The Journal of Microbiology, March 1997, p.30-39
Copyright © 1997, The Microbiological Society of Korea
Vol. 35, No. 1

# Growth on Methanol of a Carboxydobacterium, *Acinetobacter* sp. Strain JC1 DSM 3803

**Young Tae Ro[1], Jae Goo Seo, Joohun Lee, Daemyung Kim[2] In Kwon Chung, Tae Ue Kim[3] and Young Min Kim***

*Department of Biology, College of Science, Yonsei University, Seoul 120-749, Korea*
*[2]Department of Genetic Engineering, Chungjoo University, Chungjoo 360-764, Korea*
*[3]Department of Medical Technology, College of Health Science, Yonsei University, Wonju 222-701, Korea*

(Received February 11, 1997 / Accepted March 8, 1997)

***Acinetobacter* sp. strain JC1 DSM 3803, a carboxydobacterium, was found to grow methylotrophically at the expense of methanol and methylamine, but not of methane, formaldehyde, formate, dimethylamine, or trimethylamine, as the sole source of carbon and energy. The doubling times of the bacterium growing on methanol (0.5%, v/v) and methylamine (0.5%, w/v) at 30°C and pH 6.8 were 4.8 h and 5.7 h, respectively. Cells grown on methanol, however, failed to show typical methanol dehydrogenase and oxidase activities. The cell was found to contain no *c*-type cytochromes. Cells grown on methanol exhibited higher catalase activity than those grown on pyruvate or glucose. The catalase present in the cells also exhibited peroxidase activity. The catalase activity, growth on methanol of the cell, and oxygen consumption by methanol-grown cells were inhibited strongly by 0.1 mM, 1 mM, and 0.1 mM hydroxylamine, respectively. Formaldehyde dehydrogenase, formaldehyde reductase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase activities were detected from cells grown on methanol.**

**Key words: Carbon monoxide, methanol, carboxydobacteria, *Acinetobacter* sp. JC1, catalase, peroxidase**

Carboxydobacteria are a group of aerobic bacteria which are able to grow chemolithoautotrophically at the expense of carbon monoxide (CO) as sole carbon and energy sources (31, 40). The bacteria, except *Streptmyces thermoautotrophicus* which grows only on CO and $H_2/CO_2$, however, are facultative chemoautotrophs and are able to utilize several organic materials as growth substrates (31, 40). Among the carboxydobacteria, *Pseudomonas gazotropha* has been known as the only bacterium capable of growing on methanol as the sole source of carbon and energy and recognized as the first example able to grow by means of three types of nutrition, i.e. organotrophy, autotrophy, and methylotrophy (31, 41, 45, 46, 53).

In earlier work, *Acinetobacter* sp. strain JC1 DSM 3803, a carboxydobacterium isolated in Korea, was not observed to grow on methanol (12). We, however, recently recognized through careful observation that the cell can grow with methanol and methylamine as the carbon and energy sources.

In this study, we report the interesting nutrition and novel mechanism for methanol dissimilation in *Acinetobacter* sp. JC1. We could not detect the well-known methanol dehydrogenase (MDH) and methanol oxidase activities from cells grown on methanol. The cell, therefore, may adopt other enzyme for the oxidation of methanol.

## Materials and Methods

### Organism and cultivation

*Acinetobacter* sp. JC1 DSM 3803 (12) was cultivated chemoautotrophically at 30°C in mineral medium (30) with a gas mixture of 30% CO-70% air. For heterotrophic and methylotrophic growth, the cell was grown at 30°C in the mineral medium supplemented with appropriate concentration of each substrate. For several experiments, *Methylobacterium extorquens* AM1 (NCIB 9133) and *Candida boidinii* (KCTC 1712) were used as controls. Growth was measured by turbidity determined at 436 nm using a spectrophotometer.

### Cell-free extracts preparation

Cells were harvested by centrifugation for 30 min at 10,000×*g*, washed once by resuspension in 0.05 M potassium phosphate buffer (pH 7.0, standard buffer). The washed cells were resuspended in the same buffer, homogenized, and disrupted by sonic treatment (10 s per ml). The suspension was then centrifuged at 10,000×*g* for 30 min, and the result-

* To whom correspondence should be addressed
[1] Present address: Southwestern Foundation for Biomedical Research, P.O. Box 2817, San Antonio, Texas 78228-0147, USA.

ing supernatant was used as crude cell extracts. Proteins were determined by the method of Lowry *et al.* (37), using bovine serum albumin as a standard, after boiling the extracts in 20% NaOH for 10 min (30).

**Enzyme assays**

All assays were carried out at 30°C, otherwise described.

Pyrroloquinoline quinone (PQQ)-containing MDH activity, present in Gram-negative methylotrophs, was assayed by measuring the methanol-dependent decrease in the absorbancy at 600 nm of 2,6-dichlorophenol indophenol (DCPIP, $\varepsilon_{600}=22.0\times10^3$ $M^{-1}$ $cm^{-1}$; 8) by the method of Anthony and Zatman (6) with several modifications. The reaction mixture contained the following in a total volume of 1 ml: Tris-HCl (pH 9.0), 100 μmol; phenazine methosulfate (PMS), 1.1 μmol; DCPIP, 43.3 nmol; $NH_4Cl$, 15 μmol; KCN, 1 μmol; appropriate amount of cell-free extract. The reaction was started by addition of methanol (6.7 μmol) to the reaction mixture.

NAD-dependent PQQ-MDH activity, observed in *Amycolatopsis methanolica* which was previously known as *Streptomyces* sp. 239 (29) and *Nocardia* sp. 239 (23), was assayed by measuring the methanol-dependent decrease in the extinction of DCPIP by the method of Duine *et al.* (19).

NAD-dependent MDH activity, detected in methylotrophic *Bacillus* (7), was assayed by measuring the methanol-dependent NADH ($\varepsilon_{340}=6.22\times10^3$ $M^{-1}cm^{-1}$; 15) production by the method of Arfman *et al.* (7) in a 1-ml assay system.

NAD-dependent, glutathione (GSH)-requiring MDH activity which was observed in methylotrophic yeasts (39) was assayed by measuring the methanol-dependent NADH production at 340 nm following the method of Mehta (39) in a 1-ml assay system.

Cytochrome *c*-dependent MDH activity, detected in *A. methanolica* (29), was assayed by the methanol-dependent increase in the extinction at 550 nm by the method of Kato *et al.* (29) with several modifications in a reaction mixture (1 ml) containing: potassium phosphate buffer (pH 7.0), 50 μmol; cytochrome *c* (Type III, from horse heart, Sigma Chem. Co.), 0.5 mg; cell extract. The reaction was started by the addition of 50 μmol methanol.

*N,N'*-dimethyl-4-nitrosoaniline (NDMA)-dependent MDH activity, observed in *A. methanolica* (11), was assayed by measuring the methanol-dependent reduction of NDMA ($\varepsilon_{440}=35.4\times10^3$ $M^{-1}cm^{-1}$, 20) by the method of Bystrykh *et al.* (11).

Methanol oxidase which is present in methylotrophic yeasts (3) was assayed by measuring the amount of formaldehyde or $H_2O_2$ produced during enzyme reaction (50) and also by a method developed in this experiment on the basis of the peroxidase assay of Basson *et al.* (10). Formaldehyde was produced in a reaction mixture as described previously by Tani *et al.* (50). After incubation for 30 min, the reaction was stopped by the addition of 0.2 ml of 4 N HCl. Precipitates were removed by centrifugation for 5 min at 8,000×*g*, and supernatants were used for determination of formaldehyde using the methods of Nash (44) and Avigad (9). $H_2O_2$ was produced by the method of Tani *et al.* (50) with several modifications. The reaction mixture (0.9 ml) contained 50 μg peroxidase, 0.33 μmol *o*-dianisidine, 5.3 μmol methanol, 100 μmol potassium phosphate buffer (pH 7.5), and cell extracts. Reaction was carried out for 30 min and stopped by the addition of 0.1 ml of 4 N HCl. After removing precipitates by centrifugation (8,000×*g*/5 min), absorbancy of the supernatant was measured at 460 nm. The reaction mixture (1 ml) of the newly developed method based on the Basson *et al.* (10) contained 50 μmol potassium phosphate (pH 7.0), 1 μmol 2,2-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 5 μg horse radish peroxidase, and cell-free extract. The reaction was started by the addition of 50 μmol methanol and absorbance of the green $ABTS^{\cdot+}$ cation radical was measured at 660 nm.

Catalase activity was assayed by the method of Couderc and Baratti (14) with a modification. The reaction mixture (1 ml) contained 50 μmol potassium phosphate buffer (pH 7.0) and 12.5 μmol $H_2O_2$ ($\varepsilon_{240}=43.6$ $M^{-1}cm^{-1}$; 24). The reaction was started by the addition of cell-free extracts.

Peroxidase activity was measured by the method of Hochman and Goldberg (25) with several modifications. The reaction mixture (1 ml) contained 100 μmol citric acid, 12.5 μmol $H_2O_2$, and 0.25 μmol *o*-dianisidine. The reaction was started by the addition of an appropriate amount of cell-free extracts and the rate of oxidation of *o*-dianisidine was measured at 460 nm.

NAD-dependent formaldehyde dehydrogenase (FADH) activity, detected in *A. methanolica* (19), was assayed by measuring the formaldehyde-dependent reduction of DCPIP at 600 nm by the method of Duine *et al.* (19).

NAD-dependent, GSH-requiring FADH activity, observed in *C. boidinii* (27), was assayed by measuring the formaldehyde-dependent reduction of NAD by the method of Couderc and Baratti (14) with some modifications. The reaction mixture (1 ml) contained 50 μmol potassium phosphate buffer (pH 7.5), 0.4 μmol NAD, 6 μmol GSH, and cell extracts. The reaction was started by the addition of 1

μmol formaldehyde.

Formate dehydrogenase (FMDH) activity was assayed by measuring the formate-dependent formation of NADH or ferrocyanide ($\varepsilon_{420}=1.02\times10^3\ M^{-1}cm^{-1}$; 16) following the method described by Höpner *et al.* (26).

Formaldehyde reductase (FAR) activity was assayed by measuring the formaldehyde-dependent oxidation of NADH at 340 nm by the method of Arfman *et al.* (7).

Glucose-6-phosphate dehydrogenase (GPDH) and 6-phosphogluconate dehydrogenase (PGDH) activities were assayed by measuring the glucose-6-phosphate- and 6-phosphogluconate-dependent reduction of NADP at 340 nm by the method of Kato *et al.* (28).

NADH dehydrogenase activity was assayed by measuring the NADH-dependent reduction of DCPIP at 600 nm by the method of Arfman *et al.* (7).

NADH oxidase activity was measured at 340 nm following the method of Arfman *et al.* (7).

### Determination of oxygen consumption

The rate of oxygen consumption by whole cells and cell-free extracts was determined at 30°C using a biological oxygen monitor (YSI-5300, Yellow Springs Instruments) equipped with a Clark-type oxygen electrode. Cells growing on methanol or glucose at the mid-exponential growth phase were harvested, washed twice with the standard buffer, and assayed immediately. The reaction mixture (3 ml) contained the standard buffer and appropriate amount of cell suspension or cell extracts. After air saturation and temperature equilibration at 30°C, the reaction was started by the addition of an appropriate amount of methanol or glucose. The solubility of oxygen in air-saturated water at 30°C was counted as 7.559 mg per l (22).

### Determination of methanol concentration

The amount of methanol present in the culture media during growth of the cell was determined using a gas chromatograph (GC-8A, Shimadzu, Japan) equipped with a thermal conductivity detector and a Porapak Q column (stainless steel, 2 mm × 1 m). Cell-free supernatants were prepared after centrifugation of the liquid cultures and analyzed for remained methanol. The media supplemented with methanol, but was not inoculated, was used as a control. Helium was used as a carrier gas at a flow rate of 20 ml per min. The temperatures for column, injection port, and detector were 100°C, 180°C, and 180°C, respectively. The injection volume was 0.5 μl, and triplicate measurements were performed for each assay. Under the conditions used, the retention time of methanol was 2.2 min.

### Electrophoresis

Nondenaturing polyacrylamide gel electrophoresis (PAGE) was carried out in gels containing 7.5% acrylamide by the method of Laemmli (34), but without sodium dodecyl sulfate (SDS), as described by Kim and Hegeman (30). Gels were stained with 0.25% Coomassie brilliant blue R-250 (CBB) (52). Activity staining for catalase was performed by the method of Clare *et al.* (13) with some modifications. The nondenaturing gel was pre-equilibrated with cold standard buffer for 5 min. The gel was then transferred to a small box containing 50 ml of standard buffer and 0.5 mg horse radish peroxidase. After 45 min, $H_2O_2$ was added to a concentration of 10 mM and the gel was left for 5 min under the condition. The treated gel was then washed several times with distilled water and stored in 20 mM diaminobenzidine solution until the translucent catalase bands were visualized out of the dark-brown backgrounds. For peroxidase staining, the gel pre-equilibrated in cold 50 mM citrate buffer (pH 4.5) for 5 min was transferred to a small box containing 50 ml of the citrate buffer, 2.5 mM *o*-dianisidine and 10 mM $H_2O_2$. The gel was then left under the condition until the dark-reddish peroxidase bands were appeared.

The activity staining of methanol oxidase was carried out by a method based on the method for peroxidase staining. After nondenaturing PAGE, the gel was washed with distilled water and soaked for 5 min in 100 mM potassium phosphate buffer (pH 7.5) containing 2.5 mM *o*-dianisidine and horse radish peroxidase (20 μg per ml). Methanol was then added to a final concentration of 50 mM, and gels were left until the dark-reddish bands were appeared. The gels stained by activity were washed several times with distilled water and kept in 7.5% acetic acid solution.

### Restriction analysis of chromosomal DNA

Total chromosomal DNAs were prepared from cells grown on methanol, CO, and nutrient broth by the method of Morris and Lidstrom (42). The DNAs were digested with *Eco*RI (Promega, U.S.A.) according to the manufacturer's suggestions. Electrophoresis was carried out using gels containing 1.0% agarose following the method described by Sambrook *et al.* (49).

## Results

### Growth on methylotrophic substrates

Among several methylotrophic substrates tested,

**Table 1.** Growth of *Acinetobacter* sp. JC1 on various substrates[a]

| Substrates | Concn (%) | Doubling time (h) |
|---|---|---|
| CO | 30[b] | 19 |
| Methane | 30[b] | NG[c] |
| Methanol | 0.5[b] | 4.8 |
| Formaldehyde | 0.5[b] | NG |
| Formate | 0.5[d] | NG |
| Methylamine | 0.5[d] | 5.7 |
| Dimethylamine | 0.5[b] | NG |
| Trimethylamine | 0.5[b] | NG |
| Ethanol | 0.5[b] | 3.3 |
| Acetate | 0.5[d] | 3.5 |
| Pyruvate | 0.5[d] | 3.6 |
| Glucose | 0.2[d] | 8.7 |
| Nutrient broth | 0.8[d] | 3.6 |

[a]Cells were grown at 30°C in liquid mineral medium supplemented with different substrates.
[b]Volume per volume.
[c]No growth.
[d]Weight per volume.

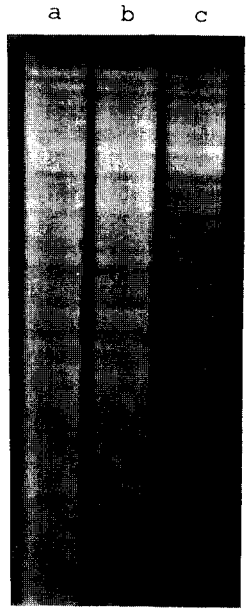


**Fig. 1.** Restriction patterns of total DNAs of *Acinetobacter* sp. JC1 grown on different substrates. DNAs were prepared from cells grown on CO (lane a), methanol (lane b), and nutrient broth (lane c) by the method of Morris and Lidstrom (42). DNAs were digested with *Eco*RI and electrophresed in gels containing 1.0% agarose as described in Materials and Methods.

*Acinetobacter* sp. JC1 was found to utilize methanol or methylamine, but not methane, formaldehyde, formate, dimethylamine, and trimethylamine, as a source of carbon and energy (Table 1). The rate of growth on methanol or methylamine was found to be slower than those on ethanol, pyruvate, or nutrient broth, but was faster than that on CO. The cells, however, required a lag period of 7 to 8 days to grow on methanol or methylamine when cells growing even at the exponential phase on other heterotrophic substrates or CO were first transferred to the medium containing methanol or methylamine. The lag period was not observed once the cells were adapted to grow on methylotrophic substrates.

Cells growing on CO, methanol, pyruvate, and glucose as a sole source of carbon and energy formed homogeneous colonies during several transfer of a single colony formed on carboxydotrophic substrate to methylotrophic and heterotrophic media, and *vice versa*. The numbers of colonies formed after spreading diluted cultures of CO-grown cells to the methanol-containing media were equal to those formed after spreading of the same cultures to the pyruvate-containing media, and *vice versa* (data not shown). Cells grown on different substrates were found to be identical in several biochemical and molecular properties including the restriction pattern of the total DNA (Fig. 1).

*Acinetobacter* sp. JC1 grew most rapidly in mineral medium containing 0.5% methanol (v/v) among several concentrations of methanol tested. The growth of the cells with 0.5% methanol ($t_d$=4.8 h) was almost twice faster than that with 2.0% ($t_d$=9.5 h) or 3.0% ($t_d$=10.0 h) methanol. Cells in the lag phase consumed little or no methanol. The cells, however, consumed methanol very rapidly once they started to grow and used it up almost completely by the beginning of the stationary phase (data not shown).

## Methanol oxidation

When cells of *Acinetobacter* sp. JC1 growing on methanol at the exponential growth phase were tested for methanol-dependent oxygen consumption, the cells were found to consume oxygen very actively at a rate of 135 nmol oxygen per mg cell (dry weight) per min. The cell extracts consumed oxygen at a rate of 2.5 nmol per mg protein per min.

Reduced (10 mM dithiothreitol) minus oxidized (10 mM ferricyanide) spectra (400 to 600 nm) obtained using Hitachi U-2000 spectrophotometer of cell-free extracts of methanol-grown cells of *Acinetobacter* sp. JC1 revealed the absence of the typical absorption peakes for *c*-type cytochromes. Cell extracts of *M. extorquens* AM1 and *M. methylotrophus*, on the other hand, showed peaks of $\alpha$, $\beta$, and $\gamma$ for *c*-type cytochromes at 550.5, 520.5, and 415.5, respectively.

All attempts to detect the ammonium-requiring, PQQ-containing, dye (PMS/DCPIP)-linked MDH ac-

**Table 2.** Activities of several enzymes in *Acinetobacter* sp. JC1 grown on methanol

| Enzymes | Sp. act.[a] |
|---|---|
| Formaldehyde dehydrogenase | |
| Dye-linked | 4.8±0.2[b] |
| Glutathione-requiring | 14.5±0.7[c] |
| Formaldehyde reductase | 20.1±1.2[d] |
| Formate dehydrogenase | 1.4±0.1[c] |
| Glucose 6-phosphate dehydrogenase | 35.4±2.8[e] |
| 6-Phosphogluconate dehydrogenase | 53.1±3.2[e] |
| NADH oxidase | 21.3±1.9[d] |
| NADH dehydrogenase | 60.5±3.1[b] |

[a]Values are the means±standard deviations of five experiments.
[b]Nanomol 2,6-dichlorophenol indophenol reduced per milligram protein per min.
[c]Nanomol NADH produced per milligram protein per min.
[d]Nanomol NADH oxidized per milligram protein per min.
[e]Nanomol NADP reduced per milligram protein per min.

tivity (6) in cell extracts prepared from cells of *Acinetobacter* sp. JC1 grown on methanol failed. The activities of the NAD-dependent, PQQ-containing, dye-linked (DCPIP) MDH (19), the cytochrome *c*-dependent MDH (29) and methanol:NDMA oxidoreductase of *A. methanolica* (11), the NAD-dependent MDH of methylotrophic *Bacillus* strains (7), and the GSH-requiring, NAD-dependent MDH of methylotrophic yeasts (39) also were not detected.

It was found that cell extracts prepared from cells of *Acinetobacter* sp. JC1 grown on methanol did not produce measurable amounts of formaldehyde and $H_2O_2$ when the extracts were incubated with methanol, whereas the extracts from *C. boidinii* grown on methanol, which was used as a control, produced large amounts of the two products (formaldehyde, 19.64 nmol per mg protein per min; $H_2O_2$, 19.63 nmol per mg protein per min). In an another experiment to detect methanol oxidase activity employing peroxidase and ABTS, the cell extracts from *Acinetobacter* sp. JC1 grown on methanol did not produce green $ABT^{\cdot+}$ radical, whereas the extracts from methanol-grown cells of *C. boidinii* exhibited methanol oxidase activity and resulted in a change in absorbancy at 660 nm of 23.6 per mg protein per min. Activity staining after non-denaturing PAGE of cell-free extracts prepared from cells grown on methanol revealed that there was no protein in cell extracts of *Acinetobacter* sp. JC1 which was stained by methanol oxidase activity; the extracts from *C. boidinii* showed the methanol oxidase band, but that from *M. extorquens* AM1 also did not.

Cell-free extracts of *Acinetobacter* sp. JC1 grown on methanol showed both the NAD-dependent, dye-linked (DCPIP) FADH and the NAD-dependent, GSH-requiring FADH activities (Table 2). The extracts also exhibited FAR, NAD-dependent FMDH, GPDH, PGDH, NADH oxidase, and NADH dehydrogenase activities.

**Table 3.** Catalase and peroxidase activities in *Acinetobacter* sp. JC1 grown on different substrates

| Substrates | Enzymes[a] | |
|---|---|---|
| | Catalase[b] | Peroxidase[c] |
| Methanol | 23.9±1.9 | 2.2±0.2 |
| Glucose | 2.8±0.3 | 0.3±0.01 |
| Pyruvate | 6.0±0.5 | 0.4±0.01 |

[a]Values are the means±standard deviations of four experiments.
[b]Micromol $H_2O_2$ disappeared per milligram protein per min.
[c]$\Delta_{460}$ of o-dianisidine per milligram protein per min.

Cell extracts of methanol-grown cells of *Acinetobacter* sp. JC1 exhibited catalase activity 4 and 8 times, respectively, higher than those of the extracts prepared from pyruvate- and glucose-grown cells (Table 3). Cells grown on methanol also exhibited higher peroxidase activity than those grown on pyruvate or glucose. Activity staining after non-denaturing PAGE revealed that cells grown on methanol contain more amount of catalase than those of the cells grown on pyruvate and glucose (Fig. 2A). The major catalase present in *Acinetobacter* sp. JC1 was found to exhibit peroxidase activity (Fig. 2B), indicating that the enzyme is a catalse-peroxidase exhibiting both catalase and peroxidase activities.

## Inhibition of growth, oxygen consumption, and enzyme activities

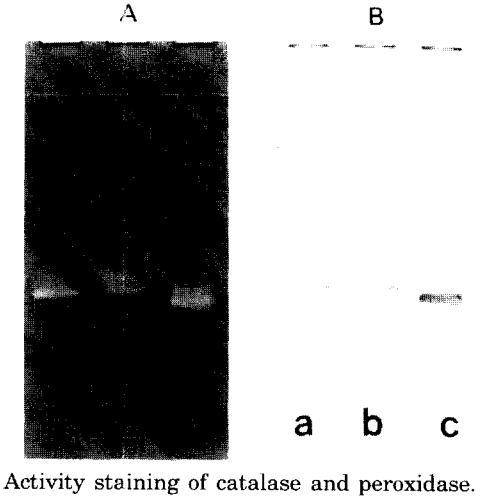


**Fig. 2.** Activity staining of catalase and peroxidase. Activity stainings for catalase (A) and peroxidase (B) after non-denaturing PAGE (7.5% acylamide) with 80 μg each of the cell-free extracts prepared from cells grown on pyruvate (a), glucose (b), and methanol (c) were performed with methanol as a substrate as described in Materials and Methods.

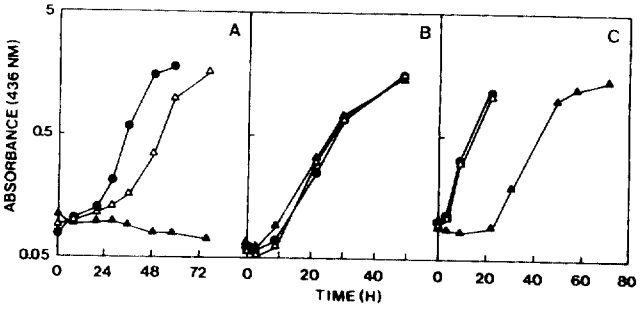


**Fig. 3.** Effect of hydroxylamine on the growth of methylotrophic bacteria. *Acinetobacter* sp. JC1 was grown on methanol (A) or glucose (B) and *M. extorquens* AM1 was grown on methanol (C) in the presence (-△-, 0.1 mM; -▲-, 1.0 mM) or absence (-●-) of hydroxylamine.

It was found that 3-amino-1,2,4-triazole (aminotriazole), an inhibitor of the typical catalase (38), added into the medium did not affect the growth on methanol or glucose of *Acinetobacter* sp. JC1 even at 20 mM. Hydroxylamine which is known to inhibit the manganese-containing catalase (2, 33), however, inhibited the growth on methanol of the cell completely at 1 mM (Fig. 3A). The growth of the cell on glucose was not affected by the presence of hydroxylamine at the same concentration in the medium (Fig. 3B). The growth of *M. extorquens* AM 1 on methanol was not inhibited by 0.1 mM hydroxylamine, but the cell showed a lag period of about 22 h when it was cultivated in the presence of 1 mM hydroxylamine (Fig. 3C). It was also found that 0.1 mM hydroxylamine present in the assay mixture for measuring the rate of oxygen consumption of methanol-grown cells inhibited 99% of the rate of methanol-dependent consumption measured in the absence of hydroxylamine (Fig. 4). When cells grown on glucose were tested for the glucose-dependent consumption of oxygen in the presence of 0.1 mM hydroxylamine, the rate of oxygen consumption was reduced only by 4% of the rate observed in the absence of hydroxylamine. When catalase in the cell extracts prepared from cells grown on methanol or glucose was assayed in the presence of hydroxylamine (0.1 mM), only less than 2% of the enzyme activity measured in the absence of hydroxylamine was detected. Aminotriazole, however, did not affect the rate of oxygen consumption and catalase activity even at 10 mM.

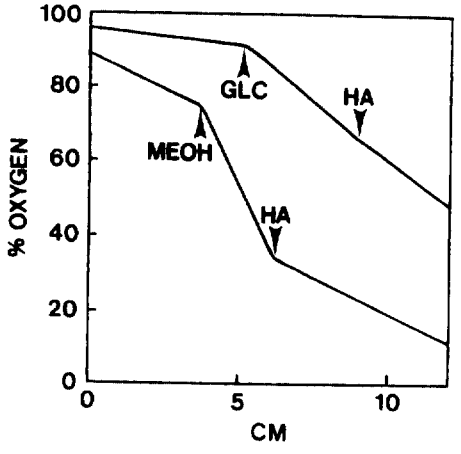


**Fig. 4.** Effect of hydroxylamine on the rate of $O_2$ consumption in whole cells of *Acinetobacter* sp. JC1. The rate of glucose- or methanol-dependent $O_2$ consumption was determined using an oxygen monitor in the presence or absence of 0.1 mM hydroxylamine (HA) as described in Materials and Methods. Arrows indicate the time when glucose (GLC; 22.3 nmol) and methanol (MEOH; 14 μmol) were injected.

## Discussion

Several groups of microorganisms are known to grow aerobically on compounds containing one or more carbons but no carbon to carbon bonds as the sole source of carbon and energy (3, 4). In carboxydobacteria, *P. gazotropha* has been known as the only bacterium able to grow on methanol as sole carbon and energy sources (31, 41, 45, 46, 53).

We found in this experiment that *Acinetobacter* sp. JC1, another carboxydobacterium, also grows on methanol and methylamine. We were suspicious at first on the purity and identity of the cultures growing on methanol and methylamine and considered about the possibility of contamination of *Acinetobacter* sp. JC1 culture by certain methylotrophic microorganisms during the week-long lag period required for the initial growth on the methylotrophic substrates. The suspicion, however, was eliminated through careful examination of the homogeneity of colonies formed on different media and of several properties including restriction patterns of total DNAs of the cells grown on different substrates. The recent observations that CO-DH in *Acinetobacter* sp. JC1 is constitutive and the enzymes present in cells grown on different substrates are identical in size and molecular properties (47) also support the present observations. It has been reported that *Thiosphaera pantotropha* which is able to grow lithotrophically with thiosulfate or molecular hydrogen as an energy source acquired the ability to grow on methanol as a sole carbon and energy sources through mutational events during incubation in methanol-containing mineral medium (21). The possibility of mutational acquisition of the methylotrophic ability was also eliminated since the CO-grown cells which were

spreaded on methanol- and pyruvate-containing media formed same numbers of colonies on the two media, and *vice versa*.

The reduction of methanol concentration in culture media during growth of the cell and the active consumption of oxygen by the methanol-grown cells in the presence of methanol confirmed that *Acinetobacter* sp. JC1 grew on methanol as a growth substrate. The growth with 2.0% or 3.0% methanol, however, was much slower than that with 0.5% methanol, the optimal concentration, indicating that methanol, possibly certain metabolites of methanol, are still inhibitory to *Acinetobacter* sp. JC 1 even though the cell is able to grow on methanol. In the previous studies, we did a 3-day experiment to test the substrate specificity and judged that the cells did not grow on methanol (12). Growth of *Acinetobacter* sp. JC1 on methanol and methylamine indicates that the bacterium is able to grow methylotrophically as well as chemoautotrophically and heterotrophically like *P. gazotropha*.

It has been reported that MDH or methanol oxidase, depending on the organisms, is the key enzyme for methanol oxidation and is responsible for the conversion of methanol to formaldehyde in cells growing on methanol (3, 4). MDH was found in all methylotrophic bacteria (3, 4, 17, 36) and also in some methylotrophic yeasts such as *C. boidinii, Pichia pinus,* and *Kloeckera* sp. 2201 (39). Methanol oxidase, however, was found only in methylotrophic yeasts (3, 4, 32, 36). We have tried all the assay methods reported to date to detect the MDH activity from cells of *Acinetobacter* sp. JC1 grown on methanol, but none of the assays were effective to measure the enzyme activity. Addition of the known MDH stabilizing agents such as methanol (1), $MgSO_4$ /dithiothreitol (7), or sucrose (7) into the standard buffer before preparation of crude cell extracts, addition of various metal ions into the reaction mixtures, and tests of various artificial electron acceptors other than the one used in the established methods were also found to be ineffective to measure these enzymes, implying that MDH is not present in *Acinetobacter* sp. JC1. The absence of MDH is further supported by the finding that *Acinetobacter* sp. JC1 grown on methanol does not contain *c*-type cytochromes since the primary electron acceptor of MDH is known as *c*-type cytochromes (3, 4). The present result that there is no *c*-type cytochromes in *Acinetobacter* sp. JC1 was implicated previously from the absence of cytochrome *c* oxidase activity in this bacterium (12).

The absence of MDH in *Acinetobacter* sp. JC1, then, implied that methanol oxidase may be a candidate responsible for the first step of methanol oxidation in this bacterium. We, however, could not detect the enzyme activity using a method based on the analysis of formaldehyde and $H_2O_2$ produced after reaction of the enzyme with methanol. We have tried several modifications of the known methods and several agents for enzyme stabilization, but all were found to be ineffective to measure the two products. This together with the failure to assay the enzyme activity using the ABTS·$^+$ method developed for the present study and to detect a protein on the polyacylamide gel stained by the enzyme activity suggested that the oxidase-type of methanol-oxidizing enzyme also may not be present in *Acinetobacter* sp. JC1.

It was very interesting that *Acinetobacter* sp. JC1 grown on methanol exhibits higher catalase activity than those grown on pyruvate or glucose. This together with the finding that the amount of catalase present in cells grown on methanol also far exceeded that in cells grown on pyruvate or glucose suggested that catalase in *Acinetobacter* sp. JC1 may play an important role during growth of the cells on methanol through decomposing extra $H_2O_2$ which may be produced in relation with methanol oxidation. This suggestion was further supported by the observations that the growth on methanol, not on glucose, of *Acinetobacter* sp. JC1 and the consumption of oxygen by methanol-grown cells, not by glucose-grown cells, were inhibited by hydroxylamine a known inhibitor of catalase. Considering the reports that the amounts and activities of methanol oxidase and catalase increased during growth on methanol of methylotrophic yeasts compared with those of the enzymes during growth on ethanol or other organic materials (35, 48), the present results indicate that an oxidase-type, not the dehydrogenase-type, enzyme has to be present in *Acinetobacter* sp. JC1 growing on methanol. There has been no report on the enzyme responsible for oxidation of methanol in *P. gazotropha*.

It has been postulated that the peroxidative activity of catalase in methylotrophic yeasts may also play a role in the oxidation of methanol, formaldehyde, and formate using $H_2O_2$ generated by methanol oxidase (18, 48). Since the catalase present in *Acinetobacter* sp. JC1 also exhibited peroxidase activity and since the amount and activity of catalase in this bacterium were increased during growth on methanol, we first assumed that the enzyme in *Acinetobacter* sp. JC1 may also be involved in the oxidation of methanol to formaldehyde. The assumption was found to be rather unlikely since the purified catalase of *Acinetobacter* sp. JC1 did not show high peroxidase activity with methanol as a substrate (9.4 nmol formaldehyde produced per mg pro-

tein per min) (Unpublished data). Kinetic studies using a catalase purified from *C. boidinii* also suggested that catalase may not be an additional enzyme involved in the direct oxidation of methanol in yeasts (43).

The presence of high activity of NAD-dependent, GSH-requiring FADH compared with the low activity of NAD-dependent, dye-linked FADH indicates that *Acinetobacter* sp. JC1 may use GSH-requiring FADH for oxidation of formaldehyde to formate as in methyotrophic yeasts (32). The presence of NADH-dependent FAR in *Acinetobacter* sp. JC1, which was also observed in yeasts (51), implies that the enzyme may play a role to control the concentration of formaldehyde in the cell and to replenish cellular NAD which is necessary for the oxidation of formaldehyde to $CO_2$ by combined action of FADH and FMDH present in this bacterium.

The absence of *c*-type cytochromes and the elevation in the amount and activity of catalase in cells grown on methanol strongly suggest the involvement of methanol oxidase in the oxidation of methanol into formaldehyde. We first assumed that the inability to detect the methanol oxidase through measuring the amount of formaldehyde and $H_2O_2$ and also by the method employing ABTS may come from the rapid decomposition of $H_2O_2$ by catalase and peroxidase activities of the catalase-peroxidase, from the rapid oxidation of formaldehyde by the GSH-requiring FADH together with the FMDH, and from the rapid reduction of formaldehyde by the FAR. The assumption, however, seemed to be unlikely since the cell extracts prepared from cells grown on methanol did not exhibit methanol oxidase activity even when the extracts were subjected to stain by activity. We, therefore, think that the oxidase-type of enzyme responsible for the oxidation of methanol to formaldehyde in *Acinetobacter* sp. JC1 may be extremely labile under air even though all efforts were failed to stabilize the enzyme activity. We, however, cannot exclude the possibility that the oxidation of methanol in *Acinetobacter* sp. JC1 is carried out by a novel enzyme of the dehydrogenase- or oxidase-type of which activity is not detectable by the known assay methods.

## Acknowledgement

This work was supported by a research grant for Basic Science Research Institute from the Ministry of Education (BSRI 95-4419), Korea.

## References

1. **Adachi, O., K. Matsushita, E. Shinagawa, and M. Ameyama.** 1990. Purification and properties of methanol dehydrogenase and aldehyde dehydrogenase from *Methylobacillus glycogenes*. *Agric. Biol. Chem.* **54**, 3123-3129.
2. **Allgood, G.S., and J.J. Perry.** 1986. Characterization of a manganese-containing catalase from the obligate thermophile *Thermoleophilum album*. *J. Bacteriol.* **168**, 563-567.
3. **Anthony, C.** 1982. The biochemistry of methylotrophs. Academic Press, New York.
4. **Anthony, C.** 1986. Bacterial oxidation of methane and methanol. *Adv. Microb. Physiol.* **27**, 113-210.
5. **Anthony, C.** 1988. Quinoproteins and energy transduction, p 293-316. *In* C. Anthony (ed.), Bacterial energy transduction. Academic Press, New York.
6. **Anthony, C., and L.J. Zatman.** 1967. The microbial oxidation of methanol. Purification and properties of the alcohol dehydrogenase of *Pseudomonas* sp. M27. *Biochem. J.* **104**, 953-959.
7. **Arfman, N., E.M. Watling, W. Clement, R.J. van Oosterwijk, G.E. de Vries, W. Harder, M.M. Attwood, and L. Dijkhuizen.** 1989. Methanol metabolism in thermotolerant methylotrophic *Bacillus* strains involving a novel catabolic NAD-dependent methanol dehydrogenase as a key enzyme. *Arch. Microbiol.* **152**, 280-288.
8. **Armstrong, J.M.** 1964. The molar extinction coefficient of 2,6-dichlorophenol indophenol. *Biochim. Biophys. Acta* **86**, 194-197.
9. **Avigad, G.** 1983. A simple spectrophotometric determination of formaldehyde and other aldehydes: Application to periodate-oxidized glycol systems. *Anal. Biochem.* **134**, 499-504
10. **Basson, K., J.M. Zyl, and J. van der Walt.** 1991. Isolation and characterization of the catalse-peroxidase enzyme of Bacille-Calmette-Guerin: effect of isoniazid. *South African J. Science* **87**, 507-513.
11. **Bystrykh, L.V., N.I. Govorukhina, P.W. van Ophem, H.J. Hektor, L. Dijkhuizen, and J.A. Duine.** 1993. Formaldehyde dismutase activities in Gram- positive bacteria oxidizing methanol. *J. Gen. Microbiol.* **139**, 1979-1985.
12. **Cho, J.W., H.S. Yim, and Y.M. Kim.** 1985. *Acinetobacter* isolates growing with carbon monoxide. *Kor. J. Microbiol.* **23**, 1-8.
13. **Clare, D.A., M.N. Duong, D. Darr, F. Archibald, and I. Fridovich.** 1984. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. *Anal. Biochem.* **140**, 532-537.
14. **Coudere, R., and J. Baratti.** 1980. Oxidation of methanol by the yeast, Pichia pastoris. Purification and properties of the alcohol oxidase. *Agr. Biol. Chem.* **44**, 2279-2289.
15. **Dawson, R.M.C., D.C. Elliott, W.H. Elliott, and K.M. Jones.** 1986. Data for biochemical research (3rd ed.), p. 122-123. Oxford Science Publications, New York.
16. **Dawson, R.M.C., D.C. Elliott, W.H. Elliott, and K.M. Jones.** 1986. Data for biochemical research (3rd ed.), p. 356-357. Oxford Science Publications, New York.
17. **Dijkhuizen, L., P.R. Levering, and G.E. de Vries.** 1992. The physiology and biochemistry of aerobic methanol-utilizing Gram-negative and Gram-positive bacteria,

p. 149-181. *In* J.C. Murrell, and H. Dalton (ed.), Methane and methanol utilizers. Plenum Press, New York and London.

18. **van Dijken, J.P., W. Harder, and J.R. Quayle.** 1981. Energy transduction and carbon assimilation in methylotrophic yeasts, p. 191-201. *In* H. Dalton (ed.), Microbial growth on $C_1$ compounds. Heydon, London.
19. **Duine, J.A., J. Frank, and M.P.J. Berkhout.** 1984. NAD-dependent, PQQ-containing methanol dehydrogenase: a bacterial dehydrogenase in a multienzyme complex. *FEBS Lett.* **168**, 217-221.
20. **Dunn, M.F., and S.A. Bernhard.** 1971. Rapid kinetic evidence for adduct formation between the substrate analog *p*-nitroso-*N*,*N*-dimethyl-aniline and reduced nicotinamide dinucleotide during enzymatic reduction. *Biochemistry* **10**, 569-4575.
21. **Egert, M., A. Hamann, R.K men, C.G. Friedrich.** 1993. Methanol and methylamine utilization result from mutational events in *Thiosphaera pantotropha*. *Arch. Microbiol.* **159**, 364-371.
22. **Franson, M.A.H., A.E. Greenberg, R.R. Trussell, and L.S. Clesceri.** 1985. Standard methods: For the examination of water and wastewater (16th ed.), p. 413-415. American Public Health Association.
23. **Hazeu, W., J.C. de Bruyn, and J.P. van Dijken.** 1983. *Nocardia* sp. 239, a facultative methanol utilizer with the ribulose monophosphate pathway of formaldehyde fixation. *Arch. Microbiol.* **135**, 205-210.
24. **Hildebrandt, A.G., and I. Roots.** 1975. Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent formation and breakdown of hydrogen peroxide during mixed function oxidation reactions in liver microsomes. *Arch. Biochem. Biophys.* **171**, 385-397.
25. **Hochman, A., and I. Goldberg.** 1991. Purification and characterization of a catalase-peroxidase and a typical catalase from the bacterium *Klebsiella pneumoniae*. *Biochim. Biophys. Acta* **1077**, 299-307.
26. **Höpner, T., U. Ruschig, U. Muller, and P. Willnow.** 1982. Formate dehydrogenase from *Pseudomonas oxalaticus*. *Methods Enzymol.* **89**. 531-537.
27. **Kato, N., H. Sahm, and F. Wagner.** 1979. Steady state kinetics of formaldehyde dehydrogenase and formate dehydrogenase from a methanol utilizing yeast, *Candida boidinii*. *Biochim. Biophys. Acta.* **566**, 12-20.
28. **Kato, N., H. Sahm, H. Sch tte, and F. Wagner.** 1979. Purification and properties of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from a methanol utilizing yeast, *Candida boidinii*. *Biochim. Biophys. Acta.* **566**, 1-11.
29. **Kato, N., K. Tsuji, Y. Tani, and K. Ogata.** 1975. Utilization of methanol by an Actinomycete, p. 91-98. *In* The organizing committee (ed.), Microbial growth on $C_1$ compounds. The Society of Fermentation Technology, Tokyo.
30. **Kim, Y.M., and G.D. Hegeman.** 1981. Purification and some properties of carbon monoxide dehydrogenase from *Pseudomonas carboxydohydrogena*. *J. Bacteriol.* **148**, 904-911.
31. **Kim, Y.M., and G.D. Hegeman.** 1983. Oxidation of carbon monoxide by bacteria. *Intl. Rev. Cytol.* **81**, 1-32.
32. **de Koning, W., and W. Harder.** 1992. Methanol-utilizing yeasts, p. 207-244. *In* J. C. Murrell, and H. Dalton (ed.), Methane and methanol utilizers. Plenum Press, London.
33. **Kono, Y., and I. Fridovich.** 1983. Isolation and characterization of the pseudocatalase of *Lactobacillus plantarum*. A new manganese containing enzyme. *J. Biol. Chem.* **258**, 6015-6019.
34. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
35. **Large, P.J.** 1983. Methylotrophy and methanogenesis. American Society of Microbiology, Washington D.C.
36. **Large, P.J., and C.W. Bamforth.** 1988. Methylotrophy and Biotechnology. Longman Scientific & Technical, Harlow.
37. **Lowry, O.H., N.J. Rosenbrough, A.L. Farr, and R.J. Randall.** 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
38. **Margoliash, E., A. Novogrodsky, and A. Schejter.** 1960. Irreversible reaction of 3-amino-1,2,4-triazole and related inhibitors with the protein of catalase. *Biochem. J.* **74**, 339-348.
39. **Mehta, R.J.** 1975. Pyridine nucleotide linked oxidation of methanol in methanol assimilating yeast. *J. Bacteriol.* **124**, 1165-1167.
40. **Meyer, O.** 1989. Aerobic carbon monoxide-oxidizing bacteria. p. 331-350. *In* H.G. Schlegel, and B. Bowien (ed.), Autotrophic bacteria. Springer-Verlag, Berlin.
41. **Meyer, O., and H. G. Schlegel.** 1983. Biology of aerobic carbon monoxide oxidizing bacteria. *Annu. Rev. Microbiol.* **37**, 277-310.
42. **Morris, C.J., and M.E. Lidstrom.** 1992. Cloning of a methanol-inducible *moxF* promoter and its analysis in *moxB* mutants of *Methylobacterium extorquens* AM1rif. *J. Bacteriol.* **174**. 4444-4449.
43. **Mozaffar, S., M. Ueda, K. Kitatsuji, S. Shimizu, M. Osumi, and A. Tanaka.** 1986. Properties of catalase purified from a methanol grown yeast, *Kloeckera* sp. 2201. *Eur. J. Biochem.* **155**, 527-531.
44. **Nash, T.** 1953. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochemistry* **55**, 416-421.
45. **Nozhevnikova, A.N., and L.N. Yurganov.** 1978. Microbiological aspects of regulating the carbon monoxide content in the earth's atmosphere. *Adv. Microbial Ecol.* **2**, 203-244.
46. **Nozhevnikova, A.N., and G.A. Zavarzin.** 1974. On the taxonomy of CO oxidizing Gram-negative bacteria. *Izv. Akad. Nauk SSSR Ser. Biol.* **3**, 436-440.
47. **Ro, Y.T., and Y.M. Kim.** 1993. Constitutive expression of carbon monoxide dehydrogenase in *Acinetobacter* sp. strain JC1 DSM 3803. *Kor. J. Microbiol.* **31**, 214-217.
48. **Roggenkamp, R., H. Sahm, W. Hinkelmann, and F. Wagner.** 1975. Alcohol oxidase and catalase in peroxisomes of methanol-grown *Candida boidinii*. *Eur. J. Biochem.* **59**, 231-236.
49. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual (2nd. ed.). Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
50. **Tani, Y., T. Miya, and K. Ogata.** 1972. The microbial metabolism of methanol: Part II. Properties of crystal-

line alcohol oxidase from *Kloeckera* sp. No. 2201. *Agr. Biol. Chem.* **36**, 76-83.

51. **Trotsenko, Y.A., L.V. Bystrykh, and V.M. Ubiyvovk.** 1984. Regulatory aspects of methanol metabolism in yeast, p. 118-122. **In** R. L. Crawford and R. S. Hanson (ed.), Microbial growth on $C_1$ compounds. American Society for Microbiology, Washington, DC.
52. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**, 4406-4412.
53. **Zavarzin, G.A., and A.N. Nozhevnikova.** 1977. CO oxidizing bacteria, p. 207-213. *In* H. G. Schlegel *et al.* (ed.), Microbial production and utilization of gases, E. Goltze KG, Göttingen.