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An Alternative Isovaleryl CoA Biosynthetic Pathway Involving a Previously Unknown 3-Methylglutaconyl CoA Decarboxylase**

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Myxobacteria are swarming bacteria characterized by their social behavior and ability to undergo a complex developmental life cycle.[1] They prey on other bacteria or fungi for food. Under starvation conditions, the vegetative cells aggregate to form multicellular fruiting bodies. To support such a remarkable lifestyle, myxobacteria exhibit an enormous metabolic potential, which is mirrored by discoveries of new compounds and unusual biochemical pathways in recent years.[2] One example is the biosynthesis of isovaleryl coenzyme A (IV-CoA) for which an additional alternative pathway has been recently suggested.[3] IV-CoA is generally derived from leucine degradation by transamination and subsequent oxidative decarboxylation by the branched-chain α-keto acid dehydrogenase complex (Bkd).[4] The Bkd complex is also involved in degradation pathways of valine and isoleucine to produce isobutyryl CoA and 2-methylbutyryl CoA. These precursors are the starter units of branched-chain (or iso-) fatty acids (FAs), which are of particular importance in myxobacteria. IV-CoA-derived iso-odd FAs are the majority of FAs, as shown in the model myxobacterium, *Myxococcus xanthus*.[5,6] These FAs maintain the membrane fluidity for thermal adaptation, and play key roles in signaling during myxobacterial developmental differentiation.[7–11] Besides its role in iso-odd FA biogenesis, IV-CoA is the precursor of a number of myxobacterial secondary metabolites, such as myxothiazol[12,13] and aurafuron.[14] During our study of myxothiazol biosynthesis, we analyzed the *bkd*− mutants of *M. xanthus* and *Stigmatella aurantiaca* and showed that iso-FAs and myxothiazol were still produced in these mutants, albeit at lower amounts,[3] which provided the first evidence for the existence of an alternative pathway to IV-CoA. The *bkd*− mutants could incorporate labeled acetate, but not leucine, into IV-CoA-derived compounds and exhibited a labeling pattern similar to that found in mevalonate-dependent isoprenoids, thus establishing a likely link between the hypothetical IV-CoA and the mevalonate pathway.[3,15] 3-Hydroxy-3-methylglutaryl CoA (HMG-CoA) was proposed to be the branching point, from which reverse reactions of leucine degradation would happen and lead to IV-CoA (Figure 1). Feeding studies and analysis of a double-mutant *bkd*−/*mvaS*− (*mvaS* encodes HMG-CoA synthase) demonstrated the involvement of HMG-CoA and the intermediacy of 3,3-dimethylacrylyl CoA (DMA-CoA; as shown by incorporation of labeled 3,3-dimethylacrylic acid, which needs to undergo activation to the CoA ester) in the alternative pathway.[7,15] Importantly, this pathway was found to be highly active in the *bkd*− mutant and during fruiting body formation when leucine-derived IV-CoA is limited,[7] reflecting essential roles of IV-CoA-derived compounds in the life cycle of myxobacteria.

To identify genes involved in the alternative pathway, we compared global gene-expression patterns in vegetative cells of the wild-type and the *bkd*− mutant of *M. xanthus* DK1622.[16] The *mvaS* gene was found to be part of a five-gene operon (MXAN_4263 to MXAN_4267) that was highly up-regulated in the *bkd*− mutant. Gene inactivation and complementation experiments confirmed their involvement in the alternative route to IV-CoA.[16] Thus the operon is here renamed to the *aib* (alternative IV-CoA biosynthesis) operon. In addition to the HMG-CoA synthase, the operon encodes proteins with significant similarity to a TetR-like transcriptional regulator (AibR), a glutaconate CoA transferase (Gct) subunit A and B (AibA/B), and a dehydrogenase (AibC). Furthermore, we identified genes employed in leucine catabolism using homologues from pseudomonads as baits and functionally analyzed them in vivo. Among those, *liuC* (MXAN_3757), encoding a 3-methylglutaconyl CoA (MG-CoA) hydratase, was shown to participate in the alternative pathway.[16] Taken together, this pathway was proposed to proceed by LiuC-catalyzed dehydration of HMG-CoA, followed by decarboxylation and subsequent reduction to form IV-CoA (Figure 1). The latter two steps would be catalyzed by a heterodimer composed of AibA/B and AibC, respectively. Herein, we confirmed the proposed function of each enzyme, leading to the complete reconstitution of the alternative biosynthesis of IV-CoA in vitro. Most importantly, we characterized AibA/B as a novel MG-CoA decarboxylase, which apparently evolved from CoA transferases, which was how AibA/B were annotated prior to this study.

To characterize the MG-CoA decarboxylase activity, we synthesized MG-CoA from (*E*,*Z*)-3-methylglutaconate using recombinant Gct from *Acidaminococcus fermentans*.[17] Only the (*E*)-isomer of MG-CoA, the natural compound in cells, was prepared because of the substrate specificity of bacterial Gcts.[18] As sequence analysis revealed that AibA and AibB

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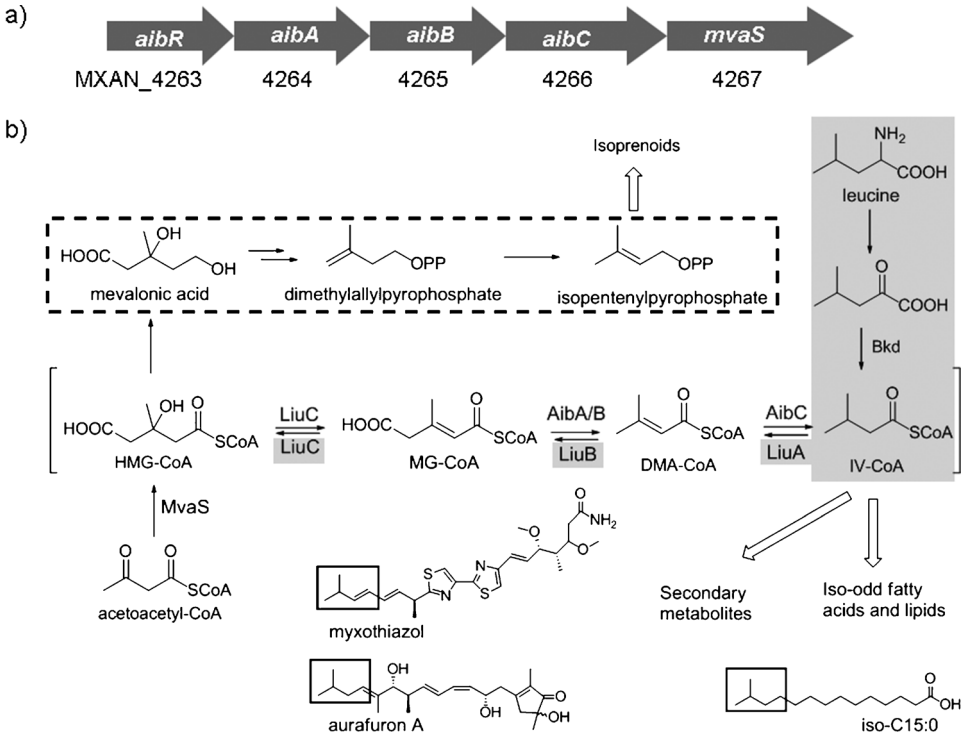

Figure 1. a) Gene organization of the *aib* operon in *M. xanthus*. b) Metabolic pathway of alternative IV-CoA biosynthesis (between parentheses), leucine degradation (gray), and mevalonate-dependent isoprenoid biosynthesis (in box with dashed lines) in myxobacteria. Structural motifs derived from isovaleryl CoA are boxed.

are related to two subunits of CoA transferases,[16] respectively, we assumed it highly likely that both enzymes act as complex. Both proteins were co-expressed with N-terminal His_6-tags in *Escherichia coli* and co-purified by nickel affinity chromatography. AibA and B eluted together in a near 1 to 1 ratio starting from 200 mM imidazole (Supporting information, Figure S1). The complex displayed a molecular mass of 57.9 Da as determined by size-exclusion chromatography, corresponding to the formation of a heterodimer (AibA 30 kDa, AibB 28 kDa). Activity assay of AibA/B was performed with MG-CoA at 37 °C for 3 h. Subsequent high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS) analysis revealed production of a new compound in the reaction with identical chromatographic properties to authentic DMA-CoA (r.t. = 17.8 min, m/z $[M+H]^+ = 850$). High-resolution MS (HR-MS) determination further confirmed its identity as DMA-CoA, the decarboxylated product of MG-CoA (calculated monoisotopic mass for $C_{26}H_{43}N_7O_{17}P_3S$ m/z $[M+H]^+ = 850.16490$, measured mass 850.16336, $\Delta = -1.8$ ppm). DMA-CoA could not be detected in the control sample with heat-inactivated AibA/B (Figure 2). When HMG-CoA was used as substrate, no conversion was observed. To clarify if the association of AibA and AibB is necessary for MG-CoA decarboxylase activity, each enzyme was expressed and purified separately. While recombinant AibA was highly soluble, AibB was produced mainly as inclusion bodies that resulted in very low yields (Supporting information, Figure S1). As expected, AibA or AibB alone showed no activity towards MG-CoA. In contrast, addition of both enzymes at a molar ratio of 1:1 restored DMA-CoA production, albeit with very low activity (Supporting information, Figure S2). This is probably due to the sub-optimal formation of the heterodimer if AibA/B are not co-expressed. Nevertheless, these results unambiguously confirm AibA and AibB as two subunits required to form MG-CoA decarboxylase.

The closest structural homologue of AibA/AibB is Gct from *A. fermentans*,[19] the catalytic residue of which is identified to be Glu54 on the B subunit, which is well conserved in other bacterial Gcts.[17,20] Sequence comparison of AibB with the B subunits of Gcts revealed the replacement of Glu54 by a Cys in AibB (Cys56 according to AibB numbering; Figure 3). Therefore, we aimed to answer the question whether Cys56 plays a role in the catalysis and whether we can change AibA/B to a functional Gct. We performed site-directed mutagenesis of *aibB* to generate expression constructs for mutants AibB_C56E and AibB_C56D. MG-CoA decarboxylase activities of the resulting complex AibA/B_C56E and AibA/B_C56D were reduced to 13 % and 1.2 %, respectively, compared to that of the wild-type enzyme. To characterize CoA transferase activity, assays were performed using (*E*,*Z*)-methylglutaconate (20 mM) and acetyl CoA (1 mM) as substrates. Neither the wild-type nor the two mutant enzymes were able to produce MG-CoA. Curiously, significant amounts of CoA were detected in reactions with mutant proteins, indicating that they have an acetyl CoA hydrolase activity. To confirm this, acetyl CoA (1 mM) was incubated with the WT or mutant AibA/B complexes for 3 h. About 60 % and 30 % of acetyl CoA were converted into CoA by the C56D and C56E mutants, respectively, while the WT led to no CoA production (Supporting information, Figure S3). These experiments demonstrate the importance of Cys56 in AibA/AibB catalysis.

Recombinant LiuC (29 kDa) was incubated with HMG-CoA in buffers with pH ranging from 6–8. In all cases, tiny amounts of MG-CoA were produced and its identity was confirmed by using synthetic MG-CoA as reference (Figure 2). HR-MS of the product was further determined and corresponded well to MG-CoA (calculated monoisotopic mass for $C_{27}H_{43}N_7O_{19}P_3S$ m/z $[M+H]^+ = 894.15473$, measured mass 894.15351, $\Delta = -1.4$ ppm). Addition of the AibA/B complex to LiuC reaction mixture led to the formation of DMA-CoA. This unambiguously proves that LiuC has HMG-

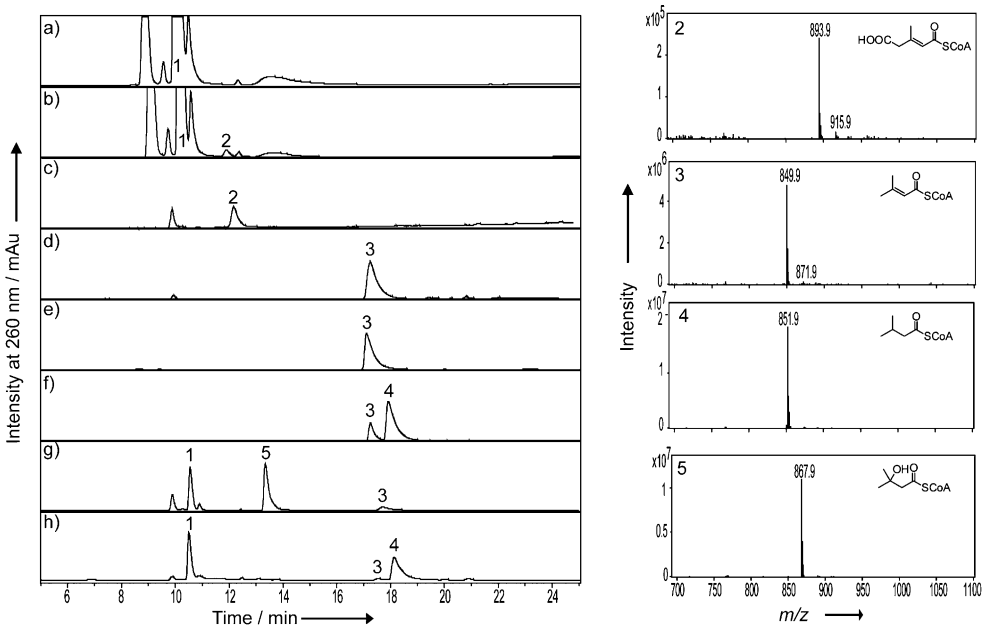

Figure 2. Characterization of Aib enzymes in vitro. Left: HPLC traces of enzymes assays monitored by UV at 260 nm. a) HMG-CoA only; b) HMG-CoA with LiuC; c) MG-CoA with inactivated AibA/AibB; d) MG-CoA with AibA/AibB; e) DMA-CoA with inactivated AibC; f) DMG-CoA with AibC; g) HMG-CoA with LiuC and AibA/AibB; h) HMG-CoA with LiuC, AibA/AibB, and AibC. Chromatographic intensities of reaction (a) and (b) are increased tenfold. 1 HMG-CoA, 2 MG-CoA, 3 DMA-CoA, 4 IV-CoA, 5 3-methylhydroxybutyryl-CoA. Right: Mass spectra of enzymatic products.

CoA dehydration activity. Surprisingly, in addition to DMA-CoA, a new peak having a mass corresponding to 3-methylhydroxybutyryl CoA was revealed by HPLC and HR-MS analysis (calculated monoisotopic mass for $C_{26}H_{45}N_7O_{18}P_3S$ m/z $[M+H]^+ = 868.17546$, measured mass 868.17476, $\Delta = -0.8$ ppm). This is most likely due to the hydration of DMA-CoA catalyzed by LiuC. We next investigated the substrate specificity of LiuC. LiuC hydrated efficiently DMA-CoA and crotonyl CoA (Supporting information, Figure S4a). This finding is consistent with previous studies on various MG-CoA hydratases.[21–24] LiuC conserves two glutamate residues that are important in catalysis, as shown for the human AUH enzyme (Gly144 and 164 according to human AUH numbering, Figure S4b),[23] and therefore it is able to accept substrates that lack the γ-carboxylate group.

The last step of the alternative pathway is proposed to involve the reduction of DMA-CoA. Recombinant AibC (38 kDa) was assayed with DMA-CoA in the presence of NADH. A new product with identical properties as authentic IV-CoA was produced (r.t. 18.2 min, HR-MS: calculated monoisotopic mass for $C_{26}H_{45}N_7O_{17}P_3S$ m/z $[M+H]^+ = 852.18055$, measured mass 852.17877, $\Delta = -2.1$ ppm; Figure 2). Thus, AibC is characterized as an IV-CoA dehydrogenase. Presence of EDTA (5 mM) did not affect AibC activity, indicating that no metal cofactor is required for AibC. To reconstitute the complete alternative biosynthesis, HMG-CoA (1 mM) was incubated with equal amounts of LiuC, AibA/B, and AibC in the presence of NADH. As expected, this yielded IV-CoA as a major product (Figure 2).

In summary, we performed characterization in vitro and provided ultimate evidence for the proposed novel pathway. Intriguingly, this route involves reverse steps of leucine degradation. Genes encoding MvaS, AibA-C, and also

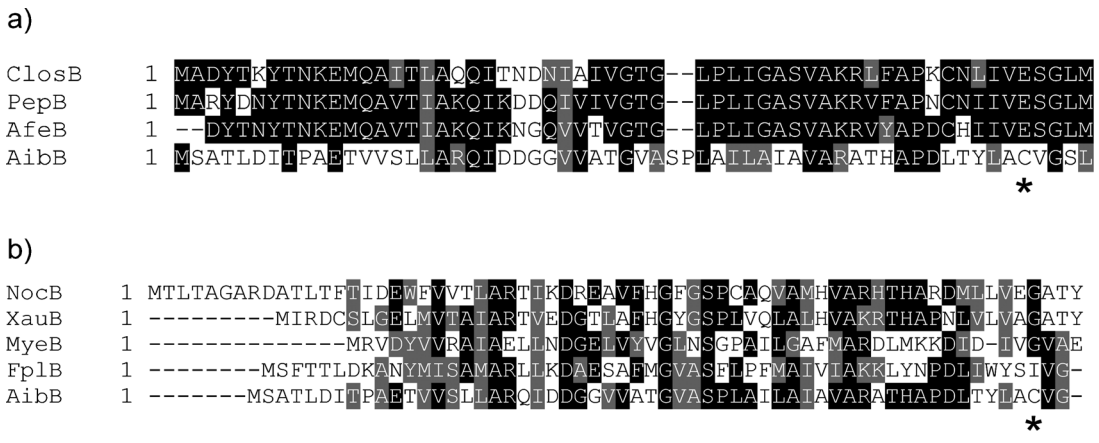

Figure 3. a) Sequence alignment of AibB with subunit B of other Gcts. AfeB: Gct from *A. fermentans* subunit B; ClosB: CBK77199.1, from *Clostridium* cf. *sacharolyticum*; PepB: EFI41745.1, from *Peptoniphilus* sp. ⋆ indicates the active-site Glu. b) Sequence alignment of AibB with homologous proteins. NocB: YP_919290.1 from *Nocardioides* sp. JS614; XauB: YP_001409505.1 from *Xanthobacter autotrophicus* Py2; MyeB: ZP_09705801.1 from *Metallosphaera yellowstonensis* MK1; FplB: YP_003435597.1 from *Ferroglobus placidus* DSM10642. ⋆ indicates Cys56 in AibB.

a putative pathway-specific regulator (AibR) are organized in an operon, which represents an advantage in coordinated regulation under leucine-limiting conditions, such as starvation that leads to fruiting body formation. On the other hand, *liuC* is encoded in another gene locus and is thus under different transcriptional controls (for example, *liuC* is not up-regulated in the *bkd*− mutant of *M. xanthus*[16]). Such differential regulation of the *aib* operon and *liuC* may be important to maintain equilibrated partitioning of HMG-CoA between the IV-CoA synthesis and the mevalonate pathway. Moreover, LiuC is an unusual bacterial MG-CoA hydratase and its low activity towards typical natural substrates is probably an intrinsic feature reflecting its importance in regulating two metabolic pathways in parallel. To investigate whether the novel shunt pathway is common in other bacteria, we used *aibA* and *aibB* as baits to search available bacterial genomes. This revealed that the *aib* operon is strictly conserved in myxobacteria, most of which belonging to the suborder Cystobacterineae (Supporting Information, Table S1). The alternative synthesis of IV-CoA is therefore likely to be unique for a set of myxobacteria, a feature related to their specific lifestyle. However, we cannot exclude the possibility of *aib* genes dispersed at various gene loci in other bacteria.

The key step in the shunt pathway is the conversion of MG-CoA into DMA-CoA catalyzed by an unprecedented MG-CoA decarboxylase (AibA/B). AibA/B functionally resembles the ECH2 domain of CurF involved in the biosynthesis of the cyanobacterial metabolite curacin A.[25] CurF ECH2 belongs to the crotonase superfamily and catalyzes decarboxylation of an acyl carrier protein (ACP) tethered 3-methylglutaconyl group to DMA-ACP. Structurally, AibA/B most likely possess similar folds as CoA transferases, although they exhibit different oligomeric states. AibA/B is a heterodimer, whereas functional Gct is a tetramer of the heterodimer as shown by Gct from *A. fermentans*.[19] Mechanistically, AibA/B would share similarity with CurF ECH2[26] and related crotonases that involve a thioester enolate anion intermediate stabilized by two backbone amide groups, forming an oxyanion hole. Such hydrogen-bonding residues have also been identified in the structure of Gct from *A. fermentans*.[19] The active site Cys56 in AibB may function as the proton donor to the C-4 position of the decarboxylated product (Supporting information, Figure S5). How AibA/B has exactly adapted a CoA transferase structure to catalyze CoA decarboxylation is an intriguing question and warrants further work of structural analysis. Gcts employ a catalytic glutamate to form alternately enzyme-bound anhydrides and thioesters during the reaction cycle.[27] Substitution of the corresponding residue in AibB (Cys56) to Glu or Asp severely reduced MG-CoA decarboxylase activity, but led to some acetyl CoA hydrolase activity, which could be attributed to an anhydride mechanism similar to that of Gcts (Supporting information, Figure S6). However, these single substitutions were not sufficient to convert AibA/B into a CoA transferase. This finding suggests that AibA/B most likely underwent active site remodeling to enable decarboxylation while maintaining the overall CoA transferase structure. AibB shows modest homology to other proteins in the database annotated as CoA transferase subunit B (around 30% sequence identity), all of which do not conserve a Glu active site, suggesting that not all of them perform CoA transferase reactions.

This study demonstrates the importance of in-depth biochemical studies, which are urgently required to make use of the enormous potential of available genome sequences, not only to understand the underlying biochemical principles but also to identify novel biocatalysts. In this respect, our findings are also of importance to biofuel research, as the novel pathway to synthesize IV-CoA provides opportunities to produce isobutene, a precursor of renewable fuels and chemicals, from glucose by synthetic biology approaches.[28]

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