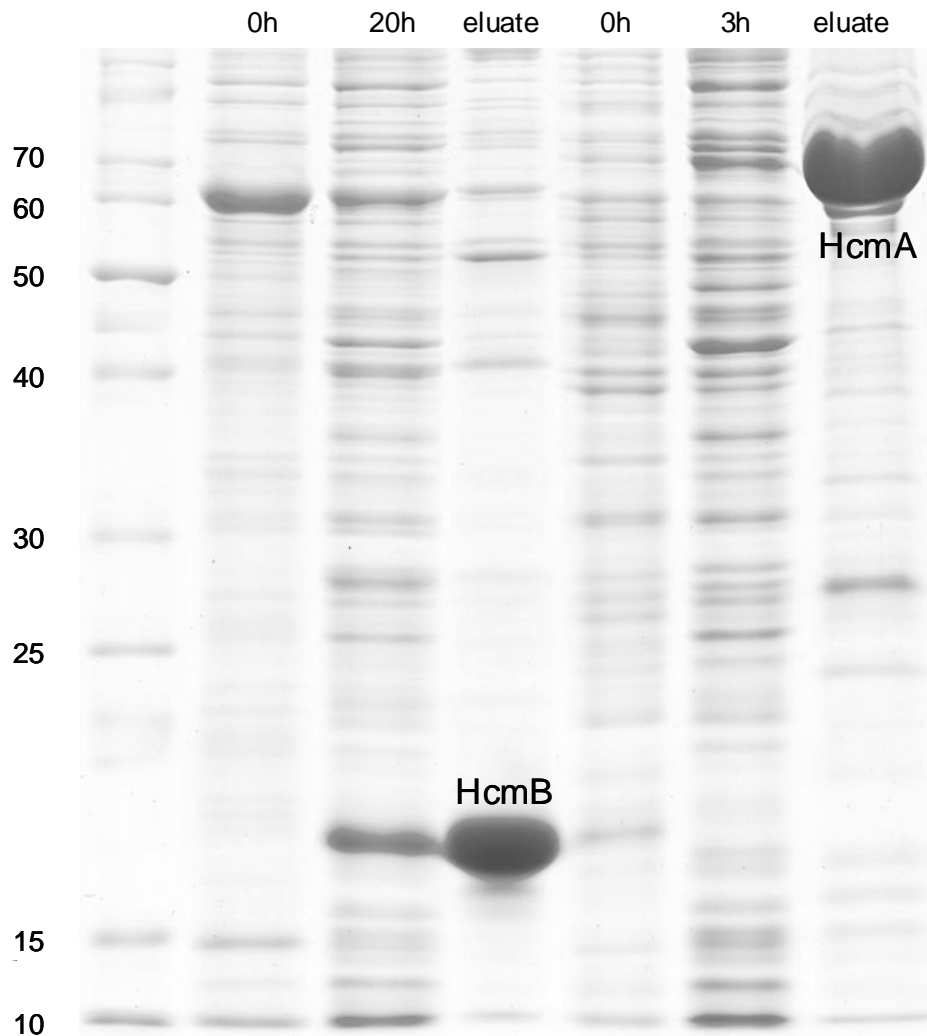
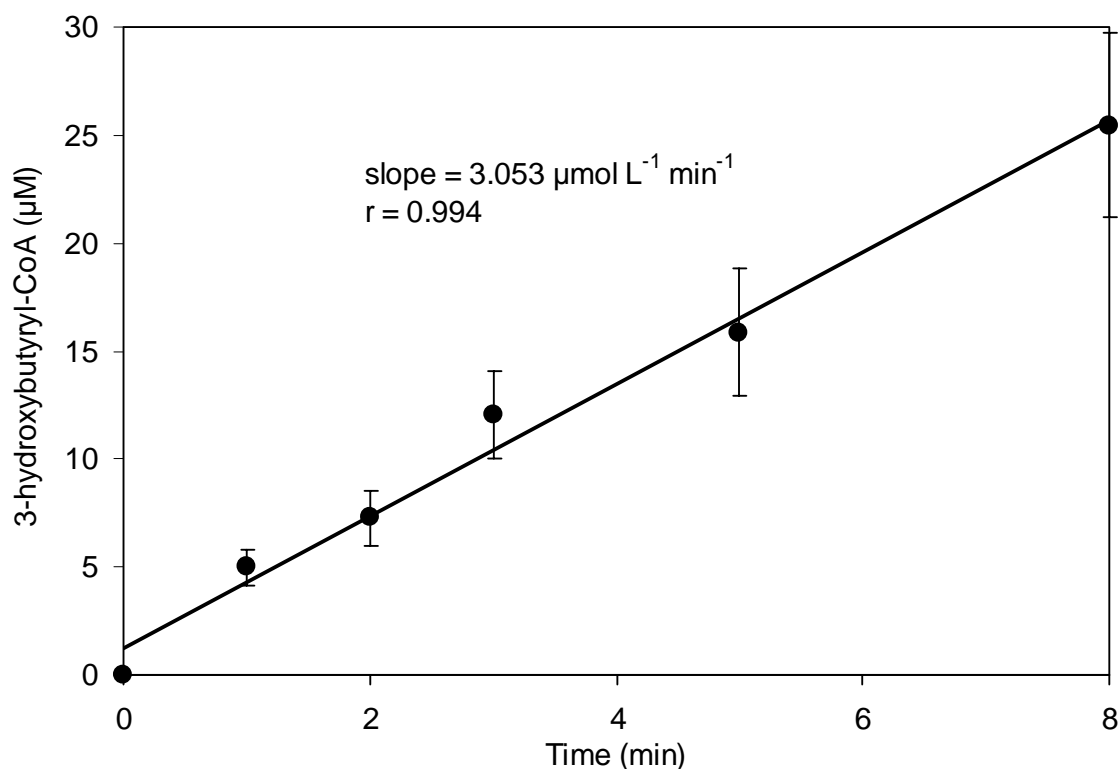


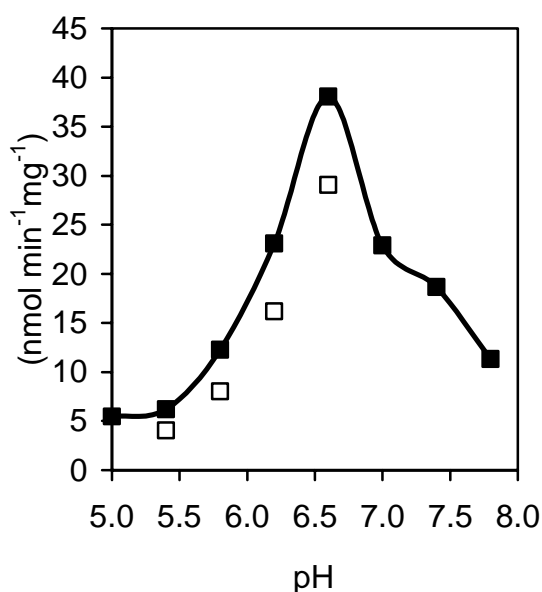
Supplementary Fig. S1. Analysis of synthesized acyl-CoA esters by ESI-MS/MS. In the Neutral Loss Scan mode (NL), masses of ionized acyl-CoA esters were detected, whereas in the Enhanced Product Ion Scan mode (EPI), also masses of smaller fragments were obtained. Characteristic fragments of 2-hydroxyisobutyryl-CoA (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.



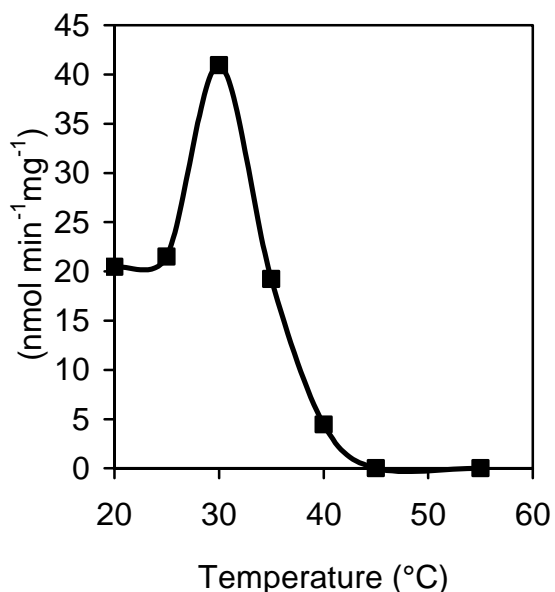
Supplementary Fig. S2. SDS PAGE analysis of crude extracts and eluates after heterologous expression of wild type HcmA and HcmB and their purification by one-step affinity chromatography, respectively (12% SDS-PAGE). Sampling times starting at induction of expression in *E. coli* strains TOP10 (HcmA) and ArcticExpress (DE3) (HcmB). Lanes: 0h, 30 μ g *E. coli* cell extract protein before induction; 20 and 3h, 30 μ g *E. coli* cell extract protein after 20 and 3 hours of induction, respectively; eluate, 30 μ g 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).



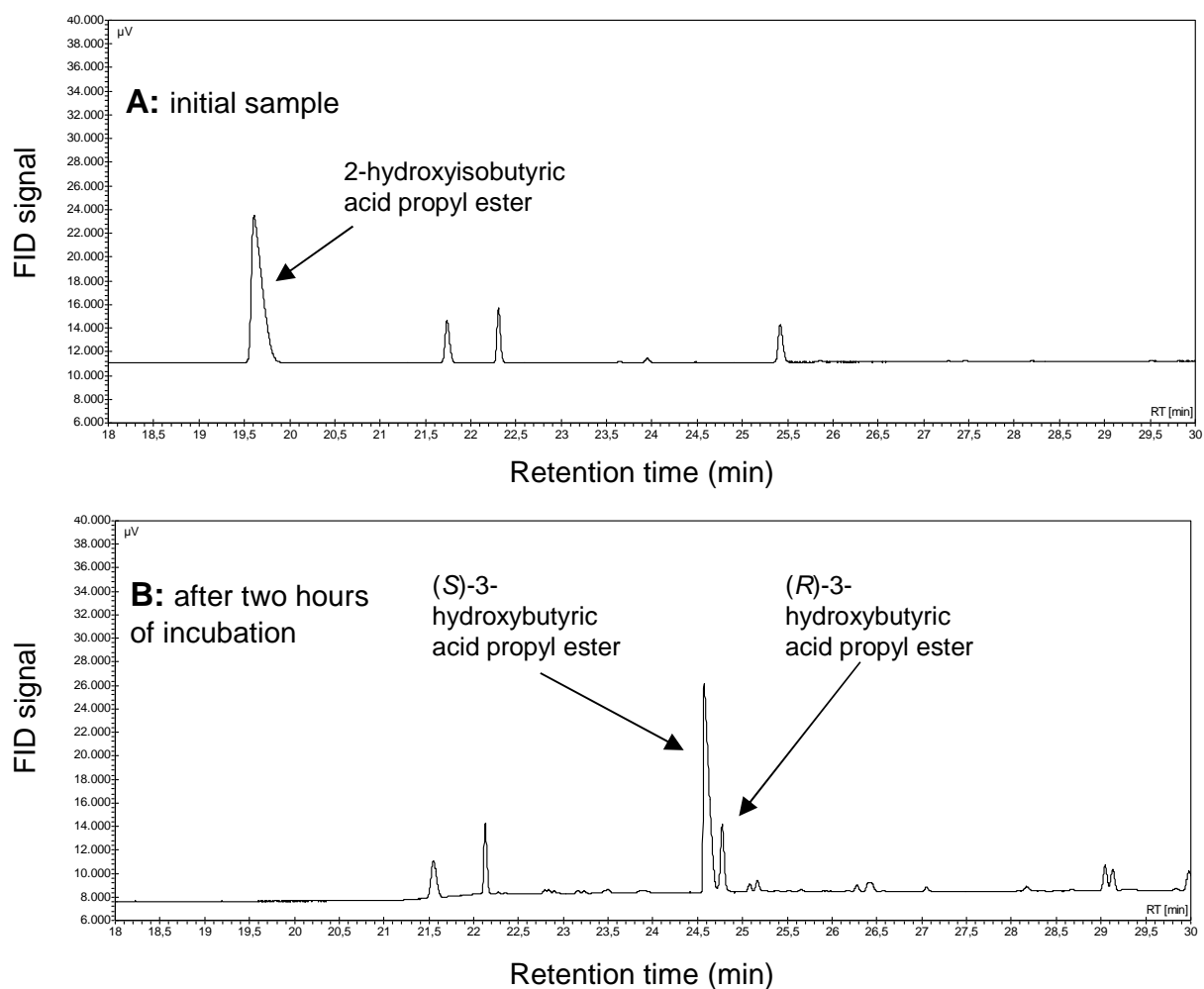
Supplementary Fig. S3. HPLC assay for quantifying HCM activity. Reconstituted wild type HcmAB (1.4 μM) was incubated with 250 μM 2-hydroxyisobutyryl-CoA at pH 6.6 and 30°C. The shown increase in 3-hydroxybutyryl-CoA represents mean values and SD of four replicates.



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.

HcmA_L108 TYTAAD-IADTPLEDIG-----LPGRYPFTRGYPPTMYRSRTWTMRQIAGFG 94
HcmA_PM1 TYTAAD-IADTPLEDIG-----LPGRYPFTRGYPPTMYRSRTWTMRQIAGFG 94
HcmA_KD131 TYTAAD-IADTPLEDIG-----LPGKYPFTRGYPPTMYRGRNWTMRQIAGFG 95
HcmA_17029 TYTAAD-IADTPLEDIG-----LPGKYPFTRGYPPTMYRGRNWTMRQIAGFG 95
HcmA_Dsm506 TYTAAD-LADTPVEDIG-----LPGRYPFTRGYPPTMYRSRTWTMRQIAGFG 100
HcmA_Py2 VYTAAD-AAATPIEDIG-----LPGRYPFTRGYPPTMYRSRNWTMRQIAGFG 94
HcmA_CCNWXJ12-2 TYIAAD-IAGTPAEDIG-----LPGRYPFTRGYPPTMYRGRNWTMRQIAGFG 95
HcmA_DG893 TYTPLD-VKNTPFEDIG-----FPGQYPFTRGYPPTMYRGRNWTMRQIAGFG 97
HcmA_JS614 VYTPAD-LPED-WNDIG-----LPGQFPFTRGYPPTMYRGRHWMTMRQIAGFG 101
IcmF_HTA426 TLSGLD-IPKVVLPKFKDYGEILRWVYKENVPGSFPYTAGVFPFKRQG-EDPKRQFAGEG 593
Icm_A3823.5 VYGPRPGDTYDGFERIG-----WPGEYPFTRGLYATGYRRTWTIROFAGFG 84
ECM_17029 -----MTQKDS-----PWLFRITVAGHS 17
MCM_CIRM-BIA1 -LFNEDVYKDMDWLDTY-----AGIPPFVHGPYATMYAFRPWTIROVAGFS 93

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HcmA_L108 TGEDTNKRFKYLIAQQQTG-ISTDFDMPTLMGYSDH-PMSDGEVGRGVAIDTLADMEA 152
HcmA_PM1 TGEDTNKRFKYLIAQQQTG-ISTDFDMPTLMGYSDH-PMSDGEVGRGVAIDTLADMEA 152
HcmA_KD131 TGEDTNKRFKFLIEQQQTG-ISTDFDMPTLMGYSDH-PMSDGEVGRGVAIDTLADMEA 153
HcmA_17029 TGEDTNKRFKFLIEQQQTG-ISTDFDMPTLMGYSDH-PMSDGEVGRGVAIDTLADMEA 153
HcmA_Dsm506 TGEDTNKRFKYLIAQQQTG-ISTDFDMPTLMGYSDH-PMSDGEVGRGVAIDTLADMEA 158
HcmA_Py2 TGEDTNKRFKYLIEQQQTG-ISTDFDMPTLMGYSDH-PMSDGEVGRGVAIDTLADMEA 152
HcmA_CCNWXJ12-2 TGEDTNRRFKFLIEQQQTG-ISTDFDMPTLMGYSDH-PMSDGEVGRGVAIDTLADMEA 153
HcmA_DG893 TARETNKRFKYLIAQQQTG-LSIDFDMPTLMGYSDH-AMSDGEVGRGVAIDTLADMEA 155
HcmA_JS614 QAEETNKRFQYLINQQQTG-LSDVDFMPTLMGLSDH-PMSLGEVGRGVAIDVLDLADMEA 159
IcmF_HTA426 TPERTNRRFHYLCKEDKAKRLSTAFDSVTLYGEDPDYRPIFGKVGSGVSVITLDDMKK 653
Icm_A3823.5 NAEQTNERFKMILANGGGG-LSVAFDMPTLMGRSDH-PRSLGEVGHCGVAIDSAADMEV 142
ECM_17029 TAKASNALYRTNLAKGQTG-LSVAFDLPTQTGYSDH-ALARGEVGVGVPICHLGDMMR 75
MCM_CIRM-BIA1 TAKESNAFYRRNLAAQKQK-LSVAFDLPTHRGYSDH-PRVAGDVGMAVADSIYDMRE 151

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HcmA_L108 LLADIDLEK--ISVSFTINPSAWILLAMYVALGEK-----RGY 188
HcmA_PM1 LLADIDLEK--ISVSFTINPSAWILLAMYVALGEK-----RGY 188
HcmA_KD131 LLDGIDLEK--ISVSLTINPTAWILLAMYIALCEE-----RGY 189
HcmA_17029 LLDGIDLEK--ISVSLTINPTAWILLAMYIALCEE-----RGY 189
HcmA_Dsm506 LFDGIDLEK--ISVSMTINPSAWILLAMYIVLAEK-----RGY 194
HcmA_Py2 LFDGIDLEK--ISVSMTINPSAWILLAMYIVLAEK-----RGY 188
HcmA_CCNWXJ12-2 LLADIDLEK--ISVSLTINPTAWILLAMYVALAEK-----RGY 189
HcmA_DG893 LFDGIDLEK--ISVSMTINPSAWILLAMYIALAEK-----RGY 191
HcmA_JS614 LFDGIDLEK--ISVSMTINPSAWILLAMYIAVAED-----KGY 195
IcmF_HTA426 LYKGFDLCDPLTISVSMTINGPAPILLAMFMNTAIDQVEKKEAELGRPLTPEEYEQVKEW 713
Icm_A3823.5 LFKDIPLDG--VTSMTISGPAVPVFCMYLVAER-----QGV 178
ECM_17029 LFDQIPLEQ--MNTSMTINATAPWLLALYIAVAEE-----QGA 111
MCM_CIRM-BIA1 LFDGIDLEK--MSVSMTMNGAVLPIALYVVTAE-----QGV 187

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HcmA_L108 DLNKLSGTVQADILKEYMAQKEYIYPIAPSVRIVRDIITYSAKNLKR-YNPINISGYHIS 247
HcmA_PM1 DLNKLSGTVQADILKEYMAQKEYIYPIAPSVRIVRDIITYSAKNLKR-YNPINISGYHIS 247
HcmA_KD131 DLNKVSGTVQADILKEYMAQKEYIFPIAPSVRIVRDIISHSRTMKR-YNPINISGYHIS 248
HcmA_17029 DLNKVSGTVQADILKEYMAQKEYIFPIAPSVRIVRDIISHSRTMKR-YNPINISGYHIS 248
HcmA_Dsm506 DLNKLSGTVQADILKEYMAQKEYVFPPIEPSVRIVRDCITYCARNMKR-YNPINISGYHIS 253
HcmA_Py2 DLNKLSGTVQADILKEYMAQKEYIYPIAPSVRIVRDCITYCAKMKR-YNPINISGYHIS 247
HcmA_CCNWXJ12-2 DLNKLSGTVQADILKEYMAQKEYIFPIAPSVRIVRDLIAYSTRMKR-YNPINISGYHIS 248
HcmA_DG893 DLNKLSGTVQADILKEYMAQKEYIFPVRPSVRLVRDCIQYGSNMNR-YNPINISGYHIS 250
HcmA_JS614 DLNKLSGTVQADILKEYMAQKEYIFPVRPSMRIVRDCIAYCAENMAR-YNPINISGYHIS 254
IcmF_HTA426 TLQTVRGTQADILKEDQGNQTCIFSTDFALKMMGDIQYEIFKHRVRYNYSVVISGYHIA 773
Icm_A3823.5 DPAVLNGTLQTDIFKEYIAQKEWLFQPEPHRLIGDLMEHCARDIPA-YKPLSVSGYHIR 237
ECM_17029 DISKLGTVQADNDLMKEYLSRGTYICPPRPSLRMITDVAAYTRVHLPK-WNPMNVCSYHLQ 170
MCM_CIRM-BIA1 KPEQLAGTVQADNDILKEFMVRYNTYIYPPQPSMRIISEIFAYTSANMPK-WNSISISGYHMQ 246

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HcmA_L108 EAGSSPLQEAFTLANLIYVNEV--TKTGMHVDEFAPRLAFFFVVSQGDFFEEVAKFRAL 305
HcmA_PM1 EAGSSPLQEAFTLANLIYVNEV--TETGMHVDEFAPRLAFFFVVSQGDFFEEVAKFRAL 305
HcmA_KD131 EAGSSPLHEAFTLANLIYVVEEV--LKTGVEVDDFAPRLAFFFVVSQADFFEEIAKFRAL 306
HcmA_17029 EAGSSPLHEAFTLANLIYVVEEV--LKTGVEVDDFAPRLAFFFVVSQADFFEEIAKFRAL 306
HcmA_Dsm506 EAGSSPLHEAFTLANLIYVVEEV--LKTGMQVDEFAPRLAFFFVVSQADFFEEIAKFRAL 311
HcmA_Py2 EAGSSPVDEVAFTLANLIYVVEEV--LKTGMKVDDFAPRLAFFFVVSQADFFEEIAKFRAL 305
HcmA_CCNWXJ12-2 EAGSSPLHEAFTLANLIYVVEEV--TKLGDVDDFAPRLAFFFVVSQADFFEEVAKFRAL 306
HcmA_DG893 EAGSTAVQEVAYTMAITMAYVVRTA--IDAGVDVNDVDFGRLSFFVVSQADFFEEIAKFRAL 308
HcmA_JS614 EAGANAVQEVAFMAITRAYVSDV--IAAGVDVDTFAPRLSFFVVSQADFFEEIAKFRAL 312
IcmF_HTA426 EAGANPIQLAFATLANLGYVVEEV--LSRGMHIDDFAPRLSFFVVSQADFFEEIAKFRAL 830
Icm_A3823.5 EAGATAAQELAYTLADGFGYVELG--LSRGLDLDVDFAPRLSFFVVSQADFFEEIAKFRAL 295
ECM_17029 EAGATPEQELAFALATGIAVLDDLRTRKVPVPAEHFPAHVGRISFFVVSQADFFEEIAKFRAL 230
MCM_CIRM-BIA1 EAGATADIE MAYTLADGVDYIRAG--ESVGLNVDVDFAPRLSFFVVSQADFFEEIAKFRAL 304

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HcmA_L108      RRCYAKIMKERFGARNPESMRLRFHCQTAAATLTKPQYMNVVVRTSLQALSAVLG---GA 362
HcmA_PM1      RRCYAKIMKERFGAKNPESMRLRFHCQTAAATLTKPQYMNVVVRTSLQALSAVLG---GA 362
HcmA_KD131    RRCYAKIMKERFGAKKESMRLRFHCQTAAASLTKPQYMNVMRTTQALAAAVLG---GA 363
HcmA_17029    RRCYAKIMKERFGAKKESMRLRFHCQTAAASLTKPQYMNVMRTTQALAAAVLG---GA 363
HcmA_Dsm506   RRCYAKIMKERFGAQNPESMRLRFHCQTAAASLTKPQYMNVVVRTAMQALAAALG---GT 368
HcmA_Py2      RRCYAKIMKERFGARNPESMRLRFHCQTAAASLTKPQFMNVVVRTTLQALAAAVLG---GC 362
HcmA_CCNWXJ12-2 RRCYAKIMKERFGARQPESMRLRFHCQTAAASLTKPQYMNVVVRTAMQALSAVLG---GT 363
HcmA_DG893    RRVYAKIMREKFGATKPEASRLRFHAQTAAATLTKPQYTNINPIRTALQALSAVLG---GA 365
HcmA_JS614    RRFYAKMMRDEFGAENQSMRLRFHAQTAAATLTKPQPMNNIIRTTLQALSAILG---GA 369
IcmF_HTA426   RRIWAIVMREKYGANE-RSQKLYHIQTSGRSLHAQEIDFNDIRTTLQALLAIYD---NC 886
Icm_A3823.5   RRIWARWLRDEYGAKTEKAQWLRFHTQTAGVSLTAQQPYNNVVRTAVEALAAAVLG---GT 352
ECM_17029     VDLWDEICRDYGIEEEKYRRFRYGVQVNSLGLTEQQPENNVYRILIEMLAVTLSKKARA 290
MCM_CIRM-BIA1 RMLWAKLVHQ-FGPKNPKSMSLRTHSQTSGSWLSLTAQDVYNNVVRTCIEAMAATQG---HT 360
:      : : *      .      : : * . *      :      * *      : : . .

HcmA_L108      QSLHTNGYDEAFAIPTEDAMKMALRTQQIIAEESGVADVIDPLGSSYVVEALTTEYEKKI 422
HcmA_PM1      QSLHTNGYDEAFAIPTEDAMKMALRTQQIIAEESGVADVIDPLGSSYVVEALTTEYEKKI 422
HcmA_KD131    QSLHTNGYDEAFAIPTEHAMQLALRTQQVIADETGVTQVVDPLGSSYFVESLTNDYEKKI 423
HcmA_17029    QSLHTNGYDEAFAIPTEHAMQLALRTQQVIADETGVTQVVDPLGSSYFVESLTNDYEKKI 423
HcmA_Dsm506   QSLHTNGYDEAFAIPTEDAMRMALRTQQVIAEETNVTQVVDPLGSSYVVESLTTEYEKRI 428
HcmA_Py2      QSLHTNGFDEAFAIPTEEAMRLALRTQQVIAEESNVTQVIDPVGSSYVVETLTTEYEKRI 422
HcmA_CCNWXJ12-2 QSLHTNGFDEAFAIPTEEAMQLALRTQQVIADETNVTQVVDPLGSSYVVEALTNEYEKRI 423
HcmA_DG893    QSLHTNGMDEAFAIPTEEAMRIALRTQQIIAYETNITQVVDPLGSSYVVENLTDEIEKEV 425
HcmA_JS614    QSLHTNGLDEAYTIPSETAMKIALRTQQVIAHETGVPSIVDPLGSSYVVEALTDEIETGI 429
IcmF_HTA426   NSLHTNAYDEAITPTEESVRRAMAIQLIITKEFGLTKNENPLGSFIIEELTDLVEEAV 946
Icm_A3823.5   NSLHTNALDETLALPSEQAEIALRTQQVLMEETGVANVADPLGSWYIEQLTDRIEADA 412
ECM_17029     RAVQLPAWNEALGLPRPWDQQWSLRMQQILAYESDLLEYEDLFDGNPAIERKVEALKDGA 350
MCM_CIRM-BIA1 QSLHTNSLDEAIALPTDFSARIARNTQLFLQQESGTTRVIDPWSGSAYVEELTWDLARKA 420
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Supplementary Fig. S7. Extended ClustalW2 multiple sequence alignment of substrate binding domains of B₁₂-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. cinnamomensis* 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.