Autotrophic Carbon Dioxide Assimilation in *Thermoproteales* Revisited[▽]

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Received 3 February 2009/Accepted 24 April 2009

For *Crenarchaea*, two new autotrophic carbon fixation cycles were recently described. *Sulfolobales* use the 3-hydroxypropionate/4-hydroxybutyrate cycle, with acetyl-coenzyme A (CoA)/propionyl-CoA carboxylase as the carboxylating enzyme. *Ignicoccus hospitalis* (*Desulfurococcales*) uses the dicarboxylate/4-hydroxybutyrate cycle, with pyruvate synthase and phosphoenolpyruvate carboxylase being responsible for CO₂ fixation. In the two cycles, acetyl-CoA and two inorganic carbons are transformed to succinyl-CoA by different routes, whereas the regeneration of acetyl-CoA from succinyl-CoA proceeds via the same route. *Thermoproteales* would be an exception to this unifying concept, since for *Thermoproteus neutrophilus*, the reductive citric acid cycle was proposed as a carbon fixation mechanism. Here, evidence is presented for the operation of the dicarboxylate/4-hydroxybutyrate cycle in this archaeon. All required enzyme activities were detected in large amounts. The key enzymes of the cycle were strongly upregulated under autotrophic growth conditions, indicating their involvement in autotrophic CO₂ fixation. The corresponding genes were identified in the genome. ¹⁴C-labeled 4-hydroxybutyrate was incorporated into the central building blocks in accordance with the key position of this compound in the cycle. Moreover, the results of previous ¹³C-labeling studies, which could be reconciled with a reductive citric acid cycle only when some assumptions were made, were perfectly in line with the new proposal. We conclude that the dicarboxylate/4-hydroxybutyrate cycle is operating in CO₂ fixation in the strict anaerobic *Thermoproteales* as well as in *Desulfurococcales*.

Two new autotrophic carbon fixation cycles have recently been discovered in the *Crenarchaea*, one of the two subgroups of the *Archaea*. The 3-hydroxypropionate/4-hydroxybutyrate cycle functions in the aerobic autotrophic *Sulfolobales* (7) and the dicarboxylate/4-hydroxybutyrate cycle (Fig. 1) in the anaerobic autotrophic *Ignicoccus hospitalis*, belonging to the *Desulfurococcales* (27). These pathways have in common the synthesis of succinyl-coenzyme A (CoA) from acetyl-CoA and two inorganic carbons, although this is accomplished in quite different ways and using different carboxylases. 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 regenerations of acetyl-CoA, the primary CO₂ acceptor, from succinyl-CoA are similar in the two pathways.

Acetyl-CoA regeneration is as follows. The CO₂ fixation product 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 radical [4Fe-4S] and flavin adenine dinucleotide-containing dehydratase (11, 37) is considered a key enzyme of the 4-hydroxybutyrate part of each pathway. 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. Therefore, two dif-

ferent autotrophic pathways in different crenarchaeal orders share many common enzymes and intermediates.

In this context, the order *Thermoproteales* would constitute an exception within the *Crenarchaea*, since the reductive citric acid cycle was proposed for *Thermoproteus neutrophilus* (6, 48–50, 55) and *Pyrobaculum islandicum* (26). *T. neutrophilus* is a strictly anaerobic hyperthermophilic archaeon growing autotrophically by reducing sulfur with hydrogen at 85°C and neutral pH (19). It can also assimilate organic compounds, such as acetate or succinate, but only in the presence of CO₂ and H₂, i.e., in a mixotrophic way (48).

In the reductive citric acid cycle, succinyl-CoA is further transformed with 2 CO₂ to citrate, followed by citrate cleavage to oxaloacetate and acetyl-CoA. This requires two characteristic enzymes, 2-oxoglutarate synthase (2-oxoglutarate-ferredoxin oxidoreductase) and ATP citrate lyase. The proposal of the functioning of the reductive citric acid cycle in *T. neutrophilus* was based on the results of a ¹³C retrobiosynthetic analysis of the central carbon metabolism, using ¹³C-labeled succinate and acetate as an additional carbon source, following its incorporation into cellular building blocks. The ¹³C enrichment data of, e.g., glutamate, which is directly derived from 2-oxoglutarate, were consistent with the operation of a reductive citric acid cycle only when further assumptions were made (55). The activities of the enzymes of this cycle were demonstrated with extracts of autotrophically grown cells. However, the measured 2-oxoglutarate synthase and ATP-citrate lyase activity levels were very low and could not support the reported growth rate under autotrophic conditions (6, 48).

The recent sequencing of the genome of *Pyrobaculum aerophilum*, belonging to the *Thermoproteales* (20), revealed a surprising feature, the presence of a 4-hydroxybutyryl-CoA dehydratase gene without the presence of an ATP-citrate lyase

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[▽] Published ahead of print on 1 May 2009.

FIG. 1. Dicarboxylate/4-hydroxybutyrate cycle for autotrophic CO_2 fixation, as proposed for *T. neutrophilus*. Enzymes: 1, pyruvate synthase (reduced MV); 2, pyruvate-water dikinase; 3, PEP carboxylase; 4, malate dehydrogenase (NADH); 5, fumarate hydratase; 6, fumarate reductase (reduced MV); 7, succinyl-CoA synthetase (ADP forming); 8, succinyl-CoA reductase (NADPH); 9, succinic semialdehyde reductase (NADPH); 10, 4-hydroxybutyrate-CoA ligase (AMP forming); 11, 4-hydroxybutyryl-CoA dehydratase; 12, crotonyl-CoA hydratase; 13, (S)-3-hydroxybutyryl-CoA dehydrogenase (NAD^+); 14, acetoacetyl-CoA β -ketothiolase. Fd_{red} , reduced ferredoxin.

gene. Similar gene patterns are found in the genomes of *T. neutrophilus* as well as *Pyrobaculum caldifontis* and *P. islandicum*, sequenced by the DOE Joint Genome Institute (<http://www.jgi.doe.gov/>). This indicates a possible functioning of the dicarboxylate/4-hydroxybutyrate cycle in *Thermoproteales* and brings into question the involvement of the reductive citric acid cycle in autotrophic CO_2 fixation. This study has reinvestigated the pathway of autotrophic CO_2 fixation in *Thermoproteus neutrophilus*. We provide different lines of evidence for the operation of the dicarboxylate/4-hydroxybutyrate cycle.

MATERIALS AND METHODS

Cell material and growth conditions. *Thermoproteus neutrophilus* (DSM 2338) was a kind gift from K. O. Stetter and H. Huber from the culture collection of the Lehrstuhl für Mikrobiologie, University of Regensburg. It was grown anaerobically and autotrophically on a defined mineral medium with elemental sulfur under gassing with a mixture of 80% H_2 and 20% CO_2 (vol/vol) at 85°C and pH 6.8, as described in reference 48. For comparative studies, cells were grown under the same conditions, but in addition, 5 mM acetate was included in the medium. *T. neutrophilus* was cultivated in a 2-liter glass fermenter. The cells were harvested by centrifugation in the exponential growth phase (approximately 1×10^9 cells per ml) and stored at -70°C until use.

Syntheses. Acetoacetyl-CoA was synthesized from diketene by the method of Simon and Shemin (53). Succinyl-CoA was synthesized from its anhydride by a slightly modified version of the method described in reference 53; the deviations from that method involved the use of anaerobic conditions and room temperature. (R)- and (S)-3-hydroxybutyryl-CoA were synthesized by the mixed-anhydride method (54). The dry powders of the CoA esters were stored at -20°C .

Preparation of cell extracts. Cell extracts were prepared under anoxic conditions. Cells were suspended in 10 mM Tris-HCl buffer (pH 7.8; 1:1), and the cell suspension was passed through a chilled French pressure cell at 137 kPa. The lysate was ultracentrifuged for 1 h ($100,000 \times g$; 4°C), and the aliquots of the supernatant (cell extract) were stored anaerobically at -70°C until use.

Enzyme measurements. Spectrophotometric enzyme assays (0.5 ml assay mixture) were performed aerobically with 0.5-ml glass cuvettes at 65°C , unless otherwise indicated. Anaerobic assays were done with N_2 as the headspace. Reactions involving NAD(P)H were measured at 365 nm ($\epsilon_{\text{NADH}} = 3.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{\text{NADPH}} = 3.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) (8). Reactions with methyl viologen (MV) were measured at 578 nm ($\epsilon_{\text{MV}} = 9.7 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) (61).

Pyruvate and 2-oxoglutarate-acceptor oxidoreductase (EC 1.2.7.1 and EC 1.2.7.3, respectively) were measured anaerobically in a mixture containing 100 mM Tris-HCl (pH 7.8), 5 mM MgCl_2 , 4 mM dithiothreitol (DTT), 4 mM MV, 1 mM CoA, and cell extract. Dithionite was added by use of a syringe until a permanent faint bluish color was obtained. The addition of pyruvate or 2-oxoglutarate (3 mM) started the reaction. The $^{14}\text{CO}_2$ exchange reaction with the carboxyl group of pyruvate was assayed radiochemically at 80°C with a reaction

mixture containing 100 mM morpholinepropanesulfonic acid (MOPS)-KOH (pH 7.2), 5 mM MgCl₂, 5 mM 1,4-DTT, 0.2 mM CoA, 10 mM pyruvate, 15 mM NaH¹⁴CO₃ (50 kBq/ml), and cell extract. The determination of acid-stable ¹⁴C was done as described previously (30).

Pyruvate-water dikinase (EC 2.7.9.2) activity was measured at 80°C using a discontinuous assay according to the method of Eyzaguirre et al. (18). The assay mixture contained 200 mM Tris-HCl (pH 7.9), 20 mM MgCl₂, 10 mM DTT, 50 mM NH₄Cl, 5 mM ATP, 0.8 mM pyruvate, and cell extract. The amounts of PEP formed in the reaction after 0, 5, and 10 min of incubation were determined by transferring the sample (0.2 ml) into an assay mixture (0.8 ml; 30°C) containing 200 mM Tris-HCl (pH 7.9), 20 mM MgCl₂, 1 mM ADP, and 0.5 mM NADH and measuring the decrease in absorbance at 365 nm after the consecutive addition of lactate dehydrogenase (25 U) and pyruvate kinase (5 U).

Pyruvate-orthophosphate dikinase (EC 2.7.9.1) was measured similarly to pyruvate-water dikinase, but the reaction mixture was supplemented with potassium phosphate (2 mM).

Pyruvate kinase (EC 2.7.1.40) activity was measured at 42°C in a coupling assay with exogenous lactate dehydrogenase. The reaction mixture (0.5 ml) contained 100 mM Tris-HCl (pH 7.8), 30 mM MgCl₂, 5 mM DTT, 5 mM PEP, 0.5 mM NADH, 5 mM ADP, 2.5 U lactate dehydrogenase, and cell extract.

PEP carboxylase (EC 4.1.1.31) was measured spectrophotometrically at 365 nm in the assay coupled with endogenous malate dehydrogenase. The reaction mixture contained 100 mM MOPS-KOH (pH 7.2), 4 mM MnCl₂, 40 mM NaHCO₃, 5 mM DTT, 0.5 mM NADH, and cell extract. The reaction was started by the addition of PEP (5 mM). The reaction was also measured radiochemically using a similar reaction mixture at 80°C with 15 mM NaH¹⁴CO₃ (50 kBq/ml).

ATP-, GTP-, and diphosphate-dependent PEP carboxykinases (EC 4.1.1.49, 4.1.1.32, and 4.1.1.38, respectively) were measured similarly to PEP carboxylase, but the reaction mixture was supplemented with ADP (5 mM), GDP (5 mM), or potassium phosphate (2 mM) for ATP-, GTP-, or pyrophosphate-dependent PEP carboxykinase, respectively.

Malate dehydrogenase (EC 1.1.1.37) was detected spectrophotometrically by oxaloacetate-dependent oxidation of NADH in an assay mixture containing 100 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 5 mM DTT, 0.3 mM NADH, and cell extract. The reaction was started by the addition of oxaloacetate (4 mM).

Fumarate hydratase (EC 4.2.1.2) was measured anaerobically at 250 nm ($\epsilon_{\text{fumarate}} = 1,479 \text{ M}^{-1} \text{ cm}^{-1}$) (42). The assay mixture contained 100 mM potassium phosphate buffer (pH 7.9), 30 mM D,L-malate, and cell extract. The reaction was started by the addition of cell extract.

The fumarate reductase (EC 1.3.99.1) assay mixture contained 100 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 4 mM DTT, 4 mM MV, and cell extract. MV was reduced with 50 mM dithionite stock solution to an optical density of about 1.5 (578 nm), and the reaction was started by the addition of fumarate (3 mM). The presence of NAD(P)H-dependent fumarate reductase (EC 1.3.1.6) was tested at 365 nm with a similar reaction mixture where MV was replaced by NADPH (0.5 mM).

The succinyl-CoA synthetase (EC 6.2.1.5) assay mixture contained 100 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 4 mM DTT, 0.6 mM CoA, 0.5 mM NADPH, 1 mM ATP, 1.0 U ($\mu\text{mol/min}$) ml⁻¹ recombinant malonyl-CoA/succinyl-CoA reductase from *Metallospira sedula* (1), and cell extract. The reaction was started by the addition of succinate (3 mM).

Succinyl-CoA reductase (EC 1.2.1.-) and succinic semialdehyde reductase (EC 1.1.1.-) were determined with a reaction mixture containing 100 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 5 mM DTT, 0.5 mM NADPH, and cell extract. The reaction was started by addition of succinyl-CoA or succinic semialdehyde (0.2 mM).

4-Hydroxybutyrate-CoA ligase (EC 6.2.1.-) activity was measured at 85°C using a discontinuous assay. The assay mixture contained 200 mM MOPS-KOH (pH 7.8), 5 mM MgCl₂, 3 mM ATP, 0.5 mM CoA, and 10 mM 4-hydroxybutyrate. The reaction was started by the addition of cell extract. After 0, 2, and 4 min of incubation, 0.1 ml of the test mixture was removed and diluted in 0.9 ml of 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, and 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at 0°C. The amount of CoA consumed during the reaction was determined by recording the decrease of absorbance at 412 nm ($\epsilon_{\text{DTNB-CoA}} = 14.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) (45).

4-Hydroxybutyryl-CoA dehydratase (EC 4.2.1.-) activity was measured anaerobically at 80°C using a discontinuous assay with recombinant crotonyl-CoA carboxylase/reductase (EC 1.3.1.-) from *Rhodobacter sphaeroides* (17). The 4-hydroxybutyryl-CoA dehydratase reaction mixture contained 100 mM MOPS-KOH (pH 7.2), 3 mM MgCl₂, 3 mM ATP, 5 mM DTT, 2 mM CoA, 10 mM 4-hydroxybutyrate, and cell extract. The amounts of crotonyl-CoA formed in the reaction after 0, 2, and 4 min of incubation were determined by transferring the sample (0.1 ml) into 0.4 ml (25°C) of 100 mM MOPS-KOH (pH 7.2), 0.5 mM NADPH,

and 40 mM NaHCO₃ and measuring the decrease in absorbance at 365 nm after the addition of 2.5 U crotonyl-CoA carboxylase/reductase.

Crotonyl-CoA hydratase (EC 4.2.1.17) was measured at 42°C by coupling the reaction to crotonyl-CoA carboxylase/reductase (7). The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 5 mM DTT, 0.3 mM NADPH, 40 mM NaHCO₃, 2.5 U crotonyl-CoA carboxylase/reductase, and cell extract. The reaction was started by the addition of (R)- or (S)-3-hydroxybutyryl-CoA (0.2 mM). Activity was found only with (S)-3-hydroxybutyryl-CoA as a substrate.

3-Hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) was measured spectrophotometrically at 65°C as (S)-3-hydroxybutyryl-CoA-dependent reduction of NAD⁺ (7) with a reaction mixture containing 100 mM Tris-HCl (pH 7.8), 5 mM DTT, 5 mM MgCl₂, 0.3 mM NAD⁺, 0.2 mM (S)-3-hydroxybutyryl-CoA, and cell extract. No activity was detected with the use of NADP⁺ or (R)-3-hydroxybutyryl-CoA as a substrate.

Acetoacetyl-CoA β -ketothiolase (EC 2.3.1.9) activity was measured at 65°C by monitoring acetyl-CoA formation from acetoacetyl-CoA (7). The reaction mixture (0.1 ml) contained 100 mM MOPS-KOH (pH 7.2), 5 mM MgCl₂, 5 mM DTT, 1 mM CoA, 1 mM acetoacetyl-CoA, and cell extract. The reaction was stopped after 0, 0.5, and 1 min by addition of 10 μ l of 1 M HCl, and the products were analyzed using RP-C₁₈ column reversed-phase high-performance liquid chromatography (HPLC), as described previously (17).

Acetate-CoA ligase (EC 6.2.1.1) activity was measured at 85°C similarly to 4-hydroxybutyrate-CoA ligase, but the reaction mixture was supplemented with acetate (10 mM) instead of 4-hydroxybutyrate.

Citrate synthase (EC 4.1.3.7) activity was detected as oxaloacetate-dependent release of CoA from acetyl-CoA, with DTNB as a CoA-detecting agent, at 65°C (30). The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 1 mM DTNB, 0.2 mM acetyl-CoA, and cell extract.

Aconitase (EC 4.2.1.3) activity was determined by following the formation of *cis*-aconitate at 240 nm ($\epsilon_{\text{cis-aconitate}} = 3.4 \text{ M}^{-1} \text{ cm}^{-1}$) (24). The assay was performed under anoxic conditions with a mixture containing 100 mM potassium phosphate buffer (pH 7.9) and 10 mM citrate. The reaction was started with the addition of cell extract.

Isocitrate dehydrogenase (EC 1.1.1.42) activity was detected as isocitrate-dependent reduction of NADP⁺ with a reaction mixture containing 100 mM Tris-HCl (pH 7.8), 5 mM DTT, 5 mM MgCl₂, 1 mM NADP⁺, 10 mM *threo*-isocitrate, and cell extract.

ATP-citrate lyase (EC 4.1.3.8) was tested in the coupled assay with endogenous malate dehydrogenase at 65°C under both oxic and anoxic conditions (30). The assay mixture contained 100 mM Tris-HCl (pH 7.8), 5 mM DTT, 5 mM MgCl₂, 3 mM ATP, 0.5 mM CoA, 0.4 mM NADH, and cell extract. The reaction was started by the addition of citrate (3 mM).

Citrate lyase (EC 4.1.3.6) activity was tested anaerobically by coupling the reaction to endogenous malate dehydrogenase (43, 44) with a reaction mixture containing 100 mM potassium phosphate (pH 7.9), 5 mM DTT, 5 mM MgCl₂, 0.5 mM NADH, and cell extract. The reaction was started by the addition of 3 mM citrate. In order to reactivate inactive deacetylated citrate lyase, the enzyme-containing samples (0.5 ml) were treated with 3% acetic anhydride in dimethyl sulfoxide before the measurement.

Malic enzyme [NAD(P)-dependent malate dehydrogenase (decarboxylating)] (EC 1.1.1.38 and EC 1.1.1.40) was tested with an assay mixture containing 100 mM Tris-HCl (pH 7.8), 5 mM DTT, 5 mM MgCl₂, 1 mM NAD⁺ or NADP⁺, and cell extract. The reaction was started with D,L-malate (30 mM).

Conversion of 4-hydroxy[1-¹⁴C]butyrate to [¹⁴C]acetyl-CoA by cell extracts of *T. neutrophilus*. 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 (160 kBq ml⁻¹), and cell extract (0.7 mg protein ml⁻¹). In a control, ATP was omitted from the mixture. The reaction was performed anaerobically at 80°C and was started by the addition of cell extract. The reaction was stopped after different time intervals by mixing the samples (50 μ l) with 10 μ l of 1 M HCl. The samples were centrifuged (4°C; 20,000 $\times$ g; 15 min) and analyzed by HPLC using an RP-C₁₈ column, as described previously (17). The identification of the CoA esters was based on cochromatography with standards.

Incorporation of 4-hydroxy[1-¹⁴C]butyrate by autotrophically growing cells of *T. neutrophilus*. When the incorporation of 4-hydroxy[1-¹⁴C]butyrate into *T. neutrophilus* cells was studied, the cells were cultivated in a 2-liter glass fermenter under a gas phase of H₂ plus CO₂ (80%/20%, vol/vol) at 500 rpm in the presence of 0.1 μ M 4-hydroxy[1-¹⁴C]butyrate (10 μ Ci/2 liters of medium). The samples (0.3 to 1.0 ml, dependent on cell density) were filtrated through a 0.2- μ m nitrocellulose filter (Schleicher & Schuell, Dassel, Germany) and washed two times with 5 ml of medium without radioactively labeled compounds. The radioactivity of the filter as well as the filtrate was determined by a liquid scintillation

counter, using Rotiszint Eco Plus scintillation fluid (Roth). Cells were counted using a Neubauer counting chamber.

Isolation and hydrolysis of ^{14}C -labeled protein. Frozen cells (1 g [wet weight]) grown autotrophically with 0.1 μM 4-hydroxy[1- ^{14}C]butyrate were resuspended in 2 ml water and passed through a chilled French pressure cell at 137 kPa. The lysate was ultracentrifuged for 30 min at $65,000 \times g$ and 4°C . The pellet was washed with 2 ml water and ultracentrifuged again. Then, 2 ml of 70% HClO_4 was added to the pooled supernatants (4 ml) to precipitate the protein. After 30 min of incubation on ice, the sample was centrifuged (15 min; $48,000 \times g$; 4°C) to remove low-molecular-mass substances. The resulting pellet was washed three times with 10 ml 0.5 M HClO_4 (15 min; $48,000 \times g$; 4°C). To remove nucleic acids, 10 ml of 0.5 M HClO_4 was added to the pellet and the solution was incubated at 70°C for 20 min and immediately on ice for 5 min. After centrifugation (15 min; $20,000 \times g$; 4°C), nucleic acid fragments were in the supernatant, and proteins were in the pellet. The treatment with perchloric acid was repeated once more, and the obtained pellet was additionally treated to remove lipids. To this effect, it was resuspended in 10 ml ethanol-diethylether (3:1, vol/vol) and incubated at 40°C for 1 h. A centrifugation step (15 min; $20,000 \times g$; 4°C) separated the protein pellet from lipids in the supernatant. Then, the pellet was washed in 10 ml diethylether and centrifuged (15 min; $20,000 \times g$; 4°C). The precipitated proteins were dried at 80°C for 10 min and hydrolyzed under N_2 gas in 1.5 ml 6 N HCl in a sealed glass ampoule at 110°C for 24 h (33, 46). The amino acid solution (hydrolysate) was mixed with 20 mg charcoal and centrifuged (10 min; $5,000 \times g$; 4°C). Then, the solution was flash evaporated three times, almost to dryness, and redissolved in 3 ml water. The pH was adjusted to pH 5.3 with 1 M NaOH. Precipitated brown material was removed by centrifugation (21, 22).

Isolation of aspartate and glutamate. Aspartate and glutamate were purified using anion exchange resin Dowex 1×8 100/200 mesh, acetate form, equilibrated with 50 mM sodium acetate pH 5.3, in accordance with references 21 and 22. A gradient of acetic acid from 0 to 2 M was applied to separate aspartate and glutamate (55). Neutral and basic amino acids eluted in the flowthrough. The elution was followed by liquid scintillation counting.

Conversion of alanine to lactate and isolation of lactate. The fractions containing the neutral and basic amino acids were pooled and flash evaporated. The amino acids were redissolved in 1 ml of 25 mM Tris-HCl (pH 7.6), and the pH was adjusted to 7.6 with 1 M NaOH. Alanine was enzymatically converted to lactate at 30°C . The reaction mixture contained 25 mM Tris-HCl (pH 7.6), 5 mM 2-oxoglutarate, 25 U lactate dehydrogenase from rabbit muscle, 5 U glutamate-pyruvate transaminase from porcine heart, 0.5 mM NADH, and alanine-containing solution. Lactate was separated from the neutral and basic amino acids by using anion exchange resin Dowex 50W $\times$ 8 100/200 mesh, H^+ form, in accordance with reference 21.

Determination of the specific radioactivities of aspartate, glutamate, and alanine (lactate). The radioactivities of the compounds were quantified by liquid scintillation counting with an external standard, as described before. Aspartate and glutamate were quantitated using the ninhydrin method (39, 59). Lactate was determined enzymatically (16). The assay mixture contained 1 M glycine, 0.4 M hydrazinium sulfate (pH 9.5), 7 mM EDTA, 3 mM NAD^+ , 50 U lactate dehydrogenase, and lactate solution. Note that this assay slightly overestimates the lactate concentration (25). Therefore, the specific radioactivity of alanine is slightly (maybe 5%) underestimated.

Extraction of lipids and determination of their specific radioactivity. Lipids were extracted from 100 mg labeled *T. neutrophilus* cells (wet mass) with chloroform by a modified Bligh and Dyer method (40, 41), with the only exception being that the water in the solvent was replaced by 5% trichloroacetic acid. Chloroform was evaporated from the sample, and the ^{14}C content was measured by liquid scintillation counting as described above.

Protein determination. Protein was measured according to the Bradford method (9), using bovine serum albumin as a standard.

Database search and phylogenetic analysis. Query sequences were obtained from the National Center for Biotechnology Information (NCBI) database. The BLAST searches were performed via the NCBI BLAST server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (3). The 16S rRNA sequences were obtained from the Ribosomal Database Project website (<http://rdp.cme.msu.edu/>) (14). The phylogenetic tree was reconstructed using a neighbor-joining algorithm (47) in the TREECONW program package (56).

Materials. Chemicals were obtained from Fluka (Neu-Ulm, Germany), Sigma-Aldrich (Deisenhofen, Germany), VWR (Darmstadt, Germany), or Roth (Karlsruhe, Germany). 4-Hydroxy[1- ^{14}C]butyrate was obtained from American Radiolabeled Chemicals, St. Louis, MO.

RESULTS

Enzyme activities of a reductive citric acid cycle in cell extracts. *T. neutrophilus* was cultivated autotrophically as well as mixotrophically in the presence of 5 mM acetate. Cells grew autotrophically with a generation time of 9 h, whereas acetate accelerated the growth rate threefold, up to a generation time of 3 h. A generation time of 9 h corresponds to a specific growth rate (μ) of 0.077 h^{-1} , with a specific carbon fixation rate of $106 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein. If two molecules of CO_2 are fixed in one turn of the autotrophic CO_2 fixation cycle, the minimal in vivo specific activity of its enzymes is $53 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein. This estimate is based on the specific substrate (S) consumption (d_s) per time unit (d_t) given by $d_s/d_t = (\mu/Y) \cdot X$, where Y represents the growth yield (1 g of dry cell mass formed per 0.5 g of carbon fixed) and X represents the cell dry mass in g (1 g of cell dry mass, corresponding to approximately 0.5 g of protein).

The activities of the enzymes of the reductive citric acid cycle in autotrophically grown cells were tested (Table 1). The results obtained were similar to those obtained in previous studies (48), including low 2-oxoglutarate synthase activity, in addition to pyruvate synthase activity. When both pyruvate and 2-oxoglutarate were added to the assay, the reaction rate was the same as for pyruvate alone. This suggests the presence of a single enzyme catalyzing both reactions and preferring pyruvate. ATP-citrate lyase or citrate lyase activity, cleaving citrate to acetyl-CoA (or acetate) and oxaloacetate, could not be detected with the spectrophotometric assays. Minimal enzyme activity (6 to $7 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein) based on the formation of labeled malate from labeled citrate had previously been reported (6).

Enzyme activities of a dicarboxylate/4-hydroxybutyrate cycle in cell extracts. However, all enzymes of the dicarboxylate/4-hydroxybutyrate cycle (Fig. 1) were detected in extracts of autotrophically grown cells (Table 1). In the first (carboxylation) part of the cycle, acetyl-CoA is transformed to oxaloacetate via pyruvate synthase, pyruvate-water dikinase, and PEP carboxylase, and oxaloacetate is further reduced to succinyl-CoA by the reactions of an incomplete reductive citric acid cycle. The regeneration of acetyl-CoA in the second (4-hydroxybutyrate) part of the cycle starts from succinyl-CoA and proceeds in the same way as in *Ignicoccus hospitalis*. There were minor differences to the situation in *I. hospitalis*. Succinyl-CoA reduction to succinic semialdehyde was NADPH dependent (Table 1), and the activity with methyl viologen (MV) was an order of magnitude lower. Although the reaction could also be measured with NADH, its activity was only 10% of that with NADPH. Similarly, for the reduction of succinic semialdehyde, NADH could replace NADPH, but only with 20% of the activity. Crotonyl-CoA hydratase and 3-hydroxybutyryl-CoA dehydrogenase were (S)-3-hydroxybutyryl-CoA specific; no activity was found with the R stereoisomer. 3-Hydroxybutyryl-CoA dehydrogenase was active only with NAD^+ , as in the cases of *Metallosphaera sedula* and *I. hospitalis*. Fumarate reductase could be measured with MV; no activity could be found with NAD(P)H as an electron donor.

Regulation of enzyme activities. The specific activities of the characteristic enzymes of the dicarboxylate/4-hydroxybutyrate cycle were strongly downregulated in cells grown under the

TABLE 1. Enzymes involved in autotrophic CO₂ assimilation in *T. neutrophilus*^a

Enzyme	Enzyme no. in Fig. 1	Assay temp (°C)	Sp act (nmol/min/mg protein) in cells grown with:				Putative gene(s) in <i>T. neutrophilus</i>
			CO ₂		Acetate plus CO ₂		
			Measured	Tentatively extrapolated to 85°C ^b	Measured	Tentatively extrapolated to 85°C ^b	
Pyruvate synthase	1	65	115 ± 15	460	110 ± 8	440	Tneu_0130/0131, Tneu_0176/0177/0178/0179, Tneu_1526/1527/1528/1529, Tneu_1795/1796/1797 ^c
Spectrophotometric assay (MV)		80	510 ± 160	720	410 ± 190	580	
¹⁴ CO ₂ exchange reaction							
Pyruvate-water dikinase	2	80	190 ± 45	270	90 ± 18	130	Tneu_1204
Pyruvate-phosphate dikinase		80	<10	NA	<10	NA	No gene found
Pyruvate kinase		42	<1	NA	<1	NA	No gene found
Phosphoenolpyruvate carboxylase	3	65	50 ± 8	200	20 ± 2	80	Tneu_0418
Spectrophotometric assay		80	580 ± 140	820	180 ± 60	260	
¹⁴ CO ₂ assay							
Phosphoenolpyruvate carboxykinase (ADP) ^d							No gene found
Spectrophotometric assay		65	30 ± 7	120	10 ± 2	40	
¹⁴ CO ₂ assay		80	197 ± 37	400	24 ± 8	90	
Malate dehydrogenase, decarboxylating (NADP ⁺)	4	65	<5	NA	<5	NA	No gene found
Malate dehydrogenase (NAD ⁺)	5	65	1,770 ± 250	9,480	1,300 ± 600	5,200	Tneu_1509
Fumarate hydratase	6	65	510 ± 90	2,040	<10	NA	Tneu_1334/1335
Fumarate reductase (MV)	7	65	130 ± 10	520	<10	NA	Tneu_0423/0424
Succinyl-CoA synthetase	8	65	510 ± 7	2,040	50 ± 16	200	Tneu_1463/1464
Succinyl-CoA reductase (NADPH)	9	65	210 ± 40	840	20 ± 1	80	Tneu_0421 ^e
Succinic semialdehyde reductase (NADPH)	10	65	7,600 ± 2,200	30,400	1,350 ± 20	5,400	Tneu_0419 ^f
4-Hydroxybutyrate-CoA ligase (AMP-forming)	11	85	440 ± 60	440	100 ± 10	100	Tneu_0420 ?
4-Hydroxybutyryl-CoA dehydratase	12	80	200 ± 55	280	10 ± 6	10	Tneu_0422
Crotonyl-CoA hydratase [(S)-3-hydroxybutyryl-CoA forming]	13	42	120 ± 20	2,360	40 ± 7	790	Tneu_0541 ^g
(S)-3-Hydroxybutyryl-CoA dehydrogenase (NAD ⁺) ^h	14	65	60 ± 14	240	20 ± 8	80	Tneu_0541 ^g
Acetoacetyl-CoA β-ketothiolase		80	8,200 ± 3,340	11,600	4,750 ± 1,180	6,720	Tneu_0249
Acetate-CoA ligase		85	730 ± 270	730	675 ± 140	675	Tneu_0385
2-Oxoglutarate:acceptor oxidoreductase (MV)		65	30 ± 12	120	20 ± 11	80	Tneu_0130/0131, Tneu_0176/0177/0178/0179, Tneu_1526/1527/1528/1529, Tneu_1795/1796/1797 ^c
(<i>st</i>)-Citrate synthase ⁱ		65	50 ± 8	200	70 ± 13	280	Tneu_0713
Aconitase		65	70 ± 55	280	40 ± 1	160	Tneu_0614
Isocitrate dehydrogenase (NADP ⁺)		65	300 ± 125	1,200	320 ± 290	1280	Tneu_0698
ATP-citrate lyase		65	<1	NA	<1	NA	No gene found
Citrate lyase		65	<2	NA	<2	NA	No gene found

^a For comparison, cells grown with CO₂ as the sole carbon source were compared with cells grown with acetate and CO₂ as carbon sources. The energy source was H₂ plus S⁰ in both cases. Because of the use of mesophilic coupling enzymes and the instability of some substrates, the indicated assay temperatures were used; the growth temperature was 85°C. Subunits of the multicomponent enzymes are listed with “/.” NA, not applicable. The values shown are means ± standard deviations of results from at least three independent measurements with different cell extracts.

^b The specific activity at 85°C was calculated based on the assumption that a 10°C rise in temperature doubles the reaction rates. At high temperatures, enzymes may become inactivated in vitro, in contrast to the protected environment of the cell.

^c The substrate specificity of the enzymes, encoded by the corresponding putative genes, cannot be determined on the basis of sequence comparison.

^d The activity with GDP was about 30% of the activity with ADP.

^e Function shown by study of the recombinant enzyme (Hugo Ramos-Vera and Georg Fuchs, unpublished results).

^f Function deduced due to the very high similarity to succinic semialdehyde reductase from *Metallophaga vedalia* (Daniel Kockelkorn and Georg Fuchs, unpublished results).

^g Fusion protein containing 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase domains.

^h No activity with (*R*)-3-hydroxybutyryl-CoA.

ⁱ Stereospecificity was determined from the data for retrobiosynthetic analysis (55) and was also based on the presence of the gene for the (*st*)- but not the (*re*)-specific enzyme.

same conditions (CO_2 , H_2 , and elemental sulfur), but only in the presence of 5 mM acetate (Table 1). For example, fumarate hydratase and fumarate reductase activities could hardly be detected in acetate-grown cells, and 4-hydroxybutyryl-CoA dehydratase and succinyl-CoA reductase had very low activity levels. This strict regulatory pattern is a strong argument in favor of the involvement of these enzymes in autotrophic CO_2 fixation.

The characteristic enzymes of the oxidative citric acid cycle, citrate synthase, aconitase, and isocitrate dehydrogenase, participating also in glutamate synthesis in heterotrophs and many autotrophs (e.g., reference 27), were also present. Their activities were approximately the same under autotrophic and mixotrophic growth conditions (Table 1), reflecting their involvement in amino acid biosynthesis rather than in autotrophy.

Conversion of 4-hydroxy[1- ^{14}C]butyrate to [1- ^{14}C]acetyl-CoA in vitro. In the dicarboxylate/4-hydroxybutyrate cycle, 4-hydroxybutyrate formed from succinyl-CoA is converted to two acetyl-CoA molecules in five reactions. This conversion was demonstrated by HPLC using 4-hydroxy[1- ^{14}C]butyrate. Extracts of autotrophically grown cells rapidly converted 4-hydroxy[1- ^{14}C]butyrate to [1- ^{14}C]acetyl-CoA, provided that MgATP, CoA, and NAD^+ were present (Fig. 2A). HPLC analysis of the reaction course showed that labeled 4-hydroxybutyryl-CoA, crotonyl-CoA, 3-hydroxybutyryl-CoA, and acetoacetyl-CoA were intermediates (Fig. 2B). A plot of the relative amounts of radioactivity in the individual products versus time showed that the intermediates appeared in the expected chronological order and that acetyl-CoA was the end product (Fig. 2C). The rate of this transformation was $110 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein. Therefore, not only could the enzymes responsible for 4-hydroxybutyrate conversion to acetyl-CoA be measured in extracts of autotrophically grown cells, but the whole reaction sequence could also be demonstrated in vitro. This implies the functioning in vivo of the corresponding enzymes of this reaction sequence in the direction of acetyl-CoA formation.

Incorporation of 4-hydroxy[1- ^{14}C]butyrate into growing cells. 4-Hydroxybutyrate is not encountered in any biosynthetic pathway apart from the proposed archaeal autotrophic CO_2 fixation cycles, and the transfer of label from 4-hydroxy[1- ^{14}C]butyrate to central biosynthetic precursors speaks in favor of the operation of the dicarboxylate/4-hydroxybutyrate cycle. To test this possibility, *T. neutrophilus* was grown autotrophically during three generations in the presence of $0.1 \mu\text{M}$ 4-hydroxy[1- ^{14}C]butyrate. The cell density obtained was 8×10^8 cells/ml, corresponding to 0.8 g cells (wet mass) per liter. After 3 h of growth, 63% of the label from 4-hydroxybutyrate was incorporated into the cell mass (Fig. 3). Part (24%) of the label remained in the medium even after 23 h, possibly in the form of γ -butyrolactone, which may not be assimilated. The total radioactivity in the culture did not decrease, indicating that 4-hydroxy[1- ^{14}C]butyrate was not oxidized to volatile $^{14}\text{CO}_2$.

In vivo incorporation of 4-hydroxy[1- ^{14}C]butyrate into central biosynthetic metabolites. The labeled cells were fractionated, and protein was separated and purified. After acid hydrolysis, aspartate, glutamate, and alanine were isolated from the obtained amino acid hydrolysate and their specific radioactivities were determined (Table 2). Approximately 1 to 2 μmol of each of the amino acids was obtained from 10 mg

FIG. 2. Conversion of 4-hydroxy[1- ^{14}C]butyrate to [1- ^{14}C]acetyl-CoA at 80°C by cell extracts of *T. neutrophilus*. (A) HPLC separation of labeled substrate and products and ^{14}C detection by flow separation scintillation counting. The figure shows samples taken after 2 and 5 min of incubation as well as those from a control experiment lacking ATP after 20 min of incubation. The radioactive peak at 3.5 min most likely represents γ -butyrolactone, which forms spontaneously from 4-hydroxybutyrate at an acidic pH or from 4-hydroxybutyryl-CoA at a neutral pH. (B) Time course of substrate consumption and product formation. One hundred percent corresponds to the total radioactivity added at the beginning. (C) Percentage of radioactivity present in the individual products, compared with the total radioactivity in all labeled products, versus time. One hundred percent corresponds to the total radioactivity contained in all products at a given time. The strong negative slope for 4-hydroxybutyryl-CoA indicates that it is the first intermediate. The strong positive slope for acetyl-CoA indicates that it is the end product. Crotonyl-CoA, 3-hydroxybutyryl-CoA, and acetoacetyl-CoA behave like intermediates between 4-hydroxybutyryl-CoA and acetyl-CoA.

FIG. 3. Growth of *T. neutrophilus* under autotrophic conditions in the presence of 4-hydroxy[1- ^{14}C]butyrate. The total 4-hydroxybutyrate concentration was 0.1 μM . ●, growth; ■, ^{14}C in cell-free culture supernatant; ▲, ^{14}C in washed cells. Note that the culture was continuously gassed and that volatile $^{14}\text{CO}_2$ would have been lost.

protein. The ratio of the specific radioactivities of aspartate/glutamate/alanine was approximately 1:2:1, indicating that oxaloacetate and pyruvate contained one labeled carbon atom and 2-oxoglutarate contained two labeled carbon atoms. The two-times-higher specific radioactivity in glutamate would be expected if 2-oxoglutarate were synthesized from singly labeled acetyl-CoA and singly labeled oxaloacetate. These results can easily be explained by the scheme shown in Fig. 4, where glutamate synthesis proceeds through citrate synthase reaction. Note that the functioning of the reductive citric acid cycle would result in a different glutamate labeling, since it would be produced through 2-oxoglutarate synthase reaction and thus would inherit the labeling pattern of oxaloacetate (aspartate) alone. The conversion of singly labeled acetyl-CoA to pyruvate and oxaloacetate via the proposed pathway would result in singly labeled alanine and aspartate, as observed. Interestingly, early ^{14}C enrichment studies of [1,4- ^{14}C]succinate incorporation by *T. neutrophilus* cells resulted in a similar labeling pattern (48, 49), in accordance with 4-hydroxybutyrate synthesis from succinate in the cycle (Fig. 4). Taken together, these results are in good agreement with the proposed functioning of the dicarboxylate/4-hydroxybutyrate cycle in *T. neutrophilus*.

According to this proposal, acetyl-CoA is formed from 4-hydroxybutyrate in the course of the dicarboxylate/4-hydroxybutyrate cycle (Fig. 4), leading to singly labeled acetyl-CoA. To estimate the label content in acetyl-CoA, archaeal lipids were extracted from 100 mg labeled cells and the total radioactivity of the lipid fraction was determined (Table 2). With the assumption that 100 mg of the wet mass corresponds to 20 mg of dry biomass with 10% of lipids, and taking into account that one molecule of diether archaeol (molecular mass, 740 Da) is synthesized from 17 molecules of [^{14}C]acetyl-CoA, the specific radioactivity of [^{14}C]acetyl-CoA was calculated (Table 2). The estimated value was very close to the values obtained for aspartate and alanine and fits perfectly to the scheme shown in Fig. 4.

DISCUSSION

Dicarboxylate/4-hydroxybutyrate cycle versus reductive citric acid cycle in *T. neutrophilus*. The results of the previous studies of autotrophic CO_2 fixation in *T. neutrophilus* were interpreted as indicating the operation of the reductive citric acid cycle. However, the measured activity levels of its key enzymes were low (6, 48). The results of the retrobiosynthetic analysis of [1,4- $^{13}\text{C}_1$]succinate labeling studies could be reconciled with a reductive carboxylation of succinyl-CoA to 2-oxoglutarate only when further assumptions were made (55).

In 2-oxoglutarate produced from carboxyl-labeled succinate, four carbons (C-2 to C-5) were equally labeled, and a small fraction of doubly labeled [3,4- $^{13}\text{C}_2$]2-oxoglutarate was formed (49, 55). This contradiction was explained by some additional, though not unreasonable, assumptions: (i) the reversibility of the interconversion of 2-oxoglutarate and citrate, (ii) the reversibility of citrate cleavage by ATP-citrate lyase, and (iii) the reversibility of the interconversion of oxaloacetate with the symmetric fumarate molecule (55). This would finally lead to some scrambling of label in 2-oxoglutarate and even double labeling. However, the dicarboxylate/4-hydroxybutyrate cycle perfectly explains the obtained labeling pattern without further assumptions. [1,4- $^{13}\text{C}_1$]succinate is reduced to 4-hydroxy[1,4- $^{13}\text{C}_1$]butyrate, and the latter is converted to [1,2- $^{13}\text{C}_1$]acetyl-CoA. Carboxylation of [1,2- $^{13}\text{C}_1$]acetyl-CoA leads first to [2,3- $^{13}\text{C}_1$]pyruvate (precursor of alanine) and then to [2,3- $^{13}\text{C}_1$]oxaloacetate (precursor of aspartate). The synthesis of 2-oxoglutarate proceeds through the incomplete oxidative citric acid cycle, where (*si*)-citrate synthase condenses [1,2- $^{13}\text{C}_1$]acetyl-CoA with [2,3- $^{13}\text{C}_1$]oxaloacetate. This results in the labeling pattern observed in the previous experiments (49, 55) (Fig. 4). Thus, the retrobiosynthetic analysis supports the functioning of the dicarboxylate/4-hydroxybutyrate cycle in *T. neutrophilus*.

The relative incorporation of the label from 4-hydroxy[1- ^{14}C]butyrate in alanine, aspartate, glutamate, and lipids also supports the dicarboxylate/4-hydroxybutyrate cycle and contradicts the reductive citric acid cycle (Fig. 4). Taking also into account (i) the demonstration of all required enzyme activities and (ii) their pronounced regulation as well as (iii) the identification of the corresponding genes in the genome, the operation of the dicarboxylate/4-hydroxybutyrate cycle in this archaeon can now be considered established.

TABLE 2. Specific radioactivities of some amino acids and acetyl units isolated from *T. neutrophilus* cells grown autotrophically for three generations in the presence of 0.1 μM 4-hydroxy[1- ^{14}C]butyrate

Compound	Specific radioactivity	
	Bq/ μmol	%
Precursor (4-hydroxybutyrate)	2×10^6	100
Isolated		
Glutamate	57.3	2.8×10^{-3}
Aspartate	28.3	1.4×10^{-3}
Alanine	24.7	1.2×10^{-3}
Acetyl-CoA (from lipids)	31.7	1.6×10^{-3}

FIG. 4. Scheme illustrating the incorporation of 4-hydroxy[1- ^{14}C]butyrate into central biosynthetic metabolites (acetyl-CoA, pyruvate/PEP, oxaloacetate, and 2-oxoglutarate) by autotrophically growing *T. neutrophilus* cells. The labeling pattern of biosynthetic metabolites is consistent with the operation of the dicarboxylate/4-hydroxybutyrate cycle in CO_2 fixation. The specific radioactivities of aspartate, glutamate, and alanine are indicated in boxes. For comparison, early ^{13}C enrichment data from [1,4- $^{13}\text{C}_1$]succinate incorporation in *T. neutrophilus* cells are shown (55). The numbers near the carbon atoms represent the ^{13}C enrichment of individual carbons of the intermediates (data from reference 55). Solid lines represent ^{14}C or ^{13}C incorporation during the first turn of the cycle, whereas dotted lines indicate the intermediates entering the second turn of the cycle. All further cycles can be extrapolated. ^{14}C carbon atoms derived from 4-hydroxybutyrate are marked by ●, and ^{13}C enrichment carbon atoms derived from succinate are marked by ▲.

Bioenergetic considerations. The formation of acetyl-CoA via the dicarboxylate/4-hydroxybutyrate cycle in *T. neutrophilus* follows the equation $1 \text{ CO}_2 + 1 \text{ HCO}_3^- + 2 \text{ reduced ferredoxin} + 2 \text{ NAD(P)H} + 3 \text{ ATP} + 1 \text{ CoA} \rightarrow 1 \text{ acetyl-CoA} + 2 \text{ oxidized ferredoxin} + 2 \text{ NAD(P)}^+ + 2 \text{ AMP} + 1 \text{ ADP} + 3 \text{ P}_i + 1 \text{ PP}_i$. This assumes that pyruvate synthase and fumarate reductase use reduced ferredoxin (each transferring two electrons). Further assimilation of acetyl-CoA to form triose phosphates follows: $\text{acetyl-CoA} + \text{CO}_2 + 1 \text{ reduced ferredoxin} + \text{NAD(P)H} + 2 \text{ ATP} \rightarrow 1 \text{ triose phosphate} + 1 \text{ oxidized ferredoxin} + \text{NAD(P)}^+ + \text{ADP} + \text{AMP} + 2 \text{ P}_i + 1 \text{ CoA}$. In total, triose phosphate formation via the *T. neutrophilus* variant of the dicarboxylate/4-hydroxybutyrate cycle follows the equation $2 \text{ CO}_2 + 1 \text{ HCO}_3^- + 3 \text{ reduced ferredoxin} + 3 \text{ NAD(P)H} + 5 \text{ ATP} \rightarrow 1 \text{ triose phosphate} + 3 \text{ oxidized ferredoxin} + 3 \text{ NAD(P)}^+ + 2 \text{ ADP} + 3 \text{ AMP} + 5 \text{ P}_i + 1 \text{ PP}_i$. The source of reduced ferredoxin is probably hydrogen gas oxidized by hydrogenase. Assuming that PP_i is

hydrolyzed, the fixation of three molecules of inorganic carbon into triose phosphates costs eight ATP equivalents.

Strict regulation of the cycle. *T. neutrophilus* grows autotrophically or mixotrophically using a limited number of organic compounds, which may be delivered to the dicarboxylate/4-hydroxybutyrate cycle (e.g., acetate or succinate) (19, 48). The experiments with labeled acetate, succinate, or 4-hydroxybutyrate showed that these substrates enter the dicarboxylate/4-hydroxybutyrate cycle (Fig. 4). Acetate, as shown here, completely turns off the carbon fixation cycle by downregulation of the activities of characteristic enzymes of the cycle, notably fumarase and fumarate reductase. The rest of the enzymes are required for the assimilation of acetate. To activate acetate, a constitutively formed acetate-CoA ligase is present. This behavior makes *T. neutrophilus* an interesting subject for the study of regulation of autotrophic carbon fixation.

Gene cluster in the genomes of *Thermoproteales* involved in CO_2 fixation. In the genome of *T. neutrophilus* (the genome

has been sequenced by the DOE Joint Genome Institute but not published yet), five genes encoding four enzymes of the dicarboxylate/4-hydroxybutyrate cycle, namely, PEP carboxylase, succinic semialdehyde reductase (Daniel Kockelkorn and Georg Fuchs, unpublished results), 4-hydroxybutyryl-CoA dehydratase, and fumarate reductase, are located in the same gene cluster (Tables 1 and 3). Interestingly, this cluster includes also the gene encoding a putative pyridine nucleotide-disulfide oxidoreductase. This gene is localized downstream of the gene encoding the two fumarate reductase subunits.

Moreover, this cluster contains genes encoding a putative organic acid-CoA ligase (Tneu_0420) and a putative aldehyde dehydrogenase (Tneu_0421). Since the activities of PEP carboxylase, succinic semialdehyde reductase, 4-hydroxybutyryl-CoA dehydratase, and fumarate reductase are regulated similarly to those of 4-hydroxybutyrate-CoA ligase and succinyl-CoA reductase (Table 1), these genes may code for 4-hydroxybutyrate-CoA ligase and succinyl-CoA reductase, respectively. The gene product of the putative CoA ligase is very similar (45% amino acid identity) to 3-hydroxypropionate-CoA ligase in *M. sedula* and *S. tokodaii* (2), which can use 4-hydroxybutyrate as alternative substrate with 10% activity (Ivan Berg and Georg Fuchs, unpublished observation). Two genes for other AMP-forming CoA ligases can be found in the *T. neutrophilus* genome (Tneu_1843 and Tneu_0385), one of which may represent the acetate-CoA ligase gene (Tneu_0385). The gene cluster therefore may contain seven genes encoding characteristic enzymes of the dicarboxylate/4-hydroxybutyrate cycle. These suggestions need to be tested experimentally in a future study.

Minor modifications of the 4-hydroxybutyrate part of the autotrophic cycles. Succinyl-CoA reductase from *T. neutrophilus* is active with both NADPH and NADH. In contrast, *M. sedula* succinyl-CoA reductase (which is identical to malonyl-CoA reductase [D. Kockelkorn and G. Fuchs, unpublished]) is strictly NADPH dependent (1, 7). This protein is a paralogue of aspartate semialdehyde dehydrogenase, which probably evolved by duplication of the aspartate semialdehyde dehydrogenase gene in the ancestor of *Sulfolobales* and mutated to its new function (1, 7). Since *T. neutrophilus* has only one copy of the aspartate semialdehyde dehydrogenase gene (Tneu_1841) clustering with genes involved in threonine biosynthesis, succinyl-CoA reductase in *T. neutrophilus* is not homologous to the enzyme of *Sulfolobales* or to the enzyme in *I. hospitalis* that uses reduced MV in vitro (and possibly ferredoxin in vivo).

Putative regulator. The cluster also comprises three genes coding for small proteins with cystathionine β -synthase (CBS) domains (5) (Table 3). CBS domains are ubiquitous in proteins from all three domains of life. The three CBS domain-containing proteins are closely related to PAE2072 of *P. aerophilum*, a protein of unknown function with two tandem CBS domains capable of building a complex with AMP (35). Interestingly, all the amino acids identified as responsible for AMP binding (35) are conserved in the proteins from *T. neutrophilus* (data not shown). Adenine nucleotides and coenzymes containing adenine (di)nucleotides are known to be good candidates for metabolic signals [AMP, ADP, ATP, CoA, CoA thioesters, NAD(P), and NAD(P)H] (58). Tandem pairs of CBS domains usually act as sensors of the cellular energy status (51) or probably perform regulatory and signaling functions (5).

TABLE 3. Homologues for the genes involved in autotrophic CO₂ assimilation by *T. neutrophilus* in the autotrophic *Thermoproteales* whose genomes are available

<i>Thermoproteales</i>	Protein (evidence value) corresponding to indicated <i>Thermoproteus neutrophilus</i> protein ^a								Reference
	Tneu_0418 (PEP carboxylase)	Tneu_0419 (succinic semialdehyde reductase)	Tneu_0420 (putative 4-hydroxybutyrate-CoA ligase)	Tneu_0421 (succinyl-CoA reductase)	Tneu_0422 (4-hydroxybutyryl-CoA dehydratase)	Tneu_0423 (putative fumarate reductase, SdhA/FrdA)	Tneu_0424 (putative fumarate reductase, SdhB/FrdB)	Tneu_0425 (putative pyridine nucleotide-disulfide oxidoreductase)	
<i>Thermoproteus tenax</i>	NA	Al621280 (6e-151)	NA	Al621277 (0.0)	NA	Al621278 (0.0)	Al621279 (0.0)	NA	52
<i>Pyrobaculum islandicum</i>	Pis1_0252 (0.0)	Pis1_0251 (0.0)	Pis1_0250 (0.0)	Pis1_0249 (0.0)	Pis1_0248 (0.0)	Pis1_0247 (0.0)	Pis1_0246 (6e-42), Pis1_0670 (3e-27)	Pis1_0245 (0.0), Pis1_0611 (4e-97)	
<i>Pyrobaculum caldifontis</i>	Pcal_1392 (e-176)	Pcal_1391 (e-141)	Pcal_1386 (0.0)	Pcal_1387 (0.0)	Pcal_1396 (0.0)	Pcal_1388 (0.0), Pcal_2127 (0.0)	Pcal_1389 (2e-30), Pcal_2128 (2e-24)	Pcal_1390 (2e-109), Pcal_1700 (4e-96)	20
<i>Pyrobaculum aerophilum</i>	PAE3416 (2e-136)	— (≥3e-53)	PAE2474 (0.0)	— (≥4e-86)	PAE2693 (0.0)	PAE0716 (0.0)	PAE0717 (4e-23), PAE2690 (2e-21)	PAE2371 (3e-94)	
<i>Pyrobaculum arsenaticum</i>	Pars_1014 (3e-175)	— (≥e-49)	— (≥8e-163)	— (≥3e-56)	— (≥2.3)	Pars_2358 (0.0)	Pars_2359 (3e-23)	Pars_1572 (4e-98)	

^a Queries for the BLASTP search. —, annotation uncertain. NA, not applicable.

FIG. 5. Postulated distribution of two autotrophic carbon fixation cycles in *Crenarchaea*. *Thermoproteales* and *Desulfurococcales* use the dicarboxylate/4-hydroxybutyrate cycle for autotrophic CO₂ fixation, whereas *Sulfolobales* and, probably, *Cenarchaeales* possess the 3-hydroxypropionate/4-hydroxybutyrate cycle. A phylogenetic tree of *Crenarchaea* based on 16S rRNA gene sequences is shown. The scale bar represents a difference of 0.1 substitutions per site. Numbers at nodes indicate the percentage bootstrap values for the clade of this group in 1,000 replications. The GenBank accession numbers of the sequences are indicated. Species able to grow autotrophically are shown in bold. Underlined species (or species closely related to them) have been studied in some detail (1, 2, 6, 7, 12, 13, 27, 30–33, 38, 48–50, 55); for studies of *Pyrobaculum islandicum*, see reference 26.

Therefore, we suspect a possible role for the proteins Tneu_0415, Tneu_0416, and Tneu_0417 in the regulation of the dicarboxylate/4-hydroxybutyrate cycle.

Autotrophic CO₂ fixation in other *Thermoproteales*. Among sequenced *Thermoproteales*, the gene for the key enzyme of the dicarboxylate/4-hydroxybutyrate cycle, the 4-hydroxybutyryl-CoA dehydratase gene, can also be found in the genomes of *P. islandicum*, *P. calidifontis*, and *P. aerophilum*. A gene cluster similar to the one found in *T. neutrophilus* is present in *P. islandicum* and *P. calidifontis* (the genomes have been sequenced by the DOE Joint Genome Institute but not published yet) but not in *P. aerophilum* (20) or *Pyrobaculum arsenaticum* (the genome has been sequenced by the DOE Joint Genome Institute but not published yet) (Table 3). In *P. calidifontis*, the arrangement of the genes is slightly different. Although the genome of *Thermoproteus tenax* has not been made public yet, the sequences of some genes involved in carbon metabolism

are available (52). Among them, there are succinic semialdehyde reductase, succinyl-CoA reductase, and fumarate reductase genes (Table 3). Thus, *T. tenax* may also have a similar gene cluster and be able to assimilate CO₂ via the dicarboxylate/4-hydroxybutyrate cycle. This proposal is in accordance with the finding that three genes of the cycle encoding fumarate reductase and succinic semialdehyde reductase (AJ621278/AJ621279 and AJ621280, respectively) were strongly upregulated in autotrophically grown cells in comparison with their levels in glucose-grown cultures (60).

T. neutrophilus is closely related to the members of the genus *Pyrobaculum* (4, 15) and may be reclassified as a true *Pyrobaculum* sp. (15). However, *T. tenax*, the type species of the genus *Thermoproteus*, does not belong to the *Pyrobaculum* cluster (4). Of the six known *Pyrobaculum* species, *P. islandicum*, *P. aerophilum*, and *P. arsenaticum* are capable of chemolithoautotrophic growth (28, 29, 57). *P. calidifontis* is considered to be

a heterotroph (4). Its capability of autotrophic growth needs to be reinvestigated in view of the presence of a gene cluster similar to the one involved in CO₂ fixation in *T. neutrophilus*.

Interestingly, although *P. aerophilum* possesses genes encoding 4-hydroxybutyryl-CoA dehydratase and putative 4-hydroxybutyrate-CoA ligase, other specific enzymes of the pathway, i.e., succinic semialdehyde reductase and succinyl-CoA reductase, seem to be absent (Table 3). It is unclear whether this species uses a modified dicarboxylate/4-hydroxybutyrate cycle, where the corresponding reactions are catalyzed by nonhomologous enzymes, or another CO₂ fixation pathway. The only *Pyrobaculum* species that lacks all the genes for the enzymes catalyzing the specific reaction of the 4-hydroxybutyrate part of the cycle is *P. arsenaticum* (Table 3). This species might have lost its autotrophic growth mode during the constant laboratory cultivation on rich media.

Hu and Holden (26) reported the presence of both ATP-dependent and ATP-independent citrate lyase activities, the key reaction of the reductive citric acid cycle, in *P. islandicum*. Also, they did not find pyruvate synthase activity in autotrophically grown cells (26). However, the genes for citrate-cleaving enzymes cannot be identified in the genome. Taking into consideration the presence of all genes required for the dicarboxylate/4-hydroxybutyrate cycle in *P. islandicum*, the functioning of this cycle seems to be more plausible.

Evolutionary considerations. The 3-hydroxypropionate/4-hydroxybutyrate cycle has also been proposed for the mesophilic ammonia oxidizing symbiotic *Cenarchaeum symbiosum* (7), a member of "marine group 1" *Crenarchaea*, which are abundant in the sea (34); the phylogenetic position of this archaeal group is currently under discussion (10). This may indicate the possible ancestry of the 4-hydroxybutyrate part of the cycle in *Crenarchaea*. As a conclusion, all autotrophic *Crenarchaea* studied so far share the 4-hydroxybutyrate part in their autotrophic CO₂ fixation pathways. They use one of the two pathways, either the 3-hydroxypropionate/4-hydroxybutyrate (*Sulfolobales* and *Cenarchaeales*) or the dicarboxylate/4-hydroxybutyrate (*Desulfurococcales* and *Thermoproteales*) cycle, depending on an aerobic or anaerobic mode of growth of the corresponding species (Fig. 5). Analysis of the sequenced archaeal genomes implies that the ancestor of *Archaea* was a chemolithoautotrophic hyperthermophile (36), and the *Thermoproteales* may branch off near the root of *Crenarchaea* (23). Therefore, the studies of autotrophic CO₂ fixation in *Archaea* are important for our understanding of the evolution of early life.

ACKNOWLEDGMENTS

Thanks are due to Nasser Gad'on and Christa Ebenau-Jehle, Freiburg, Germany, for help with growing cells and maintaining the laboratory running. We thank K.O. Stetter and H. Huber, Regensburg, Germany, for the kind gift of archaeal strains. The DOE Joint Genome Institute is acknowledged for the early release of archaeal genomic sequence data.

This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

- Alber, B., M. Olinger, A. Rieder, D. Kockelkorn, B. Jobst, M. Hügler, and G. Fuchs. 2006. Malonyl-coenzyme A reductase in the modified 3-hydroxypropionate cycle for autotrophic carbon fixation in archaeal *Metallosphaera* and *Sulfolobus* spp. *J. Bacteriol.* **188**:8551–8559.
- Alber, B. E., J. W. Kung, and G. Fuchs. 2008. 3-Hydroxypropionyl-coenzyme A synthetase from *Metallosphaera sedula*, an enzyme involved in autotrophic CO₂ fixation. *J. Bacteriol.* **190**:1383–1389.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Amo, T., M. L. Paje, A. Inagaki, S. Ezaki, H. Atomi, and T. Imanaka. 2002. *Pyrobaculum caldifontis* sp. nov., a novel hyperthermophilic archaeon that grows in atmospheric air. *Archaea* **1**:113–121.
- Bateman, A. 1997. The structure of a domain common to archaeobacteria and the homocystinuria disease protein. *Trends Biochem. Sci.* **22**:12–13.
- Beh, M., G. Strauss, R. Huber, K. O. Stetter, and G. Fuchs. 1993. Enzymes of the reductive citric acid cycle in the autotrophic eubacterium *Aquifex pyrophilus* and in the archaebacterium *Thermoproteus neutrophilus*. *Arch. Microbiol.* **160**:306–311.
- Berg, I. A., D. Kockelkorn, W. Buckel, and G. Fuchs. 2007. A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in *Archaea*. *Science* **318**:1782–1786.
- Bergmeyer, H. U. 1975. Neue Werte für die molaren Extinktions-Koeffizienten von NADH und NADPH zum Gebrauch im Routine-Laboratorium. *Z. Klin. Chem. Klin. Biochem.* **13**:507–508.
- Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Brochier-Armanet, C., B. Boussau, S. Gribaldo, and P. Forterre. 2008. Mesophilic Crenarchaea: proposal for a third archaeal phylum, the Thaumarchaeota. *Nat. Microbiol. Rev.* **6**:245–252.
- Buckel, W., and G. T. Golding. 2006. Radical enzymes in anaerobes. *Annu. Rev. Microbiol.* **60**:27–49.
- Burton, N. P., T. D. Williams, and P. R. Norris. 1999. Carboxylase genes in *Sulfolobus metallicus*. *Arch. Microbiol.* **172**:349–353.
- Chuaakrut, S., H. Arai, M. Ishii, and Y. Igarashi. 2003. Characterization of a bifunctional archaeal acyl coenzyme A carboxylase. *J. Bacteriol.* **185**:938–947.
- Cole, J. R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R. J. Farris, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, T. Marsh, G. M. Garrity, and J. M. Tiedje. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* **37**(Database Issue): D141–D145.
- Cozen, A. E., M. T. Weirauch, K. S. Pollard, D. L. Bernick, J. M. Stuart, and T. M. Lowe. 2009. Transcriptional map of respiratory versatility in the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*. *J. Bacteriol.* **191**:782–794.
- Engel, P. C., and J. B. Jones. 1978. Causes and elimination of erratic blanks in enzymatic metabolite assays involving the use of NAD⁺ in alkaline hydrazine buffers: improved conditions for the assay of L-glutamate, L-lactate, and other metabolites. *Anal. Biochem.* **88**:475–484.
- Erb, T. J., I. A. Berg, V. Brecht, M. Müller, G. Fuchs, and B. E. Alber. 2007. Synthesis of C₅-dicarboxylic acids from C₂-units involving crotonyl-CoA carboxylase/reductase: the ethylmalonyl-CoA pathway. *Proc. Natl. Acad. Sci. USA* **104**:10631–10636.
- Eyzaguirre, J., K. Jansen, and G. Fuchs. 1982. Phosphoenolpyruvate synthetase in *Methanobacterium thermoautotrophicum*. *Arch. Microbiol.* **132**:67–74.
- Fischer, F., W. Zillig, K. O. Stetter, and G. Schreiber. 1983. Chemolithoautotrophic metabolism of anaerobic extremely thermophilic archaebacteria. *Nature* **301**:511–513.
- Fitz-Gibbon, S. T., H. Ladner, U. J. Kim, K. O. Stetter, M. I. Simon, and J. H. Miller. 2002. Genome sequence of the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*. *Proc. Natl. Acad. Sci. USA* **99**:984–989.
- Fuchs, G., E. Stupperich, and R. K. Thauer. 1978. Acetate assimilation and the synthesis of alanine, aspartate and glutamate in *Methanobacterium thermoautotrophicum*. *Arch. Microbiol.* **117**:61–66.
- Fuchs, G., and E. Stupperich. 1978. Evidence for an incomplete reductive carboxylic acid cycle in *Methanobacterium thermoautotrophicum*. *Arch. Microbiol.* **118**:121–125.
- Gribaldo, S., and C. Brochier-Armanet. 2006. The origin and evolution of Archaea: a state of the art. *Philos. Trans. R. Soc. Lond. B* **361**(1470):1007–1022.
- Henson, C. P., and W. W. Cleland. 1967. Purification and kinetic studies of beef liver cytoplasmic aconitase. *J. Biol. Chem.* **242**:3833–3838.
- Hohorst, H.-J. 1962. L-(+)-Lactat Bestimmung mit Lactat-Dehydrogenase und DPN, p. 266–270. In H. U. Bergmeyer (ed.), *Methoden der enzymatischen Analyse*. Verlag Chemie, Weinheim, Germany.
- Hu, Y., and J. F. Holden. 2006. Citric acid cycle in the hyperthermophilic archaeon *Pyrobaculum islandicum* grown autotrophically, heterotrophically, and mixotrophically with acetate. *J. Bacteriol.* **188**:4350–4355.
- Huber, H., M. Gallenberger, U. Jahn, E. Eylert, I. A. Berg, D. Kockelkorn, W. Eisenreich, and G. Fuchs. 2008. A dicarboxylate/4-hydroxybutyrate autotrophic carbon assimilation cycle in the hyperthermophilic archaeum *Ignicoccus hospitalis*. *Proc. Natl. Acad. Sci. USA* **105**:7851–7856.
- Huber, R., J. K. Kristjansson, and K. O. Stetter. 1987. *Pyrobaculum* gen. nov., a new genus of neutrophilic rod-shaped archaebacteria from continental solfatara growing optimally at 100°C. *Arch. Microbiol.* **149**:95–101.

29. Huber, R., M. Sacher, A. Vollmann, H. Huber, and D. Rose. 2000. Respiration of arsenate and selenate by hyperthermophilic archaea. *Syst. Appl. Microbiol.* **23**:305–314.
30. Hügler, M., H. Huber, K. O. Stetter, and G. Fuchs. 2003. Autotrophic CO₂ fixation pathways in archaea (Crenarchaea). *Arch. Microbiol.* **179**:160–173.
31. Hügler, M., R. S. Krieger, M. Jahn, and G. Fuchs. 2003. Characterization of acetyl-CoA/propionyl-CoA carboxylase in *Metallosphaera sedula*. Carboxylating enzyme in the 3-hydroxypropionate cycle for autotrophic carbon fixation. *Eur. J. Biochem.* **270**:736–744.
32. Ishii, M., T. Miyake, T. Satoh, H. Sugiyama, Y. Oshima, T. Kodama, and Y. Igarashi. 1996. Autotrophic carbon dioxide fixation in *Acidianus brierleyi*. *Arch. Microbiol.* **166**:368–371.
33. Jahn, U., H. Huber, W. Eisenreich, M. Hügler, and G. Fuchs. 2007. Insights into the autotrophic CO₂ fixation pathway of the archaeon *Ignicoccus hospitalis*: comprehensive analysis of the central carbon metabolism. *J. Bacteriol.* **189**:4108–4119.
34. Karner, M. B., E. F. DeLong, and D. M. Karl. 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* **409**:507–510.
35. King, N. P., T. M. Lee, M. R. Sawaya, D. Cascio, and T. O. Yeates. 2008. Structures and functional implications of an AMP-binding cystathionine beta-synthase domain protein from a hyperthermophilic archaeon. *J. Mol. Biol.* **380**:181–192.
36. Makarova, K. S., A. V. Sorokin, P. S. Novichkov, Y. I. Wolf, and E. V. Koonin. 2007. Clusters of orthologous genes for 41 archaeal genomes and implications for evolutionary genomics of archaea. *Biol. Direct* **2**:33.
37. Martins, B. M., H. Dobbek, I. Cinkaya, W. Buckel, and A. Messerschmidt. 2004. Crystal structure of 4-hydroxybutyryl-CoA dehydratase: radical catalysis involving a [4Fe-4S] cluster and flavin. *Proc. Natl. Acad. Sci. USA* **101**:15645–15649.
38. Menendez, C., Z. Bauer, H. Huber, N. Gad'on, K.-O. Stetter, and G. Fuchs. 1999. Presence of acetyl coenzyme A (CoA) carboxylase and propionyl-CoA carboxylase in autotrophic *Crenarchaeota* and indication for operation of a 3-hydroxypropionate cycle in autotrophic carbon fixation. *J. Bacteriol.* **181**:1088–1098.
39. Moore, S., and W. H. Stein. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. Biol. Chem.* **211**:907–913.
40. Nishihara, M., and Y. Koga. 1987. Extraction and composition of polar lipids from the Archaeobacterium, *Methanobacterium thermoautotrophicum*: effective extraction of tetraether lipids by an acidified solvent. *J. Biochem.* **101**:997–1005.
41. Nishihara, M., H. Morii, and Y. Koga. 1987. Structure determination of a quartet of novel tetraether lipids from *Methanobacterium thermoautotrophicum*. *J. Biochem.* **101**:1007–1015.
42. O'Hare, M. C., and S. Doonan. 1985. Purification and structural comparison of the cytosolic and mitochondrial isoenzymes of fumarase from pig liver. *Biochim. Biophys. Acta* **827**:127–134.
43. Quentmeier, A., and G. Antranikian. 1985. Characterization of citrate lyase from *Clostridium sporosphaeroides*. *Arch. Microbiol.* **141**:85–90.
44. Quentmeier, A., A. Holzenburg, F. Mayer, and G. Antranikian. 1987. Reevaluation of citrate lyase from *Escherichia coli*. *Biochim. Biophys. Acta* **913**:60–65.
45. Riddles, P. W., R. L. Blakeley, and B. Zerner. 1983. Reassessment of Ellman's reagent. *Methods Enzymol.* **91**:49–60.
46. Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britton. 1957. Studies of biosynthesis in *Escherichia coli*. Carnegie Institute, Washington, DC.
47. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
48. Schäfer, S., C. Barkowski, and G. Fuchs. 1986. Carbon assimilation by the autotrophic thermophilic archaeobacterium *Thermoproteus neutrophilus*. *Arch. Microbiol.* **146**:301–308.
49. Schäfer, S., M. Götz, W. Eisenreich, A. Bacher, and G. Fuchs. 1989. ¹³C-NMR study of autotrophic CO₂ fixation in *Thermoproteus neutrophilus*. *Eur. J. Biochem.* **184**:151–156.
50. Schäfer, S., T. Paalme, R. Vilu, and G. Fuchs. 1989. ¹³C-NMR study of acetate assimilation in *Thermoproteus neutrophilus*. *Eur. J. Biochem.* **186**:695–700.
51. Scott, J. W., S. A. Hawley, K. A. Green, M. Anis, G. Stewart, G. A. Scullion, D. G. Norman, and D. G. Hardie. 2004. CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *J. Clin. Invest.* **113**:274–284.
52. Siebers, B., B. Tjaden, K. Michalke, C. Dörr, H. Ahmed, M. Zaparty, P. Gordon, C. Sensen, A. Zibat, H. P. Klenk, S. C. Schuster, and R. Hensel. 2004. Reconstruction of the central carbohydrate metabolism of *Thermoproteus tenax* by use of genomic and biochemical data. *J. Bacteriol.* **186**:2179–2194.
53. Simon, E. J., and D. Shemin. 1953. The preparation of S-succinyl coenzyme-A. *J. Am. Chem. Soc.* **75**:2520.
54. Stadtman, E. R. 1957. Preparation and assay of acyl coenzyme A and other thiol esters; use of hydroxylamine. *Methods Enzymol.* **3**:931–941.
55. Strauss, G., W. Eisenreich, A. Bacher, and G. Fuchs. 1992. ¹³C-NMR study of autotrophic CO₂ fixation pathways in the sulphur-reducing archaeobacterium *Thermoproteus neutrophilus* and in the phototrophic eubacterium *Chloroflexus aurantiacus*. *Eur. J. Biochem.* **205**:853–866.
56. Van de Peer, Y., and R. De Wachter. 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput. Appl. Biosci.* **10**:569–570.
57. Völkl, P., R. Huber, E. Drobner, R. Rachel, S. Burggraf, A. Trincone, and K. O. Stetter. 1993. *Pyrobaculum aerophilum* sp. nov., a novel nitrate-reducing hyperthermophilic archaeum. *Appl. Environ. Microbiol.* **59**:2918–2926.
58. Wilson, W. A., S. A. Hawley, and D. G. Hardie. 1996. Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. *Curr. Biol.* **6**:1426–1434.
59. Yokoyama, S., and J. Hiramatsu. 2003. A modified ninhydrin reagent using ascorbic acid instead of potassium cyanide. *J. Biosci. Bioeng.* **95**:204–205.
60. Zaparty, M., A. Zaigler, C. Stamme, J. Soppe, R. Hensel, and B. Siebers. 2008. DNA microarray analysis of central carbohydrate metabolism: glycolytic/gluconeogenic carbon switch in the hyperthermophilic crenarchaeum *Thermoproteus tenax*. *J. Bacteriol.* **190**:2231–2238.
61. Zeikus, J. G., G. Fuchs, W. Kenealy, and R. K. Thauer. 1977. Oxidoreductases involved in cell carbon synthesis of *Methanobacterium thermoautotrophicum*. *J. Bacteriol.* **132**:604–613.