

## Short Communication

# Prokaryotic type III pantothenate kinase enhances coenzyme A biosynthesis in *Escherichia coli*

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**Key Words:** coenzyme A; *Escherichia coli*; metabolic engineering; pantothenate; pantothenate kinase

Coenzyme A (CoA) is a carrier of acyl groups, and is a cofactor in numerous metabolic pathways. This molecule is biosynthesized in five enzymatic steps from vitamin B<sub>5</sub>, which is also known as pantothenate (Abiko, 1975; Jackowski, 1996). The first reaction, catalyzed by pantothenate kinase (CoaA), is a committed step in *Escherichia coli* (Fig. 1), and subject to feedback inhibition by CoA and acyl-CoAs (Vallari et al., 1987). As a result, intracellular CoA content is strictly regulated (Chohnan et al., 1997, 1998). On the other hand, Vadali and coworkers (2004a) successfully increased the intracellular CoA pool by expressing *E. coli* pantothenate kinase from a high-copy number plasmid. This strategy was highly effective in producing useful compounds such as isoamyl acetate (Vadali et al., 2004b), succinate (Lin et al., 2004), antibiotics (Mandakh et al., 2010), and porphyrin (Lee et al., 2013).

Bacterial CoAs are categorized based on their amino acid sequences into three types, namely prokaryotic type I, II, and III CoAs. The eukaryotic enzyme called PanK comprises a fourth category (Choudhry et al., 2003; Leonardi et al., 2005b). The eukaryotic and prokaryotic type I enzymes are highly regulated through end-product inhibition by CoA and its derivatives (Calder et al., 1999; Vallari et al., 1987). In contrast, type II and III enzymes are insensitive to CoA and its thioesters (Brand and Strauss, 2005; Leonardi et al., 2005a). In addition, the type III enzyme requires monovalent cations such as K<sup>+</sup> and NH<sub>4</sub><sup>+</sup> for activity (Hong et al., 2006). Thus, bacterial CoAs differ not only in primary structure, but also in sensitivity to CoA derivatives.

In this paper, we investigated the possibility of using enzymes other than type I CoaA to enhance CoA biosyn-

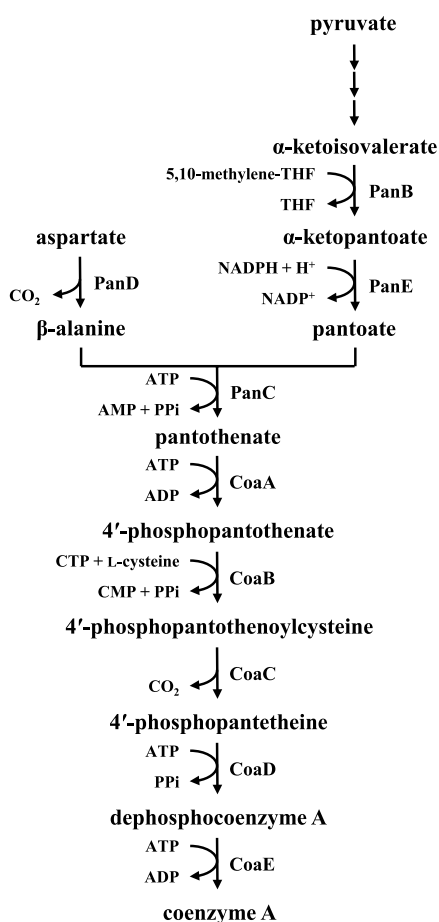
thesis in *E. coli*. We compared the ability of type I, II, and III enzymes to increase the CoA content by expressing, respectively, the *coaA* gene from *E. coli* (*Ec-coaA*), *Staphylococcus aureus* (*Sa-coaA*), and *Pseudomonas putida* (*Pp-coaA*) using recombinant plasmids. Strains and plasmids are listed in Table S1. Briefly, each *coaA* was amplified by PCR using primer sets summarized in Table S2. Amplified fragments were inserted into pUC118, a high-copy number plasmid, or into pSTV28, a low-copy number plasmid, downstream of the *lac* promoter. Constructs were then transformed into *E. coli* strain W3110. Transformants were grown aerobically at 30°C for 16 h in LB medium containing 5% glucose and 50 µg/ml ampicillin or 25 µg/ml chloramphenicol, as appropriate. Media were supplemented with or without 10 mM pantothenate, the initial substrate in CoA biosynthesis (Fig. 1).

Pantothenate kinase activity was estimated based on phosphorylation of D-[1-<sup>14</sup>C]-labeled pantothenic acid into 4'-phosphopantothenate (Ogata et al., 2014). The intracellular CoA pool was analyzed using the acyl-CoA cycling method (Chohnan and Takamura, 2004; Ogata et al., 2014; Takamura et al., 1985), except that the commercially available enzymes acetate kinase, citrate synthase, and phosphate acetyltransferase were replaced with recombinant enzymes prepared in our laboratory (Figs. S1 and S2). The acyl-CoA cycling method enables the determination of nonesterified CoA (CoASH), acetyl-CoA, and malonyl-CoA, the three major molecular species of cellular CoA. The method requires malonate decarboxylase, which was partially purified from *P. putida* JCM 20089 according to a published method (Takamura and Kitayama, 1981), with some modification. Briefly, the enzyme was prepared by ammonium sulfate precipitation at 30–45% saturation,

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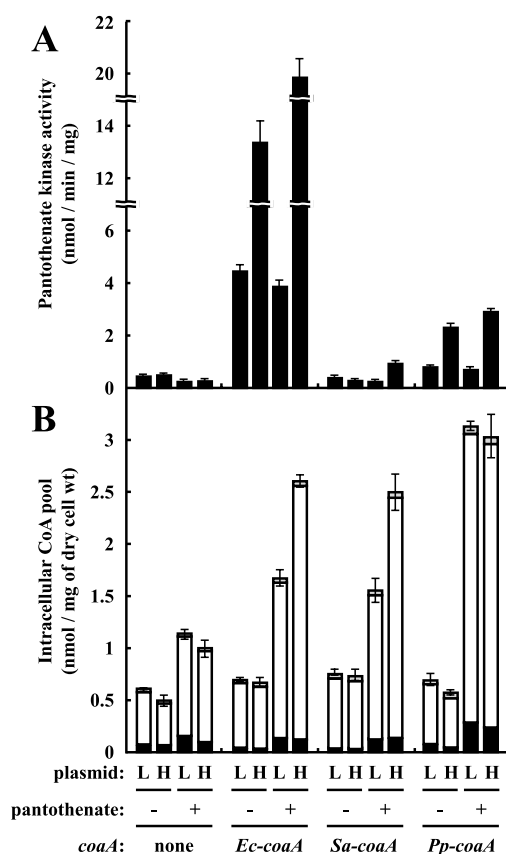


**Fig. 1.** Pantothenate and coenzyme A biosynthesis in bacteria.

PanB, ketopantoate hydroxymethyltransferase; PanC, pantothenate synthetase; PanD, L-aspartate- $\alpha$ -decarboxylase; PanE, ketopantoate reductase; CoaA, pantothenate kinase; CoaB, 4'-phosphopantothenoylcysteine synthase; CoaC, 4'-phosphopantothenoylcysteine decarboxylase; CoaD, phosphopantetheine adenyltransferase; CoaE, dephospho-CoA kinase.

stepwise elution (0–100 mM NaCl) from a DEAE-Toyopearl 650M column, and gel filtration on a Sephacryl S-300 HR column. Purification increased the specific activity 10.1-fold to 6.97 units/mg protein, with a yield of 46.9%. To measure the sum of acetyl-CoA and malonyl-CoA, 1 unit of malonate decarboxylase was first added to 400  $\mu$ l of the mixture containing *E. coli* cell extracts, and the mixture was incubated at 30°C for 20 min, followed by the addition of 1 unit of recombinant acetate kinase. After 20 min, 200  $\mu$ l of 2.5 M neutralized hydroxylamine was added, and the reaction was continued for an additional 20 min. The reaction was terminated by adding 600  $\mu$ l of 10 mM ferric chloride dissolved in 25 mM trichloroacetic acid-1 M HCl. Finally, the absorbance at 540 nm due to acetohydroxamate was measured. The malonyl-CoA content was determined by depleting acetyl-CoA from cellular extracts using 4  $\mu$ g/ml citrate synthase (Fig. S3), while the CoASH content was determined using 1  $\mu$ g/ml phosphate acetyltransferase to convert CoASH to acetyl-CoA (Fig. S4).

*E. coli* expressing an exogenous *coaA* gene grew with similar kinetics as cells transformed with an empty plasmid. As shown in Fig. 2A, *Ec-coaA* and *Pp-coaA* increased pantothenate kinase activity in a dose-dependent manner,



**Fig. 2.** Intracellular pantothenate kinase activity (panel A) and CoA content (panel B) in *E. coli* W3110 cells transformed with type I, II, or III CoaA.

Cells were aerobically cultivated at 30°C for 16 h in LB medium containing 5% glucose and supplemented with (+) or without (–) 10 mM pantothenate. In panel B, CoA pools were analyzed for CoASH (black bar), acetyl-CoA (open bar), and malonyl-CoA (gray bar) content. Data are mean  $\pm$ SD,  $n = 3$ . “L” and “H” indicate low-copy number pSTV28 and high-copy number pUC118 plasmids, respectively, carrying *coaA* from *E. coli* (*Ec-coaA*), *S. aureus* (*Sa-coaA*), or *P. putida* (*Pp-coaA*). “none” indicates empty pSTV28 or pUC118.

the dose being a function of plasmid copy number. In the absence of pantothenate, pantothenate kinase activity was highest in recombinant *E. coli* expressing an extra copy of *Ec-coaA* in a high-copy number plasmid. The activity in these cells was 29.4-fold higher than in cells transformed with the empty plasmid (Fig. 2A), although the size of the entire CoA pool was only slightly elevated from 0.497 to 0.673 nmol/mg of dry cell weight (Fig. 2B). In contrast, the addition of 10 mM pantothenate boosted the cellular CoA pool 3.9-fold in cells overexpressing *Ec-coaA* (Fig. 2B), indicating that *de novo* pantothenate synthesis in cells with exogenously expressed CoaA was a significant limiting factor to augment CoA content. An increase was also observed in cells expressing *Ec-coaA* from a low-copy number plasmid, even though the resulting CoA pool was not as large, presumably due to lower kinase activity. Although type I CoaA is highly sensitive to CoA and its thioesters, robust pantothenate kinase activity due to exogenously expressed *Ec-coaA* overcame this regulation, resulting in the increase of CoA pools as large as those observed in cells expressing other CoaA as described below.

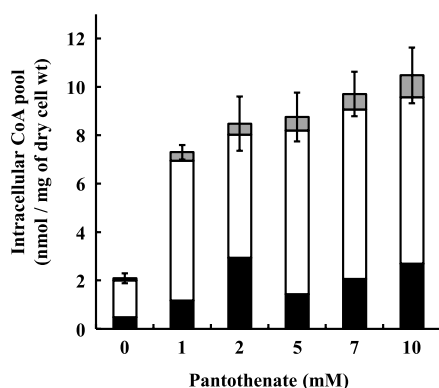


Fig. 3. Effect of supplementation with pantothenate.

*E. coli* W3110 cells transformed with *Pp-coaA* in a low-copy number plasmid were aerobically grown at 30°C for 24 h in M9 minimal medium supplemented with 2% glucose, 0.01% thiamine, 25 µg/ml chloramphenicol, and pantothenate at the indicated concentration. Cellular CoASH (black bar), acetyl-CoA (open bar), and malonyl-CoA (gray bar) were estimated by acyl-CoA cycling. Data are mean ±SD,  $n = 3$ .

Type II *Sa-coaA* increased pantothenate kinase activity only negligibly, regardless of copy number (Fig. 2A). Nevertheless, the CoA pool was significantly increased to the same levels as in cells overexpressing *Ec-coaA* (Fig. 2B). Furthermore, CoA biosynthesis was remarkably enhanced in cells overexpressing type III *Pp-coaA*, even though kinase activity in the presence of 10 mM pantothenate was less than 20% of the activity in the *Ec-coaA*-expressing strain. Surprisingly, *E. coli* cells with a low dose of *Pp-coaA* accumulated CoA to 3.14 nmol/mg of dry cell weight, the highest concentration measured. These results reflect the sensitivity of bacterial pantothenate kinases to CoA and its derivatives. Thus, *Pp-coaA*, a type III enzyme insensitive to end-product inhibition, raised the intracellular CoA pool most substantially.

Since supplementation with pantothenate was found to enhance the CoA pool, the effect of the pantothenate dose was examined (Fig. 3). *E. coli* cells overexpressing *Pp-coaA* from pSTV28 were aerobically grown at 30°C for 24 h in flasks containing M9 minimal medium with 2% glucose, 0.01% thiamine, 25 µg/ml chloramphenicol, and increasing concentrations of pantothenate. Intracellular CoA content increased with the concentration of pantothenate added (Fig. 3), even though cultures uniformly produced pantothenate kinase activity at around 1 nmol/min/mg of protein (data not shown). For example, supplementation with 10 mM pantothenate increased the CoA content 5-fold. The CoA content in *E. coli* cells grown in M9 medium is more than 3-fold higher than in cells grown in LB medium (Fig. 2B). Indeed, as the growth rate in M9 medium is much lower than in LB medium, CoA levels in M9 medium would approach maximal levels possibly because intracellular CoA content is known to peak at the middle of the logarithmic growth phase (Takamura and Nomura, 1988).

As pantothenate is produced in cells by the condensation of  $\beta$ -alanine and D-pantoate (Fig. 1), the effects of these precursors were analyzed, as well as that of L-aspartate, the precursor to  $\beta$ -alanine (Fig. 4). As pantoate is not available commercially, it was prepared by hydrolyzing

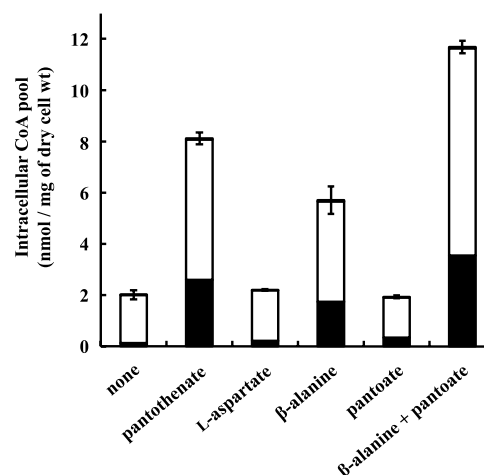


Fig. 4. Effect of supplementation with pantothenate precursors.

*E. coli* W3110 cells expressing *Pp-coaA* exogenously were aerobically cultivated at 30°C for 24 h in M9 minimal medium supplemented with 2% glucose, 0.01% thiamine, 25 µg/ml chloramphenicol, and 5 mM of the indicated pantothenate precursor. Cellular CoASH (black bar), acetyl-CoA (open bar), and malonyl-CoA (gray bar) were measured. Data are mean ±SD,  $n = 3$ .

D-pantolactone in 200 mM KOH (King et al., 1974). Aspartate did not affect the CoA pool in *E. coli* overexpressing *Pp-coaA*, while  $\beta$ -alanine elevated cellular CoA nearly 3-fold, to 70% of the level accumulated in the presence of 5 mM pantothenate. This result clearly implies that the supply of  $\beta$ -alanine is limited by L-aspartate- $\alpha$ -decarboxylase (PanD). Combining  $\beta$ -alanine with pantoate resulted in another 2-fold increase in CoA content, even though pantoate did not elicit more CoA production by itself. When  $\beta$ -alanine and pantoate are exogenously supplied, the resulting CoA pool is 44% more than the pool accumulated in the presence of 5 mM pantothenate. This difference is likely due to the inhibition of pantothenate uptake rather than the efficient utilization of exogenously supplied precursors. Indeed, the uptake of extracellular pantothenate through pantothenate permease is sensitive to pantetheine, a decomposition product derived from an intermediate in CoA biosynthesis (Vallari and Rock, 1985). Taken together, the data indicate that in *E. coli* cells with enhanced CoA biosynthesis due to overexpressed type III CoaA, supplementation with both  $\beta$ -alanine and pantoate augments the CoA pool, as pantothenate synthetase (PanC) seems able to fully utilize exogenously added precursors.

In this study, prokaryotic type III CoaA was demonstrated to enhance CoA biosynthesis in *E. coli*. Notably, over 70% of the resulting CoA pool is acetyl-CoA (Figs. 2–4), which can be used to synthesize other useful compounds. Furthermore, supplementation with  $\beta$ -alanine and pantoate was necessary to maximize the CoA pool. Alternatively, we hypothesize that a combination of *Pp-coaA* with enhanced pantothenate biosynthesis may also elevate CoA content without requiring an exogenous supply of precursors. It has been reported that pantothenate biosynthesis is enhanced by overexpression of ketopantoate hydroxymethyltransferase (PanB) from *Salmonella enterica* serovar Typhimurium (Rubio and Downs, 2002), PanD from *Corynebacterium glutamicum* (Dusch et al.,

1999; Shen et al., 2014), or ketopantoate reductase (PanE) from *E. coli* (Elischewski et al., 1999).

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## Supplementary Materials

**Fig. S1.** Schemes for expression and purification of recombinant acetate kinase (A), citrate synthase (B), and phosphate acetyltransferase (C) used to analyze intracellular CoA by the acyl-CoA cycling method.

**Fig. S2.** SDS-PAGE analysis of recombinant acetate kinase (A), citrate synthase (B), and phosphate acetyltransferase (C) in whole cells and after purification.

*E. coli* cells transformed with pET-ackA (A), pQE-citZ (B), or pQE-pta (C) were grown aerobically at 30°C in 100 mL LB media supplemented with an appropriate antibiotic. At  $A_{600} \sim 0.5$ , IPTG was added to induce expression. Lane 1, standard molecular mass markers; lane 2, a 0.1-ml sample of *E. coli* cultures at  $A_{600}$  2.5 before induction; lane 3, *E. coli* cells after 6 h of induction; lane 4, soluble fraction obtained after sonication; lane 5, insoluble fraction; lane 6, 5  $\mu$ g of purified enzyme.

**Fig. S3.** Elimination of acetyl-CoA by recombinant citrate synthase.

A reaction mixture containing 50 mM Tris-HCl (pH 7.2), 200 pmol acetyl-CoA, 2 mM oxaloacetate, and recombinant citrate synthase at the indicated concentration in a total volume of 1 ml was incubated at 25°C for 20 min, and residual acetyl-CoA was measured using the acyl-CoA cycling method.

**Fig. S4.** Conversion of CoASH to acetyl-CoA by recombinant phosphate acetyltransferase.

Reactions were performed at 25°C for 20 min in 1 ml of 50 mM Tris-HCl (pH 7.2), 10 mM  $MgSO_4$ , 1 mM 2-mercaptoethanol, 200 pmol CoASH, 0.1 mM acetylphosphate, and recombinant phosphate acetyltransferase at the indicated concentration. The acetyl-CoA formed was measured using the acyl-CoA cycling method after the His-tagged recombinant enzyme was filtered out by Ni-Sepharose 6 Fast Flow spin columns (GE Healthcare).

**Table S1.** Strains and plasmids.

**Table S2.** Primers.

Supplementary figures and tables are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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