Enzymes Catalyzing Crotonyl-CoA Conversion to Acetoacetyl-CoA During the Autotrophic CO\textsubscript{2} Fixation in \textit{Metallosphaera sedula}

Li Liu\textsuperscript{1}, Harald Huber\textsuperscript{2} and Ivan A. Berg\textsuperscript{1*}

\textsuperscript{1} Institute for Molecular Microbiology and Biotechnology, University of Münster, Münster, Germany, \textsuperscript{2} Institute for Microbiology and Archaeal Center, University of Regensburg, Regensburg, Germany

Autotrophic Crenarchaeota use two different cycles for carbon dioxide fixation. Members of the Sulfolobales use the 3-hydroxypropionate/4-hydroxybutyrate (HP/HB) cycle, whereas Desulfurococcales and Thermoproteales use the dicarboxylate/4-hydroxybutyrate cycle. While these two cycles differ in the carboxylation reactions resulting in the conversion of acetyl-CoA + 2 CO\textsubscript{2} to succinyl-CoA, they have a common regeneration part in which succinyl-CoA is reconverted to two acetyl-CoA molecules. This common part includes crotonyl-CoA conversion to acetoacetyl-CoA, which has unequivocally been shown in \textit{Ignicoccus hospitalis} (Desulfurococcales) and \textit{Pyrobaculum neutrophilus} (Thermoproteales) to be catalyzed by a bifunctional crotonase/3-hydroxybutyryl-CoA dehydrogenase. It is a fusion protein consisting of an enoyl-CoA hydratase and a dehydrogenase domain. As the homologous bifunctional protein is present in Sulfolobales as well, its common functioning in the conversion of crotonyl-CoA to acetoacetyl-CoA was proposed. Here we show that a model autotrophic member of Sulfolobales, \textit{Metallosphaera sedula}, possesses in addition to the bifunctional protein (Msed_0399) several separate genes coding for crotonyl-CoA hydratase and (S)-3-hydroxybutyryl-CoA dehydrogenase. Their genes were previously shown to be transcribed under autotrophic and mixotrophic conditions. The dehydrogenase Msed_1423 (and not the bifunctional protein Msed_0399) appears to be the main enzyme catalyzing the (S)-3-hydroxybutyryl-CoA dehydrogenase reaction. Homologs of this dehydrogenase are the only (S)-3-hydroxybutyryl-CoA dehydrogenases present in all autotrophic Sulfolobales, strengthening this conclusion. Two uncharacterized crotonase homologs present in \textit{M. sedula} genome (Msed_0336 and Msed_0384) were heterologously produced and characterized. Both proteins were highly efficient crotonyl-CoA hydratases and may contribute (or be responsible) for the corresponding reaction in the HP/HB cycle in vivo.

Keywords: 3-hydroxypropionate/4-hydroxybutyrate cycle, Sulfolobales, \textit{Metallosphaera sedula}, crotonyl-CoA hydratase, 3-hydroxybutyryl-CoA dehydrogenase
INTRODUCTION

Autotrophic CO₂ fixation is the most important biosynthetic process in nature, being responsible for the primary production of organic matter. Autotrophy is arguably the oldest type of metabolism (Wächtershäuser, 1988; Russell and Martin, 2004; Fuchs, 2011), and the first metabolic pathway was probably the (autotrophic) reductive acetyl-CoA pathway (Russell and Martin, 2004). During evolution, several fundamentally different CO₂ fixation pathways evolved. They vary not only in their reaction sequences, but also in their types of carboxylases, cofactors, and electron donors used to fix inorganic carbon into biomass (Berg et al., 2010a; Berg, 2011; Fuchs, 2011; Hügler and Sievert, 2011). Conversion of CO₂, a greenhouse gas, into value-added products attracts great attention to autotrophic metabolism, rendering it one of the popular topics for fundamental and applied research.

The 3-hydroxypropionate/4-hydroxybutyrate (HP/HB) cycle was discovered in thermophilic aerobic Crenarchaeota of the order Sulfolobales (Figure 1; Ishii et al., 1996; Menendez et al., 1999; Berg et al., 2007). Later, a formally similar, but phylogenetically unrelated pathway was shown in aerobic ammonia-oxidizing Thaumarchaeota (Köneke et al., 2014; Otte et al., 2015). While the autotrophic cycle in Thaumarchaeota is less studied, with some characteristic enzymes of the cycle still being unidentified, all enzymes of the crenarchaeal cycle were identified and biochemically characterized (Table 1).

In former studies, the model crenarchaeon Metallosphaera sedula was used; it is capable of growing autotrophically with hydrogen, sulfidic ores or S⁰ under aerobic conditions. Furthermore, heterotrophic growth with, e.g., peptone or yeast extract and mixotrophic growth in the presence of organic compounds and inorganic electron donors is possible. The transcriptomic studies of M. sedula revealed that most of the enzymes of the cycle were up-regulated under mixotrophic and autotrophic growth conditions (Auernik and Kelly, 2010; Hawkins et al., 2013a; Ai et al., 2019). Nevertheless, there were several notable exceptions, as 3-hydroxypropionyl-CoA dehydratase, 4-hydroxybutyryl-CoA synthetase and bifunctional crotonyl-CoA hydratase/3-hydroxybutyryl-CoA dehydrogenase were not regulated, or even down-regulated in the presence of inorganic electron donors (Table 1; Auernik and Kelly, 2010; Hawkins et al., 2013a; Ai et al., 2019). Two of these enzymes, 4-hydroxybutyryl-CoA synthetase and bifunctional crotonyl-CoA hydratase/3-hydroxybutyryl-CoA dehydrogenase, stand out in comparative analysis of specific activities of the purified enzymes with the corresponding activities in cell extracts of M. sedula. This analysis allows to estimate the amount of the individual enzyme proteins required to account for autotrophic growth, based on the specific activities of the purified enzymes and the measured specific enzyme activities in cell extract. This estimate led to the surprising high number of 58% of total cellular protein required to operate the HP/HB cycle (Table 1). 4-Hydroxybutyryl-CoA synthetase and crotonyl-CoA hydratase/3-hydroxybutyryl-CoA dehydrogenase alone were responsible for more than half of this amount (12.5 and 19.7%, respectively). Whereas 4-hydroxybutyryl-CoA dehydratase appears to constitute a large fraction of cellular protein as well, it slowly catalyzes a mechanistically difficult radical reaction (Martins et al., 2004; Buckel, 2019) and is indeed present in high amounts in other organisms (Scherf and Buckel, 1993). Furthermore, the posttranscriptional inactivation of AMP-producing organic acid CoA-ligases by acetylation has been frequently reported and may result in down-estimation of the activity of recombinant 4-hydroxybutyryl-CoA synthetase (Crosby et al., 2010; Ramos-Vera et al., 2011). However, crotonyl-CoA hydratase catalyzes a relatively simple reaction, and its postulated high abundance in the cell is surprising.

Here we studied enzymes catalyzing the conversion of crotonyl-CoA to acetoacetyl-CoA in M. sedula. We found that this conversion might be catalyzed by a number of isoenzymes with different kinetic properties, and not by a single bifunctional enzyme, as was previously thought.

MATERIALS AND METHODS

Materials

Chemicals and biochemicals were obtained from Fluka (Neu-Ulm, Germany), Sigma-Aldrich (Deisenhofen, Germany), Merck (Darmstadt, Germany), VWR (Darmstadt, Germany), Roth (Karlsruhe, Germany), Applichem (Darmstadt, Germany), IBA (Göttingen, Germany) or Cell Signaling Technology (Frankfurt, Germany). Materials for cloning and expression were purchased from MBI Fermentas (St. Leon-Rot, Germany), New England Biolabs (Frankfurt, Germany), and Novagen (Schwalbach, Germany). Materials and equipment for protein purification were obtained from GE Healthcare (Freiburg, Germany), Macherey-Nagel (Düren, Germany), Pall Corporation (Dreieich, Germany) or Millipore (Eschborn, Germany). Primers were synthesized by Sigma-Aldrich (Steinheim, Germany).
TABLE 1 | Overview of the enzymes involved in the 3-hydroxypropionate/4-hydroxybutyrate cycle of autotrophic CO$_2$ assimilation in M. sedula.

| Reaction (Nr. in Figure 1) | Genes, according to Berg et al. (2007) | Genes, according to Loder et al. (2016) | Min. act. in cell extract (Berg et al., 2007) | $V_{\text{max}}$ or specific activity, µmol min$^{-1}$ mg$^{-1}$ protein | Regulation: auto/mixo. versus heterotob | Min. % of cellular protein | References |
|---------------------------|----------------------------------------|----------------------------------------|---------------------------------|---------------------------------|---------------------------------|----------------------|----------------|
| Acetyl-CoA carboxylase (1) | Msed_0147/ Msed_0148/ Msed_1375        | Msed_0147/ Msed_0148/ Msed_1375        | 0.11                            | $-3.2^{3,e}$                   | Up                              | 3.4                  | Hügler et al., 2003 |
| Malonyl-CoA reductase (2)  | Msed_0709                               | Msed_0709                               | 0.42                            | $-44^{9}g$/$-40^{1,g}$         | Up                              | 0.5                  | Kockelkorn and Fuchs, 2009; Loder et al., 2016 |
| Malonic semialdehyde reductase (3) | Unknown                                | Msed_1993                               | 3.0                             | $-200^{1,g}$                   | Up                              | 0.5                  | Kockelkorn and Fuchs, 2009 |
| 3-Hydroxypropionyl-CoA synthetase (4) | Msed_1456                              | Msed_1456                               | 0.24                            | 18$^{9,2}$                      | Up                              | 0.7                  | Alber et al., 2008 |
| 3-Hydroxypropionyl-CoA dehydratase (5) | Msed_2001                              | Msed_2001                               | 0.24                            | $-151^{1,g}$/$-272^{1,g}$     | No                              | 0.04                 | Teufel et al., 2009; Loder et al., 2016 |
| Acryloyl-CoA reductase (6) | Msed_1426                               | Msed_1426                               | 0.24                            | $-7.6^{9,3}$/$-18.7^{9,3}$    | Up                              | 0.9                  | Teufel et al., 2009; Loder et al., 2016 |
| Propionyl-CoA carboxylase (7) | Msed_0147/ Msed_0148/ Msed_1375        | Msed_0147/ Msed_0148/ Msed_1375        | 0.12                            | $-3.3^{4,a}$                   | Up                              | 3.6                  | Hügler et al., 2003 |
| Methylmalonyl-CoA epimerase (8) | Msed_0639                              | Msed_0639                               | 0.06$^i$                        | $-218^{8,9}$                   | (Up)                            | 0.1                  | Han et al., 2012   |
| Methylmylonyl-CoA isomerase (9) | Msed_0638/ Msed_2055                   | Msed_0638/ Msed_2055                   | 0.06$^i$                        | $-2.2^{7,3}$                   | Up                              | 1.4                  | Han et al., 2012   |
| Succinyl-CoA reductase (10) | Msed_0709                               | Msed_0709                               | 1.0                             | $-40^{9,9}$                    | Up                              | 1.3                  | Kockelkorn and Fuchs, 2009 |
| Succinic semialdehyde reductase (11) | Msed_1424                              | Msed_1424                               | 5.0                             | $-700^{9,9}$/$-695^{9,3}$     | Up                              | 0.4                  | Kockelkorn and Fuchs, 2009; Loder et al., 2016 |
| 4-Hydroxybutyryl-CoA synthetase (12) | Msed_1422                              | Msed_0406                               | 0.3                             | ND$^i$/$-1.7^{8,2,h}$         | Up                              | 12.5                 | Berg et al., 2007; Hawkins et al., 2013a, 2014 |
| 4-Hydroxybutyryl-CoA dehydratase (13) | Msed_1220                              | Msed_1321                               | 0.39                            | ND$^i$/$-2.2^{8,2,h}$         | No                              | Up                   | 12.5                 | Hawkins et al., 2014 |
| Crotonyl-CoA hydratase (14) | Msed_0399                               | Msed_0399                               | 15.0                            | $13.8^{9,9}/38^{9,9}$/$-20^{8,2,h}$ | ND                              | Down                 | 19.7                 | Ramos-Vera et al., 2011; Hawkins et al., 2014 |
| (S)-3-Hydroxybutyryl-CoA dehydrogenase (15) | Msed_1423                              | Msed_0399                               | 1.6                             | ND$^i$/$-16^{8,2,h}$/$-j$     | ND                              | No                   | (2.1)$^k$            | Kockelkorn and Fuchs, 2009; Hawkins et al., 2014 |

(Continued)
TABLE 1 | Continued

| Reaction (Nr. in Figure 1) | Genes, according to Berg et al. (2007) | Genes, according to Loder et al. (2016) | Min. act. in cell extract (Berg et al., 2007) | \( V_{\text{max}} \) or specific activity, \( \mu \) mol min\(^{-1}\) mg\(^{-1}\) protein | Regulation: auto/mixo. versus hetero\(^{b}\) | Min.% of cellular protein\(^{c}\) | References |
|-----------------------------|--------------------------------------|--------------------------------------|----------------------------------------|------------------------------------------|---------------------------------|---------------------------------|-----------------|
| Acetoacetyl-CoA \( \beta \)-ketothiolase (16) | Msed_0656 | Msed_0656 | 1.06 | Up | 0.5 | Hawkins et al., 2014 |
| Msed_1647 | | | | | |
| Msed_1290 | | | | | |
| Msed_0096 | | | | | |
| Msed_0386 | | | | | |
| Msed_0271 | | | | | |
| Msed_0270 | | | | | |
| Summe | – | – | – | – | 58.04 | – | – |

\(^{a}\)Minimal activity in cell extracts of M. sedula was tentatively extrapolated to 75°C. \(^{b}\)Up-regulation in mixotrophically and autotrophically grown cells, based on transcriptomic analysis (Kerms and Kelly, 2010; Hawkins et al., 2013a; Ai et al., 2019); up, clear up-regulation; (up), slightly up-regulated; down, down-regulated; no, no regulation; ?, no obvious trend. \(^{c}\)The relative abundance of individual protein in the cell (%).
0.5 ml min⁻¹ to a 1-ml Protino Ni-NTA column (Macherey-Nagel) that had been equilibrated with 20 mM Tris–HCl containing 150 mM KCl (pH 7.8 for Msed_1423 and Msed_0389, pH 7.6 for Msed_0384, and pH 8.0 for Msed_0336). The column was washed with the same buffer containing 50 mM imidazole at a flow rate of 0.5 ml min⁻¹ to elute unwanted protein. The recombinant enzymes were eluted with the same buffer containing 500 mM imidazole. The enzymes were concentrated using 10K Vivaspin Turbo 4 (Sartorius, Göttingen, Germany) and stored at −20°C with 50% glycerol. The identity of the purified recombinant proteins was confirmed using gel digestion by trypsin followed by LC-MS/MS (Supplementary Table S2).

**Enzyme Assays**

The 3-hydroxybutyryl-CoA dehydratase and 3-hydroxypropionyl-CoA dehydratase activities were measured at 42°C. All other enzyme activities were measured at 65°C.

**Crotonyl-CoA Hydratase**

Crotonyl-CoA hydratase activity was measured using ultra-high-performance liquid chromatography (UHPLC) by monitoring crotonyl-CoA-dependent formation of 3-hydroxybutyryl-CoA. The reaction mixture (20 µl) contained 100 mM Tris–HCl (pH 7.8), 1 mM crotonyl-CoA, and purified enzyme. The reaction was stopped after 1 min by the addition of 20 µl of 1 M HCl/10% acetonitrile. Protein was removed by centrifugation, and the samples were analyzed by UHPLC using a reverse-phase (RP) C₁₈ column. For $K_m$ determination, the concentration of crotonyl-CoA was varied (0.01–5 mM). For the measurement of crotonyl-CoA hydratase activity of Msed_0399, 100 mM Tris–HCl (pH 9) was used.

**3-Hydroxybutyryl-CoA Dehydratase**

3-Hydroxybutyryl-CoA dehydratase activity was measured spectrophotometrically by coupling the reaction to crotonyl-CoA carboxylase/reductase (Ccr) from *Rhodobacter sphaeroides* which reductively carboxylates crotonyl-CoA and acryloyl-CoA to ethylmalonyl-CoA and methylmalonyl-CoA, respectively (Erb et al., 2009). The reaction mixture (20 µl) contained 100 mM Tris–HCl (pH 7.8), 1 mM 3-hydroxybutyryl-CoA, 1 mM NADPH, 0.1 mg ml⁻¹ Ccr, 50 mM NaHCO₃, and purified enzyme. The reaction was stopped as described above, and the samples were analyzed by RP-C₁₈ UHPLC. For $K_m$ determination, the concentration of 3-hydroxybutyryl-CoA was varied (0.01–4 mM).

**3-Hydroxypropionyl-CoA Dehydratase**

3-Hydroxypropionyl-CoA dehydratase was measured spectrophotometrically as 3-hydroxypropionyl-CoA-dependent acryloyl-CoA formation by coupling the reaction to Ccr. The reaction mixture (20 µl) contained 100 mM MOPS/KOH (pH 7.0), 1 mM 3-hydroxypropionyl-CoA, 1 mM NADPH, 0.1 mg ml⁻¹ Ccr, 50 mM NaHCO₃, and purified enzyme. The reaction was stopped as described above, and the samples were analyzed by RP-C₁₈ UHPLC. For $K_m$ determination, the concentration of 3-hydroxypropionyl-CoA was varied (0.01–1.5 mM).

**Acryloyl-CoA Hydratase**

Acryloyl-CoA hydratase activity was measured using UHPLC by monitoring 3-hydroxypropionyl-CoA formation from acryloyl-CoA. The reaction mixture (20 µl) contained 100 mM Tris–HCl (pH 7.8), 1 mM acryloyl-CoA, and purified enzyme. The reaction was stopped as described above, and the samples were analyzed by RP-C₁₈ UHPLC. For $K_m$ determination, the concentration of acryloyl-CoA was varied (0.02–4 mM).

**Methacryloyl-CoA Hydratase**

Methacryloyl-CoA hydratase activity was measured using UHPLC by monitoring 3-hydroxy-2-methylpropionyl-CoA formation from methacryloyl-CoA. The reaction mixture (20 µl) contained 100 mM Tris–HCl (pH 7.8), 1 mM methacryloyl-CoA, and purified enzyme. The reaction was stopped as described above, and the samples were analyzed by RP-C₁₈ UHPLC. For $K_m$ determination, the concentration of methacryloyl-CoA was varied (0.01–5 mM).

**(E)-2-Octenoyl-CoA Hydratase**

(E)-2-octenoyl-CoA dehydratase activity was measured using UHPLC by monitoring 3-hydroxyoctanoyl-CoA formation from (E)-2-octenoyl-CoA. The reaction mixture (20 µl) contained 100 mM Tris–HCl (pH 7.8), 1 mM (E)-2-octenoyl-CoA, and purified enzyme. The reaction was stopped as described above, and the samples were analyzed by RP-C₁₈ UHPLC. For $K_m$ determination, the concentration of (E)-2-octenoyl-CoA was varied (0.01–2 mM).

**Analytical UHPLC**

CoA and CoA-esters were detected with Agilent 1290 Infinity II UHPLC using a reversed-phase C₁₈ column (Agilent InfinityLab Poroshell 120 EC-C₁₈ 1.9 µm 2.1 × 50 mm column). The following acetonitrile gradient in 10 mM potassium phosphate buffer (pH 7), with a flow rate of 0.55 ml min⁻¹, was used: from 2 to 8% at 0–2.66 min; from 8% to 30% at 2.66–3.33 min; from 30% to 3% at 3.33–3.68 min; 2% at 3.68–5 min. Retention times were: 2-methylmalonyl-CoA, 0.9 min; 3-hydroxypropionyl-CoA, 1.4 min; acetyl-CoA, 1.6 min; 3-hydroxybutyryl-CoA, 1.8 min; 3-hydroxy-2-methylpropionyl-CoA, 2.0 min; acryloyl-CoA, 2.2 min; propionyl-CoA, 2.4 min; crotonoyl-CoA, 2.6 min; methacryloyl-CoA, 2.9 min. For the conversions with (E)-2-octenoyl-CoA, the following acetonitrile gradient in 10 mM potassium phosphate buffer (pH 6.8), with a flow rate of 0.55 ml min⁻¹, was used: from 2 to 30% at 0–3.33 min; from 30% to 3% at 3.33–3.68 min; 2% at 3.68–5 min. Retention times were: 3-hydroxyoctanoyl-CoA, 2.3 min; 3-oxo-octanoyl-CoA, 2.4 min; (E)-2-octenoyl-CoA, 3.2 min. Reaction products and standard compounds were detected by UV absorbance at 260 nm with 1290 Infinity II diode array detector (Agilent), and the amount of product was calculated from the relative peak area. The identification of the CoA esters was based on chromatography with standards and analysis of the UV spectra of the products.
Other Methods
Apparent $K_M$ and $V_{max}$ values were calculated using GraphPad Prism5 software. Protein concentration was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. DNA sequence determination was performed by Eurofins (Ebersberg, Germany). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5%) was performed as described by Laemmli (1970). An unstained protein MW marker (Thermo Scientific, 14.4-116 kDa) and a prestained protein marker (Cell Signaling, 11-190 kDa) were used as molecular mass standard. Proteins were visualized by Coomassie blue staining (Zehr et al., 1989). Protein identification was performed at the IZKF Core Unit Proteomics Münster based on tryptic in-gel digestion and mass spectrometric analysis using Synapt G2 Si coupled to M-Class (Waters Corp.). Query sequences for the database searches were obtained from the National Center for Biotechnology Information (NCBI) data base. The BLAST searches were performed via NCBI BLAST server1 (Altschul et al., 1990).

RESULTS
Catalytic Properties of Bifunctional Crotonyl-CoA Hydratase/(S)-3-Hydroxybutyryl-CoA Dehydrogenase Msed_0399
Msed_0399 is a bifunctional crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase consisting of two domains, a C-terminal enoyl-CoA hydratase domain (~20 kDa) and an N-terminal dehydrogenase domain (~40 kDa) (Ramos-Vera et al., 2011). This enzyme is widely distributed among Archaea and has been predicted to be present in the common ancestor of the TACK superphylum, i.e., it was already present in the common ancestor of Cren- and Thaumarchaeota (Williams et al., 2017). The enzyme was purified formerly from autotrophically grown M. sedula cells, following the reduction of NAD with crotonyl-CoA, i.e., the presence of both crotonase and dehydrogenase reactions simultaneously (Ramos-Vera et al., 2011). The identified msed_0399 was cloned and heterologously produced, and the recombinant enzyme was characterized (Ramos-Vera et al., 2011; Hawkins et al., 2014). However, the characterization was preliminary, as the activity was measured only as crotonyl-CoA or (S)-3-hydroxybutyryl-CoA-dependent NAD reduction (Ramos-Vera et al., 2011; Hawkins et al., 2014). The measured hydratase activity in the coupled assay was necessarily limited by the level of the much lower dehydrogenase activity.

In order to fill this gap, we heterologously produced and purified the bifunctional protein Msed_0399, and characterized the enzyme by measuring dehydrogenase and crotonase reactions separately. In hydratase reaction, Msed_0399 was promiscuous and could hydrate not only crotonyl-CoA, but also (E)-2-octenoyl-CoA and acrylyl-CoA (Table 2). Similarly, it acted as dehydrogenase with both C4 and C8 (S)-3-hydroxyacyl-CoAs (Table 2). Interestingly, the enzyme was most active with octenoyl-CoA, thus suggesting that Msed_0399 may participate in β-oxidation, in addition to autotrophic CO2 fixation. Importantly, Msed_0399 is the only 3-hydroxyacyl-CoA dehydrogenase that was active with C8 (3-hydroxy)acyl-CoAs (Tables 2, 3), further suggesting its participation in heterotrophic metabolism.

Crotonyl-CoA Hydratase and (S)-3-Hydroxybutyryl-CoA Dehydrogenase Activities in M. sedula Cell Extract
The apparent $K_m$ value of Msed_0399 for crotonyl-CoA in the crotonase/(S)-3-hydroxybutyryl-CoA dehydrogenase reaction was four times higher than the corresponding value in M. sedula cell extract (Table 2) suggesting the presence of additional crotonase(s). Furthermore, the apparent $K_m$ value of Msed_0399 for (S)-3-hydroxybutyryl-CoA in the dehydrogenase reaction was three times higher than the corresponding value in M. sedula cell extract (Table 2). These data suggest that Msed_0399 may not be the only enzyme responsible for crotonyl-CoA hydratase and (S)-3-hydroxybutyryl-CoA dehydrogenase activity in autotrophically grown M. sedula. Therefore, we cloned and expressed other candidate genes for these enzymes (Table 1) in E. coli and characterized the catalytic activities of the resulting enzymes.

3-Hydroxybutyryl-CoA Dehydrogenases Msed_0389 and Msed_1423
The purified recombinant Msed_0389 and Msed_1423 catalyzed the NAD-dependent oxidation of (S)-3-hydroxybutyryl-CoA to acetoacetyl-CoA and were highly specific for their substrate ($K_m$ 2.6 and 5 µM, respectively; Table 3). Both enzymes were not active with (S)-3-hydroxyoctanoyl-CoA. Their $k_{cat}/K_m$ values were 3- and 20-fold higher than the corresponding value of Msed_0399 in (S)-3-hydroxybutyryl-CoA dehydrogenase reaction (Table 3). Interestingly, both these genes were shown to be up-regulated in auto- and mixotrophically grown cell (see Table 1; Auernick and Kelly, 2010; Ai et al., 2019). The Msed_0389 gene clustered together with various β-oxidation genes in the M. sedula genome. In contrast, msed_1423 clustered with several genes encoding specific enzymes of the HP/HB cycle, suggesting its involvement in autotrophic CO2 fixation (Figure 2).

Crotonyl-CoA Hydratases Msed_0336 and Msed_0384
The purified recombinant Msed_0336 and Msed_0384 catalyzed the hydration of crotonyl-CoA to (S)-3-hydroxybutyryl-CoA (Table 4); the $k_{cat}/K_m$ values of both enzymes for the reverse reaction were ~2.5 times higher. Both enzymes were also active with (E)-2-octenoyl-CoA and, to a lesser extent, with acrylyl-CoA and methacrylyl-CoA. The $V_{max}$ values of these enzymes for crotonyl-CoA hydration were lower than the $V_{max}$ of Msed_0399; yet their apparent crotonyl-CoA $K_m$ values were also much lower, resulting in a catalytic constant $k_{cat}/K_m$ similar to that

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1http://www.ncbi.nlm.nih.gov/BLAST/
acetyl-CoA to crotonyl-CoA are present in all fully sequenced Dehydrogenase Genes in Sulfolobales (Distribution of Crotonase and k).

| Substrate | Msed_0399 | M. sedula (cell extract) |
|-----------|-----------|-------------------------|
|           | $V_{\text{max}}$ (U mg$^{-1}$ protein) | $K_{\text{m}}$ (mM) | $k_{\text{cat}}/K_{\text{m}}$ | $V_{\text{max}}$ (U mg$^{-1}$ protein) | $K_{\text{m}}$ (mM) |
|           | Measured (65°C) | Extrapolated to 75°C |           |           | Measured (65°C) | Extrapolated to 75°C |
| Crotonyl-CoA hydratase | 263 ± 7 | 526 ± 14 | 0.97 ± 0.07 | 640 | 16 ± 4 | 32 ± 8 |
| (S)-3-Hydroxybutyryl-CoA dehydratase | 25 ± 1<sup>b</sup> | 246 ± 10 | 0.86 ± 0.12 | 338 | 2 ± 1<sup>b</sup> | 20 ± 10 |
| Acrylyl-CoA hydratase | 186 ± 11 | 372 ± 22 | 1.1 ± 0.20 | 399 | 132 ± 4 | 264 ± 8 |
| 3-Hydroxypropionyl-CoA dehydratase<sup>c</sup> | 0.49 ± 0.04<sup>d</sup> | 4.8 ± 0.39 | 0.06 ± 0.02 | 94 | 1 ± 0.2<sup>b</sup> | 10 ± 2 |
| Methacrylyl-CoA hydratase | 1.3 ± 0.1 | 2.6 ± 0.2 | 0.79 ± 0.12 | 4 | 2 ± 0.1 | 4 ± 0.2 |
| (E)-2-Octenoyl-CoA hydratase | 76 ± 2 | 152 ± 4 | 0.08 ± 0.01 | 2,243 | 12 ± 4 | 24 ± 8 |
| Crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydratase<sup>c</sup> | 35 ± 1 | 70 ± 2 | 0.30 ± 0.03<sup>d</sup> | 275 | 0.8 ± 0.06 | 1.6 ± 0.1 |
| (S)-3-hydroxybutyryl-CoA dehydratase<sup>c</sup> | 38 ± 1 | 76 ± 2 | 0.12 ± 0.01<sup>d</sup> | 748 | 1.3 ± 0.05 | 2.6 ± 0.1 |
| (E)-2-octenoyl-CoA hydratase<sup>c</sup> | 5.2 ± 0.4 | 10.4 ± 0.8 | 0.02 ± 0.01 | 614 | 0.75 ± 0.07 | 1.5 ± 0.1 |

The $V_{\text{max}}$ specific activity values are normalized to 75°C based on the assumption that a 10°C rise in temperature doubles the reaction rate. ND, not determined. <sup>a</sup>$k_{\text{cat}}$ was calculated in s$^{-1}$ mM$^{-1}$ for the activities at 75°C. <sup>b</sup>Activity was measured at 42°C. <sup>c</sup>The reaction was measured with the corresponding enoyl-CoA as a substrate. <sup>d</sup>The following $K_{\text{m}}$ values were detected previously: crotonyl-CoA, 0.3 and 0.07 mM, (S)-3-hydroxybutyryl-CoA, 0.2 and 0.06 mM (Ramos-Vera et al., 2011; Hawkins et al., 2014).

| Substrate | Msed_1423 | Msed_0389 |
|-----------|-----------|-----------|
|           | $V_{\text{max}}$ (U mg$^{-1}$ protein) | $K_{\text{m}}$ (mM) | $k_{\text{cat}}/K_{\text{m}}$ | $V_{\text{max}}$ (U mg$^{-1}$ protein) | $K_{\text{m}}$ (mM) | $k_{\text{cat}}/K_{\text{m}}$ |
|           | Measured (65°C) | Extrapolated to 75°C |           |           | Measured (65°C) | Extrapolated to 75°C |
| (S)-3-Hydroxybutyryl-CoA (with 0.5 mM NAD) | 48 ± 3 | 96 ± 6 | 0.005 ± 0.002 | 14,947 | 4.8 ± 0.2 | 9.6 ± 0.4 | 0.0026 ± 0.001 | 2,476 |
| NAD | 41 ± 1 | 82 ± 2 | 0.03 ± 0.003 | 2,128 | 4.8 ± 0.1 | 9.6 ± 0.2 | 0.012 ± 0.002 | 536 |
| NADP | 43 ± 1 | 86 ± 2 | 5.1 ± 0.5 | 13 | 2.9 ± 0.2 | 5.8 ± 0.4 | 1.5 ± 0.3 | 3 |

The $V_{\text{max}}$ values are normalized to 75°C based on the assumption that a 10°C rise in temperature doubles the reaction rate. No activity was detected with (S)-3-hydroxyoctanoyl-CoA (with 0.5 mM NAD; detection limit <20 U mg$^{-1}$ for Msed_1423 and <50 U mg$^{-1}$ for Msed_0389). <sup>a</sup>$k_{\text{cat}}$ was calculated in s$^{-1}$ mM$^{-1}$ for the activities at 75°C.

of Msed_0399. The clustering of msed_0384 with β-oxidation genes conforms with its higher $k_{\text{cat}}/K_{\text{m}}$ value for octenoyl-CoA. Nevertheless, it is Msed_0399 which has the highest $k_{\text{cat}}/K_{\text{m}}$ value for this substrate. In the published transcriptomic analyses, Msed_0384 was shown to be down-regulated in autotrophic and mixotrophically grown cell, while Msed_0336 showed no significant regulation in these cells (see Table 1; Auernik and Kelly, 2010; Ai et al., 2019).

**Distribution of Crotonase and (S)-3-Hydroxybutyryl-CoA Dehydrogenase Genes in Sulfolobales**

The genes of the HP/HB cycle catalyzing reactions from acetyl-CoA to crotonyl-CoA are present in all fully sequenced Sulfolobales genomes (Supplementary Table S3), indicating that they have the potential to grow autotrophically. The only exception was Sulfolobus acidocaldarius and Saccharolobus solfataricus that nevertheless encode all key genes of the HP/HB cycle in their genomes (Supplementary Table S3).

Homolog(s) of bifunctional crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase Msed_0399 genes were found in 75% of the genomes, whereas four autotrophic
DISCUSSION

In vivo proteins suggest that crotonyl-CoA hydratase and (S)-3-hydroxybutyryl-CoA dehydratase, in turn, have crotonase activity (Teufel et al., 2009). Although analysis of deletion mutants would be very helpful to clarify the role of individual proteins in autotrophic CO₂ fixation, genetic system is unfortunately not available for autotrophic Sulfolobales.

In contrast to the current understanding, our data show that the bifunctional crotonyl-CoA hydratase/(S)-3-hydroxybutyryl-CoA dehydrogenase is not the main enzyme that catalyzes these reactions in *M. sedula*. This is corroborated by the fact that its gene is absent in many other autotrophic Sulfolobales. In fact, *msed_0399* is located in the cluster with the genes homologous to various β-oxidation genes (Figure 2A) and may thus be primarily involved in, e.g., fatty acid metabolism rather than CO₂ fixation (Table 2). Although the growth of *M. sedula* on fatty acids has not been shown, some Sulfolobales are capable to use these compounds as a sole carbon source (Wang et al., 2019). Furthermore, reversibility of β-oxidation enzymes in Archaea and their involvement in fatty acid synthesis was proposed (Dibrova et al., 2014).

Interestingly and in contrast to Sulfolobales, the homologous bifunctional protein is the only enzyme capable to covert crotonyl-CoA into acetoacetyl-CoA in autotrophic Desulfurococcales (*Iignicoccus hospitalis*) and Thermoproteales (*Pyrobaculum neutrophilus*) that use the dicarboxylate/4-hydroxybutyrate cycle for CO₂ fixation (Huber et al., 2008; Ramos-Vera et al., 2009, 2011). This cycle differs from the HP/HB cycle in the carboxylation reactions (ferredoxin-dependent pyruvate synthase and phosphoenolpyruvate carboxylase) but shares the regeneration part, i.e., the conversion of succinyl-CoA into acetyl-CoA that includes crotonase and (S)-3-hydroxybutyryl-CoA dehydrogenase reactions. The anaerobic dicarboxylate/4-hydroxybutyrate cycle is probably the ancestral CO₂ fixation pathway in Crenarchaeota, and the presence of the bifunctional protein widely distributed among Archaea seems to be an ancestral feature for the pathway.

Nevertheless, even if several enzymes catalyze one and the same reactions in *M. sedula*, our biochemical data (Table 3) and the results of our gene distribution analysis (Table 5) together with the published transcriptomic data (see Table 1) show that *Msed_1423* is the main (S)-3-hydroxybutyryl-CoA dehydrogenase in the HP/HB cycle in Sulfolobales. This protein is clustered together with two specific enzymes of the HP/HB cycle, succinyl semialdehyde reductase *Msed_1424* and acryloyl-CoA reductase *Msed_1426* (Figure 2B), further supporting its involvement in autotrophic CO₂ fixation. The

### TABLE 4 | Catalytic properties of crotonyl-CoA hydratases Msed_0336 and Msed_0384.

| Substratea | Msed_0336 | Msed_0384 |
|------------|-----------|-----------|
|            | *V*ₘₐₓ (U mg⁻¹ protein) | *K*ₘ (mM) | *k*ₗₐₚ/ₘₐₓ | *V*ₘₐₓ (U mg⁻¹ protein) | *K*ₘ (mM) | *k*ₗₐₚ/ₘₐₓ |
|            | Measured (65°C) | Extrapolated to 75°C | Measured (65°C) | Extrapolated to 75°C |
| Crotonyl-CoA | 26 ± 1 | 52 ± 2 | 0.08 ± 0.01 | 317 | 227 ± 7 | 454 ± 14 | 0.22 ± 0.02 | 1,043 |
| (S)-3-hydroxybutyryl-CoA | 12 ± 0.8 | 119 ± 8 | 0.07 ± 0.02 | 830 | 25 ± 1 | 242 ± 12 | 0.05 ± 0.01 | 2,445 |
| Acrylyl-CoA | 9 ± 0.2 | 18 ± 0.4 | 0.07 ± 0.005 | 126 | 25 ± 1 | 50 ± 2 | 0.18 ± 0.03 | 140 |
| 3-Hydroxypropionyl-CoA | 0.4c,d | 4c | NA | NA | 0.4c,d | 4c | NA | NA |
| Methacrylyl-CoA | 17 ± 1 | 34 ± 2 | 0.29 ± 0.05 | 57 | 0.7 ± 0.1 | 1.4 ± 0.2 | 0.07 ± 0.03 | 10 |
| (E)-2-octenoyl-CoA | 12 ± 0.4 | 24 ± 0.8 | 0.09 ± 0.01 | 130 | 123 ± 5 | 246 ± 10 | 0.5 ± 0.06 | 249 |

*V*ₘₐₓ values are normalized to 75°C based on the assumption that a 10°C rise in temperature doubles the reaction rate. NA, not applicable. *k*ₗₐₚ was calculated in s⁻¹ mM⁻¹ for the activities at 75°C. *No activity was detected with (R)-3-hydroxybutyryl-CoA (detection limit ≤0.3 U mg⁻¹ Msed_0336, ≤1 U mg⁻¹ Msed_0384). *Activity was measured at 42°C. *Specific activity.

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The *S. ambivalens*, *Acidianus hospitalis*, *Acidianus sulfitivorans*, and *Stygiolobus azoricus* do not possess the corresponding gene (Table 5). Notably, the functioning of the HP/HB cycle was experimentally shown for *S. azorius* (Berg et al., 2010b), indicating that the presence of the bifunctional protein is not essential for autotrophic CO₂ fixation. The only two genes that are (almost) universally distributed in Sulfolobales are the crotonase Msed_0566 gene and the dehydrogenase Msed_1423 gene (Table 5 and Supplementary Table S3). They are absent only in the genome of *S. acidocaldarius*. 

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**Table 4** | Catalytic properties of crotonyl-CoA hydratases Msed_0336 and Msed_0384.
only species that does not possess the corresponding gene, *S. acidocaldarius*, is not capable to grow autotrophically (Zeldes et al., 2019).

The crotonase homolog, Msed_0566, was the only monofunctional crotonase (besides the promiscuous 3-hydroxypropionyl-CoA dehydratase) that was present in all Sulfolobales except for the heterotrophic *S. acidocaldarius* (**Table 5**). Unfortunately, all our attempts to heterologously express msed_0566 (as well as msed_0385) were not successful and resulted in insoluble protein. It is to be shown whether this protein is the primary crotonyl-CoA hydratase in *M. sedula* and especially in *S. azoricus, A. ambivalens*, and *A. hospitalis* (**Table 5**), or this reaction is also catalyzed by a promiscuous 3-hydroxypropionyl-CoA dehydratase, as was shown for the HP/HB cycle in Thaumarchaeota (Könneke et al., 2014).
The inability of *S. acidocaldarius* to grow autotrophically is well-known (Berg et al., 2010b; Zeldes et al., 2019). It has recently been suggested that the obligate heterotrophy of this organism is rooted rather in gene regulation than in the biochemical capacity (Zeldes et al., 2019). Our data suggest that this inability is likely due to the absence of the Msed_1423 (and possibly Msed_0566) homologs in this species.

Comparison of the activities of the characterized (S)-3-hydroxybutyryl-CoA dehydrogenase Msed_1423 and crotonase Msed_0384 with the activities of these reactions in cell extract of *M. sedula* leads to the values 1.7 and 7.1% of total cellular protein, respectively. The last value was calculated based on the activity of crotonase found in our work (Table 2), which is twice higher than the previously measured activity (Berg et al., 2007; see Table 1). With these corrected values, the estimated abundance of the HP/HB cycle enzymes is ~47% of total cellular protein, i.e., 11% less than was calculated in Table 1. This value may further be corrected (reduced?) after the characterization of the further crotonase homologs in *M. sedula*.

The studied enzymes were promiscuous, differing in their kinetic properties. Their simultaneous presence not only increases the activity of particular reactions, but also allows fast and efficient fine-tuning of metabolism and adaptation to different growth conditions, varying metabolite concentrations and growth rates. This strategy may be especially advantageous for metabolically versatile organisms that are not limited in energy source.

The HP/HB cycle is a perspective route for the conversion of CO$_2$ into liquid fuels and industrial chemicals and can be engineered for the production of value-added compounds like 3-hydroxypropionate or n-butanol (Hawkins et al., 2011, 2013b; Keller et al., 2013; Fuchs and Berg, 2014; Loder et al., 2016). The identification of highly specific thermophilic enzymes involved in this pathway expands opportunities for the application of the pathway. Simultaneous usage of several isoenzymes differing in their kinetic properties is an interesting concept for biotechnology that may allow stable production under varying conditions.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study are available on request to the corresponding author.

**AUTHOR CONTRIBUTIONS**

IB designed the experiments. LL and HH performed the experiments. LL, HH, and IB analyzed the data. LL and IB wrote the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00354/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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