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Enzyme Activities Related to the Methanol Oxidation of *Mycobacterium* sp. strain JC1 DSM 3803

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***Mycobacterium* sp. strain JC1 DSM 3803 grown in methanol showed no methanol dehydrogenase or oxidase activities found in most methylotrophic bacteria and yeasts, respectively. Even though the methanol-grown cells exhibited a little methanol-dependent oxidation by cytochrome *c*-dependent methanol dehydrogenase and alcohol dehydrogenase, they were not the key enzymes responsible for the methanol oxidation of the cells, in that the cells contained no *c*-type cytochrome and the methanol oxidizing activity from the partially purified alcohol dehydrogenase was too low, respectively. In substrate switching experiments, we found that only a catalase-peroxidase among the three types of catalase found in glucose-grown cells was highly expressed in the methanol-grown cells and that its activity was relatively high during the exponential growth phase in *Mycobacterium* sp. JC1. Therefore, we propose that catalase-peroxidase is an essential enzyme responsible for the methanol metabolism directly or indirectly in *Mycobacterium* sp. JC1.**

Key words: *Mycobacterium* sp. JC1, methanol oxidation, catalase-peroxidase

Carboxydobacteria are a group of aerobic bacteria which are able to grow chemolithoautotrophically at the expense of carbon monoxide (CO) as the sole carbon and energy source. Also, they are also able to utilize several organic materials as growth substrates, except for *Streptomyces thermoautotrophicus* (35, 44). Among carboxydobacteria, *Pseudomonas gazotropha* is the only bacterium capable of growing in methanol as a source of carbon and energy and was recognized as the first organism utilizing three nutrient types, i.e., organotrophy, autotrophy, and methylotrophy (45, 48, 54) until it was reported that *Mycobacterium* sp. strain JC1 DSM 3803 (recently reclassified from *Acinetobacter* sp. strain JC1 DSM 3803 through analysis of its 16S rRNA sequence (17) and fatty acid composition [unpublished data]), can grow methylotrophically on both methanol and methylamine as sole carbon and energy sources (15, 51).

Interestingly, methanol-grown *Mycobacterium* sp. JC1 showed neither the classical methanol dehydrogenase (MDH) activity, which is detected in most methylotrophic Gram-negative bacteria (2, 3, 4, 32, 33) nor the well-known methanol oxidase reported in methanol-oxidizing yeast (30, 53). Instead, *Mycobacterium* sp. JC1 was found to possess dihydroxyacetone synthase (DHAS), a key enzyme of the xylulose monophosphate (XuMP) pathway (50), which is known to be present only in methanol-oxidizing yeast (29). In addition, *Mycobacterium* sp. JC1 grown in methanol has ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity, indicating the presence of a Calvin reductive pentose phosphate cycle for CO_2 fixation (31). Also, DHAS has been found to be expressed only in cells grown in methanol and to be expressed earlier than the RubisCO (50), suggesting that *Mycobacterium* sp. JC1 assimilates methanol via both the XuMP pathway and Calvin cycle, depending on their expressions during cell growth.

Besides *Mycobacterium* sp. JC1, *Mycobacterium gastri* MB19 is the only mycobacterium which dissimilates methanol via a novel enzyme, methanol:*N,N'*-dimethyl-4-nitrosoaniline oxidoreductase (MNO) and assimilates methanol via the ribulose monophosphate (RuMP) pathway (13, 14, 47). However, *Mycobacterium* sp. JC1 grown in methanol did not show any MNO activity at all (51). Furthermore, as described above, JC1 assimilated methanol into cell carbon via totally different pathways, the XuMP pathway and Calvin cycle (31, 50). These facts mean that mycobacterial methanol oxidation occurs by diverse mechanisms.

Catalase-peroxidase in mycobacterial metabolism plays a crucial enzymatic role by increasing the chance of survival inside macrophages (42). Previously we reported that the methanol-grown *Mycobacterium* sp. JC1 pos-

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sesses only a catalase-peroxidase in contrast to the glucose-grown cell contains three different types of catalase including the catalase-peroxidase (52) and that hydroxylamine, a manganese-containing catalase inhibitor (36), inhibited the growth in methanol of *Mycobacterium* sp. JC1 completely at 1 mM (51), suggesting that the catalase-peroxidase may play an important role in methanol oxidation of *Mycobacteirum* sp. JC1.

In this study, we report that a couple of enzymes, cytochrome *c*-dependent MDH and NAD-dependent alcohol dehydrogenase (ADH), show little methanol-oxidizing activities, but they are not the key enzymes responsible for methanol dissimilation in *Mycobacterium* sp. JC1. Also, we report that the catalase-peroxidase activity in methanol-grown *Mycobacterium* sp. JC1 is relatively higher than that in pyruvate- or glucose-grown cells, suggesting that the catalase-peroxidase of *Mycobacterium* sp. JC1 plays an important role in methanol metabolism.

Materials and Methods

Organisms and cultivation

Mycobacterium sp. strain JC1 DSM 3803 was cultivated methylotrophically at 30°C in standard mineral base (SMB) medium (34) with 0.5% methanol (v/v). For growth substrate switching experiments, the fully adjusted cells in the designated growth substrates (at least five consecutive passaged cells) were inoculated in SMB media supplemented with methanol, and grown at 30°C for regular time intervals. Subsequently, the methanol-grown cells were re-inoculated and grown in SMB media supplemented with each original growth substrate. The concentration of CO was provided as a gas mixture of 30% CO-70% air (v/v), and the concentrations of pyruvate and glucose used for growth were 0.5% (w/v) each. For several experiments, *Methylobacterium extorquens* AM1 (NCIB 9133) and *Methylobacterium methylotrophus* (NCIB 10515) were used as controls. Growth was measured by turbidity determined spectrophotometrically at 436 nm.

Preparation of cell-free extracts

Cells were collected by centrifugation at 10,000 × g for 10 min and washed once with 50 mM potassium phosphate buffer (pH 7.0, standard buffer). Cell disruption was done by sonic treatment (10 s per ml). The suspension was then centrifuged at 10,000 × g for 10 min, and the resulting supernatant was used as cell-free crude extracts for enzyme assays. Protein concentration was determined by the method of Lowry *et al.*, (39), using bovine serum albumin (BSA) as a standard.

Determination of oxygen consumption

Oxygen consumption rates of whole cells and cell-free extracts were determined by a biological oxygen monitor (YSI-5300, Yellow Spring Instruments) equipped with a Clark-type oxygen electrode as previously described (51). The solubility of oxygen in air-saturated water at 30°C was counted as 7.559 mg per liter (23).

Enzyme assays

All assays were carried out at 30°C using a Hitachi U-2000 spectrophotometer.

Pyrroloquinoline quinone (PQQ)-containing MDH activity, present in most Gram-negative methylotrophs, was assayed by measuring the methanol-dependent decrease in the absorbancy at 600 nm of 2,6-dichlorophenol indophenol (DCPIP, $\varepsilon_{600} = 22.0 \times 10^3\ M^{-1}cm^{-1}$; 7) as described in Anthony and Zatman (5) with minor modifications (51). NAD-dependent PQQ-MDH and NAD-dependent MDH activities were assayed using NAD/DCPIP (20) and NAD itself, respectively, as electron accepters by the method of Arfman *et al.* (6). NAD-dependent glutathione (GSH)-requiring MDH activity was assayed by measuring the methanol-dependent NADH (ε_{340}=6.22 $mM^{-1}cm^{-1}$) production at 340 nm following Mehta's methol (43). Tetrazolium dye-linked alcohol dehydrogenase (TD-ADH) activity reported in a Gram positive *Amycolatopsis methanolica* (49) was assayed using *p*-iodonitrotetrazolium violet (INT, ε_{549}=17.981 $mM^{-1}cm^{-1}$).

The *N,N'*-dimethyl-4-nitrosoaniline (NDMA)-dependent MDH activity reported in *A. methanolica* and *M. gastri* MB19 (13, 14) was assayed by measuring the methanol-dependent reduction of NDMA (ε_{440}=35.4 $mM^{-1}cm^{-1}$; 21) by the method of Bystrykh *et al.* (14).

Cytochrome *c*-dependent MDH activity was assayed by the methanol-dependent increase of absorption at 550 nm by the method of Kato *et al.* (28) with minor modifications (51).

Methanol oxidase present in methylotrophic yeast (30) was assayed by measuring the methanol-dependent oxidation of 2,2-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at 660 nm following the method of Basson *et al*, (12).

Methylamine dehydrogenase (MADH) activity, the key enzyme of the main pathway of methylamine oxidation (22), was assayed using phenazine methosulfate (PMS) and DCPIP by the method of Eady and Large (22).

N-methylalanine dehydrogenase and *N*-methylglutamate dehydrogenase activities, the main enzymes for an alternative pathway of methylamine oxidation, were assayed as described before (38 and 9, respectively). Alanine dehydrogenase activity was assayed by the method for detecting the *N*-methylalanine dehydrogenase activity (38) with some modifications. Briefly, the concentration of sodium pyruvate used was 5 mM instead of 20 mM and methylamine (200 mM) as a substrate was replaced by 50 mM of NH_4Cl.

Alcohol dehydrogenase (ADH) activity was assayed by measuring the ethanol-dependent NADH (ε_{340}=6.22 mM^{-1} cm^{-1}) production (6).

The catalase activity was assayed by the method of Couderc and Baratti (18) with a modification. The reaction mixture (1 ml) contained 50 μM potassium phosphate buffer (pH 7.0) and 12.5 μM H_2O_2 (ε_{240}=43.6 $M^{-1}cm^{-1}$; 26). The reaction was started by the addition of cell-free extracts.

The peroxidase activity was determined spectrophotometrically by measuring the rate of oxidation of 0.25 mM o-dianisidine at 460 nm (ε_{460}=11.3 $mM^{-1}cm^{-1}$) in the presence of 12.5 mM hydrogen peroxide (40)

Electrophoresis and catalase activity staining

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out in gels containing 7.5% acrylamide by Laemmli's method (37). Proteins were stained with Coomassie brilliant blue (CBB) R-250. Activity staining for catalase was performed by a procedure described before (16) with some modifications (51).

Partial purification of ADH

All purification steps were carried out at 4°C except when noted otherwise.

A 30-g portion of methanol-grown *Mycobacterium* sp. JC1 was suspended in 90 ml of cold standard buffer and disrupted by sonic treatment (10 s per ml). The suspension was clarified at 12,000 × g for 30 min. The resulting supernatant (crude extract) was then treated with protamine sulfate to a final concentration of 0.054%, left on ice for 10 min, and then sedimented at 100,000 × g for 90 min. The resulting supernatant (soluble fraction) was next made 20% saturated with respect to ammonium sulfate. After 30 min, this fraction was centrifuged at 12,000 × g for 30 min. The resulting supernatant was further treated with ammonium sulfate to achieve a final concentration of 60% saturation. After 30 min, the solution was centrifuged again at 12,000 × g for 30 min, and the sediment was resuspended in a 5 ml of cold standard buffer. The suspension was then dialyzed against three 1-liter changes of standard buffer for 15 h. The dialyzed fraction was applied to a Sepharose 6B column (1.8 by 125 cm, Sigma) and eluted with standard buffer at a flow rate of 8.5 ml/cm^2 per h. Fractions with high ADH activity were pooled and then applied to a DEAE-Sephacel column (2.8 by 8.5 cm, Sigma) pre-equilibrated with standard buffer. The elution was performed with 300 ml of standard buffer containing 0.1 M of KCl followed by a linear KCl gradient (300 ml, 0.1 to 0.7 M) in standard buffer at a flow rate of 4.5 ml/cm^2 per h. Fractions with high ADH activity were pooled, dialyzed against 10 mM of phosphate buffer (pH 6.8), and then concentrated with an ultrafiltration membrane (Amicon YM10) under an atmosphere of nitrogen gas. The concentrated fraction was applied to a hydroxylapatite column (2.3 by 10 cm, Biorad). The elution was performed with 120 ml of 10 mM phosphate buffer followed by a liner phosphate gradient (240 ml, 10 to 100 mM) in phosphate buffer (pH 6.8) at a flow rate of 7.2 ml/cm^2 per h. Fractions with the highest ADH activity were pooled and used as the partially purified ADH for some biochemical analysis.

Substrate specificity of the partially purified ADH

To determine the substrate specificity of ADH expressed in *Mycobacterium* sp. JC1, the partially purified ADH was pre-incubated in the reaction mixture at 37°C for 1 min. The reaction was then started by the addition of 50 mM of each of the substrates listed in Table 3.

Results

Methanol oxidation activities in Mycobacterium sp. JC1

Mycobacterium sp. JC1 actively grew on methanol-containing medium. However, we could not detect any known MDH or methanol oxidase activities in the methanol-grown cells (Table 1), To know whether the cell truly utilized and oxidized methanol as a growth substance, we determined its oxygen consumption rate on methanol using the whole cells and the cell-free extract. The result showed that whole cells of *Mycobacterium* sp. JC1 showed a high oxygen consumption rate, 720.8 nmol per mg cells (dry weight) per min upon methanol addition to assay mixture. However, the cell extract prepared from the methanol-grown *Mycobacterium* sp. JC1 has, relatively, a very low oxygen consumption rate (4.5 nmol/mg protein per minute) (Table 1), revealing that there is a great difference in methanol oxidation rate before and after disrupting cells. Interestingly, there was a little cytochrome *c* reduction observed when methanol was added to the cell-free extract (Table 1). This reduction was completely abolished when the extract was denatured by a heat treatment (data not shown), indicating that a heat-

Table 1. Methanol consumption and methanol oxidizing activities in *Mycobacterium* sp. JC1

Assays	Electron acceptor	Methanol oxidation rate	Reference
Whole cells	O_2	720.8[a]	
Cell-free extracts			
Methanol dehydrogenase	PMS-DCPIP	ND[b]	(5)
	NAD-DCPIP	ND	(20)
	INT	ND	(49)
	NAD	ND	(6)
	NAD-GSH	ND	(43)
	NDMA	ND	(14)
	Cytochrome *c*	0.01[c]	(28)
Methanol Oxidase	O_2	4.5[d]	
	ABTS	ND	(12)

[a]nmol of oxygen consumed per mg cell (dry weight) per minute
[b]not detected
[c]ΔA_{550} per mg protein per minute
[d]nmol of oxygen consumed per mg protein per minute

Table 2. Enzyme activities related to methylamine oxidation in *Mycobacterium* sp. JC1 and *M. extorquens* AM1

Strains	*M. extroquens* AM1	*Mycobacterium* sp. JC1	
Growth Substrates	Methanol	Methanol	Methylamine
Enzyme activities			
Methylamine dehydrogenase[a]	38.3	ND[b]	ND
N-methylalanine dehydrogenase[c]	ND	ND	0.2
Alanine dehydrogenase[d]	3.7	17.9	42.1
N-methylglutamate dehydrogenase[e]	0.2	3.0	24.7

[a]nmol of DCPIP reduced per mg protein per minute
[b]not detected
[c]nmol of NADPH oxidized per mg protein per minute
[d]nmol of NADH oxidized per mg protein per minute
[e]nmol of DCPIP reduced per mg protein per minute

labile substance is involved in cytochrome *c*-dependent methanol oxidation. However, it is unlikely that *Mycobacterium* sp. JC1 uses cytochrome *c*-dependent MDH as a main methanol oxidation enzyme because the observed activity was too low and the typical absorption peaks for *c*-type cytochromes, a distinct property of other known methylotrophic bacteria, was not detected in *Mycobacterium* sp. JC1 (51). All other attempts to detect any methanol oxidizing enzyme activities, including uses of other artificial electron acceptors, reducing agents, and enzyme stabilizing agents, failed to generate positive data.

Methylamine oxidation in Mycobacterium sp. JC1

Mycobacterium sp. JC1 can also use methylamine as a carbon and energy source (51). To determine whether methylamine-grown *Mycobacterium* sp. JC1 can oxidize methylamine using the well-known methylamine oxidizing system found in methylotrophic bacteria, we examined methylamine dehydrogenase (MADH) and other methylamine oxidizing enzyme activities in the cell extract prepared from the methylamine-grown cells. A typical methylotrophic bacterium, *M. extorquens* AM1 showed the MADH activity. However, *Mycobacterium* sp. JC1 has no MADH activity at all, but it possesses *N*-methylalanine dehydrogenase and *N*-methylglutamate dehydrogenase activities (Table 2), indicating that it uses not a direct methylamine oxidation pathway via MADH, but an alternative *N*-methylglutamate pathway for the methylamine oxidation.

ADH in Mycobacterium sp. JC1

Methanol-grown *Mycobacterium* sp. JC1 possesses NAD-dependent ADH activity. To test the possibility that this enzyme can oxidize methanol and serve as the key enzyme in methanol oxidation, we partially purified the ADH using a couple of column chromatographies. The partially purified ADH is a heat-stable enzyme and its optimum pH and temperature for the enzyme activity were pH 9.5 and 60°C, respectively. Also the Km and Vmax for ethanol as a substrate were found to be 6.8 mM and 548 nmole per min per mg protein, respectively, and those for NAD of the enzyme were 0.14 mM and 452 nmole per min per mg protein, respectively. The main purpose of purifying the ADH partially is to see whether this enzyme can oxidize methanol sufficient for bacterial growth. The substrate specificity of the partially purified ADH was examined in an optimal assay condition. As shown in Table 3, the ADH had a broad range of substrate specificities including methanol and formaldehyde. However, the enzyme activity in methanol was much lower than those in other alcohols and it could not serve as the key enzyme of methanol oxidation in *Mycobacterium* sp. JC1.

Table 3. Substrate specificities of the partially purified alcohol dehydrogenase in *Mycobacterium* sp. JC1

Substrates[a]	Relative activity[b](%)
Methanol	5
Ethanol	100
1-Propanol	320
2-Propanol	175
1-Butanol	85
2-Butanol	160
Isoamyl alcohol	60
Allyl alcohol	185
1-Octanol	10
1-Nonanol	0
N-Heptane	0
Benzyl alcohol	0
Glycerol	0
Formaldehyde	15
Acetaldehyde	60
Glutaraldehyde	0

[a]Final concentration of all the substrates used was 50 mM.
[b]A specific activity for ethanol (404.5 nmol of NDA reduced per mg protein per minute) was set as 100%.

A catalase-peroxidase in methanol-grown Mycobacterium sp. JC1

Our previous results showed that cell-free extracts of methanol-grown *Mycobacterium* sp. JC1 exhibit catalase activity 4 and 8 times higher, respectively, than those of the cell extracts from pyruvate- and glucose-grown cells (51). Glucose-grown *Mycobacterium* sp. JC1 exhibits three-types of catalase (Cat1, 2, and 3) and Cat2 exhibits dual catalatic and peroxidatic (catalase-peroxidase) activities, but the methanol-grown cells express only a catalase-peroxidase protein (Cat2) (52). To examine the possible involvement of the catalase-peroxidase in methanol oxidation of *Mycobacterium* sp. JC1, we conducted substrate switching experiments and checked the catalase activity. The CO-adjusted cells were inoculated and grown in methanol-containing media to late-stationary phase. Then, the cells were re-inoculated in media containing CO gas

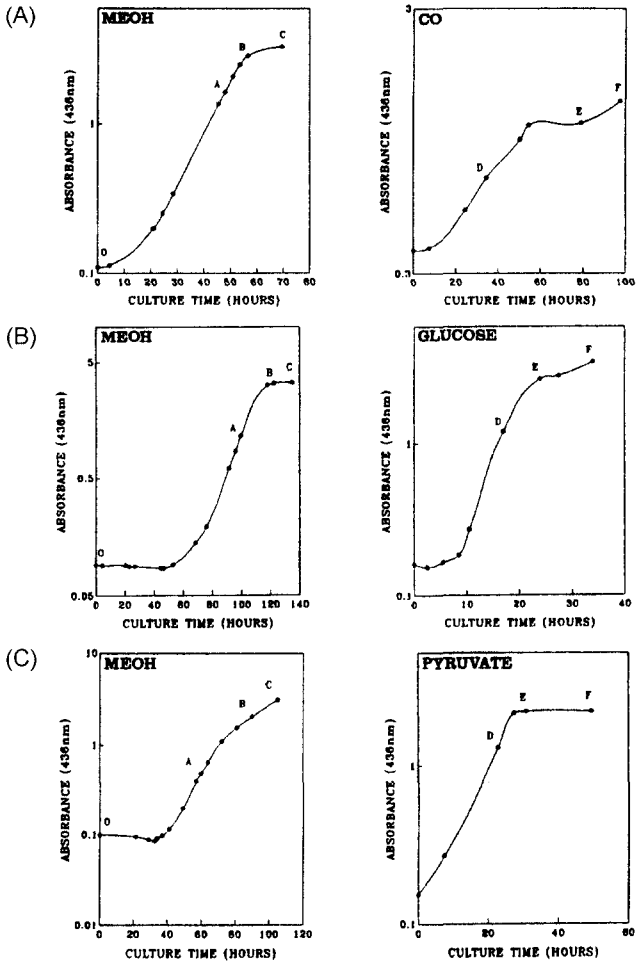

Fig. 1. Growth curves of *Mycobacterium* sp. JC1 during substrate switching experiments. Capital letters in each figure indicate the harvest points for catalase activity assay. (A) Cells growing in the CO-methanol-CO switching media. (B) Cells growing in the glucose-methanol-glucose switching media. (C) Cells growing in pyruvate-methanol-pyruvate switching media.

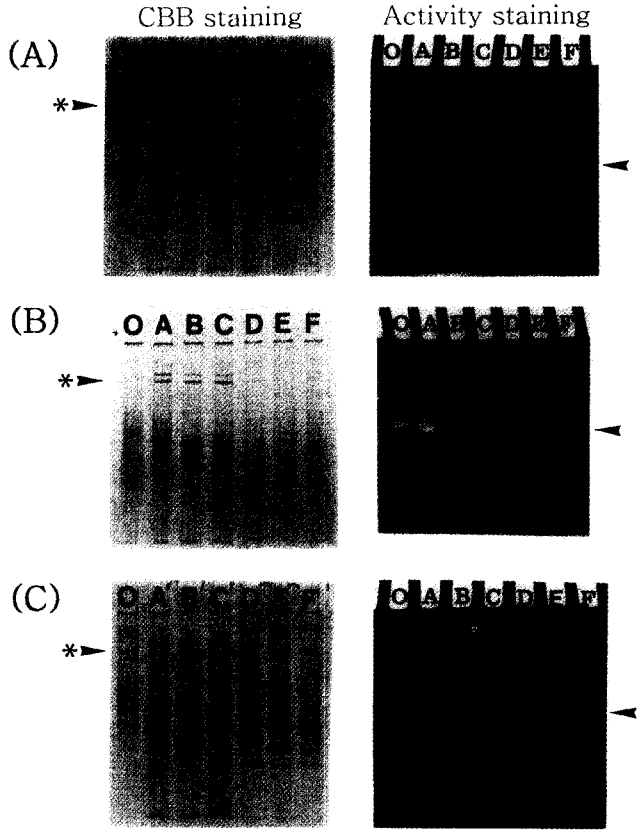

Fig. 2. CBB and catalase activity staining in cells growing in each substrate switching media. Capital letters in the upper panels of each picture indicate each harvest point shown in Fig. 1. The catalase-peroxidase band stained and a major protein responsible for growth only in methanol are highlighted with an arrow head and an asterisk, respectively. (A) Cells growing in the CO-methanol-CO switching media. (B) Cells growing in the glucose-methanol-glucose switching media. (C) Cells growing in pyruvate-methanol-pyruvate switching media.

(30%, v/v) and allowed to grow to stationary phase. In each experiment, some portions of the cells were harvested at the indicated time-points and catalase activity was assayed by spectrophotometric and catalase activity staining methods. Also, similar experiments were conducted in pyruvate-methanol-pyruvate switching and glucose-methanol-glucose switching media. When the CO-adjusted cells were inoculated into methanol-containing media, they grew immediately without a lag period. However, glucose- or pyruvate-adjusted cells showed over 40 hours of lag time in methanol-containing media (Fig. 1). These results imply that *Mycobacterium* sp. JC1 use common enzymes for CO and methanol metabolism, and that the cells grown in multi-carbon growth substrates needs *de novo* synthesis of protein(s) for utilizing methanol as a source of carbon and energy. The glucose-grown cells expressed three different types of catalase, similar to findings in previous work. (52). However, when the growth substrate was switched from glucose to methanol, the cells expressed only the catalase-peroxidase and the total catalase activity detected in methanol-grown cells was relatively higher than that in glucose-grown cells (Fig. 2 and Table 4). This result reveals that the catalase-peroxidase is the main catalase, especially in the cells growing in methanol. In the pyruvate-methanol-pyruvate switching experiment, the result clearly showed that the catalase-peroxidase was highly expressed in actively growing cells in methanol (Fig. 2 and Table 4), indicating that its expression is closely related to the cell growth in methanol and this enzyme may play an important role in the methanol metabolism of *Mycobacterium* sp. JC1. The cells grown in CO also expressed only the catalase-peroxidase and its catalatic activity was as high as that in the methanol-grown cells, implying that the role of the catalase-peroxidase is similar in C_1 compounds metabolism of *Mycobacterium* sp. JC1. The activity staining result from

Table 4. Changes of catalase activity in *Mycobacterium* sp. JC1 during growth under substrate switching conditions

Growth substrates	Harvest point[a]	Catalase activity[b]
CO	O	20.9
Methanol	A	9.2
Methanol	B	4.4
Methanol	C	23.0
CO	D	15.4
CO	E	13.3
CO	F	9.9
Glucose	O	10.8
Methanol	A	21.6
Methanol	B	7.3
Methanol	C	22.9
Glucose	D	2.8
Glucose	E	2.1
Glucose	F	1.2
Pyruvate	O	3.6
Methanol	A	17.7
Methanol	B	13.8
Methanol	C	4.7
Pyruvate	D	7.3
Pyruvate	E	6.2
Pyruvate	F	2.6

[a]Each harvest point was indicated in Fig. 2 as capital letters
[b]μmol of H_2O_2 disappeared per mg protein per minute

glucose-methanol-glucose switching experimental group showed some discrepancy with the catalase activity assay. It can be explained by the fact that the condition we used for the catalase assay is optimal for the catalatic activity of the catalase-peroxidase among three different types of catalase found in the glucose-grown cells. It is noteworthy that at least a protein marked with an asterisk in Fig. 2 is greatly induced only in methanol-grown cells, indicating that this induced protein may play an important role in methanol oxidation of *Mycobacterium* sp. JC1.

Discussion

Among carboxydobacteria, only *Mycobacterium* sp. JC1 and *P. gazotropha* are able to grow in methanol as sole carbon and energy sources (48, 51, 54). Even though *Mycobacterium* sp. JC1 was found to consume oxygen very actively with methanol addition in whole cell mixtures (51), no MDH or methanol oxidase activities found to date in most methylotrophic bacteria and yeast (2, 5, 24, 30, 53) are detected in the cell extracts prepared from the methanol-grown cells, suggesting that *Mycobacterium* sp. JC1 may adopt other enzyme(s) or system(s) not reported in most methylotrophic bacteria or yeasts for methanol oxidation.

It has been reported that a mycobacterium, *M. gastri* MB19, can utilize methanol as a sole carbon and energy source (13, 14). This bacterium possesses enzymes catalyzing the oxidation of methanol with the concomitant reduction of an artificial electron acceptor, NDMA, and the dismutation of formaldehyde, producing methanol and formate (13). The enzymes are referred to as methanol : NDMA oxidoreductases (MNOs) and they are decameric proteins of 49 to 50 kDa subunits (13). Also this bacterium may assimilate methanol via the ribulose monophosphate (RuMP) pathway, based on a finding that the gene cluster of the RuMP pathway for formaldehyde fixation was identified in the chromosome of the bacterium (47). *Mycobacterium* sp. JC1, a mycobacterium recently re-grouped into the genus *Mycobacterium*, does not possess MNO activity and does assimilate methanol via both the XuMP pathway found only in methylotrophic yeast and the Calvin cycle (31, 50), suggesting that the methanol oxidation in mycobacteria may operate in diverse mechanisms.

All the attempts to find a key enzyme for the methanol dissimilation in *Mycobacterium* sp. JC1 have failed using enzyme assay methods reported to date, except that the cells show a little cytochrome *c*-dependent MDH activity and that the ADH partially purified from the methanol-grown cells can use methanol and formaldehyde as substrates. However, we exclude the possible involvement of these enzymes in methanol oxidation of *Mycobacterium* sp. JC1 because the cells do not contain *c*-type cytochromes (51) and the methanol oxidation rate by the partially purified ADH is too low.

We also tried to detect the reaction products generated as a result of methanol oxidation in *Mycobacterium* sp. JC1 using nuclear magnetic resonance (NMR) spectrum analysis. When ^{13}C-labeled methanol was added to the cell suspension mixture, the cells rapidly used ^{13}C-labeled methanol during cell growth, completely consuming the added methanol in 27 h. The only product detected as the result of methanol oxidation was bicarbonate molecules (160.28 δppm). Concentrations of the bicarbonate molecules were increased in only 3 hours after methanol addition and then decreased steadily until the methanol was used up (data not shown). The reason why no formaldehyde was detected in NMR analysis as an intermediate of methanol oxidation may be caused by the existence of NAD-dependent formaldehyde dehydrogenase and DHA synthase in *Mycobacterium* sp. JC1 (51 and 50, respectively). The produced formaldehyde may be rapidly incorporated into a multicarbon source by DHA synthase for the XuMP assimilation pathway and further oxidized to formate by formaldehyde dehydrogenase. Subsequently, the resulting formate is converted to bicarbonate for the Calvin cycle by NAD-dependent formate dehydrogenase (31, 51).

In substrate switching experiments, we found that at least three types of catalase exist in glucose-grown cells and one of them is a catalase-peroxidase as described

before (52). Interestingly, the methanol-grown cells express only the catalase-peroxidase and the catalase activity in methanol-grown cells is relatively higher than that in glucose- or pyruvate-grown cells, indicating that the catalase-peroxidase may play an important role in methanol oxidation in *Mycobacterium* sp. JC1. In fact, it has been postulated that the peroxidative activity of catalase in methylotrophic yeasts may also play a role in the oxidation of methanol using H_2O_2 generated by methanol oxidase (8, 24).

Previously we reported that this catalase-peroxidase activity was not affected by a treatment of 3-amino-1,2,4-triazole (AT), an inhibitor for typical catalases (41, 52). However, hydroxylamine which inhibits the manganese-containing catalase (1, 36) inhibited the cell growth in methanol completely at 1 mM, but did not affect the growth in glucose at the same concentration of hydroxylamine (51). The enzyme activity of the purified catalase-peroxidase from the methanol-grown cells was also strongly inhibited by hydroxylamine treatment, but not by AT treatment (unpublished results) indicating that the catalase-peroxidase activity is required for methanol utilization of *Mycobacterium* sp. JC1.

Mycobacterium tuberculosis, the causative agent of tuberculosis (19), has been shown to have a high resistance to killing by H_2O_2 (27, 46) and this resistance is believed to be mediated by the mycobacterial catalase-peroxidase (KatG) and the alkyl hydroperoxide reductase (AhpC) proteins (25). Isoniazid, a frontline antimycobacteria agent, requires activation by KatG before exerting a lethal effect (11, 55) and its resistance in a majority of clinical isolates results from point mutations in *katG* (10). When the growing *Mycobacteium* sp. JC1 in methanol was treated with isoniazid to a concentration of 25 μg per ml, the cell growth was significantly retarded and the catalase-peroxidase activity was also greatly reduced. However, isoniazid did not affect the cell growth and the catalase-peroxidase activity of the cells growing in pyruvate-containing media (unpublished results), suggesting that the catalase-peroxidase of *Mycobacterium* sp. JC1 plays an important role for methanol utilization of the cells and its action mechanism may be diverse depending on growth substrates.

We have no direct evidence yet that the catalase-peroxidase is the key enzyme for methanol oxidation in *Mycobacterium* sp. JC1 and we cannot exclude a possibility that another enzyme, not found yet, is truly responsible for the methanol oxidation of the cell. It should be noted that a protein (marked in Fig. 2 with an asterisk) is highly induced when the cells are subjected to growth only in methanol. This protein is also expressed in the ethanol-grown cells (unpublished observation), indicating that the protein expression is closely related to alcohol utilization including methanol.

From these results, we propose a model that the catalase-peroxidase may be an essential enzyme responsible for the methanol oxidation directly or indirectly in *Mycobacterium* sp. JC1 (Fig. 3). A possibility that a greatly induced protein in *Mycobacterium* sp. JC1 growing in methanol is responsible for methanol oxidation will be addressed via N-terminal amino acid sequencing and homology searching with all known proteins. Also, the possibility that the catalase-peroxidase is important for methanol oxidation of *Mycobacterium* sp. JC1 is under investigation via further study of the purified catalase-peroxidase, and the selection and characterization of the catalase-peroxidase negative mutants using isoniazid.

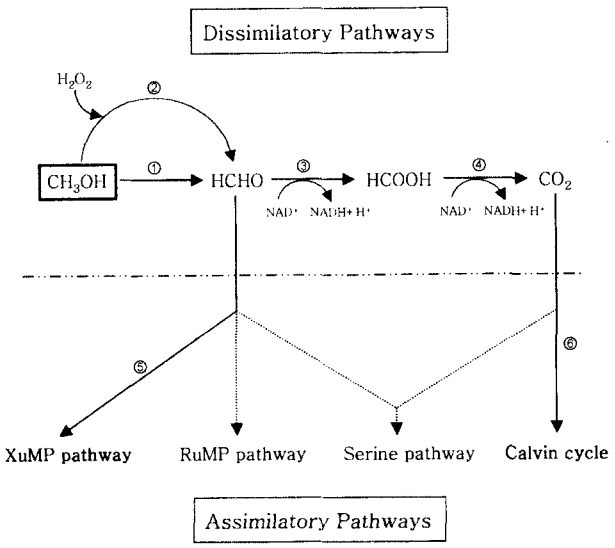

Fig. 3. A proposed model for methanol metabolism in *Mycobacterium* sp. strain JC1 DSM 3803. Symbols : unknown methanol oxidizing enzyme (①), catalase-peroxidase (②), formaldehyde dehydrogenase (③), formate dehydrogenase (④), dihydroxyacetone synthase (⑤), and ribulose bisphosphate carboxylase (⑥).

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