Isolation of Facultative Methanol-utilizing Bacteria Which Produce Electrophoretically Different Methanol Dehydrogenase

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Received August 29, 1977

Bacterial strains capable of assimilating methanol as the sole source of carbon and energy have been isolated. These bacteria produced phenazine methosulfate-dependent methanol dehydrogenase (primary alcohol dehydrogenase, EC 1.1.99.8). The enzyme activity was detected in electrophoresis on polyacrylamide gels by activity staining technique. The dehydrogenase can be grouped to at least three types based on their mobility in electrophoresis: (1) enzyme which migrates toward the anode slowly, (2) enzyme which remains at the top of the gel at pH 9.3 and (3) enzyme which migrates to the anode at faster mobility. The three bacteria were selected as producers of the respective three types of dehydrogenase. They were facultative methanol-utilizing bacteria and two of them were identified, one as *Pseudomonas* sp. No. 2941 and the other as *Pseudomonas* sp. S25. Most isolated bacteria produced the first type of enzyme and several strains produced the second type of enzyme. Strain S50 was the only strain which produced the third type of enzyme. These groupings of methanol dehydrogenase may contribute to the classification of methylotrophic bacteria.

Since Anthony and Zatman¹⁾ first demonstrated a methanol dehydrogenase (primary alcohol dehydrogenase, EC 1.1.99.8) from *Pseudomonas* sp. M27 in 1964, this dehydrogenase has been found in many methanol-utilizing bacteria, e.g., *Pseudomonas* sp. AM1, *Pseudomonas methanica*, *Pseudomonas extroquens*,²⁾ *Methylococcus capsulatus*,³⁾ *Pseudomonas oleovorans*,⁴⁾ *Pseudomonas* 1 and *Pseudomonas* C.^{5~7)} This enzyme was further purified^{8~14)} and crystalline preparations were obtained from *Methylosinus sporium*¹⁵⁾ and *Pseudomonas* sp. No. 2941.¹⁶⁾ A good similarity among these enzyme preparations can be demonstrated especially on their physiological properties: e.g., substrate specificity, electron acceptor and some of molecular properties. But one evident difference is their electrophoretic behavior on polyacrylamide gels.

In order to isolate active methanol-utilizing

bacteria, we assayed primary alcohol dehydrogenase activity in crude extracts of methanol-grown cells of newly isolated bacteria. Then, the enzyme was detected after electrophoresis on polyacrylamide gels by activity staining technique. In this paper, the grouping of dehydrogenase is described based on electrophoretic mobility. Three methanol-utilizing bacterial strains were selected from three groups and some characteristics of these strains are discussed.

MATERIALS AND METHODS

Isolation of methanol utilizing bacteria. Methanol utilizing bacteria were isolated by the enrichment culture technique from sewage, active sludge, sediment and soil. The medium was composed of 0.26% (NH₄)₂SO₄, 0.24% KH₂PO₄, 0.56% K₂HPO₄, 0.1% yeast extract, 0.05% MgSO₄·7H₂O and 1% methanol. The pH was adjusted to 7.0 before autoclaving. Magnesium sulfate was autoclaved separately and added to the medium. Methanol was also aseptically supplemented to the medium. Culture was made on a reciprocal shaker at 30°C for 3~5 days. The organisms were isolated and purified further by alternate streaking technique on methanol-agar plates.

Preparation of cell-free extracts. Each isolated

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strain was grown on 100 ml of methanol medium in a 500-ml flask at a shaking rate of 120 rev/min at 30°C for 24 hr. The cells were harvested by centrifugation at $10,000\times g$, washed twice and suspended in ice-cold 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM methanol. This was exposed for 5~10 min to ultrasonic oscillation at 20 kHz. Cell-free extracts were obtained after centrifugation at $14,800\times g$ for 20 min.

Enzyme assays. All spectrophotometric enzyme assays were made with a Hitachi spectrophotometer, model 139 or a Gilford recording spectrophotometer, model 240, thermostated at 30°C. Phenazine methosulfate-linked dehydrogenase activities were assayed at pH 9.6 according to the procedure of Anthony and Zatman¹¹ with methanol, ethanol, formaldehyde, formate or methylamine as substrate. NAD^+ - or NADP^+ -linked dehydrogenase activities were assayed in the assay mixture (1.0 ml) containing 50 μmol phosphate buffer, pH 8.0; 10 μmol substrate (methanol, formate, methylamine or ethanol) or 1 μmol formaldehyde; 0.5 μmol NAD^+ or NADP^+ with or without 0.5 μmol GSH.* Hydroxypyruvate reductase(D-glycerate: NAD^+ oxidoreductase, EC 1.1.1.29) was assayed with Tris-HCl buffer, pH 7.5 as described by Large and Quayle¹⁷ and activity was calculated after subtracting NADH oxidase activity. One unit of enzyme activity is defined as the amount of enzyme utilizing 1 μmol of substrate or NAD(P)^+ or producing 1 μmol of product per hr. Specific activity is defined as units per mg of protein. Protein was determined spectrophotometrically from ultraviolet absorption at 280 and 260 nm.

Polyacrylamide gel electrophoresis. Electrophoresis was carried out on 7.5% acrylamide gels in Tris-HCl buffer, pH 9.4, according to the procedure of Davis.¹⁸ Protein was stained in 2.5% Coomassie blue in acetic acid-methanol (46:454) for 1 hr. Dehydrogenase activity in the gels was detected and visualized by coupling with reduction of nitroblue tetrazolium chloride or with reduction of 2,6-dichlorophenol indophenol, as described in a previous paper.¹⁰

Materials. NAD^+ , NADP^+ and NADH were purchased from Oriental Yeast Co., Osaka and β -hydroxypyruvic acid (lithium salt) was obtained from Sigma Chemicals Co., St. Louis, Mo., U.S.A.

RESULTS

Detection of methanol dehydrogenase activity

Methanol dehydrogenase activity was assayed on extracts of methanol-grown cells of

newly isolated bacteria. This activity was determined from the rate of reduction of 2,6-dichlorophenol indophenol. Active strains which produced relatively high levels of enzyme activity were selected. The enzyme activity from all selected strains linked directly to phenazine methosulfate and required ammonium chloride. Therefore, these activities were also demonstrated on polyacrylamide gels after electrophoresis immersing the gels with methanol, phenazine methosulfate and dye (nitroblue tetrazolium chloride or 2,6-dichlorophenol indophenol). The location of enzyme protein was easily visualized as a purple-blue band in the presence of nitroblue tetrazolium chloride or as a colorless band in deep blue stained gels in the presence of 2,6-dichlorophenol indophenol. Therefore, this dehydrogenase can be referred to the primary alcohol dehydrogenase.

Electrophoretic behavior of methanol dehydrogenase

All methanol-grown cells tested produced one band of methanol dehydrogenase by electrophoresis on polyacrylamide gels, but their mobilities were distinctly different. Majority of bacteria produced enzyme which migrated slowly into gels. This type was designated as the first type of dehydrogenase, and strain No. 2941 was selected as the representative strain of this type. The second type of enzyme

FIG. 1. Electrophoretic Pattern of Crude Extracts of Methanol-grown Cells on Polyacrylamide Gels.

1,2: strain S25; 3,4: strain No. 2941; 5,6: strain S50. 1,3,5: protein stain; 2,4,6: activity stain on methanol dehydrogenase.

* Abbreviation used: GSH, reduced glutathione and PMS, phenazine methosulfate.

was characterized as not migrating into gels at pH 9.3. The enzyme activity remained on top of the gel during electrophoresis, while many protein bands were detected in the gel. Several strains were included in this group and strain S25 was selected as the typical strain. The third type of enzyme moved into the gel during electrophoresis. Only strain S50 showed this migration behavior.

Unlike isozyme, one enzyme band was always detected in each strain, and no bacterial strain was found to produce two or more enzyme bands. Therefore, the difference on the isoelectric point of the protein may reflect differences on migration behavior. Figure 1 shows the electrophoretic patterns of proteins and methanol dehydrogenase activities of the three selected strains, No. 2941, S25 and S50.

FIG. 2. Electronmicrograph of Strain No. 2941.

TABLE I. SPECIFIC ACTIVITIES OF ENZYMES IN EXTRACTS OF METHANOL-GROWN BACTERIA

Enzymes	Specific activity ^a		
	Strain 2941	Strain S25	Strain S50
Methanol dehydrogenase (PMS)	12.5	3.6	2.1
Methanol dehydrogenase (NAD ⁺)	0	0	0
Methanol dehydrogenase (NADP ⁺)	0	0	0
Formaldehyde dehydrogenase (NAD ⁺)	0	0.06	0.08
Formaldehyde dehydrogenase (NAD ⁺ , GSH)	0	0.17	1.20
Formaldehyde dehydrogenase (NADP ⁺)	0	0	0
Formaldehyde dehydrogenase (NADP ⁺ , GSH)	0	0	0
Formate dehydrogenase (NAD ⁺)	0.4	0.4	6.9
Formate dehydrogenase (NADP ⁺)	0.4	0.3	7.4
Methylamine dehydrogenase (NAD ⁺)	0	0	0
Ethanol dehydrogenase (NAD ⁺)	0	0.07	0.01
NADH oxidase	0.04	0.08	1.58
Hydroxypyruvate reductase (NADH)	11.6	0.03	6.0

^a Specific activity = μ moles/60 min/mg of protein.

FIG. 3. Electronmicrograph of Strain S25.

FIG. 4. Electronmicrograph of Strain S50.

Enzyme activities in extracts

Table I compared the phenazine methosulfate-linked dehydrogenase activities of the three strains in crude extracts of methanol-grown cells. Methanol dehydrogenase (prima-

ry alcohol dehydrogenase) was the most active enzyme of these strains. But the activities of NAD^+ - or NADP^+ -linked methanol dehydrogenase, ethanol dehydrogenase were not demonstrated in the three organisms. The

activity of NAD^+ -dependent formaldehyde dehydrogenase was demonstrated in strain S50, and this activity was accelerated in the presence of reduced glutathione. NADP^+ could not replace NAD^+ . Formate dehydrogenase (NAD^+ - or NADP^+ -linked) was also detected in strain S50, but in trace amounts in strains sp. No. 2941 and S25. This dehydrogenase activity disappeared after a short period of refrigerated storage (less than 5 hr). NADP^+ showed the same activity for this dehydrogenase. All three organisms did not have NAD^+ -linked methanol and methylamine dehydrogenase activities. These results are included in Table I.

Taxonomic characteristics of the three active strains

Electronmicrographs of three strains, sp. No. 2941, S25 and S50 are shown in Figs. 2~4. Their morphological, cultural and physiologi-

cal properties were studied and summarized in Table II. Identification studies were performed by referring to the 8th Edition of Bergey's Manual of Determinative Bacteriology.¹⁹⁾ All three strains are aerobic and non-spore forming Gram-negative short rods. In liquid culture, strains No. 2941 and S25 appeared mostly singly and motile with polar flagella, but strain S50 was plump rod with round ends, usually occurring in pairs or occasionally in short chains and being non-motile. They grew well on bouillon agar and colonies were entire, smooth and circular. Strain No. 2941 produced reddish pink pigment. Two strains, S25 and S50 were non-colored, but old cultures became opaque or pale yellowish white. Table III summarized the assimilation of carbon sources by the three isolates. Methanol is apparently the most utilizable carbon source for the three strains. However, growth was not observed on for-

TABLE II. TAXONOMICAL CHARACTERISTICS OF THREE ISOLATED STRAINS

	2941	S25	S50
1. Morphological characteristics			
Shape	Rods	Short rods	Plump rods
Size	1.0 to 1.1 by 2.0 to 2.9 μm	0.4 to 0.7 by 1.1 to 1.5 μm	1.0 by 1.2 to 1.5 μm
Motility	Motile by polar flagellum	Motile by polar flagella	Non-motile
Spore formation	Negative	Negative	Negative
Gram-stain	Negative	Negative	Negative
2. Physiological characteristics			
Growth temperature	20~37°C	25~37°C	20~37°C
Growth pH	6~8	6~9	6~9
Litmus milk	No changed	Alkaline	No changed
Nitrate reduction	Negative	Negative	Negative
Denitrification	Negative	Positive	Negative
Methyl red test	Negative	Negative	Negative
Voges-Proskauer test	Negative	Negative	Negative
Indole production	Negative	Negative	Negative
Hydrogen sulfide formation	Negative	Negative	Negative
Hydrolysis of starch	Negative	Negative	Negative
Liquefaction of gelatin	Negative	Positive	Negative
Citrate utilization	Negative	Positive	Positive
Urease	Positive	Positive	Negative
Catalase	Positive	Positive	Positive
Oxidase	Positive	Positive	Positive
Pigment production			
<i>Pseudomonas</i> F agar	Negative	Positive	Negative
<i>Pseudomonas</i> P agar	Negative	Negative	Negative

TABLE III. GROWTH OF THREE ISOLATES ON VARIOUS CARBON SOURCES

Carbon source	2941	S25	S50
Methanol	++	++	+
Formaldehyde	—	—	—
Methylamine	+	±	++
Dimethylamine	—	—	+
Trimethylamine	—	—	+
Ethanol	+	++	+
<i>n</i> -Propanol	±	++	—
<i>n</i> -Butanol	—	+	—
D-Glucose	±	++	++
D-Fructose	++	++	++
D-Mannose	±	++	++
D-Galactose	±	++	++
Sucrose	±	++	++
Maltose	±	++	++
D-Sorbitol	±	++	++
D-Mannitol	±	++	++
D-Xylose	±	++	++
D-Ribose	±	++	++
L-Arabinose	±	++	+
D-Arabinose	±	++	—
Glycerol	++	++	++
Glycine	—	—	—

Symbols denote: ++, good growth; +, weak growth; ±, variable; —, no growth.

maldehyde in batch culture. As for C₁-compounds, methylamine and dimethylamine were also assimilated with different degrees of assimilation. Strain S50 assimilated trimethylamine, as well as monomethylamine. Among primary alcohols, ethanol was the second utilizable carbon source for the three strains, but *n*-propanol was assimilated by S25. Higher primary alcohols, *n*-butanol, *n*-hexanol, *n*-octanol, *n*-decanol and higher alcohols were not assimilated.

Strain No. 2941 assimilated D-fructose and glycerol, but growth was variable or weak on 11 other tested sugars. Strains S25 and S50 grew on a series of hexoses, pentoses and polyalcohols. Therefore, all three strains were facultative methylotroph. As strains No. 2941 and S25 were aerobic Gram-negative rod and motile with polar flagella, they should fall into the genus *Pseudomonas*. Strain S25 may correspond to *Pseudomonas fluorescens* type and strain No. 2941 was a typical "red methanol utilizer." Strain S50 was non-motile and had

no flagellum, as shown in Fig. 4. This strain did not produce pigments and was positive in catalase and oxidase, but negative in urease. This strain can be classified as a member of group VI in Kouno's classification system of methanol utilizing bacteria.²⁰⁾ Further taxonomic studies will be required on strain S50.

DISCUSSION

The phenazine methosulfate-dependent methanol dehydrogenase activity is commonly demonstrable in both obligate and facultative methylotrophs and also in both assimilation pathways: serine and ribulose monophosphate. Therefore, it is the key enzyme for all types of methylotrophs. Electrophoresis of methanol dehydrogenase was reported on the partially purified preparations to determine their purity. While *Methylococcus capsulatus* and *Pseudomonas* sp. M27 were not taxonomically related, there were some similarity on biochemical and physical properties between the enzymes of two bacteria.^{11,12,21)} However, electrophoretic behavior of two enzymes was clearly different: *M. capsulatus* enzyme moved to anode faster than *Pseudomonas* enzyme. *Hyphomicrobium* WC enzyme and *Pseudomonas* TP-1 enzyme migrated toward the anode at pH 8.9, whereas *Pseudomonas* W1 enzyme migrated toward the cathode.¹²⁾ Two purified methanol dehydrogenase from soluble fraction and from solubilized fraction of *Methylococcus capsulatus* migrated to the anode with identical mobility in electrophoresis on polyacrylamide gels.

Comparing these results and the present study, it can be proposed that phenazine methosulfate-dependent methanol dehydrogenase can be grouped to at least three types based on electrophoretic mobility. The first type of enzyme migrates toward the anode at relative slow mobility. Enzymes from *Pseudomonas* sp. M27,^{11,12)} *Pseudomonas* TP-1,¹²⁾ *Hyphomicrobium* WC,¹²⁾ *Pseudomonas* C¹⁴⁾ and *Pseudomonas* sp. No. 2941¹⁶⁾ may belong to this group. The second type of enzyme does not migrate to the anode at pH 8.9~9.3. Enzyme of *Pseudomonas* S25 belong to this

group and *Pseudomonas* W1 enzyme¹²⁾ may be included in this group. The third type is the most positively charged protein and it migrates toward the anode faster than the first type of enzyme. The isolated bacterium sp. S50 is the only strain among over 50 strains tested which produces this type of enzyme. The enzyme from *Methylococcus capsulatus* may be classified into this group.^{11,12)} All three methanol dehydrogenases from *Pseudomonas* sp. No. 2941, *Pseudomonas* S25 and a strain S50 were purified and crystallized.^{18,22)} Properties of these crystalline enzymes will be published in detail later. The classification of methanol dehydrogenase may contribute to establishing the classification system for methylotrophic bacteria.

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