# Unprecedented acetoacetyl-coenzyme A synthesizing enzyme of the thiolase superfamily involved in the mevalonate pathway

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**Acetoacetyl-CoA is the precursor of 3-hydroxy-3-methylglutaryl (HMG)-CoA in the mevalonate pathway, which is essential for terpenoid backbone biosynthesis. Acetoacetyl-CoA is also the precursor of poly-β-hydroxybutyrate, a polymer belonging to the polyester class produced by microorganisms. The de novo synthesis of acetoacetyl-CoA is usually catalyzed by acetoacetyl-CoA thiolase *via* a thioester-dependent Claisen condensation reaction between two molecules of acetyl-CoA. Here, we report that *nphT7*, found in the mevalonate pathway gene cluster from a soil-isolated *Streptomyces* sp. strain, encodes an unusual acetoacetyl-CoA synthesizing enzyme. The recombinant enzyme overexpressed in *Escherichia coli* catalyzes a single condensation of acetyl-CoA and malonyl-CoA to give acetoacetyl-CoA and CoA. Replacement of malonyl-CoA with malonyl-(acyl carrier protein) resulted in loss of the condensation activity. No acetoacetyl-CoA synthesizing activity was detected through the condensation of two molecules of acetyl-CoA. Based on these properties of NphT7, we propose to name this unusual enzyme of the thiolase superfamily acetoacetyl-CoA synthase. Coexpression of *nphT7* with the HMG-CoA synthase gene and the HMG-CoA reductase gene in a heterologous host allowed 3.5-fold higher production of mevalonate than when only the HMG-CoA synthase and HMG-CoA reductase genes were expressed. This result suggests that *nphT7* can be used to significantly increase the concentration of acetoacetyl-CoA in cells, eventually leading to the production of useful terpenoids and poly-β-hydroxybutyrate.**

acetoacetyl-CoA | *Streptomyces* | terpenoid

Carbon–carbon bond formation is an essential step in natural product biosynthesis. Thiolase superfamily enzymes catalyze the formation of this covalent bond *via* a thioester-dependent Claisen condensation reaction in various metabolic pathways, such as fatty acid, polyketide, and mevalonate biosyntheses (1, 2). The superfamily enzymes are categorized into two groups according to their mechanism of α-anion generation in the Claisen condensation: one consists of nondecarboxylative enzymes, including acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase; and the other includes decarboxylative enzymes, such as β-ketoacyl-(acyl carrier protein (ACP)) synthase (KAS) I, KAS II, KAS III, β-ketoacyl-CoA synthase (KCS), and chalcone synthase (CHS) family enzymes (1, 2). The nondecarboxylative enzymes generate an anion by proton elimination from the α-carbon of acyl-CoA, whereas the decarboxylative enzymes generate an anion by β-carbon decarboxylation of a di-keto moiety in malonyl-CoA or malonyl-ACP (1–4). An extensive body of biochemical studies concerning decarboxylative KAS III enzymes and their crystal structures has identified a catalytic triad involved in the Claisen condensation that includes Cys, His, and Asn residues (5–9). The Cys residue is involved in acyl loading from a primer substrate acyl-CoA to give an acyl-cysteine intermediate, and the other two residues are involved in decarboxylation of the malonyl moiety of malonyl-ACP to yield a carbanion. The resulting carbanion performs a nucleophilic attack on the carbonyl carbon of the acyl-cysteine intermediate to yield β-ketoacyl-ACP such as acetoacetyl-ACP for fatty acid synthesis.

Recently, we identified a mevalonate pathway gene cluster (*nphT1* to *nphT6*) in *Streptomyces* sp. strain CL190 (10–12). Mevalonate pathway gene clusters have also been cloned from *Actinoplanes* sp. strain A40644 (13), *Streptomyces* sp. strain KO-3988 (14), and *Streptomyces anulatus* (15). Each gene cluster contains six open reading frames encoding enzymes that catalyze the formation of isopentenyl diphosphate and dimethylallyl diphosphate for terpenoids *via* mevalonate from acetoacetyl-CoA (Fig. 1). This CoA derivative is usually biosynthesized *via* a thioester-dependent Claisen condensation reaction between two molecules of acetyl-CoA catalyzed by acetoacetyl-CoA thiolase (EC 2.3.1.9). Therefore, acetoacetyl-CoA thiolase was expected to exist in the previously mentioned mevalonate pathway gene clusters. However, homologs of acetoacetyl-CoA thiolase, involved in the conversion of acetyl-CoA to acetoacetyl-CoA, are missing in the gene clusters. Instead, an open reading frame (for example, *nphT7* of *Streptomyces* sp. strain CL190) that shares homology with KAS III flanks each gene cluster (Fig. 1).

In this paper, we demonstrate that NphT7 catalyzes a single condensation of acetyl-CoA and malonyl-CoA to give acetoacetyl-CoA and CoA. Based on the enzymatic properties of NphT7, we propose that it is an unprecedented acetoacetyl-CoA synthase of the thiolase superfamily. This paper also describes the effect of *nphT7* expression on mevalonate production in the heterologous host *Streptomyces albus*. Coexpression of *nphT7* with the HMG-CoA synthase gene and the HMG-CoA reductase gene in *S. albus* resulted in 3.5-fold higher production of mevalonate than when only the HMG-CoA synthase and HMG-CoA reductase genes were expressed. This result suggests that *nphT7* can be used to significantly increase the production of useful terpenoids such as carotenoids, taxol, and artemisinin.

## Results

**Expression and Purification of Recombinant *nphT7* Gene Product.** The *nphT7* gene from *Streptomyces* sp. strain CL190 was overexpressed in *Escherichia coli* as an N-terminal $His_8$-tagged protein, and the recombinant protein was purified to apparent homogeneity. The molecular mass of the *nphT7* product was estimated to be 37 kDa by SDS-PAGE (Fig. S1) and 63 kDa by gel filtration chromatography, suggesting that NphT7 is most likely a dimer.

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Data deposition: The sequence reported in this paper has been deposited in the DDBJ/EMBL/GenBank database (accession no. AB540131).

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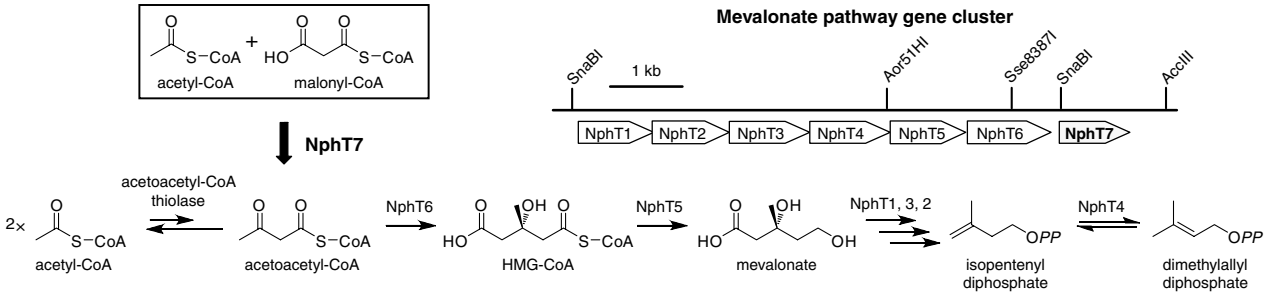


**Fig. 1.** The mevalonate pathway gene cluster from *Streptomyces* sp. strain CL190 and the mevalonate pathway. The structures of the mevalonate pathway gene clusters from *Actinoplanes* sp. strain A40644, *Streptomyces* sp. strain KO-3988, and *S. anulatus* are identical to that of CL190. The NphT7-catalyzed condensation of acetyl-CoA and malonyl-CoA to form acetoacetyl-CoA is an unprecedented reaction elucidated in the present study. NphT genes: 1, mevalonate kinase; 2, diphosphomevalonate decarboxylase; 3, phosphomevalonate kinase; 4, type 2 isopentenyl diphosphate isomerase; and 5, HMG-CoA reductase; and 6, HMG-CoA synthase.

**Assay for KAS III Activity of NphT7.** KAS III enzymes possess a catalytic triad of Cys, His, and Asn residues involved in the Claisen condensation of the primer substrate acetyl-CoA and the extender substrate malonyl-ACP. Alignment of NphT7 and the well characterized *E. coli* KAS III (5, 6) identified the highly conserved catalytic triad at residues Cys115, His256, and Asn286 in NphT7 (Fig. S2). Therefore, we first determined whether NphT7 displays KAS III activity, in which the acetyl group of acetyl-CoA is transferred to malonyl-ACP to yield acetoacetyl-ACP. To detect this acetyl transfer in the KAS III reaction, we used [1-$^{14}$C]acetyl-CoA as the primer substrate. A high level of radioactivity arising from [3-$^{14}$C]acetoacetyl-ACP was detected when malonyl-ACP (Fig. S3) was incubated with FabH, a KAS III protein from *Streptomyces coelicolor* A3(2) (positive control) (Fig. 2). By contrast, the radioactive signal of the ACP derivatives incubated with NphT7 was no greater than the signal in the absence of enzyme (negative control). Interestingly, the NphT7 protein itself was labeled at low levels with the acetyl moiety of [1-$^{14}$C]acetyl-CoA, suggesting that the acetyl moiety remained on the Cys115 residue of NphT7 due to the lack of an appropriate extender substrate for NphT7. We thus concluded that NphT7 cannot use malonyl-ACP as the extender substrate and therefore exhibits no KAS III activity. This conclusion is consistent with the fact that an important Arg residue responsible for recognition of ACP by KAS III enzymes (16) is replaced by Val261 in NphT7 (Fig. S2).

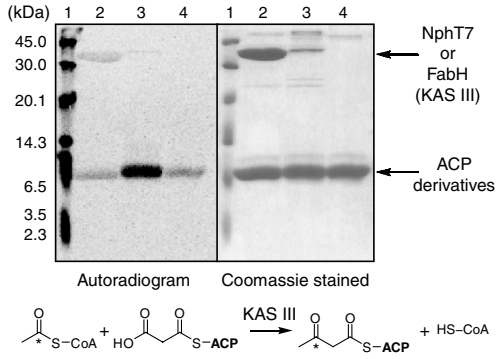


**Fig. 2.** Assay for KAS III activity. [1-$^{14}$C]acetyl-CoA and malonyl-ACP were incubated with NphT7 or FabH (*S. coelicolor* KAS III) as described in *Materials and Methods* and then separated by SDS-18% PAGE. *Lanes*: *1*, molecular mass markers; *2*, NphT7 reaction; *3*, KAS III reaction as a positive control; and *4*, negative control. A high level of radioactivity arising from [3-$^{14}$C]acetoacetyl-ACP was observed in the KAS III reaction (*lane 3*). The radioactive signal from the ACP derivatives in the NphT7 reaction (*lane 2*) was as faint as that in the absence of enzyme (*lane 4*). Some faint radioactivity of the NphT7 protein itself was also observed (*lane 2*). Asterisks in the KAS III-catalyzed reaction

**Assay for Acetoacetyl-CoA Synthesizing Activity and the Kinetic Parameters of NphT7.** As described above, NphT7 displayed no KAS III activity, which raised the possibility of NphT7 using malonyl-CoA as an extender substrate condensing it into acetyl-CoA to yield acetoacetyl-CoA. Thus, we used malonyl-CoA as the extender substrate in an assay to assess if NphT7 exhibited acetoacetyl-CoA synthesizing activity. When the assay mixture was subjected to HPLC coupled to high-resolution MS, the formation of two products was observed: CoA (**1**): retention time = 9.6 min; $[M(C_{21}H_{36}N_7O_{16}P_3S) - H]^-$ $m/z$ 766.1096, $C_{21}H_{35}N_7O_{16}P_3S$, calculated 766.1074; $[M(C_{21}H_{36}N_7O_{16}P_3S) + Na - 2H]^-$ $m/z$ 788.0876, $C_{21}H_{34}N_7O_{16}P_3SNa$, calculated 788.0893); and acetoacetyl-CoA (**2**): retention time = 9.8 min; $[M(C_{25}H_{40}N_7O_{18}P_3S) - H]^-$ $m/z$ 850.1284, $C_{25}H_{39}N_7O_{18}P_3S$, calculated 850.1285; $[M(C_{25}H_{40}N_7O_{18}P_3S) + Na - 2H]^-$ $m/z$ 872.1092, $C_{25}H_{38}N_7O_{18}P_3SNa$, calculated 872.1105). Product formation was concomitant with consumption of malonyl-CoA (**3**): retention time = 9.2 min); and acetyl-CoA (**4**): retention time = 10.0 min) (Fig. 3 *A*, *B*, and *D*). In contrast, no acetoacetyl-CoA synthesizing activity was detected in the absence of malonyl-CoA. These NphT7 in vitro assays unambiguously elucidated the function of NphT7, which catalyzes the condensation of acetyl-CoA and malonyl-CoA to yield CoA and acetoacetyl-CoA. Furthermore, the stoichiometry of the NphT7 reaction clearly indicates that NphT7 catalyzes a single condensation of one molecule of acetyl-CoA and one molecule of malonyl-CoA to yield one molecule of CoA and one molecule of acetoacetyl-CoA (Fig. 3*E* and Fig. S4*A*).

The steady-state kinetic constants for NphT7 with acetyl-CoA and malonyl-CoA as substrates (bisubstrate conditions) were determined (Fig. S5). The apparent $K_m^{\text{acetyl-CoA}}$ was estimated to be $68 \pm 4$ μM at a fixed concentration of malonyl-CoA (100 μM), whereas the apparent $K_m^{\text{malonyl-CoA}}$ was estimated to be $28 \pm 2$ μM at a fixed concentration of acetyl-CoA (200 μM). The $V_{\max}$ value was $8.9 \pm 0.3$ $\mu$mol · min$^{-1}$ · mg$^{-1}$.

As described previously, *E. coli* KAS III catalyzes the formation of acetyl-ACP from malonyl-ACP by decarboxylating malonyl-ACP in the absence of the primer substrate acetyl-CoA (2, 17). This fact led us to assume that NphT7 catalyzes the formation of acetyl-CoA from malonyl-CoA by decarboxylating malonyl-CoA. To test this assumption, we incubated NphT7 with malonyl-CoA alone (monosubstrate conditions). HPLC of the NphT7 reaction with the addition of only malonyl-CoA revealed the formation of CoA (**1**) and acetoacetyl-CoA (**2**) concomitant with consumption of malonyl-CoA (**3**) (Fig. 3*C*). The stoichiometry of the NphT7 reaction under monosubstrate conditions indicates that one molecule of CoA and one molecule of acetoacetyl-CoA were formed concomitantly with the consumption of two molecules of malonyl-CoA (Fig. 3*F* and Fig. S4*B*). The steady-state kinetic constants for NphT7 under monosubstrate conditions were also determined (Fig. S5*C*): $K_m^{\text{malonyl-CoA}} = 320 \pm 20$ μM and $V_{\max} = 1.8 \pm 0.3$ $\mu$mol · min$^{-1}$ · mg$^{-1}$.

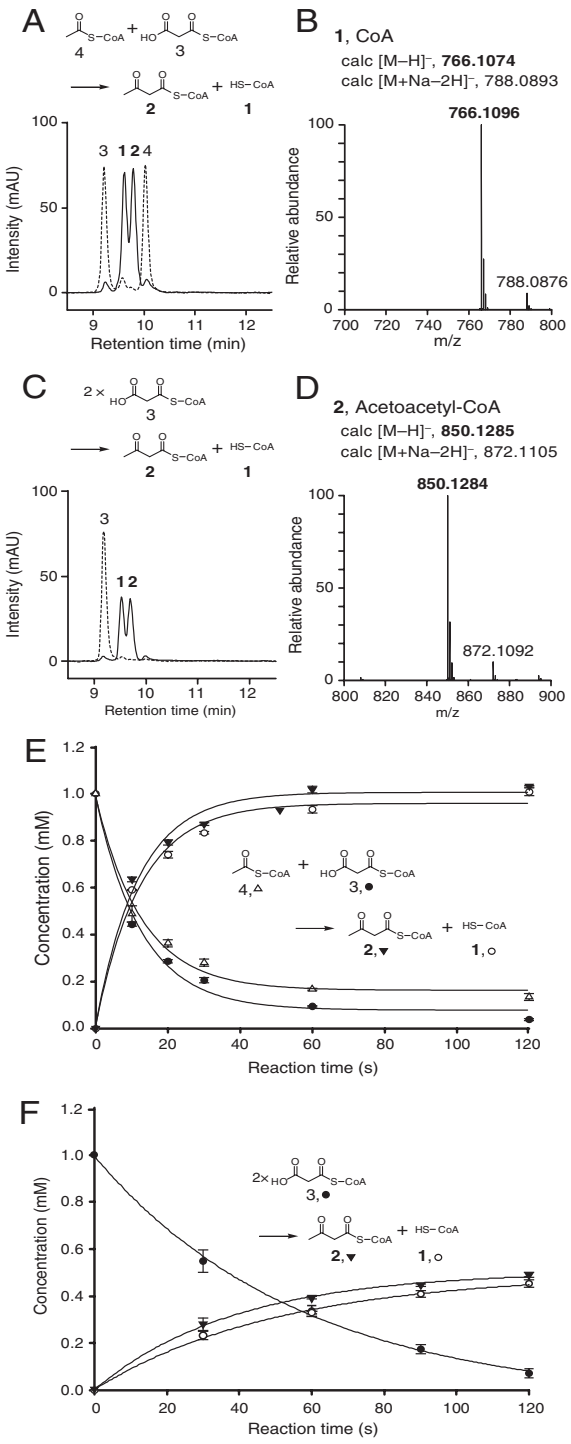


**Fig. 3.** Assay for acetoacetyl-CoA synthesizing activity. All reactions were performed as described in *Materials and Methods*. HPLC analysis was performed for the NphT7-catalyzed reactions (*A*) with acetyl-CoA and malonyl-CoA and (*C*) with malonyl-CoA alone. Chromatograms monitored at 259 nm. Peaks: 1, CoA; 2, acetoacetyl-CoA; 3, malonyl-CoA; and 4, acetyl-CoA. *A*: *solid line*, after a 1-min incubation with NphT7; *dotted line*, after a 2-min incubation with heat denatured NphT7. *C*: *solid line*, after a 2-min incubation with NphT7; *dotted line*, after a 2-min incubation with heat denatured NphT7. *B* and *D*: high-resolution mass spectra (negative mode) of peaks 1 and 2, respectively. *E* and *F*: stoichiometries of the acetoacetyl-CoA synthesizing activity of NphT7 under bisubstrate and monosubstrate conditions, respectively. *Filled triangles*, acetoacetyl-CoA **2**; *circles*, CoA **1**; *triangles*, acetyl-CoA **4**; and *filled circles*, malonyl-CoA **3**. The standard errors of each point were generated by repeating the experiment three times. The NphT7-catalyzed reaction formulas are described inside. Also see chromatograms in Fig. S4.

**Prediction of the Reaction Mechanism of NphT7.** The cysteine residue, which functions as an anchor for the acyl moiety of primer substrates, is highly conserved and exists in a catalytic Cys-His-Asn triad in KAS III and other decarboxylative condensing enzymes such as KCS and CHS. Substitution of the cysteine to another amino acid results in the loss of the condensation activity. However, the substituted enzyme retains its decarboxylation activity through the actions of the His and Asn residues to produce acetyl-ACP from malonyl-ACP by decarboxylation of the malonyl moiety (5). To determine the function of Cys115 in NphT7, we constructed the mutant NphT7 (C115A) (Fig. S1) and evaluated its activity. HPLC of the NphT7 (C115A) reaction mixture in the presence of acetyl-CoA and malonyl-CoA (bisubstrate conditions) confirmed the full consumption of malonyl-CoA and an increase in acetyl-CoA (Fig. 4*A*). Meanwhile, there was no formation of CoA or acetoacetyl-CoA. This result indicates that the C115A mutant enzyme yields acetyl-CoA *via* its malonyl-CoA decarboxylation activity, presumably by the His256 and Asn286 residues, while it has lost its condensation activity. This loss of function is most likely due to the lack of Cys115, which anchors the acetyl moiety of acetyl-CoA. These results strongly suggest that Cys115 in NphT7 functions as a key catalytic residue for the condensation reaction.

To determine the function of the His256 and Asn286 residues in NphT7, we attempted to construct the mutants NphT7 (H256A) and NphT7 (N286A) (Fig. S1). However, we could not obtain NphT7 (N286A) as a soluble protein. Therefore, we investigated the activity of only NphT7 (H256A). HPLC of the NphT7 (H256A) reaction mixture revealed that this mutant enzyme exhibited detectable acetoacetyl-CoA synthesizing activity, but its specific activity ($0.23\ \mu mol \cdot min^{-1} \cdot mg^{-1}$) was approximately 40-fold lower than that of wild-type NphT7 ($8.9\ \mu mol \cdot min^{-1} \cdot mg^{-1}$) (Fig. 4*B*). This low activity of the NphT7 (H256A) suggests that His256 functions as a catalytic residue for the decarboxylation of the extender substrate malonyl-CoA.

Taken together, the results suggest that the NphT7-catalyzed reaction presumably proceeds through an almost identical reaction mechanism to that of KAS III.

**in Vivo Acetoacetyl-CoA Synthesizing Activity of NphT7.** To verify the in vivo acetoacetyl-CoA synthesizing activity of NphT7, we evaluated the effect of *nphT7* expression on mevalonate production by constructing two plasmids (pSEMV25 and pSEMV40) and then transforming each into *S. albus*. pSEMV25 contains *nphT5* and *nphT6* (18), whereas pSEMV40 contains *nphT5*, *nphT6*, and

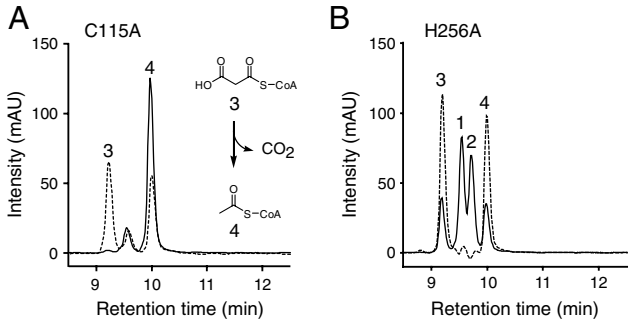


**Fig. 4.** Assays for the acetoacetyl-CoA synthesizing activity of NphT7 (C115A) and NphT7 (H256A). Peaks: 1, CoA; 2, acetoacetyl-CoA; 3, malonyl-CoA; and 4, acetyl-CoA. *A*: HPLC analysis of the NphT7 (C115A)-catalyzed reaction monitored at 259 nm. *Solid line*, after a 10-min incubation of the reaction mixture with NphT7 (C115A); *dotted line*, after a 10-min incubation of the reaction mixture with heat denatured NphT7 (C115A). *B*: HPLC analysis of the NphT7 (H256A)-catalyzed reaction monitored at 259 nm. *Solid line*, after a 5-min incubation of the reaction mixture with NphT7 (H256A); *dotted line*, after a 5-min incubation of the reaction mixture with heat denatured NphT7 (H256A).

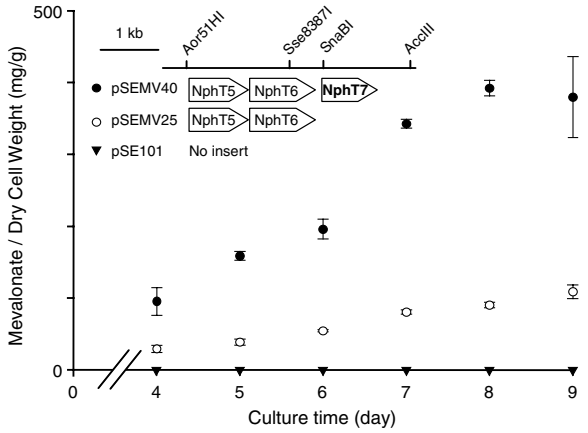


**Fig. 5.** Mevalonate production in the transformants. *Filled circles*, *S. albus*/pSEMV40 containing *nphT5*, *nphT6*, and *nphT7*; *circles*, *S. albus*/pSEMV25 containing *nphT5* and *nphT6*; *filled triangles*, *S. albus*/pSE101 (negative control). The standard errors of each point were generated from mevalonate production of three independent cultures for each transformant.

*nphT7* (Figs. 1, 5). All *nphT* genes in the two plasmids are most likely transcribed as a single operon by read-through from the promoter of the replication protein in the vector pSE101 (19–21).

*S. albus* possesses an intrinsic acetoacetyl-CoA thiolase but neither HMG-CoA synthase nor HMG-CoA reductase (accession number ABYC00000000), and therefore it cannot produce mevalonate. Conversely, the resulting transformants harboring pSEMV25 or pSEMV40 should produce mevalonate by the actions of HMG-CoA synthase (the *nphT6* gene product) and HMG-CoA reductase (the *nphT5* gene product) on these plasmids. In addition, *S. albus*/pSEMV40 should presumably produce more mevalonate than *S. albus*/pSEMV25 because expression of *nphT7* on pSEMV40 should increase the acetoacetyl-CoA supply for mevalonate production. Each transformant was grown, and the amount of mevalonate produced from these transformants was determined. Mevalonate production was detected on the fourth day of culture, and a gradual increase in production was observed until the eighth day (Fig. 5). Ultimately, the *S. albus*/pSEMV40 and *S. albus*/pSEMV25 cultures produced $380 \pm 60$ mg of mevalonate per gram of dry cells (day 8) and $110 \pm 10$ mg of mevalonate per gram of dry cells (day 9), respectively. As we expected, *S. albus*/pSEMV40 exhibited a 250% greater yield of mevalonate, indicating that NphT7 functions not as a KAS III but as an acetoacetyl-CoA synthesizing enzyme in vivo as well as in vitro, and it thereby increases the acetoacetyl-CoA supply for mevalonate production.

## Discussion

We demonstrated in the present study that NphT7, a KAS III homolog flanking the mevalonate pathway gene cluster of *Streptomyces* sp. strain CL190, displays an unusual acetoacetyl-CoA synthesizing activity. Synthesis of acetoacetyl-CoA is usually catalyzed by acetoacetyl-CoA thiolase *via* the condensation of two molecules of acetyl-CoA, yet NphT7 synthesizes acetoacetyl-CoA *via* the condensation of acetyl-CoA and malonyl-CoA. Importantly, NphT7 cannot use malonyl-ACP as its extender substrate, whereas KAS III enzymes can. We therefore propose to name NphT7 "acetoacetyl-CoA synthase," an unprecedented enzyme of the thiolase superfamily.

We demonstrated in this study that NphT7 can synthesize acetoacetyl-CoA in assay mixtures in which only malonyl-CoA is added as the substrate (monosubstrate conditions). Furthermore, a C115A mutant enzyme loses its condensation activity while retaining its malonyl-CoA decarboxylation activity to yield acetyl-CoA. These results indicate that NphT7 converts malonyl-CoA into acetyl-CoA *via* decarboxylation of malonyl-CoA. This finding explains why NphT7 displays acetoacetyl-CoA synthesizing activity even with no addition of acetyl-CoA into the reaction mixture, as it alternatively generates acetyl-CoA primer substrate by decarboxylating malonyl-CoA in an irreversible reaction. A similar reaction has been reported for the plant type III polyketide synthase 2-pyrone synthase (2-PS) (22). 2-PS catalyzes two Claisen condensations of one molecule of acetyl-CoA with two molecules of malonyl-CoA to yield 6-methyl-4-hydroxy-2-pyrone. When acetyl-CoA is not present in this reaction, 2-PS synthesizes the primer substrate acetyl-CoA *via* decarboxylation of malonyl-CoA and further synthesizes 6-methyl-4-hydroxy-2-pyrone. In contrast, a bacterial type III polyketide synthase, 1,3,6,8-tetrahydroxynaphthalene (THN) synthase, synthesizes THN by four Claisen condensations of a single malonyl-CoA extender substrate with no incorporation of an acetyl-CoA primer substrate, although THN synthase can also convert malonyl-CoA into acetyl-CoA (23). It is known that THN synthase does not load acetyl-CoA as a primer substrate despite possessing the ability to decarboxylate malonyl-CoA. In view of the substrate structures and the condensation number of NphT7, the NphT7-catalyzed reaction is simplest among the decarboxylative condensing enzymes possessing the catalytic triad (Fig. S6). Therefore, a crystal structure of NphT7 will provide new insights into the substrate recognition mechanism and the reaction mechanism of not only KAS III, 2-PS, and THN synthase, but also other decarboxylative enzymes possessing the Cys-His-Asn catalytic triad.

To gain insight into the substrate recognition of NphT7, we compared amino acid sequences of the NphT7 homologs with that of well-characterized *E. coli* KAS III (5, 6, 16). N-terminal architectures and recognition residues for acetyl-CoA are relatively conserved among the NphT7 homologs and *E. coli* KAS III (ecoKASIII), whereas partial C-terminal architectures and ACP recognition residues are not conserved (Fig. 6 and Fig. S2). Recognition residues Trp32 and Arg151 for the adenine ring of acetyl-CoA (5, 6) are completely conserved, but Thr28 is replaced with Val or Ile in the NphT7 homologs. Residues Thr37, Arg151, Met207, Gly209, Ala246, and Asn247, which are responsible for the recognition of phosphopantetheine of ACP or CoA (5, 6), are also completely conserved, but a variety of residues equivalent to Arg36, Asn210, Arg249, and Ile250 in ecoKASIII are found in the NphT7 homologs. The Gly305–307 residues that form an oxyanion hole (5, 6) are highly conserved. Residues Phe87, Leu142, Phe157, Leu188, and Leu205 form a hydrophobic pocket for recognition of the acetyl methyl group of acetyl-CoA (5, 6). Of the five residues, Phe157 and Leu188 are highly conserved, but both Leu142 and Leu205 are replaced with the bulky residue Tyr in the NphT7 homologs. In addition, the Phe87 residue that dictates primer substrate specificity in ecoKASIII (5, 6) is replaced with Gln in the NphT7 homologs. Since the NphT7 homologs share a STPDXPQ sequence motif in the predicted L3 loop region containing the important Gln residue, the motif may be a determinant site of the primer substrate specificity of the NphT7 homologs. The Cα1 and Cα2 regions of ecoKASIII interact with the helix II region of ACP to recognize the ACP molecule (16). The ACP recognition residues Lys214, Arg249, Lys256, and Lys257 in the Cα1 and Cα2 regions (16) are replaced with various amino acids in the NphT7 homologs. Most importantly, the predicted L9 loops in the NphT7 homologs are inserted by extra sequences and share an (A/G)GGSR sequence motif (Fig. 6). This (A/G)GGSR motif, characteristic of the NphT7 homologs, may serve as one of recognition sites for the CoA moiety of the extender substrate malonyl-CoA. Further insights into the structural basis for the substrate recognition mechanism require crystal structures of NphT7 (acetoacetyl-CoA synthase) complexed with the substrate or the product.

We determined the steady-state kinetic constants for NphT7 under both monosubstrate and bisubstrate conditions to determine which condition the NphT7 enzyme prefers. The $K_m$ value

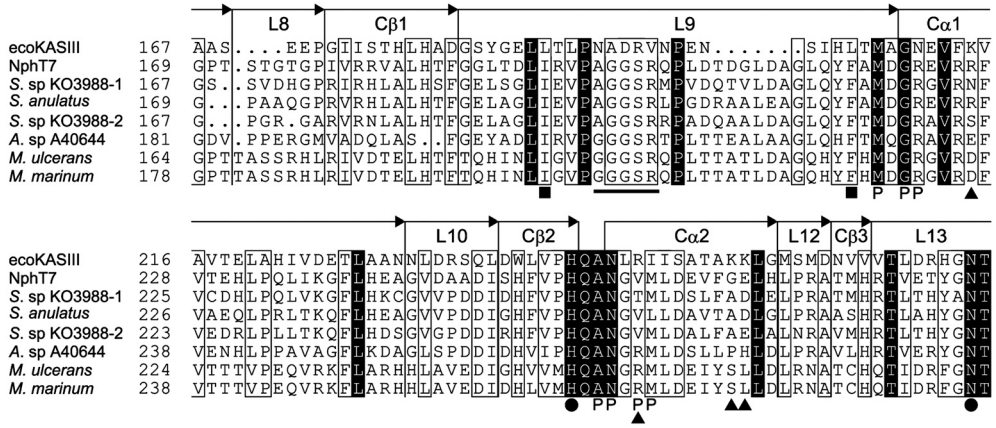


**Fig. 6.** Alignment of the C-terminal amino acid sequences of *E. coli* KAS III, and NphT7 homologs. This alignment was constructed using ClustalW ver. 1.82. *Dots* represent gaps introduced for optimization of the alignment. ecoKASIII, *E. coli* KAS III (PDB ID code 1EBL); *S.* sp KO3988-1 and *S.* sp KO3988-2, NphT7 homologs from *Streptomyces* sp. strain KO-3988 (Protein IDs, BAD86806 and BAE78983, respectively); *S. anulatus*, NphT7 homolog from *S. anulatus* strain 9663 (CAX48662); *A.* sp A40644, NphT7 homolog from *Actinoplanes* sp. strain A40644 (BAD07381); *M. ulcerans*, NphT7 homolog from *Mycobacterium ulcerans* Agy99 (YP_907152); and *M. marinum*, NphT7 homolog from *M. marinum* M (YP_001851502). The (A/G)GGSR motif found in the present study is underlined. Secondary structure of *E. coli* KAS III is indicated above the alignment (5, 6). The symbols represent the following functional amino acid residues in ecoKASIII: *filled circles*, catalytic triad; *filled squares*, hydrophobic residues responsible for recognition of the acetyl moiety; *filled triangles*, residues responsible for recognition of ACP; *bold letters* "P", residues responsible for recognition of phosphopantetheine. The full alignment is shown in Fig. S2.

for malonyl-CoA under monosubstrate conditions was approximately 10-fold higher than that for malonyl-CoA under bisubstrate conditions. In addition, the $V_{max}$ value under monosubstrate conditions was approximately 5-fold lower than that under bisubstrate conditions. These notable differences of the kinetic constants imply that NphT7 synthesizes acetoacetyl-CoA *via* the predominant bisubstrate reaction in vivo.

We demonstrated in this study that NphT7 functions as an acetoacetyl-CoA synthase involved in the mevalonate pathway. Although acetoacetyl-CoA thiolase (EC 2.3.1.9) also produces acetoacetyl-CoA by reversible nondecarboxylative condensation of two molecules of acetyl-CoA, this enzyme prefers acetoacetyl-CoA thiolysis to acetoacetyl-CoA synthesis (2). Because NphT7 exhibits no thiolysis activity against acetoacetyl-CoA, and since NphT7-catalyzed acetoacetyl-CoA synthesis is essentially an energy-favored reaction, NphT7 could be an ideal enzyme to supply acetoacetyl-CoA in cells. As previously reported, an increase in the supply of acetoacetyl-CoA or mevalonate induces increased carotenoid production in an engineered *E. coli* (24–26). Therefore, *nphT7* could be used for heterologous production of biofuel (butanol), biodegradable plastic (poly-β-hydroxybutyrate), and useful terpenoids, such as carotenoids, taxol, and artemisinin (27), all of which use acetoacetyl-CoA as common precursors.

BLAST searches using the NphT7 sequence as a query sequence found 55 sequences from protein databases of the National Center for Biotechnology Information annotated as putative KAS III sequences displaying E-values less than $3 \times e^{-59}$. The 55 sequences include 14 actinobacterial sequences (Table S1). Of the 14 sequences annotated as putative KAS III sequences, six NphT7 homologs flank the mevalonate pathway gene clusters for terpenoid biosynthesis (Fig. 6), whereas the others are likely located in the biosynthetic gene clusters of as-yet-unidentified natural products, suggesting that the NphT7-like enzymes are widely involved in natural product biosynthesis. Future work following up on the present data will help to elucidate the function of the NphT7 homologs.

## Materials and Methods

**Preparation of NphT7, NphT7 (C115A), and NphT7 (H156A).** Using previously prepared pCLC7 (accession numbers AB037666 and AB540131) (11) including *nphT7* as a template, a PCR amplification for ligation into the *E. coli* expression vector pHIS8 (28) was carried out with the forward primer 5′-GGGGGATCCACCGACGTCCGATTCCGC-3′ (*Bam*HI site underlined) and the reverse primer 5′-GGGGAATTCCTACCACTCGATCAGGGCG-3′ (*Eco*RI site underlined) to generate pHis_*nphT7*. Using pHis_*nphT7* as a template, site-directed mutagenesis for NphT7 (C115A) and NphT7 (H256A) was performed to construct pHis_*nphT7*_C115A and pHis_*nphT7*_H256A, respectively, following the QuickChange XL (Stratagene) protocol. The primers for mutagenesis were: forward for NphT7 (C115A), 5′-GACGTCAACGCGGTCGCGTCCGGCACCGTGTTC-3′; reverse for NphT7 (C115A), 5′-GAACACGGTGCCGGACGCGACCGCGTTGACGTC-3′; forward for NphT7 (H256A), 5′-AGCCACTTCGTGCCGGCGCAGGCCAACGGTGTC-3′; and reverse for NphT7 (H256A), 5′-GACACCGTTGGCCTGCGCCGGCACGAAGTGGCT-3′. For full experimental details of protein expression and purification of NphT7, NphT7 (C115A), and NphT7 (H256A), see *SI Text*.

**Assay for KAS III Activity.** The assay contains 4 μg NphT7, 84.7 μM [2-$^{14}$C]acetyl-CoA, and 13.5 μM malonyl-ACP in 10 μL reaction buffer (100 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol). As a positive control, 0.3 μg FabH (*S. coelicolor* KAS III) was added instead of NphT7. As a negative control, the reaction buffer was added instead of NphT7. The reaction was carried out at 25 °C for 2 h and terminated by boiling with 1× loading dye for 5 min. Proteins were separated by SDS-18% PAGE. After gel drying, the gel was exposed to an imaging plate (Fujifilm) for 18 h and the radiolabeled ACP derivatives were visualized on an FLA-3000 imaging system (Fujifilm). For full experimental details of the preparation of malonyl-ACP (29), see *SI Text* and Table S2.

**Assay for Acetoacetyl-CoA Synthesizing Activity.** Under bisubstrate conditions, acetoacetyl-CoA synthesizing activity was assayed in 100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM acetyl-CoA, and 1 mM malonyl-CoA in a total volume of 50 μL. After the reaction mixture devoid of enzyme was incubated at 30 °C for 1 min, the reaction was started by adding 15 μg NphT7, 50 μg NphT7 (C115A), or 70 μg NphT7 (H256A). The reactions, in the case of NphT7, were carried out at 30 °C for 10, 20, 30, 60, and 120 sec. In the case of NphT7 (C115A), the reaction was carried out for 10 min. For NphT7 (H256A), the reactions were carried out for 30, 60, 120, 180, and 300 sec. Under monosubstrate conditions, acetoacetyl-CoA synthesizing activity was assayed in 100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM malonyl-CoA, and 30 μg NphT7 in a total volume of 50 μL. The reactions under monosubstrate conditions were carried out at 30 °C for 30, 60, 90, and 120 sec. The reactions were all terminated by adding 5 μL of 100% saturated trichloroacetate. The sample was immediately diluted 10-fold with 1 M MES buffer (pH 6.0) and centrifuged for 10 min at 15,000 x *g* and 4 °C to remove precipitated protein. A total of 10 μL of the resulting supernatant was analyzed on an HPLC system equipped with an MD-2010 Plus photodiode array (JASCO) with CAPCELL PAK C18 MGII (4.6$\phi$ × 250 mm, 5 μm, column temperature 25 °C, Shiseido) under the following conditions: mobile phase ($A$ = methanol, $B$ = 5 mM di-*n*-butylammonium acetate +5 mM ammonium acetate, pH 6.5), 20% to 90% A over 15 min, 90% to 20% A over 1 min, 20% A for 20 min; flow rate, 1.0 mL/ min. The concentrations of the remaining substrates and the generated products in the reaction mixtures were determined from each peak area in the chromatograms detected at 259 nm. The specific activities of NphT7

and NphT7 (H256A) were calculated from respective consumption of acetyl-CoA. Reaction products arising from CoA derivatives were collected and identified using a Thermo Fisher Accela HPLC system linked to a Thermo Fisher Scientific LTQ Orbitrap XL™ mass spectrometer. The collected products were loaded onto and separated by a CAPCELL PAK C18 MGII column (2.0$\phi \times$ 150 mm, 5 μm, column temperature 40 °C; Shiseido) under the conditions described above, except for a flow rate of 0.2 mL/min. MS analysis was performed by electrospray ionization in negative ion mode (30). Under the HPLC-MS condition, the authentic standards malonyl-CoA, CoA, acetoacetyl-CoA, and acetyl-CoA were detected at 9.2 min, 9.6 min, 9.8 min, and 10.0 min, respectively.

**Steady-State Kinetic Parameters.** A spectrophotometric NphT7 assay based on the formation of a $Mg^{2+}$-acetoacetyl-CoA enolate complex was employed for steady-state kinetic studies of NphT7 (31). Under bisubstrate conditions, acetoacetyl-CoA synthesizing activity was assayed in 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM dithiothreitol, acetyl-CoA, and malonyl-CoA in a total volume of 1 mL. When the concentration of malonyl-CoA was fixed at 100 μM, the concentrations of acetyl-CoA were varied (10, 20, 50, 100, and 500 μM). With a fixed concentration of 200 μM acetyl-CoA, the concentrations of malonyl-CoA were varied (10, 20, 30, 50, and 100 μM). Under monosubstrate conditions, acetoacetyl-CoA synthesizing activity was assayed in 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM dithiothreitol, and malonyl-CoA in a total volume of 1 mL. The concentrations of malonyl-CoA were varied (20, 50, 100, 200, 500, 1,000, and 2,000 μM). After the reaction mixture devoid of enzyme was incubated at 30 °C for 1 min, the reaction was started by adding 5 μg of NphT7. NphT7-dependent formation of the enolate complex was monitored at 303 nm in a UV-1600PC spectrophotometer (Shimadzu) equipped with a CPS-240A cell holder (Shimadzu) adjusted to 30 °C. Initial velocities were determined from the slope of a plot of the enolate complex formation vs. incubation time. The molar extinction coefficient ($\varepsilon$) of the enolate complex at 303 nm was 8,300. Steady-state kinetic parameters were calculated using the SigmaPlot 10.0 software and Enzyme Kinetics Module 1.3 (Systat Software).

**Mevalonate Production.** A supernatant (1 mL) of *S. albus* transformant culture was adjusted to pH 2 by adding phosphoric acid and then extracted twice with 2 mL ethyl acetate. After drying over $Na_2SO_4$, the organic layer was evaporated in vacuo and redissolved in 100 μL of 2-propanol. Mevalonate in the 2-propanol solution was analyzed on an HPLC system (JASCO) equipped with a refractive index detector RI-74 (Shodex) with AQUASIL C18 (4.0$\phi \times$ 150 mm, 5 μm, column temperature 40 °C; Thermo Fisher) employing an isocratic elution of 0.1% acetate and 0.1% triethyl amine in water at a flow rate of 1.0 mL/min. For full experimental details of plasmid construction and transformant culture, see *SI Text*.

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