Synthesis of C_5-dicarboxylic acids from C_2-units involving crotonyl-CoA carboxylase/reductase: The ethylmalonyl-CoA pathway

Tobias J. Erb*, Ivan A. Berg*†, Volker Brecht‡, Michael Müller‡, Georg Fuchs*, and Birgit E. Alber*§

*Mikrobiologie, Institut für Biologie II and ‡Pharmazeutische und Medizinische Chemie, Fakultät für Chemie, Pharmazie und Geowissenschaften, Albert-Ludwigs-Universität Freiburg, Schänzlestrasse 1, 79104 Freiburg, Germany

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Fifty years ago, Kornberg and Krebs established the glyoxylate cycle as the pathway for the synthesis of cell constituents from C_2-units. However, since then, many bacteria have been described that do not contain isocitrate lyase, the key enzyme of this pathway. Here, a pathway termed the ethylmalonyl-CoA pathway operating in such organisms is described. Isotopically labeled acetate and bicarbonate were transformed to ethylmalonyl-CoA by cell extracts of acetate-grown, isocitrate lyase-negative *Rhodobacter sphaeroides* as determined by NMR spectroscopy. Crotonyl-CoA carboxylase/reductase, catalyzing crotonyl-CoA + CO_2 + NADPH → ethylmalonyl-CoA$^-$ + NADP$^+$ was identified as the key enzyme of the ethylmalonyl-CoA pathway. The reductive carboxylation of an enoyl-thioester is a unique biochemical reaction, unprecedented in biology. The enzyme from *R. sphaeroides* was heterologously produced in *Escherichia coli* and characterized. Crotonyl-CoA carboxylase/reductase (or its gene) can be used as a marker for the presence of the ethylmalonyl-CoA pathway, which functions not only in acetyl-CoA assimilation. In *Streptomyces* sp., it may also supply precursors (ethylmalonyl-CoA) for antibiotic biosynthesis. For methylotrophic bacteria such as *Methylobacterium extorquens*, extension of the serine cycle with reactions of the ethylmalonyl-CoA pathway leads to a simplified scheme for isocitrate lyase-independent C_1 assimilation.

acetyl-CoA assimilation | glyoxylate cycle | methylotrophy | polyketide | serine cycle

This year marks the 50th anniversary of the discovery of the glyoxylate cycle by Kornberg and Krebs (1). This anaplerotic reaction sequence enables an organism to use substrates, which enter the central carbon metabolism on the level of acetyl-CoA, as sole carbon source. Examples of such substrates are fatty acids, alcohols, and esters, including various fermentation products, but also waxes, alkenes, and methylated compounds. Originally delineated for bacteria, the glyoxylate cycle is also required for the metabolism of storage oil by plants during germination of seedlings (2) and for the conversion of triacylglycerols to carbohydrates in developing eggs of nematodes (3). Isocitrate lyase, the first key enzyme of the glyoxylate cycle, together with enzymes of the citric acid cycle, is responsible for the oxidation of acetyl-CoA to glyoxylate (Fig. 1). The second key enzyme, malate synthase, condenses glyoxylate and another molecule of acetyl-CoA to malate. The subsequent oxidation of malate regenerates the initial acetyl-CoA acceptor molecule oxaloacetate in the citric acid cycle. Therefore, any intermediate of the citric acid cycle can be withdrawn from the cycle and used for cell carbon biosynthesis.

However, the glyoxylate cycle cannot be the sole solution for acetyl-CoA assimilation, because several organisms that require such an anaplerotic reaction sequence lack isocitrate lyase activity (4–8) or show a labeling pattern after growth on acetate inconsistent with the operation of the glyoxylate cycle in acetate assimilation (9, 10). These organisms include many purple nonsulfur bacteria, for example *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* (4, 5, 11), and other α-proteobacteria, like the methylotroph *Methylobacterium extorquens* (6, 12), the facultative denitrifier *Paracoccus versutus* (7), and, inferred by genome sequence analysis, several aerobic anoxygenic phototrophs (13). In addition, the diverse group of streptomycetes uses an alternate, isocitrate lyase-independent route for acetyl-CoA assimilation, and there appears to be a direct link to antibiotic biosynthesis (8, 14).

We have recently shown that acetate assimilation by *R. sphaeroides* requires the conversion of a C_4-compound, acetoacetyl-CoA, derived from two acetyl-CoA molecules, to the C_5-compound mesaconyl-CoA (13). β-Methylmalyl-CoA, formed by hydration of mesaconyl-CoA, is cleaved to glyoxylate and propionyl-CoA. Condensation of glyoxylate and another molecule of acetyl-CoA yields malate; propionyl-CoA is carboxylated and yields succinate. The connecting steps between the C_4- and C_5-branch of the postulated pathway have not been elucidated but had been proposed to include a carboxylation step (13). This study aimed at determining the substrate and product of this carboxylation step. Much to our surprise, an enzyme was present in cell extracts of *R. sphaeroides*, which catalyzed an ATP-independent reductive carboxylation of the enoyl-CoA ester, crotonyl-CoA. The gene encoding this enzyme, representing the key reaction in the proposed pathway for acetyl-CoA assimilation, was identified and heterologously expressed, and the enzyme was studied.

Results

Carboxylation Activities in Cell Extracts of *Rhodobacter sphaeroides*. Incorporation of [^{14}C]bicarbonate by cell extracts of aerobically acetate-grown *R. sphaeroides* in the presence of different C_4-thioesters of CoA was examined. Given that mesaconyl-CoA, the first identified C_5-compound in the pathway, is expected to be reductively formed from two molecules of acetyl-CoA and CO_2, NADPH was added as a source of reducing equivalents. Acetoacetyl-CoA (0.08 U mg^{-1}, where U is μmol min^{-1}) as well as (*R*)-3-hydroxybutyryl-CoA- (0.2 U mg^{-1}) dependent [^{14}C]bicarbonate fixation was found, which required NADPH but, surprisingly, was independent of ATP. The NADPH-dependent carboxylation activity with crotonyl-CoA was severalfold higher

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Abbreviation: U, μmol min^{-1}.

†On leave from the Department of Microbiology, Moscow State University, Moscow, Russia.

§To whom correspondence should be sent at the present address: Department of Microbiology, Ohio State University, 484 West 12th Avenue, Columbus, OH 43210. E-mail: alber.8@osu.edu.

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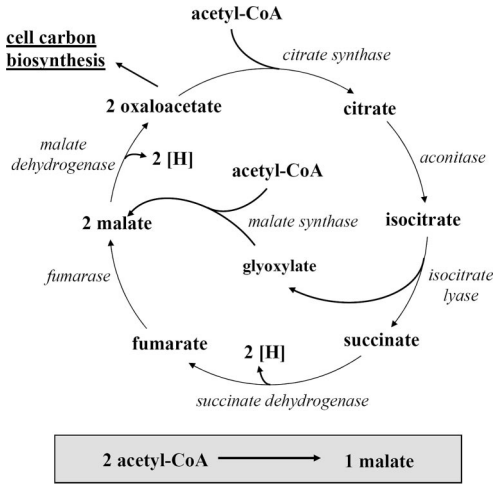

Fig. 1. The glyoxylate cycle as proposed by Kornberg and Krebs 50 years ago. The citric acid cycle is modified to bypass the two decarboxylation steps by the action of its two key enzymes isocitrate lyase and malate synthase. This allows the net synthesis of malate from two molecules of acetyl-CoA.

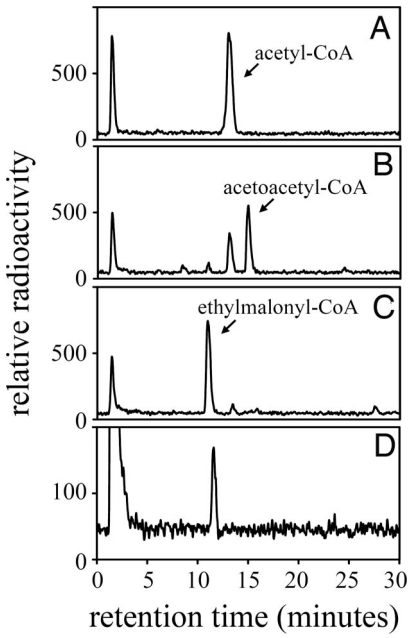

Fig. 2. HPLC analysis of CoA thioesters formed from acetyl-CoA and $NaHCO_3$ by cell extracts of *R. sphaeroides* in the presence of NADPH at 30°C. [U-^{14}C]acetyl-CoA was synthesized by incubation of 1 mM [U-^{14}C]acetate (320 kBq ml^{-1}), 4 mM ATP, 1 mM CoA, 5 mM KCl, and 4 mM $MgCl_2$ with 6 U·ml^{-1} of acetyl-CoA synthetase in 70 mM Tris·HCl buffer (pH 7.9). (*A*) The reaction was started by addition of 0.5 mM 5–5′-dithiobis(2-nitrobenzoic acid), 35 mM $NaHCO_3$, 6.5 mM NADPH, and 0.8 mg of cell-extract protein. (*B* and *C*) After 5 min (*B*) and 45 min (*C*), incubation samples were withdrawn from the assay mixture and analyzed by reverse-phase HPLC. (*D*) Products formed in the presence of $NaH^{14}CO_3$ (480 kBq·ml^{-1}) after 45 min incubation; unlabeled acetate was used. Products were identified by MS-HPLC, and the elution times were as follows: free CoA, 6.2 min; ethylmalonyl-CoA, 10.6 min; acetyl-CoA, 12.6 min; acetoacetyl-CoA, 14.6 min; crotonyl-CoA, 23 min; and butyryl-CoA, 25 min.

(1.1 U mg^{-1}). Butyryl-CoA was carboxylated only in the presence of ATP, albeit with very low rates (0.005 U mg^{-1}). The butyryl-CoA carboxylase activity was inhibited by avidin and, by partial purification of the enzyme, found to be a side activity of the ATP- and biotin-dependent propionyl-CoA carboxylase, which is needed in a later part of the pathway.

Determination of Products of the Carboxylation Reaction in Cell Extracts of *R. sphaeroides*. Products of the carboxylation reaction were analyzed by reversed-phase high-performance liquid chromatography (HPLC) with ^{14}C monitoring (Fig. 2). [U-^{14}C]acetyl-CoA, NADPH, and bicarbonate were incubated with cell extracts of *R. sphaeroides* at 30°C. After 45 min of incubation, a prominent ^{14}C-labeled CoA-thioester was formed; this compound was derived from a transient product formed after 5 min of incubation (Fig. 2 *B* and *C*). Identification of the CoA-esters was based on cochromatography with standards (acetoacetyl-CoA, ethylmalonyl-CoA, and butyryl-CoA) and on the detection of their molecular masses by using HPLC-MS. The transient product was identified as acetoacetyl-CoA with a mass of 851 Da, the second product was identified as ethylmalonyl-CoA with a mass of 881 Da. When [^{14}C]bicarbonate was incubated with unlabeled acetyl-CoA, ethylmalonyl-CoA was the sole labeled product formed (Fig. 2*D*). To confirm the identity of ethylmalonyl-CoA as the product of the reductive carboxylation reaction, cell extract of *R. sphaeroides* was incubated with [^{13}C]bicarbonate and [U-^{13}C]acetyl-CoA in the presence of NADPH for 30 min. CoA-esters were isolated from the reaction mixture by using solid-phase extraction and analyzed by 2D NMR spectroscopy [supporting information (SI) Fig. 6]. The major compound identified was characterized through ^{13}C NMR-signals (SI Fig. 6 *A* and *B*) at 198.7 ppm [C = O, doublet (d), J = 44.4 Hz], 174.7 (C = O, d, J = 48.8 Hz), 65.2 [CH, multiplet (m)], 24.6 [CH_2, doublet of doublets (dd), J = 33.6 Hz], and 12.6 (CH_3, d, J = 35.2 Hz). The corresponding signals in the proton NMR (SI Fig. 6*C*) at 4.3 ppm (CH, m), 1.9 (CH_2, m), and 0.9 (CH_3, m) were identified through a gHSQC experiment (SI Fig. 6*E*). An INADEQUATE experiment verified the proposed structure of a 2-ethylmalonate-thioester (SI Fig. 6*F*). The incorporation of [^{13}C]bicarbonate was ≈80%, deduced from additional ^{13}C NMR signals at 174.7 ppm and 24.6 (d, J = 34.6 Hz) (SI Fig. 6*B*). Minor amounts of butyryl-CoA and 3-hydroxybutyryl-CoA were identified through ^{13}C NMR. Moreover, at least three more compounds showing the characteristic doublet signal (J ≈ 50 Hz) in the range of 173–179 ppm gave a hint for carboxylated derivatives. All of these gave a cross signal to signals at 40 ppm, suggesting the presence of acetyl-CoA and derivatives.

Identification of Crotonyl-CoA Carboxylase/Reductase. Crotonyl-CoA was the best substrate for NADPH-dependent carboxylation in cell extracts of *R. sphaeroides*, suggesting that it might be the direct carboxylation substrate. Crotonyl-CoA reductase (encoded by the gene *ccr*) has been previously purified from *Streptomyces collinus* and shown to be involved in assimilation of C_1- and C_2-compounds (8, 15). Mutation of the *ccr*-like gene in *M. extorquens* resulted in the inability to oxidize acetyl-CoA to glyoxylate (16). To test whether crotonyl-CoA reductase not only catalyzes the reduction of crotonyl-CoA to butyryl-CoA as suggested (15) but, rather, the reductive carboxylation of crotonyl-CoA to ethylmalonyl-CoA, the *ccr*-like gene of *R. sphaeroides* was heterologously expressed in *Escherichia coli*. Cell extract of recombinant *E. coli* catalyzed the crotonyl-CoA-dependent oxidation of NADPH in the presence of bicarbonate (Fig. 3*B*). No activity was found in the presence of either acetoacetyl-CoA or (*R*)-3-hydroxybutyryl-CoA. The enzyme (120 mg) was purified from 9 g of *E. coli* cells in two steps by using DEAE-Sepharose and Cibacron Blue chromatography (Fig. 3*A*). The purified protein catalyzed the fixation of [^{14}C]-bicarbonate in the presence of NADPH and crotonyl-CoA with a specific activity of 103 units mg^{-1} and was named crotonyl-CoA carboxylase/reductase; its properties are summarized in SI Table 1. Gel-filtration chromatography of the native enzyme gave a

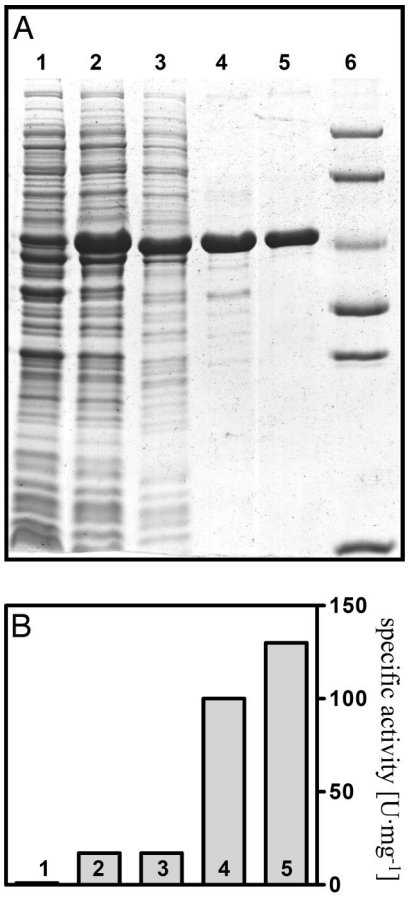

Fig. 3. Recombinant crotonyl-CoA carboxylase/reductase. (*A*) Denaturing PAGE of various steps during purification. Lane 1, 25 μg of *E. coli* cell-extract protein before induction; lane 2, 25 μg of *E. coli* cell-extract protein after 4 h of induction; lane 3, 20 μg of protein after ultracentrifugation; lane 4, 15 μg of protein from the DEAE column step; lane 5, 8 μg of protein from the Cibacron Blue column step; lane 6, molecular mass marker (97 kDa, phosphorylase B; 67 kDa, BSA; 45 kDa, ovalbumin; 34 kDa, lactate dehydrogenase; 29 kDa, carbonic anhydrase; 14 kDa, lysozyme). (*B*) Specific activity of the crotonyl-CoA carboxylase/reductase at various steps during purification. The specific activity was determined spectrophotometrically by the crotonyl-CoA-dependent oxidation of NADPH at 360 nm. Numbering is according to *A*.

molecular mass of 105 kDa, suggesting a homodimeric structure. Comprehensive metal analysis (30 elements) of crotonyl-CoA carboxylase/reductase by plasma emission spectroscopy indicated the absence of any metals.

The product of the reductive carboxylation of crotonyl-CoA was identified as ethylmalonyl-CoA by HPLC-MS. The reaction followed Michaelis–Menten kinetics with apparent K_m values of 0.4 mM for crotonyl-CoA, 0.7 mM for NADPH, and 14 mM for $NaHCO_3$. CO_2 and not bicarbonate was determined to be the reactive carbon species by using a modified method by Thauer *et al.* (ref. 17 and data not shown). The reaction is reversible, with an apparent rate of 12 U mg^{-1}. Crotonyl-CoA carboxylase/reductase also catalyzed the reduction of crotonyl-CoA to butyryl-CoA (the product was identified by HPLC-MS) at low rate (12 U mg^{-1}), but only in the absence of bicarbonate/CO_2; in the presence of bicarbonate/CO_2 ethylmalonyl-CoA was formed exclusively. This observation could be explained by the following mechanism: first, the β-carbon of crotonyl-CoA accepts a hydride from NADPH, then the α-carbon is either carboxylated to yield ethylmalonyl-CoA or, in the absence of CO_2, a proton replaces the electrophilic substrate, forming butyryl-CoA. According to biochemical data, we suggest the following enzymatic overall reaction:

$$\text{crotonyl-CoA} + \text{NADPH} + CO_2 \rightarrow$$

$$\text{ethylmalonyl-CoA}^- + \text{NADP}^+.$$

Crotonyl-CoA Carboxylase/Reductase Activity in Cell Extracts of Isocitrate Lyase-Negative Bacteria. The unique reductive carboxylation of crotonyl-CoA serves as a key reaction of the pathway, which is named after its characteristic intermediate ethylmalonyl-CoA. Extracts of *R. sphaeroides* grown photoheterotrophically with acetate catalyzed the crotonyl-CoA- and CO_2-dependent oxidation of NADPH (0.7 U mg^{-1}); this activity was down-regulated at least 60-fold in cells grown photoheterotrophically with succinate (< 0.01 U mg^{-1}), giving support to the suggested functioning of the ethylmalonyl-CoA pathway in acetate assimilation. High crotonyl-CoA carboxylase/reductase activity (0.8 U mg^{-1}) was present in extracts of *M. extorquens* grown with methanol, suggesting that the key enzyme of the ethylmalonyl-CoA pathway is participating in C_1 assimilation. Extracts of *Streptomyces coelicolor* grown with butyrate also catalyzed the crotonyl-CoA- and CO_2-dependent oxidation of NADPH (0.4 U mg^{-1}). Incorporation of [^{14}C]bicarbonate into ethylmalonyl-CoA by extracts of butyrate-grown cells (0.4 U mg^{-1}) was down-regulated 20-fold in extracts of succinate-grown cells (0.02 U mg^{-1}). These results suggest that crotonyl-CoA reductase of *Streptomyces* (15) catalyses not only the reduction but also the concurrent carboxylation of crotonyl-CoA and that the ethylmalonyl-CoA pathway is functioning in these organisms.

Discussion

Crotonyl-CoA carboxylase/reductase catalyzes the central reaction in an acetyl-CoA assimilation pathway, which is distinct from the glyoxylate cycle (Fig. 1). The pathway, shown in Fig. 4, was named the ethylmalonyl-CoA pathway after its characteristic intermediate and product of its key enzyme, crotonyl-CoA carboxylase/reductase. This enzyme catalyzes the reductive carboxylation of an enoyl-CoA ester, a reaction unprecedented in biology. Therefore, the presence of its unique activity in cell extracts of organisms utilizing substrates as sole carbon source, which are metabolized by means of acetyl-CoA, can be used as an indication for the presence of the ethylmalonyl-CoA pathway. The gene encoding crotonyl-CoA carboxylase/reductase (*ccr*) is present in the genomes of all organisms proposed to use an acetyl-CoA assimilation pathway distinct from the glyoxylate cycle (13). We could recently show that *meaA*, which is found clustered with *ccr* in genomes, encodes coenzyme B_{12}-dependent ethylmalonyl-CoA mutase forming methylsuccinyl-CoA (T.J.B., G.F., and B.E.A., unpublished results). An acyl-CoA dehydrogenase up-regulated in acetate- versus glucose-grown *R. sphaeroides* may catalyze the oxidation of methylsuccinyl-CoA to mesaconyl-CoA (13), thereby closing the remaining gap in the pathway (Fig. 4). A homologous gene is part of the same gene cluster encoding *ccr* and *meaA* in *Streptomyces* species, and mutation of the corresponding gene in *M. extorquens* results in a methanol-minus phenotype (12). Whether this acyl-CoA dehydrogenase indeed encodes for methylsuccinyl-CoA dehydrogenase remains to be shown.

All genes implicated in the ethylmalonyl-CoA pathway for acetate assimilation of *R. sphaeroides* are conserved for *M. extorquens* (13). This isocitrate lyase-negative methylotroph uses the serine cycle for C_1-assimilation (type II methylotroph) and for this requires a pathway for the oxidation of acetyl-CoA to glyoxylate (6, 19). Here, we have shown high crotonyl-CoA carboxylase/reductase activity in cell extracts of methanol-grown *M. extorquens*. The presence of the ethylmalonyl-CoA pathway (excluding the condensation of acetyl-CoA and glyoxylate) in *M.*

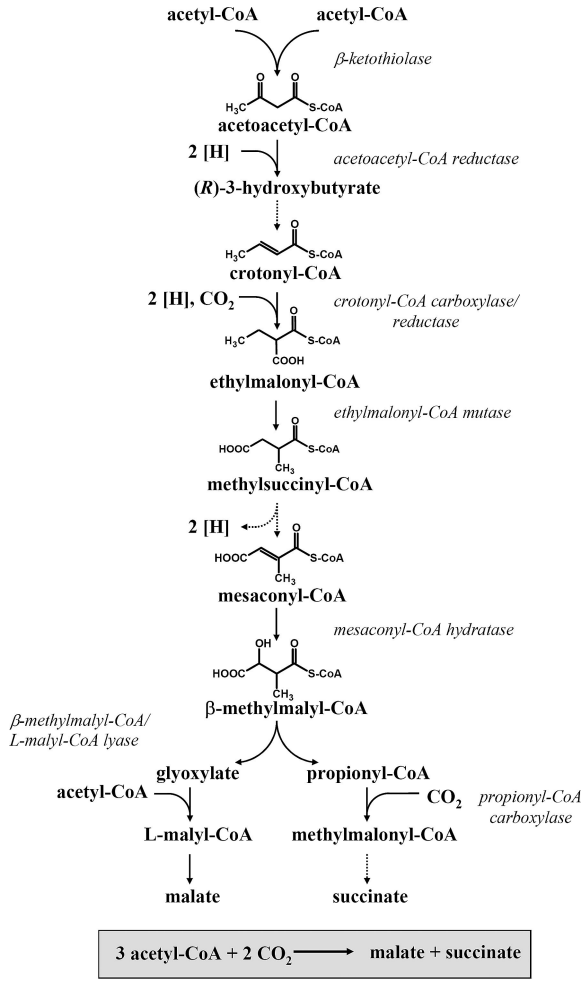

Fig. 4. The ethylmalonyl-CoA pathway as studied in isocitrate lyase-negative *R. sphaeroides*. Crotonyl-CoA carboxylase/reductase was identified as the key enzyme of the herein-described acetyl-CoA assimilation pathway distinct from the glyoxylate cycle. Mutations in the genes encoding β-ketothiolase and mesaconyl-CoA hydratase were previously shown to result in an acetate-minus phenotype (13). The bifunctional β-methylmalyl-CoA/malyl-CoA lyase catalyses the cleavage of β-methylmalyl-CoA as well as the condensation of acetyl-CoA and glyoxylate to form malyl-CoA (18). Dotted lines indicate steps that have not been elucidated so far. Exogenous CO_2 is not required for growth with acetate as sole carbon source, indicating that the two molecules of CO_2 fixed in the ethylmalonyl-CoA pathway are derived from the oxidation of acetyl-CoA.

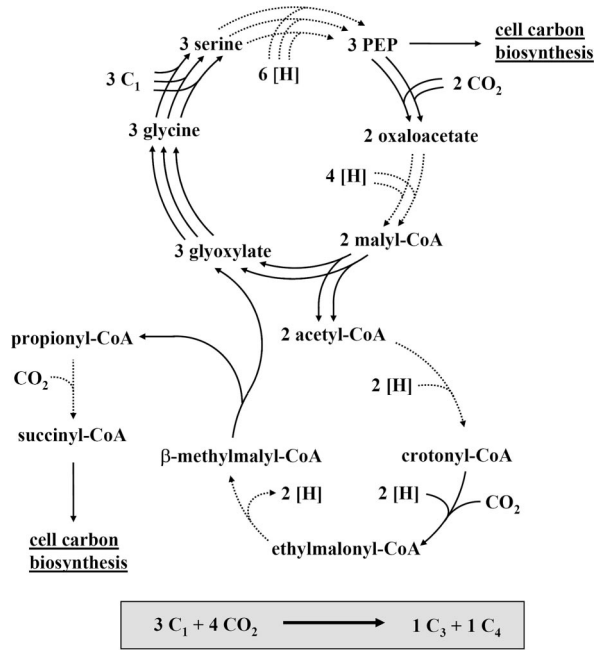

Fig. 5. Proposed pathway for the assimilation of C_1 compounds by isocitrate lyase-negative type II methylotrophs by using the serine cycle for formaldehyde fixation. The ethylmalonyl-CoA pathway described here (excluding the condensation of acetyl-CoA and glyoxylate) is integrated in the serine cycle (upper part) and is involved in assimilation of acetyl-CoA and regeneration of glyoxylate during growth on C_1 compounds. It is assumed that during growth on C_2 compounds the ethylmalonyl-CoA pathway (Fig. 4) is used exclusively. Dotted lines indicate more than one reaction step.

extorquens simplifies the scheme for isocitrate lyase-negative C_1-assimilation (Fig. 5) compared with former proposals (12). Genes of the ethylmalonyl-CoA pathway, including *ccr*, were affected in mutants of *M. extorquens* unable to form glyoxylate from acetyl-CoA. However, different catalytic roles were initially assigned to the corresponding gene products (12, 16, 20).

It has been reported that marine species of the *Roseobacter* clade, which constitute a substantial fraction of ocean bacterioplankton involved in sulfur cycling, are capable of demethylation reactions and therefore are likely to be facultative methylotrophs (21). However, the use of C_1-compounds by these aerobic phototrophs has not been explicitly shown. All genes implicated in the herein-described ethylmalonyl-CoA pathway, including *ccr*, now known to encode the key enzyme crotonyl-CoA carboxylase/reductase, are conserved in sequenced representatives of this phylogenetic group (13), suggesting that the extended serine cycle (Fig. 5) may be involved in metabolism of further ecologically important compounds such as dimethylsulfoniopropionate.

For *S. coelicolor*, the gene encoding crotonyl-CoA carboxylase/reductase (*ccr*) is part of a gene cluster containing additional genes involved in acetyl-CoA assimilation (8, 13). Similar gene clusters are found in other *Streptomyces* species (22), suggesting that the ethylmalonyl-CoA pathway functions in several streptomycetes during growth on compounds like butyrate, requiring a pathway for acetyl-CoA assimilation. Interestingly, homologues of *ccr* are present in other actinomycetes (SI Fig. 7) and are part of gene clusters encoding polyketide synthases. This finding provides support for the observed involvement of *ccr* in antibiotic biosynthesis by supplying ethylmalonyl-CoA as an extender unit (14, 23).

We conclude that the ethylmalonyl-CoA pathway is not a "stand-alone" pathway but, instead, integrates different metabolic routes such as assimilation of compounds, which would require the glyoxylate cycle, methylotrophy, antibiotic biosynthesis, and also synthesis and utilization of polyhydroxybutyrate, a major storage compound of all organisms mentioned here.

Materials and Methods

Bacterial Strains and Growth Conditions. *R. sphaeroides* 2.4.1 (DSMZ 158) and *M. extorquens* AM1 (DSMZ 1338) were grown aerobically in the dark at 30°C and pH 6.7 in a 200 l fermenter (Bioengineering, Wald, Switzerland; air flow, 20–60 liters min^{-1}; 150–220 rpm) on a defined minimal medium (13) supplemented with 10 mM sodium acetate (*R. sphaeroides*) or 0.5% methanol (*M. extorquens*). For regulatory studies, *R. sphaeroides* was also grown anaerobically in incandescent light (8,000 lux) in 2-liter bottles in minimal medium supplemented with 10 mM acetate or 10 mM succinate. *S. coelicolor* A3 (2) (DSMZ 40783) was grown aerobically on minimal medium supplemented with 10 mM succinate or 10 mM butyrate in 2-liter Erlenmeyer flasks with baffles, filled with 500 ml of medium containing a metal

spiral spring to break up cell clumps. Cells were harvested at midexponential growth phase at an OD_{578} = 0.5 − 1.0 (*R. sphaeroides* and *S. coelicolor*) or 2.5 (*M. extorquens*). Cells were stored at −80°C until use.

Syntheses. Acetoacetyl-CoA was synthesized from diketene as described (24). Butyryl-CoA and crotonyl-CoA were synthesized from their anhydrides (24). (*R*)-3-hydroxybutyryl-CoA and (*R*/*S*)-ethylmalonyl-CoA were synthesized by the mixed-anhydride method (25).

Cell Extracts and Enzyme Measurement. Frozen cells (300–400 mg) were resuspended in 0.5 ml of 100 mM Tris·HCl (pH 7.9) and 50 μg ml^{-1} of DNase I. After addition of 1.1 g of glass beads (diameter 0.1–0.25 mm), the cell suspension was treated in a mixer mill (type MM2; Retsch, Haare, Germany) for 9 min at 30 Hz. The supernatant obtained after centrifugation (10 min, 14,000 × *g*, 4°C) was used for assays. The protein content of the cell extracts was determined by the Bradford method (26) by using BSA as a standard and was ≈10 mg ml^{-1} for *R. sphaeroides* and *M. extorquens* or 2 mg ml^{-1} for *S. coelicolor*. For specific activities, one unit (1 U) corresponds to one μmol product formed per min.

Incorporation of [^{14}C]bicarbonate into acid-stable compounds. Carboxylation reaction in the presence of different CoA-thioesters and NADPH was tested radiochemically. The reaction mixture (0.35 ml) contained 80 mM Tris·HCl buffer (pH 7.9), 33 mM $NaHCO_3$, 1 MBq ml^{-1} $NaH^{14}CO_3$ (Amersham, Braunschweig, Germany), 5 mM NADPH, 2 mM CoA-ester, and cell extract (0.3–0.5 mg of protein per ml^{-1}). To study ATP-dependence, 5 mM ATP and 5 mM $MgCl_2$ were added to the reaction mixture. The reaction was stopped at different time points by transferring 100 μl of the reaction mixture to 500 μl of 5% trichloroacetic acid. The samples were shaken overnight to remove nonincorporated $^{14}CO_2$ and the amount of remaining ^{14}C was determined by liquid scintillation counting by using 3 ml of Rotiszint 2200 scintillation mixture (Roth).

Crotonyl-CoA carboxylase/reductase. The crotonyl-CoA-dependent oxidation of NADPH was followed spectrophotometrically at 360 nm (ε_{NADPH} = 3,400 M^{-1} cm^{-1}) by using a cuvette with a path length of 0.1 cm. The reaction mixture (0.2 ml) contained 100 mM Tris·HCl buffer (pH 7.9), 4 mM NADPH, 2 mM crotonyl-CoA, and 0.04–0.8 mg of cell extract protein or 1–5 μg of purified crotonyl-CoA carboxylase/reductase. The reaction was started by the addition of 33 mM $KHCO_3$ or $NaHCO_3$. The apparent K_m values of crotonyl-CoA and $NaHCO_3$ were determined by varying the concentration of $NaHCO_3$ (0.4–66.6 mM) or crotonyl-CoA (0.125–2.0 mM), while keeping the other substrates at saturating concentrations. The apparent K_m value of NADPH was determined by incorporation of [^{14}C]bicarbonate into (acid stable) ethylmalonyl-CoA. The reaction mixture (0.33 ml) contained 100 mM Tris·HCl buffer (pH 7.9), 3 mM crotonyl-CoA, 3 mM $NaHCO_3$, 64 kBq ml^{-1} $NaH^{14}CO_3$, and 7 μg of purified crotonyl-CoA carboxylase. The reaction was started by adding NADPH (0.125–5 mM) to the assay mixture. The reaction was stopped at different time points by transferring 50 μl of the reaction mixture to 50 μl of 1.5 M $HClO_4$. The samples were shaken overnight to remove nonincorporated $^{14}CO_2$, and the amount of remaining ^{14}C was determined by liquid scintillation.

Backreaction. The ethylmalonyl-CoA-dependent reduction of $NADP^+$ was followed spectrophotometrically at 360 nm by using a cuvette with a path length of 1 cm. The reaction mixture (0.4 ml) contained 90 mM Tri·HCl buffer (pH 7.9), 5.7 mM $NADP^+$, and 23 μg of purified crotonyl-CoA carboxylase/reductase. The reaction was started by the addition of 1.5 mM ethylmalonyl-CoA. The apparent K_m value of ethylmalonyl-CoA was determined by varying the concentration of ethylmalonyl-CoA (0.038–1.5 mM) while keeping the concentration of $NADP^+$ constant.

HPLC Analysis. At different time points, the conversion of acetyl-CoA and bicarbonate in cell extracts of *R. sphaeroides* was stopped by transferring 100 μl of the reaction mixture to 400 μl of methanol. Protein was removed by centrifugation, methanol was evaporated in a Speedvac concentrator, and samples were analyzed for CoA-thioesters by reversed-phase HPLC on a Waters 2690 separation module (Waters, Eschborn, Germany) by using a RP-C_{18} column (LiChrospher 100, end-capped, 5 μm, 125 × 4 mm; Merck, Darmstadt, Germany); 100 μl of centrifuged sample were injected onto the column. A 30-min gradient from 2% to 10% (vol/vol) acetonitrile in 50 mM potassium phosphate buffer (pH 6.7) at a flow rate of 1 ml min^{-1} was used. Simultaneous detection of UV absorbance and radioactivity in standard compounds and reaction products was done with a Waters 996 photodiode array detector and a Ramona 2000 radioactive monitor (Raytest, Straubenhardt, Germany) in series.

HPLC-MS was performed on an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) interfaced with an Applied Biosystems API 2000 triple-quadrupole mass spectrometer by using the same separation conditions as on the Waters system but with 40 mM ammonium acetate (pH 6.7) instead of phosphate buffer. The temperature of the Turbo-Ionspray auxiliary gas was 400°C, and the ionization voltage was −4,500 V. The samples were analyzed with a mass range of 100–1,600 Da.

NMR Spectroscopy. The ^{13}C-enriched products of the conversion of [U-^{13}C]acetyl-CoA (Cambridge Isotope Laboratories Andover, MA) and [^{13}C]bicarbonate (Spectra Stable Isotopes, Columbia, MD) in cell extracts of *R. sphaeroides* were analyzed by NMR spectroscopy. [U-^{13}C]acetyl-CoA was synthesized by incubation of 1.1 mM [U-^{13}C]acetate, 4.4 mM ATP, 1.1 mM CoA, 4.4 mM KCl, and 4.4 mM $MgCl_2$ with 0.15 U ml^{-1} of acetyl-CoA synthetase (Sigma–Aldrich, Deisenhofen, Germany) in 22.4 ml of 78 mM Tris·HCl buffer (pH 7.9). After 60 min of incubation at 37°C, the reaction mixture was transferred to 37°C and supplemented with 0.5 mM 5–5′-dithiobis(2-nitrobenzoic acid), 31.6 mM $NaH^{13}CO_3$, and 5.9 mM NADPH. The reaction was started by the addition of 21 mg of cell-extract protein of *R. sphaeroides* to a final volume of 31.6 ml. The reaction was stopped after 30 min by transferring the whole mixture to 160 ml of methanol. Protein was removed by centrifugation, and the supernatant was concentrated by flash evaporation at 40°C (150 mbar) to a volume of 20 ml. The pH of the yellowish solution was adjusted with formic acid to 3.0, and the solution was centrifuged to remove the precipitate. The supernatant was applied onto a 25-ml (2 mg) ISOLUTE C_{18}(EC) solid phase extraction column (Separtis, Grenzach Whylen, Germany) which had been activated with 20 ml of methanol and equilibrated with 40 ml of ammonium formate buffer (100 mM, pH 4.0). The column was washed with 20 ml of ammonium formate buffer, followed by elution of the products with 60 ml of methanol. The organic solvent was concentrated again by flash evaporation, followed by lyophilization of the remaining liquid. NMR spectra were recorded with an Avance DRX-400 spectrometer (Bruker, Rheinstetten, Germany) at 27°C. Chemical shifts were recorded and reported in ppm relative to MeOH-d4 (^{1}H: δ = 3.31, ^{13}C: δ = 49.15) as internal standard. INADEQUATE measurements were performed with SF = 100.624 MHz, D1 = 6 sec, Aq = 0.1 sec, PW90 = 8.8 μs −3db, NS = 160, TD – F2 = 4096 and TD – F1 = 128.

Heterologous Expression of the *ccr* Gene from *R. sphaeroides* and Production of the Protein in *E. coli*. The gene encoding crotonyl-CoA carboxylase/reductase was amplified by PCR from *R.*

sphaeroides chromosomal DNA by using the forward primer (5′-GGAGGCAA*CCATG*GCCCTCGACGTGCAGAG-3′) introducing a NcoI site (italicized) at the initiation codon and reverse primer (5′-GAGACTTGC*GGATCC*CTCCGATCAGGCCTTGC-3′) introducing a BamHI site (italicized) after the stop codon. The PCR product was isolated and cloned into the pET3d expression vector (Merck), generating pTE13. Competent *E. coli* BL21(DE3) cells (27) were transformed with pTE13 and grown at 37°C in a 200-liter fermenter (air flow, 80 liter min^{-1}; 300 rpm) containing Luria-Bertani broth and 100 μg ml^{-1} ampicillin. Expression was induced at $OD_{578} = 0.75$ with 0.5 mM isopropylthio-galactopyranoside. After additional growth for 3.5 h, the cells were harvested and stored in liquid nitrogen until use.

Purification of Heterologously Produced Crotonyl-CoA Carboxylase/Reductase. The purification was performed at 4°C. Crotonyl-CoA carboxylase/reductase activity was measured by using the spectrophotometric assay described above.

Preparation of cell extracts. Frozen cells were suspended in the double volume of buffer A (20 mM Tris·HCl, pH 7.9) containing 0.1 mg of DNase I per ml. The suspension was passed twice through a French pressure cell at 137 MPa and then centrifuged (100,000 × *g*) at 4°C for 1 h.

DEAE chromatography. Supernatants [15 ml (1.6 g of protein)] after the centrifugation step were applied at a flow rate of 2.5 ml min^{-1} onto a 30-ml DEAE-Sepharose Fast Flow column (Amersham Biosciences), which had been equilibrated with 60 ml of buffer A. The column was washed with 90 ml of buffer A and thereafter with 135 ml of buffer A containing 50 mM KCl. Activity was eluted with 100 mM KCl in buffer A in a volume of 195 ml. Active fractions were pooled, desalted, and concentrated to a final volume of 20 ml by ultrafiltration (Amicon YM 10 membrane; Millipore, Bedford, MA).

Affinity chromatography. Concentrated protein solution [1.5 ml (17 mg of protein)] obtained by DEAE chromatography were applied at a flow of 0.5 ml min^{-1} onto a 10-ml Cibacron blue 3GA agarose 3000 CL column (Sigma–Aldrich), which had been equilibrated with 20 ml of buffer A. The column was washed with 22 ml of buffer A, followed by 37 ml of buffer A containing 100 mM KCl and 37 ml of buffer A containing 200 ml of KCl. Activity was eluted with 500 mM KCl in buffer A in a volume of 30 ml. Active fractions were pooled, desalted, and concentrated to a final volume of 1.5 ml by ultrafiltration (Amicon YM 10 membrane; Millipore). The protein (7.5 mg) was stored at −20°C with 50% glycerol to avoid precipitation. The affinity chromatography step was scaled up to purify 15 ml (153 mg of protein) of the concentrated DEAE fractions by using a 60-ml Cibacron Blue 3GA agarose 3000 CL column (Sigma–Aldrich) and a flow of 2.5 ml min^{-1}.

Protein Analyzing Methods. ***Standard techniques.*** Protein fractions were analyzed by SDS-12.5% polyacrylamide gel electrophoresis (28).

Determination of native molecular mass. The purified protein was applied at a flow rate of 1 ml min^{-1} onto a 120-ml Highload Superdex 200 16/60 column (Amersham Biosciences) equilibrated with buffer A containing 125 mM KCl. Activity eluted with a retention volume of 18.2–18.8 ml. The native molecular mass of the enzyme was estimated by using the same gel-filtration column, calibrated with thyroglobulin (660 kDa), ferritin (450 kDa), catalase (240 kDa), aldolase (150 kDa), BSA (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), and RNaseA (13.7 kDa) as molecular mass standards.

Metal analysis. Purified recombinant crotonyl-CoA carboxylase/reductase (4.0 mg) was analyzed for metals by inductively coupled plasma emission spectroscopy (ICP-OES) in the Chemical Analysis Laboratory of R. Auxier (University of Georgia, Athens, GA).

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