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Elevating 4-hydroxycoumarin production through alleviating thioesterase-mediated salicoyl-CoA degradation

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ABSTRACT

Acyl-CoAs are essential intermediates in the biosynthetic pathways of a number of industrially and pharmaceutically important molecules. When these pathways are reconstituted in a heterologous microbial host for metabolic engineering purposes, the acyl-CoAs may be subject to undesirable hydrolysis by the host's native thioesterases, resulting in a waste of cellular energy and decreased intermediate availability, thus impairing bioconversion efficiency. 4-hydroxycoumarin (4HC) is a direct synthetic precursor to the commonly used oral anticoagulants (e.g. warfarin) and rodenticides. In our previous study, we have established an artificial pathway for 4HC biosynthesis in *Escherichia coli*, which involves the thioester intermediate salicoyl-CoA. Here, we utilized the 4HC pathway as a demonstration to examine the negative effect of salicoyl-CoA degradaton, identify and inactivate the responsible thioesterase, and eventually improve the 4HC production. We screened a total of 16 *E. coli* thioesterases and tested their hydrolytic activity towards salicoyl-CoA in vitro. Among all the tested candidate enzymes, YdiI was found to be the dominant contributor to the salicoyl-CoA degradation in *E. coli*. Remarkably, the *ydiI* knockout strain carrying the 4HC pathway exhibited an up to 300% increase in 4HC production. An optimized 4HC pathway construct introduced in the *ydiI* knockout strain led to the accumulation of 935 mg/L of 4HC in shake flasks, which is about 1.5 folds higher than the wild-type strain. This study demonstrates a systematic strategy to alleviate the undesirable hydrolysis of thioester intermediates, allowing production enhancement for other biosynthetic pathways with similar issues.

1. Introduction

Coenzyme A-activated acyl groups (or acyl-CoAs) are building blocks or essential intermediates for the metabolism of all living organisms. The acyl-CoA molecules possess a high-energy thioester bond between the acyl group and the CoA moiety. These energy-rich CoA thioesters allow for efficient transfer of an acyl group to the receptor molecule, thus facililtating biochemical activities in a thermodynamically favorable way. On one hand, acyl-CoAs such as acetyl-CoA, malonyl-CoA and fatty acyl-CoA are involved in primary biochemical transformations, including the citric acid cycle (TCA) and fatty acid metabolism (Lehninger et al., 2008). On the other hand, some acyl-CoAs are the intermediates of secondary metabolism that can generate a number of industrially and pharmaceutically important molecules. For example, salicoyl-CoA is a direct biosynthetic precursor to 4-hydroxycoumarin (4HC) (Lin et al., 2013; Liu et al., 2010), a critical pharmacophore used to manufacture oral anticoagulants (e.g. warfarin, acenocoumarol and phenprocoumon) and rodenticides; while *p*-coumaroyl-CoA is a common intermediate to the health promoting plant polyketides (e.g. resveratrol and flavonoids)(Falcone Ferreyra et al., 2012; Halls and Yu, 2008). As another example, the biosynthesis of the new-generation biofuel n-butanol involves a series of 4-carbon acyl-CoAs (Huang et al., 2010). Other examples include the biosynthesis of isoprenoid (Lange et al., 2000), poly(hydroxyalkanoates) (Poirier et al., 1995), carboxylic esters (Rodriguez et al., 2014), etc.

Recent advances in metabolic engineering allow the reconstitution of many non-native and non-natural biosynthetic pathways in heterologous microbial hosts such as *Escherichia coli* to achieve easy manipulation and efficient biomanufacturing (Ajikumar et al., 2010; Atsumi et al., 2008; Lin et al., 2014a; Ma et al., 2011; Santos et al., 2011; Sun

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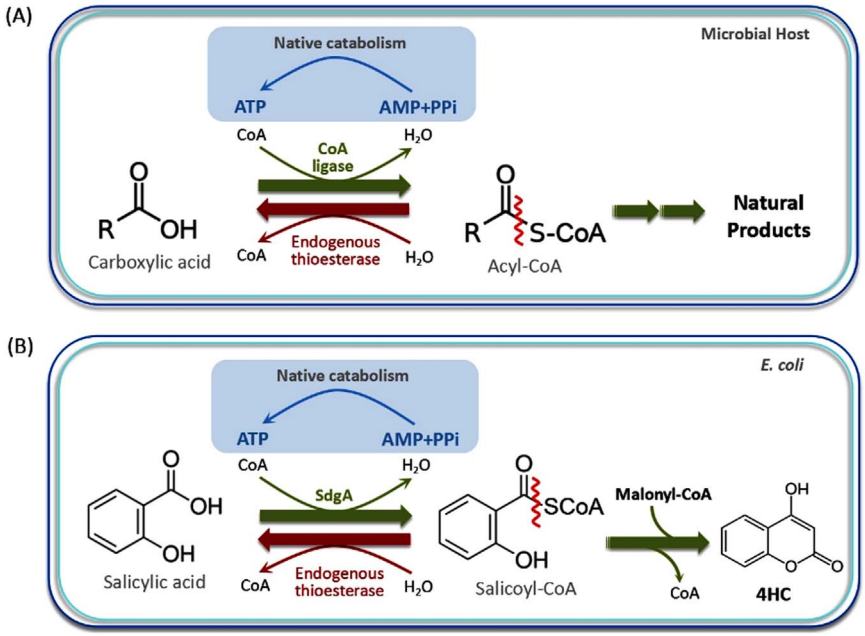

Fig. 1. Catalytic reactions of the formation and hydrolysis of (A) Acyl-CoA thioester in the biosynthesis of natural products and (B) salicoyl-CoA in the biosynthesis of 4HC.

et al., 2015; Tai et al., 2015; Xu et al., 2013). However, the assembly of biosynthetic pathways that contain acyl-CoA intermediates may often encounter the issue of undesirable hydrolysis due to the side activity of the host's endogenous thioesterase(s), leading to poor acyl-CoA availability for biosynthesis use. Even worse, acyl-CoA hydrolysis results in a waste of cellular energy. To be specific, formation of an acyl-CoA molecule is either through the thioesterification of CoA with an organic acid or through the transformation from another acyl-CoA molecule. Formation of a thioester bond is an thermodynamically unfavorable reaction, which has to be coupled with ATP hydrolysis. Although microbial cells can generate ATP via glycolysis, the TCA cycle and respiration chain (Lehninger et al., 2008), simultaneous occurance of hydrolysis and esterification leads to a futile cycle for the interconversion of ATP and AMP + PPi (Fig. 1). As acyl-CoA hydrolysis is a exothermic reaction, the net effect is that the chemical energy obtained from catabolism is consumed on generating heat rather than supporting cell growth and biosynthesis. As a result, large-scale microbial processes need the input of extra cooling energy to offset the generated heat, thus resulting in higher electricity consumption and production cost.

Previous studies have indicated that unknown thioesterase(s) native to *E.coli* are able to hydrolyze acyl-CoA intermediates of heterologous pathways (Beuerle and Pichersky, 2002; Watts et al., 2004). However, no systematic study was performed to identify the contributing enzymes for a specific acyl-CoA intermediate, let alone exploring the relavant effects on microbial production. In *E. coli*, there are a number of thioesterases, many of which have unknown physiological functions. It is usually difficult to predict the thioesterase(s) responsible for the hydrolysis of a specific thioester intermediate because many of them have broad substrate specificity and even catalytic promiscuity. It is common that one hydrolytic reaction is catalyzed by two or more thioesterases, and inactivation of a single thioesterase usually does not show any negative effect on cell growth.

In our previous study, we have established an artificial pathway for 4HC biosynthesis in *E. coli*, which contains a thioester intermediate salicoyl-CoA (Lin et al., 2013). Some preliminary experimental evidences suggested the occurance of salicoyl-CoA degradation. Herein, we utilize the established 4HC pathway as a demonstration to examine the negative effect of salicoyl-CoA degradaton, identify and inactivate the responsible thioesterase, and eventually improve the 4HC production. This study demonstrates a systematic strategy to alleviate the undesirable hydrolysis of thioester intermediates, allowing for the improvement in production efficiency for other biosynthetic pathways with similar issues.

2. Materials and methods

2.1. Experimental materials

Luria-Bertani (LB) medium was used to grow *E. coli* cells for plasmid construction and inoculum preparation. The biosynthesis medium M9Y contains (per liter): M9 minimal salts (11.28 g), Yeast Extract (5 g), Glycerol (20 g), $CaCl_2{\cdot}2H_2O$ (0.1 mmol), and $MgSO_4{\cdot}7H_2O$ (2 mmol). Ampicillin (100 mg/L) or kanamycin (50 mg/L) were added to cultures as needed. *E. coli* strains XL1-Blue and BL21 Star (DE3) were used as host strains for gene cloning and protein expression, respectively. Wild-type *E. coli* strain BW25113/F′ and the mutant strain *ΔydiI::frt* were used for biosynthesis of 4-hydroxycoumarin. High-copy number plasmid pETDuet-1 was used for protein expression. pCP20 was used to delete the kanamycin resistance gene from the Keio strain. Phusion High-Fidelity DNA polymerase, restriction endonucleases and Quick Ligation Kit were purchased from New England Biolabs (Beverly, MA, USA). Zyppy™ Plasmid Miniprep Kit, Zymoclean™ Gel DNA Recovery Kit, and DNA Clean & Concentrator™-5 were purchased from Zymo Research (Irvine, CA, USA). 4-Hydroxycoumarin was purchased from ACROS ORGANICS (Bridgewater, NJ, USA). All tested mutant strains were obtained from the Keio Knockout Strain Library (The Coli Genetic Stock Center, Yale University, New Haven, CT, USA). All of strains and plasmids used in this study are listed in Table 1.

2.2. Plasmid construction and protein purification

All 16 thioesterase genes were PCR-amplified from *E. coli* Bw25113/F′ genomic DNA. All genes but *tesB* were cloned and digested between BamHI and SalI; SacI and HindIII were used to digest *tesB*. The digested gene fragments were inserted into the plasmid backbone of pETDuet-1 and subsequently transferred into BL21 Star (DE3) for His-tagged protein expression. pETDuet-sdgA constructed in previous study was used to the SdgA protein expression (Lin et al., 2013). For protein purification, an overnight inoculum was prepared in 3 mL LB broth. 1% of inoculum was transferred to 50 mL of LB where expression was induced via 0.5 mM IPTG after 3 h. The cells were pelleted and resuspended in