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Thermophilic Coenzyme B_{12}-Dependent Acyl Coenzyme A (CoA) Mutase from *Kyrpidia tusciae* DSM 2912 Preferentially Catalyzes Isomerization of (*R*)-3-Hydroxybutyryl-CoA and 2-Hydroxyisobutyryl-CoA

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The recent discovery of a coenzyme B_{12}-dependent acyl-coenzyme A (acyl-CoA) mutase isomerizing 3-hydroxybutyryl- and 2-hydroxyisobutyryl-CoA in the mesophilic bacterium *Aquincola tertiaricarbonis* L108 (N. Yaneva, J. Schuster, F. Schäfer, V. Lede, D. Przybylski, T. Paproth, H. Harms, R. H. Müller, and T. Rohwerder, J Biol Chem 287:15502–15511, 2012, http://dx.doi.org/10.1074/jbc.M111.314690) could pave the way for a complete biosynthesis route to the building block chemical 2-hydroxyisobutyric acid from renewable carbon. However, the enzyme catalyzes only the conversion of the stereoisomer (*S*)-3-hydroxybutyryl-CoA at reasonable rates, which seriously hampers an efficient combination of mutase and well-established bacterial poly-(*R*)-3-hydroxybutyrate (PHB) overflow metabolism. Here, we characterize a new 2-hydroxyisobutyryl-CoA mutase found in the thermophilic knallgas bacterium *Kyrpidia tusciae* DSM 2912. Reconstituted mutase subunits revealed highest activity at 55°C. Surprisingly, already at 30°C, isomerization of (*R*)-3-hydroxybutyryl-CoA was about 7,000 times more efficient than with the mutase from strain L108. The most striking structural difference between the two mutases, likely determining stereospecificity, is a replacement of active-site residue Asp found in strain L108 at position 117 with Val in the enzyme from strain DSM 2912, resulting in a reversed polarity at this binding site. Overall sequence comparison indicates that both enzymes descended from different prokaryotic thermophilic methylmalonyl-CoA mutases. Concomitant expression of PHB enzymes delivering (*R*)-3-hydroxybutyryl-CoA (beta-ketothiolase PhaA and acetoacetyl-CoA reductase PhaB from *Cupriavidus necator*) with the new mutase in *Escherichia coli* JM109 and BL21 strains incubated on gluconic acid at 37°C led to the production of 2-hydroxyisobutyric acid at maximal titers of 0.7 mM. Measures to improve production in *E. coli*, such as coexpression of the chaperone MeaH and repression of thioesterase II, are discussed.

Carbon skeleton rearrangement of carboxylic acids via a chemically challenging radical mechanism is catalyzed by coenzyme B_{12}-dependent acyl-coenzyme A (acyl-CoA) mutases (1). During catalysis, both acyl-CoA and B_{12} molecules are completely buried within the enzyme. This extensive interaction is mediated by highly conserved amino acid residues, forming a characteristic triose phosphate isomerase (TIM) barrel and a Rossman fold. The best-studied member of this enzyme family is methylmalonyl-CoA mutase (MCM), specifically catalyzing the isomerization of succinyl- and (*R*)-methylmalonyl-CoA (2). Several genetic defects impairing mitochondrial MCM activity are associated with methylmalonic aciduria, an inborn error of branched-chain amino acid metabolism (3, 4). Another mutase playing a role in central carbon metabolism is ethylmalonyl-CoA mutase (ECM), involved in acetic acid assimilation in bacteria lacking the glyoxylate cycle (5). In addition, isobutyryl-CoA mutase (ICM) appears to function mainly in secondary metabolism, e.g., the bacterial synthesis of polyketide antibiotics (6). Recently, a fourth subfamily of coenzyme B_{12}-dependent acyl-CoA mutases has been characterized, specifically catalyzing the interconversion of 3-hydroxybutyryl-CoA enantiomers and 2-hydroxyisobutyryl-CoA (7) (Fig. 1). Initially, the 2-hydroxyisobutyryl-CoA mutase (HCM) has been discovered in the bacterial strains *Aquincola tertiaricarbonis* L108 and *Methylibium petroleiphilum* PM1, operating in the degradation of the fuel oxygenate methyl *tert*-butyl ether (MTBE) for the conversion of the unusual 2-hydroxyisobutyric acid intermediate to common metabolites (8). However, closely related HCM sequences have also been predicted from genomes of other bacteria not associated with fuel oxygenate degradation (7), e.g., *Rhodobacter sphaeroides* strains ATCC 17029, *Starkeya novella* DSM 506, *Xanthobacter autotrophicus* Py2, *Marinobacter algicola* DG893, and *Nocardioides* sp. strain JS614, indicating different biological roles of HCM.

Besides their natural involvement in primary and secondary carbon metabolism, B_{12}-dependent mutases may also have biotechnological potential for the synthesis of commodity and specialty chemicals (9, 10). In principle, their highly specific catalysis may be used one day for the stereospecific synthesis of some short-

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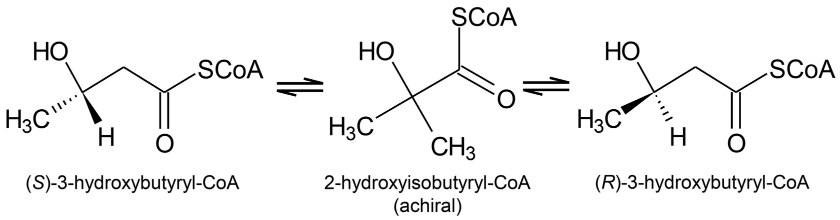

FIG 1 Reversible HCM-catalyzed B_{12}-dependent carbon skeleton rearrangement of achiral 2-hydroxyisobutyryl-CoA resulting in either (*R*)- or (*S*)-3-hydroxybutyryl-CoA formation.

and medium-chain carboxylic acids not easily attainable by chemical synthesis (10). Currently most interesting, however, is a biotechnological synthesis route employing HCM for the industrial production of the poly(methyl methacrylate) precursor 2-hydroxyisobutyric acid from renewable carbon (11), having great potential to completely replace the established petrochemical processes. Accordingly, mutase-dependent synthesis of this important building block from simple sugars and carboxylic acids as well as from carbon dioxide has already been demonstrated at the lab scale (12–15). Implementation of an industrial HCM process seems to be particularly feasible, as the metabolic route delivering the mutase substrate 3-hydroxybutyryl-CoA is part of a well-studied overflow metabolism in bacteria. In fact, an HCM process could be considered a slight modification of the established microbial production of poly(3-hydroxybutyrate) (PHB) (Fig. 2). By replacing the PHB polymerase with HCM, not the polyester but the monomeric 2-hydroxyisobutyric acid will be formed (11). However, a major hurdle for an implementation of the HCM process is the different stereospecificity of HCM and the PHB route. The latter provides exclusively (*R*)-3-hydroxybutyryl-CoA, whereas the former is highly specific for (*S*)-3-hydroxybutyryl-CoA, isomerizing it with a nearly 800-times-higher catalytic efficiency than the (*R*)-enantiomer (7).

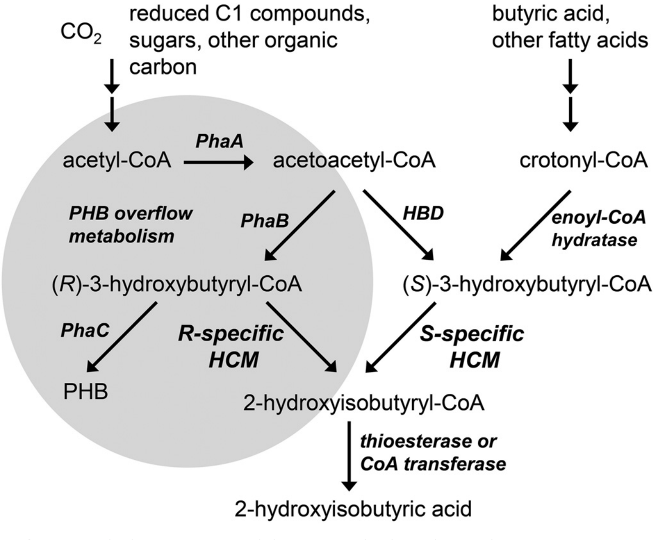

FIG 2 Metabolic sequences delivering 3-hydroxybutyryl-CoA enantiomers for HCM-dependent 2-hydroxyisobutyric acid synthesis. Bacterial PHB overflow metabolism (highlighted in gray) resulted in production of the (*R*)-enantiomer via beta-ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB) activity. By replacing PHB polymerase PhaC with an HCM enzyme specific for (*R*)-3-hydroxybutyryl-CoA conversion ("*R*-specific"), overflow carbon could be exclusively channeled to 2-hydroxyisobutyric acid. In contrast, (*S*)-3-hydroxybutyryl-CoA dehydrogenase activity (HBD) and beta-oxidation of fatty acids could deliver the substrate for an HCM enzyme specifically converting (*S*)-3-hydroxybutyryl-CoA ("*S*-specific").

In this study, we have characterized an acyl-CoA mutase from the thermophilic knallgas bacterium *Kyrpidia tusciae* DSM 2912 (16, 17). Although the sequences of the large and small HCM subunits A and B, binding the acyl-CoA substrate and coenzyme B_{12}, respectively, from strain L108 and the new mutase share only 43 and 44% identical residues, respectively, the enzyme from strain DSM 2912 likewise catalyzes the isomerization of 3-hydroxybutyryl- and 2-hydroxyisobutyryl-CoA. For distinguishing between the two enzymes, however, we termed the L108-type mutase HCM and the new DSM 2912-type mutase RCM in this study. Kinetic analysis with purified enzyme revealed that RCM, in contrast to HCM, converted both (*R*)- and (*S*)-3-hydroxybutyryl-CoA at high rates. More interesting, with catalytic efficiencies of about 3.4 and 0.3 μM^{-1} min^{-1}, respectively, the (*R*)-enantiomer is slightly favored by RCM. In line with this, concomitant expression of the new mutase and an (*R*)-3-hydroxybutyryl-CoA-delivering route in *Escherichia coli* strains resulted in the production of 2-hydroxyisobutyric acid.

MATERIALS AND METHODS

Chemicals. 2-Hydroxyisobutyric acid (>98% pure), dicyclohexylcarbodiimide (≥99% pure), and thiophenol (≥99% pure) were purchased from Merck Schuchardt. Coenzyme B_{12} (≥97% pure), CoA (≥93% pure), (*R*)-3-hydroxybutyric acid sodium salt (96% pure), and (*S*)-3-hydroxybutyric acid (>97% pure) were purchased from Sigma. Tetrabutylammonium hydrogen sulfate (>98% pure) was from AppliChem. All organic solvents used, such as methanol, acetonitrile, N,N-dimethylformamide, and diethyl ether, were of analysis or high-performance liquid chromatography (HPLC) grade or at comparable high purity. Inorganic salts, bases, and acids were of analysis grade or at the highest purity available. 2-Hydroxyisobutyryl-CoA and enantiopure (*R*)- and (*S*)-3-hydroxybutyryl-CoA were synthesized from the free acids and CoA via thiophenyl esters by the method of Padmakumar and coworkers (18).

Bacterial strains, plasmids, primers, and growth conditions. All bacterial strains, plasmids, and primers used in this study are listed in Table 1. *Kyrpidia tusciae* DSM 2912 (16), previously isolated from ponds in a solfatara in the geothermal area of Tuscany, Italy (17), was routinely cultivated at 55°C in liquid mineral salt medium 1 containing the following (in milligrams $liter^{-1}$): NH_4Cl, 760; KH_2PO_4, 680; K_2HPO_4, 970; $CaCl_2 \cdot 6H_2O$, 27; $MgSO_4 \cdot 7H_2O$, 71.2; $FeSO_4 \cdot 7H_2O$, 14.94; $CuSO_4 \cdot 5H_2O$, 0.785; $MnSO_4 \cdot 4H_2O$, 0.81; $ZnSO_4 \cdot 7H_2O$, 0.44; $Na_2MoO_4 \cdot 2H_2O$, 0.25; and $CoCl_2 \cdot 6H_2O$, 0.040; it also contained the following vitamins (in micrograms $liter^{-1}$): biotin, 20; folic acid, 20; pyridoxine-HCl, 100; thiamine-HCl, 50; riboflavin, 50; nicotinic acid, 50; DL-Ca-pantothenate, 50; *p*-aminobenzoic acid, 50; lipoic acid, 50; and cobalamin, 50. The pH was 7.5, and the medium was supplied with 2-hydroxyisobutyric acid at a concentration of 1 g $liter^{-1}$. *Cupriavidus necator* DSM 428 was incubated at 30°C in mineral salt medium 2 consisting of the following (in milli-

TABLE 1 Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence	Reference or source
Strains		
Kyrpidia tusciae DSM 2912	Wild type	DSMZ
Cupriavidus necator DSM 428	Wild type	DSMZ
E. coli TOP10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL*(Str^r) *endA1* λ$^-$	Novagen
E. coli DH5-α	*fhuA2* Δ(*argF lacZ*)*U169 phoA glnV44* φ80*lacZ*ΔM15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Novagen
E. coli BL21(DE3)	F^- *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) λ(DE3)	Invitrogen
E. coli JM109 (DE3)	*recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 glnV44 mcrB*$^+$ Δ(*lac-proAB*) e14$^-$ [F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] λ(DE3)	Promega
Plasmids		
pASG-IBA43	Expression vector, *tetA* promoter, Amp^r	IBA Goettingen
pCDFDuet-1	Expression vector, T7 promoter, Sm^r	Novagen
pBBR1MCS-3	Expression vector, *P*lac, Tc^r	21
pASG-IBA43 [rcmA]	*rcmA* from *K. tusciae* inserted into pASG-IBA43	This study
pASG-IBA43 [rcmB]	*rcmB* from *K. tusciae* inserted into pASG-IBA43	This study
pCDFDuet-1 [phaA-phaB]	*phaA-phaB* from *C. necator* inserted into pCDFDuet-1	This study
pBBR1MCS-3 [rcmA-rcmB]	*rcmA-rcmB* from *K. tusciae* inserted into pBBR1MCS-3	This study
pBBR1MCS-3 [meaH2-rcmA-rcmB]	*meaH2-rcmA-rcmB* from *K. tusciae* inserted into pBBR1MCS-3	This study
Primers		
RcmA_F	5′-AGC GGC TCT TCA ATG GCT GAT CAA GAG AAG CTC TTT A-3′	This study
RcmA_R	5′-AGC GGC TCT TCT CCC AAC CAA AGG GAA CTG CCA CA-3′	This study
RcmB_F	5′-AGC GGC TCT TCA ATG GAG AAA AAG ATC AAG GTG A-3′	This study
RcmB_R	5′-AGC GGC TCT TCT CCC ATC CCG ATC CGG AAA CCG G-3′	This study
PstI_phaAB_F	5′-ATA TAT ATC TGC AGG TTC CCT CCC GTT TC-3′	This study
PstI_phaAB_R	5′-ATA TAT ATC TGC AGC CTC GCC CCC GCG-3′	This study
XbaI_meaH2_F	5′-ATA TTC TAG AAA TGC AAG AGC TTC TCT CGC GAT TC-3′	This study
XbaI_rcmA_F	5′-ATA TTC TAG AAA TGG CTG ATC AAG AGA AGC TCT TTA-3′	This study
SacI_rcmB_R	5′-ATA TGA GCT CTC AAT CCC GAT CCG GAA ACC GG-3′	This study

grams liter^{-1}): NH_4Cl, 1,000; KH_2PO_4, 1,500; Na_2HPO_4, 3,570; $MgSO_4$, 200; $CaCl_2$, 20; cobalamin, 0.05; Fe(III) citrate, 8.1; $CuSO_4 \cdot 5\ H_2O$, 0.785; $MnSO_4 \cdot 4H_2O$, 0.81; $ZnSO_4 \cdot 7H_2O$, 0.44; $Na_2MoO_4 \cdot 2H_2O$, 0.25; $CoSO_4$, 0.026; and $NiCl_2 \cdot 6H_2O$, 0.0166. The medium (pH 6.9) was supplemented with 2 g liter^{-1} of fructose. *E. coli* TOP10 and DH5-α were grown at 37°C in Luria-Bertani broth. *E. coli* strains BL21(DE3) and JM109 (DE3) were cultivated at 37°C in EZ-Rich defined medium (19). The growth of strains was routinely monitored by measuring the optical density of cultures at 700 nm.

Analytics. Carboxylic acids, including 2-hydroxyisobutyric and 3-hydroxybutyric acid, were routinely quantified using high-performance liquid chromatography with refractive index (RI) detection (13) applying an eluent of 0.01 N sulfuric acid at 0.5 ml per min and a Nucleogel ION 300 OA column (300 by 7.7 mm; Macherey-Nagel). In addition, 2-hydroxyisobutyric acid was identified as the corresponding methyl ester by gas chromatography (GC) as described before (8) by applying mass detection (5973 mass selective detector [MSD]; Agilent). The resulting mass spectra of samples were compared with results obtained with pure standards and with most-probable matches by the GC mass spectral National Institute of Standards and Technology (NIST) database. Acyl-CoA esters were analyzed by HPLC with photometric detection at 260 nm, applying a Nucleosil 100-5 C_{18} column (250 by 3 mm by 5 μm; Macherey-Nagel) and a mobile phase of 14.5 vol% acetonitrile, 10 mM tetrabutylammonium hydrogen sulfate, and 100 mM sodium phosphate at pH 4.5 (7).

Heterologous expression of Strep-tagged *rcmA* and *rcmB* and protein purification. Genomic DNA from *K. tusciae* strain DSM 2912 was extracted using the DNA extraction kit (Macherey-Nagel). Then, both *rcm* genes were amplified by applying forward primer RcmA_F and reverse primer RcmA_R for *rcmA* and forward primer RcmB_F and reverse primer RcmB_R for *rcmB*. PCR was accomplished with proofreading OneTaq DNA polymerase (NEB) according to the protocol of the manufacturer. For 30 cycles, the DNA was incubated at 94°C for 20 s, at 57°C for 30 s, and at 68°C for 3 min for *rcmA* and 1.5 min for *rcmB*. The PCR products were cloned into the expression vector pASG-IBA43 according to the protocol of IBA Goettingen, and chemocompetent cells of *E. coli* TOP10 were subsequently transformed with pASG-IBA43 [rcmA] or pASG-IBA43 [rcmB]. After growth on Luria-Bertani medium supplemented with 100 mg liter^{-1} ampicillin, induction was performed at an optical density of 0.5 with 200 μg liter^{-1} anhydrotetracycline for 3 h at 30°C. Cells were harvested by centrifugation at 13,000 × *g* and 4°C for 10 min, suspended in Tris buffer (100 mM Tris, pH 7, adjusted with HCl), and disrupted using a mixer mill (MM 400; Retsch GmbH) with glass beads (212 to 300 μm; Sigma) at 30 s^{-1} for 30 min. Then, crude extracts of *E. coli* TOP10 pASG-IBA43 [rcmA] and TOP10 pASG-IBA43 [rcmB] were loaded onto Strep-Tactin Superflow high-capacity 10-ml columns (IBA Goettingen). After washing with 20 column volumes of Tris buffer, the RCM subunits were eluted with Tris buffer containing 2.5 mM desthiobiotin. Heterologous expression and protein purification by affinity chromatography were analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with samples of crude extracts before and after induction as well as with the column eluates. RcmA and RcmB were concentrated from collected protein fractions using 30- and 10-kDa Viva Spin columns (GE Healthcare), respectively, and then diluted with conservation buffer (50 mM potassium phosphate, 10% glycerol, pH 7.4).

TABLE 2 Kinetic parameters of reconstituted RcmA and RcmB subunits incubated with the HCM main substrates at pH 7.8 and 55°C[a]

Substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
(*R*)-3-Hydroxybutyryl-CoA	3.53 ± 0.04	87 ± 3	293 ± 3	3.4 ± 0.1
2-Hydroxyisobutyryl-CoA	2.64 ± 0.10	90 ± 13	219 ± 8	2.4 ± 0.4
(*S*)-3-Hydroxybutyryl-CoA	1.13 ± 0.08	313 ± 88	94 ± 7	0.3 ± 0.1

[a] Values are means ± SD.

Acyl-CoA mutase assay. Activity of purified mutase, consisting of heterologously expressed RcmA and RcmB subunits purified by affinity chromatography, was measured by directly quantifying CoA ester transformations via HPLC ion-pair chromatography. For determining the pH optimum, activity for the isomerization of 2-hydroxyisobutyryl-CoA to 3-hydroxybutyryl-CoA was measured by incubating recombinant RCM in phosphate-acetate (50 mM potassium phosphate, 50 sodium acetate) and phosphate-Tris (50 mM potassium phosphate, 50 mM Tris) buffers amended with 833 μM coenzyme B_{12}, 10 mM $MgCl_2$, and 10% glycerol at pH values between 5.8 and 9.5 at 50°C. For determining the temperature optimum, recombinant RCM was incubated with 2-hydroxyisobutyryl-CoA in the phosphate-Tris buffer at pH 7.8 and temperatures between 30 and 75°C. The determination of kinetic parameters was carried out with 0.2 μM recombinant RCM in phosphate-Tris buffer at pH 7.8 and 55°C. The reaction was started by adding acyl-CoA ester substrate. For stopping the reaction, samples were mixed with an equal volume of 100 mM acetate buffer (pH 3.5) and cooled at 0°C for 5 min prior to HPLC analysis. Kinetic parameters were calculated by nonlinear regression analysis applying the Michaelis-Menten equation (OriginPro 9.0). Errors given for the maximum rate of metabolism (V_{max}), K_m, and k_{cat} (Table 2) represent the regression-derived standard deviation values based on 10 experiments. The standard deviation value of the catalytic efficiency k_{cat}/K_m was calculated considering the propagation of uncertainty by applying the common variance formula (20).

Cloning of *phaAB* and *rcm* genes for hydroxybutyric acid production in *E. coli*. For cloning the genes of beta-ketothiolase (CAJ92573) and NADP-dependent (*R*)-3-hydroxybutyryl-CoA dehydrogenase (acetoacetyl-CoA reductase) (CAJ92574), *phaA* and *phaB*, respectively, genomic DNA was isolated from *C. necator* DSM 428 using the Master Pure DNA purification kit (Biozym). Forward primer PstI_phaAB_F and reverse primer PstI_phaAB_R were applied to amplify a DNA fragment encoding both enzymes using Q5 High Fidelity DNA polymerase (NEB) according to the manufacturer's protocol. The PCR program included incubation for 30 s at 94°C, 30 s at 68°C, and 2.5 min at 72°C for 30 cycles. The resulting fragment was cut with PstI (NEB) and cloned into the pCDFDuet-1 expression vector, and chemocompetent cells of *E. coli* DH5-α were transformed with the final plasmid.

For cloning RCM genes, the extracted genomic DNA of *K. tusciae* strain DSM 2912 was used as a template to amplify DNA fragments either encoding only the mutase genes *rcmA* and *rcmB* or also including the adjacent gene *meaH2* (see Fig. S1 in the supplemental material), encoding a putative G-protein chaperone. Forward primers XbaI_rcmA_F and XbaI_meaH2_F were applied for amplification of fragments *rcmA-rcmB* and *meaH2-rcmA-rcmB*, respectively. As reverse primer, SacI_rcmB_R was used for both reactions. For PCR, the *phaA-phaB* amplification program was used, extending the elongation time to 3.5 min. PCR fragments were cut with XbaI and SacI-HF (NEB) and cloned into expression vector pBBR1MCS-3 (21). The resulting plasmids pBBR1MCS-3 [rcmA-rcmB] and pBBR1MCS-3 [meaH2-rcmA-rcmB] were introduced into *E. coli* DH5-α via transformation.

PhaAB- and RCM-dependent production of 3-hydroxybutyric and 2-hydroxyisobutyric acid in *E. coli*. Production of 3-hydroxybutyric acid was studied in *E. coli* BL21(DE3) and JM109 (DE3) transformed with pCDFDuet-1 [phaA-phaB]. For establishing 2-hydroxyisobutyric acid production, both strains were additionally transformed either with pBBR1MCS-3 [rcmA-rcmB] or with pBBR1MCS-3 [meaH2-rcmA-rcmB]. Strains carrying an empty pBBR1MCS-3 vector functioned as negative control. One colony of each resulting strain was transferred to 50 ml of Luria-Bertani broth containing 50 mg liter^{-1} of streptomycin and, in the case of the pBBR1MCS-3 constructs, 10 mg liter^{-1} of tetracycline. After overnight growth at 37°C and 200 rpm, these cultures were used to inoculate 100 ml of EZ-Rich defined medium containing 2 g liter^{-1} of fructose and the respective antibiotics to yield an initial optical density of 0.1. In the case of the pBBR1MCS-3 constructs, the medium was additionally supplemented with 15 mg liter^{-1} of vitamin B_{12}. Incubation was continued at 37°C and 200 rpm. As pCDFDuet-1 and pBBR1MCS-3 carry a T7 and a *lac* promoter, respectively, 1 g liter^{-1} of lactose was added to induce expression at an optical density of 0.8. Then, cultures bearing only pCDFDuet-1 [phaA-phaB] were supplemented daily with 0.5 g liter^{-1} of lactose and 2 g liter^{-1} of either fructose, gluconic acid, pyruvate, or acetate. Cultures transformed with pCDFDuet-1 and pBBR1MCS-3 plasmids were initially fed once with 3 g liter^{-1} of gluconic acid after induction and then daily supplemented with 0.5 g liter^{-1} of lactose and 2 g liter^{-1} of gluconic acid. The data shown in this study represent the mean values and standard deviations from at least four replicate experiments.

Sequence analysis. The comparison of protein sequences in databases was performed with BLASTP (22). Multiple sequence alignments of B_{12}-dependent acyl-CoA mutases were performed with ClustalW2 (23).

RESULTS

Growth of *Kyrpidia tusciae* DSM 2912 on 2-hydroxyisobutyric acid. When screening for 2-hydroxyisobutyric acid-degrading microorganisms, we also tested the thermophilic Gram-positive *Kyrpidia tusciae* DSM 2912 (16), which has been isolated from a solfatara sample by Bonjour and Aragno (17) as an autotrophic hydrogen-oxidizing bacterium. Surprisingly, strain DSM 2912 grew well on 2-hydroxyisobutyric acid as sole source of carbon and energy. At 55°C, generation times were about 9 h. As the capability of autarkic growth on 2-hydroxyisobutyric acid has previously been associated with HCM activity (7, 8), the three gene clusters of the annotated genome of strain DSM 2912 (NC_014098) predicted to encode B_{12}-dependent mutases (see Fig. S1 in the supplemental material) were inspected. The proteins Btus_1313 and Btus_1314 likely represent the large and small subunits of an MCM, as characteristic key active-site residues are present (10). The Btus_1053 sequence is closely related to those of ICM variants in which the two mutase subunits are fused with the G-protein chaperone MeaI, e.g., sharing 71% identical residues with the IcmF enzyme from *Geobacillus kaustophilus* (24). However, the third putative mutase of strain DSM 2912, consisting of Btus_0469 and Btus_0470, which represent 563-amino-acid acyl-CoA-binding and 132-amino-acid B_{12}-binding mutase subunits A and B, respectively (see Fig. S1C in the supplemental material), seems to be unrelated to known mutase subfamilies. BLASTP analysis revealed only less than 50% identity to sequences of characterized mutases. Based on these findings, we chose this likely HCM candidate, termed RCM in this study, for further characterization.

Kinetic parameters of purified RCM from strain DSM 2912. The genes of the two mutase subunits, *rcmA* and *rcmB*, were cloned and expressed in *E. coli* TOP10 strains. Both subunits were purified with the help of their Strep tags by one-step affinity chromatography. The expression and the molecular weights of the purified proteins were verified via SDS-PAGE (see Fig. S2 in the supplemental material). For the isomerization of 2-hydroxy-

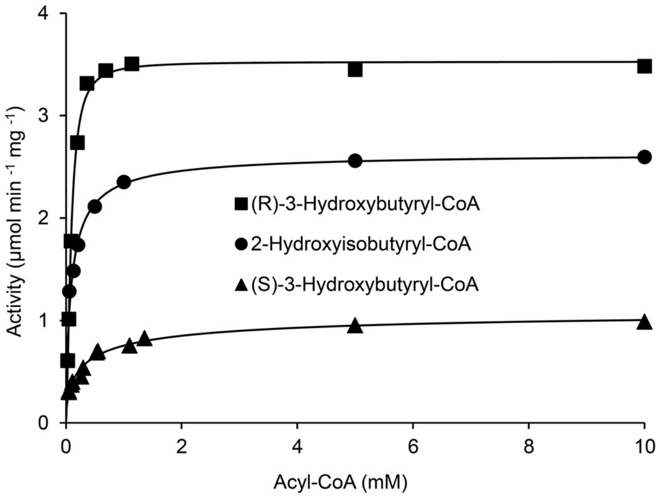

FIG 3 Kinetic plots of acyl-CoA rearrangement activities of reconstituted RcmA and RcmB subunits. Conversion of (*R*)-3-hydroxybutyryl-, 2-hydroxyisobutyryl-, and (*S*)-3-hydroxybutyryl-CoA was measured at pH 7.8 and 55°C.

isobutyryl-CoA, pH and temperature optima of reconstituted mutase subunits RcmA and RcmB were 7.8 and 55°C, respectively (see Fig. S3 in the supplemental material). At 30 and 75°C, activity was reduced to only 25 and 55% of the optimum, respectively, suggesting that the enzyme is suitable for incubation under both mesophilic and thermophilic conditions. The corresponding Arrhenius plot revealed a linear dependence in the temperature range between 30 and 55°C with an activation energy of about 43 kJ mol^{-1} (see Fig. S4 in the supplemental material). In contrast to the previously characterized HCM from strain L108, the purified RCM showed high isomerization activity with all three HCM substrates, i.e., 2-hydroxyisobutyryl-CoA as well as (*R*)-3-hydroxybutyryl- and (*S*)-3-hydroxybutyryl-CoA (Table 2 and Fig. 3). Surprisingly, with a V_{max} of about 3.5 μmol min^{-1} mg^{-1}, (*R*)-3-hydroxybuytryl-CoA was converted 3.1 times faster than the (*S*)-enantiomer, indicating an inverted stereospecificity in the catalysis of RCM compared to HCM. Likewise, rather than (*S*)-3-hydroxybutyryl-CoA, 2-hydroxyisobutyryl-CoA was also preferred, although only with a 2.3-times-higher V_{max}. As RCM also possesses the lowest K_m value for (*R*)-3-hydroxybutyryl-CoA (Table 2), the catalytic efficiency for this substrate is about 11 times higher than for the (*S*)-enantiomer.

Production of 3-hydroxybutyric and 2-hydroxyisobutyric acid in *E. coli* expressing *phaAB* and *rcm* genes. The observed preference of RCM catalysis for the isomerization of (*R*)-3-hydroxybutyryl-CoA to 2-hydroxyisobutyryl-CoA motivated us to establish a 2-hydroxyisobutyric acid synthesis route in *E. coli* by concomitantly expressing the RCM genes and *phaA* and *phaB*, encoding beta-ketothiolase and NADP-dependent (*R*)-3-hydroxybutyryl-CoA dehydrogenase (i.e., acetoacetyl-CoA reductase) from *C. necator* DSM 428 (25). Thus, the complete route from the common metabolite acetyl-CoA via acetoacetyl-CoA and (*R*)-3-hydroxybutyryl-CoA to 2-hydroxyisobutyryl-CoA would be introduced. As already demonstrated previously (26), *E. coli* cultures expressing *phaAB* readily accumulated 3-hydroxybutyric acid. A concentration of 3-hydroxybutyric acid of up to 17.7 mM formed within 8 days of feeding pCDFDuet-1 [phaA-phaB]-transformed strain BL21(DE3) with gluconic acid as the main carbon source (Fig. 4A), while cultures bearing only an empty pCDFDuet-1 did not produce 3-hydroxybutyric acid (data not shown). Similar results were obtained with transformed *E. coli* strain JM109(DE3) (data not shown). However, with both strains expressing *phaAB*, incubation on acetate, pyruvate, and fructose as main carbon sources yielded only up to 2.5, 7.9, and 9.3 mM 3-hydroxybutyric acid, respectively. Therefore, for 2-hydroxyisobutyric acid production via 3-hydroxybutyric acid, *E. coli* strains expressing both the *phaAB* and *rcm* genes were incubated only with gluconic acid besides the inducer lactose. In cultures of BL21(DE3) bearing the pCDFDuet-1 [phaA-phaB] and pBBR1MCS-3 [meaH2-rcmA-rcmB] plasmids, again nearly 18 mM 3-hydroxybutyric acid was produced. In addition, about 0.66 mM 2-hydroxyisobutyric acid accumulated within 8 days (Fig. 4A). The latter compound did not significantly accumulate in cultures bearing only pBBR1MCS-3 [rcmA-rcmB] or pBBR1MCS-3

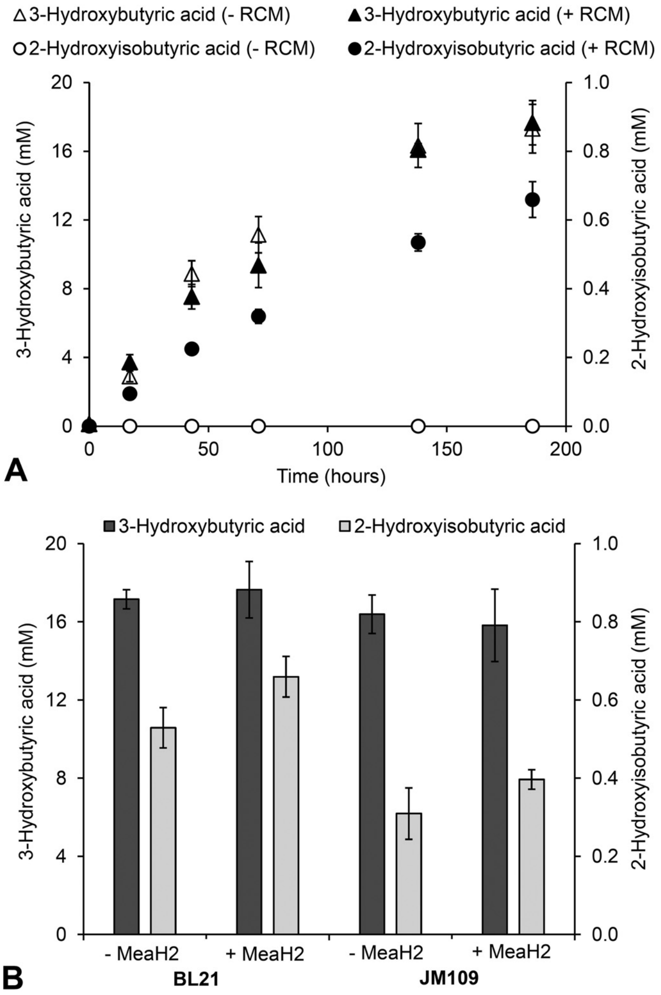

FIG 4 Production of 3-hydroxybutyric and 2-hydroxyisobutyric acids by *phaAB*- and *rcm*-engineered *E. coli* strains incubated on gluconic acid plus lactose at 37°C. (A) Accumulation of hydroxybutyric acids in cultures of pCDFDuet-1 [phaA-phaB]-bearing strain BL21(DE3) without (− RCM) and additionally transformed with pBBR1MCS-3 [meaH2-rcmA-rcmB] (+ RCM). (B) Final hydroxybutyric acid titers after 8 days of incubation found in cultures of pCDFDuet-1 [phaA-phaB]-bearing strains BL21(DE3) and JM109(DE3) additionally transformed with either pBBR1MCS-3 [rcmA-rcmB] (− MeaH2) or pBBR1MCS-3 [meaH2-rcmA-rcmB] (+ MeaH2).

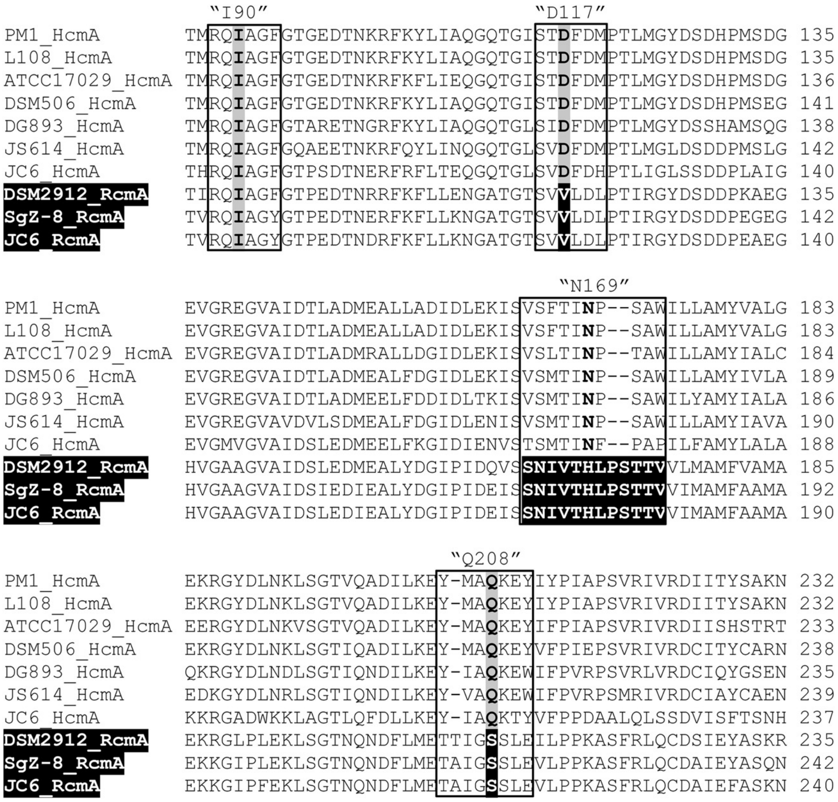

FIG 5 ClustalW2 alignment of HcmA-like sequences from *A. tertiaricarbonis* L108 (AFK77668), *M. petroleiphilum* PM1 (Mpe_B0541), *R. sphaeroides* ATCC 17029 (Rsph17029_3657), *S. novella* DSM 506 (Snov_2770), *M. algicola* DG893 (MDG893_09606), *Nocardioides* sp. strain JS614 (Noca_2131), *B. massiliosenegalensis* JC6 (HcmA, BMSHG_03085), *K. tusciae* DSM 2912 (Btus_0469), *B. thermotolerans* SgZ-8 (QY97_3034), and *B. massiliosenegalensis* JC6 (RcmA, BMSHG_03065). Four regions of active-site residues likely involved in determining substrate specificity are highlighted (boxes). Gray shading indicates residues highly conserved in HCM, while black shading marks sequences presumed to be specific for RCM-type enzymes.

[meaH2-rcmA-rcmB] (data not shown), indicating that expression of both *phaAB* and RCM mutase genes are necessary to produce 2-hydroxyisobutyric acid in *E. coli*. Cultures of BL21(DE3) with *phaAB* and *rcmAB* accumulated about 25% less 2-hydroxyisobutyric acid than the BL21 strain also expressing the gene for the G-protein chaperone MeaH2 (Fig. 4B). The same effect was observed with transformed JM109 strains, producing about 0.4 mM 2-hydroxyisobutyric acid with, and only 0.3 mM without, expression of the *meaH2* gene (Fig. 4B).

DISCUSSION

Investigation of 2-hydroxyisobutyric acid degradation in *K. tusciae* strain DSM 2912 led to the discovery of the new B_{12}-dependent HCM-type mutase RCM. Kinetic parameters of the purified enzyme determined for the isomerization of the three HCM main substrates clearly distinguish RCM from the previously characterized HCM found in strain *A. tertiaricarbonis* L108. Above all, the preference of the new enzyme for the conversion of (*R*)-3-hydroxybutyryl-CoA paves the ground for a biotechnological route to the building block 2-hydroxyisobutyric acid employing the established overflow metabolism for bacterial PHB synthesis.

The catalytic efficiency of RCM for (*R*)-3-hydroxybuytryl-CoA is only about 11 times higher than for the (*S*)-enantiomer, indicating that the stereospecificity is not fully complementary to HCM, by which (*S*)-3-hydroxybutyryl-CoA conversion is nearly 800-fold more efficient (7). Consequently, the active-site architecture of RCM seems not to be an exact mirror image of that of HCM. In the latter, residues Ile^{A90} and Gln^{A208} (numbering as in the HcmA subunit of strain *A. tertiaricarbonis* L108) seem to be characteristic for the HCM subfamily (7, 8). Recently, by elucidating the protein structure of HCM from strain L108 (27), it could be demonstrated that Ile^{A90} allows orientation of Asp^{A117} toward the active site and thus enables formation of hydrogen bonds with the HCM main substrates (*S*)-3-hydroxybutyryl- and 2-hydroxyisobuytryl-CoA. Inspection of homologous residues in a couple of HCM enzymes, in RCM from *K. tusciae* strain DSM 2912, and in closely related mutase sequences from *Bacillus thermotolerans* SgZ-8 and *Bacillus massiliosenegalensis* JC6 revealed that residue Ile^{A90} is conserved in all mutases (Fig. 5). However, Asp^{A117} is changed to Val in RCM-type enzymes, thus reverting the polarity at position 117. Accordingly, an RCM-like but less-pronounced reversion of stereospecificity has recently been obtained with an HCM variant possessing the single point mutation Asp^{A117} to Val^{A117}. Although having the same amino acid residue at position HcmA 117 as found in RCM, conversion of (*R*)-3-hydroxybuytryl-CoA by this enzyme, i.e., HcmA D117V reconstituted with wild-type HcmB, is not as efficient as by RCM, but only a 5-times-higher catalytic efficiency than for the (*S*)-enantiomer has been observed (27). This weaker reversion indicates that not only the HcmA residue at position 117 but likely also substrate interactions with other active site amino acids contribute to stereospecificity in HCM enzymes. In line with this, HcmA subunit

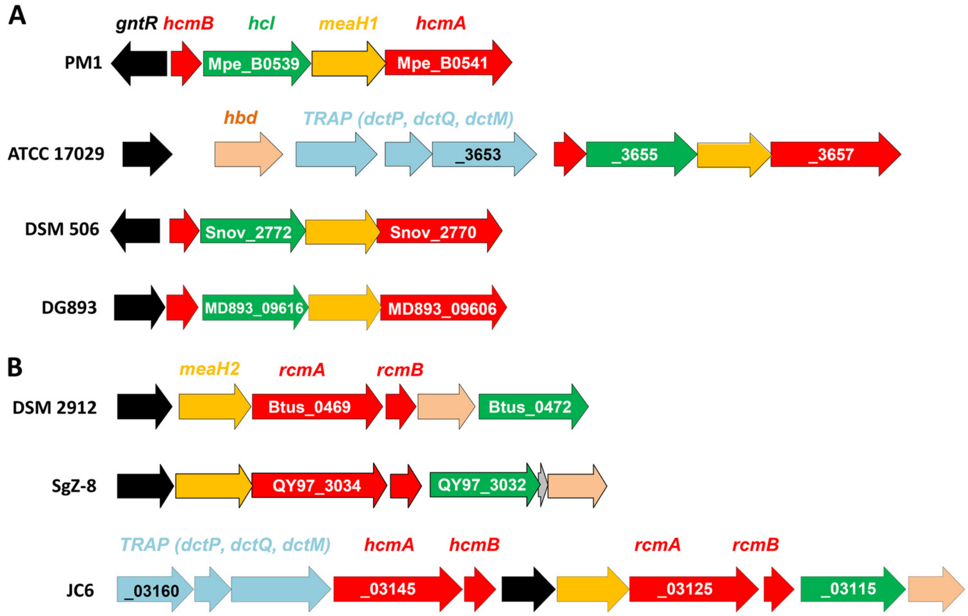

FIG 6 Operonic association of *gntR*, *hcl*, *meaH*, and *hcm* genes. (A) *hcm* gene clusters from *M. petroleiphilum* PM1 (Mpe_B0537 to Mpe_B0541), *R. sphaeroides* ATCC 17029 (Rsph17029_3649 to Rsph17029_3657), *S. novella* DSM 506 (Snov_2774 to Snov_2770), and *M. algicola* DG893 (MD893_09626 to MD893_09606). (B) *rcm* gene clusters from *K. tusciae* DSM 2912 (Btus_0467 to Btus_0472), *B. thermotolerans* SgZ-8 (QY97_3036 to QY97_3030), and *B. massiliosenegalensis* JC6 (BMSHG_RS03160 to BMSHG_RS03110). Annotations: *gntR*, transcriptional regulator; *hcmA* and *hcmB*, subunits of HCM; *rcmA* and *rcmB*, subunits of RCM; *hcl*, 2-hydroxyisobutyrate-CoA ligase; *meaH1* and *meaH2*, HCM-associated G protein chaperone; *hbd*, 3-hydroxybutyryl-CoA dehydrogenase; *TRAP* (*dctP*, *Q*, *M*), TRAP C_4-dicarboxylate transport system.

sequences of HCM and RCM significantly deviate in two other regions, as indicated in Fig. 5. Likely most interesting, Gln^{A208} of HCM is replaced by Ser in RCM. Thus, considering the active-site architecture of HCM (27), changes to Val and Ser at positions 117 and 208, respectively, might result in a preferred binding of (*R*)-3-hydroxybutyryl-CoA as observed for RCM in this study. However, a precise description of the active-site architecture of RCM cannot simply be derived from sequence alignments; models based on crystal structure analysis are required.

Interestingly, when directly comparing HCM and RCM kinetics, the latter is superior for both 3-hydroxybutyryl-CoA enantiomers. With the preferred substrate (*R*)-3-hydroxybutyryl-CoA, RCM catalysis at 55°C is about 28,000-fold more efficient than that of HCM at 30°C. In addition, even the preferred substrate of HCM, (*S*)-3-hydroxybutyryl-CoA, is isomerized about 3 times more efficiently by the new enzyme. Even with the 4.0-fold reduction of $V_{\max}$ at 30°C (see Fig. S3B in the supplemental material), RCM is still about 7,000-fold more efficient for the conversion of the (*R*)-enantiomer, while the catalytic efficiency for the (*S*)-enantiomer is almost equal, with 90 and 75 $\text{mM}^{-1}\,\text{min}^{-1}$ for HCM and RCM, respectively. Consequently, employing RCM in combination with the PHB route seems to be very promising for an efficient biotechnological production of 2-hydroxyisobutyric acid (Fig. 2). Accordingly, *E. coli* strains concomitantly expressing *phaAB* and the *rcm* genes were able to produce 2-hydroxyisobutyric acid, although only at concentrations below 1 mM. The low production rates established thus far may result from the incubation restriction of *E. coli* cultures to 37°C, which is significantly below the temperature optimum of the thermophilic RCM enzyme tested. In addition, competing metabolic routes, e.g., thioesterase II (TesB) activity hydrolyzing 3-hydroxybutyryl-CoA (28, 29), might diminish product yield in *E. coli*.

In contrast to a previous study on *phaAB*- and *tesB*-engineered *E. coli* strains reporting that strain K-12 derivative MG1655 is a better (*R*)-3-hydroxybutyric acid producer than B strain BL21 (30), we found to our surprise that 3-hydroxybutyric acid titers were almost identical in BL21 and K-12-derived strain JM109 (Fig. 4B). However, 2-hydroxyisobutyric acid production in the latter was nearly 1.7 times higher than in the former. This may indicate that, at least in the case of mutase-dependent production, the better expression performance of heterologous genes in B strains (31) seems to be more important than metabolic differences, e.g., distribution of precursors, such as acetyl-, acetoacetyl-, and 3-hydroxybutyryl-CoA, in B- and K-12-derived *E. coli* strains (30). Furthermore, not only efficient expression of the mutase genes but also other variables may influence the *in vivo* rearrangement activity. In this connection, it has been previously reported that 2-hydroxyisobutyric acid production in a *phaAB*-bearing *E. coli* strain expressing *hcmAB* and additionally the putative G protein chaperone *meaH1* significantly improves rates and titers (32). However, in our study the corresponding chaperone MeaH2 seems not to improve RCM activity, as only a slight increase of about 20 to 30% in product formation was observed in BL21 as well as in JM109 (Fig. 4B).

As RCM isomerizes both enantiomers of 3-hydroxybutyryl-CoA with high efficiency, not only PHB overflow metabolism but also other metabolic routes delivering the 2-hydroxyisobutyryl-CoA mutase substrate could be applied (Fig. 2), such as the oxidation of butyric acid, which has already been tested for the HCM-dependent building block production in *C. necator* DSM 428 (13). Considering the temperature optimum of RCM, however, expression in a thermophilic production strain should be favored in the future to tap the full potential of the enzyme.

The occurrence of two HCMs with different stereospecificities is quite surprising. For 2-hydroxyisobutyric acid degradation alone, HCM would be sufficient, as the (*S*)-3-hydroxybutyryl-CoA produced could be readily assimilated or dissimilated by metabolic sequences present in almost all living beings. BLAST analysis detected HCM in 24 bacterial strains (see Table S1 in the supplemental material), while RCM is present only in strains DSM 2912, SgZ-8, and JC6, and two other *Bacillaceae* strains. Sharing only less than 44% identical residues, the two mutases are not closely related, which rules out a gene duplication event as a common origin. More likely, HCM and RCM descended from different mutases, i.e., thermophilic archaeal and bacterial MCMs, respectively (see Table S1 in the supplemental material). However, in line with catalyzing carbon skeleton rearrangement of the same branched acyl-CoA ester, genes encoding putative 2-hydroxyisobutyryl-CoA synthetase (HCL), G-protein chaperone MeaH, and a GntR-like transcriptional regulator seem to be well conserved in all *hcm* gene clusters (Fig. 6). This indicates that at least other functions essential for 2-hydroxyisobutyric acid degradation, e.g., specific CoA activation of the free carboxylic acid and regulation of *hcm* gene expression, might have the same origin. In *R. sphaeroides* ATCC 17029 and in *B. massiliosenegalensis* JC6, *hcm* gene clusters are flanked by genes of the tripartite ATP-independent periplasmic (TRAP) C_4-dicarboxylate transport system (33), indicating that a DctPQM permease may be involved in 2-hydroxyisobutyric acid uptake in these strains. Surprisingly, besides the *rcm* cluster, strain JC6 possesses also the *hcm* genes; however, these are not directly associated with *meaH* and *hcl* genes (Fig. 6B). This "mixed" *hcm* and *rcm* gene cluster might represent an evolutionary snapshot still showing characteristics of both mutase clusters due to acquisition of genes from different sources.

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