Ethylmalonyl-CoA Mutase from *Rhodobacter sphaeroides* Defines a New Subclade of Coenzyme B_{12}-dependent Acyl-CoA Mutases*[^5]

Received for publication, July 18, 2008, and in revised form, September 23, 2008 Published, JBC Papers in Press, September 25, 2008, DOI 10.1074/jbc.M805527200

Tobias J. Erb[^1], Janos Rétey[^1], Georg Fuchs[^1], and Birgit E. Alber[^1]

From ^1^Mikrobiologie, Institut für Biologie II, Albert-Ludwigs-Universität Freiburg, Schänzlestrasse 1, 79104 Freiburg im Breisgau, Germany, ^9^Institut für Organische Chemie, Universität Karlsruhe (Technische Hochschule), Gebäude 30.42, Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany, and ^6^Department of Microbiology, The Ohio State University, Columbus, Ohio 43210

Coenzyme B_{12}-dependent mutases are radical enzymes that catalyze reversible carbon skeleton rearrangement reactions. Here we describe *Rhodobacter sphaeroides* ethylmalonyl-CoA mutase (Ecm), a novel member of the family of coenzyme B_{12}-dependent acyl-CoA mutases, that operates in the recently discovered ethylmalonyl-CoA pathway for acetate assimilation. Ecm is involved in the central reaction sequence of this novel pathway and catalyzes the transformation of ethylmalonyl-CoA to methylsuccinyl-CoA in combination with a second enzyme that was further identified as promiscuous ethylmalonyl-CoA/methylmalonyl-CoA epimerase. In contrast to the epimerase, Ecm is highly specific for its substrate, ethylmalonyl-CoA, and accepts methylmalonyl-CoA only at 0.2% relative activity. Sequence analysis revealed that Ecm is distinct from (2R)-methylmalonyl-CoA mutase as well as isobutyryl-CoA mutase and defines a new subfamily of coenzyme B_{12}-dependent acyl-CoA mutases. In combination with molecular modeling, two signature sequences were identified that presumably contribute to the substrate specificity of these enzymes.

Recently we proposed the so-called ethylmalonyl-CoA pathway for acetyl-CoA assimilation by *Rhodobacter sphaeroides*, which lacks isocitrate lyase, the key enzyme of the glyoxylate cycle (3, 4). The new pathway converts three molecules of acetyl-CoA, one molecule of CO_{2}, and one molecule of bicarbonate to the citric acid cycle intermediates malate and succinyl-CoA (see Fig. 1). Initially two molecules of acetyl-CoA are converted into crotonyl-CoA involving steps that are common to polyhydroxybutyrate synthesis. Crotonyl-CoA is further transformed in a unique reaction sequence to β-methylmalyl-CoA that is cleaved in the latter part of the pathway to glyoxylate and propionyl-CoA. Glyoxylate condenses with another molecule of acetyl-CoA to form l-malyl-CoA that is in turn hydrolyzed by a so far unknown thioesterase to malate. Propionyl-CoA is assimilated by carboxylation followed by a carbon skeleton rearrangement step catalyzed by coenzyme B_{12}-dependent (2R)-methylmalonyl-CoA mutase yielding succinyl-CoA. This novel assimilation pathway is not only limited to *R. sphaeroides* but seems to operate in a number of other bacteria that have been reported to lack a functional glyoxylate cycle, like *Methyllobacterium extorquens* and *Streptomyces coelicolor* (3, 4, 6, 7).

The central and characteristic part of the ethylmalonyl-CoA pathway, the conversion of the C_{4} compound crotonyl-CoA to the C_{6} compound β-methylmalyl-CoA, is not fully understood. The first step in this reaction sequence is a reductive carboxylation of crotonyl-CoA to ethylmalonyl-CoA, an unusual reaction catalyzed by crotonyl-CoA carboxylase/reductase (Ccr)^[^2] (4). This study aimed at elucidating the steps following the formation of ethylmalonyl-CoA. Here we report the identification and characterization of two enzymes, ethylmalonyl-CoA epimerase and ethylmalonyl-CoA mutase, that are involved in the carbon rearrangement of ethylmalonyl-CoA to methylsuccinyl-CoA (Fig. 1). Although the epimerase is a promiscuous enzyme acting equally well on ethylmalonyl-CoA and methylmalonyl-CoA, ethylmalonyl-CoA mutase is highly specific for its substrate and distinct from the well studied (2R)-methylmalonyl-CoA mutase. Sequence and structural model comparisons of ethylmalonyl-CoA mutase with known methylmalonyl-CoA mutases and isobutyryl-CoA mutase from *Streptomyces*

[^1]: To whom correspondence should be addressed: Dept. of Microbiology, The Ohio State University, 484 West 12th Ave., Columbus, OH 43210. Tel.: 614-247-4443; Fax: 614-292-8120; E-mail: alber.8@osu.edu

[^5]: This work was supported by Deutsche Forschungsgemeinschaft Grant AL677/1-1 and by Evonik-Degussa GmbH. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[^2]: The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

[^1]: §1 The abbreviations used are: Ccr, crotonyl-CoA carboxylase/reductase; Ecm, ethylmalonyl-CoA mutase; HPLC, high pressure liquid chromatography; LB, Luria-Bertani; kan, kanamycin resistance cassette; Epi, ethylmalonyl-CoA/methylmalonyl-CoA epimerase.
**EXPERIMENTAL PROCEDURES**

**Materials**

Chemicals were obtained from Fluka (Neu-Ulm, Germany), Sigma-Aldrich, Merck, Serva (Heidelberg, Germany), or Roth (Karlsruhe, Germany). Biochemicals were from Roche Diagnostics, Applichem (Darmstadt, Germany), or Gerbu (Craiberg, Germany). Materials for cloning and expression were purchased from MBI Fermentas (St. Leon-Rot, Germany), New England Biolabs (Frankfurt, Germany), Novagen (Schwalbach, Germany), Genaxxon Bioscience GmbH (Biberach, Germany), MWG Biotech AG (Ebersberg, Germany), or Qiagen (Hilden, Germany). Materials and equipment for protein purification were obtained from GE Healthcare or Millipore (Eschborn, Germany). NaH$^{14}$CO$_3$ was obtained from Hartmann Analytic (Braunschweig, Germany).

**Bacterial Strains and Growth Conditions**

*R. sphaeroides* strain 2.4.1 (DSMZ 158) was grown at pH 6.7 and 30 °C aerobically in the dark or in 2-liter bottles anaerobically in the light (3,000 lux) on defined media supplemented with a 10 mM concentration of the appropriate carbon source as described previously (3). Growth was followed by determining the absorbance at 578 nm ($A_{578}$), and cells were harvested in midexponential phase at an optical density of 0.5–1.0. For growth studies, *R. sphaeroides* mutant ecm::kan and wild type were pregrown anaerobically in 10 ml of minimal medium containing 10 mM sodium succinate, and 0.1 ml was transferred to stoppered screw-capped (Hungate) tubes with 10 ml of minimal medium and the appropriate carbon source. The mutant was grown in the presence of 20 μg ml$^{-1}$ kanamycin. *Escherichia coli* strains DH5α,
BL21(DE3), Rosetta 2(DE3), and S17-1/λpir were grown in Luria-Bertani (LB) broth. For conjugation experiments R. sphaeroides was also grown aerobically on LB medium in the dark.

**Syntheses**

Crotonyl-CoA was synthesized from its anhydride (8). Acrylyl-CoA was synthesized from the free acid by the method of Stadtman (9). CoA-esters were quantified by determining the absorption at 260 nm ($\varepsilon = 22,000 \text{ m}^{-1} \text{ cm}^{-1}$) (10), and the purity was analyzed by a previously described HPLC method (4).

**Mutant Construction and Strain Isolation**

Chromosomal DNA from R. sphaeroides was isolated using standard techniques. A fragment containing 500–600 nucleotides flanking regions on either side of the PstI sites within the ecm gene was amplified using the forward primer, 5′-ATG GTA AGC TTC CTG TAG AA AAT TCT GTC TCA AAA TC-3′, to introduce HindIII and XbaI sites (underlined) and the reverse primer, 5′-TCC GGT AC CAG TCT AGA TAT GGG GTA GCT CTT C-3′, to introduce KpnI and XbaI sites (underlined). PCR was performed using Pfu polymerase (Genaxxon Bioscience GmbH) for 25 cycles, including denaturation for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 5 min. The PCR product was isolated and cloned into pUC19 to introduce HindIII and XbaI sites (underlined) and the reverse primer, 5′-AAC TCG AGT TAG AAA AAC TCA TCG AGC-3′, to introduce KpnI and XbaI sites (underlined). PCR was performed using Pfu polymerase (Genaxxon Bioscience GmbH) for 25 cycles, including denaturation for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 5 min. The PCR product was isolated and cloned into pUC19 to obtain plasmid pAS10. A kanamycin resistance cassette (kan) was amplified using forward primer 5′-AAC TCG AGT TAG AAA AAC TCA TCG AGC-3′, reverse primer 5′-TCC GGT AC CAG TCT AGA TAT GGG GTA GCT CTT C-3′, and pUC4KSAC (Promega) as a template. The 159-nucleotide PstI fragment within the ecm gene was replaced by the kanamycin resistance cassette, resulting in plasmid pASK101. The plasmid was digested with XbaI, and the fragment containing the interrupted ecm gene was ligated into pQF200mp18 (11), resulting in plasmid pLS8meAKJ. This plasmid was transferred into R. sphaeroides by conjugation with E. coli S17-1/λpir carrying pLS8meAKJ. Donor and recipient strains were grown on LB medium to midexponential phase ($A_{778} = 0.4–0.5$) with 20 μg ml$^{-1}$ kanamycin and 15 μg ml$^{-1}$ gentamycin in the case of the donor strain. Cells (from a 15-ml culture) were collected by centrifugation at 6,000 × g for 10 min, washed with LB medium, mixed in a 1:1 ratio, and centrifuged again. The pellet was resuspended in 200 μl of LB medium and placed as a single drop on an LB agar plate for mating. After incubation at 30 °C in the dark for 24 h, the cells were suspended in 200 μl of LB medium, diluted from 10$^{-1}$ to 10$^{-6}$, and plated on LB agar with 15 μg ml$^{-1}$ kanamycin (positive selection for crossover event) and 10% sucrose (negative selection for single crossover and donor). Conjugants were picked after 4–6 days of incubation (30 °C anaerobically in the light). The deletion/insertion mutation of the ecm gene was verified by PCR analyses.

**Cell Extracts and Qualitative Enzymatic Measurements**

Cell extracts of R. sphaeroides were prepared by suspending 0.3–0.5 g of cells in 0.5 ml of 20 mM Tris-HCl (pH 7.8) containing 50 μg ml$^{-1}$ DNase I. Glass beads (1.1 g, 0.1–0.25-mm diameter) were added, and the suspension was treated for 9 min at 30 Hz and 4 °C in a mixer mill (Retsch, Haare, Germany). After centrifugation (10 min, 20,000 × g, 4 °C), the supernatant (cell extract, 5–10 mg ml$^{-1}$ protein) was tested for ethylmalonyl-CoA or methylmalonyl-CoA transforming activity. Protein concentrations were determined by the method of Bradford using bovine serum albumin as the standard (12).

**Ethylmalonyl-CoA Mutase Assay**—Radioactive labeled ethylmalonyl-CoA was synthesized in situ from crotonyl-CoA, H$^{14}$CO$_3^-$, NADPH, and recombinant Ccr and subsequently used as substrate: the “substrate mixture” (3.07 ml) contained 76 mM Tris-HCl (pH 7.8), 4.9 mM NADPH, 2.0 mM crotonyl-CoA, 17.7 mM NaHCO$_3$, 0.8 MBq ml$^{-1}$ NaH$^{14}$CO$_3$, and 120 μg of recombinant Ccr. After 10–30 min of incubation at 30 °C, 90 μl of the substrate mixture were added to 20 μl of cell extract protein that had been preincubated for 10 min at 30 °C in the presence of 0.025 μmol of coenzyme B$_{12}$. An aliquot of 30-μl samples was taken at different time points and stopped with 5 μl of 4× KOH to follow the fate of ethylmalonyl-CoA. The samples were incubated for 20 min at 80 °C to hydrolyze the CoA-esters and then acidified with 10 μl of H$_2$SO$_4$. Separation of the free acids was performed with thin layer chromatography on silica gel 60 F$^{254}$ plates (Merck) with CHCl$_3$:acetic acid (5:1, v/v) as solvent and subsequent detection of radioactivity by phosphorimaging. The dependence on coenzyme B$_{12}$ was examined using the suprantant of a 35% ammonium sulfate precipitation of cell extract protein that had been dialyzed overnight against 20 mM Tris-HCl and preincubated for 10 min in the presence (0.025 μmol) or absence of coenzyme B$_{12}$.

**Methylmalonyl-CoA Mutase Assay**—Conversion of methylmalonyl-CoA was measured analogously to ethylmalonyl-CoA transforming activity, replacing crotonyl-CoA by acrylyl-CoA. Non-radioactive labeled ethylmalonate, methylmalonate, methylsuccinate, and succinate were used as standards for thin layer chromatography and were visualized by bromoresol green solution (0.05% w/v) in 1:4 ethanol:water.

**Product Isolation and Identification by NMR**

Cell extract of R. sphaeroides was prepared by suspending 3 g of cells in 6 ml of 20 mM Tris-HCl (pH 7.8) containing 0.1 mg μl$^{-1}$ DNase I. The suspension was passed twice through a French pressure cell at 137 megapascals and 4 °C, and the cell lysate was centrifuged (100,000 × g, 4 °C, 1 h). Coenzyme B$_{12}$ (0.635 μmol) was added to 1.6 ml of the supernatant (cell extract protein, approximately 60 mg ml$^{-1}$) and incubated at 30 °C. 3-Carboxy-13/14C-labeled ethylmalonyl-CoA was produced in a substrate mixture (15.7 ml) that contained 79 mM Tris-HCl (pH 7.8), 5.1 mM NADPH, 2.0 mM crotonyl-CoA, 31.8 mM NaH$^{14}$CO$_3$, 29 kBq ml$^{-1}$ NaH$^{14}$CO$_3$, and 6 mg of recombinant Ccr. After 12 min of incubation at 30 °C, the substrate mixture was combined with the B$_{12}$-incubated cell extract. After incubation for 90 min at 30 °C, 1.6 ml of 4 M KOH were added, and the mixture was incubated for 30 min at 80 °C to hydrolyze the CoA ester into free acids. Protein was removed by adding 25% HCl to pH = 0 followed by a centrifugation step (38,000 × g, 4 °C, 20 min). The free acids were extracted from the supernatant by solvent extraction with ethyl acetate (four times, 1:1, v/v), and the organic phase was concentrated to a volume of 1.5 ml by evaporation. The acids were separated by preparative thin layer chromatography on silica gel 60 F$^{254}$.
plates (Merck) with CHCl₃/acidic acid (5:1, v/v) as solvent and subsequent detection of radioactivity. The radioactive labeled compound co-eluting with methylsuccinate was scratched from the plate. The material was extracted five times with 10 ml of methanol. The organic solvent was concentrated by evaporation and lyophilized overnight, and the remainder was dissolved in MeOH-δ₄ for NMR spectroscopy. NMR spectra were recorded with a Bruker Avance DRX-400 spectrometer at 27 °C. Chemical shifts were recorded and reported in ppm relative to MeOH-δ₄ (δ: δ = 3.31, 13C: δ = 49.15) as internal standard.

Cloning and Heterologous Expression of ecm and epi from *R. sphaeroides* in *E. coli*

The gene encoding ethylmalonyl-CoA mutase (*ecm*) was amplified from *R. sphaeroides* chromosomal DNA by using the forward primer 5′-CCG GAC TAT GCC GCA GAA GGA TAG CCC CTG-3′ introducing an Ndel site (underlined) and the reverse primer 5′-CGG GAA GCT TGG GAT CCT ATT CCG CC-3′ introducing a HindIII site (underlined). PCR was performed with PfuTurbo polymerase (Stratagene, Cedar Creek, TX) for 33 cycles, including denaturation for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 2.5 min. The PCR product was isolated and cloned into pET16b (Invitrogen) to obtain pTE33 for expression of ecm and production of an N-terminal deca-His tag fusion protein. The gene encoding ethylmalonyl-CoA/methylmalonyl-CoA epimerase (*epi*) was amplified with forward primer 5′-GGA GAG CAT ATG ATC GGA CGC and reverse primer 5′-TGC TCG AGC TCC ACG-3′ introducing an NdeI site (underlined) and reverse primer 5′-TGC AAC CAT GTG G-3′ introducing a HindIII site (underlined). PCR was performed with PfuTurbo polymerase (Stratagene, Cedar Creek, TX) for 33 cycles, including denaturation for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 2.5 min. The PCR product was isolated and cloned into pET16b (Invitrogen) to obtain pTE33 for expression of ecm and production of an N-terminal deca-His tag fusion protein. The gene encoding ethylmalonyl-CoA/methylmalonyl-CoA epimerase (*epi*) was amplified with forward primer 5′-GGG GAG CAT ATG ATC GGA CGC CTG AAC CAT GTG G-3′ introducing a HindIII site (underlined) and reverse primer 5′-GCC GGT GAA GCT TGG GAT CCT ATT CCG CC-3′ introducing a HindIII site (underlined) and a PCR program of 34 cycles with denaturation for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 80 s. The PCR product was cloned into pET16b resulting in pTE45 to express an N-terminal deca-His tag fusion protein.

**Purification of Recombinant His-tagged Ecm and Epi**

All purification steps were performed at 4 °C. Frozen cells were suspended in a double volume of 20 mM Tris-HCl (pH 7.8) containing 0.1 mg ml⁻¹ DNase I. The suspension was passed twice through a chilled French pressure cell at 137 megapascals, and the cell lysate was centrifuged (100,000 × g) at 4 °C for 1 h. An aliquot of the supernatant (2–5 ml, 70–100 mg of protein) was applied at a flow rate of 1 ml min⁻¹ onto a 1-ml nickel-Sepharose Fast Flow column (HisTrap FF; Amersham Biosciences) that had been equilibrated with buffer A (20 mM Tris-HCl and 200 mM KCl (pH 7.8)). After application of cell extract, the column was washed with buffer A and buffer A containing 75 mM imidazole at a flow rate of 1 ml min⁻¹ to elute unwanted protein. Recombinant Ecm or Epi was eluted with buffer A containing 500 mM imidazole. The enzymes were desalted and concentrated by ultrafiltration with an Amicon XM 50 (Ecm) or an Amicon YM 10 (Epi) membrane (Millipore, Eschborn, Germany). The protein (8–10 mg) was stored at −20 °C in 10 mM Tris-HCl (pH 7.8) with 50% glycerol. Recombinant Ccr was purified from cell extracts as described before (4).

**Determination of Molecular Mass**

Sodium dodecyl sulfate-polyacrylamide (12.5%) gel electrophoresis was performed as described previously (14). The native molecular mass of Ecm was estimated by gel filtration at a flow rate of 0.4 ml min⁻¹ on a 120-ml Superdex 200 16/60 column (Amersham Biosciences) equilibrated with 10 mM Tris-HCl (pH 7.9) and 125 mM KCl. Ecm eluted with a retention volume of 16.9 ml. Ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and RNase A (13 kDa) were used as molecular mass standards for calibration.

**Quantitative Enzymatic Measurements of Recombinant Enzymes**

Ecm and Epi were measured quantitatively by an HPLC-based assay.

**Ethylmalonyl-CoA Mutase**—Radioactive labeled ethylmalonyl-CoA was synthesized from crotonyl-CoA, H¹⁴CO₃⁻, and recombinant Ccr and subsequently used as substrate. The substrate mixture (1.07 ml) contained 80 mM Tris-HCl (pH 7.8), 3.7 mM NADPH, 1.9 mM crotonyl-CoA, 7.9 mM NaHCO₃, 0.4 MBq ml⁻¹ NaH¹⁴CO₃, and 230 μg of recombinant Ccr. After incubation at 30 °C for 10 min, crotonyl-CoA was completely transformed to ethylmalonyl-CoA as shown by HPLC, and the reaction was stopped by boiling the reaction mixture for 5 min. To start the ethylmalonyl-CoA mutase assay, 212 μl of the substrate mixture were added to 20 μl of a “protein solution” containing 0.12 μmol of coenzyme B₁₂, an excess of partially purified methylmalonyl-CoA racemase fraction from *Propionibacterium freudenreichii* subsp. *shermanii* (15), and 3–6 μg of recombinant Ecm. The reaction was stopped at different time points by removing 50–100 μl samples and adding 5 μl of 20% formic acid; acidification also avoids hydrolysis of the unstable methylsuccinyl-CoA. The samples were diluted with 50 μl of water and centrifuged (15 min, 20,000 × g) to remove denatured protein. The supernatant was analyzed by reversed-phase HPLC on a C₁₈ column (LiChrospher 100, end-capped, 5 μm, 125 × 4 mm; Merck). The column was developed for 7 min under isocratic conditions with 100 mM NaH₂PO₄ (pH 4.0) in 7.5% methanol (v/v) followed by a linear 10-min gradient from 0 to 60% 100 mM sodium acetate (pH 4.2) in 90% methanol (v/v) at a flow rate of 1 ml min⁻¹. Reaction products and standard compounds were detected by UV absorbance with a Waters 996 photodiode array detector. Radioactivity of eluting compounds was also monitored by a Ramona 2000 radioactive monitor (Raytest, Straubenhardt, Germany) connected in series. Retention times were 11.7 min for methylmalonyl-CoA, 12.3 min for succinyl-CoA, 12.5 min for ethylmalonyl-CoA, and 13.1 min for methy-
ethylsuccinyl-CoA (radioactive monitoring). The amount of product formed was calculated from the relative peak area. The apparent $K_m$ value of ethylmalonyl-CoA was determined by varying the end concentration of ethylmalonyl-CoA from 0.12 to 1.8 mM while keeping coenzyme B$_{12}$ at a saturating concentration (506 μM). The apparent $K_d$ of coenzyme B$_{12}$ was determined by varying the end concentration of coenzyme B$_{12}$ from 0.25 to 506 μM at a saturating ethylmalonyl-CoA concentration (1.8 mM). The pH optimum was determined using 16 mM Tris(1.8 mM). The pH optimum was determined using 16 mM Tris (pH 7.8) instead of 80 mM in the substrate mixture. The pH was subsequently adjusted by addition of 92 μl of the substrate mixture to 55 μl of a protein solution containing 0.13 μmol of coenzyme B$_{12}$, excess amounts of ethylmalonyl-CoA racemase from P. freudenreichii subsp. shermanii, 3 μg of recombinant Ecm, and an Ellis and Morrison (16) buffer of constant ionic strength composed of 363 mM N-(2-acetamido)-2-aminoethanesulfonic acid, 189 mM Tris, and 189 mM ethanolamine. The pH value of the buffer was varied from pH 6.0 to 10.0 with HCl or NaOH (0.5 pH units per step).

**Ethylmalonyl-CoA Epimerase**—To measure ethylmalonyl-CoA epimerase activity, recombinant Ecm was used in excess, and the amount of Epi was kept rate-limiting. Also the substrate mixture for the synthesis of ethylmalonyl-CoA (from crotonyl-CoA) was not stopped by boiling to avoid spontaneous epimerization of ethylmalonyl-CoA. Instead 212 μl of the substrate mixture were added directly to 38 μl of a protein solution containing 0.13 μmol of coenzyme B$_{12}$, 0.20 μmol of CoCl$_2$, 0.08 – 0.24 μg of recombinant Epi, and 32 – 64 μg of recombinant Ecm. The apparent $K_m$ value of ethylmalonyl-CoA was examined by varying the end concentration of ethylmalonyl-CoA (0.06 – 0.88 mM) in the assay at a saturating coenzyme B$_{12}$ concentration (506 μM). Additionally NaH$^{14}$CO$_3$ was increased to 3.2 MBq ml$^{-1}$ for the synthesis of ethylmalonyl-CoA by Ccr. To test the influence of divalent cations, EDTA was added to a 1.3 mg ml$^{-1}$ Epi solution to a final concentration of 2 mM and incubated overnight. An aliquot of this solution containing 0.03 – 0.3 μg of Epi was diluted into protein solution (final volume, 38 μl) containing 0.2 μmol of CoCl$_2$, MgCl$_2$, MnCl$_2$, or NiCl$_2$ or no divalent cation before the reaction was started by adding 212 μl of substrate mixture.

**Methylmalonyl-CoA Epimerase**—Methylmalonyl-CoA epimerizing activity of recombinant Epi was measured analogously to ethylmalonyl-CoA epimerizing activity. Crotonyl-CoA was replaced by acrylyl-CoA for substrate synthesis resulting in the formation of radioactively labeled methylmalonyl-CoA. Recombinant methylmalonyl-CoA mutase$^3$ was used in excess, and the amount of Epi (10 – 40 ng) was kept rate-limiting. The apparent $K_m$ value of methylmalonyl-CoA was examined by varying the concentration of methylmalonyl-CoA from 0.06 to 0.88 mM while keeping coenzyme B$_{12}$ at saturating concentrations (506 μM).

**Phylogenetic Analysis**

The genomic BLAST interface (February 13, 2008) at the National Center for Biotechnology Information (NCBI) was used to search 592 fully sequenced genomes with the amino acid sequences of methylmalonyl-CoA mutase (large subunit) from P. freudenreichii subsp. shermanii (NCBI accession number P11653.3), isobutyryl-CoA mutase (large subunit) from S. cinnamononensis (NCBI accession number AAC08713.1), and ethylmalonyl-CoA mutase from R. sphaeroides (NCBI accession number YP_354045). All hits with an expectation value $>e^{-20}$ were controlled for the presence of a substrate binding domain. In absence of either domain, the genomic context was analyzed for the presence of an open reading frame encoding the missing subunit. When both subunits were present, their sequences were linked to a concatamer. All hits where the substrate binding domain was fused to an argK-like domain were excluded because these proteins are not characterized yet and their function as bona fide mutases remains to be shown. The amino acid sequences were aligned using ClustalW as implemented in the BioEdit 7.0.9.0. software package, and the sequences obtained were used for phylogenetic analysis. Phylogenetic trees were constructed using neighbor-joining algorithms as implemented in the TreeConW 1.3b software package or maximum parsimony and distance matrix algorithms as implemented in the PHYLYP 3.67 software package.

**Computational Modeling**

Ethylmalonyl-CoA mutase was modeled using the SWISS-MODEL Automated Comparative Protein Modeling Server (November 6, 2007) with the structure of the methylmalonyl-CoA mutase substrate complex from P. freudenreichii subsp. shermanii (Protein Data Bank code 4REQ) as template. The software packages of DeepView 3.7 and University of California San Francisco Chimera 1 were used to visualize and overlay the structure models.

**RESULTS**

Coenzyme B$_{12}$-dependent Conversion of Ethylmalonyl-CoA to Methylsuccinyl-CoA in Cell Extracts of R. sphaeroides—To follow the fate of ethylmalonyl-CoA in cell extracts of R. sphaeroides, 3-carboxy-$^{14}$C-labeled ethylmalonyl-CoA was produced enzymatically from crotonyl-CoA, H$^{14}$CO$_3$, and NADPH by Ccr from R. sphaeroides and used in subsequent experiments. Cell extracts of acetate-grown R. sphaeroides were able to convert this [3-carboxy-$^{14}$C]ethylmalonyl-CoA into a radioactive labeled product, and the conversion was dependent on the presence of coenzyme B$_{12}$. After hydrolysis of CoA-thioesters, the product of the conversion co-eluted with methylsuccinate on thin layer chromatography plates (Fig. 2) and was isolated from preparative scale using 3-carboxy-$^{14}$C-labeled ethylmalonyl-CoA. One-dimensional ($^1$H, $^{13}$C) and two-dimensional (correlation spectroscopy, heteronuclear multiple bond correlation) NMR experiments confirmed the product as [4-carboxy-$^{13}$C]methylsuccinate, suggesting that a rearrangement of the carbon skeleton had taken place (supplemental Fig. S1). We conclude that a coenzyme B$_{12}$-dependent ethylmalonyl-CoA mutase catalyzed this reaction in cell extracts of R. sphaeroides.

**Insertional Inactivation of ecm, the Putative Ethylmalonyl-CoA Mutase Gene, and Characterization of the**
Ethylmalonyl-CoA Mutase from R. sphaeroides

Mutant ecm::kan—Sequence analysis identified two genes with coenzyme B$_{12}$ binding motifs encoding putative mutases in the complete genome of R. sphaeroides (GenBank™ accession numbers CP000143 and CP000144). One gene (mcm) was located downstream of genes annotated to encode both subunits of propionyl-CoA carboxylase. The corresponding protein (Mcm, NCBI accession number ABA78347) had 61% sequence identity to the well-characterized methylmalonyl-CoA mutase from P. freudenreichii subsp. shermanii (17–20) and was shown to catalyze the rearrangement of methylmalonyl-CoA, the product of propionyl-CoA carboxylation, to succinyl-CoA (3). The other gene (NCBI accession number ABA80144) clustered with ccr and showed 66% amino acid sequence identity to meaA from M. extorquens. meaA was able to rescue a chemical-induced methanol and ethanol assimilation-deficient mutant of M. extorquens (21–24), and mutation of a homologous gene in Streptomyces coelicolor also abolished the assimilation of acetyl-CoA (7). The function of this enzyme in acetyl-CoA assimilation and its physiological substrate, however, is not known. To investigate its function in the ethylmalonyl-CoA pathway of R. sphaeroides, this gene, called ecm (for ethylmalonyl-CoA mutase), was inactivated by homologous recombination. Thereby part of ecm—Sequence analysis identified two genes

FIGURE 2. Coenzyme B$_{12}$-dependent conversion of ethylmalonyl-CoA by cell extract of acetate grown R. sphaeroides. Radioactive labeled ethylmalonyl-CoA was formed in vitro by recombinant Ccr from crotonyl-CoA, NADPH, and H$^{14}$CO$_3$ (t(0)). The reaction was started by cell extract protein of acetate-grown R. sphaeroides (supernatant of a 35% ammonium sulfate fraction dialedyzed overnight) in the presence (+coenzyme B$_{12}$) or absence (−coenzyme B$_{12}$) of coenzyme B$_{12}$. Samples were taken after 90 min and stopped with KOH to hydrolyze the corresponding coenzyme A-thioesters. Free acids were separated by thin layer chromatography, and radioactivity was detected. The positions of non-radioactive labeled authentic ethylmalonate (EM) and methylsuccinate (MS) were detected by bromcresol green solution and are marked.

R. sphaeroides ecm::kan was able to grow with carbon substrates that did not require the operation of the (complete) ethylmalonyl-CoA pathway (succinate, propionate/HCO$_3^-$, or acetate plus glyoxylate) but was unable to use acetate or acetoacetate as the sole carbon source (Fig. 3). The latter compounds rely on the central reactions of ethylmalonyl-CoA (Fig. 1), indicating that ecm is involved on the level of C$_5$ compounds and further supporting the hypothesis that the protein might act as an ethylmalonyl-CoA mutase. The fact that growth on propionate/HCO$_3^-$, which is known to involve methylmalonyl-CoA mutase, was not affected by the mutant ecm::kan suggested that two distinct coenzyme B$_{12}$-dependent mutases are operating in the ethylmalonyl-CoA pathway.

FIGURE 3. Phenotypic characterization of the R. sphaeroides ecm::kan mutant. Growth curves of R. sphaeroides mutant ecm::kan (A) and R. sphaeroides wild type (B) are shown for comparison. Both strains were pregrown on minimal medium with succinate and transferred on minimal medium containing succinate (■), propionate/HCO$_3^-$ (●), acetate plus glyoxylate (●), acetate (■), or acetoacetate (●). For the positions of these organic acids as CoA-thioesters, see Fig. 1.

Expression of ecm and Purification of Ethylmalonyl-CoA Mutase—To study its biochemical properties, ecm was heterologously expressed in E. coli, and the N-terminal His-tagged protein was purified from crude extracts in an one affinity chromatographic step. Denaturing gel electrophoresis revealed the presence of a major protein band (75 kDa; supplemental Fig. S2A) and two additional minor bands with lower molecular masses (66 and 63 kDa; supplemental Fig. S2A). According to...
Ethylmalonyl-CoA Mutase from R. sphaeroides

Ethylmalonyl-CoA Mutase Depends on an Epimerase-like Protein—Purified recombinant Ecm was unable to form methylsuccinyl-CoA from ethylmalonyl-CoA when ethylmalonyl-CoA was produced by reductive carboxylation of crotonyl-CoA and recombinant Ccr. However, addition of cell extract of R. sphaeroides ecm::kan mutant to the enzyme assay restored ethylmalonyl-CoA mutase activity (Fig. 5A). This indicated that the enzyme itself is competent but that an additional factor present in cell extract is required for Ecm to be active. Note that ethylmalonyl-CoA exists in two stereoisomeric forms, one of which is probably selectively formed by Ccr. Hence the situation might be similar to methylmalonyl-CoA mutase that is dependent on methylmalonyl-CoA epimerase to convert (2S)-methylmalonyl-CoA, the product of propionyl-CoA carboxylation, to (2R)-methylmalonyl-CoA, the substrate for Mcm (25, 26). We tested the ability of methylmalonyl-CoA epimerase to accept ethylmalonyl-CoA as a substrate and to complement the activity of Ecm. Indeed addition of a methylmalonyl-CoA epimerase fraction from P. freudenreichii subsp. shermanii to Ecm restored methylmalonyl-CoA converting activity (Fig. 5B), suggesting the involvement of a similar enzymatic activity in the ethylmalonyl-CoA pathway.

Identification, Molecular Expression, and Characterization of Ethylmalonyl-CoA/Methylmalonyl-CoA Epimerase—A homology search in the genome of R. sphaeroides using the amino acid sequence from methylmalonyl-CoA epimerase of P. freudenreichii subsp. shermanii (NCBI accession number AAL57846) identified only one possible candidate annotated to encode glyoxylase I (NCBI accession number ABA79990), an enzyme of structural and functional homology to methylmalonyl-CoA epimerase (27). The gene called epi (for epimerase) was cloned and expressed, and the protein was heterologously produced as a histidine fusion protein in E. coli (supplemental Fig. S2B). The purified protein was able to substitute Propionibacterium epimerase in the overall conversion of crotonyl-CoA/H\(^{13}\)CO\(_3\)\(^{-}\) to methylsuccinyl-CoA using recombinant Ccr and Ecm (data not shown), indicating that Epi acts as an ethylmalonyl-CoA epimerase for R. sphaeroides.

A coupled assay was developed to determine kinetic parameters of Epi. Ethylmalonyl-CoA was synthesized from crotonyl-CoA/H\(^{13}\)CO\(_3\)\(^{-}\) by Ccr and used as substrate with excess amounts of Ecm and rate-limiting amounts of Epi (Fig. 6A). The dependence of Epi for ethylmalonyl-CoA followed Michaelis-Menten kinetics with an apparent \(K_m\) of 40 \(\mu\)M and \(v_{max}\) of 110 units mg\(^{-1}\). To test whether Epi also catalyzes the epimerization of methylmalonyl-CoA, the assay was modified. Methylmalonyl-CoA was synthesized from acrylyl-CoA/H\(^{13}\)CO\(_3\)\(^{-}\) using recombinant Ccr and Ecm was replaced with excess recombinant methylmalonyl-CoA mutase encoded by mcm from R. sphaeroides\(^3\) (Fig. 6B). Interestingly with a \(v_{max}\) of 120 units mg\(^{-1}\) and an apparent \(K_m\) of 80 \(\mu\)M, the kinetic parameters for Epi toward methylmalonyl-CoA are comparable to its kinetic properties with ethylmalonyl-CoA, suggesting that Epi is a promiscuous enzyme catalyzing both isomerization reactions in vivo. The activities of methylmalonyl-CoA epimerases from different sources have been reported to be stimulated by Co\(^{2+}\) (28, 29). Incubation of Epi for 20 min with Co\(^{2+}\) (0.4 mM) increased enzymatic activity 4-fold (440 units mg\(^{-1}\)), whereas incubation of Epi with 2 mM EDTA overnight led to a complete loss of activity. The dependence on divalent cations was studied by incubation of EDTA-inactivated Epi with different metal
Ethylmalonyl-CoA mutase from R. sphaeroides

A. Ethylmalonyl-CoA epimerase activity. Radioactively labeled ethylmalonyl-CoA was synthesized using recombinant Ccr and crotonyl-CoA. The assay was started by addition of ethylmalonyl-CoA mutase and ethylmalonyl-CoA/methylmalonyl-CoA epimerase (+epi). In a control experiment, ethylmalonyl-CoA/methylmalonyl-CoA epimerase was omitted from the assay (−epi). Samples were withdrawn from the assay after 1 min and analyzed by reversed-phase HPLC. The retention times were 2.7 min for H14CO3−, 12.5 min for ethylmalonyl-CoA, and 13.1 min for methylsuccinyl-CoA. The activity of ethylmalonyl-CoA/methylmalonyl-CoA epimerase was quantified from the amount of product formed at several time points.

B. Ethylmalonyl-CoA/methylmalonyl-CoA epimerase activity. Radioactively labeled ethylmalonyl-CoA/methylmalonyl-CoA epimerase activity. In a control experiment, ethylmalonyl-CoA/methylmalonyl-CoA epimerase was omitted (−epi). Samples were taken after 1 (-epi) or 5 min (+epi) and analyzed by reversed-phase HPLC. The retention times were 11.7 min for methylmalonyl-CoA and 12.3 min for succinyl-CoA.

Molecular Properties of Recombinant Ethylmalonyl-CoA Mutase—The newly developed coupled assay (see above) also enabled the kinetic characterization of Ecm using excess amounts of Epi and rate-limiting amounts of Ecm. The specific activity of ethylmalonyl-CoA mutase in cells of R. sphaeroides grown on acetate was 50 nmol min−1 mg−1 of protein. The purified recombinant ethylmalonyl-CoA mutase followed Michaelis-Menten kinetics with apparent \( K_m \) values of 60 μM for ethylmalonyl-CoA. Half-maximal activity for Ecm toward coenzyme \( B_{12} \) was 2 μM. This value can be taken as the dissociation constant because Ecm is only active with coenzyme \( B_{12} \) bound. The maximal specific activity \( (v_{max}) \) was determined as 7 units mg−1, a value that represents a lower limit because truncated and therefore inactive protein is present in the purified enzyme fraction (supplemental Fig. S2A). The enzyme was tested in a pH range of 6.0–10.0 (0.5 pH units/step) and had a broad pH optimum around 6.5–8.0. The calculated molecular mass of Ecm is 74 kDa, and gel filtration chromatography of the native enzyme gave an apparent molecular mass of 153 kDa, suggesting an apparently homodimeric subunit structure (α2). Ethylmalonyl-CoA mutase accepted methylmalonyl-CoA as substrate with only 0.2% relative activity.

Identification of Ethylmalonyl-CoA Mutase Defines a New Subfamily of \( B_{12} \)-dependent Acyl-CoA Mutases—The sequence distance relationship of known acyl-CoA mutases with ethylmalonyl-CoA mutase from R. sphaeroides and closely related proteins was studied. For this analyses, sequences of biochemically characterized (homo- and heterodimeric) methylmalonyl-CoA, isobutyryl-CoA, and 2-hydroxybutyryl-CoA mutases (17, 18, 30–36) or corresponding acyl-CoA mutases from fully sequenced genomes were used in which the physiological role has been reasonably suggested from the genomic context or experimental studies. In the case of heterodimeric methylmalonyl-CoA mutases that are composed of an active α and an inactive β subunit, only the sequences of the catalytically competent α subunit were included in the analysis.

Interestingly all coenzyme \( B_{12} \)-dependent acyl-CoA mutases were clearly clustered according to their function/substrate specificity and not to the proposed phylogenetic positioning of the corresponding species (Fig. 7A; for a more detailed analysis see supplemental Fig. S3). The only exceptions are archaeal methylmalonyl-CoA mutases that appear more closely related to isobutyryl-CoA mutases than to bacterial and eukaryotic/mitochondrial methylmalonyl-CoA mutases.

According to this functional clustering, ethylmalonyl-CoA mutase from R. sphaeroides formed a distinct subclade with proteins encoded by the genomes of various \( \alpha \)-proteobacteria and actinomycetes (Fig. 7B), including MeaA from M. extorquens and Streptomyces sp. (7, 21, 24). In each case, the corresponding gene clustered with a gene likely to encode ccr on the chromosome, and all other genes involved in the ethylmalonyl-CoA pathway were also present in the genomes of these organisms (data not shown). We concluded that all proteins of this subclade are ethylmalonyl-CoA mutases that function in the ethylmalonyl-CoA pathway of the respective bacteria.

Signature Sequences of Ethylmalonyl-CoA Mutase May Have Mechanistic Implications—The identification of the ethylmalonyl-CoA mutase subfamily provided the opportunity to study the molecular basis of the very narrow substrate specificity of acyl-CoA dependent mutases. Primary structure comparisons of methylmalonyl-CoA- and ethylmalonyl-CoA mutases identified two signature sequences that were unique to either...
zyme B12-dependent acyl-CoA mutases.

three-dimensional structure, and functional context in coen-

tributes considerably to the altered substrate specificity,

the histidine to glycine exchange of signature sequence I con-

mental Fig. S5). Moreover the structure model suggested that

CoA complex of

with the structure of a methylmalonyl-CoA succinyl-

sequences and the sequence of human methylmalonyl-CoA mutase; boot-

FIGURE 7. Comparison of coenzyme B12-dependent ethylmalonyl-CoA

mutase homologs. A, neighbor-joining tree for 181 prokaryotic amino acid

sequences and the sequence of human methylmalonyl-CoA mutase; boot-

strap values for 100 samples are given. The sequence of the catalytically inac-

quences and the sequence of human methylmalonyl-CoA mutase; boot-

methylmalonyl-CoA epimerase therefore

raises the question whether broad substrate specificity is a com-

mon feature of this enzyme family. Alternatively the promiscu-

ity observed could reflect a specific feature of those enzymes

involved in the ethylmalonyl-CoA pathway.

Ethylmalonyl-CoA Mutase: a New Acyl-CoA Mutase—In

contrast to the epimerase, ethylmalonyl-CoA mutase is highly

specific for ethylmalonyl-CoA and accepts methylmalonyl-

CoA only with very low activity (0.2%). Ethylmalonyl-CoA

mutase is therefore distinct from its paralog methylmalonyl-

CoA mutase (54% sequence similarity) that is also up-regulated

during growth of R. sphaeroides on acetate (3) and catalyzes the

conversion of methylmalonyl-CoA to succinyl-CoA in later in the

pathway.\(^3\)

Despite their high sequence similarity, ethylmalonyl-CoA

mutase (this study) and methylmalonyl-CoA mutase (19)\(^3\) are

highly specific for their respective substrate; this can be

explained by the radical reaction mechanism of those enzymes.

This mechanism implies the tight binding of the substrate to

the active center of the enzyme to exclude water and to prevent

any unwanted side reaction. The CoA-ester is therefore bound

along a cleft in the substrate binding domain that is associated

with a large conformational change to bury the substrate, close

up the active site, and initiate the radical reaction as shown for

methylmalonyl-CoA mutase (18). Consistent with this model of

“negative catalysis” (40, 41), alteration of the substrate specific-

ity should then require a substantial change in the active site

that otherwise would result in suboptimal binding of the altered

substrate and, therefore, in a non-functional enzyme. A first

proof of principle was provided by changing a specific active

site amino acid in methylmalonyl-CoA mutase to the corre-

sponding residue present in isobutyryl-CoA mutase: the turn-

over number for methylmalonyl-CoA was decreased by 10\(^4\)

(42), and isobutyryl-CoA was accepted by the variant. But

instead of becoming a substrate and being converted into

butyryl-CoA, isobutyryl-CoA acted as a suicide inhibitor

enzyme and located in the central part of the proteins (supple-

mental Fig. S4). Signature sequence I displayed a conserved

exchange of a histidine in Mcm to a glycine in Ecm, whereas an

additional stretch of three amino acids was observed in signature

sequence II, indicating a possible correlation of amino acid

sequence/structure and substrate specificity.

These results were supported by homology modeling of Ecm

with the structure of a methylmalonyl-CoA mutase succinyl-

CoA complex of P. freudenreichii subsp. shermanii as template

(Protein Data Bank code 4REQ; sequence identity, 61%). In this

model, both signature sequences were localized in direct vicin-

ity to the succinyl moiety bound to the active center (supple-

mental Fig. S5). Moreover the structure model suggested that

the histidine to glycine exchange of signature sequence I con-

tributes considerably to the altered substrate specificity,

emphasizing the striking correlation of sequence conservation,

three-dimensional structure, and functional context in coen-

zyme B12-dependent acyl-CoA mutases.

DISCUSSION

Rearrangement of Ethylmalonyl-CoA to Methylsuccinyl-

CoA—The assimilation of C\(_1\) and C\(_2\) compounds via the

recently described ethylmalonyl-CoA pathway depends on

the conversion of ethylmalonyl-CoA into methylsuccinyl-

CoA, a central reaction in the unique reaction sequence from
crotonyl-CoA to \(\beta\)-methylmalyl-CoA (Fig. 1). This challeng-
ing carbon skeleton rearrangement is catalyzed by the com-
bined action of ethylmalonyl-CoA/methylmalonyl-CoA

epimerase and ethylmalonyl-CoA mutase, a new coenzyme

B\(_{12}\)-dependent enzyme described in this study.

Ethylmalonyl-CoA/Methylmalonyl-CoA Epimerase: a Pro-

miscuous Enzyme with a Dual Role in the Ethylmalonyl-CoA

Pathway—The formation of methylsuccinyl-CoA from ethyl-

malonyl-CoA \textit{in vivo} requires not only ethylmalonyl-CoA

mutase but also an additional enzyme that could be identified as

ethylmalonyl-CoA epimerase. This suggests the following reac-
tion sequence in which crotonyl-CoA is first carboxylated to

(2S)-ethylmalonyl-CoA that is then converted by ethylmalonyl-

CoA epimerase into its 2R-stereoisomer. (2R)-Ethylmalonyl-

CoA in turn serves as \textit{bona fide} substrate for ethylmalonyl-CoA

mutase as proposed from its homology to methylmalonyl-CoA

mutase, which has been shown to be specific for (2R)-methyl-

malonyl-CoA (25, 26).

Interestingly ethylmalonyl-CoA epimerase is a promiscuous

enzyme accepting methylmalonyl-CoA with comparable

kinetic properties as a substrate. This implicates a dual role of

ethylmalonyl-CoA/methylmalonyl-CoA epimerase in the ethyl-

malonyl-CoA pathway because the later conversion of propi-

onyl-CoA to succinyl-CoA is dependent on the action of a

methylmalonyl-CoA epimerase (37, 38). The identification of

ethylmalonyl-CoA/methylmalonyl-CoA epimerase therefore

raises the question whether broad substrate specificity is a com-

mon feature of this enzyme family. Alternatively the promiscu-

ity observed could reflect a specific feature of those enzymes

involved in the ethylmalonyl-CoA pathway.

Ethylmalonyl-CoA Mutase: a New Acyl-CoA Mutase—In

contrast to the epimerase, ethylmalonyl-CoA mutase is highly

specific for ethylmalonyl-CoA and accepts methylmalonyl-

CoA only with very low activity (0.2%). Ethylmalonyl-CoA

mutase is therefore distinct from its paralog methylmalonyl-

CoA mutase (54% sequence similarity) that is also up-regulated

during growth of R. sphaeroides on acetate (3) and catalyzes the

conversion of methylmalonyl-CoA to succinyl-CoA in later in the

pathway.\(^3\)
because of irreversible inactivation of the enzyme by uncontrolled electron transfer (42).

Although methylmalonyl-CoA and isobutyryl-CoA are comparable with respect to the C-2 atom where the rearrangement reaction takes place (30), ethylmalonyl-CoA is a more bulky substrate (supplemental Fig. S6). A change in enzyme specificity from methylmalonyl-/isobutyryl-CoA to ethylmalonyl-CoA should therefore require a major change of the active site to tightly bind this bulkier acyl moiety (at the C-2 atom) that would allow a controlled course of the rearrangement reaction. Such an adaptation of ethylmalonyl-CoA mutase to its more spacious substrate may be reflected in both signature sequences that have been identified as part of the active site and are conserved within the subfamily of ethylmalonyl-CoA mutases. Especially the substitution of a conserved histidine (position 328 in Mcm of P. freudenreichii subsp. shermanii) to a less spacious glycine (homologous position 255 in Ecm of R. sphaeroides) in signature sequence I could provide more space for the preferential binding of the additional CH$_3$ group in the acyl moiety of ethylmalonyl-CoA. The situation for signature sequence II is less clear. Here an additional stretch of three amino acids was observed for ethylmalonyl-CoA mutase, possibly resulting in a structural change in this part of the protein. This insertion is accompanied by the exchange of a conserved asparagine (position 366 in Mcm of P. freudenreichii subsp. shermanii) to a proline (homologous position 296 in Ecm of R. sphaeroides). Prolines are known to have a strong impact on the three-dimensional structure of proteins, emphasizing a possible structural difference between ethylmalonyl-CoA and methylmalonyl-CoA mutase. Considering the similarity of methylmalonyl-CoA to isobutyryl-CoA, it is notable that all residues discussed above are conserved between methylmalonyl-CoA mutase and isobutyryl-CoA mutase of S. cinnamonensis (30).

The model of negative catalysis predicts multiple interactions of a substrate with its enzyme to direct the radical reaction and suppress any unwanted side reactions (41, 42). This should in turn require a strict amino acid conservation of the respective protein that would explain why mutases with different substrate specificities strongly cluster together and form distinct subclades (Fig. 7 and supplemental Fig. S3). This also has consequences for the emergence of mutases with novel substrate specificities. A change in substrate specificity would demand several very specific amino acid substitutions together with conservation of the remaining residues, which in evolutionary terms must be an extremely rare event and likely only occurred once. The following scenario is proposed. During a singular event, ethylmalonyl-CoA and isobutyryl-CoA mutases evolved from an ancestral methylmalonyl-CoA mutase and were distributed by lateral gene transfer. Consistent with this is the fact that methylmalonyl-CoA mutases are widely distributed, and their clustering follows phylogenetic lines. An exception is archaical methylmalonyl-CoA mutases, which are located in the isobutyryl-CoA mutase cluster. The only reported function of these methylmalonyl-CoA mutases so far is the involvement in a novel pathway of CO$_2$ fixation (43). One possibility is that an archaical methylmalonyl-CoA mutase has evolved from an isobutyryl-CoA mutase-like ancestor.

**Ethylmalonyl-CoA Mutase from R. sphaeroides**

**Occurrence and Proposed Function of Ethylmalonyl-CoA Mutase**—Genes coding for ethylmalonyl-CoA mutases were found in completely sequenced and fully assembled genomes of a number of bacteria belonging to $\alpha$-proteobacteria, streptomycetes, and two *Leptospira* species (for a detailed list see Fig. 7B). With the exception of the *Frankia* and *Leptospira* species, all bacteria also contain copies of genes encoding the other characteristic enzymes of the ethylmalonyl-CoA pathway: crotonyl-CoA carboxylase/reductase (4), meサconyl-CoA hydratase (3, 5), 1-malyl-CoA/$\beta$-methylmalonyl-CoA lyase (44), and an acyl-CoA dehydrogenase proposed to encode methylsuccinyl-CoA dehydrogenase. The presence of all those genes suggests a functional ethylmalonyl-CoA pathway in these organisms. In addition, Ccr activity has already been demonstrated in selected representatives, such as *R. sphaeroides*, *M. extorquens*, and *S. coelicolor*, grown on substrates requiring a glyoxylate cycle-independent assimilation pathway (4). In the case of *M. extorquens*, a well known type II methylotroph lacking the glyoxylate cycle (6, 23, 45), the ethylmalonyl-CoA pathway is part of C$_1$ assimilation, whereas in *S. coelicolor* it functions in the assimilation of butyrate.

In all the bacteria mentioned above, the genes encoding Ecm and Ccr are always clustered on the chromosome, whereas the other genes involved in the pathway are not necessarily grouped. For some *Frankia*, *Salinospora*, and *Streptomycetes* species, additional copies of ccr genes are present elsewhere on the genome and are not clustered with ecm but with genes encoding polyketide synthases. The corresponding enzymes likely provide ethylmalonyl-CoA as precursors for the biosynthesis of secondary metabolites, including antibiotics (4, 46, 47). In these cases, ecm is absent from the gene clusters because methylsuccinyl-CoA does not represent an $\alpha$-alkylmalonyl-CoA ester and, therefore, does not serve as an extender unit for polyketide synthesis (39, 48). The presence of ecm in a given genomic context clustering together with ccr therefore strongly indicates an operating acetate assimilation pathway and makes ecm more than ccr a specific marker for the presence of the complete ethylmalonyl-CoA pathway in an organism.

In this context it is noteworthy that genes encoding putative Ecm and Cccr are present in the genomes of the *Frankia* and *Leptospira* species listed above, but there they are not grouped in an apparent genetic entity. Furthermore obvious candidates for genes of other characteristic enzymes of the ethylmalonyl-CoA pathway are also missing. Therefore, the possible involvement of ethylmalonyl-CoA mutase in other metabolic pathways in these bacteria as well as the substrates, requiring the ethylmalonyl-CoA pathway in all organisms listed above, remains to be elucidated.

**Acknowledgments**—We thank J. Quandt (Vegreville, Alberta, Canada) for providing plasmid pJ200mp18, Srijan Satagopan for help in structure modelling, and Jasmin Schleimer and Katharina Pfeil for technical assistance. We also thank Michael Müller and Volker Brecht, both from the Institut für Pharmazeutische Wissenschaften, Albert-Ludwigs Universität Freiburg (Freiburg, Germany), for NMR spectroscopy and stimulating discussions.
REFERENCES

1. Kornberg, H. L. (1965) *Angew. Chem. Int. Ed. Engl.* 4, 558–565
2. Kornberg, H. L., and Krebs, H. A. (1957) *Nature* 179, 988–991
3. Alber, B. E., Spanheimer, R., Ebenau-Jehle, C., and Fuchs, G. (2006) *Mol. Microbiol.* 61, 297–309
4. Erb, T. I., Berg, I. A., Brecht, V., Müller, M., Fuchs, G., and Alber, B. E. (2007) *Proc. Natl. Acad. Sci. U. S. A.* 104, 10631–10636
5. Barzczek, J., Schlachtig, A., Strychalski, N., Müller, M., Alber, B. E., and Fuchs, G. (2008) *J. Bacteriol.* 190, 1366–1374
6. Anthony, C. (1982) The *Biochemistry of Methylotrophs*, p. 103, Academic Press, London
7. Han, L., and Reynolds, K. A. (1997) *Eur. J. Biochem.* 259, 142, 128, 107–115
8. Manso, F., Smith, G. A., and Evans, P. R. (1999) *J. Biol. Chem.* 274, 9212–9221
9. Miyamoto, E., Watanabe, F., Charles, T. C., Yamaji, R., Inui, H., and Nakano, Y. (2003) *Arch. Microbiol.* 180, 151–154
10. Ramirez, J., Breuer, U., Benndorf, D., Lechner, U., and Müller, R. H. (2006) *Appl. Environ. Microbiol.* 72, 4128–4135
11. Taoka, S., Padmakumar, R., Lai, H. W., Liu, H. W., and Banerjee, R. (1994) *J. Biol. Chem.* 269, 31630–31634
12. Thomas, N. H., Evans, P. R., and Leadlay, P. F. (2000) *Biochemistry* 39, 9212–9221
13. Vrijbloed, J. W., Zerbe-Burkhardt, K., Ratnatileke, A., Grubelink-Leister, A., and Robinson, J. A. (1999) *J. Bacteriol.* 181, 5600–5605
14. Allen, S. H., Kellermeyer, R., Sternholm, R., Jacobson, B., and Wood, H. G. (1963) *J. Biol. Chem.* 238, 1637–1642
15. Korotkova, N., Chistoserdova, L., Kuksa, K., and Lidstrom, M. E. (2002) *J. Bacteriol.* 184, 1750–1758
16. Xue, Q., Ashley, G., Hutchinson, C. R., and Santi, D. V. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 11740–11745
17. Retey, J. (1990) *Angew. Chem.* 102, 373–379
18. Vrijbrodt, J. W., Zerbe-Burkhardt, K., Ratnatileke, A., Grubelink-Leister, A., and Robinson, J. A. (1998) *Biochemistry* 37, 558–565
19. Meister, M., Saum, S., Alber, B. E., and Fuchs, G. (2005) *J. Bacteriol.* 187, 1415–1425
20. Chistoserdova, L., Chen, S. W., Lapidus, A., and Lidstrom, M. E. (2003) *J. Bacteriol.* 185, 2980–2987
21. Liu, H., and Reynolds, K. A. (1999) *J. Bacteriol.* 181, 6806–6813
22. Wu, K., Chung, L., Revill, W. P., Katz, L., and Reeve, C. D. (2000) *Gene (Amst.)* 251, 81–90
23. Hopwood, D. A., and Sherman, D. H. (1990) *Annu. Rev. Genet.* 24, 37–66

**Ethylmalonyl-CoA Mutase from R. sphaeroides**