INTRODUCTION

Six autotrophic CO₂ fixation pathways are known: the reductive pentose phosphate cycle (Calvin–Benson–Bassham cycle), the reductive citric acid cycle (Arnon–Buchanan cycle), the reductive acetyl-CoA pathway (Wood–Ljungdahl pathway), the 3-hydroxypropionate/malyl-CoA cycle, the 3-hydroxypropionate/4-hydroxybutyrate cycle, and the dicarboxylate/4-hydroxybutyrate cycle. The last two pathways were described recently in the (hyper)thermophilic autotrophic Crenarchaeota and, up to now, are restricted to this group of Archaea (Norris et al., 1989; Ishii et al., 1996; Burton et al., 1999; Menendez et al., 1999; Berg et al., 2007; Jahn et al., 2007; Huber et al., 2008; Ramos-Vera et al., 2009; Hügler et al., 2003a, b).

The archael phylum Crenarchaeota comprises currently four orders of (hyper)thermophilic organisms, namely Sulfolobales, Thermoproteales, Desulfurococcales and ‘Caldisphaerales’ (Fig. 1) (Garrity & Holt, 2001; Garrity et al., 2005). Mesophilic Archaea belonging to the so-called ‘marine group’-1 Crenarchaeota, which are abundant in the sea (Karner et al., 2001), were initially described as representatives of the Crenarchaeota, and the taxonomic entities ‘Cenarchaeales’ (Preston et al., 1996) or ‘Nitrosopumilales’ (Könneke et al., 2005) have been proposed. However, the phylogenetic position of this archael group is currently under discussion, and its description as a separate archael phylum has been suggested (Brochier-Armanet et al., 2008). More recently, additional members of this group have been enriched from thermophilic environments (de la Torre et al., 2008; Hatzenpichler et al., 2008).

Among Crenarchaeota, the ability to grow autotrophically is widespread and can be found in representatives of the Sulfolobales, Thermoproteales and Desulfurococcales (Fig. 1). However, it should be noted that although many Sulfolobales species were initially described as autotrophs or mixotrophs (e.g. Sulfolobus acidocaldarius, Sulfolobus sulfataricus), several strains deposited in public culture collections (including the type strains) have lost the ability to grow autotrophically after continuous laboratory cultivation in nutrient-rich media (Huber et al., 1989; Huber & Prangishvili, 2006). This is consistent with the presence of a large number of transposable elements in the
genomes of some Sulfolobales and underlines the caution required in working experimentally with these organisms (Redder & Garrett, 2006). Nevertheless, very closely related strains have been isolated which are still able to grow strictly chemolithoautotrophically (e.g. S. solfataricus strain Ron 12/III; Fuchs et al., 1996).

All Crenarchaeota studied so far use either the 3-hydroxypropionate/4-hydroxybutyrate cycle or the dicarboxylate/4-hydroxybutyrate cycle, as has been experimentally shown for Metallosphaera sedula (Sulfolobales), Ignicoccus hospitalis (Desulfurococcales, Desulfurococcales) and Thermoproteus neutrophilus (Thermoproteales), and generalized based on whole-genome comparisons (Berg et al., 2007; Jahn et al., 2007; Huber et al., 2008; Ramos-Vera et al., 2009). These pathways have in common the synthesis of succinyl-CoA from acetyl-CoA and two inorganic carbons, although this is accomplished in quite different ways and using different carboxylases (Fig. 2). In the 3-hydroxypropionate/4-hydroxybutyrate cycle, acetyl-CoA propionyl-CoA carboxylase fixes two molecules of bicarbonate, and in the dicarboxylate/4-hydroxybutyrate cycle pyruvate synthase and phosphoenolpyruvate (PEP) carboxylase are the two carboxylating enzymes. Yet the regeneration of acetyl-CoA, the primary CO₂ acceptor, from succinyl-CoA is similar in both pathways. Succinyl-CoA is reduced to 4-hydroxybutyrate, which is activated to 4-hydroxybutyryl-CoA and then dehydrated to crotonyl-CoA by 4-hydroxybutyryl-CoA dehydratase. This \([4Fe-4S] \) and FAD-containing dehydratase (Martins et al., 2004; Buckel & Golding, 2006) is considered a key enzyme of the 4-hydroxybutyrate part of both cycles. Its product, crotonyl-CoA, is further converted to acetoacetyl-CoA and then to two acetyl-CoA molecules, closing the cycle and generating an additional molecule of acetyl-CoA for biosynthesis.

Although these two autotrophic pathways share many common enzymes and intermediates, the fundamental difference is their different sensitivity to oxygen. The enzymes of the 3-hydroxypropionate/4-hydroxybutyrate cycle tolerate oxygen. Although 4-hydroxybutyryl-CoA dehydratase is inactivated by oxygen (Scherf et al., 1994), it may be sufficiently stable at low oxygen tensions to...
maintain activity, especially in the protected environment of the cell. In contrast, the oxygen sensitivity of some of the enzymes and electron carriers of the dicarboxylate/4-hydroxybutyrate cycle such as pyruvate synthase and ferredoxin seems to restrict this cycle to organisms growing under anoxic or microoxic conditions. Indeed, Thermoproteus and Ignicoccus species grow as strict anaerobes by reducing elemental sulfur with hydrogen gas, whereas most of the Sulfolobales need oxygen as an electron acceptor in their metabolism.

However, some autotrophic members of the Sulfolobales (Acidianus sp., Stiyloglobus azorius) are capable of growth under strictly anaerobic conditions (Segerer et al., 1985, 1986, 1991; Huber & Stetter, 2001). All sequenced Sulfolobales contain putative pyruvate synthase, pyruvate: water dikinase and PEP carboxylase genes required for the dicarboxylate/4-hydroxybutyrate cycle (Auernik et al., 2008; Chen et al., 2005; Kawarabayasi et al., 2001; She et al., 2001; Reno et al., 2009). These genes seem to be expressed at a low level even in M. sedula (Berg et al., 2007; Hügler et al., 2003a).

In contrast, Pyrolobus fumarii is a strictly autotrophic member of the Pyrodictiaceae (Desulfurococcales). It can grow aerobically or anaerobically by nitrate respiration (Blöchl et al., 1997). The sequence and the annotation of its genome are not available yet, and it is not known if this species contains the genes encoding biotin-dependent carboxylases, though the sequenced genomes of members of the Desulfurococcales lack them (Anderson et al., 2009; Brügger et al., 2007; Kawarabayashi et al., 1999; Podar et al., 2008; Ravin et al., 2009). Moreover, autotrophic pathways functioning in Pyrodictiaceae are completely unknown.

This study investigated the pathways of autotrophic CO₂ fixation in Crenarchaeota. (a) The 3-hydroxypropionate/4-hydroxybutyrate cycle, as proposed for the Sulfolobales (Berg et al., 2007); (b) the dicarboxylate/4-hydroxybutyrate cycle, as proposed for the Desulfurococcales and Thermoproteales (Huber et al., 2008; Ramos-Ver et al., 2009). Note that the succinyl-CoA reductase in Thermoproteus neutrophilus (Thermoproteales) uses NADPH, and in I. hospitalis uses reduced methyl viologen (possibly as a substitute for reduced ferredoxin). Enzymes: 1, acetyl-CoA carboxylase; 2, malonyl-CoA reductase (NADPH); 3, malonate semialdehyde reductase (NADPH); 4, 3-hydroxypropionate-CoA ligase (AMP-forming); 5, 3-hydroxypropionyl-CoA dehydratase; 6, acryloyl-CoA reductase (NADPH); 7, propionyl-CoA carboxylase; 8, methylmalonyl-CoA epimerase; 9, methylmalonyl-CoA mutase; 10, succinyl-CoA reductase (NADPH or reduced methyl viologen); 11, succinic semialdehyde reductase (NADPH); 12, 4-hydroxybutyrate-CoA ligase (AMP-forming); 13, 4-hydroxybutyryl-CoA dehydratase; 14, crotonyl-CoA hydratase; 15, (S)-3-hydroxybutyl-CoA dehydrogenase (NAD⁺); 16, acetoacetyl-CoA β-ketoisovalerate; 17, pyruvate synthase (reduced methyl viologen); 18, pyruvate: water dikinase; 19, PEP carboxylase; 20, malate dehydrogenase (NAD); 21, fumarate hydratase; 22, fumarate reductase (reduced methyl viologen); 23, succinyl-CoA synthetase (ADP-forming). Fd_red, reduced ferredoxin; MV, methyl viologen.
high temperatures even under (micro)aerobic conditions. Therefore, the distribution of these two cycles follows a 16S rRNA-sequence-derived phylogenetic pattern.

**METHODS**

**Materials.** Chemicals were obtained from Fluka, Sigma–Aldrich, VWR or Roth. 4-Hydroxy-1[14C]butyrate was obtained from American Radiolabelled Chemicals. NaH[14CO3] was obtained from Hartmann Analytic. Primers were from Bionmers.net. DNA isolation, PCR and PCR fragment purification were performed with kits from GE Healthcare, Qiagen and Genaxxon BioScience.

**Cell material and growth conditions.** *P. fumarii* strain DSM 11204 and *Acidulans infernus* strain DSM 3191 were obtained from the culture collection of the Lehrstuhl für Mikrobiologie, University of Regensburg. *P. fumarii* was grown autotrophically under anaerobic conditions in 1/2SMF medium (‘synthetisches Meerwasser’; Huber & Stetter, 2006) at 106 °C and pH 5.5 ( Bölch et al., 1997) using KNO3 (1 g l−1) as electron acceptor. In the 300 l fermenter a gassing rate of 5 l min−1 was applied using a gas mixture of 80% H2 and 20% CO2 (v/v). *A. infernus* was grown as described by Segerer et al. (1953). *S. azovicus* strain DSM 6296 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). It was grown in a 10 l fermenter autotrophically under anaerobic conditions on a defined mineral medium (Allen, 1959) with sulfur (9 g l−1) and vitamin solution (1 ml l−1; Balch et al., 1979) under a gassing mixture of 80% H2 and 20% CO2 (v/v) at 85 °C and pH 2.5. The cells were harvested by centrifugation during the exponential growth phase and stored at −70 °C until use. *Thermoproteus neutrophilus* and *M. sedula* were grown autotrophically as reported previously (Alber et al., 2006; Ramos-Vera et al., 2009).

**Syntheses.** Acetyl-CoA, propionyl-CoA, succinyl-CoA and crotonyl-CoA were synthesized from their anhydrides, and acetoacetyl-CoA from diketene by the method of Simon & Shemin (1953). The dry powders of the CoA-esters were stored at −20 °C.

**Preparation of cell extracts.** Cell extracts were prepared under anaerobic conditions. Cells were suspended in an equal volume of 10 mM Tris/His buffer (pH 7.8), and the cell suspension was passed through a chilled French pressure cell at 137 kPa. The lysate was ultracentrifuged for 1 h (100 000 g, 4 °C), and aliquots of the supernatant (cell extract) were stored anoxically at −70 °C until use.

To obtain a membrane fraction, the pellet was resuspended in the supernatant (cell extract) were stored anoxically at −70 °C until use.

**Preparation of cell extracts.** Cell extracts were prepared under anaerobic conditions. Cells were suspended in an equal volume of 10 mM Tris/His buffer (pH 7.8), and the cell suspension was passed through a chilled French pressure cell at 137 kPa. The lysate was ultracentrifuged for 1 h (100 000 g, 4 °C), and aliquots of the supernatant (cell extract) were stored anoxically at −70 °C until use.

**Enzyme measurements.** Spectrophotometric enzyme assays (0.5 ml assay mixture) were performed in 0.5 ml glass cuvettes at the temperatures indicated in Table 1. Anoxic assays were done with N2 in the headspace. Reactions involving NADP+ were measured at 365 nm (εNADP+=3.4×104 M−1 cm−1, εNADPH=3.5×104 M−1 cm−1; Bergmeyer, 1975). Reactions with methyl viologen were measured under anaerobic conditions at 578 nm (ε=9.7×103 M−1 cm−1; extrapolated from Trudinger, 1970). Reactions with 5,5′-dithiobis-(2-nitrobenzoic acid) were measured at 412 nm (ε=1.4×104 M−1 cm−1; Riddles et al., 1983).

Pyrurate: water dikinase (EC 2.7.9.2), succinyl-CoA synthetase (EC 6.2.1.4, 6.2.1.5), 4-hydroxybutyrate-CoA ligase (EC 6.2.1.1), acetoacetyl-CoA β-ketothiolase (EC 2.3.1.9), malic enzyme [NAD(P)−dependent malate dehydrogenase (decarboxylating)] (EC 1.1.1.38, 1.1.1.40) were measured as described previously (Ramos-Vera et al., 2009). 3-Hydroxypropionate-CoA ligase (EC 6.2.1.2) was measured as described for 4-hydroxybutyrate-CoA ligase, but with 3-hydroxypro- pionate (10 mM) instead of 4-hydroxybutyrate as substrate.

Acetyl-CoA and propionyl-CoA carboxylases (EC 6.4.1.2 and 6.4.1.3, respectively) were measured radiochemically by determining propionyl-CoA or acetyl-CoA-dependent fixation of 14CO2. The reaction mixture (0.35 ml) contained 100 mM Tris/His (pH 7.8), 5 mM DTT, 10 mM MgCl2, 5 mM ATP, 15 mM NaH[14CO3] (3.3 kBq μmol−1) and cell extract. The reaction was started by the addition of CoA-ester (1 mM). Acid-stable 14C was determined as described previously (Hügler et al., 2003a).

Malonyl-CoA reductase (EC 1.2.1.1), NAD(P)H-dependent succinyl-CoA reductase (EC 1.2.1.1), and succinic semialdehyde reductase (EC 1.1.1.) were determined in a reaction mixture containing 100 mM MOPS/KOH (pH 7.0), 5 mM DTT, 10 mM MgCl2, 0.5 mM NAD(P)H, and cell extract. The reaction was started by the addition of malonyl-CoA (0.2 mM), succinyl-CoA (1 mM) or succinic semialdehyde (2 mM). Methyl viologen-dependent succi- nyl-CoA reductase (EC 1.2.1.1) was measured anoxically following the oxidation of reduced methyl viologen in a reaction mixture containing 100 mM potassium phosphate (pH 7.5), 4 mM DTT, 1 mM MgCl2, 4 mM methyl viologen and cell extract. Methyl viologen was reduced by addition of dithionite from a 10 mM stock solution to OD578 −1.5, and the reaction was started with succinyl-CoA (2 mM).

Malonic semialdehyde reductase was measured in a reaction mixture containing 100 mM MOPS/KOH (pH 7.0), 5 mM DTT, 10 mM MgCl2, 0.7 mM NAD(P)H, 0.2 mM malonyl-CoA and 0.2 U malonaldehyde reductase from Sulfolobus tokodaii (Alber et al., 2006). The mixture was incubated for 5 min, allowing the formation of malonate semialdehyde, and then started by addition of cell extract.

The reductive conversion of 3-hydroxypropionate to propionyl-CoA was measured as 3-hydroxypropionate-, ATP- and CoA-dependent oxidation of NADPH in the following reaction mixture: 100 mM Tris/His (pH 7.8), 5 mM DTT, 5 mM MgCl2, 100 mM KCl, 0.5 mM NADPH, 0.8 mM CoA, 3 mM ATP, 4 mM 3-hydroxypropionate and cell extract.

Pyruvate- and 2-oxoglutarate:acceptor oxidoreductases (EC 1.2.7.1 and EC 1.2.7.3, respectively) were measured anoxically by two methods. The pyruvate- or 2-oxoglutarate-dependent reduction of methyl viologen was followed in a reaction mixture containing 100 mM potassium phosphate (pH 7.5), 4 mM DTT, 1 mM MgCl2, 4 mM methyl viologen, 0.5 mM CoA and cell extract. Dithionite was added with a syringe until a permanent faint bluish colour was obtained, and the reaction was started by the addition of pyruvate or 2-oxoglutarate (10 mM). The 14CO2 exchange reaction with the carboxyl group of pyruvate or 2-oxoglutarate was followed in a reaction mixture (0.35 ml) containing 100 mM MOPS/KOH (pH 7.0), 5 mM MgCl2, 5 mM DTT, 0.2 mM CoA, 15 mM NaH[14CO3] (3.3 kBq μmol−1) and cell extract. The reaction was started by the addition of pyruvate or 2-oxoglutarate (10 mM), and the acid-stable 14C was determined after appropriate time intervals (Hügler et al., 2003a).

PEP carboxylase (EC 4.1.1.31) was measured radiochemically as PEP-dependent fixation of bicarbonate (Ramos-Vera et al., 2009). ATP-, GTP- and diphosphate-dependent PEP carboxykinases (EC 4.1.1.49, 4.1.1.32 and 4.1.1.38, respectively) were measured in a similar manner to PEP carboxylase, but the reaction mixture was supplemented with ADP, GDP or potassium phosphate for ATP-, GTP- or diphosphate-dependent PEP carboxykinase, respectively (Ramos-Vera et al., 2009).

NAD(P)-dependent malate dehydrogenase (EC 1.1.1.37 and 1.1.1.82 for NAD- and NADP-dependent enzymes, respectively) was detected.
Table 1. Enzymes involved in autotrophic CO₂ assimilation in *S. azoricus* and *P. fumarii*

Because of the use of mesophilic coupling enzymes and the instability of some substrates, the indicated assay temperatures were used, although the growth temperatures were 85 and 105 °C, respectively. The specific activity at 85 °C was calculated based on the assumption that a 10 °C rise in temperature doubles the reaction rate. At high temperatures, enzymes may become inactivated *in vitro*, in contrast to the protected environment of the cell. ND, Not detectable; NA, not applicable; MV, methyl viologen.

<table>
<thead>
<tr>
<th>Enzyme Enzyme no. in Fig. 1</th>
<th>Assay temperature (°C)</th>
<th>Specific activity [nmol min⁻¹ (mg protein)⁻¹]</th>
<th>Assay temperature (°C)</th>
<th>Specific activity [nmol min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Measured</td>
<td>Tentatively extrapolated to 85 °C</td>
<td>Measured</td>
</tr>
<tr>
<td><strong>3-Hydroxypropionate part (part 1) of the 3-hydroxypropionate/4-hydroxybutyrate cycle</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Acetyl-CoA carboxylase</td>
<td>1</td>
<td>65</td>
<td>21</td>
<td>84</td>
</tr>
<tr>
<td>Malonyl-CoA reductase (NADPH)</td>
<td>2</td>
<td>65</td>
<td>1700</td>
<td>6800</td>
</tr>
<tr>
<td>Malonate semialdehyde reductase (NADPH)</td>
<td>3</td>
<td>65</td>
<td>3100</td>
<td>12 400</td>
</tr>
<tr>
<td>3-Hydroxypropionate-CoA ligase</td>
<td>4</td>
<td>65</td>
<td>270</td>
<td>1100</td>
</tr>
<tr>
<td>3-Hydroxypropionate conversion to propionyl-CoA (NADPH)</td>
<td>4–6</td>
<td>65</td>
<td>60</td>
<td>240</td>
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<tr>
<td>Propionyl-CoA carboxylase</td>
<td>7</td>
<td>65</td>
<td>21</td>
<td>84</td>
</tr>
<tr>
<td>Propionyl-CoA conversion to succinyl-CoA</td>
<td>7–9</td>
<td>65</td>
<td>≥21</td>
<td>≥84</td>
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<tr>
<td><strong>Dicarboxylate part (part 1) of the dicarboxylate/4-hydroxybutyrate cycle</strong></td>
<td></td>
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<td>Pyruvate synthase</td>
<td>MV reduction</td>
<td>17</td>
<td>65</td>
<td>20</td>
</tr>
<tr>
<td>14CO₂ exchange reaction</td>
<td>65</td>
<td>6</td>
<td>24</td>
<td>85</td>
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<td>Pyruvate:water dikinase</td>
<td>18</td>
<td>65</td>
<td>42</td>
<td>170</td>
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<td>PEP carboxylase</td>
<td>19</td>
<td>65</td>
<td>7</td>
<td>28</td>
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<td>Malate dehydrogenase (NAD+/NADP+)</td>
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<td>65</td>
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<td>NA</td>
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<td>Fumarate hydratase</td>
<td>21</td>
<td>65</td>
<td>180</td>
<td>720</td>
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<td>Fumarate reductase (cell extract/membrane fraction; MV)</td>
<td>22</td>
<td>65</td>
<td>23/90</td>
<td>92/360</td>
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<tr>
<td>Succinyl-CoA synthetase</td>
<td>23</td>
<td>65</td>
<td>ND</td>
<td>NA</td>
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<td><strong>4-Hydroxybutyrate part (part 2) of the dicarboxylate/4-hydroxybutyrate and 3-hydroxypropionate/4-hydroxybutyrate cycles</strong></td>
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<tr>
<td>Succinyl-CoA reductase*</td>
<td>10</td>
<td>65</td>
<td>3100</td>
<td>12 400*</td>
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<td>Succinic semialdehyde reductase (NADH/ NADPH)</td>
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<td>4400/3100</td>
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<td>12</td>
<td>65</td>
<td>380</td>
<td>1500</td>
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<td>4-Hydroxybutyryl-CoA dehydratase</td>
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<td>585</td>
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<td>Crotonyl-CoA conversion to acetoacetyl-CoA (NAD⁺)</td>
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<td>380</td>
<td>1500</td>
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<td>Acetoacetyl-CoA β-ketothiolase</td>
<td>16</td>
<td>65</td>
<td>3500</td>
<td>14 000</td>
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<tr>
<td><strong>Other enzymes</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>PEP carboxykinase (ADP/GDP)</td>
<td>–</td>
<td>65</td>
<td>5/5</td>
<td>20/20</td>
</tr>
<tr>
<td>Malate dehydrogenase, decarboxylating (NAD+/NADP⁺)</td>
<td>–</td>
<td>65</td>
<td>150/55</td>
<td>600/220</td>
</tr>
<tr>
<td>2-Oxoglutarate:acceptor oxidoreductase</td>
<td>MV reduction</td>
<td>–</td>
<td>65</td>
<td>&lt;1</td>
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<tr>
<td>14CO₂ exchange reaction</td>
<td>65</td>
<td>≤1.2</td>
<td>NA</td>
<td>85</td>
</tr>
<tr>
<td>Phosphoribulokinase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>85</td>
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<tr>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase</td>
<td>–</td>
<td>85</td>
<td>0.5</td>
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</table>

*Succinyl-CoA reductase is NADPH-dependent in *S. azoricus* and methyl viologen-dependent in *P. fumarii*
spectrophotometrically by the oxaloacetate-dependent oxidation of NAD(P)H under oxic conditions in an assay mixture containing 100 mM Tris/HCl (pH 7.8), 5 mM MgCl₂, 5 mM DTT, 0.3 mM NAD(P)H and cell extract. The reaction was started by the addition of oxaloacetate (4 mM). For S. azoricus, the enzyme was measured both oxically and anoxically in cell extracts as well as in the membrane fraction. Dye-linked malate dehydrogenase (EC 1.1.99.16) was measured with 2,6-dichlorophenolindophenol according to Schauder et al. (1987).

Fumarate hydratase (EC 4.2.1.2) was measured anaerobically at 250 nm (εfumarate=1479 M⁻¹ cm⁻¹) (O’Hare & Doonan, 1985). The assay mixture contained 100 mM potassium phosphate (pH 7.9), 30 mM D,L-malate and cell extract. The reaction was started by the addition of cell extract. In the case of S. azoricus, the enzyme was activated before the measurement by incubating cell extract for 30 min at room temperature with ferrous ammonium sulfate (0.5 mM) and DTT (25 mM) (Suzuki et al., 1977).

Fumarate reductase (EC 1.3.99.1) was measured as described for methyl violenogen-dependent succinyl-CoA reductase, but with fumarate (10 mM) instead of succinyl-CoA as substrate.

In S. azoricus and M. sedula, 4-hydroxybutyryl-CoA hydrahydrate (EC 4.2.1.2) activity was measured spectrophotometrically at 42 °C in an assay coupled with the recombinant crotonyl-CoA carboxylase/reductase from Rhodobacter sphaeroides (Erb et al., 2007), as described previously (Berg et al., 2007). Since the growth temperature of P. fumarii is 20 °C higher than that of S. azoricus, a discontinuous assay was used in this case (Ramos-Vera et al., 2009).

Crotonyl-CoA hydrolase (EC 4.2.1.17) and 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) were measured together as crotonyl-CoA-dependent reduction of NAD⁺ in the following reaction mixture: 100 mM Tris/HCl (pH 7.8), 5 mM DTT, 5 mM MgCl₂, 1 mM NAD⁺, 0.8 mM crotonyl-CoA and cell extract.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) was determined as ribulose 1,5-bisphosphate-dependent fixation of NaH¹⁴CO₃ into acid-stable products under anaerobic conditions (Hügler et al., 2003a). The reaction mixture (0.35 ml) contained 100 mM Tris/HCl (pH 7.8), 5 mM DTT, 5 mM MgCl₂, 15 mM NaH¹⁴CO₃ (18 kBg μmol⁻¹) and cell extract. After preincubation for 5 min, the reaction was started by the addition of ribulose 1,5-bisphosphate (1 mM).

Phosphoribulokinase (EC 2.7.1.19) was measured by coupling ribulose 5-phosphate conversion to ribulose 1,5-bisphosphate with the endogenous ribulose 1,5-bisphosphate carboxylase/oxygenase. The reaction was performed as described for ribulose-1,5-bisphosphate carboxylase/oxygenase, except that ribulose 1,5-bisphosphate was replaced by ribulose 5-phosphate (1 mM) and ATP (3 mM). The reaction was started by the addition of ribulose 5-phosphate.

**Enzyme inactivation under oxic conditions.** When the oxygen sensitivity of pyruvate synthase, fumarase, fumarate reductase and 4-hydroxybutyryl-CoA dehydratase was tested, cell extracts were incubated for various periods of time at 25 °C under oxic conditions with stirring, and then used for anoxic spectrophotometric enzyme activity measurements, as described above. The assay temperature was 65 °C for pyruvate synthase, fumarase and fumarate reductase, and 42 °C for 4-hydroxybutyryl-CoA dehydratase.

**Conversion of 4-hydroxy[1-¹⁴C]butyrate to [¹⁴C]acetyl-CoA by cell extracts of S. azoricus and P. fumarii.** The assay mixture (0.5 ml) contained 100 mM MOPS/KOH (pH 7.2), 3 mM MgCl₂, 3 mM ATP, 5 mM DTT, 2 mM CoA, 2 mM NAD⁺, 1 mM 4-hydroxy[1-¹⁴C]butyrate (10 kBg μmol⁻¹) and cell extract (1.4–1.8 mg protein ml⁻¹). In control, ATP was omitted. The reaction was performed anoxically at 65 °C (S. azoricus) or 85 °C (P. fumarii) and was started by the addition of cell extract. The reaction was stopped after different time intervals by mixing the samples (50 μl) with 10 μl 1 M HCl. The samples were centrifuged (4 °C, 20000 g, 15 min) and analysed by HPLC using an RP-C₁₈ column, as described previously (Erb et al., 2007). The identification of the CoA esters was based on co-chromatography with standards (Erb et al., 2007; Berg et al., 2007).

**Conversion of 3-hydroxypropionate to propionyl-CoA by cell extract of S. azoricus.** The reaction mixture (0.35 ml) contained 100 mM Tris/HCl (pH 7.8), 100 mM KCl, 5 mM MgCl₂, 0.5 mM NADPH, 1 mM CoA, 3 mM ATP and 1.4 mg protein ml⁻¹. In a control, ATP was omitted. The reaction was performed aerobically at 65 °C and was started by the addition of cell extract. The analysis of the samples was performed as described above for the 4-hydroxybutyrate conversion.

**Analysis of the products of the propionyl-CoA carboxylase reaction.** [¹⁴C]-labelled products of the propionyl-CoA carboxylase reaction were analysed by TLC with CH₄Cl₂-acetic acid (5:1, v/v) and detected by phosphorimaging (Erb et al., 2008).

**Detection of biotinylated proteins.** Biotinylated proteins in cell extracts were detected with peroxidase-conjugated avidin (Menendez et al., 1999).

**Protein determination.** Protein was measured according to the Bradford method (Bradford, 1976), using BSA as standard.

**DNA extraction, PCR amplification and sequencing of 4-hydroxybutyryl-CoA dehydratase gene fragments.** DNA was extracted with the illustra bacteria genomicPrep Mini Spin kit (GE Healthcare) according to the manufacturer’s protocol. For amplification of the 4-hydroxybutyryl-CoA dehydratase genes, different primer sets designed from available archaeal sequences in GenBank were used in a 36-cycle PCR with RadTag Polymerase PCR Master Mix (Genaxxon BioScience), and the annealing temperature was 52 °C. The primers used were abfD_s1_F [5’-TTC CA(AG) AG(AG) TG(CT) GTI GGI TGG GA-3’], where I is inosine and abfD_s1_R [5’-GG(AG) CTI CCI GCI CC(AG) TG(AGT) AC-3’]. PCR products were purified from agarose gels using a QIAquick gel extraction kit (Qiagen) and sequenced by GATC Biotech using the ABI 3730xl DNA analyser.

**Database search and phylogenetic analysis.** The 16S rRNA sequences were obtained from the Ribosomal Database Project website (http://rdp.cme.msu.edu; Cole et al., 2009). The 4-hydroxybutyryl-CoA dehydratase amino acid sequences were obtained from the National Center for Biotechnology Information (NCBI) database. The BLAST searches were performed via the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990).

The newly obtained 4-hydroxybutyryl-CoA dehydratase amino acid sequences were aligned with those from GenBank using CLUSTAL W (Thompson et al., 1994) as implemented in the BioEdit 7.0.9 software package (http://www.ncbi.nlm.nih.gov/BLAST/). The positions with gaps and ambiguous sequences were removed, and the remaining 312 amino acids were used for further phylogenetic analysis. The phylogenetic tree was reconstructed using a neighbour-joining algorithm (Saitou & Nei, 1987) in the TREECONW program (Van de Peer & De Wachter, 1994).

**Nucleotide sequence accession numbers.** Accession numbers for the sequences used for the construction of the 4-hydroxybutyryl-CoA dehydratase phylogenetic tree as well as those used for the 16S rRNA gene phylogenetic tree are given in the Supplementary Material.
RESULTS

Autotrophic growth of S. azoricus and P. fumarii

S. azoricus grew anaerobically by reducing molecular sulfur with H₂ to a density of 1 × 10⁸ cells ml⁻¹ with a generation time of 10 h, which corresponds to a specific growth rate (μ) of 0.069 h⁻¹ and a specific autotrophic carbon fixation rate of 96 nmol min⁻¹ (mg protein)⁻¹ [calculated as described in Ramos-Vera et al. (2009)]. If two molecules of CO₂ are fixed in one turn of the autotrophic CO₂ fixation cycle, the minimal in vivo specific activity of its enzymes is 48 nmol min⁻¹ (mg protein)⁻¹. P. fumarii was cultivated under denitrifying conditions with molecular hydrogen as an electron donor. Cells grew to a density of about 1 × 10⁷ cells ml⁻¹ with a generation time of 10 h (μ = 0.069 h⁻¹), which is the same as for S. azoricus and therefore corresponds to the same minimal in vivo specific activity of the CO₂ fixation cycle enzymes.

Detection of biotin-containing proteins in cell extracts

Proteins from cell extracts of S. azoricus and P. fumarii were separated by SDS-PAGE and blotted to detect biotin-containing proteins using the avidin technique. Cell extracts of autotrophically grown M. sedula were used as a positive control for the presence of biotin carrier protein of acetyl-CoA-propionyl-CoA carboxylase. A single biotin-containing protein was detected in S. azoricus as well as in M. sedula, whereas no signal was found in P. fumarii (Fig. 3). These data are in line with the presence of the biotin-dependent carboxylases in all Sulfolobales studied so far (Norris et al., 1989; Burton et al., 1999; Ishii et al., 1996; Chaukrut et al., 2003; Hugler et al., 2003a, b; Menendez et al., 1999) as well as with the absence of these proteins and the corresponding genes in the sequenced Desulfurococcaceae genomes (Hugler et al., 2003a; Jahn et al., 2007; Anderson et al., 2009; Brügger et al., 2007; Kawarabayasi et al., 1999; Podar et al., 2008; Ravin et al., 2009).

Enzyme activities in S. azoricus

The results obtained were similar to those for M. sedula (Menendez et al., 1999; Hugler et al., 2003a; Berg et al., 2007). High activities of all specific enzymes of the 3-hydroxypropionate part of the 3-hydroxypropionate/4-hydroxybutyrate cycle were found in S. azoricus, namely acetyl-CoA-propionyl-CoA carboxylase, malonyl-CoA reductase, malonate semialdehyde reductase, and the enzymes responsible for the conversion of 3-hydroxypropionate to propionyl-CoA (Table 1). In contrast, the activities of most of the enzymes of the dicarboxylate part of the dicarboxylate/4-hydroxybutyrate cycle were low or not detectable (Table 1). Notably, the activity of PEP carboxylase involved in the latter cycle was very low, and the activities of malate dehydrogenase and succinyl-CoA synthetase could not be detected at all with the methods used in our study (Table 1).

Biotin dependence of the measured acetyl-CoA and propionyl-CoA carboxylases was confirmed in the experiments with avidin. Incubation of the extract with avidin (5 μg ml⁻¹) before measurement completely inhibited both acetyl-CoA- and propionyl-CoA-dependent fixation of ¹⁴CO₂. Addition of biotin (0.2 mg ml⁻¹) prevented inactivation of the carboxylases.

The products of the biotin-dependent propionyl-CoA carboxylase reaction were analysed by TLC and detected by phosphorimaging. The only detected product was succinate (Fig. 4). Therefore, the product of propionyl-CoA carboxylase, (S)-methylmalonyl-CoA, is immediately further converted to succinyl-CoA, implying that methylmalonyl-CoA epimerase and mutase activities were at least as high as the propionyl-CoA carboxylase activity (Table 1).

Cell extracts catalysed the 3-hydroxypropionate-, MgATP- and CoA-dependent oxidation of NADPH (Table 1), which was interpreted as the conversion of 3-hydroxypropionate to propionyl-CoA. The reaction leads to propionyl-CoA formation via 3-hydroxypropionyl-CoA as intermediate (Fig. 5). No propionyl-CoA was formed in a control experiment without 3-hydroxypropionate.

The enzymes of the 4-hydroxybutyrate part of the 3-hydroxypropionate/4-hydroxybutyrate cycle were also...
Succinyl-CoA reductase was NADPH-dependent (5% activity with NADH, no activity with reduced methyl viologen), as in the case of the enzyme from \textit{M. sedula} (Alber et al., 2006; Berg et al., 2007). Succinic semialdehyde reductase was similarly active with both NADPH and NADH (Table 1), and 3-hydroxybutyryl-CoA dehydrogenase only with NAD$^+$, as in the case of other autotrophic Crenarchaeota (Berg et al., 2007; Huber et al., 2008; Ramos-Vera et al., 2009).

The conversion of 4-hydroxybutyrate to two acetyl-CoA molecules in the presence of MgATP, CoA and NAD$^+$ was demonstrated by HPLC. Extracts rapidly formed 4-hydroxybutyryl-CoA, crotonyl-CoA, 3-hydroxybutyryl-CoA, and finally acetyl-CoA (Fig. 6a, b). Similar results were obtained when 4-hydroxy[1-14C]butyrate was used, and the radioactive products of the conversion were detected (Fig. 6c, d). Therefore, in addition to the enzymes responsible for 4-hydroxybutyrate conversion to acetyl-CoA, the whole reaction sequence could be demonstrated \textit{in vitro}.

2-Oxoglutarate synthase, one of the key enzymes of the reductive citric acid cycle, was not found, and the activity of ribulose-1,5-bisphosphate carboxylase, the key enzyme of the Calvin–Benson–Bassham cycle, was extremely low, if present at all (Table 1).

\textbf{Enzyme activities in \textit{P. fumarii}}

All enzymes of the dicarboxylate/4-hydroxybutyrate cycle were detected in autotrophically grown \textit{P. fumarii} cells at high activity, notably pyruvate synthase and PEP carboxylase (Table 1). The relatively low activity of fumarate reductase may be explained by the use of an artificial electron donor (reduced methyl viologen). Its natural electron donor is unknown. In contrast, none of the enzymes of the 3-hydroxypropionate part of the 3-hydroxypropionate/4-hydroxybutyrate cycle was detected. The differences between \textit{P. fumarii} and \textit{S. azoricus} in the activities of pyruvate synthase, pyruvate : water dikinase, PEP carboxylase, malate dehydrogenase and succinyl-CoA synthetase, the enzymes of the dicarboxylate part of the cycle, were also noteworthy.

Interestingly, succinyl-CoA reduction in \textit{P. fumarii} was not NAD(P)H-dependent, but required reduced methyl viologen (Table 1), as in \textit{I. hospitalis} (Huber et al., 2008). Fumarate reductase could also be measured only with reduced methyl viologen; no activity could be found with NAD(P)H as an electron donor.

Extracts rapidly converted 4-hydroxybutyrate to acetyl-CoA, provided that MgATP, CoA and NAD$^+$ were present, and 4-hydroxybutyryl-CoA was the only detected intermediate of this conversion (Fig. 6e, f). 4-Hydroxy-
butyryl-CoA and acetyl-CoA were formed when 4-hydroxy[1-14C]butyrate was used (Fig. 6h, i). When NAD$^+$ was omitted, acetyl-CoA was not formed. Instead, crotonyl-CoA and 3-hydroxybutyryl-CoA accumulated, in addition to 4-hydroxybutyryl-CoA (Fig. 6g).

A very low ribulose-1,5-bisphosphate carboxylase activity was detected in *P. fumarii* (Table 1), as in *Pyrodictium* sp. (Hügler *et al.*, 2003a). However, the second key enzyme of the Calvin–Benson–Bassham cycle, phosphoribulokinase, was not detected (Table 1).

**Fig. 6.** Conversion of 4-hydroxy[1-14C]butyrate to [14C]acetyl-CoA by cell extracts of *S. azoricus* [at 65 °C (a–d)] and *P. fumarii* [at 85 °C (e–i)]. Substrates and products were separated by HPLC and visualized either by measurement of A$_{260}$ (a, b, e–g) or by flow-through scintillation counting ($^{14}$C detection) (c, d, h, i). (a)/(e), (b)/(f) A$_{260}$ analysis of the samples taken after 2 and 5 min incubation, respectively; (c, h) $^{14}$C detection of the products and substrates in the reaction mixture after 5 min incubation; (d, i) $^{14}$C detection of the products and substrates in a control experiment lacking ATP after 10 min incubation; (g) A$_{260}$ analysis of a control sample lacking NAD$^+$ taken after 5 min incubation. The radioactive peak at 3.5 min most likely represents $\gamma$-butyrolactone, which forms spontaneously from 4-hydroxybutyrate at acidic pH or from 4-hydroxybutyryl-CoA at neutral pH. Polar products elute within the first 3 min. Note that crotonyl-CoA and 3-hydroxybutyryl-CoA behave like intermediates between 4-hydroxybutyryl-CoA and acetyl-CoA. The transformation was catalysed at specific activities of 67 and 200 nmol min$^{-1}$ (mg protein)$^{-1}$ for *S. azoricus* and *P. fumarii* cell extracts, respectively.

**Detection and phylogenetic analysis of the 4-hydroxybutyryl-CoA dehydratase genes from *S. azoricus* and *A. infernus***

The in silico-designed primers for the ‘crenarchaea type-1’ 4-hydroxybutyryl-CoA dehydratase gene of the hyperthermophilic Crenarchaeota (Berg *et al.*, 2007) were used to amplify the 4-hydroxybutyryl-CoA dehydratase gene fragments from *S. azoricus* and *A. infernus*. Gene fragments of the expected size (about 1050 bp) were successfully obtained and sequenced. The results of the BLAST analysis
revealed a high identity (77–97 %) between the newly determined nucleotide sequences and the genes available in GenBank, confirming their affiliation to the same family of genes. The deduced amino acid sequences were aligned with the analogous sequences from GenBank, and the phylogenetic tree was constructed on the basis of this alignment (Fig. 7). All attempts to amplify the corresponding gene product from *P. fumarii* were unsuccessful. This is not unexpected, since the only available 4-hydroxybutyryl-CoA dehydratase gene sequence from the Desulfurococcales is that from *I. hospitalis* (Podar et al., 2008). More sequences are probably required for the design of a functional primer pair.

**Sensitivity to oxygen of the enzymes of the dicarboxylate/4-hydroxybutyrate cycle and 3-hydroxypropionate/4-hydroxybutyrate cycle**

Because of the low cell yields of *P. fumarii* and *S. azoricus* cultures, the oxygen sensitivity of the enzymes involved in autotrophic CO₂ assimilation was tested in cell extracts of *M. sedula* (4-hydroxybutyryl-CoA dehydratase) and *Thermoproteus neutrophilus* (pyruvate synthase, fumarase and fumarate reductase). Other enzymes of these cycles could be measured under aerobic conditions and thus are probably robust to oxygen. Only pyruvate synthase had a high oxygen sensitivity (half-life 9 min), and the half-life of fumarase under anaerobic conditions was ~40 min. The incubation of cell extracts with air for 2 h did not result in any significant decrease of 4-hydroxybutyryl-CoA dehydratase and fumarate reductase activity. Interestingly, purified 4-hydroxybutyryl-CoA dehydratase from *Clostridium kluyveri* was more oxygen-labile and had a half-life of approximately 30 min (Scherf et al., 1994).

**DISCUSSION**

**3-Hydroxypropionate/4-hydroxybutyrate cycle in *S. azoricus***

We have presented evidence that the strictly anaerobic *S. azoricus* fixes CO₂ via the 3-hydroxypropionate/4-hydroxybutyrate cycle. Since a single biotin-containing protein was detected by avidin staining (Fig. 3), this archaeon probably has a bifunctional acetyl-CoA/propionyl-CoA carboxylase, as does *M. sedula* (Hügler et al., 2003b). All enzymes of the cycle were detected (Table 1), and their specific activities are much higher than the minimal *in vivo* activity calculated from the growth rate [48 nmol min⁻¹ (mg protein)⁻¹]. Strangely enough, succinyl-CoA synthetase and malate dehydrogenase activities could not be detected; these would be essential for the dicarboxylate/4-hydroxybutyrate cycle, but not for the 3-hydroxypropionate/4-hydroxybutyrate cycle functioning in *S. azoricus*. Oxaloacetate, the precursor of the aspartate family of amino acids, could be synthesized from C₃ compounds, e.g. by PEP carboxylase. However, the existence of an unusual malate dehydrogenase cannot be ruled out.

*S. azoricus* is, to our knowledge, the first strict anaerobe shown to use the 3-hydroxypropionate/4-hydroxybutyrate cycle for autotrophic CO₂ fixation. Although the *S. azoricus*
In contrast to S. azoricus, Dicarboxylate/4-hydroxybutyrate cycle in P. fumarii and anoxic conditions. Although I. hospitalis is a strict anaerobe, P. fumarii is a facultative aerobe, and O2 can serve as electron acceptor for H2 oxidation, but only in trace amounts (up to 0.3 % in the gas phase) (Blochl et al., 1997). The actual conditions in the cytoplasm of actively respiring cells are probably anoxic at these low oxygen concentrations and at the optimal growth temperature of 106 °C. Similarly, hyperthermophilic autotrophic representatives of the bacterial phylum Aquificae grow aerobically using the reductive citric acid cycle with ferredoxin-dependent pyruvate and 2-oxoglutarate synthases (Shiba et al., 1985; Ikeda et al., 2006; Yamamoto et al., 2006; Aoshima, 2007; Beh et al., 1993; Hügler et al., 2007). However, it cannot be ruled out that the enzymes from other Archaea exhibit different sensitivities to oxygen.

**Dicarboxylate/4-hydroxybutyrate cycle in P. fumarii**

In contrast to S. azoricus, P. fumarii does not synthesize enzymes of the 3-hydroxypropionate part of the 3-hydroxypropionate/4-hydroxybutyrate cycle (Table 1). Instead, all enzymes of the dicarboxylate/4-hydroxybutyrate cycle were found in this species. Their specific activities exceeded by far the minimal in vivo activity calculated from the growth rate [48 nmol min⁻¹ (mg protein)⁻¹]. Trace amounts of RubisCO activity and the lack of phosphoribulokinase activity do not support the operation of the Calvin–Bassham–Benson cycle. Possible functions of the archaeal RubisCO have been discussed (Tabita et al., 2007; Sato et al., 2007; Ashida et al., 2008). P. fumarii is a representative of the family Pyrodictiaceae (Desulfurococcales). This family contains other autotrophic species (Fig. 1), and we postulate that the dicarboxylate/4-hydroxybutyrate cycle operates in all autotrophic Desulfurococcales as well as Thermoproteales. However, variants of this cycle exist. One instance is succinyl-CoA reductase. The P. fumarii and I. hospitalis enzyme is methyl viologen-dependent, the natural electron donor probably being reduced ferredoxin. In contrast, the Thermoproteus enzyme is NADPH-dependent.

**Oxygen sensitivity of key enzymes of the cycles**

Crenarchaeal 4-hydroxybutyryl-CoA dehydratase seems to be robust to oxygen, making the 3-hydroxypropionate/4-hydroxybutyrate cycle fully oxygen-tolerant. On the other hand, pyruvate synthase, a primary carboxylase in the dicarboxylate/4-hydroxybutyrate cycle, is inactivated by exposure to air. Moreover, this enzyme requires reduced ferredoxin. Fumarate reductase and succinyl-CoA reductase (in I. hospitalis and P. fumarii) may also be ferredoxin-dependent. The usage of this low-potential electron donor, in addition to the oxygen sensitivity of pyruvate synthase, may restrict the dicarboxylate/4-hydroxybutyrate cycle to anaerobic conditions. Although I. hospitalis is a strict anaerobe, P. fumarii is a facultative aerobe, and O2 can serve as electron acceptor for H2 oxidation, but only in trace amounts (up to 0.3 % in the gas phase) (Blochl et al., 1997). The actual conditions in the cytoplasm of actively respiring cells are probably anoxic at these low oxygen concentrations and at the optimal growth temperature of 106 °C. Similarly, hyperthermophilic autotrophic representatives of the bacterial phylum Aquificae grow aerobically using the reductive citric acid cycle with ferredoxin-dependent pyruvate and 2-oxoglutarate synthases (Shiba et al., 1985; Ikeda et al., 2006; Yamamoto et al., 2006; Aoshima, 2007; Beh et al., 1993; Hügler et al., 2007). However, it cannot be ruled out that the enzymes from other Archaea exhibit different sensitivities to oxygen.

**4-Hydroxybutyryl-CoA dehydratase: evolutionary considerations**

The topology of the phylogenetic tree of the 4-hydroxybutyryl-CoA dehydratase gene (Fig. 7) corresponds to that of the 16S rRNA gene tree (Fig. 1). All sequences of the ‘crenarchaea type-1’ genes from Crenarchaeota grouped together and formed a monophyletic clade. In this clade, the sequences formed two clusters corresponding to the crenarchaeal orders Sulfolobales and Thermoproteales, and a separate branch corresponding to the 4-hydroxybutyryl-CoA dehydratase gene from I. hospitalis belonging to the Desulfurococcales. Although members of the Sulfolobales have a second copy of the 4-hydroxybutyryl-CoA dehydratase gene [‘crenarchaea type-2’ gene (Berg et al., 2007)], the gene product lacks some conserved amino acids for the iron–sulfur-cluster pocket, and its function remains to be shown. Analysis of this tree suggests that the 4-hydroxybutyryl-CoA dehydratase gene was transferred vertically during the evolution of the hyperthermophilic Crenarchaeota, and the ancestor of all three crenarchaeal orders most probably had this gene. Taken together with the presence of this gene in marine Crenarchaeota, this implies the antiquity of the 4-hydroxybutyrate part of the CO2 fixation pathways characteristic of Crenarchaeota.

**ACKNOWLEDGEMENTS**

Thanks are due to Nasser Gad’on and Christa Ebenau-Jehle, Freiburg, for help with growing cells and maintaining the running of the laboratory, and Michael Thomm, Regensburg, for ongoing support. The DOE Joint Genome Institute is acknowledged for the early release of archaeal genomic sequence data. This work was supported by grants of the Deutsche Forschungsgemeinschaft to G.F. and H.H.
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Edited by: J. A. Vorholt