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Autotrophic CO_2 Fixation in *Pseudomonas* sp. Strain HD-1

(Fiksasi CO, secara Autotrof pada *Pseudomonas* sp. Strain HD-1)

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Pseudomonas **sp. strain HD-1, was reported to grow autotrophically on H_2-CO_2 by Morikawa and Imanaka (1993). However, there is no report on the mechanism of CO, fixation this strain. In our study the strain was cultivated autotrophically. The result shows no significant growth of the strain under autotrophic condition. In further study the enzyme involved in autotrophic growth was induced by inoculating heterotrophically grown cells in a complete inorganic medium in the presence of H_2-CO_2. Cell-free extract was prepared from the induced cells by sonicatioa, then the activities of the key enzymes of autotrophic CO, fixation pathways in the cell-free extract were investigated. The activities of the key enzymes of the reductive citric acid cycle (ATP:Citrate lyase) and acetyl-CoA pathway (Carbon monoxide dehydrogenase) could not be detected. In contrast, the activity of the key enzyme of the reductive pentose phosphate cycle (Ribulose-1,5- Bisphosphate Carboxylase/Oxygenase; RubisCO) was detected. The activity was in the range of 0.94 to 5.32 nmol min^{-1} mg protein-'. The data implies that the strain assimilates CO, autotrophically via reduced pentose phosphate cycle.**

INTRODUCTION

The concentration of atmospheric carbon dioxide has been increased presently, due to various human activities which may lead to a number of environmental destructions. The amount of the atmospheric CO_2 can be reduced through biological activities, such as microbial CO, fixation.

Pseudomonas sp. strain HD-1 was isolated by Morikawa and Imanaka in 1993. They reported that (i) the strain is gram negative, facultative anaerobic bacterium, (ii) the strain can grow anaerobically on CO_2 as the sole carbon source in the presence of H_2, and (iii) the strain can also degrade hydrocarbon under anaerobic condition (Morikawa and Imanaka 1993). However until now, nothing has been reported on the autotrophic CO_2 fixation of the strain. Therefore, we attempted to study the pathway for autotrophic CO_2 fixation of the strain.

Three biological CO_2 fixation pathways have been found in autotrophic microorganisms: The first mechanism is a reductive pentose phosphate cycle (Calvin cycle) (a). The key enzymes of the cycle are Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase (RubisCO) and Phosphoribulokinase (PRK) (Tabita 1988, McFadden 1989, and Gutteridge 1990). The second is CO_2 fixation through reductive citric acid cycle (b) which is a reversed process of the Tricarboxylic Acid cycle, involving key enzymes namely ATP:Citrate lyase, pyruvate synthase, and 2-oxoglutarate-synthase (Ivanovsky *et al.* 1980 and Fuchs 1989). Another mechanism is reductive non cyclic Acetyl-CoA pathway (c) which involves the key enzymes Carbon monoxide dehydrogenase (CODH) and Acetyl-CoA synthase (Jansen and Thauer 1984, Eikman *et* al. 1985, and Wood *et al.* 1986).

Pathway (a) is the only autotrophic pathway occuring in plants and occurs mainly in aerobic bacteria. Pathways (b) and (c) are present in anaerobic bacteria or in microaerophilic bacteria whose metabolism exhibits anaerobic traits (Strauss and Fuch 1993). Our definition of an autotrophic organisms is organisms which use CO_2 (or CO) as the source of carbon for growth. This definition does not include all organisms which utilize one-carbon organic compounds, such as format, metanol, metil amin or metan as the source of carbon.

In this study, we cultivate the strain under autotrophic condition, and measure the activity of the enzymes involved in each pathways.

MATERIAL AND METHODS

Autotrophic cultivation. *Pseudomonas* sp. strain HD-1 was cultivated at 37°C in 50 ml of Basal Medium (BM) which consists of 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgCl_2.6H_2O$ in dionized water. The pH was adjusted to 7.0. After inoculation, gas exchange was conducted by flowing the gas mixture of CO, and H_2 (CO, :H_2=1:1) at 500 ml/min. The gas exchange was performed at every other day with the total cultivation period of 1-10 days. Autotrophic cultivation was also performed by using solidified medium prepared by the addition of 1.5% agar onto the BM. For solid cultivation, we used vials and plates. The gas phase of the vials was mixed to newly prepared mixed gas (CO_2:H_2= 1:1) daily. The plates were set in a glass container, gas exchange was performed by the gas mixture (CO_2:H_2= (1:1)) for 45 minutes every other day.

Heterotrophic cultivation. The strain was cultivated at 37°C in 50 ml of Lactose Broth Medium (LB) consisted of 10% Tryptone, 5% Yeast extract, 5% NaCl in 1 liter deionized water.

Induction of the enzymes for autotrophic growth. The following two-stages cultivation was performed to induce enzymes involved in autotrophic growth after heterotrophic cultivation. The strain was cultivated in 11 of

LB by using a shaking flask (3l-volume) at 37°C overnight. After collecting the cells by centrifugation at 7000 rpm (2-4°C) for 10 min, the cell pellets were washed by using BM and centrifuged at 7000 rpm (2-4°C) for 20 min. The cell pellets were resuspended in BM and 1 ml of the cell suspension was put into a vial (100 ml-volume) containing 5 ml of BM. The gas mixture of CO_2 and H_2 (CO_2 :H_2=1:1) was supplied for 3 min into the vials to exchange the gas phase. The vials were shaken at 37°C with total cultivation period of 1-10 days. The cells prepared by this procedure were used in the following section.

Preparation of cell-free extract. After collecting the cells by centrifugation at 6000 rpm (2-4°C) for 10 min, the cell pellets were resuspended in 100 mM KH_2PO_4 buffer (pH 7.0). After sonication of the cell suspension (Branson Sonifier 250 D, Pulse: 50%, Out put: 3), the suspension was used directly for the measurement of enzyme activities.

Measurement of ATP:Citrate lyase. In the following assay scheme, the products of ATP:Citrate lyase, oxaloacetate, acts as a subrate for endogenous malate dehydrogenase to bring about the oxidation of NADH, which can be measured spectrophotometrically. The assay mixtures contained in final volume of 2 ml were 20 µl of 20 mM NADH, 20 ml of 600 mM citrate, 10 µl of 150 mM ATP, 20 µl of 0.08 mM CoA, 20 µl of 360 mM $MgCl_2.6H_2O$, 1.41 ml of 100 mM Tris-HCl (pH 8.5) and 500 µl of cell free extract (1.06 - 16,2 mg of protein/ml). Optical assays were performed by using a spectrophotometer at 340 nm and 37°C for 30 min.

Measurement of Carbon monoxide dehydrogenase (CODH). In the following assay, an artificial electron carrier, methyl viologen, was used as an electron carrier. Three hundred microliters of cell free extract (1.06 - 16.2 mg protein/ml), 1150 µl of 100 mM KH_2PO_4 (pH=7.0) and 50 µl of 600 mM methyl viologen were added into a vial flask which was closed with a rubber and an aluminium cap. Oxygen was removed by purging N_2 gas for 3 minutes. Then, CO gas was introduced for 5 seconds. Following this, vials were incubated at 45°C for 1 hour for qualitative analysis.

Measurement of Ribulose Bisphosphate Carboxylase/Oxygenase (RubisCO). Fifty micro liters of cell-free extract, 50 µl of BEMD buffer (50 mM Bicine, 0.1 mM EDTA, 10 mM $MgCl_2.6H_2O$, 1 mM Dithiothreitol, 25 mM $NaHCO_3$, pH 7.8), and 35 µl of 100 mM $NaHCO_3$ (consist of 1.93 mM $NaH^{14}CO_3$ and 98.05 mM $NaHCO_3$) were incubated at 37°C for 10 minutes in a small test tube. The radioactive specific activity was measured in dpm (disintegration per minute). After incubation, 20 µl of 10 mM RuBP (Ribulose 1,5-Bisphosphate) was added and reaction was carried out at 37°C for 30 minutes. The enzyme reaction was stopped by addition of 100 µl of glacial acetic acid. Then, 200 µl of the solution was transferred into a vial and incubated at 70°C for 1 hour to eliminate unfixed $NaH^{14}CO_3$. After this procedure, the remaining radioactivity was measured by scintillation counting. The activity of the enzyme was calculated in nmol min^{-1} mg $protein^{-1}$.

Measurement of Hydrogenase. The activity of hydrogenase was measured using methyl viologen as an electron acceptor. Four hundred microliters of cell-free extract, 50 µl of 600 mM methyl viologen and 1.5 ml of 100 mM KH_2PO_4 (pH 7.0) were added into a vial. Then, H_2 was supplied into vials by bubbling with the gas for 3 minutes. Qualitative assay was performed at 37°C for 40 minutes.

Measurement of Protein Content. Protein was determined according to Bio-Rad protein assay procedure. Several dilution of protein standard (Bovine serum albumin) was prepared containing from 1 to 25 µg/ml. As much as 0.8 ml of standards and appropriately diluted samples were mixed in clean, dry test tube. Sample buffer (0.8 ml) was used as blank. The Dye Reagant Concentrate (0.2 ml) was added and the test tube was vortex briefly. Protein content was measured by using a spectrometer at 595 nm.

RESULTS

Autotrophic cultivation. To study the carbon dioxide fixation pathway, it was necessary to cultivate the strain autotrophically. However, unlike the previous report (Morikawa and Imanaka 1993), we could not succeed in cultivating the strain autotrophically in a liquid medium.

Our next strategy was to induce the enzymes involved in autotrophic CO_2 fixation by changing the culture conditions from heterotroph to autotroph. The enzyme responsible for hydrogen metabolisms (hydrogenase) would be expected to be induced as well. Therefore we measured the hydrogenase activity in order to check the induction. The data indicated that the strain HD-1 has hydrogenase activity (Table 1).

Measurement of enzymatic activity. The strain showed RubisCO activity under induction cultivation (Table 2). The activity was in the range of 0.94 to 5.32 nmol min^{-1} mg $protein^{-1}$. In contrast, under non-induction condition, none of the enzyme activities (RubisCO, ATP:Citrate lyase and CODH) was detected (Table 3).

DISCUSSION

Autotrophic cultivation. Because we could not succeed in cultivating the strain autorophically in a liquid medium, we further cultivated the strain autotrophically on solidified medium. However, in spite of all of these efforts, we could not get enough amount of autotrophically grown cells. Our next strategy was to let the strain induces hydrogen metabolisms, by changing the culture conditions from heterotrophic to autotrophic. In the second-stage cultivation (autotrophic cultivation), the strain showed limited growth (Table 1). This means that the strain has at least the ability to grow autotrophically. During autotrophic cultivation, the gas phase was H_2-CO_2. This means that the enzyme involved in autotrophic growth were induced, the enzyme catalyzing hydrogen metabolisms, namely hydrogenase, should also be induced. By using the cell-free extract prepared from various stages of autotrophic cultivation, hydrogenase activity was measured. Hydrogenase activity in this experiment implied that other enzymes may be induced.

Measurement of enzymatic activity. We used the cell-free extract from the induced cells (second stage

Tabel 1. OD_{540} and Hydrogenase activity during the second-stage cultivation

Incubation time (days)	OD 540	Hydrogenase activity
2	4.08	+
4	4.23	+
6	4.36	+
8	4.42	+
10	4.47	+

+; activity deteceted

Tabel 2. Enzymatic activities in induced cells

Number of cultivation	RubisCO (nmol min-1 mg-1)	ATP:Citrate lyase	CODH
F	5.30	N.D	N.D
G	0.94	N.D	N.D
H	4.95	N.D	N.D
I	5.32	N.D	N.D
P	1.12	N.D	N.D

N.D.: Not detected

Tabel 3. Enzyme activities in non-induced cells

Number of cultivation	OD 540	RubisCO	ATP:Citrate lyase	CODH
X	9.11	N.D	N.D	N.D
Y	5.42	N.D	N.D	N.D

N.D.: Not detected

cultivation) for the measurement of RubisCO, ATP:Citrate lyase and Carbon monoxide dehydrogenase activities. These are key enzymes in biological CO_2 fixation pathways.

RubisCO is key enzyme for Calvin Benson Cycle and catalyzes the following reaction: Ribulose 1,5-Bisphospate + CO_2 -------> 2 x 3-Phosphoglyceric acid. The fixation of CO_2 occurs through its condensation with a C-5 acceptor (Ribulose 1,5-Bisphospate) to form two molecules of 3-phosphoglyceric acid (Tabita 1994). ATP:Citrate lyase is the key enzyme for reductive TCA cycle and catalyzes the following reaction: Citrate + ATP + CoA -------> Acetyl-CoA + Oxaloacetate + ADP P1 (Ivanovsky *et al.* 1980). The reaction scheme of CODH, the key enzyme of acetyl-CoA pathway, is as follows: CO + H_2O + X_{ox} --------> CO_2 + $2H^+$ + X_{red} (X:being electron carrier) (Wood *et al.* 1986). Under noninduced conditions, none of the enzymatic activities (RubisCO, ATP:Citrate lyase and CODH) was detected (Table 3). Table 2 shows that induced cells of *Pseudomonas* sp. strain HD-1 has RubisCO activity. Chung *et al.* (1994) reported that cell-free extract of *P. hydrogenothermophila* strain TH-1 has RubisCO activity at 0.12 μmol min-1 mg protein-1 under the optimal condition. The inability of the strain to produce enough activity of RubisCO (Table 2) is probably reflected by the slow autotroph growth rate (Table 1). Under the same condition, the activities of ATP:citrate lyase and carbon monoxide dehydrogenase could not be detected (Table 2). This implies that the strain is not using reductive TCA cycle or acetyl-CoA pathway but using Calvin Cycle for autotrophic CO_2 fixation.

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REFERENCES

Chung, S.Y., K. Yokoyama, M. Gomi, N. Tearoong, M. Ishii, Y. Igarashi & T. Kodama. 1994. Purification and some properties of ribulose-1,5-bisphosphate carboxylase/Oxygenase from a thermophilic hydrogen oxidizing Bacterium, *Pseudomonas hydrogeno-thermophila* Strain TH-1. *J. Ferment. Bioeng.* **78:** 469-471.

Eikmans, B., G. Fuchs & R.K. Thauer. 1985. Formation of carbon monoxide from CO_2 and H_2. Eur. *J. Biochem.* **146:** 149-154.

Fuch, G. 1989. Alternative phatways of autotrophic CO_2 fixation, hlm. 369-372. In H.G. Schlegel and B. Bowien (ed) *Autotrophic Bacteria*, Science Tech Publishers, Wisconsin.

Gutteridge, S. 1990. Limitation of the primary events of CO_2 fixation in photosynthetic organisms: the sructure and mechanism of RubisCO. *Biochem. Biophys. Acta.* **1015:** 1-14.

Ivanovsky, R.N., N.V. Sinstov & E.N. Kondratieva. 1980. ATP-Linked citrate lyase activity in the green sulfur bacterium *Chlorobium limicola* Forma *thio-sulfatophilum*. *Arch. Microbiol.* **128:** 239-241.

Jansen, K. & R.K. Thauer. 1984. Carbon assimilation pathways in sulfate reducing bacteria. Formate, Carbon dioxide, Carbon monoxide, and acetate assimilation by *Desulfovibrio baarsii. Eur. J. Biochem.* **146:** 149-154.

McFadden, B.A. 1989. The Ribulose Bisphosphate pathway of CO_2 fixation, hlm. 353-363. Di dalam H.G. Schlegel and B. Bowien (ed), *Autotrophic bacteria*, Science Tech Publisher, Wisconsin.

Morikawa, M. & T. Imanaka. 1993. Isolation of a new mixotrophic bacterium which can fix CO_2 and assimilate aliphatic and aromatic hydrocarbons anaerobically. *J. Ferment. Bioeng.* **76:** 280-283.

Tabita, F.R. 1988. Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. *Microbiological Rev.*: 155-189.

Wood, H.G., S.W. Ragsdale & E. Pezacka. 1986. The acetyl-CoA pathway of autotrophic growth (CO dehydrogenase; acetogenesis; methanogenesis; sulfate-reducers). *FEMS Microbiol. Rev.* **39:** 345-362.