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Metabolic Coupling of Dehydration and Decarboxylation in the Curacin A Pathway: Functional Identification of a Mechanistically Diverse Enzyme Pair

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Curacin A (**1**, Figure 1), a marine cyanobacterial metabolite from *Lyngbya majuscula*, is a mixed polyketide−nonribosomal peptide natural product with potent antiproliferative and cytotoxic activity against colon, renal, and breast-cancer-derived cell lines.[1] Because of its unusual structure, which includes a cyclopropane group, thiazoline moiety, *cis*-alkenyl group, and terminal double bond, we were motivated to characterize the unique enzymes responsible for its assembly. Recently, we identified and characterized a gene cluster (*cur*) from *Lyngbya majuscula* L19, whose architecture and domain organization correlate directly with that expected for curacin A biosynthesis.[2] Within the *cur* gene cluster that spans ∼65 kbp is an ∼8 kbp region (CurA−CurF) with striking similarity (80−95% amino acid identity) to a comparably sized section of the jamaicamide **2** (*jam*) biosynthetic pathway (JamE−JamJ).[3] The genes from this region are translated into a set of enzymes containing (1) a predicted α-ketoglutarate-dependent halogenase,[4] (2) a set of three tandem ACPs, and (3) a 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase (HCS)-like gene cassette that collectively are likely involved in the introduction of the cyclopropane group in curacin A and the vinyl chloride substituent in jamaicamide.[2,3] The HCS-like gene cassette encodes five biosynthetic enzymes: an acyl carrier protein (CurB), a putative ketosynthase (CurC), an HMG-CoA synthase (CurD), and two putative enoyl-CoA hydratases (CurE [ECH_1] and CurF [ECH_2]). This novel cassette has recently been identified in several additional gene clusters, including the *jam* pathway from *L. majuscula*, the mupirocin **3** (*Mup*) pathway from *Pseudomonas fluorescens*, and the "PksX" pathway from *Bacillus subtilis*[2−5] and is proposed to catalyze addition of C-2 from acetate onto the polyketide chain to generate a pendant functional group (Figure 1).

In this paper, we provide evidence for the specific function of the CurE/CurF ECH_1−ECH_2 enzyme pair (Figure 1) in curacin A biosynthesis and demonstrate that they catalyze the successive dehydration and decarboxylation of (*S*)-HMG-ACP **5** to generate a 3-methylcrotonyl-ACP **7** intermediate for subsequent formation of the cyclopropane ring in **8** (Scheme 1). Significantly, it is now evident from bioinformatic analysis that the ECH_1−ECH_2 enzyme pair exists widely in different microbial species and is not necessarily limited to HCS-like gene cassettes (see Supporting Information). Therefore, we believe that the ECH_1−ECH_2 of the curacin A system represents an excellent example of chemistry-directed enzyme evolution, giving rise to "mechanistic diversity"[6] within a single protein family.

In the *cur* gene cluster, ECH_1 is encoded by *curE*, and the ECH_2 gene is embedded in *curF* as the first domain of the CurF hybrid PKS/NRPS bimodular polypeptide (Figure 1). Although the sequence identity between *cur* ECH_1 and ECH_2 is only 17%, they are predicted to belong to the crotonase superfamily and contain two consensus sequences (see Supporting Information) essential for the "oxyanion hole" to stabilize enolate anions.[7] In contrast, the sequence identities in the ECH_1 and ECH_2 groups compared to that of various biosynthetic systems (see Supporting Information) are generally above 40%, which suggests that ECH_1 and ECH_2 have different functional roles. To identify their precise function, we reasoned that the HCS motif would follow a path involving initial assembly of HMG-ACP via the reaction of C-2 of acetyl-ACP with acetoacetyl-ACP **4** (catalyzed by HMG-CoA synthase (CurD)). The collinearity of the Cur biosynthetic pathway suggested that the ECH_1−ECH_2 pair would operate in late stage construction of the presumed acyl-ACP precursor for cyclopropane ring formation.

To probe the function of ECH_1, *curE* was cloned, overexpressed, and purified as a 30.5 kDa N-His-tagged fusion protein. Next, we sought to excise a functional form of ECH_2 from the amino terminus of the CurF multifunctional enzyme. This was accomplished by choosing an optimized cutting site in the linker region of ECH_2 (259 amino acids) to yield a 29.5 kDa C-His-tagged fusion protein. Additionally, *curB* was cloned, overexpressed, and purified in the apo-CurB (acyl carrier protein) form as an 11 kDa N-His-tagged fusion protein. ECH_1, ECH_2, and apo-CurB were isolated in soluble form from *E. coli* and purified by Ni-NTA agarose resin. The apo-CurB protein (75 μM) was subsequently loaded using HMG-CoA

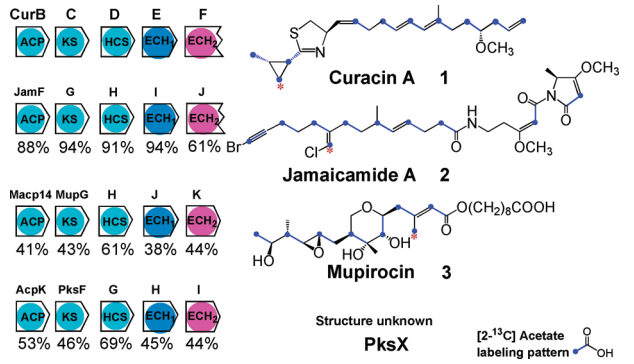
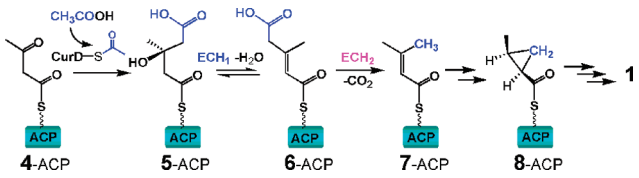


Figure 1. HMG-CoA synthase containing gene cassettes are proposed to introduce a pendant carbon (indicated by red asterisk) from C-2 of acetate to the polyketide chains. Amino acid identities of enzymes are shown (%), and acetate labeling patterns are highlighted in blue.

Scheme 1



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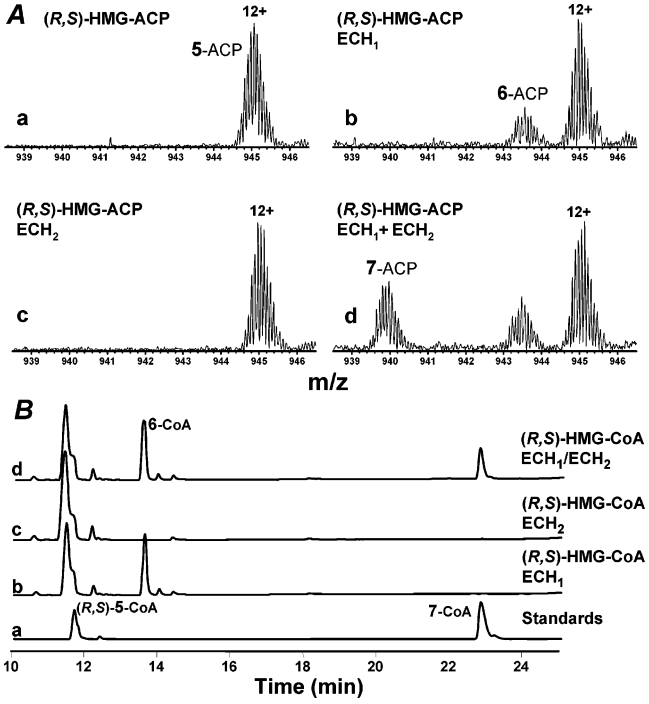

Figure 2. ECH_1 and ECH_2 assays for the substrates in CoA and ACP forms. (A) FTICR spectra, 50 μM (*R*,*S*)-HMG-ACP, 2 μM ECH_1, ECH_2, or both, at 37 °C for 3 h. Experimental most abundant mass: **5**-ACP, 11325.8; **6**-ACP, 11307.8; **7**-ACP, 11264.8. (B) UV 275 nm traces of (a) standards: **5**−CoA and **7**−CoA (MC−CoA), 0.5 mM (*R*,*S*)-**5**−CoA treated with (b) 2 μM ECH_1, (c) 2 μM ECH_2, (d) 2 μM ECH_1 and ECH_2 at 37 °C for 3 h. The CoA peak shoulders and the following minor peaks are possibly due to CoA aggregation.

via in vitro phosphopantetheinylation through in situ incubation with (*R*,*S*)-HMG-CoA (500 μM) and Sfp (4 μM).[8] The HMG-holo-CurB was dialyzed against the ECH assay buffer to adjust pH and remove excess HMG-CoA.

The in vitro activities of ECH_1 and ECH_2 were investigated by incubating ECH_1, ECH_2, or both (2 μM each), with (*R*,*S*)-HMG-holo-CurB (50 μM) in a series of buffers at 37 °C. ESI-FTMS (Apex-Q instrument, Bruker Daltonics) was applied to detect mass change of the acyl group covalently linked to the holo-CurB. We found that, in the presence of ECH_2 alone (Figure 2A, c), no new reaction products were observed. In contrast, in the presence of ECH_1, a peak corresponding to 18 Da loss in molecular mass occurred (Figure 2A, b), and when both ECH_1 and ECH_2 were employed, two peaks corresponding to 18 and 62 Da loss in molecular mass were observed (Figure 2A, d). These results suggest that ECH_1 catalyzes dehydration of HMG-ACP **5** to 3-methylglutaconyl-ACP **6** (Scheme 1), and ECH_2 catalyzes subsequent decarboxylation to **7**.

Next, we also demonstrated that ECH_1 and ECH_2 are able to accept and catalyze dehydration and decarboxylation of (*R*,*S*)-HMG-CoA (Figure 2B), which suggests that the two enzymes recognize the phosphopantetheine arm from CoA as well as holo-ACP. Importantly, this finding facilitated structural identification of the dehydration and decarboxylation products from the reaction. Specifically, 0.5 mM (*R*,*S*)-HMG-CoA was incubated with the ECH_1, ECH_2, or both (2 μM each), in 30 mM bis-Tris buffer, pH 6.5 at 37 °C for 3 h, and the reaction mixtures were analyzed by HPLC equipped with a photodiode array detector and an ESI-LTQ mass spectrometer (ThermoFinnigan). UV spectral analysis of the HMG-CoA substrate, and the following dehydration and decarboxylation steps, revealed a significant absorption increase at 260 nm for both dehydration and decarboxylation products (see Supporting Information). This increase is consistent with an α,β C=C bond formation in an acyl-CoA thioester.[9] In addition, MS/MS analysis confirmed that the dehydration product was **6**−CoA, and we did not pursue the further clarification of the C=C configuration. To determine regiochemistry of the double bond in the decarboxylation product, we synthesized 3-methyl-3-butenoyl-CoA and used commercially available **7**−CoA (Sigma) as authentic standards. HPLC co-injection showed that the decarboxylation product is **7**−CoA (see Supporting Information).

Finally, we investigated the substrate preference of ECH_1 by comparing the conversion ratio of (*R*,*S*)-HMG-CoA and (*S*)-HMG-CoA, which was generated by HMG-CoA reductase.[10] On the basis of HPLC traces, the conversion ratio of (*S*)-HMG-CoA is 2-fold higher than that of (*R*,*S*)-HMG-CoA (see Supporting Information), which indicates that (*S*)-HMG is the natural ECH_1 substrate.[11]

In summary, CurE/CurF ECH_1−ECH_2 polypeptides from the curacin A biosynthetic pathway were functionally identified as a mechanistically diverse enzyme pair. We demonstrated that CurE ECH_1 catalyzes dehydration of (*S*)-HMG-ACP **5** to form 3-methylglutaconyl-ACP **6**, and CurF ECH_2 catalyzes decarboxylation of **6** to generate 3-methylcrotonyl-ACP **7**, the presumed precursor for cyclopropyl-ACP **8** formation in curacin A. The detailed steps leading from 3-methylcrotonyl-ACP to the cyclopropane ring are the subject of ongoing studies in our laboratory. It is noteworthy that, to date, only two members of the crotonase enzyme superfamily, methylmalonyl CoA decarboxylase (YgfG)[12] and CarB,[13] were reported to catalyze loss of carbon dioxide. Thus, identification of the reaction catalyzed by CurF ECH_2 provides a new example of this novel biotin-independent decarboxylase in secondary metabolism.

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Supporting Information Available: Experimental details and supplementary data. This material is available free of charge via the Internet at http://pubs.acs.org.

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