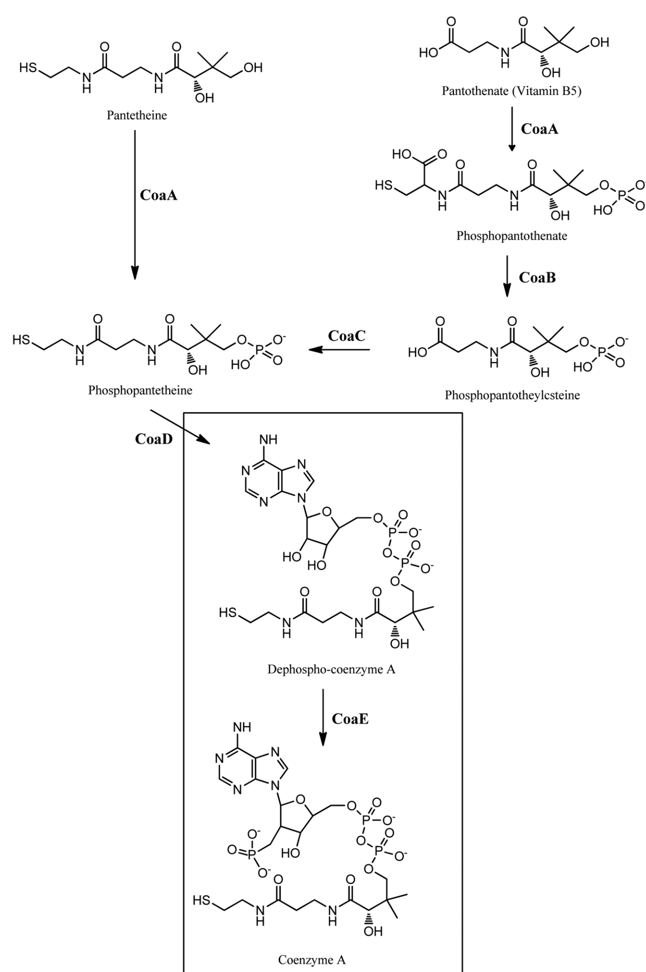

Fig. 1. CoA-related biosynthetic pathway.

E. coli and *in vitro* enzyme coupling assay. Thus, we applied *coaE* gene heterologous overexpression, which could lead to enhanced doxorubicin production.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

S. peucetius ATCC27952 was cultured in R2YE medium (0.01% Difco casamino acid, 1% glucose, 1% magnesium chloride, 0.02% potassium sulfate, 5% sucrose, and 0.5% yeast extract) for 3 days at the optimum temperature (28°C), for the preparation of genomic DNA [7]. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium, provided with the proper amount of antibiotics (ampicillin, 100 µg/ml). pGEM-T easy (Promega, USA) and pET-32a(+) (Novagen, Germany) were used as cloning and expression vectors, respectively. *E. coli* XL1-Blue (MRF') (Stratagene, USA) was used as a cloning host, and *E. coli* BL21 (DE3) (Stratagene, USA) was used as an overexpression host. During the cloning experiments, isopropyl-β-thiogalactopyranoside (IPTG) and 5-bromo-5-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were used in blue-white screening of cloned genes. In order to obtain the non-methylated DNA, plasmids were propagated in JM110 for the transformation into *S. peucetius.* For protoplast transformation, every strain was cultured separately in R2YE medium containing 5% sucrose, 1% glucose, 0.02% potassium sulfate, 1% magnesium chloride, 0.6% yeast extract, and 0.01% Difco casamino acid, at 28°C for 24 h, using a shaker at 250 rpm. The growing seed culture was later transferred to 50 ml of R2YE medium, while maintaining the growing temperature and shaking speed constant, for 36 h. *S. peucetius* transformants were provided with 5.0, 12.5, and 25.0 µg/ml) of thiostrepton for the production of secondary metabolites. All bacterial strains and vectors used in this study are listed in Table 1.

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

Strains/vectors	Relevant features	Sources or references
Bacterial strains		
S. peucetius	Wild-type, DOX producer	ATCC27952
E. coli		
XL-1 Blue	Generic cloning host	Stratagene PBL Company
BL21 (DE3)	Generic expression host	Stratagene, La Jolla, CA, USA
JM110	Generic cloning host	Invitrogen
Plasmid vectors		
pGEM-T easy vector system	Generic cloning vector, Amp	Promega, USA
pET32a	His6-tagged overexpression vector	Novagen
pCE32a	pET32a with *coaE*	This study
pIBR25	*Streptomyces* overexpression vector with an *ermE** promoter	[20]
pIBR-cA	pIBR25 with *coaA*	This study
pIBR-cE	pIBR25 with *coaE*	This study
pIBR-cAE	pIBR25 with *coaAE*	This study

Table 2. Oligonucleotide primers used in this study.

Oligonucleotide primers	Sequence	Function
RT-PCR		
CoaA-F	AAGAGGTCGAGCAGCTCC	5′-CoaA RT primer
CoaA-R	TCGTACGACTCGGGGAAG	3′-CoaA RT primer
CoaBC-F	AGGCCGAAGGTCGTTCTC	5′-CoaBC RT primer
CoaBC-R	TGTTGGTCAGCAGGTCGT	3′-CoaBC RT primer
CoaD-F	TACACGTACCAGCCGCAG	5′-CoaD RT primer
CoaD-R	TTCGTACCGGTTGAGCTG	3′-CoaD RT primer
CoaE-F	GCAAGAGCGAAGTGTCCC	5′-CoaE RT primer
CoaE-R	ATGCACGATGCCGTTCAG	3′-CoaE RT primer
Overexpression-PCR		
CoaA-F-1	GGATCCGAGACTATGATCACTTCGCCG	5′-CoaA overexpression primer
CoaA-R-1	GCGAATTCCTAGAGCTTGCGCAGTGACAG	3′-CoaA overexpression primer
CoaE-F-2	AATGAATTCGGAGCCATGCTGACAGTGGGC	5′-CoaE overexpression primer
CoaE-R-2	GACAAGCTTGCGCTATTCCGTCGGCCA	3′-CoaE overexpression primer

DNA Manipulation and Construction of Recombinant Plasmids

Standard methods were followed for cloning, plasmid isolation, and restriction enzyme digestion [7, 16]. An alkaline lysis method was deployed for the isolation of plasmid from *E. coli*. The BLAST, ClustalX, Clustalw2, and GeneDoc programs were used for the analysis of nucleotide and protein sequences [1]. Table 2 lists the primers of *coaE* used for the amplification of nucleotide sequences. The polymerase chain reaction (PCR) was performed by initial denaturation at 94°C for 7 min, followed by 30 cycles of 94°C for 1 min, annealing at 55°C for 1 min, polymerization at 72°C for 1 min, and finally gap filling at 72°C for 10 min. The PCR product was purified and cloned into pGEM-T easy vector. The sequence analysis was performed before cloning into the overexpression vector, in order to confirm that no mutation had occurred during the PCR. The gene was digested with the respective restriction enzymes and ligated into the overexpression vector pET32a(+), which was named pCE32a. For the expression, plasmid pCE32a was introduced into *E. coli* BL21 (DE3) by heat pulse transformation [17]. Similarly, the PCR products of *coaA*, *coaE*, and *coaAE* containing RBS only with the forward primer were cloned into the pIBR25 *Streptomyces*-overexpression vector under the control of the *ermE** promoter. The constructed recombinant overexpression plasmids pIBR-cA, pIBR-cE, and pIBR-cAE of *coaA*, *coaE*, and *coaAE*, respectively, were used for expression in *Streptomyces* strains [20].

Overexpression and Purification of CoaE

After the transformation of plasmid pCE32a(+) into the overexpression host *E. coli* BL21, a single colony was picked out and cultured overnight in a 15 ml tube with appropriate antibiotics, as a seed culture. The seed culture of 1 ml was transferred into a 50 ml flask, and the growth temperature was maintained at 37°C. After the cell density reached 0.6 at 600 nm of wavelength, the protein overexpression was induced by adding IPTG at the final concentration of 0.4 mM. The incubation was continued at 20°C for 30 h. The cell pellets obtained were centrifuged at 300 rpm for 10 min, and were washed with 50 mM Tris-HCl buffer at pH 7.4 containing 10% glycerol. Finally, the cell pellets were mixed with the respective buffer and lysed by using ultrasonication. The soluble fraction was separated from the cell debris by centrifugation at 12,000 rpm for 20 min at 4°C. The molecular masses of proteins were observed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with standard molecular weight protein markers (Novagen, USA). His-tagged protein in crude cell extracts was purified by an immobilized Co^{2+}-affinity column. Protein concentration was determined by the Bradford assay, using BSA as a standard [3].

CoaE Specific Activity Assay

The activity of CoaE was checked with substrate, dephospho-CoA, and ATP, using a slightly modified version of the standard enzyme coupling method [4]. The final volume of 400 µl reaction mixture contained 50 mM ATP, 50 mM Tris-HCl (pH 7.4), 2 mM magnesium chloride, 20 mM potassium chloride (pH 7.0), 50 µl of dephospho-CoA, and 2 mM of phosphoenolpyruvate. Finally, 300 µM of NADH was added into the reaction mixture, and the absorbance was checked at 340 nm. About 30 units of DPCK (dephospho-CoA kinase) and 25 units of PK (pyruvate kinase)/LDH (lactic dehydrogenase) were added, after the sample had been running for 20 and 40 sec, respectively. The reaction was observed at 340 nm for 6 min at 20 sec intervals. The activity of protein and the depletion of NADH were observed with descending absorbance at 340 nm in a UV monitor (Shimadzu, UV-1601PC), which confirms the activity of enzyme to be the

ATP-dependent phosphorylation of dephospho-CoA.

DNA Sequence Accession Number

The nucleotide sequence of *coaE* reported in this paper has been deposited in the NCBI nucleotide sequence database, under Accession No. KF500403.

RT-PCR Analysis in CoA Biosynthetic Genes

First, *S. peucetius* was cultured in R2YE medium. The culture of 5 ml at about 48 h was suspended in RNA to protect the bacteria reagent (Qiagen) for 5 min. An RNeasy mini kit (Qiagen) was used for RNA isolation, according to the methods provided by the manufacturer. The sample was freed from DNA by using RNase-free DNase (Qiagen), and was verified by PCR analysis with the RNA as the template. The concentration and purity of total RNA were determined by observing the optical density at 260 and 280 nm, respectively, using a spectrophotometer. RT-PCR was carried out with a QuaniTech SYBER Green RT-PCR kit (Qiagen), using a set of primers (Table 2). The same amount of RNA (1.5 μg) was deployed for RT-PCR in every experiment. RT-PCR first-strand cDNA synthesis took place at 50°C for 30 min; initial denaturation at 95°C for 15 min; and 45 cycles of 1 min at 94°C, 1 min at 63°C, and elongation at 72°C for 2 min. The reaction products were electrophoresed on a 0.8% agarose gel, and visualization was performed using ethidium bromide staining. Negative controls were carried out with *Taq* DNA polymerase without reverse transcripts, in order to confirm contamination of the RNA preparations. The 16S rRNA gene from *S. peucetius* was used as a positive internal control. For further confirmation, the results obtained were cross-checked by running RT-PCR with decreased PCR cycles.

Transformation into *S. peucetius*, and Analysis of Doxorubicin Production

The protoplast transformation and the selection of thiostreptone-resistant transformants were performed, using an already defined protocol [7]. *S. peucetius* ATCC27952 wild-type (50 ml) was cultured in a flask for 36 h, at the optimum temperature of 28°C. The culture with broth was transferred into a 50 ml tube and washed with 10.3% sucrose. The protoplast was prepared after the incubation of mycelia for 55 min at 37°C, with the addition of 3 ml lysozyme (5 mg/ml). The recombinant DNA was transformed into *S. peucetius* ATCC27952 using protoplast transformation methods, and it was overlaid with 3 ml of soft agar (0.4%) with 40 μg/ml thiostreptone, which was confirmed by PCR.

To determine the growth rate, *S. peucetius* ATCC27952 was grown in NDYE production medium (0.2% ammonium sulfate, 0.2% calcium carbonate, 1% corn starch, 0.2% magnesium sulfate, 0.1% potassium phosphate, 0.1% sodium chloride, and 0.2% tryptone), supplemented with 1 ml inorganic solution (1 mg ferrous sulfate, 1 mg magnesium chloride, and 1 mg zinc sulfate per 1,000 ml distilled water) at 28°C, after incubating the seed culture in R2YE medium for 36 h. With the time interval of 12 h, 50 ml of culture broth of each *S. peucetius* strain was centrifuged for 15 min at 3,000 rpm. Then, the cell pellets plates were washed with distilled water and dried at 70°C in a vaccum oven, for the analysis of dry cell weight. Again, 50 ml of the supernatant was extracted with two volumes of $CHCl_3$:CH_3OH (9:1), dried by a rotary evaporator, and mixed with 1 ml of methanol. A 20 μl aliquot of the extract was analyzed by high-performance liquid chromatography (HPLC), using a reverse-phase C18 column with 100% methanol (solvent B) and distilled water (solvent A, pH 2.34 by trifluoroacetic acid) for 30 min, with a flow rate of 1 ml/min, by gradient methods. A UV absorbance detector at 254 nm was used with doxorubicin as a standard reference.

Statistical Analysis

All analyses were carried out in triplicate. Experimental values are the mean ± standard deviation (SD). Statistical comparisons using one-way analysis of variance (ANOVA) with $p < 0.05$ were regarded as significant.

Results and Discussion

Expression and Purification of CoaE

After the transformation of the recombinant plasmid pCE32 into *E. coli* BL21 (DE3), it was subjected to overexpression, as described in the Materials and Methods section. Protein overexpression was done by induction with 0.3 mM IPTG for 30 h at 20°C. For the stability of protein, 10% glycerol was used along with 1 mM DTT, 1 mM PMSF, and 0.1 mM EDTA in 50 mM Tris-HCl buffer (pH 7.4). By using 15% SDS-PAGE, the exact size of the His-tagged protein was confirmed, and it was found to be the predicted size of 28.6 kDa. The purification of enzyme was performed, using immobilized metal chromatography (Co^{2+})-affinity resin. The target protein was eluted with 500 mM imidazole, in the elution buffer provided by the manufacturer. The obtained protein was concentrated by using a centricon (Millipore), and analyzed again by SDS-PAGE (Fig. 2A).

Characterization of *S. peucetius* CoaE

If there is no change in the absorbance of enzyme during the reaction, a coupling assay is applied by using a spectrophotometer. The desired enzymatic reaction is paired with a second enzymatic reaction that can be easily measured, in order to cope with the potential risks caused by multiple enzymes. Generally, in a coupling assay, the substrate for enzymatic reaction is used to couple for convenient measurement by using UV spectra. To test the role of CoaE in dephospho-CoA phosphorylation, we performed a coupling assay. The variations in pH, substrate

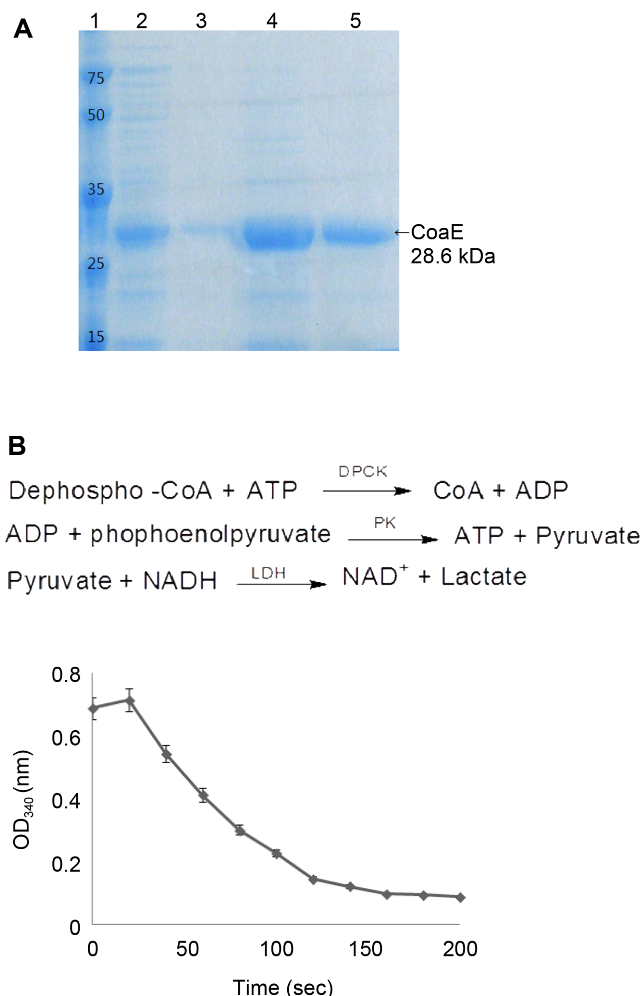

Fig. 2. Characterization of CoaE (28.6 kDa).

(**A**) SDS–PAGE of pSPCE32a overexpressed in *E. coli* BL21(DE3). Lane 1, protein ladder; lane 2, crude extracts; lane 3, eluted protein from 10 mM immidazole washing step; lane 4, purified CoaE from 100 mM immidazole elution step; and lane 5, purified CoaE from 500 mM immidazole elution step. (**B**) Scheme for double-reaction assay and saturation plot of NADH absorption (340 nm), optimized at 50 mM to assess the phosphorylating activity of CoaE. DPCK, dephospho-CoA kinase; PK, pyruvate kinase; and LDH, lactic dehydrogenase.

concentration, and enzyme concentration were used. The optimum pH was found to be at 7.4. The best dephospho-CoA concentration was 50 mM, and the optimal concentration of enzyme was 1 μM. The same experiment was repeated without protein, in order to cross-check the role of the enzyme. All the experiments were repeated three times in order to determine the convincible value. To determine the activity of CoaE with dephospho-CoA, a greater amount a NADH was added into the reaction mixture to study the NADH consumption rate during phosphorylation. During

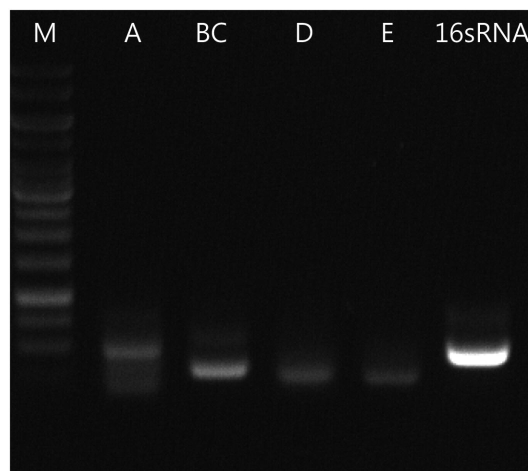

Fig. 3. RT-PCR data of coenzyme A biosynthesis-related genes.

M, DNA ladder; A, CoaA; BC, CoaBC; D, CoaD; E, CoaE; and 16sRNA, 16S RNA from *Streptomyces peucetius*.

this process, the gradual consumption of NADH was observed in the reaction (Fig. 2B).

Effects of CoaA, CoaE, and CoaAE in Doxorubicin Production

We checked the expression levels of CoaA, BC, D, and E in the *S. peucetius* CoA biosynthesis pathway, using RT-PCR. As a result, the CoaE level was found to be lower than that of the other genes (Fig. 3). In a previous study, we confirmed that secondary metabolites like doxorubicin production can be enhanced with the overexpression of CoaA [11]. Since CoaA and CoaE exist in the same biosynthetic pathway of CoA, we hypothesized that CoaA and CoaE expression might be related to doxorubicin production. Although several kinetic studies on the same enzymes have already established a significant regulatory role of CoA biosynthesis [13], the role of dephospho-CoA kinase in the production of secondary metabolites is of significant interest. Hence, we overexpressed CoaE in *S. peucetius*, to study its influence on doxorubicin production. After the transformation of plasmids pIBR25, pIBR-cA, pIBR-cE, and pIBR-cAE into *S. peucetius* (Fig. 4A), the recombinant strains of *S. peucetius* were selected to be observed for any changes in the production level of doxorubicin by HPLC (Fig. S1 and Fig. 4B). RT-PCR results showed that only a small amount of CoaE was generated. However, when we overexpressed CoaE, the production level of CoaA was increased, and at the same time, doxorubicin as a final secondary metabolite was found to

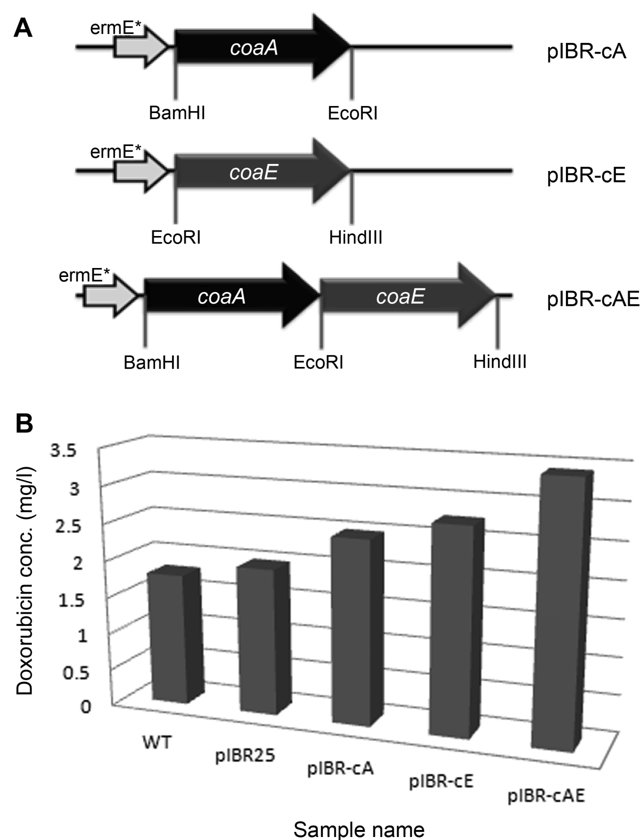

Fig. 4. Plasmid maps and doxorubicin production.
(**A**) Plasmid maps in this study. Plasmids pIBR-cA, pIBR-cE, and pIBR-cAE included *coaA*, *coaE*, and *coaAE*, respectively. (**B**) Production of doxorubicin from *S. peucetius* transformants containing vectors pIBR25, pIBR-cA, pIBR-cE, and pIBR-cAE, respectively.

be increased by 1.5-fold (2.45 ± 0.02 ml/l) with respect to the wild type. In the same way, the expression level was observed to increase 2.1-fold (3.34 ± 0.03 ml/l), when both *coaA* and *coaE* genes were overexpressed together.

In the process of primary metabolites production, it was found that five enzymes play a crucial role in the formation of CoA [15]; among them, pantothenate kinase activity was regulated by CoaA and its thioesters [22]. CoaE also has a significant role in the production of CoA from dephospho-CoA [12]. Identification from the study of CoaE may provide genetic information regarding CoA biosynthesis [2]. The CoA biosynthetic pathway is omnipresent, and it is an essential pathway in most organisms. Therefore, in order to produce essential metabolites, the five steps to CoA biosynthesis is very compactly controlled and regulated by multiple mechanisms, which are designed for the optimum utilization of cellular resources [23]. Since the production of secondary metabolites is related to the CoA biosynthesis pathway, we can easily predict the role of CoaE in the production of secondary metabolites such as doxorubicin [9]. The enhancement in doxorubicin production after the overexpression of both CoaA and CoaE clearly supports our prediction. In this study, we found that the production level of doxorubicin can be increased, if there is enhanced overexpression of both CoaA and CoaE. Although overexpression of only the CoaE also enhanced the production level, it would be most suitable if both CoaA and CoaE were overexpressed together. Thus, these data showed that for the enhancement of the cellular CoA pool, the two different regulatory genes *coaA* and *coaE* played a positive role in the improvement of doxorubicin production.

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