Autotrophic CO₂ fixation via the reductive tricarboxylic acid cycle in different lineages within the phylum *Aquificae*: evidence for two ways of citrate cleavage

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Summary

Autotrophic carbon fixation was characterized in representative members of the three lineages of the bacterial phylum *Aquificae*. Enzyme activity measurements and the detection of key genes demonstrated that *Aquificae* use the reductive tricarboxylic acid (TCA) cycle for autotrophic CO₂ fixation. This is the first time that strains of the *Hydrogenothermaceae* and ‘*Desulfurobacteriaceae*’ have been investigated for enzymes of autotrophic carbon fixation. Unexpectedly, two different mechanisms of citrate cleavage could be identified within the *Aquificae*. *Aquificae* use citryl-CoA synthetase and citryl-CoA lyase, whereas *Hydrogenothermaceae* and ‘*Desulfurobacteriaceae*’ use ATP citrate lyase. The first mechanism is likely to represent the ancestral version of the reductive TCA cycle. Sequence analyses further suggest that ATP citrate lyase formed by a gene fusion of citryl-CoA synthetase and citryl-CoA lyase and subsequently became involved in a modified version of this pathway. However, rather than having evolved within the *Aquificae*, our phylogenetic analyses indicate that *Aquificae* obtained their ATP citrate lyase through lateral gene transfer. *Aquificae* play an important role in biogeochemical processes in a variety of high-temperature habitats. Thus, these findings substantiate the hypothesis that autotrophic carbon fixation through the reductive TCA cycle is widespread and contributes significantly to biomass production particularly in hydrothermal habitats.

Introduction

Autotrophy is a requirement for self-sustaining life and chemolithoautotrophs—i.e. organisms that acquire energy from the oxidation of inorganic compounds and that use inorganic carbon as the source for cell carbon— are likely to have been among the first types of organisms on Earth (e.g. Huber and Wächtershäuser, 1997; Russell and Hall, 1997). Phylogenetic analyses based on 16S rRNA and whole genomes place autotrophic hyperthermophiles at the base of the evolutionary tree of life, lending evidence to the hypothesis that in addition to being autotrophs, the first life forms might have been hyperthermophilic (Pace, 1991; House and Fitz-Gibbon, 2002). Microorganisms carrying out autotrophic carbon fixation at high temperatures, i.e. above 70°C, utilize CO₂ fixation pathways other than the Calvin-Benson-Bassham (Calvin) cycle, which is well known from cyanobacteria, plants and a variety of chemolithoautotrophic Proteobacteria (Fuchs, 1989; Madigan et al., 2003). Presently, three of these so-called alternative pathways are known: the 3-hydroxypropionate cycle, the reductive acetyl-CoA pathway and the reductive tricarboxylic acid (TCA) cycle (Madigan et al., 2003). The reductive TCA cycle, which has been proposed as a likely candidate for the earliest autotrophic pathway that evolved on Earth (Wächtershäuser, 1990; Cody et al., 2001; Smith and Morowitz, 2004), was first shown to function in the green sulfur bacterium Chlorobium limicola (Evans et al., 1966; Fuchs et al., 1980; Ivanovski et al., 1980). Subsequently, the operation of the reductive TCA cycle was also confirmed in a variety of anaerobic and microaerobic bacteria, including the sulfate-reducing bacterium Desulfobacter hydrogenophilus (Schauer et al., 1987), and most recently various ε-Proteobacteria (Hügler et al., 2005; Takai et al., 2005). Interestingly, two species of the *Aquificaceae*, *Aquifex pyrophilus* (Beh et al., 1993) and *Hydrogenobacter thermophilus* (Shiba et al., 1985) have also been shown to use the reductive TCA cycle for autotrophic carbon fixation.
Within the bacteria, the phylum *Aquificae* is the only group that contains hyperthermophilic autotrophs. Presently it is divided into three major lineages (Eder and Huber, 2002; Takai et al., 2003a), (i) the *Aquificaceae* comprising the genera *Aquifex*, *Hydrogenobacter*, *Hydrogenobaculum*, *Hydrogenivirga* and *Thermocrinis*; (ii) the *Hydrogenothermaceae* comprising the genera *Hydrogenothermus*, *Persephonella* and *Sulfurihydrogenibium*; and (iii) several genera incertae sedis (‘*Desulfurobacteriaceae*’) including the genera *Balnearium*, *Desulfurobacterium* and *Thermovibrio* (Fig. 1). Whereas the *Aquificaceae* and *Hydrogenothermaceae* contain predominantly microaerophilic chemolithoautotrophs that obtain energy by oxidizing molecular hydrogen or reduced sulfur compounds, all members of the ‘*Desulfurobacteriaceae*’ are strict anaerobes, obtaining energy by coupling the oxidation of *H*₂ either to the reduction of elemental sulfur or nitrate.

In recent years, several studies have shown that members of the *Aquificae* are likely to play an important role as primary producers in various high-temperature environments (e.g. Reysenbach et al., 1994; 2000a,b; Harmsen et al., 1997; Yamamoto et al., 1998; Eder and Huber, 2002; Blank et al., 2002; Inagaki et al., 2003; Spear et al., 2005), suggesting that carbon fixation through the reductive TCA cycle might be significant in these habitats. However, at present no information exists on what carbon fixation pathway members of the ‘*Desulfurobacteriaceae*’ might use and only limited information, in form of carbon isotopic analyses, exists for the *Hydrogenothermaceae* (Zhang et al., 2002).

The reductive TCA cycle is essentially a reversal of the well-known oxidative TCA or Krebs cycle. Three molecules of CO₂ are fixed, forming acetyl-CoA and subsequently pyruvate, the precursor of all other central metabolites. While most of the enzymes are shared between the reductive and oxidative TCA cycle, three enzymes are essential to run this cycle in reverse: 2-oxoglutarate : ferredoxin oxidoreductase, fumarate reductase and ATP citrate lyase. The latter enzyme catalyses the ATP- and the CoA-dependent cleavage of citrate into acetyl-CoA and oxaloacetate. ATP citrate lyase is encoded by two separated genes *aclA* and *aclB*, arranged in the same way in all organisms studied to date (Fig. 2A). ATP citrate lyase genes have so far only been found in prokaryotes using the reductive TCA cycle for autotrophic carbon fixation, such as the green sulfur bacteria (Antranikian et al., 1982; Wahlund and Tabita, 1997; Kanao et al., 2001), and e-Proteobacteria (Campbell et al., 2003; Hügler et al., 2005; Takai et al., 2005), and thus can at present be considered as indicator genes.
for this pathway. In contrast to Chlorobiaceae and ε-Proteobacteria, the ATP-dependent citrate cleavage in *H. thermophilus* is catalysed by two enzymes, citryl-CoA synthetase and citryl-CoA lyase, which catalyse the ATP-dependent formation of citryl-CoA from citrate and CoA and the subsequent cleavage of citryl-CoA into acetyl-CoA and oxaloacetate respectively (Aoshima *et al.*, 2004a,b) (Fig. 2B). The sequence similarity between citryl-CoA synthetase, succinyl-CoA synthetase and ATP citrate lyase on one hand and between citryl-CoA lyase, citrate synthase and ATP citrate lyase on the other hand suggests that these enzymes share an evolutionary history (Aoshima *et al.*, 2004b). Furthermore, it has been argued that the reductive TCA cycle as identified in *H. thermophilus* constitutes the ancient form, whereas the reductive TCA cycle in *Chlorobium* involving ATP citrate lyase represents a secondary adaptation that evolved from the oxidative TCA cycle (Aoshima *et al.*, 2004b). In this context, we were interested in investigating which citrate cleavage mechanism might operate in the two other lineages of the *Aquificae*.

In the present study we have investigated the carbon fixation pathways in representative organisms of all major lineages within the phylum *Aquificae*. We could measure the activities of the enzymes of the reductive TCA cycle and amplify the genes coding for the respective citrate cleaving enzymes, suggesting that all investigated members of the *Aquificae* utilize the reductive TCA cycle for autotrophic carbon fixation. Unexpectedly, we found that there exist two ways of citrate cleavage within the *Aquificae*. The evolutionary and ecological implications of these findings are discussed.

### Results and discussion

#### Activities of enzymes of the reductive TCA cycle in *Aquificae*

We studied carbon fixation in representatives of the three different lineages of the phylum *Aquificae*. Table 1 provides an overview of the strains that were used in this investigation, including their isolation site and growth requirements. Cell extracts of *Aquifex aeolicus*, *Thermocrinis ruber* (both *Aquificaceae*), *Thermovibrio ammonificans*, *Thermovibrio ruber* (both *Desulfurobacteriaceae*) and *Sulfurihydrogenibium subterraneum* (*Hydrogenothermaceae*) were tested for the activity of enzymes of the reductive and oxidative TCA cycle. In addition, cell extract of *Desulfurobacterium thermolithotrophicum* (*Desulfurobacteriaceae*) was tested for citrate cleavage, 2-oxoglutarate and pyruvate : benzyl viologen (BV) oxidoreductase activities. The results of these enzyme measurements are presented in Table 2. In general, the activities of all enzymes of a reductive TCA cycle could be detected in all tested organisms, including the key enzymatic activities of ATP-dependent citrate cleavage, fuma-
rate reductase, 2-oxoglutarate : BV oxidoreductase, and pyruvate : BV oxidoreductase. Enzymes of the oxidative TCA cycle, which are not shared with the reductive version of the pathway, could not be detected or exhibited only very low rates. These enzymes are 2-oxoglutarate, succinate and pyruvate dehydrogenase. In the following sections, the results for the three families are described and discussed in more detail.

Table 1. Strains used in this study and some characteristics.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Family</th>
<th>Isolation site</th>
<th>Growth temperature (°C) (optimum)</th>
<th>Electron donor/ carbon source</th>
<th>Electron acceptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquifex aeolicus</td>
<td>Aquificaeae</td>
<td>Hydrothermal system (Vulcano, Italy)</td>
<td>85</td>
<td>H₂/CO₂</td>
<td>O₂</td>
</tr>
<tr>
<td>Balnearium lithotrophicum</td>
<td>‘Desulfurobacteriaceae’</td>
<td>Deep-sea hydrothermal vent chimney (Sulio Seamount, Japan) (70–75)</td>
<td>45–80</td>
<td>H₂/CO₂</td>
<td>S</td>
</tr>
<tr>
<td>Desulfurobacterium crinifex</td>
<td>‘Desulfurobacteriaceae’</td>
<td>Deep-sea hydrothermal vent chimney (Juan de Fuca Ridge) (60–65)</td>
<td>50–70</td>
<td>H₂/CO₂</td>
<td>S</td>
</tr>
<tr>
<td>Desulfurobacterium thermolithotrophum</td>
<td>‘Desulfurobacteriaceae’</td>
<td>Deep-sea hydrothermal vent chimney (Mid-Atlantic Ridge) (70)</td>
<td>40–75</td>
<td>H₂/CO₂</td>
<td>S</td>
</tr>
<tr>
<td>Hydrogenobacter hydrogenophilus</td>
<td>Aquificaeae</td>
<td>Hot spring (Geyser Valley, Kamchatka, Russia) (74–76)</td>
<td>50–82</td>
<td>H₂/CO₂</td>
<td>O₂</td>
</tr>
<tr>
<td>Hydrogenobacter thermophilus</td>
<td>Aquificaeae</td>
<td>Hot spring (Mine, Izu, Japan) (70)</td>
<td>50–79</td>
<td>H₂/CO₂</td>
<td>O₂</td>
</tr>
<tr>
<td>Persephonella marina</td>
<td>Hydrogenothermaceae</td>
<td>Deep-sea hydrothermal vent chimney (East Pacific Rise) (73)</td>
<td>55–80</td>
<td>H₂/CO₂</td>
<td>O₂</td>
</tr>
<tr>
<td>Thermocinus ruber</td>
<td>Aquificaeae</td>
<td>Octopus Spring (Yellowstone National Park) (80)</td>
<td>44–89</td>
<td>Formate</td>
<td>O₂</td>
</tr>
<tr>
<td>Thermovibrio ammonificans</td>
<td>‘Desulfurobacteriaceae’</td>
<td>Deep-sea hydrothermal vent chimney (East Pacific Rise) (75)</td>
<td>60–80</td>
<td>H₂/CO₂</td>
<td>NO₃⁻</td>
</tr>
<tr>
<td>Thermovibrio ruber</td>
<td>‘Desulfurobacteriaceae’</td>
<td>Submarine hydrothermal vent system (Lihir Island, Papua New Guinea) (68)</td>
<td>50–80</td>
<td>H₂/CO₂</td>
<td>NO₃⁻</td>
</tr>
<tr>
<td>Sulfurihydrogenibium azorense</td>
<td>Hydrogenothermaceae</td>
<td>Hot spring (Furnas, Azores, Portugal) (68)</td>
<td>50–73</td>
<td>H₂/CO₂</td>
<td>NO₃⁻</td>
</tr>
<tr>
<td>Sulfurihydrogenibium subterraneum</td>
<td>Hydrogenothermaceae</td>
<td>Terrestrial, subsurface hot aquifer water (Hishikari gold mine, Japan) (65)</td>
<td>40–70</td>
<td>H₂/CO₂</td>
<td>NO₃⁻</td>
</tr>
</tbody>
</table>

Table 2. Specific activities [nmol min⁻¹ (mg cell protein)⁻¹] of enzymes of the reductive and oxidative TCA cycle.

<table>
<thead>
<tr>
<th>Enzyme activity tested</th>
<th>Aquifex aeolicus</th>
<th>Thermocinus ruber</th>
<th>Sulfurihydrogenibium subterraneum</th>
<th>Desulfurobacterium thermolithotrophum</th>
<th>Thermovibrio ammonificans</th>
<th>Thermovibrio ruber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay temperature (°C)</td>
<td>85</td>
<td>80</td>
<td>65</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>Citrate cleavage</td>
<td>71</td>
<td>82</td>
<td>55</td>
<td>485</td>
<td>275</td>
<td>340</td>
</tr>
<tr>
<td>2-Oxoglutarate : BV oxidoreductase</td>
<td>420</td>
<td>345</td>
<td>1600</td>
<td>330</td>
<td>175</td>
<td>400</td>
</tr>
<tr>
<td>Pyruvate : BV oxidoreductase</td>
<td>160</td>
<td>140</td>
<td>160</td>
<td>360</td>
<td>175</td>
<td>160</td>
</tr>
<tr>
<td>Fumarate reductase</td>
<td>1300</td>
<td>1010</td>
<td>2690</td>
<td>–</td>
<td>635</td>
<td>1200</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD⁺</td>
<td>385</td>
<td>145</td>
<td>400</td>
<td>–</td>
<td>115</td>
<td>185</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>11</td>
<td>8</td>
<td>52100</td>
<td>–</td>
<td>11750</td>
<td>45500</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>9500</td>
<td>3350</td>
<td>6040</td>
<td>–</td>
<td>9120</td>
<td>13780</td>
</tr>
<tr>
<td>NADPH</td>
<td>1310</td>
<td>1710</td>
<td>2920</td>
<td>–</td>
<td>4500</td>
<td>85600</td>
</tr>
<tr>
<td>Fumarate hydratase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fumarate</td>
<td>n.d.</td>
<td>n.d.</td>
<td>455</td>
<td>–</td>
<td>190</td>
<td>4500</td>
</tr>
<tr>
<td>Malate</td>
<td>100</td>
<td>81</td>
<td>235</td>
<td>–</td>
<td>77</td>
<td>350</td>
</tr>
<tr>
<td>Succinyl-CoA synthase</td>
<td>600</td>
<td>975</td>
<td>380</td>
<td>–</td>
<td>580</td>
<td>1460</td>
</tr>
<tr>
<td>2-Oxoglutarate dehydrogenase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD⁺</td>
<td>10</td>
<td>11</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>67</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>11</td>
<td>7</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>52</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NADP⁺</td>
<td>7</td>
<td>2</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formate dehydrogenase</td>
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</table>

a. Mean values were obtained from at least five measurements. Standard errors were less than ± 20%. n.d., no activity detected, detection limit < 1 nmol min⁻¹ (mg cell protein)⁻¹; –, not determined.
Aquificaceae. The operation of the reductive TCA cycle for carbon fixation had previously been demonstrated in A. pyrophilus and H. thermophilus, two members of the Aquificaceae (Shiba et al., 1985; Beh et al., 1993). We could measure all the enzymes of the reductive TCA cycle in A. aeolicus and Tc. ruber. Based either on the whole genome sequence (A. aeolicus) or carbon isotopic measurements (Tc. ruber) both organisms were previously suspected to use this pathway, but direct evidence had been lacking (Deckert et al., 1998; Jahnke et al., 2001). Isocitrate dehydrogenase, as well as malate dehydrogenase, was found to be NAD(H)-dependent. ATP-dependent citrate lyase activity in cell extracts was found to be optimal around pH 8.3 (data not shown) exhibiting values of 70 nmol min⁻¹ (mg protein⁻¹) for A. aeolicus and 80 nmol min⁻¹ (mg protein⁻¹) for Tc. ruber. These activities are low compared with the values obtained for S. subterraneum, D. thermolithotrophum or the Thermovibiromon species. This might reflect difficulties in measuring the coupled reactions of three different enzymes in vitro, namely citryl-CoA synthetase, citryl-CoA lyase and malate dehydrogenase rather than two as in the case of ATP citrate lyase and malate dehydrogenase. Although Tc. ruber was grown with formate as carbon source, the organism still uses the enzymes of the reductive TCA cycle to fix the CO₂ derived from formate. In addition, formate dehydrogenase activity could be measured, probably catalysing the formation of CO₂ from formate.

Hydrogenothermaceae. Sulfurihydrogenibium subterraneum, a representative member of the Hydrogenothermaceae, showed activities of all enzymes of the reductive TCA cycle, including high activities of ATP-dependent citrate lyase and 2-oxoglutarate:BV oxidoreductase. The pH optimum of ATP citrate lyase was determined to be pH 8.3 (data not shown). In contrast to members of the Aquificaceae, isocitrate dehydrogenase was NADP-dependent. Activities of the enzymes of the oxidative TCA cycle were not detected. The demonstration of the reductive TCA cycle in a member of the Hydrogenothermaceae is in agreement with stable carbon isotope analyses and the amplification of a small fragment of aclB from Persephonella marina (Zhang et al., 2002; Campbell et al., 2003).

‘Desulfurobacteriaceae’. All tested members of this family showed high activities of ATP-dependent citrate lyase and 2-oxoglutarate/pyruvate:BV oxidoreductase. The pH optimum of ATP citrate lyase was determined to be pH 8.3 in both T. ammonificans and T. ruber (data not shown). In both strains isocitrate dehydrogenase was NADP-dependent. This is the first report describing the autotrophic carbon fixation mode in this lineage, which has been proposed to represent an additional order within the phylum Aquificae (L’Haridon et al., 1998; Huber et al., 2002). Based on our data, the use of the reductive TCA cycle for autotrophic carbon fixation emerges as a common feature among the different lineages within the Aquificae.

Amplification of citryl-CoA lyase and ATP citrate lyase genes

Citrate cleavage is a key reaction of the reductive TCA cycle. It can be catalysed either through ATP citrate lyase (e.g. Chlorobium, Thiomicrospira denitrificans) (Kanao et al., 2001; Hügler et al., 2005), or by the combined action of citryl-CoA synthetase and citryl-CoA lyase (H. thermophilus) (Aoshima et al., 2004a,b) (Fig. 2). To determine which mechanism is present in the different lineages of the Aquificae, we isolated DNA and amplified, cloned and sequenced both subunits of ATP citrate lyase as well as large parts of citryl-CoA lyase using previously published and newly designed primer pairs (see Experimental procedures). Genomic DNA of A. aeolicus, Hydrogenobacter hydrogenophilus, H. thermophilus, Tc. ruber (all Aquificaceae); S. subterraneum (Hydrogenothermaceae); Balnearamium lithotrophicum, Desulfurobacterium crinifex, D. thermolithotrophum, T. ammonificans and T. ruber (all ‘Desulfurobacteriaceae’) was used. In addition, the draft genomes of P. marina and Sulfurohydrogenibium azorense were searched for ATP citrate lyase, citryl-CoA lyase and citryl-CoA synthetase genes (preliminary sequence data were obtained from The Institute for Genomic Research through the website at http://www.tigr.org). Surprisingly, both mechanisms for citrate cleavage seem to be present within the Aquificae.

Aquificaceae. As expected, we were not able to amplify fragments of the ATP citrate lyase genes (acl) from A. aeolicus, H. hydrogenophilus, H. thermophilus and Tc. ruber. Instead, we could amplify a fragment of the gene coding for citryl-CoA lyase (ccl) from all four strains using the newly designed primer pairs cclf1/cclr1 or cclf2/cclr2. This resulted in ~620 bp of sequence for ccl (representing ~80% of the expected ~780 bp). A phylogenetic tree based on the alignment of the protein sequence of citryl-CoA lyase is presented in Fig. 3. The ccl gene has previously been sequenced from H. thermophilus (Aoshima et al., 2004b). Based on this sequence, gene aq_150 (AAC06486) from A. aeolicus (annotated as coding for citrate synthase) could be identified as a ccl gene (Deckert et al., 1998; Aoshima et al., 2004b). The sequenced gene fragments from H. hydrogenophilus and Tc. ruber are closely related to these genes. As no acl gene could be amplified from H. hydrogenophilus and Tc. ruber and no acl genes are found in the genome of A. aeolicus, we assume that these three organisms use the combined action of citryl-CoA synthetase and citryl-
CoA lyase for the cleavage of citrate into oxaloacetate and acetyl-CoA. Genes coding for citryl-CoA synthetase can be found within the genome sequence of *A. aeolicus*. They have been assigned to encode succinyl-CoA synthetase *(sucC1* (AAC07285) and *sucD1* (AAC07686)). Consequently, *A. aeolicus*, *H. hydrogenophilus* and *Tc. ruber* seem to fix CO₂ via the same set of enzymes as *H. thermophilus*. These include citryl-CoA synthetase, citryl-CoA lyase as well as a NAD-dependent isocitrate dehydrogenase (Aoshima et al., 2004a,b,c).

**Hydrogenothermaceae.** Like in *Aquificaceae*, we could successfully amplify and sequence a gene fragment of citryl-CoA lyase (~620 bp, representing ~80% of the expected ~780 bp). In addition, we also amplified, cloned and sequenced the genes coding for the α- and β-subunit of ATP citrate lyase (*aclA* and *aclB*) from *S. subterraneum* using the primer pairs F2/R5, 892F/R5 and F1/R1. This way we obtained the complete gene sequence for *aclB* and a ~1.600 bp fragment of *aclA* (~90% of the expected ~1.800 bp). The gene arrangement is identical to all known ATP citrate lyase genes (*aclBA*). BLAST searches of the draft genomes of *P. marina* and *S. azorense* revealed that both organisms also possess genes coding for both ATP citrate lyase subunits, strongly suggesting that they also use the reductive TCA cycle for carbon fixation. The ATP citrate lyase genes of all three investigated members of *Hydrogenothermaceae* show high sequence identities, clustering together in the phylogenetic tree. Interestingly, BLAST searches also revealed a putative gene in the draft genomes of *P. marina* and *S. azorense* that could code for citryl-CoA lyase. However, a homologue of the citryl-CoA synthetase could not be identified, indicating that these organisms use ATP citrate lyase to catalyse citrate cleavage. The physiological role of citryl-CoA lyase is presently unclear and warrants further investigations.

**‘Desulfurobacteriaceae’.** We could amplify the ATP citrate lyase genes from all isolated strains of the ‘*Desulfurobacteriaceae*’. The entire sequence of *aclB* and ~90% of *aclA* was obtained from *B. lithotrophicum*, *D. crinifex* and *D. thermolithotrophum*. In addition, we amplified and sequenced a ~1.000 bp fragment of *aclA* (primer pair F2/R5) from *Tv. ammonificans*, as well as ~450 bp of the 5’ end of *aclB* and ~90% of *aclA* from *Tv. ruber* (primer pair 892F/R5). The translated sequences obtained from these strains show high identities between each other, as well as high identities and similarities to the sequences from *Persephonella* and *Sulfurihydrogenibium*. Using our newly designed primers we failed to amplify a citryl-CoA lyase gene from members of the ‘*Desulfurobacteriaceae*’. However, at this point we cannot exclude the presence of a citryl-CoA gene within this lineage, as the primers were designed only on a limited number of *ccl* sequences and thus might not be appropriate for this group. Regardless, the presence of *aclBA* and the high citrate cleavage activities strongly suggest that ‘*Desulfurobacteriaceae*’ use ATP citrate lyase to accomplish citrate cleavage.

ATP citrate lyase (*aclBA*) and citryl-CoA lyase (*ccl*) as phylogenetic and functional gene markers

Phylogenetic analyses of available *aclA* and *aclB* sequences reveal that the sequences obtained from
members of the phylum *Aquificae* form a monophyletic group clearly separated from sequences of *ε*-Proteobacteria, *Chlorobiaceae* and eukaryotes (fungi, plants, animals) (Fig. 4). The ATP citrate lyase sequences from *Aquificae* are most closely related to sequences from *ε*-Proteobacteria, with which they form a clade of their own. Phylogenetic trees calculated for the individual subunits of ATP citrate lyase show the same overall pattern (data not shown). The ATP citrate lyase based tree is largely in congruence with 16S rRNA phylogeny, with the ATP citrate lyase based tree is largely in congruence with 16S rRNA phylogeny, with (data not shown). 

The ATP citrate lyase sequences from members of the *Aquificae* build a framework that allows us to assign *acl* sequences obtained from the environment to particular lineages. Along these lines, we compared the *acl* sequences obtained in this study with environmental sequences obtained from different hydrothermal vent sites (Campbell and Cary, 2004) (Fig. 5). This way we can clearly classify some sequences as *Persephonella*-like, while *Sulfurihydrogenibium* species apparently seem to be absent. This is not too surprising, considering that all *Sulfurihydrogenibium* species to date have been identified in terrestrial environments. However, no sequences were found that could be assigned to the *‘Desulfurobacteriaceae’*, although these organisms have been isolated from hydrothermal vents, and were found to account for 40% of the microorganisms present in chimney walls (e.g. Harmsen et al., 1997). An explanation for this unexpected finding could be the specificity of the primers used by Campbell and Cary (2004), which in our hands failed to amplify *aclB* from *‘Desulfurobacteriaceae’*. In addition, our sequence analyses revealed a cluster of *acl* sequences that seem to be affiliated with *Hydrogenothermaceae*, but cannot be directly linked to sequences obtained from either *Persephonella* or *Sulfurihydrogenibium*. This strongly suggests that these sequences represent a new group of so far uncultivated *Aquificae*. Thus, our data provide further evidence for the usefulness of the ATP citrate lyase genes as both phylogenetic and functional gene markers. Based on our limited number of sequences, it appears that citryl-CoA lyase may have the potential to be used in the same way (Fig. 3).

**Evolutionary implications**

Surprisingly, two different pathways of citrate cleavage exist within the *Aquificae*. Members of the genera *Aquifex*, *Hydrogenobacter* and *Thermocrinis* use two enzymes, namely citryl-CoA synthetase and citryl-CoA lyase to accomplish the ATP-dependent cleavage of citrate into acetyl-CoA and oxaloacetate. This has been proposed to represent the ancestral version of the reductive TCA cycle (Aoshima et al., 2004a,b). Furthermore, succinyl-CoA synthetase and citryl-CoA synthetase are believed to have evolved via gene duplication and diversification of an enzyme that possibly catalysed both reactions.
(Aoshima et al., 2004a). On the other hand, members of the genera *Persephonella*, *Sulfurihydrogenibium* (both *Hydrogenothermaceae*), *Balnearium*, *Desulfurobacterium* and *Thermovibrio* (all ‘*Desulfurobacteriaceae*’) are using ATP citrate lyase for citrate cleavage. Based on sequence similarity, the origin of the ATP citrate lyase might be the result of a gene fusion of citryl-CoA synthetase and citryl-CoA lyase, which themselves are homologues of succinyl-CoA synthetase and citrate synthase (Fatland et al., 2002; Aoshima et al., 2004a,b). The small subunit of ATP citrate lyase is a homologue of the large subunit of citryl-CoA synthetase, whereas the large subunit of ATP citrate lyase seems to have formed by a fusion of the small subunit of citryl-CoA synthetase and citryl-CoA lyase (Fig. 2). These findings raise the question on the origin of ATP citrate lyase in *Hydrogenothermaceae* and ‘*Desulfurobacteriaceae*’. In one possible scenario, ATP citrate lyase might have evolved within the *Aquificae* from an ancient form of the reductive TCA cycle involving a two-step citrate cleavage, as exemplified presently by *H. thermophilus*, to a reductive TCA cycle utilizing only one enzyme, like it is operating in the ‘*Desulfurobacteriaceae*’. In another scenario, ATP citrate lyase might have been transferred into *Hydrogenothermaceae* and ‘*Desulfurobacteriaceae*’ via lateral gene transfer. Based on our data, the second scenario seems more likely, because ATP citrate lyase from *Hydrogenothermaceae* and ‘*Desulfurobacteriaceae*’ appears to be a derived form of the ATP citrate lyase since it is more similar to the one from ε-Proteobacteria than to the evolutionary ancestral enzyme (Figs 3 and 4). Our phylogenetic analyses confirm earlier results of Fatland and colleagues (2002), who concluded that the sequence from *C. limicola* appears to be closest to the evolutionary ancestral form. Thus, ATP citrate lyase appears to have been acquired by *Aquificae* via horizontal gene transfer, most likely from ε-Proteobacteria. Along these lines it is interesting to note that both groups occur in similar environments, providing an ecological basis for such an event.

**Ecological implications**

The results obtained in this study lend further support to the hypothesis that autotrophic carbon fixation through
the reductive TCA cycle is widespread and contributes significantly to biomass production in certain environments, particularly in hydrothermal habitats. Members of the Aquificae are important in many hot environments, e.g. shallow and deep-sea marine vents, terrestrial hot springs, as well as the terrestrial subsurface (e.g. Harmsen et al., 1997; Reysenbach et al., 2000a,b; Blank et al., 2002; Takai et al., 2002; Huber et al., 2003; Inagaki et al., 2003), and have been isolated from all around the world (Table 1). Extensive research has been particularly carried out at Yellowstone National Park, USA, where *Tc. ruber* has been identified as an ubiquitous member of microbial communities in silica-depositing hot springs (Blank et al., 2002) and Aquificae in general may dominate the microbial communities at temperatures higher than 70°C (Spear et al., 2005). Our finding that *Tc. ruber* and other Aquificae do fix CO₂ via the reductive TCA cycle suggests that primary production at temperatures higher than 70°C in hot springs in Yellowstone and probably elsewhere occurs mainly via the reductive TCA cycle.

Members of the Aquificae have also been shown to constitute an important component of the microbial communities at deep-sea hydrothermal vents, where these organisms seem to be mainly associated with sulfide structures and most likely also colonize the subsurface portion of vents (Harmsen et al., 1997; L’Haridon et al., 1998; Reysenbach et al., 2000a; Götz et al., 2002; Alain et al., 2003; Huber et al., 2003; Takai et al., 2003a; Vetriani et al., 2004). Besides members of the Aquificae, *ε*-Proteobacteria have been identified as a dominant bacterial group at deep-sea vents (e.g. Reysenbach et al., 2000b; Huber et al., 2003; Nakagawa et al., 2005). Interestingly, autotrophic members of this group also use the reductive TCA cycle for carbon fixation (Campbell et al., 2003; Hügler et al., 2005; Takai et al., 2005). As a whole, both groups exhibit similar metabolisms – i.e. the oxidation of reduced sulfur compounds and hydrogen with both oxygen and nitrate or the oxidation of hydrogen with elemental sulfur coupled to the fixation of inorganic carbon – and thus occupy a similar ecological niche. However, they seem to be partitioned by their temperature preference, with *ε*-Proteobacteria dominating the microbial communities at temperatures from 20°C to 60°C, whereas Aquificae seem to be the predominant autotrophs at temperatures higher than 60°C. Taken together, this suggests that a significant fraction of the biomass at deep-sea hydrothermal vents might be produced via carbon fixation through the reductive TCA cycle. It remains to be determined whether this production rivals the fixation occurring through the Calvin cycle, which in the current paradigm forms the base of deep-sea hydrothermal ecosystems. Ongoing studies in our laboratory that aim to couple the identity of the microorganisms carrying out CO₂ fixation in situ with concurrent rate measurements are addressing this question.

### Experimental procedures

#### Bacteria, growth conditions and preparation of cell extracts

Table 1 gives an overview about the strains used in this investigation, including their isolation site and growth requirements. *Aquifex aeolicus* VF5 (Eder and Huber, 2002), *B. lithothrophicum* 17S (DSMZ 16304) (Takai et al., 2003a), *D. crinifex* NE1206 (DSMZ 15218) (Alain et al., 2003), *D. thermolithothrophicum* BSA (DSMZ 11699) (L’Haridon et al., 1998), *H. hydrogenophilus* Z-829 (DSMZ 2913) (Kryukov et al., 1983), *H. thermophilus* TK-6 (DSMZ 6543) (Kawasumi et al., 1984), *Tv. ammonificans* HB-1 (DSMZ 15698) (Vetriani et al., 2004), *Tv. ruber* ED11/3LLK (DSMZ 14644) (Huber et al., 2002) and *S. subterraneum* HGMK1 (DSMZ 15120) (Takai et al., 2003b) were grown autotrophically as described in references. *Thermocrinis ruber* OC1/4 (DSMZ 12173) (Huber et al., 1998) was grown with formate as sole carbon source. Biomass was harvested under the exclusion of oxygen. Cell extracts were prepared using a mixer mill (type MM2; Retsch, Haare, Germany) according to Hügler and colleagues (2005). Protein concentration in cell extracts was determined by the method of Bradford (1976) using bovine serum albumin as standard.

#### Enzyme assays

Enzyme assays (0.5 ml assay mixture) were performed in stoppered 0.5 ml glass cuvettes. The assay temperature differed according to the organisms and is given in Table 2. Reactions involving pyridine nucleotides were followed spectrophotometrically at 365 nm [NAD(P)H = 3.4 × 10⁻³ M⁻¹ cm⁻¹]. Reactions involving BV were followed spectrophotometrically at 578 nm [BV = 8.6 × 10⁻³ M⁻¹ cm⁻¹].

The citrate cleavage activity of ATP citrate lyase or the combined activities of citrilly-CoA synthetase and citrilly-CoA lyase were determined by coupling the reactions to malate dehydrogenase, which reduces the produced oxaloacetate with NADH. The citrate-, CoA- and MgATP-dependent oxidation of NADH was monitored. The assay mixture contained 100 mM HEPES (N-2-Hydroxyethyl)piperazine-2'-ethanesulfonic acid)/NaOH, pH 8.3, 5 mM DTE, 5 mM MgCl₂, 3 mM ATP, 0.5 mM CoA, 0.4 mM NADH, and 3 mM β-citrate. The reaction was started by the addition of citrate. Buffers used to determine the pH optimum were HEPES/NaOH (pH 6.5–8.5), and MOPS [3-(N-Morpholino)-2-hydroxypropanesulfonic acid]/NaOH (pH 6.5–8.5). 2-Oxoglutarate : BV oxidoeductase, pyruvate : BV oxidoeductase, fumarate reductase, malate dehydrogenase, isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, and pyruvate dehydrogenase were measured according to references (Hügler et al., 2003; 2005). Formate dehydrogenase and succinyll-CoA synthetase were measured as described in Beh and colleagues (1993). Succinate dehydrogenase was determined spectrophotometrically as the reduction of...
2,6-dichlorodiphenol with succinate at 578 nm according to Schauder and colleagues (1987). Fumarate hydratase was measured in a direct spectrophotometrical assay by following the consumption or the production of fumarate at 240 nm using 0.5 mM fumarate or 5 mM malate as substrate (Schauder et al., 1987; Hügler et al., 2003).

**DNA extraction, PCR amplification, cloning and sequencing of aciBA and col and phylogenetic analyses**

DNA was extracted with the UltraClean microbial DNA Isolation kit (Mo Bio Laboratories, Solana Beach, CA) according to the manufacturer's protocol. For amplification of the ATP citrate lyase genes, different primer sets were used in a 35-cycle PCR at an annealing temperature of 42–50°C. The used primers were F2/R5 (annealing at 42°C) (Hügler et al., 2005), 892f/R5 (annealing at 50°C) (Campbell et al., 2003; Hügler et al., 2005) and F1 (5′-ATG GCT CAA AAG GCA ATT AGA GAA T-3′)/R1 (5′-GCA CCA GCA TGA CCA AAC TGA -3′) (annealing at 46°C). For the amplification of the citryl-CoA lyase gene we used newly designed primers cclf1 (5′-CGG CTT CTT GAT CTT GTA GG-3′)/cclr1(5′-TTC TTG TCT GGT ATG AGT TCT TC-3′) or cclf2(5′-CCT TTA ACC CAG ATG ATA GG-3′)/cclr2(5′-GGT TCT GGA TGT AGT TCT TC-3′) in a 30-cycle PCR at an annealing temperature of 50°C. PCR products were purified from agarose gels using a Qiaquick gel extraction kit (QIAGEN, Chatswood, CA) and cloned using the TOPO TA cloning kit for sequencing (Invitrogen, Carlsberg, CA) as described by the manufacturer. Colonies were picked, and the plasmid DNA was purified with the QIAprep spin miniprep kit (QIAGEN) as described by the user manual. Plasmids were sent to the BioResource Center at Cornell University (Ithaca, NY) for sequencing. DNA sequencing was performed using the Applied Biosystems Automated 3730 DNA analyser. The big dye terminator chemistry and AmiTaq-FS DNA polymerase were used. Preliminary sequence data of *P. marina* and *S. azorense* were obtained from The Institute for Genomic Research through the website at http://www.tigr.org. The sequences were analyzed using MacVector (Accelrys, San Diego, CA) and PAUP, version 4.0b10, as described previously (Hügler et al., 2005).

**Nucleotide sequence accession numbers**

The nucleotide sequences have been deposited in GenBank. The accession numbers for the ATP citrate lyase genes are: DQ853417 (*B. lithotrophicum*), DQ853418 (*D. crinifex*), DQ853419 (*D. thermolithothrophicum*), DQ853420 (*T. ammonificans*), DQ853421 (*T. ruber*), and DQ853422 (*S. subterraneum*). The accession numbers for the citryl-CoA lyase gene fragments are: DQ853423 (*H. hydrogenophilus*), DQ853424 (*S. subterraneum*) and DQ853425 (*Tc. ruber*).

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**References**


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