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The CO_2 assimilation via the reductive tricarboxylic acid cycle in an obligately autotrophic, aerobic hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus*

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Abstract. The incorporation of $^{14}CO_2$ by the cell suspensions of an extremely thermophilic, aerobic hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus* was studied. After short time incubation of the cell suspensions with $^{14}CO_2$, the radioactivity was initially present in aspartate, glutamate, succinate, phosphorylated compounds, citrate, malate and fumarate. All of these compounds except phosphorylated compounds were related to the members of the tricarboxylic acid cycle. The proportion of labelled aspartate or glutamate in total radioactivity on each chromatogram decreased with incubation time, while the percentage of the radioactivity incorporated in phosphorylated compounds increased with time up to 10 s. These indicated that aspartate and glutamate is derived from primary products of CO_2 fixation.

In cell-free extracts of *Hydrogenobacter thermophilus*, the two key enzymes in the Calvin cycle, ribulose-1,5-bisphosphate carboxylase and phosphoribulokinase could not be detected. The key enzymes of the reductive tricarboxylic acid cycle, fumarate reductase and ATP citrate lyase were present. Activities of phosphoenolpyruvate synthetase and pyruvate carboxylase were also detected. The reverse reactions (dehydrogenase reactions) of α-ketoglutarate synthase and pyruvate synthase could be detected by using methyl viologen as an electron acceptor.

These findings strongly suggested that a new type of the reductive tricarboxylic acid cycle operated as the CO_2 fixation pathway in *Hydrogenobacter thermophilus*.

Key words: *Hydrogenobacter thermophilus* – Autotrophic CO_2 assimilation – The reductive tricarboxylic acid cycle – Pyruvate carboxylase – Phosphoenolpyruvate synthetase – ATP citrate lyase – Fumarate reductase – Pyruvate synthase – α-Ketoglutarate synthase

In most green plants and autotrophic microorganisms, the reductive pentose-phosphate cycle (Calvin cycle, Bassham and Calvin 1957) is operative for autotrophic CO_2 assimilation. But some autotrophic bacteria assimilate CO_2 via non-Calvin type pathways (Fuchs and Stupperich 1983). Among those are, a phototrophic green-sulfur eubacterium, *Chlorobium limicola* (Evans et al. 1966; Fuchs et al. 1980a, b); methanogenic archaebacteria, *Methanobacterium thermoautotrophicum* (Fuchs and Stupperich 1978, 1980, 1982; Daniels and Zeikus 1978; Fuchs et al. 1978, 1983; Stupperich and Fuchs 1981, 1983; Stupperich et al. 1983; Jansen et al. 1982) and *Methanosarcina barkeri* (Zeikus 1983); and a strictly anaerobic homoacetogenic eubacterium, *Acetobacterium woodii* (Eden and Fuchs 1982, 1983). Comparing to these anaerobes, evidence for the operation of a non-Calvin type CO_2 assimilation pathway is very poor in aerobes. Kandler and Stetter (1981) studied $^{14}CO_2$ pulse labelling in cells of *Sulfolobus brierleyi*, a thermoacidophilic archaebacterium and suggested the autotrophic CO_2 assimilation via a reductive carboxylic acid pathway might be functioning in that bacterium, but further detailed study has not been presented.

Hydrogenobacter thermophilus, an extremely thermophilic, aerobic hydrogen-oxidizing bacterium isolated from soil of a hot spring in Japan (Kawasumi et al. 1980, 1984), is the only obligate autotroph among all aerobic hydrogen-oxidizing bacteria so far reported (Bowien and Schlegel 1981). In a previous paper (Shiba et al. 1982), we reported none of the following enzyme activities listed below was detected in cell-free extracts of this bacterium: (a) phosphofructokinase and pyruvate kinase, key enzymes of the Embden-Meyerhof pathway, (b) 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase, key enzymes of the Entner-Doudoroff pathway and (c) α-ketoglutarate dehydrogenase and pyruvate dehydrogenase. We also reported that addition of small amounts of acetate, pyruvate or malate into the medium stimulated the autotrophic growth of this bacterium (Shiba et al. 1984). Because of their metabolic defects in carbohydrate catabolizing pathways, we doubted the involvement of the Calvin cycle in this bacterium and assumed that acetate, pyruvate and malate might be the central intermediates in the autotrophic CO_2 assimilation pathway of this bacterium. In this paper, we report our studies on short term fixation of $^{14}CO_2$ into cells and enzyme activities of CO_2 fixation and some related reactions in cell-free extracts of *H. thermophilus*. From the results of this study, we propose that the reductive tricarboxylic acid cycle is operative in *H. thermophilus*.

Materials and Methods

Organism and growth conditions. Hydrogenobacter thermophilus type strain TK-6 IAM 12695 (Kawasumi et al. 1984) was grown at 70°C on a gas mixture of H_2-O_2-CO_2 (75:15:10) in an inorganic medium at pH 7.0 (Kawasumi et al. 1980). Cultivation in a 2 l jar fermentor (Labotec Co.,

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Japan) was as previously described (Shiba et al. 1982). Cells were harvested in the late exponential phase by centrifugation and washed twice in 10 mM phosphate buffer (pH 7.0) for $^{14}CO_2$-labelling studies, or 0.1 M HEPES-NaOH (pH 7.0) for enzyme assay studies, and resuspended in the same buffer.

$^{14}CO_2$ labelling procedure. Carbon dioxide fixation was performed with 2 ml of cell suspension (2 mg dry weight) placed in 25 ml Warburg vessels. Each Warburg vessel containing 10 mM phosphate buffer (pH 7.0) under the gas phase of H_2-O_2 (7:1) was preincubated at 70° C, then $NaH^{14}CO_3$ (54 mCi/mol) was added at final concentration of 2 mM. CO_2 fixation reaction was started by injection of 0.1 ml of cell suspensions preincubated at 70° C under the same gas phase. Vessels were shaken during the above procedures. Reaction was stopped by adding 8 ml of 100% cold methanol (final concentration of 80%) and cooling in an ethanol-dry ice bath. The total amount of fixed ^{14}C was determined by measuring the radioactivity of this reaction mixture after free CO_2 was expelled (see below). The cells were collected by centrifugation and washed by 5 ml of 80% methanol and resuspended with 3 ml of water as the methanol insoluble fraction. The methanol solutions were collected as the methanol soluble fraction. The radioactivities of the methanol soluble and insoluble fractions were also measured.

Analysis of fixation products. The methanol soluble fraction was evaporated and spotted on Whatman No. 1 filter paper (39 cm × 46 cm). Two dimensional chromatography was performed with the following solvent mixtures: (1) 88% phenol, water, acetic acid, 0.2 M EDTA (831:154.2:9.8:5); (2) n-butanol, water (370:25), propionic acid, water (18:22), both solutions are mixed 1:1 immediately before use. Then radioautograms were prepared by exposing the chromatograms to RX type X-ray film (Fuji Photo Film Co., Japan) and the radioactivity of each spot was determined by counting with a Geiger-Müller tube (Aloka GMH5001). Individual amino and organic acids were identified by co-chromatography on TLC plate (Myers and Huang 1969) or co-electrophoresis on the filter paper with authentic compounds.

Determination of radioactivity. Radioactivity in solution was determined by liquid scintillation counting. The samples were placed in scintillation vials and dried by illumination of an infrared lamp and aerating for 10 min to release unfixed $^{14}CO_2$, after which 5 ml of the liquid scintillation cocktail (1 l of dioxane, 100 g of naphthalene, 10 g of PPO and 250 mg of POPOP) was added. A Packard Tricarb 3255 scintillation counter was used for counting.

Preparation of cell-free extracts. The cell suspensions of *Hydrogenobacter thermophilus* in HEPES buffer containing deoxyribonuclease I (10 − 20 μg/ml) were passed through a French pressure cell (Aminco, USA) at 103 MPa. The ruptured cells were centrifuged at 10,000 × *g* for 20 min at 4° C and supernatants were ultracentrifuged at 144,000 × *g* for 1 h. These latter supernatants were used as cell-free extracts of *H. thermophilus* for common enzyme assays. For the assay of CO_2 fixation enzymes, the supernatants were further passed through Sephadex G-25 column and the brown color fractions were used as cell-free extracts.

Enzyme assays. Ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) was assayed radiometrically at 70° C in 0.3 ml reaction mixtures containing HEPES-NaOH (pH 7.8) or Tricine-NaOH (pH 7.8), 0.1 M; $MgCl_2$, 10 mM; $NaH^{14}CO_3$, 16 mM (10 μCi); D-ribulose-1,5-bisphosphate, 1.7 mM and cell-free extracts. Reaction was initiated by addition of ribulosebisphosphate and stopped by addition of 0.2 ml of 100% acetic acid or 5 N H_2SO_4. After illumination and aeration to release unfixed $^{14}CO_2$, samples were counted in a liquid scintillation counter.

Phosphoribulokinase (EC 2.7.1.19) was assayed by the radiometric two-stage assay method (Siebert et al. 1981) with some modifications: the primary reaction was carried out at 70° C; in the secondary reaction which was carried out at 30° C, ribulose-1,5-bisphosphate carboxylase from spinach leaves was used.

Pyruvate and phosphoenolpyruvate dependent CO_2 fixation enzymes were assayed radiometrically at 70° C. For malic enzyme (EC 1.1.1.38), the reaction mixture (0.5 ml, pH 7.8) contained HEPES-NaOH, 0.1 M; $MgCl_2$, 5 mM; $NaH^{14}CO_3$, 20 mM (10 μCi); NADH, 2.5 mM; pyruvate, 5 mM and cell-free extracts. For pyruvate carboxylase (EC 6.4.1.1), ATP was added at 2.5 mM. For phosphoenolpyruvate carboxylase (EC 4.1.1.31), reaction mixture was the same as that of malic enzyme except for using phosphoenolpyruvate (5 mM) instead of pyruvate, and for phosphoenolpyruvate carboxykinase (EC 4.1.1.49) and phosphoenolpyruvate carboxytransphosphorylase (EC 4.1.1.38), ADP (2.5 mM) or KH_2PO_4 (2.5 mM), respectively, was added. Reactions were started by injection of pyruvate or phosphoenolpyruvate, and stopped by addition of 0.2 ml of 5 N H_2SO_4 or 0.5 ml of 100% acetic acid. The radioactivities of acid stable products were measured by liquid scintillation counting.

Pyruvate carboxylase was also assayed spectrophotometrically at 340 nm by following the oxidation of NADH coupled to oxalacetate formation from pyruvate. The reaction mixture (3.0 ml, pH 7.8) contained Tricine-KOH, 0.1 M; $MgCl_2$, 5 mM; $KHCO_3$, 167 mM; potassium pyruvate, 5 mM; ATP, 2.5 mM; NADH, 0.15 mM and cell-free extracts. Malate dehydrogenase was not added to the reaction mixture, because activity of endogenous malate dehydrogenase in the cell-free extracts was sufficiently high (Shiba et al. 1982).

Isocitrate dehydrogenase (EC 1.1.1.41, 1.1.1.42) was assayed spectrophotometrically by following the reduction of NAD(P) in the presence of isocitrate. Mn^{2+} was used as the metal co-factor. Reaction mixture (3 ml, pH 7.8) contained Tricine-KOH, 0.1 M; $MnSO_4$, 2 mM; NAD(P), 0.67 mM; isocitrate, 4 mM and cell-free extracts.

ATP citrate lyase (EC 4.1.3.8) was assayed spectrophotometrically according to Takeda et al. (1969) by following the oxidation of NADH coupled to oxalacetate formation from citrate. Endogenous malate dehydrogenase activity in cell-free extracts served as coupling enzyme activity.

Phosphoenolpyruvate synthetase (EC 2.7.9.2) activity was measured in a discontinuous assay by the enzymatic determination of phosphoenolpyruvate formed using pyruvate kinase and lactate dehydrogenase (Eyzaguirre et al. 1982).

In the assay of fumarate reductase (EC 1.3.99.1), the oxidation of reduced methyl viologen was followed in the presence of fumarate as described by Zeikus et al. (1977) using septum capped anaerobic cuvettes. The reaction was

measured at 578 nm (ε_{578} of reduced methyl viologen: 9.7 mM^{-1} cm^{-1}).

Pyruvate dehydrogenase (EC 1.2.–.–) and α-ketoglutarate dehydrogenase (EC 1.2.–.–) were assayed by following the reduction of methyl viologen in the presence of the respective α-keto acid (Zeikus et al. 1977).

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Chemicals and a radioactive compound. All chemicals were of reagent grade. Enzymes and coenzymes were purchased from Sigma Chemical Co. (St. Louis, USA). $NaH^{14}CO_3$ was obtained from Amersham Japan (Japan).

Results

1. Short-term $^{14}CO_2$ fixation into cells of Hydrogenobacter thermophilus

The incorporation of $^{14}CO_2$ into washed cells of *Hydrogenobacter thermophilus* was measured at 70° C for short time periods between 0.5 s and 60 s. The rate of total $^{14}CO_2$ fixation was linear with time up to 60 s, and the incorporation of ^{14}C into the methanol soluble fraction of the cells was more than 90% of total $^{14}CO_2$ fixed (data not shown). The distribution of ^{14}C among methanol soluble cell compounds showed that aspartate, glutamate and succinate were the earliest ^{14}C labeled compounds. The percentage of ^{14}C incorporation into aspartate and glutamate initially decreased (Fig. 1), indicating that both amino acids were derived directly from primary $^{14}CO_2$ fixation products. Labelling of succinate increased with time up to 30 s, whereas ^{14}C incorporation into phosphorylated compounds was initially low and increased with time up to 10 s. The ^{14}C incorporation into citrate, malate and fumarate was also low and showed an early maximum. Incorporation of ^{14}C into alanine, glutamine and other several unidentified compounds was observed only after 30 s (Fig. 2).

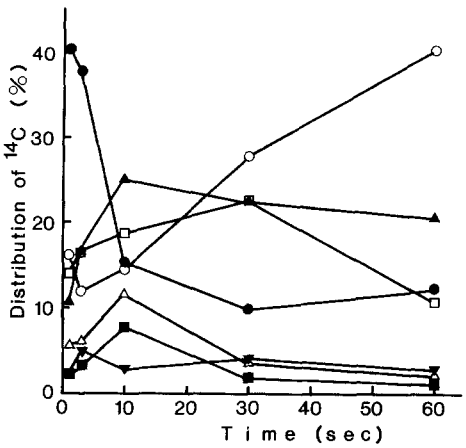

Fig. 1. Time course of ^{14}C distribution from $^{14}CO_2$ incorporated into methanol-soluble intermediates of *Hydrogenobacter thermophilus*. ●, Aspartate; ○, glutamate; ▲, phosphorylated compounds; △, malate; ■, fumarate; □, succinate; ▼, citrate

2. Key enzyme activities of the reductive pentose-phosphate cycle

Both of ribulose-1,5-bisphosphate carboxylase and phosphoribulokinase were assayed several times, but neither of the enzyme activities were detectable in cell-free extracts of this bacterium.

3. Activities of enzymes of the reductive tricarboxylic acid cycle

Activities of enzymes related to the reductive tricarboxylic acid cycle were assayed in cell-free extracts of *Hydrogenobacter thermophilus*. Table 1 shows activities of enzymes of the tricarboxylic acid cycle (Shiba et al. 1982) and of the additionally assayed enzyme activities related to the reductive tricarboxylic acid cycle.

Enzymes of CO_2 fixation to form C_4 dicarboxylic acid from pyruvate or phosphoenolpyruvate

Enzyme activities of CO_2 fixation, that are involved in the synthesis of C_4 dicarboxylic acid were assayed. Among five enzymes known up to date (pyruvate carboxylase, malic enzyme, phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase and phosphoenolpyruvate carboxytransphosphorylase), only pyruvate carboxylase was detected at a significant activity (16 nmol · min^{-1} · mg^{-1} protein) by the radiometric method. We could also measure this enzyme activity (164 nmol · min^{-1} · mg^{-1}protein) by the spectrophotometric method in an improved reaction mixture (see Methods).

Phosphoenolpyruvate synthetase

An activity catalyzing an ATP dependent synthesis of phosphoenolpyruvate from pyruvate was detected in the extracts. Two enzymes, phosphoenolpyruvate synthetase and pyruvate, phosphate dikinase are known to catalyze this reaction (Cooper and Kornberg 1974). Phosphoenolpyruvate synthetase was considered to be present in this bacterium, because requirement of phosphate was not observed in this reaction.

ATP citrate lyase

The extracts of *H. thermophilus* also contained a high activity of ATP citrate lyase. Formation of acetyl-CoA in this reaction was confirmed by the method using reaction with hydroxylamine (Takeda et al. 1969). Activity of citrate synthase, which catalyzes the reverse reaction, was very low.

Fumarate reductase

The extracts exhibited a high activity of fumarate reductase with reduced methyl viologen as electron donor.

Pyruvate dehydrogenase and α-ketoglutarate dehydrogenase

Reduction of methyl viologen in the presence of pyruvate and coenzyme-A or α-ketoglutarate and coenzyme-A was observed in the extracts. Whereas, activities of neither NAD(P)-linked pyruvate or NAD(P)-linked α-ketoglutarate dehydrogenase could be detected in *H. thermophilus* in a previous paper (Shiba et al. 1982).

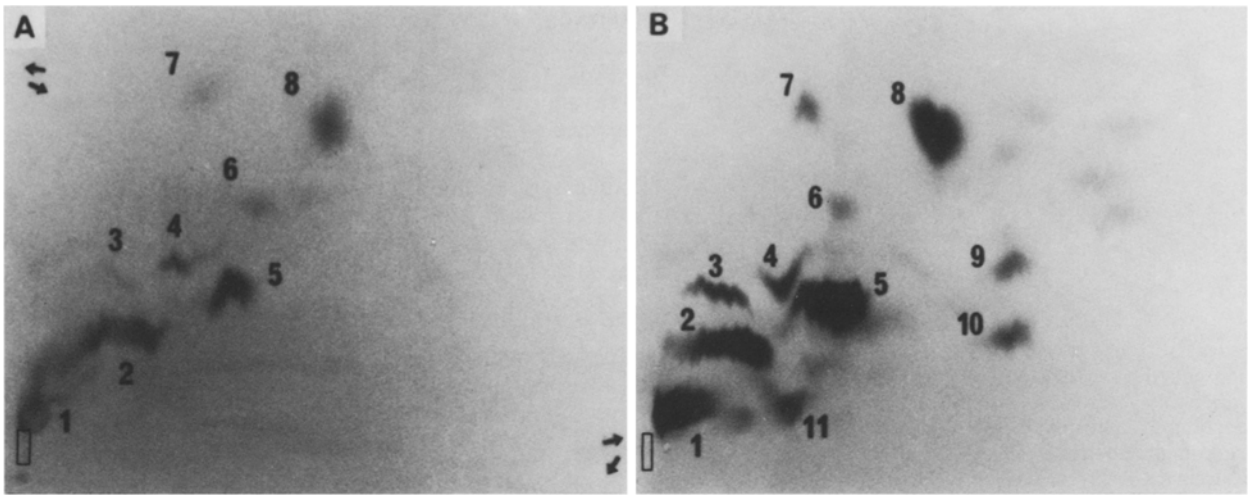

Fig. 2 A, B. Two-dimensional paper chromatography and radioautography of the methanol-soluble fractions of *Hydrogenobacter thermophilus* cells after incubation with $^{14}CO_2$ for 0.5 s (**A**) and 60 s (**B**). The quadrangle at left bottom indicates the origin position and the arrows indicate the marker for radioautography. *1* Phosphorylated compounds; *2* aspartate; *3* malate; *4* citrate; *5* glutamate; *6* an unidentified compound; *7* fumarate; *8* succinate; *9* alanine; *10* glutamine; *11* an unidentified compound

Table 1
Specific activities of enzymes of the reductive tricarboxylic acid cycle and some related enzymes in cell-free extracts of *Hydrogenobacter thermophilus*[a]

Enzyme	Specific activity (nmol · min^{-1} · mg^{-1} protein)		
	30° C	50° C	70° C
Citrate synthase[b]	1.0	1.3	4.2
ATP citrate lyase		270	443
Aconitase[b]	20.3	54.5	24.8
Isocitrate dehydrogenase (NAD)	2.4	24.6	209
Isocitrate dehydrogenase (NADP)	8.0	50.1	192
α-Ketoglutarate dehydrogenase (NAD)[b]	ND	ND	ND
α-Ketoglutarate dehydrogenase (MV)	Trace	30.3	80.0
Succinyl-CoA synthetase[b]	83.6	208	372
Succinate dehydrogenase (PMS, Cyt. c)[b]	5.6	12.0	
Fumarate reductase (MV)	78.0		178
Fumarase[b]	2.4	16.1	43.3
Malate dehydrogenase (NAD)[b]	309	1,270	3,660
Pyruvate dehydrogenase (NAD)[b]	ND	ND	ND
Pyruvate dehydrogenase (MV)	Trace	10.3	30.3
Phosphoenolpyruvate synthetase			46.8
Pyruvate carboxylase[c]			164

[a] Abbreviations: ND, not detected; MV, methyl viologen; PMS, phenazine methosulfate; Cyt. c, cytochrome c
[b] The data were taken from Shiba et al. (1982)
[c] The activity was measured spectrophotometrically

Isocitrate dehydrogenase

In a previous paper, we reported that *H. thermophilus* contains both NAD- and NADP-specific isocitrate dehydrogenase activities (Shiba et al. 1982). For those assays we used Mg^{2+} as a co-factor. In the present work Mn^{2+} instead of Mg^{2+} was employed, because Mn^{2+} was the more effective divalent cation to stimulate the activity of isocitrate dehydrogenase.

Discussion

The distribution of the short term $^{14}CO_2$ fixation products in cells of *Hydrogenobacter thermophilus*, was far different from those of autotrophic organisms known to fix CO_2 via the reductive pentose-phosphate cycle. Activities of key enzymes of the reductive pentose-phosphate cycle, ribulose 1,5-bisphosphate carboxylase and phosphoribulokinase were absent from cell-free extracts of *H. thermophilus*. We thus concluded that the reductive pentose-phosphate cycle does not operate in *H. thermophilus*.

The main products of short term $^{14}CO_2$ fixation were aspartate, glutamate, succinate, malate, citrate, fumarate and phosphorylated compounds. Especially, aspartate and glutamate showed the negative slope in ^{14}C incorporation kinetics. All of these products except phosphorylated compounds are intermediates or indirect products of the tricarboxylic acid cycle, since oxalacetate and α-ketoglutarate are considered to be the direct precursors of aspartate and glutamate, respectively. This finding, in addition to the observed stimulative effect of acetate, pyruvate and malate on the autotrophic growth (Shiba et al. 1984), strongly supported the view that CO_2 assimilation in this bacterium occurs by reversing the oxidative tricarboxylic acid cycle.

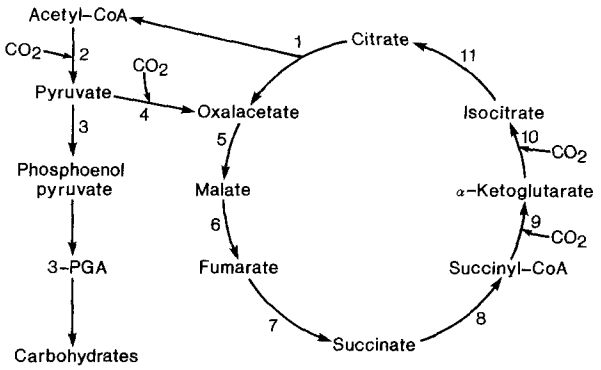

Fig. 3. Proposed reductive tricarboxylic acid cycle for CO_2 assimilation in *Hydrogenobacter thermophilus*. Enzymes: *1* ATP citrate lyase; *2* pyruvate synthase; *3* phosphoenolpyruvate synthetase; *4* pyruvate carboxylase; *5* malate dehydrogenase; *6* fumarase; *7* fumarate reductase; *8* succinyl-CoA synthetase; *9* α-ketoglutarate synthase; *10* isocitrate dehydrogenase; *11* aconitase

Several key enzymes of the reductive tricarboxylic acid cycle were detected in cell-free extracts of this bacterium. In the reductive tricarboxylic acid cycle of *Chlorobium limicola*, citrate cleavage is catalyzed by an ATP- and coenzyme-A-dependent reversible enzyme, the ATP citrate lyase (Ivanovsky et al. 1980; Antranikian et al. 1982). *H. thermophilus* also possesses this enzyme activity. The presence of malate dehydrogenase, fumarase, fumarate reductase and succinyl-CoA synthetase activities enable the organism to synthesize succinyl-CoA from oxalacetate (a CO_2 fixation product from C_3 compound) via the reversal operation of the tricarboxylic acid cycle.

Pyruvate synthase and α-ketoglutarate synthase are other key enzymes for CO_2 fixation by the reductive tricarboxylic acid cycle. In the reductive tricarboxylic acid cycle of *C. limicola*, a specific electron donor, ferredoxin, participates in these two enzyme reactions (Evans and Buchanan 1965; Buchanan and Evans 1965). In case of *Methanobacterium thermoautotrophicum*, $Factor_{420}$ (F_{420}) functions as electron donor (Zeikus et al. 1977). We were unable to detect both of these CO_2 fixation activities in *H. thermophilus*, because a native electron donor such as ferredoxin or F_{420}, was not yet found in this bacterium. But, we could demonstrate pyruvate- or α-ketoglutarate-dependent methyl viologen reduction in cell-free extracts of this bacterium. In *M. thermoautotrophicum* and *Acetobacterium woodii* (Zeikus et al. 1977; Eden and Fuchs 1983), assays of methyl viologen-dependent pyruvate or α-ketoglutarate dehydrogenase activities could be substituted for assays of pyruvate of α-ketoglutarate synthase activities, respectively. Since *H. thermophilus* contains neither NAD(P)-dependent pyruvate dehydrogenase nor NAD(P)-dependent α-ketoglutarate dehydrogenase activities (Shiba et al. 1982), we presume that the pyruvate- and α-ketoglutarate-dependent methyl viologen reductions were catalyzed by pyruvate synthase and α-ketoglutarate synthase, respectively. Reduced methyl viologen could not serve as the electron donor in the carboxylating reaction of these enzymes. To firmly establish the existence of these enzymes in *H. thermophilus*, the still unknown physiological electron donor participating in these reactions will have to be identified.

From the results of the present enzyme study, we suggest that the autotrophic CO_2 assimilation in *H. thermophilus* proceeds via the reductive tricarboxylic acid cycle outlined in Fig. 3. This cycle differs from the one occurring in *C. limicola* (Evans et al. 1966) in that *H. thermophilus* uses pyruvate carboxylase instead of phosphoenolpyruvate carboxylase to form oxalacetate from C_3 compounds.

Fuchs and Stupperich (1982, 1983) discussed that non-Calvin type carbon dioxide fixation pathways function in archaebacteria and in anaerobic eubacteria. This paper showed that a non-Calvin type CO_2 fixation pathway is also operative in an aerobic eubacterium. Now, further work is in progress to obtain the complete evidence of the proposed CO_2 fixation pathway in *H. thermophilus*. We have also been interested in the phylogenetic position of *H. thermophilus* (Kawasumi et al. 1984; Ishii et al. 1983), but still do not have enough data to discuss evolutionary aspects of this bacterium.

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