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ORIGINAL PAPER

Carboxylation reaction catalyzed by 2-oxoglutarate:ferredoxin oxidoreductases from *Hydrogenobacter thermophilus*

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Abstract *Hydrogenobacter thermophilus* TK-6 is a thermophilic, chemolithoautotrophic, hydrogen-oxidizing bacterium that fixes carbon dioxide via the reductive tricarboxylic acid (rTCA) cycle. 2-Oxoglutarate:ferredoxin oxidoreductase (OGOR) is the key enzyme in this cycle that fixes carbon dioxide. The genome of strain TK-6 encodes at least two distinct OGOR enzymes, termed For and Kor. We report here a method for measuring the carboxylation of succinyl-CoA catalyzed by OGORs. The method involves the in vitro coupling of OGOR with ferredoxin and pyruvate:ferredoxin oxidoreductase from strain TK-6, and glutamate dehydrogenase from *Sulfolobus tokodaii*. Using this method, we determined both the apparent maximum velocities and the K_m values of For and Kor for the carboxylation of succinyl-CoA. This is the first reported kinetic analysis of carbon fixation catalyzed by OGOR enzymes from the rTCA cycle.

Keywords *Hydrogenobacter thermophilus* · 2-Oxoglutarate:ferredoxin oxidoreductase · Reductive tricarboxylic acid cycle · Carboxylation · Pyruvate:ferredoxin oxidoreductase

Abbreviations

rTCA	Reductive tricarboxylic acid
OGOR	2-Oxoglutarate:ferredoxin oxidoreductase
OR	2-Oxoacid oxidoreductase
POR	Pyruvate:ferredoxin oxidoreductase
GDH	Glutamate dehydrogenase

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Introduction

The biological fixation of inorganic carbon is the primary means of production of biomass and organic compounds, and all heterotrophic organisms depend on this autotrophic 'primary production' as a source of energy and carbon. When we think about energy and material flux in the environment, biological carbon dioxide fixation is key to the input of carbon into the biomass. The reductive tricarboxylic acid (rTCA) cycle is an important pathway mediating carbon fixation in many autotrophic *Bacteria* and *Archaea* (Beh et al. 1993; Fuchs et al. 1980; Hu and Holden 2006; Hugler et al. 2005, 2007).

Members of the *Aquificaceae* family, which is phylogenetically located in the oldest branch in the domain Bacteria, are found in harsh environments such as hot springs, sulfur pools, and hydrothermal vents where they grow autotrophically using the rTCA cycle (Burggraf et al. 1992). *Hydrogenobacter thermophilus* strain TK-6 (IAM 12695, DSMZ 6534) is a thermophilic, aerobic, obligate chemolithotrophic, hydrogen-oxidizing bacterium within the family *Aquificaceae* (Kawasumi et al. 1980, 1984; Pitulle et al. 1994). Strain TK-6 can grow rapidly with a high final cell density, facilitating experimental study of its biochemistry (Ishii et al. 2000; Kameya et al. 2006; Kameya et al. 2007; Suzuki et al. 2006; Ueda et al. 2007). Analyses of the enzymes of the rTCA cycle in this organism has revealed various significant differences compared to those in other organisms, e.g. novel citrate

cleavage reactions catalyzed by citryl-CoA synthetase and citryl-CoA lyase (Aoshima et al. 2004a, b), novel carboxylation reaction of 2-oxoglutarate catalyzed by CFI (carboxylating factor for ICDH) (Aoshima and Igarashi 2006), two novel five-subunits type 2-oxoacid oxidoreductase (Ikeda et al. 2006; Yamamoto et al. 2003), and a NADH-dependent fumarate reductase (Miura et al. 2008).

2-Oxoglutarate:ferredoxin oxidoreductase (OGOR) is a key enzyme in the rTCA cycle (Evans et al. 1966), and a member of the 2-oxoacid oxidoreductase (OR) family of enzymes (Adams and Kletzin 1996). These enzymes catalyze the oxidative decarboxylation of 2-oxoacids to their acyl- or aryl-CoA derivatives. In the oxidative TCA cycle, OGOR catalyzes the oxidative decarboxylation of 2-oxoglutarate to succinyl-CoA. Conversely, in the rTCA cycle, the enzyme acts as a 2-oxoglutarate synthase and assimilates carbon dioxide. Reducing energy is required for this carboxylation reaction, and ferredoxin is believed to be the electron donor.

We reported previously that strain TK-6 expresses two different OGORs, designated For and Kor (Yoon et al. 1996a; Yun et al. 2001, 2002). Expression analysis and gene disruption experiments showed that Kor expresses both under anaerobic and aerobic growth conditions, and For does only under aerobic conditions (Yamamoto et al. 2003, 2006). Expression of For assists the aerobic robust growth of the strain. These recombinant OGORs were purified and characterized following expression in *Escherichia coli* (Yamamoto et al. 2003). The catalytic properties of For and Kor were determined with respect to the oxidative decarboxylation reaction. Various reports have studied the role of OGORs in the decarboxylation reaction, but not in the carboxylation reaction (Dorner and Boll 2002; Gehring and Arnon 1972). From the viewpoint of the metabolic function of the enzymes and its role in the ecosystem, characterization of the OGORs should be based on the reductive carboxylation reaction.

We previously reported that Kor catalyzes the incorporation of [^{14}C]-labeled bicarbonate into succinyl-CoA to produce radioactive 2-oxoglutarate in vitro (Yoon et al. 1997a). These studies made use of cell extract prepared from TK-6 strain cells, however, and similar studies using only the purified OGOR and ferredoxin have not been successful. The carboxylation reaction requires a low-potential electron donor such as reduced ferredoxin. However, addition of the amount of strong reducing agent (e.g. sodium dithionite), required for the in vitro reduction of ferredoxin, causes an unsuitable reductive potential for the overall enzymatic reaction in the mixture. Therefore, a mechanism for the re-reduction of ferredoxin without the use of a low potential reducing agent is important. The cell extract from the TK-6 strain probably contains some system for the re-reduction of ferredoxin. Although the reduction of ferredoxin has been suggested to be catalyzed by NADH:ferredoxin reductase in strain TK-6 (Yoon et al. 1996b), the latter enzyme has not been purified yet.

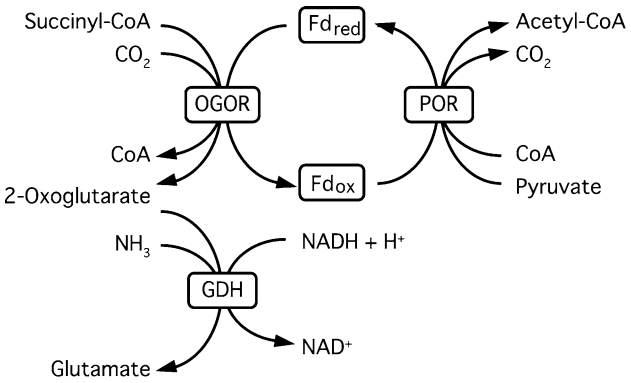

Fig. 1 Schematic view of the OGOR enzyme assay coupled with POR and GDH. *OGOR* 2-oxoglutarate:ferredoxin oxidoreductase, *POR* pyruvate:ferredoxin oxidoreductase, *GDH* glutamate dehydrogenase, Fd_{ox} oxidized ferredoxin, Fd_{red} reduced ferredoxin

Pyruvate:ferredoxin oxidoreductase (POR) also belongs to the OR family of enzymes. In strain TK-6, POR catalyzes a distinct carbon dioxide fixation reaction in the rTCA cycle**.** POR has been purified from strain TK-6 and characterized (Ikeda et al. 2006; Yoon et al. 1997b). This enzyme can catalyze decarboxylation of pyruvate concurrently with the reduction of ferredoxin. Therefore, POR should be able to provide reduced ferredoxin for the OGOR-catalyzed carboxylation of succinyl-CoA in vitro. In this paper, we developed an efficient carboxylation assay system comprising OGOR coupled to POR (Fig. 1). Glutamate dehydrogenase (GDH), which catalyzes the binding of the ammonium nitrogen to 2-oxoglutarate accompanied by the consumption of NADH, was also coupled so as to monitor the progress of the reaction. This assay system facilitates the kinetic analysis of OGOR activity as a key enzyme of the autotrophic carbon assimilation pathway.

Materials and methods

Bacterial strains, plasmids, and enzymes

Table 1 shows the plasmids and host strains used in this work. Two OGORs (For and Kor), POR, and ferredoxin derived from *H. thermophilus* TK-6 were expressed in *E. coli*, and purified as in our previous studies (Ikeda et al. 2005, 2006; Yamamoto et al. 2003). For the preparation of glutamate dehydrogenase (GDH), the *gdh* gene (ST2241) was amplified from chromosomal DNA from *Sulfolobus tokodaii* strain 7 using two PCR primers (STGDH-N, 5′-GGAATTC*CATATG*AATTCAGCCGTC-3′ and STGDH-C, 5′-CCG*CTCGAG*TAACATACCTCTAGCT-3′) (Kawarabayasi et al. 2001). The amplified PCR product was cloned

Table 1 Plasmids and bacterial strains used in this work to produce recombinant proteins

Plasmids	Genes (accession numbers[a]) and origins	Proteins	Replicons	Host strains	References
pYNA103	*korAB* (AB046568) *H. thermophilus* TK-6	Kor (OGOR)	pUC19	*E. coli*, JM109	Yun et al. (2001)
pYNA202	*ForDABGE* (AB054643) *H. thermophilus* TK-6	For (OGOR)	pUC19	*E. coli*, JM109	Yun et al. (2002)
pUC-POR	*PorEDABG* (AB042412) *H. thermophilus* TK-6	POR	pUC19	*E. coli*, JM109	Ikeda et al. (2006)
pET-Fd1	*fdx1* (AB185162) *H. thermophilus* TK-6	Ferredoxin (Fd1)	pET11a	*E. coli*, BL21 (DE3)	Ikeda et al. (2005)
pET-GDH	ST2241 (BA000023-2410) *S. tokodaii* 7	GDH	pET21c	*E. coli*, BL21 (DE3)	This work

[a] Accession numbers have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database

into the *Nde*I and *Xho*I sites of pET21c (Novagen), resulting in pET-GDH. The recombinant protein fused to a histidine tag was produced in *E. coli*, and purified after heat treatment (80°C, 15 min) using a Ni-affinity column (HisTrap FF column, GE Healthcare).

GDH enzyme assay

GDH activity was determined at 70°C by measuring the oxidation of NADH (reductive amination) by spectrophotometer at 340 nm. The reaction mixture contained 10 mM MOPS (pH 6.8), 5 mM 2-oxoglutarate, 50 mM ammonia and 0.2 mM NADH. The reaction was initiated by addition of 2-oxoglutarate. The absorption coefficient of NADH at 340 nm is 6.2 cm^2 μmol^{-1}.

Reduction of ferredoxin by POR

Ferredoxin reduction, which was dependent upon pyruvate oxidation catalyzed by POR, was measured by spectrophotometer at 70°C. The standard reaction mixture comprised POR, ferredoxin, 10 mM sodium pyruvate, 0.25 mM CoA, 1 mM $MgCl_2$, 1 mM dithiothreitol in 10 mM MOPS (pH 6.8). A quartz cell containing the mixture was sealed with a screw cap, and then argon gas was injected into the cell for 5 min. The reaction was initiated by addition of pyruvate and the absorption at 425 nm was monitored. The absorption coefficient of the Fd1 at 425 nm is 5.5 cm^2 μmol^{-1}. One electron is transferred by a molecule of Fd1.

Measurement of the carboxylation reaction

The standard assay mixture contained 10 mM MOPS (pH 6.8), 1 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM $NaHCO_3$, 5 mM NH_4Cl, 0.25 mM CoA, 0.26 mM NADH, 100 mM pyruvate, 1 mM succinyl-CoA, and proteins (OGOR, POR, ferredoxin, and GDH). The gas phase in the quartz cell was replaced with argon. The reaction was initiated by addition of succinyl-CoA. The change in A_{340} (representing a decrease in the consumption of NADH) was measured by spectrophotometer at 70°C. The measurement was taken 30 s following succinyl-CoA addition.

Results

Preparation of ferredoxin

The genome of *H. thermophilus* TK-6 contains at least two genes encoding [4Fe–4S]-type ferredoxin (*fdx1* and *fdx2*, DDBJ/EMBL/GenBank accession numbers AB185162) (Ikeda et al. 2005). These genes are located in tandem in the chromosomal DNA, and are co-transcribed. Fd1, encoded by the *fdx1*gene, has been shown to couple with OGOR and POR (Yoon et al. 1996a, 1997a, b). In contrast, coupling between Fd2, encoded by the *fdx2* gene, and either OGOR or POR has not been reported. Western blot analysis has documented the expression of Fd1 but not Fd2 in cell extracts prepared from strain TK-6 (unpublished results), so we used Fd1 for this work. Ferredoxin is one of the substrates of the carboxylation reaction catalyzed by OGOR. Maximal OGOR activity requires a high concentration of ferredoxin in the reaction mixture. We first attempted to measure the K_m of OGORs using Fd1 in the decarboxylation reaction. The apparent K_m value for Fd1 of For and Kor was 2.5 μM and 0.55 μM, respectively. One U of OGOR activity was defined as the reduction of 2 μmol ferredoxin per minute, in which 1 μmol of 2-oxoglutarate was decarboxylated. When assaying the activity of either For or Kor, the reduction of Fd1 nearly reached saturation at a concentration of 10 μM. The absorption measured in the reaction mixture at 340 nm in the presence of 10 μM Fd1 was 0.15, which was in a negligible range for the quantification of NADH. The apparent V_{max} of the decarboxylation reaction (reduction of ferredoxin) was calculated to be 0.47 (For) and 6.9 (Kor) units per mg of protein.

Preparation of POR

Recombinant POR was purified from *E. coli* cells under anaerobic conditions (Ikeda et al. 2006). It is important that the reduced ferredoxin is saturating in the OGOR assay mixture. Reduced ferredoxin (via decarboxylation of pyruvate catalyzed by POR) must be supplied at a greater rate than its consumption (carboxylation of succinyl-CoA

catalyzed by OGOR). We determined the velocity of the reduction of ferredoxin by POR to be 0.26 units per mg of protein at 10 μM Fd1. The quantity of POR in 500 μl of the OGOR reaction mixture was fixed at approximately 0.01 U (50 μg of protein).

Preparation and characterization of GDH

Recombinant GDH protein encoded by *Sulfolobus tokodaii* strain 7 was produced as a histidine fusion protein in *E. coli* cells, and purified after heat treatment using a Ni-affinity column (data not shown). The GDH enzyme activity was measured under the same conditions used for the OGOR assay (70°C, pH 6.8). The K_m value when using 2-oxoglutarate as substrate was 0.16 mM and the V_{max} was 26.3 U/mg of protein (data not shown). No activity was detected when using pyruvate as substrate. The quantity of GDH in 500 μl of the OGOR reaction mixture was fixed at 0.1 U (3.8 μg of protein).

Measurement of the carboxylation reaction

We next monitored the carboxylation activity of OGOR through coupling with POR (>0.01 U) and GDH (0.1 U). Both For and Kor were able to fix carbon dioxide as evidenced by a reduction in absorption of the reaction mixtures at 340 nm (Fig. 2). This activity was dependent upon the presence of all proteins (OGORs, POR, Fd1, and GDH), succinyl-CoA, and pyruvate in the reaction mixture (Fig. 3). However, CoA and $NaHCO_3$ were not necessary (Fig. 3) probably because CoA and carbon dioxide were produced as byproducts in the reaction mixture (Fig. 1). The kinetic analysis of OGOR activity was performed using succinyl-CoA as substrate (Fig. 4). The apparent K_m of For and Kor for succinyl-CoA was 77 μM and 32 μM, respectively. The apparent V_{max} was calculated to be 0.45 and 2.5 U per mg of protein of For and Kor, respectively. However, an increase in the initial concentration of succinyl-CoA (200 μM) caused a decrease in the initial velocity of the reaction, and the activity exhibited a maximum at approximately 100 μM succinyl-CoA.

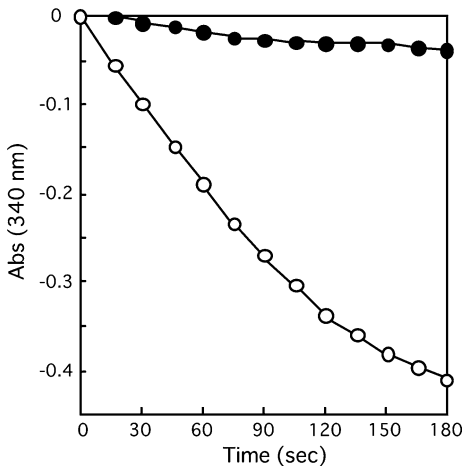

Fig. 2 Change in absorbance at 340 nm in the OGOR carboxylation assay. *Open circle* complete reaction mixture including Kor; *closed circle* reaction mixture in which OGOR was omitted

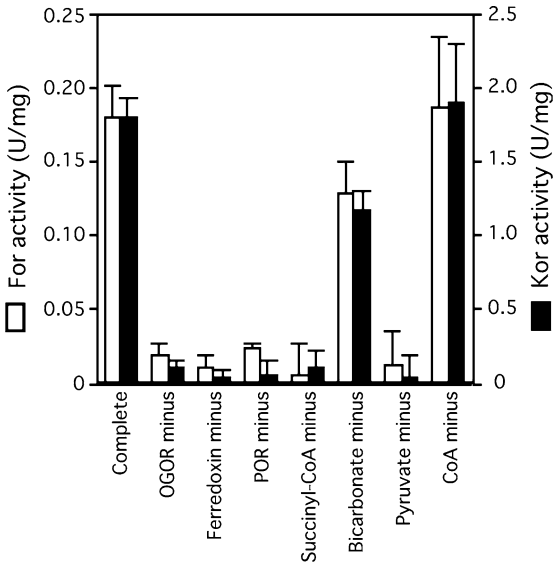

Fig. 3 Carboxylation activities of OGORs under various conditions. *Open bar* activity of For; *closed bar* activity of Kor. The assay conditions are indicated on the *horizontal axis*. Measurements were performed three times at each reaction condition. Activity of the 'OGOR minus' sample was calculated as if the same amount of OGOR had been added

Discussion

We have previously detected the carboxylation of succinyl-CoA catalyzed by OGOR using [^{14}C]-labeled bicarbonate as a substrate (Yoon et al. 1997a). However, this assay system required cell extract prepared from strain TK-6. The cell extract was thought to be necessary to allow the re-reduction of oxidized ferredoxin by NADH:ferredoxin oxidoreductase, although the latter enzyme has yet to be identified in strain TK-6. Furdui et al. used CO dehydrogenase to reduce the ferredoxin in the carboxylation reaction catalyzed by Por (Furdui and Ragsdale 2000). In this study, we made use of purified POR to reduce ferredoxin in the reaction mixture. OGOR and POR catalyze the carboxylation and decarboxylation of succinyl-CoA, respectively, such that ferredoxin was cycled between reduced and oxidized states. We could not use [^{14}C]-bicarbonate as a substrate in this assay, because OR enzymes exchange the carboxyl group of 2-oxoacid for free carbonate irrespective of carbon fixation (Gehring and Arnon 1972). Therefore, we coupled GDH with the OGOR assay. The

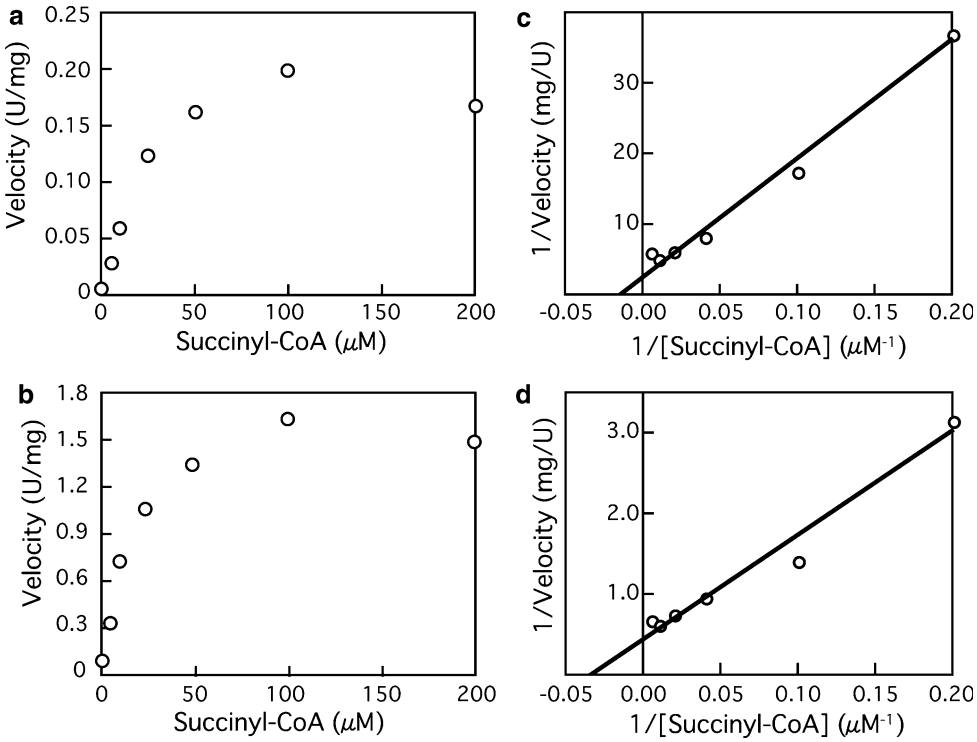

Fig. 4 Kinetic analysis of carboxylation catalyzed by OGOR. **a** and **c** For; **b** and **d** Kor. **a** and **b** direct plots; **c** and **d** double reciprocal plots. Each value represents the average of three measurements

activity of OGOR was monitored by oxidation of NADH in conjunction with the amination of 2-oxoglutarate.

Results of the coupling assay showed a decrease in absorption at 340 nm, suggesting that the carboxylation reaction by OGOR. This reaction required the presence of all enzymes, cofactors and substrates, with the exception of bicarbonate and CoA, which were produced as reaction byproducts in the assay mixture. In this assay system, the K_m for carbon dioxide could not be measured. The K_m for succinyl-CoA of For and Kor was 77 and 32 μM, respectively. A high initial concentration of succinyl-CoA inhibited enzyme activity. This phenomenon is similar to what is observed for the reverse reaction, which is inhibited by a high concentration of CoA (Yamamoto et al. 2003). The maximum velocities of For and Kor were 0.45 and 2.5 U/mg, respectively. In the reverse reaction, the maximum velocities of For and Kor were 0.47 and 6.9 U/mg, respectively. The V_{max} of the carboxylation and decarboxylation reactions catalyzed by For and Kor were similar. If the concentrations of the substrates are high, these OGORs do not determine the direction of the reaction. Because the K_m for 2-oxoglutarate (2.9 mM, For; 1.4 mM, Kor) is higher than that for CoA (26 μM, For; 80 μM, Kor) and succinyl-CoA (77 μM, For; 32 μM, Kor), low concentration (in the μM range) of the substrates promotes the carboxylation reaction. It has been reported that NADH-dependent fumarate reductase preferentially catalyzes the reaction in the direction of fumarate reduction in strain TK-6 (Miura et al. 2008). Therefore, this upstream enzyme may be key to the direction of the rTCA cycle.

The molecular mass of For and Kor has been measured as 230k and 104k, respectively, by using gel-filtration chromatography (Yamamoto et al. 2003). The K_{cat} values were calculated in the carboxylation reaction (1.7 s^{-1}, For; 4.3 s^{-1}, Kor). The catalytic efficiency (K_{cat}/K_m for succinyl-CoA) of For (0.022 s^{-1} μM^{-1}) was smaller than that of Kor (0.13 s^{-1} μM^{-1}). However, it has been cleared that the tolerance to oxygen of For is higher than that of Kor, and that For is only expressed under the aerobic conditions (Yamamoto et al. 2003, 2006). *H. thermophilus* strain TK-6 not only shows small doubling time (1.5 h) in the chemoautotrophs (Igarashi et al. 1998), but also adapts to a wide range of oxygen concentrations (0–40%; unpublished data). The presence of two distinct OGORs in the strain might contribute to higher productivity and flexibility.

The OGOR assay developed in this study made use of POR as a coupling partner. The coupling of OGOR and POR activities may also find application in an assay where lactate dehydrogenase is employed to oxidize NADH.

In this work, we used a single electron donor, Fd1. The identity of the physiological electron donor in this pathway is unclear. Identification of this donor will require a survey of the various electron donors encoded within the DNA of this bacterium and a study of the ability of these donors to interact with OGORs.

A key point of novelty in this study is the kinetic analysis of OGORs in the direction of the carboxylation reaction of the rTCA cycle. In the recent years, the importance of the carbon fixation pathway in organisms

within extreme environments has been pointed out for the primary production of biomass (Hugler et al. 2005; Takai et al. 2005). The sequences of the genomes of extremophiles are accumulating, and analyses of the microbial composition and the key-functional genes are progressing in various environments. However, our current understanding of metabolic pathways in these organisms is insufficient for a more dynamic understanding of the cycle between energy and mass in the ecosystem. Kinetic analyses of the carbon fixation would become still more important. This assay that we presented to analyze the kinetics of this reaction can help in developing a more dynamic understanding of the cycle between energy and mass.

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