Conversion of 4-Hydroxybutyrate to Acetyl Coenzyme A and Its Anapleurosis in the *Metallosphaera sedula* 3-Hydroxypropionate/4-Hydroxybutyrate Carbon Fixation Pathway

Aaron B. Hawkins,^a Michael W. W. Adams,^b Robert M. Kelly^a

Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, North Carolina, USA^a; Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia, USA^b

The extremely thermoacidophilic archaeon *Metallosphaera sedula* (optimum growth temperature, 73°C, pH 2.0) grows chemolithoautotrophically on metal sulfides or molecular hydrogen by employing the 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) carbon fixation cycle. This cycle adds two CO₂ molecules to acetyl coenzyme A (acetyl-CoA) to generate 4HB, which is then rearranged and cleaved to form two acetyl-CoA molecules. Previous metabolic flux analysis showed that two-thirds of central carbon precursor molecules are derived from succinyl-CoA, which is oxidized to malate and oxaloacetate. The remaining one-third is apparently derived from acetyl-CoA. As such, the steps beyond succinyl-CoA are essential for completing the carbon fixation cycle and for anapleurosis of acetyl-CoA. Here, the final four enzymes of the 3HP/4HB cycle, 4-hydroxybutyrate-CoA ligase (AMP forming) (Msed_0406), 4-hydroxybutyryl-CoA dehydratase (Msed_1321), crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase (Msed_0399), and acetoacetyl-CoA β -ketothiolase (Msed_0656), were produced recombinantly in *Escherichia coli*, combined *in vitro*, and shown to convert 4HB to acetyl-CoA. Metabolic pathways connecting CO₂ fixation and central metabolism were examined using a gas-intensive bioreactor system in which *M. sedula* was grown under autotrophic (CO₂-limited) and heterotrophic conditions. Transcriptomic analysis revealed the importance of the 3HP/4HB pathway in supplying acetyl-CoA to anabolic pathways generating intermediates in *M. sedula* metabolism. The results indicated that flux between the succinate and acetyl-CoA branches in the 3HP/4HB pathway is governed by 4-hydroxybutyrate-CoA ligase, possibly regulated posttranslationally by the protein acetyltransferase (Pat)/Sir2-dependent system. Taken together, this work confirms the final four steps of the 3HP/4HB pathway, thereby providing the framework for examining connections between CO₂ fixation and central metabolism in *M. sedula*.

Metallosphaera sedula is an extremely thermoacidophilic archaeon (optimum growth temperature, 73°C, and optimum pH, 2.0) that grows heterotrophically on peptides and chemolithoautotrophically on metal sulfides or hydrogen gas (1). For chemolithotrophic growth, it uses a unique carbon fixation pathway known as the 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) cycle (2), so far found only in members of the order *Sulfolobales*. This cycle is one of two such cycles found exclusively in thermophilic archaea, the other being the dicarboxylate/4-hydroxybutyrate (DC/4HB) cycle present in the orders *Desulfurococcales* and *Thermoproteales* (3–5). In the first part of the 3HP/4HB cycle, acetyl coenzyme A (CoA) (C₂) is converted into succinyl-CoA (C₄) by two successive carboxylation steps (5–7). In the second half of this cycle, succinyl-CoA is then converted to 4HB, which is rearranged and cleaved to produce two molecules of acetyl-CoA. Both the 3HP/4HB and DC/4HB cycles use the same set of enzymes to convert succinyl-CoA to acetyl-CoA. However, the enzymes used in the first half of each cycle reflect the physiologic lifestyles of their host organisms. The DC/4HB cycle uses electron carriers [NAD(P)H and ferredoxin] and enzymes (such as pyruvate synthase) that are characteristic of anaerobic organisms, while the 3HP/4HB cycle operates within an aerobic organism and so does not use the same carboxylating enzymes or electron carriers (specifically ferredoxin) (4).

Labeling studies using 4-hydroxy[1-¹⁴C]butyrate and [1,4-¹³C]₂succinate have indicated how the 3HP/4HB pathway might be connected to *M. sedula* central metabolism (8). The results suggested that about two-thirds of the carbon flux en-

ters central metabolism via succinate (succinate branch) rather than through reductive carboxylation of acetyl-CoA to pyruvate. The remaining third of the carbon flux enters central metabolism via acetyl-CoA (acetyl-CoA branch) (Fig. 1). This further distinguishes organisms that contain the 3HP/4HB cycle from those with the DC/4HB cycle, in which all flux apparently enters the central metabolism through reductive carboxylation of acetyl-CoA (4).

The 3HP/4HB cycle consists of 13 enzymes; all but two of them have now been confirmed and characterized biochemically (Table 1). The remaining two are 4-hydroxybutyryl-CoA dehydratase (Msed_1321) and β -ketothiolase (Msed_0656), which are part of the acetyl-CoA branch of the pathway that converts succinyl-CoA to acetyl-CoA. Here, we sought to characterize this anapleurotic branch by characterizing the remaining two enzymes and by demonstrating *in vitro*, using four recombinant enzymes, that the final part of the 3HP/4HB cycle efficiently converts 4HB to acetyl-CoA.

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Address correspondence to Robert M. Kelly, rmkelly@ncsu.edu.

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FIG 1 Enzymes and substrates in final reaction steps of 3HP/4HB cycle in *M. sedula*. Enzymes: HBCL, 4-hydroxybutyrate-CoA ligase; HBDC, 4-hydroxybutyryl-CoA dehydratase; CCH/HBDC, crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase; ACK, acetoacetyl-CoA β -ketothiolase; PfACS, acetyl-CoA synthetase (nonnative, used for HPLC assay).

That these enzymes are likely involved in the functioning cycle *in vivo* was further confirmed by the results of global transcriptomic analysis of *M. sedula* cells grown under autotrophic and heterotrophic conditions. Taken together, the results demonstrate that the final four enzymes of the 3HP/4HB cycle function to replenish acetyl-CoA.

MATERIALS AND METHODS

Materials. The gas mixtures (80% H₂, 20% CO₂ and 80% H₂, 20% N₂) were purchased from Airgas-National Welders (Charlotte, NC). Chemicals for media and buffers, including CoA esters for enzyme assays, were purchased from Sigma-Aldrich (St. Louis, MO) and Thermo Fischer Scientific (Pittsburgh, PA). Strains and vectors for cloning were purchased from Novagen (San Diego, CA) and Stratagene (La Jolla, CA).

Growth of *M. sedula* in a gas-intensive bioreactor. *M. sedula* (DSMZ 5348) was grown aerobically on DSMZ medium 88 at pH 2.0 in a 70°C shaking oil bath. For routine small cultures (30 ml), heterotrophically grown cells were supplemented with 0.1% tryptone, while autotrophically grown cells were grown with the addition of 50 ml gas mix (80% H₂, 20% CO₂) to the headspace (121 ml total headspace volume). Cell growth was scaled up from 300 ml in sealed 1-liter bottles (350 ml gas mix added; 835 ml total headspace volume) to 2 liters in a stirred bench-top glass fermentor (Applikon), agitated at 250 rpm. Two separately regulated gas feeds

were used, one for the H₂-CO₂ mixture and one for air. The flow rates were held constant for all conditions at 1 ml/min for the different H₂-CO₂ gas mixtures and 100 ml/min for air (composition, 78% N₂, 21% O₂, 0.03% CO₂). The gas mixture compositions were as follows: autotrophic carbon limited (ACL), 80% H₂ and 20% N₂; heterotrophic (HTR), 80% N₂ and 20% CO₂ (with 0.1% tryptone added to the medium). Tandem bioreactors were run simultaneously and started with the same inoculum to generate biological repeats. Cells were harvested at mid-exponential phase by rapid cooling with dry ice and ethanol and then centrifuged at 6,000 $\times$ g for 15 min at 4°C.

***M. sedula* oligonucleotide microarray transcriptional response analysis.** A spotted whole-genome oligonucleotide microarray was used for transcriptional analysis, as previously described (9). Total RNA was extracted and purified using an RNeasy kit (Qiagen), reverse transcribed with Superscript III (Invitrogen), repurified, and labeled with either Cy3 or Cy5 dye (GE Healthcare). Labeled cDNA was then hybridized to the microarray slide (Corning) at 42°C. Slides were scanned on a GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA), and raw intensities were quantitated using GenePix pro version 6.0. Data normalization and statistical analysis were performed using JMP Genomics 5 (SAS, Cary, NC). In general, significant differential transcription was defined to be a relative change in expression of ≥ 2 -fold (where a log₂ value of ± 1 means a 2-fold change)

TABLE 1 Enzymes in the 3HP/4HB cycle in *Metallosphaera sedula*

Enzyme	ORF(s)	Protein(s) used ^a (reference[s] or source)
Acetyl-CoA/propionyl-CoA carboxylase	Msed_0147, Msed_0148, Msed_1375	NCE (6, 34)
Malonyl-CoA/succinyl-CoA reductase (NADPH)	Msed_0709	R (35, 36)
Malonate semialdehyde reductase (NADPH)	Msed_1993	R (36)
3-Hydroxypropionate-CoA ligase (AMP forming)	Msed_1456	NP (37)
3-Hydroxypropionyl-CoA dehydratase	Msed_2001	NP, R (38)
Acryloyl-CoA reductase (NADPH)	Msed_1426	NP (38)
Methylmalonyl-CoA epimerase	Msed_0639	R (39)
Methylmalonyl-CoA mutase	Msed_0638, Msed_2055	R (39)
Succinate semialdehyde reductase (NADPH)	Msed_1424	NP, R (36)
4-Hydroxybutyrate-CoA ligase (AMP forming)	Msed_0406	R (9)
4-Hydroxybutyryl-CoA dehydratase	Msed_1321	NCE (2, 40, 41), R (this work)
Crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase (NADH)	Msed_0399	R (17)
Acetoacetyl-CoA β -ketothiolase	Msed_0656	NCE (2), R (this work)

^a NCE, native cell extract; NP, native purified enzyme; R, recombinant protein.

having a $-\log_{10} P$ value of ≥ 5.4 (Bonferroni correction equivalent to a P value of 4.0×10^{-6} for this microarray).

Heterologous expression of *M. sedula* genes in *E. coli*. Open reading frames (ORFs) Msed_0406, Msed_1321, Msed_0399, and Msed_0656 were amplified from genomic DNA using primers from IDT Technologies (Coralville, IA). 4-Hydroxybutyrate-CoA ligase (Msed_0406) was cloned into pET46-Ek/LIC with an N-terminal His₆ tag as described previously (9). 4-Hydroxybutyryl-CoA dehydratase (Msed_1321) was cloned with an N-terminal His₆ tag into a modified pETA vector into which the anaerobic *hya* promoter from *Escherichia coli* had been inserted to allow for anaerobically regulated expression (10). Crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase (Msed_0399) was cloned into pET21b without a His tag, and β -ketothiolase (Msed_0656) was cloned into pCDF-Ek/LIC with an N-terminal His₆ tag. All four constructs were individually cloned into NovaBlue GigaSingles *E. coli* competent cells and selected by growth on LB agar supplemented with antibiotic. The sequences were confirmed by Eton Biosciences, Inc. (Durham, NC). Next, the plasmids were transformed into *E. coli* Rosetta 2 (DE3) cells for protein expression. Rosetta strains containing pET46-0406, pET21b-0399, and pCDF-0656 were grown and expressed aerobically at 37°C for 16 h in Studier's autoinducing medium ZYM-5052 (11). Rosetta cells containing plasmid pETA-1321 were grown in a 2-liter Applikon bioreactor (37°C, 800 rpm, pH 6.7, 0.5 standard liter per minute air) in Studier's noninducing medium ZYM-505 with 0.5 mM FeCl₃. Cells were grown until the dissolved oxygen reached ~30% of the initial level, at which point 50 mM glucose was added and the air feed was switched to N₂ to induce anaerobic expression. The cells were grown for another 3 h before harvest.

Enzyme purification and biochemical assays. Lysis of cells containing aerobically expressed proteins began with harvesting cells and centrifuging them at $6,000 \times g$ for 15 min at 4°C. Cell pellets were resuspended in lysis buffer (50 mM sodium phosphate, 100 mM NaCl, 0.1% Nonidet P-40, pH 8.0) and lysed with a French pressure cell (two passes at 18,000 lb/in²). The lysate was centrifuged at $25,000 \times g$ for 15 min at 4°C to remove insoluble material. Native *E. coli* proteins were removed by heat treating the extract at 65°C for 20 min. Nucleic acids were precipitated by the addition of streptomycin sulfate (1%, wt/vol), and then the lysate was centrifuged again at $25,000 \times g$ for 15 min at 4°C to remove precipitated nucleic acids and heat-labile proteins. The soluble, heat-treated cell extract was sterile filtered (0.22 μ m) before chromatographic purification. Cells containing recombinant 4-hydroxybutyryl-CoA dehydratase were lysed, and the enzyme was purified in a Coy anaerobic chamber (95% N₂, 5% H₂). The cell pellet was resuspended in lysis buffer (20 mM Tris, 20 mM NaCl, 3.5 mM dithiothreitol [DTT], 1 mg/ml lysozyme, pH 8.0) and incubated for 30 min at 37°C, followed by heat treatment at 65°C for 30 min. Streptomycin sulfate (1%, wt/vol) was added, and the lysate was centrifuged at $25,000 \times g$ for 15 min at 4°C. The soluble, heat-treated cell extract was sterile filtered (0.22 μ m) before purification by column chromatography.

4-Hydroxybutyrate-CoA ligase and acetoacetyl-CoA β -ketothiolase. 4HB-CoA ligase and acetoacetyl-CoA β -ketothiolase were purified using a 1-ml HiTrap nickel column (GE Healthcare). The soluble, heat-treated lysate was loaded onto the column with binding buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 7.4), and the His-tagged enzyme removed with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 500 mM imidazole, pH 7.4). The elution fractions containing enzyme were collected, concentrated, and dialyzed into reaction buffer (100 mM morpholinepropanesulfonic acid [MOPS], pH 7.5) and then either stored at 4°C for immediate use or mixed with glycerol to 20% and stored at -20°C . For 4-hydroxybutyrate-CoA ligase, a discontinuous assay was used to measure substrate-dependent disappearance of CoA at 70°C using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), as described previously (9). For the β -ketothiolase, enzyme activity was measured using the discontinuous DTNB assay to measure the substrate-dependent disappearance of CoA at 70°C. The reaction buffer contained

20 mM MOPS, pH 7.5, 5 mM MgCl₂, 0.2 mM CoA, 0.3 mM acetoacetyl-CoA, and purified enzyme.

Crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase. Crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase was purified first using a Q-Sepharose HiLoad 16/10 anion exchange column (GE Healthcare). The protein was loaded onto the column with 20 mM Tris, pH 8.0, and eluted with 20 mM Tris, 1 M NaCl, pH 8.0, using a linear elution gradient. Fractions containing crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase were confirmed with SDS-PAGE, collected, and dialyzed into buffer for size exclusion chromatography (50 mM potassium phosphate, 150 mM NaCl, pH 7.0). The partially purified protein was further separated on a HiLoad 26/600 Superdex 200 prep grade column, and the elution fractions containing crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase were collected, concentrated, and dialyzed into reaction buffer (100 mM MOPS, pH 7.5) and then either stored at 4°C for immediate use or mixed with glycerol to 20% and stored at -20°C . Enzyme activity was measured spectrophotometrically at 70°C by following NAD⁺ reduction at 340 nm ($E_{340} = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$) (2). The assay mixture contained 20 mM MOPS (pH 7.0), 5 mM MgCl₂, 2 mM NAD⁺, 0.5 mM crotonyl-CoA or (S)-3-hydroxybutyryl-CoA, and purified enzyme. The reaction mixture was preheated for 2 min at 70°C, and the reaction initiated by the addition of the substrate. The kinetic parameters were calculated using KaleidaGraph (Synergy Software).

4-Hydroxybutyryl-CoA dehydratase. 4-Hydroxybutyryl-CoA dehydratase (Msed_1321) was purified using a 5-ml Bio-Scale Mini Profinity IMAC cartridge (Bio-Rad). The soluble, heat-treated lysate was loaded onto the column with binding buffer (50 mM Tris, 300 mM NaCl, 3.5 mM DTT, 20 mM imidazole, pH 8.0), and the His-tagged enzyme removed with elution buffer (50 mM Tris, 300 mM NaCl, 3.5 mM DTT, 500 mM imidazole, pH 8.0). The fraction collector was positioned inside the anaerobic chamber, and fractions containing enzyme were collected, concentrated, and dialyzed into reaction buffer (100 mM potassium phosphate, pH 7.5, 1 mM DTT). The enzyme solution was either stored at room temperature inside the anaerobic chamber for immediate use or mixed with glycerol to 20% and stored at -20°C in a sealed vacuum desiccator. The quaternary structure was determined using an SD200 10/300 GL size exclusion column (GE Healthcare). The enzyme (~50 μ g) was dialyzed into mobile-phase buffer (50 mM potassium phosphate, 150 mM sodium chloride, pH 7.0) and loaded onto the column (flow rate, 0.5 ml/min for mobile phase), and elution was tracked by UV absorption at 280 nm. Molecular mass was calculated using a standard curve generated with the following proteins: blue dextran (2,000 kDa), thyroglobulin (670 kDa), apoferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa).

Enzyme activity was measured aerobically in a coupled spectrophotometric assay at 70°C. The assay mixture contained 20 mM sodium phosphate, 5 mM MgCl₂, 2 mM 4HB, 2 mM ATP, 1 mM CoA, 2 mM NAD⁺, 1 mM DTT, 1 mg/ml purified Msed_0406, and 42 ng/ml Msed_0399. The reaction mixture was preheated for 5 min at 70°C to allow accumulation of 4HB-CoA, and then the reaction was initiated by the addition of purified 4-hydroxybutyryl-CoA dehydratase. For the oxygen sensitivity assay, 4-hydroxybutyryl-CoA dehydratase was washed with 100 mM potassium phosphate, pH 7.5, to remove any DTT from the reaction buffer. An aliquot of 4-hydroxybutyryl-CoA dehydratase was exposed to air, vortexed well, and tested at regular intervals. During the intervening time, the enzyme and reaction buffer were stored at 4°C. The reaction buffer was made without ATP, CoA, or NAD⁺; these were kept at -20°C and added to the reaction mixture at time of use. The kinetic parameters were calculated using KaleidaGraph (Synergy Software).

Analysis of *in vitro* acetyl-CoA production. Enzymatic production of acetyl-CoA from 4HB was performed *in vitro* at 70°C. The recombinant form (produced in *E. coli*) of the acetyl-CoA synthase (ACS) from *Pyrococcus furiosus* (12) (Pf-ACS) was used to form acetate from acetyl-CoA, and the resultant mixture was derivatized to form the phenacyl ester using

TABLE 2 Kinetic properties of selected *M. sedula* enzymes from the acetyl-CoA branch of the 3HP/4HB pathway

Enzyme	ORF	Substrate	Mean $V_{\max} \pm \text{SD}$ ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	Mean $K_m \pm \text{SD}$ (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{M}^{-1}$)
4-Hydroxybutyrate-CoA ligase	Msed_0406	4-Hydroxybutyrate	1.69 ± 0.11	2.0 ± 0.4	1.8	910
4-Hydroxybutyryl-CoA dehydratase	Msed_1321	4-Hydroxybutyryl-CoA	2.20 ± 0.16	0.15 ± 0.04	2.1	1.4×10^4
Crotonyl-CoA hydratase	Msed_0399	Crotonyl-CoA	20.2 ± 2.3	0.07 ± 0.03	19	2.6×10^5
(S)-3-Hydroxybutyryl-CoA dehydrogenase	Msed_0399	(S)-3-Hydroxybutyryl-CoA	16.0 ± 1.5	0.06 ± 0.02	15	2.6×10^5
Acetoacetyl-CoA β -ketothiolase	Msed_0656	Acetoacetyl-CoA	141 ± 10	0.18 ± 0.03	1,000	5.6×10^6

dibromoacetophenone (DBAP) (adapted from reference 13) and assayed using reversed-phase high-performance liquid chromatography (HPLC) (Waters).

The reaction mixture (100 μl) consisted of 100 mM sodium phosphate, pH 7.9, 5 mM MgCl_2 , 3 mM ATP, 3 mM CoA, 3 mM NAD^+ , 3 mM 4HB, 1 mM DTT, 3 mM ADP, purified recombinant Msed_0406 (500 ng/ μl), Msed_1321 (50 ng/ μl), Msed_0399 (50 ng/ μl), and Msed_0656 (50 ng/ μl). The reaction mixture was incubated at 70°C for 20 min, after which 10 μl of Pf-ACS extract was added before incubating for an additional 10 min at 95°C. The sample was cooled to room temperature, acidified with 50% H_2SO_4 to pH 2, and ether extracted twice with 750 ml diethyl ether. The ether fraction was neutralized with 50 μl 20 mM bicarbonate and dried down in a vacuum centrifuge for 2 h at 30°C. The sample was resuspended in 50 μl acetonitrile with 0.5 μl 0.5% phenolphthalein. A solution of 100 mM KOH was added until the sample turned pink (pH $\sim$ 9 to 10), after which 100 μl of acetonitrile, 50 μl of 1 μM 15-crown-5-ether, and 200 μl of 20 mM 2,4-dibromoacetophenone were added. The solution was heated to 80°C for 30 min, cooled back to room temperature, and injected (5 μl) onto a C_{18} silica-based column (4.6- by 250-mm Shodex C18-4E) at 30°C with a flow rate of 1 ml/min. The initial mobile-phase composition was 60% buffer A (0.05% trifluoroacetic acid) and 40% buffer B (acetonitrile). Samples were eluted with a 10-min linear gradient to a final composition of 20% buffer A and 80% buffer B. Products were detected by following the absorption spectrum at 254 nm using a single channel of the Waters 2998 photodiode array detector.

Microarray data accession number. Microarray data are available through the NCBI Gene Expression Omnibus (GEO) under accession number GSE39944.

RESULTS AND DISCUSSION

Conversion of 4-hydroxybutyrate to acetyl-CoA in the 3HP/4HB cycle. The final four steps in the acetyl-CoA branch of the 3HP/4HB pathway (which converts 4HB to acetyl-CoA) are catalyzed by 4-hydroxybutyrate-CoA ligase (AMP forming), 4-hydroxybutyryl-CoA dehydratase, crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase, and acetoacetyl-CoA β -ketothiolase (Fig. 1). In order to ascertain whether these enzymes could function together and convert 4HB to acetyl-CoA and to identify possible rate-limiting steps, recombinant versions of each were produced, purified to homogeneity, and characterized biochemically (Fig. 1; Table 2). Recombinant versions were readily produced for three of them, but initial attempts to produce recombinant 4-hydroxybutyryl-CoA dehydratase were unsuccessful. This was likely due to the oxygen sensitivity of the enzyme, based on properties of a well-studied homolog from an anaerobic bacterium, *Clostridium aminobutyricum* (14–16). As such, both the expression and purification of 4-hydroxybutyryl-CoA dehydratase were conducted under anaerobic conditions: an expression system based on the *hya* promoter from *E. coli* was used (10), and recombinant cell lysis and protein purification were carried out in an anaerobic chamber (95% N_2 , 5% H_2). This approach resulted in the production of soluble, active enzyme that was pu-

rified to electrophoretic homogeneity after metal affinity chromatography. Quaternary structure analysis using size exclusion chromatography showed a single elution peak at 221.5 kDa. With an individual subunit mass of 56.6 kDa for Msed_1321, this suggests that the enzyme associates as a homotetramer. However, subsequent testing of 4-hydroxybutyryl-CoA dehydratase from *M. sedula* revealed it to be much less oxygen sensitive than its clostridial counterpart. With a half-life of roughly 4 days, 4-hydroxybutyryl-CoA dehydratase from *M. sedula* proved to be surprisingly robust in the presence of oxygen (see Fig. S1 in the supplemental material). This increased oxygen tolerance relative to that of the *C. aminobutyricum* enzyme could be an adaptive trait associated with the aerobic environments inhabited by *M. sedula*.

The 4-hydroxybutyrate-CoA ligase activity in the 3HP/4HB is thought to be encoded by Msed_0406 (Table 1). In a previous study, Msed_0394 was also found to encode an acyl-CoA synthetase that used 4HB as a substrate, but the $V_{\max}$ value was an order of magnitude lower than that of the product of the Msed_0406 gene (9). Given the kinetic properties of the other enzymes of the pathway, it is unlikely that Msed_0394 plays a significant role in the cycle. For example, the reaction rate for 4-hydroxybutyryl-CoA dehydratase (Msed_1321), the subsequent enzyme in the cycle, is comparable to the activity of 4-hydroxybutyrate-CoA ligase on 4HB (Msed_0406). Thus, either of these two steps could be rate limiting for the production of acetyl-CoA in the 3HP/4HB pathway. Their activities are approximately 10-fold lower than those for the subsequent two reactions [crotonyl-CoA hydration and (S)-3-hydroxybutyryl-CoA dehydrogenation] catalyzed by crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase (20 and 16 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ for the hydration and dehydrogenation reactions, respectively), and more than 100-fold lower than that of the β -ketothiolase (Msed_0656) (140 $\mu\text{mol min}^{-1} \text{mg}^{-1}$). The standard deviations for the hydratase and dehydrogenase activities of Msed_0399 are such that the two measurements are not statistically different (Table 2). This likely shows that the hydration reaction is very fast compared to the subsequent dehydrogenation reaction and that the latter probably constitutes the rate-limiting step between the two. The high K_m value for Msed_0406 on 4HB (2.0 mM) stands out from the K_m values for the other enzymes on their respective substrates (Msed_1321 on 4-hydroxybutyryl-CoA, 0.15 mM; Msed_0399 on crotonyl-CoA, 0.07 mM; and Msed_0656 on acetoacetyl-CoA, 0.18 mM). These data suggest that posttranscriptional mechanisms impact substrate entry into the acetyl-CoA branch, which is discussed further below.

The activity measurements for Msed_0399 reported here are in line with previous reports of recombinant Msed_0399 activity produced in *E. coli* (13.8 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ for both the hydratase and dehydrogenase reactions); the previously reported K_m values

FIG 2 *In vitro* production of acetate from 4-hydroxybutyrate. Samples and standards were derivatized using dibromoacetophenone (DBAP) and run on a reversed-phase column to show production of acetate. Chromatograms shown are as follows: 4HB standard (1), acetate standard (2), control reaction mixture containing buffer, cofactors, and 4HB but no enzymes (3), and complete reaction using recombinant enzymes (4). Retention times were 6.9 min for 4HB and 9.1 min for acetate.

are also close—0.3 mM for crotonyl-CoA and 0.2 mM for (S)-3-hydroxybutyryl-CoA, only 2 to 3 times larger than the K_m values reported here (17). An acetoacetyl-CoA β -ketothiolase from *Thermoproteus neutrophilus*, a hyperthermophilic archaeon that utilizes the DC/4HB pathway, was recombinantly produced in *E. coli*, and the reported activity ($V_{\max} = 55 \mu\text{mol min}^{-1} \text{mg}^{-1}$) was three times lower than that reported here for the *M. sedula* β -ketothiolase (17). However, this value is not unreasonable, as other ketothiolases have been characterized from rat liver peroxisomes with activities ranging from 150 to 170 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ (18).

The availability of all four recombinant enzymes allowed the reconstitution of the last four steps of the 3HP/4HB pathway *in vitro*. The acetyl-CoA that was produced was converted to acetate by the ACS of *P. furiosus* (Fig. 1). The reaction mixture was derivatized with 2,4-dibromoacetophenone (DBAP) and then separated on a reverse-phase HPLC C_{18} column. The reaction mixture, containing 4HB and all the necessary cofactors and enzymes, was first incubated at 70°C, followed by the addition of Pf-ACS and a second incubation at 95°C to convert all the acetyl-CoA to acetate. 4-Hydroxybutyrate-CoA ligase (Msed_0406) was added in 10-fold excess of the other enzymes in order to overcome the initial kinetic barrier to the reaction. The HPLC chromatograms for the reaction mixture, control, and standards (Fig. 2) confirm the *in vitro* conversion of 4HB to acetate using recombinant versions of these enzymes, with approximately 25% conversion of 4HB to acetate.

Refined autotrophic growth conditions for *M. sedula* transcriptomic analysis. In order to obtain insight into the function of the acetyl-CoA branch of the 3HP/4HB pathway, a global transcriptomic analysis of *M. sedula* was carried out. In a previous study, autotrophic cultures were grown in sealed bottles with a known headspace gas composition and gas mass transfer (H_2 , CO_2 , and O_2) into the liquid medium was not controlled and growth was subject to significant diffusional limitations (1). To

overcome this issue in the work described herein, gas-intensive autotrophic growth of *M. sedula* was optimized by controlling the gas feed to a bioreactor (3 liter) using rotameters, and a micro-bubble sparging stone (2- μm pore size) was used to increase the dissolution of sparingly soluble gases, H_2 in particular. The doubling time for *M. sedula* exponential growth for H_2 - CO_2 autotrophy decreased from 11 to 13 h in sealed bottles to 5 to 6 h in the gas-intensive bioreactor, indicative of significant gas-liquid mass transfer limitations in the static cultures. These improved doubling times for autotrophic growth were comparable to heterotrophic growth on 0.1% tryptone (doubling time [t_d] = 5 to 6 h), suggesting that metabolic limitations from the gas supply were alleviated to a significant extent.

To investigate the role of the acetyl-CoA branch, *M. sedula* was grown under two different conditions: autotrophic carbon limited (ACL) (80% H_2 and 20% N_2) and heterotrophic (HTR) (80% N_2 and 20% CO_2 , with 0.1% tryptone supplemented to the medium). Under the ACL condition, all available inorganic carbon came from atmospheric CO_2 in the air feed. The observed growth rate for HTR cultures ($t_d = 6.7$ h) was faster than that of the ACL culture ($t_d = 9.4$ h), which indicates that the CO_2 levels for the ACL condition were low enough to be limiting to growth.

Of the 2,293 protein-coding genes in the *M. sedula* genome, 984 responded 2-fold or more when comparing HTR with the ACL condition. While the trends were consistent with previous results from less defined growth conditions (1), in many cases, they were more pronounced here. Among the most highly up-regulated genes for the HTR versus ACL contrast were those directly involved in CO_2 fixation in the 3HP/4HB pathway, especially the genes encoding acetyl-CoA/propionyl-CoA carboxylase (ACCA β) (Msed_0147, Msed_0148, Msed_1375; 7- to 30-fold), 4-hydroxybutyryl-CoA dehydratase (Msed_1321; 27-fold), and carbonic anhydrase (Msed_0390; 29-fold). The transcript levels were induced nearly 30-fold for the carbon-limited condition (ACL) compared to the results in heterotrophy. This indicates that

increasing the rate of bicarbonate formation from CO₂ is essential for autotrophic growth. The intracellular pH of *M. sedula* has been measured to be around 5.5 (19). At that pH, the rapid ionization of carbonic acid to bicarbonate would provide the necessary substrate for the operation of the 3HP/4HB pathway. Carbonic anhydrase serves to drive the conversion of gaseous CO₂ to carbonic acid inside the cytoplasm. The transcriptomics data, combined with the lower growth rates of CO₂-limited cultures, indicate that bicarbonate availability and the subsequent carboxylation are crucial rate-determining steps for autotrophic growth.

Carbon fixation via 3HP/4HB also requires reducing power, which is provided in *M. sedula* by soluble and membrane-bound hydrogenases. The transcriptional response data from growth under gas-intensive conditions provided a clearer picture of the role of genes associated with *M. sedula* hydrogenases in H₂-CO₂ autotrophy. The genes at Msed_0913 to Msed_0950 (Msed_0913–0950) were all upregulated: this locus encodes the two Ni-Fe hydrogenases (Msed_0923–0924 and Msed_0944–0945), multiple accessory proteins (HypABCD), a maturation protease (Msed_0916), and additional hypothetical proteins. Transcripts for both Ni-Fe hydrogenases were upregulated 5- to 10-fold under autotrophy, although their absolute transcript levels differed significantly; Msed_0944–0945 was transcribed at ~30-fold higher levels than Msed_0923–0924 for both heterotrophy and autotrophy. Msed_0943–0950, which encodes a membrane-associated hydrogenase, was also strongly upregulated under autotrophic conditions (Msed_0949, 48-fold increase; Msed_0948, 42-fold increase; and Msed_0947, 19-fold increase).

Assimilation and anapleurosis of acetyl-CoA during H₂-CO₂ autotrophy. The transcriptomics data acquired through the gas-intensive bioreactor provided a more complete perspective on the assimilation and anapleurosis of acetyl-CoA during growth of *M. sedula* under H₂-CO₂ autotrophy. Figure 3 summarizes these data for genes implicated in CO₂ fixation and central metabolism (adapted from reference 8).

The transcriptional response data showed that most genes directly involved in the 3HP/4HB CO₂ fixation cycle (Fig. 3, top right) were upregulated under limiting CO₂ concentrations. However, the genes encoding the incomplete tricarboxylic acid (TCA) cycle were not as responsive, suggesting other mechanisms of regulation. These data support previous carbon flux analysis for the 3HP/4HB pathway that showed that carbon from CO₂ enters the central metabolism via succinate (8). Genes encoding succinate dehydrogenase (Msed_0674–0677) (Fig. 3, number 19) were constitutively transcribed at high levels (>75% of the transcriptome based on transcript abundance), along with a gene whose product is annotated as fumarate hydratase (Msed_1462) (Fig. 3, number 20) (>70% based on transcript abundance). No strong transcriptional response was observed for potential candidates for succinic semialdehyde dehydrogenase (Msed_0367, Msed_1298, or Msed_1774) (Fig. 3, number 18) or succinyl-CoA synthetase (Msed_1581–1582) (Fig. 3, number 17), whose expression levels were actually downregulated under autotrophy (6-fold and 5-fold, respectively). This is consistent with the low activity reported for succinyl-CoA synthase in extracts of heterotrophically grown cells (146 nmol min^{−1} mg^{−1}), which was actually higher than the activity in extracts from autotrophically grown cells (36 nmol min^{−1} mg^{−1}) (8). The transcript levels for possible succinic semialdehyde dehydrogenase (Fig. 3, number 18) candidates varied: Msed_1774 decreased under autotrophy (down 3.6-fold),

while Msed_0367 and Msed_1298 showed no differential response and average transcript levels relative to the entire transcriptome. Another potential candidate for succinic semialdehyde dehydrogenase in *M. sedula* is Msed_1119, which has 52% amino acid identity and 72% amino acid similarity to the recently characterized succinic semialdehyde dehydrogenase from *Sulfolobus tokodaii* (ST0064) (20). Msed_1119 was transcribed at extremely low levels (<5% based on transcript abundance) under both autotrophy and heterotrophy and showed no transcriptional response between the two conditions. Whether these four ORFs (which have all been annotated as aldehyde dehydrogenases) are involved in connecting the 3HP/4HB pathway to the central metabolism or whether other unidentified genes are responsible for these biotransformations remains to be seen.

In a previous study, metabolic flux analysis of *M. sedula* metabolism using labeled 4-hydroxy[1-¹⁴C]butyrate and [1,4-¹³C]₂succinate showed an unexpected route linking the carbon fixation cycle to the central metabolism (8). Initially, it was suggested that acetyl-CoA was reductively carboxylated directly to pyruvate by pyruvate synthase (2). However, the labeling patterns of the amino acids did not support this hypothesis, and instead it was argued that the major flux from the carbon fixation pathway happens via succinyl-CoA. Oxidation of succinyl-CoA to malate (Fig. 3, numbers 19 and 20) and oxaloacetate (Fig. 3, number 21) ultimately yields pyruvate (25 in Fig. 3) and phosphoenolpyruvate (PEP) (Fig. 3, number 27), respectively. Therefore, to make one molecule of pyruvate with the 3HP/4HB pathway requires 1.5 turns of the cycle—one full turn to make acetyl-CoA and another half-turn to make succinyl-CoA. In the anaerobic DC/4HB pathway, pyruvate can be formed directly from acetyl-CoA by reductive carboxylation. This makes the aerobic 3HP/4HB pathway nearly twice as expensive energetically, requiring nine ATP equivalents to make one molecule of pyruvate compared to five for the DC/4HB pathway (5, 8).

Although the genes encoding succinic semialdehyde dehydrogenase and succinyl-CoA synthetase were not regulated transcriptionally, the data do not preclude their involvement. In the case of succinic semialdehyde dehydrogenase, it may be that there are as-yet-unidentified genes responsible for the conversion. Clearly, the activity levels are sufficient for the transformations, and the labeling data unambiguously support the primacy of the succinate branch for carbon flux into the central metabolism (8).

Beyond succinyl-CoA, it appears that acetyl-CoA assimilation still has an important role as a biosynthetic precursor, based on the total cell carbon measured in the labeling studies (8). This does not occur through reductive carboxylation of acetyl-CoA to pyruvate but, instead, through incorporation into other biomolecules. There are three main pathways by which this can occur. First, acetyl-CoA is used for the formation of some central carbon intermediates, such as citrate and malate (Fig. 3). Second, acetyl-CoA is also essential for isoprenoid-based lipid biosynthesis in *Archaea* (21, 22), and indeed, 33% of the 4-hydroxy[1-¹⁴C]butyrate label fed to autotrophically growing *M. sedula* ended up in the lipid and pigment fraction (8). Finally, evidence has begun to emerge that protein acetylation, which until recently was believed not to occur in *Archaea*, plays an important role in cotranslational N-terminal acetylation, posttranslational modification, and gene regulation through histone modification (23).

The initial steps of isoprenoid biosynthesis require the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA (Fig. 3).

FIG 3 Transcriptional heat map for proposed 3HP/4HB cycle and central metabolism in *M. sedula*. Metabolic diagram shows 3HP/4HB pathway (top center), incomplete tricarboxylic acid (center), gluconeogenesis (bottom center), and isoprenoid-based lipid biosynthesis pathways (top left), with normalized transcription levels for relevant genes. The heat map legend (bottom left) shows color values for transcription levels, with high transcription levels shown in red and low transcription in green. The corresponding numbers represent least squares mean values of normalized log₂-transformed transcription levels relative to the overall average transcription level of 0 (depicted as black). For all heat maps, the autotrophic carbon-limited condition appears on the left and the heterotrophic

When growing autotrophically, acetoacetyl-CoA could be recruited directly from the 3HP/4HB pathway or, alternatively, formed by acetyl-CoA acetyltransferase (Msed_1647) (Fig. 3, number 36). 3-Hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) is formed by HMG-CoA synthase (Msed_1646) (Fig. 3, number 37) and reduced to mevalonic acid (24). Interestingly, both Msed_1646 and Msed_1647 are expressed at 5-fold higher levels under the HTR than under the ACL condition. However, HMG-CoA is also produced during the catabolism of leucine, which may account for the increased expression levels of these two genes. The change in transcription level could also be related to the differences in growth rates between the HTR and ACL conditions. The remaining enzyme in the synthesis of mevalonate (Msed_1649) (Fig. 3, number 38) did not show any differential transcription between the growth conditions tested.

The assimilation of acetyl-CoA directly into central carbon intermediates occurs both through citrate and malate synthesis. There are two genes in *M. sedula* annotated as encoding citrate synthase (Msed_0281 and Msed_1522) (Fig. 3, number 22) that, under autotrophy, were downregulated 7-fold and upregulated 3.8-fold, respectively. The role of malate synthase in *M. sedula* metabolism is still uncertain. There is a gene in the *M. sedula* genome annotated as encoding malate synthase, Msed_1042, that is constitutively expressed at high levels (80%), and malate synthase activity has been measured in autotrophic cell extracts ($58 \text{ nmol min}^{-1} \text{ mg}^{-1}$) (8). Typically, malate synthase operates in conjunction with isocitrate lyase to form the glyoxylate bypass and allow the assimilation of acetate using the TCA cycle (25). However, *M. sedula* does not have a gene annotated as an isocitrate lyase and activity was found neither in autotrophic nor heterotrophic extracts, which suggests that glyoxylate is not being formed from isocitrate. Thus, it is unclear what role malate synthase has in *M. sedula* or how the glyoxylate is being formed. The recent report of malate synthase participation in pentose metabolism in *Sulfolobales* does not appear to be related, as *M. sedula* does not grow on pentose sugars (26).

Regulation of flux between the succinate and acetyl-CoA branches. Succinyl-CoA, therefore, represents a branching point where carbon flux either proceeds toward malate or continues through the cycle to 4HB and acetyl-CoA. Based on the enzyme kinetic data presented herein, flux through the acetyl-CoA branch appears to be limited by the activity of 4-hydroxybutyrate-CoA ligase. Previous work with acetyl-CoA synthetase from *Salmonella enterica* has established that acetyl-CoA synthetase is posttranslationally regulated by deacetylation of a conserved lysine residue by CobB, a sirtuin of the Sir2 family (27). Sirtuins are NAD^{+} -dependent deacetylases, known to be involved in chromosome stability

STM4275	600	D	S	L	P	K	T	R	S	G	K	I	M	R	612
Msed_0394	521	G	P	I	P	M	T	A	T	G	K	M	Q	K	533
Msed_0406	529	D	E	L	P	K	T	I	S	G	K	I	R	R	541
Msed_1456	608	D	K	L	P	K	T	R	S	G	K	V	M	R	620
SSO1314	508	N	E	I	P	K	T	A	T	G	K	I	Q	R	520
AF0366	626	N	T	L	P	K	T	R	S	G	K	I	M	R	638

FIG 4 Amino acid sequence alignments of the conserved P(X₄)GK motif in ACS from *Salmonella enterica*, *M. sedula*, *Sulfolobus solfataricus*, and *Archaeoglobus fulgidus*. Sequence alignments were generated with ClustalW.

and gene silencing (28), that have now also been implicated in posttranslational regulation of acyl-CoA synthetases. In *S. enterica*, the corresponding protein acetyltransferase (Pat) that acetylates the conserved lysine and inactivates the protein has also been identified (29). Archaeal acyl-CoA synthetases contain the same conserved lysine residue (Fig. 4). In *Sulfolobus solfataricus* P2, this same Sir2/Pat system is used to regulate Alba, a conserved chromatin protein (30, 31). The conserved lysine in Alba also shares the same sequence motif as the ACS sequence, P(X₄)GK, and it has been speculated, therefore, that the Sir2/Pat system in *Archaea* could also function for ACS regulation (31). Homologs to the *S. solfataricus* P2 Sir2/Pat system are also present in the *M. sedula* genome (Sir2, Msed_1096, 63% identity to SSO2478; Pat, Msed_1411, 55% identity to SSO2813). If the Sir2/Pat system in *M. sedula* serves to regulate the activity of acyl-CoA ligases like Msed_0406, then it would serve to modulate activity across all substrates of the enzyme, not just 4HB.

Regulation of 4-hydroxybutyrate-CoA ligase activity makes sense from an energetic standpoint, since this reaction requires an investment of 2 ATP to activate 4HB and form the thioester bond. Thermodynamically, this investment is not essential for the transformation of 4HB to acetate. However, the formation of the high-energy thioester bond serves to help overcome other, less thermodynamically favorable reactions elsewhere in the carbon fixation pathway, such as carboxylation and carbonyl reduction reactions (32). The high Michaelis-Menten constant (2 mM) for Msed_0406 on 4HB indicates that intracellular levels of 4HB must be high to overcome the activity barrier. The reaction rate for the subsequent transformation, the dehydration by 4-hydroxybutyryl-CoA dehydratase, is also low ($2.2 \mu\text{mol min}^{-1} \text{ mg}^{-1}$), and hence, these two reactions form the rate-limiting steps for the acetyl-CoA branch. The final three reactions, catalyzed by the bifunctional crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase (Msed_0399) and β -ketothiolase (Msed_0656), have much higher reaction rates, 20/16 $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ and 140 $\mu\text{mol min}^{-1} \text{ mg}^{-1}$, respectively. Taken together, these indicate that 4-hydroxybutyrate-CoA ligase activity serves as the entry

condition on the right. Acetyl-CoA (Ac-CoA) is shown in red boxes to highlight where it is produced or required; amino acid biosynthesis groups are shown in black boxes. The metabolic network shown here is adapted from Estelmann et al. (8). Enzymes: 1, acetyl-CoA/propionyl-CoA carboxylase; 2, malonyl-CoA reductase (NADPH); 3, malonic semialdehyde reductase (NADPH); 4, 3HP-CoA synthetase (AMP forming); 5, 3-hydroxypropionyl-CoA dehydratase; 6, acryloyl-CoA reductase (NADPH); 7, acetyl-CoA/propionyl-CoA carboxylase; 8, methylmalonyl-CoA epimerase; 9, methylmalonyl-CoA mutase; 10, succinyl-CoA reductase (NADPH); 11, succinic semialdehyde reductase (NADPH); 12, 4HB-CoA ligase (AMP forming); 13, 4-hydroxybutyryl-CoA dehydratase; 14 and 15, crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase (NAD^{+}); 16, acetoacetyl-CoA β -ketothiolase; 17, succinyl-CoA synthetase (ADP forming); 18, succinic semialdehyde dehydrogenase; 19, succinate dehydrogenase; 20, fumarate hydratase; 21, malate dehydrogenase; 22, (si)-citrate synthase; 23, aconitase; 24, isocitrate dehydrogenase; 25, malic enzyme; 26, pyruvate:water dikinase (ATP); 27, PEP carboxylase; 28, PEP carboxykinase (GTP); 29, enolase; 30, phosphoglycerate mutase; 31, phosphoglycerate kinase; 32, glyceraldehyde-3-phosphate dehydrogenase; 33, triosephosphate isomerase; 34, fructose 1,6-bisphosphate aldolase/phosphatase; 35, malate synthase; 36, acetyl-CoA acetyl-transferase; 37, HMG-CoA synthase; 38, HMG-CoA reductase. Abbreviations: 3HP, 3-hydroxypropionate; Suc-CoA, succinyl-CoA; Suc.semi., succinic semialdehyde; 4HB, 4-hydroxybutyrate; AcAc-CoA, Acetoacetyl-CoA; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; PEP, phosphoenolpyruvate; F6P, fructose-6-phosphate.

point both kinetically and energetically to the acetyl-CoA branch and, as such, is the primary determinant of carbon flux distribution.

Conclusion. Here, the recombinant expression and characterization of two enzymes in the final steps of the 3HP/4HB pathway are reported and *in vitro* production of acetate from 4HB is confirmed. The level of biochemical detail of the 3HP/4HB pathway in relationship to the central metabolism continues to develop, which will inform future metabolic engineering prospects for microbial biosynthesis of fuels and organic chemicals (33).

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