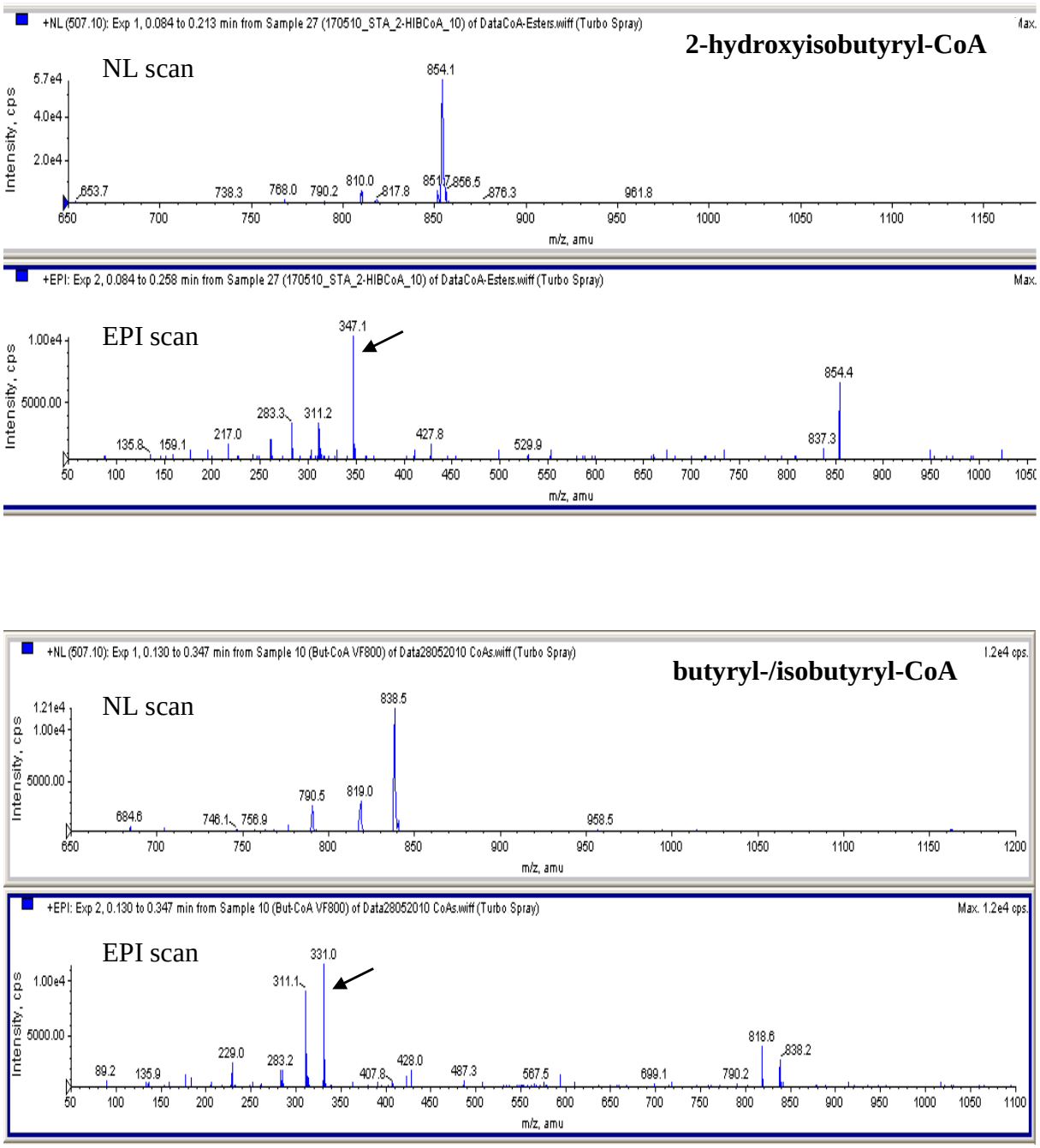
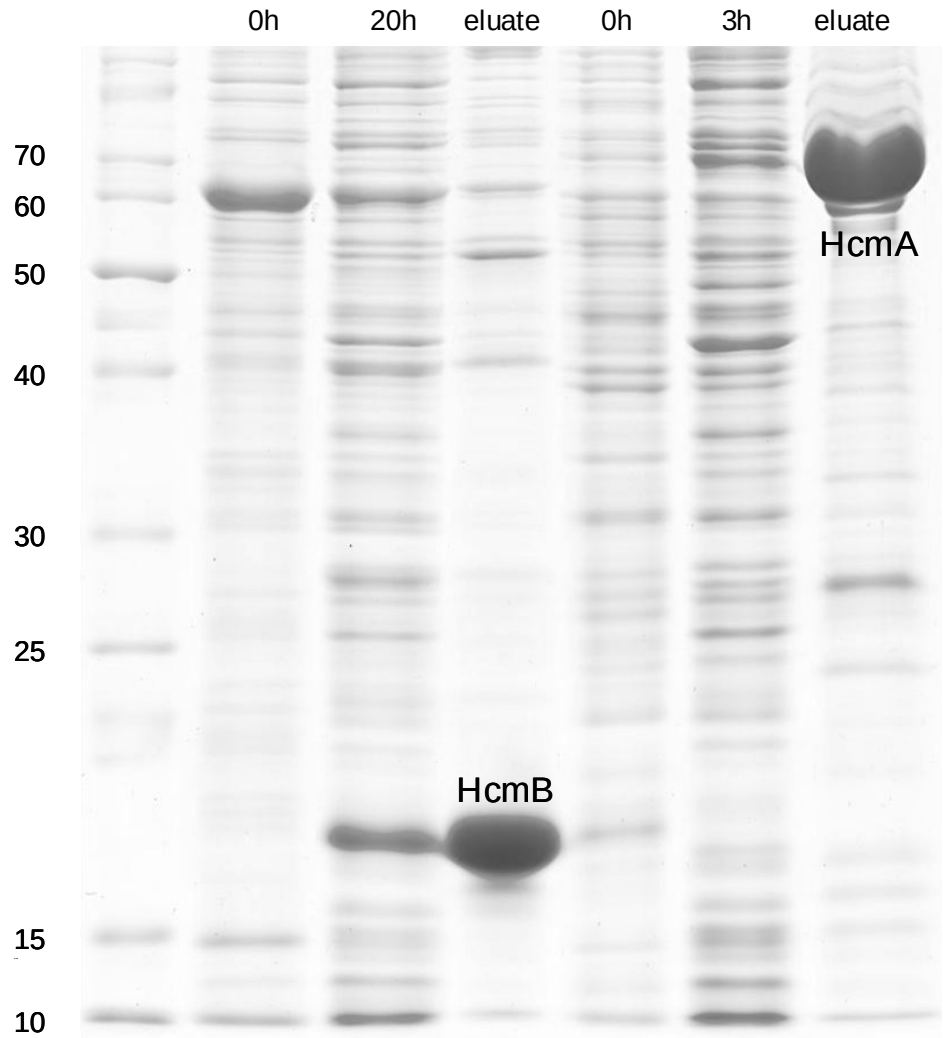
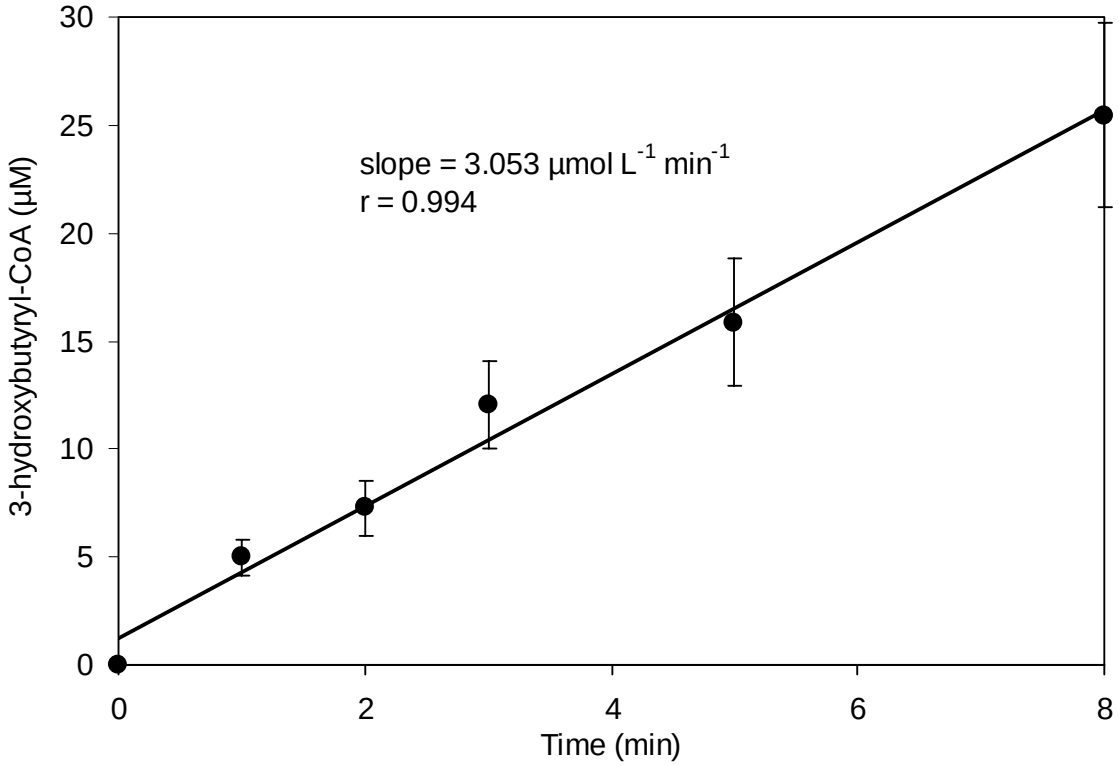


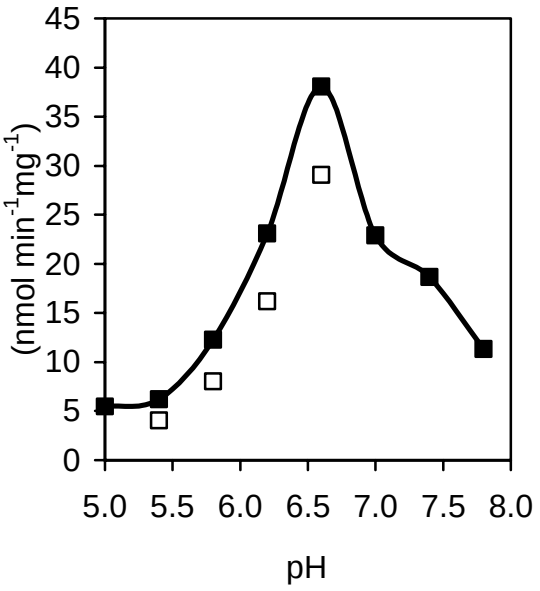
Supplementary Fig. S1. Analysis of synthesized acyl-CoA esters by ESI-MS/MS. In the Neutral Loss Scan mode (NL), masses of ionized acyl-CoA esters were detected, whereas in the Enhanced Product Ion Scan mode (EPI), also masses of smaller fragments were obtained. Characteristic fragments of 2-hydroxyisobutyryl-CoA (853.5 g mol^{-1}) and butyryl-/isobutyryl-CoA (837.5 g mol^{-1}) are the corresponding acyl-pantethein moieties of 347.4 and 331.4 g mol^{-1}, respectively, as indicated by arrows.



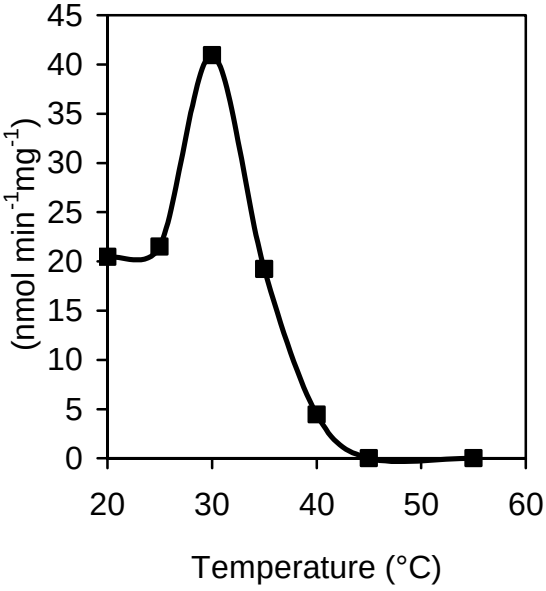
Supplementary Fig. S2. SDS PAGE analysis of crude extracts and eluates after heterologous expression of wild type HcmA and HcmB and their purification by one-step affinity chromatography, respectively (12% SDS-PAGE). Sampling times starting at induction of expression in *E. coli* strains TOP10 (HcmA) and ArcticExpress (DE3) (HcmB). Lanes: 0h, 30 µg *E. coli* cell extract protein before induction; 20 and 3h, 30 µg *E. coli* cell extract protein after 20 and 3 hours of induction, respectively; eluate, 30 µg purified protein. The molecular masses (kDa) of standard proteins are indicated. SDS PAGE analysis of the expression and purification of the HcmA mutants I90Y, I90F and I90V gave similar results (not shown).



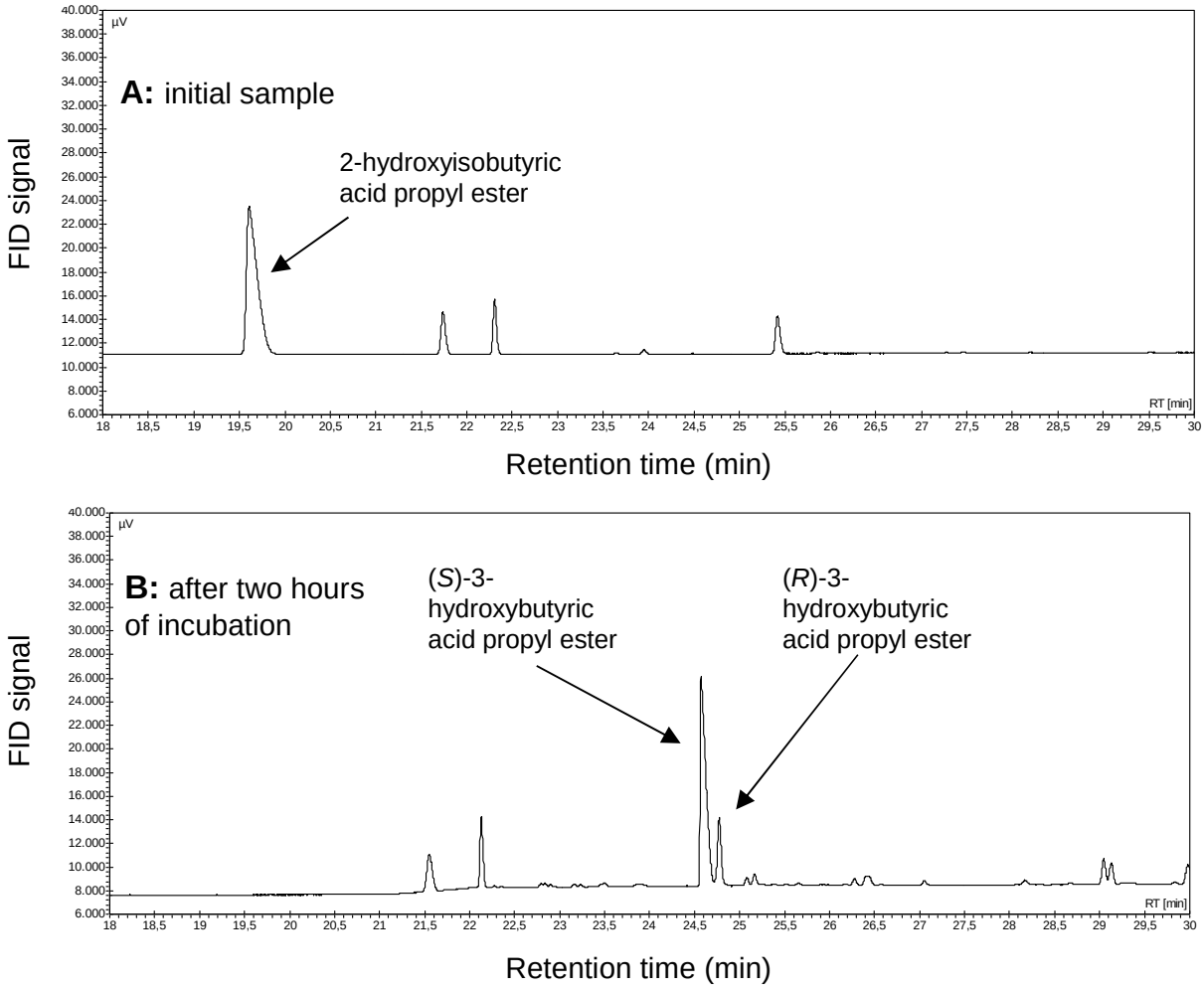
Supplementary Fig. S3. HPLC assay for quantifying HCM activity. Reconstituted wild type HcmAB (1.4 µM) was incubated with 250 µM 2-hydroxyisobutyryl-CoA at pH 6.6 and 30°C. The shown increase in 3-hydroxybutyryl-CoA represents mean values and SD of four replicates.



Supplementary Fig. S4. pH optimum of HCM activity (conversion of 2-hydroxyisobutyryl-CoA) obtained with reconstituted wild type HcmA and HcmB subunits incubated at 30°C in phosphate buffer (solid symbols) or phosphate/acetate buffer (open symbols).



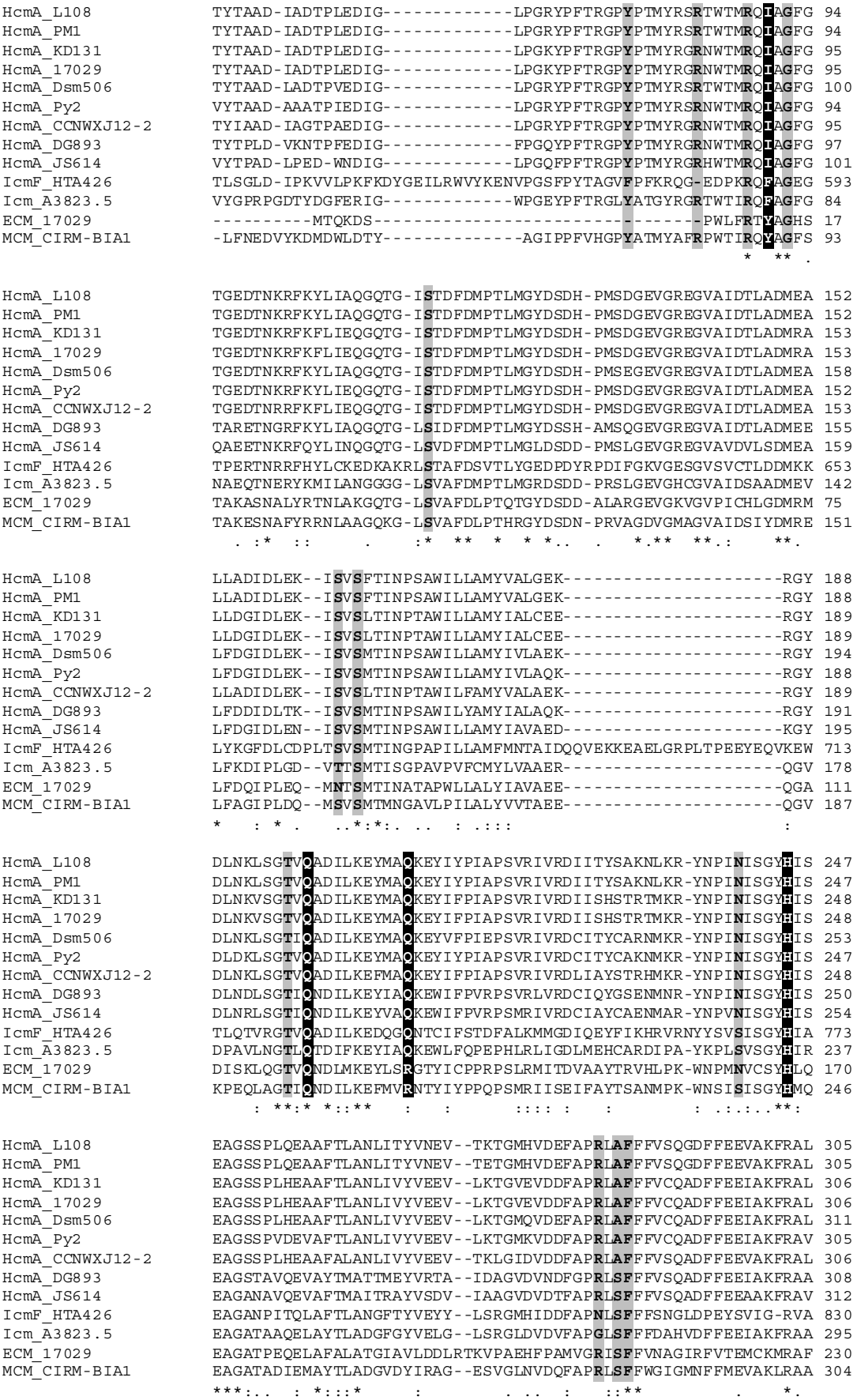
Supplementary Fig. S5. Temperature optimum of HCM activity (conversion of 2-hydroxyisobutyryl-CoA) obtained with reconstituted wild type HcmA and HcmB subunits incubated at pH 6.6.



Supplementary Fig. S6. Analysis of stereospecificity of 2-hydroxyisobutyryl-CoA conversion to 3-hydroxybutyryl-CoA esters by reconstituted wild type HcmA and HcmB. The acyl-CoAs produced were determined as propyl esters by gas chromatography (GC) using a HP 6890 system from Agilent Technologies with a Chirasil-DEX CB column (25 m by 0.25 mm by 0.25 µm) and flame ionization detector (FID). The carrier gas was helium and the injector and detector temperature was 250°C. The following GC oven temperature program was applied. The initial temperature was 35°C increasing to finally 170°C at 3°C min^{-1} and holding this temperature for 5 min. Samples were dried under vacuum and then incubated with 1 mL propanolic HCl (3 M) at 110°C for 45 min. The propyl esters formed were dissolved in 150 µL toluene prior to injection (1 µL, split 1:100).

A: Initial assay sample, immediately after addition of 12 mM 2-hydroxyisobutyryl-CoA.

B: For generating a sufficient amount of 3-hydroxybutyryl-CoA esters, the enzyme was incubated two hours with 12 mM 2-hydroxyisobutyryl-CoA under optimal assay conditions.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HcmA_KD131 TYTAAD-IADTPLEDIG----------LPGKYPFTRGPYPTMYRGRNWTMRQIAGFG 95
HcmA_17029 TYTAAD-IADTPLEDIG----------LPGKYPFTRGPYPTMYRGRNWTMRQIAGFG 95
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HcmA_DG893 TYTPLD-VKNTPFEDIG----------FPGQYPFTRGPYPTMYRGRNWTMRQIAGFG 97
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Icm_A3823.5 VYGPRPGDTYDGFERIG----------WPGEYPFTRGLYATGYRGRTWTIRQFAGFG 84
ECM_17029 ----------MTQKDS----------PWLFRTYAGHS 17
MCM_CIRM-BIA1 -LFNEDVYKDMDWLDTY----------AGIPPFVHGPYATMYAFRPWTIRQYAGFS 93
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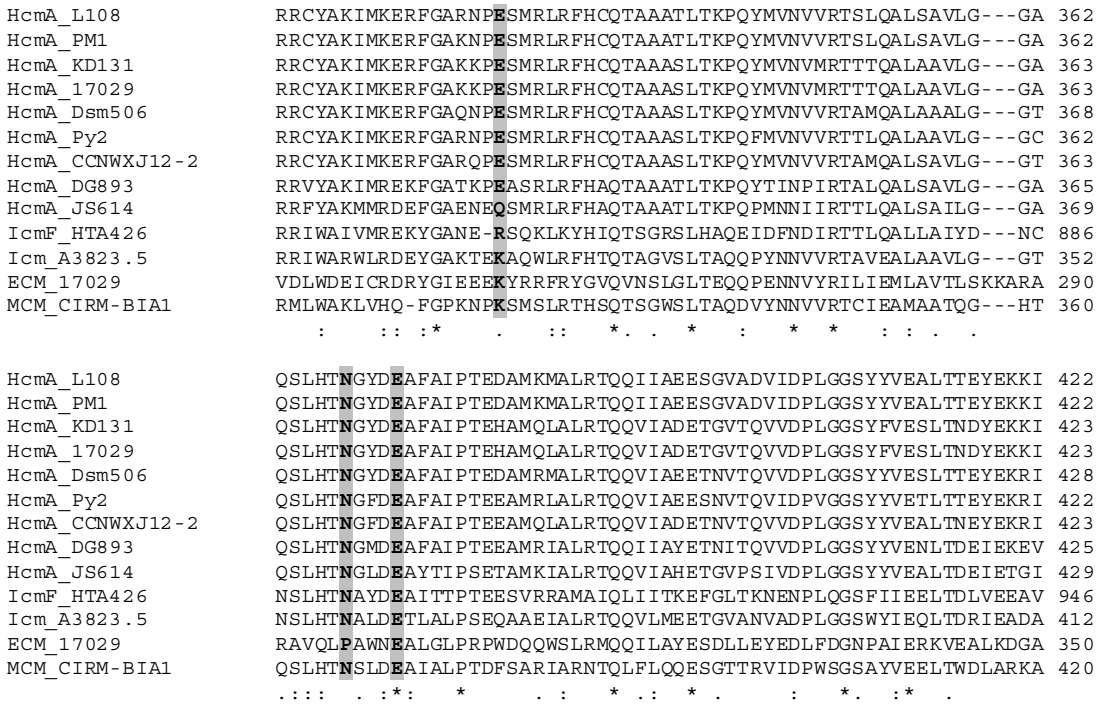

Supplementary Fig. S7. Extended ClustalW2 multiple sequence alignment of substrate binding domains of B_{12}-dependent acyl-CoA mutases shown in Fig. 7. Comparison of HcmA from *A. tertiaricarbonis* L108 to orthologous sequences from *M. petroleiphilum* PM1 (Mpe_B0541), *R. sphaeroides* KD131 (RSKD131_3116), *R. sphaeroides* ATCC 17029 (Rsph17029_3657), *S. novella* DSM 506 (Snov_2770), *X. autotrophicus* Py2 (Xaut_5021), *M. alhagi* CCNWXJ12-2 (ZP_09295256), *M. algicola* DG893 (MDG893_09606) and *Nocardioides sp*. JS614 (Noca_2131). In addition, the paralogous domains of ICM from *S. cinnamonensis* A3823.5 (*icm*, AAC08713), IcmF from *G. kaustophilus* HTA426 (GK3391), MCM from *P. freudenreichii* subsp. *shermanii* CIRM-BIA1 (YP_003687736) and ECM from *R. sphaeroides* ATCC 17029 (Rsph17029_2621) were aligned. Bold amino acids show conserved residues directly involved in substrate binding. Residues highlighted on *gray* background are known to interact specifically with the CoA moiety. White-typed amino acids on black highlight the residues thus far identified to interact specifically with the acyl group of the substrates. *Asterisks* indicate identical residues in all sequences, ":" mark conserved substitutions and "." assign semi-conserved substitutions with similar shapes.