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Microbial Oxidation of Methane and Methanol: Isolation of Methane-Utilizing Bacteria and Characterization of a Facultative Methane-Utilizing Isolate

RAMESH N. PATEL,* CHING T. HOU, AND A. FELIX

Corporate Research Laboratory, Exxon Research and Engineering Company, Linden, New Jersey 07036

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A methane-utilizing organism capable of growth both on methane and on more complex organic substrates as a sole source of carbon and energy, has been isolated and studied in detail. Suspensions of methane-grown cells of this organism oxidized C-1 compounds (methane, methanol, formaldehyde, formate); hydrocarbons (ethane, propane); primary alcohols (ethanol, propanol); primary aldehydes (acetaldehyde, propionaldehyde); alkenes (ethylene, propylene); dimethylether; and organic acids (acetate, malate, succinate, isocitrate). Suspensions of methanol- or succinate-grown cells did not oxidize methane, ethane, propane, ethylene, propylene, or dimethylether, suggesting that the enzymatic systems required for oxidation of these substrates are induced only during growth on methane. Extracts of methane-grown cells contained a particulate reduced nicotinamide adenine dinucleotide-dependent methane monooxygenase activity. Oxidation of methanol, formaldehyde, and primary alcohols was catalyzed by a phenazine methosulfate-linked, ammonium ion-requiring methanol dehydrogenase. Oxidation of primary aldehydes was catalyzed by a phenazine methosulfate-linked, ammonium ion-independent aldehyde dehydrogenase. Formate was oxidized by a nicotinamide adenine dinucleotide-specific formate dehydrogenase. Extracts of methane-grown, but not succinate-grown, cells contained the key enzymes of the serine pathway, hydroxypyruvate reductase and malate lyase, indicating that the enzymes of C-1 assimilation are induced only during growth on C-1 compounds. Glucose-6-phosphate dehydrogenase was induced during growth on glucose. Extracts of methane-grown cells contained low levels of enzymes of the tricarboxylic acid cycle, including α-keto glutarate dehydrogenase, relative to the levels found during growth on succinate.

Among the methylotrophic organisms are both obligate and facultative methylotrophs. Obligate methylotrophs are capable of utilizing only carbon compounds containing no carbon-carbon bonds (methane, methanol, dimethylether, methylamine, etc.) as sole sources of carbon and energy. Facultative methylotrophs can use compounds containing no carbon-carbon bonds as well as complex organic compounds with carbon-carbon bonds as sole sources of carbon and energy (6).

Almost all methane-utilizing bacteria isolated in pure culture exhibit obligate growth requirement for methane, methanol, or dimethylether as a source of carbon and energy (5, 9, 11, 19, 32, 35). Organic compounds were unable to serve as a carbon and energy source. Recently, Patt et al. (25) reported the isolation and characterization of bacteria in pure culture that utilize methane as well as the complex organic compounds as sources of carbon and energy.

In this paper we report on the isolation of both obligate and facultative methane-utilizing bacteria and on the characterization of one of the facultative isolates.

MATERIALS AND METHODS

Bacterial strain. *Methylobacterium organophilum* strain xx (ATCC 27886) (27) was obtained from the American Type Culture Collection, Rockville, Md.

Media and growth condition. The salt medium of Foster and Davis (11) was used for isolation and growth of organisms. When agar (Difco, purified) was present, it was added to a 1.5% (wt/vol) concentration. Methane as a carbon and energy source was supplied by filtering (through glass-wool filter) a mixture of methane and air (1:1, vol/vol) into closed flasks containing salt medium. Methanol, ethanol, or propanol (0.1%, vol/vol) was added directly to the media and was also provided via continuous release of vapors. All other carbon sources were sterilized separately in water and were added directly to the growth medium at

a final concentration of 10 mM. Agar cultures were maintained and grown on mineral salt agar plates in a desiccator jar under an atmosphere of methane and air (1:1, vol/vol) at 30°C. Liquid cultures were grown at 30°C on a rotary shaker.

Experiments with cell-suspensions. Cells were grown at 30°C in 2.8-liter flasks containing 700 ml of salt medium (11) with methane (methane and air, 1:1), methanol (0.2%, vol/vol) or succinate (10 mM) as the sole carbon and energy source. Cells were harvested by centrifuging for 15 min at 15,000 × *g* and were washed twice in 25 mM potassium phosphate buffer, pH 7.0, and suspended in the same buffer. Oxidation of various compounds by methane-, methanol-, or succinate-grown cell suspensions was studied polarographically with a Clark oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio). Reaction mixtures contained the following in a final volume of 3.0 ml: air-saturated 25 mM potassium phosphate buffer (2.4 or 2.8 ml), 0.1 M substrate (0.1 ml), and cell suspensions (0.1 ml). When methane, ethane, propane, dimethylether, carbon monoxide, propylene, or ethylene was used as the substrate, 0.4 ml of 25 mM phosphate buffer, pH 7.0, saturated with various substrate was used in the reaction mixture. Protein in the cell suspension was estimated by the method of Lowry et al. (20) with bovine serum albumin as a standard.

Preparation of cell extracts. Cell-suspensions (2 g [wet weight]) in 10 ml of 25 mM potassium phosphate buffer, pH 7.0, containing 5 mM $MgCl_2$ were sonically disrupted intermittently for 5 min at 2°C with a Megason ultrasonic disintegrator. Sometimes cell suspensions were disintegrated by two passes through a French press at 15,000 lb/in^2 pressure. The broken cell preparations were centrifuged at 8,000 × *g* for 15 min to remove intact cells and debris. The supernatant extracts (crude extract) were fractionated by centrifugation at 40,000 × *g* for 30 min to obtain soluble and particulate fractions.

Enzyme assays. Methane monooxygenase activity was assayed polarographically as described previously (10, 28). Methanol dehydrogenase was assayed spectrophotometrically as described previously (21, 22). Aldehyde dehydrogenase was assayed spectrophotometrically by measuring oxidation of various aldehydes in the presence of the auxiliary electron acceptor phenazine methosulfate. The reaction mixture, in a total of 3.0 ml, contained 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.0; 0.5 μmol of 2,6-dichlorophenol indophenol, 2 μmol of phenazine methosulfate, and soluble fraction. The reaction was started by addition of 50 μmol of substrate, and reduction of dye was measured at 600 nm. Specific activities were expressed as nanomoles of 2,6-dichlorophenol indophenol reduced per minute per milligram of protein. Formate dehydrogenase was assayed spectrophotometrically in the soluble fraction by measuring the rate of reduction of oxidized nicotinamide adenine dinucleotide (NAD^+) at 340 nm as described previously (23). The following enzymes were assayed as described previously (3, 4, 12, 13, 15–18): citrate synthase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase, hydroxypyruvate reductase, hexulose phosphate synthase, and malate lyase. The changes in absorbance were monitored with a Gilford recording spectrophotometer. Protein was determined by the Folin phenol reagent method (20) with bovine serum albumin as a standard. Specific activities were expressed as nanomoles per minute per milligram of protein.

Electron microscopy. Electron microscopic experiments were performed by Structure Probe, Inc., West Chester, Pa., by the following method. For a thin section of the organism, preparations were fixed with 0.5% paraformaldehyde and glutaraldehyde buffered with cacodylate at pH 6.3 for a duration of 1 h at 26°C. After washing and centrifugation, the pelleted agar-enrobed cells were fixed in 1% OsO_4 for 1 h. After dehydration, all preparations were embedded in Epon-Araldite. Thin sections were cut with a diamond knife on a MT-2 Sorvall microtome, placed on Parlodion and carbon-coated grids, and stained with uranylacetate and lead citrate before electron microscopic examination.

RESULTS

Isolation of methane-utilizing bacteria. Enrichment and isolation techniques used were similar to those described earlier (35). Soil samples collected from various sites at the Exxon Bayway Refinery were used as inocula. The 1-g soil samples were suspended in 10 ml of mineral salt medium and allowed to stand at room temperature for 1 h to settle soil particles. The supernatant solution was inoculated into 300-ml flasks containing 50 ml of mineral salt medium. The enrichment flasks were incubated at 30°C on a shaker under an atmosphere of methane and air (1:1, vol/vol). Within 72 to 96 h the culture medium became turbid, presumably due to the growth of methane-utilizing bacteria.

Serial dilutions of the enrichment cultures were made and spread onto mineral salt agar plates. The plates were incubated in a desiccator under an atmosphere of methane and air (1:1, vol/vol) at 30°C. Isolated colonies were picked and restreaked. It was discovered that one of these eight morphologically different single-colony isolates would grow on nutrient agar as well as on methane.

Carbon and energy sources. Various substrates were tested as sole sources of carbon and energy for our isolates as well as *M. organophilum* strain ATCC 27886 (Table 1). Seven of our isolates grew only on methane or methanol, whereas isolate R6 was able to use methane, methanol, and a variety of organic compounds, including tricarboxylic acid cycle intermediates, glucose, and nutrient agar. The seven obligate methane-utilizing isolates were morphologically similar to obligate methane utilizers isolated and described previously by Whittenbury et al. (35). Morphological and biochemical properties of isolate R6 were further characterized.

TABLE 1. *Utilization of carbon and energy sources by methylotrophic organisms*

Carbon and energy source	Isolate no.[a]								*M. organophilum* strain XX[b]
	R1	R2	R3	R4	R5	R6	R7	R8	
Methane	+	+	+	+	+	+	+	+	+
Methanol	+	+	+	+	+	+	+	+	+
Methylamine	−	−	−	−	−	−	−	−	−
Formaldehyde	−	−	−	−	−	−	−	−	NT
Formate	−	−	−	−	−	−	−	−	NT
Ethanol	−	−	−	−	−	+	−	−	−
Acetate	−	−	−	−	−	+	−	−	+
Succinate	−	−	−	−	−	+	−	−	+
Fumarate	NT	NT	NT	NT	NT	NT	NT	NT	+
Malate	NT	NT	NT	NT	NT	NT	NT	NT	+
Glucose	−	−	−	−	−	+	−	−	+
Galactose	−	−	−	−	−	−	−	−	+
Sucrose	−	−	−	−	−	−	−	−	+
Lactose	−	−	−	−	−	−	−	−	+
Propanol	−	−	−	−	−	−	−	−	−
Nutrient broth	−	−	−	−	−	+	−	−	+

[a] +, Growth; −, no growth; NT, not tested.
[b] Results of Patt et al. (25).

Morphological characterization of isolate R6. The cells of isolate R6 were gram-negative motile rods producing light brown colonies on methane, methanol, succinate, glucose, or nutrient agar plates. Cells grown on methane as the sole source of carbon and energy had extensive intracytoplasmic membranes arranged at the periphery of the cell (Fig. 1). This membrane type has previously been designated as type II. The organism measured 0.6 by 2 μm.

Experiments with cell suspension. Suspensions of cells grown on methane-oxidized C-1 compounds (methane, methanol, formaldehyde, formate and carbon monoxide); hydrocarbons (ethane, propane); alkenes (ethylene, propylene); primary alcohols (ethanol, propanol, butanol); primary aldehydes (acetaldehyde, propionaldehyde, butyraldehyde); and organic acids (acetate, malate, succinate) (Table 2). Methanol- or succinate-grown cell suspensions do not oxidize methane, ethane, propane, ethylene, propylene, dimethylether, or carbon monoxide, suggesting that the enzymes required for oxidation of these substrates are induced only during growth on methane. Cells grown on succinate do not oxidize C-1 compounds, indicating that the enzymes of C-1 oxidation are induced only during growth on C-1 compounds. Organic acids are oxidized at higher rates by succinate-grown cells than methane- or methanol-grown cells.

Enzymes of C-1 oxidation. Table 3 shows the activities of enzymes of C-1 oxidation in isolate R6 grown at the expense of methane or methanol. Particulate fractions of methane-, but not methanol-grown, cells exhibited a low activity of a reduced NAD-linked methane monooxygenase. Extracts of methane- and methanol-grown cells contained a phenazine methosulfate-linked, ammonium ion-requiring methanol dehydrogenase activity. Methanol dehydrogenase also catalyzed the oxidation of other primary alcohols, substituted primary alcohols, and formaldehyde. Rates of oxidation of various primary alcohols are shown in Table 4. The enzyme has a high pH optimum (pH 9.5). A similar alcohol dehydrogenase with these properties has been reported from other obligate (21, 22, 34) and facultative methylotrophs (1, 2, 31). Oxidation of formaldehyde by methanol dehydrogenase is presumably due to the fact that in aqueous solution formaldehyde is predominantly hydrated (>99.6%) and appears as an analog of methanol for the dehydrogenase (31).

Oxidation of primary aldehydes was catalyzed by a phenazine methosulfate-linked soluble aldehyde dehydrogenase (Table 5). The optimum pH determined with propionaldehyde as a substrate was 6.5. Aldehyde dehydrogenase does not require ammonium ion as an activator, and potassium ferricyanide could replace phenazine methosulfate as an electron acceptor. NAD^+, NADP, and flavine adenine dinucleotide did not act as electron acceptors.

Extracts contained a soluble formate dehydrogenase, similar to that found in other methane- or methanol-utilizing organisms (Table 3). Enzyme activity was demonstrated spectrophotometrically with potassium ferricyanide or NAD^+.

Methanol dehydrogenase, aldehyde dehydrogenase, and formate dehydrogenase activities could not be detected in succinate-grown cells,

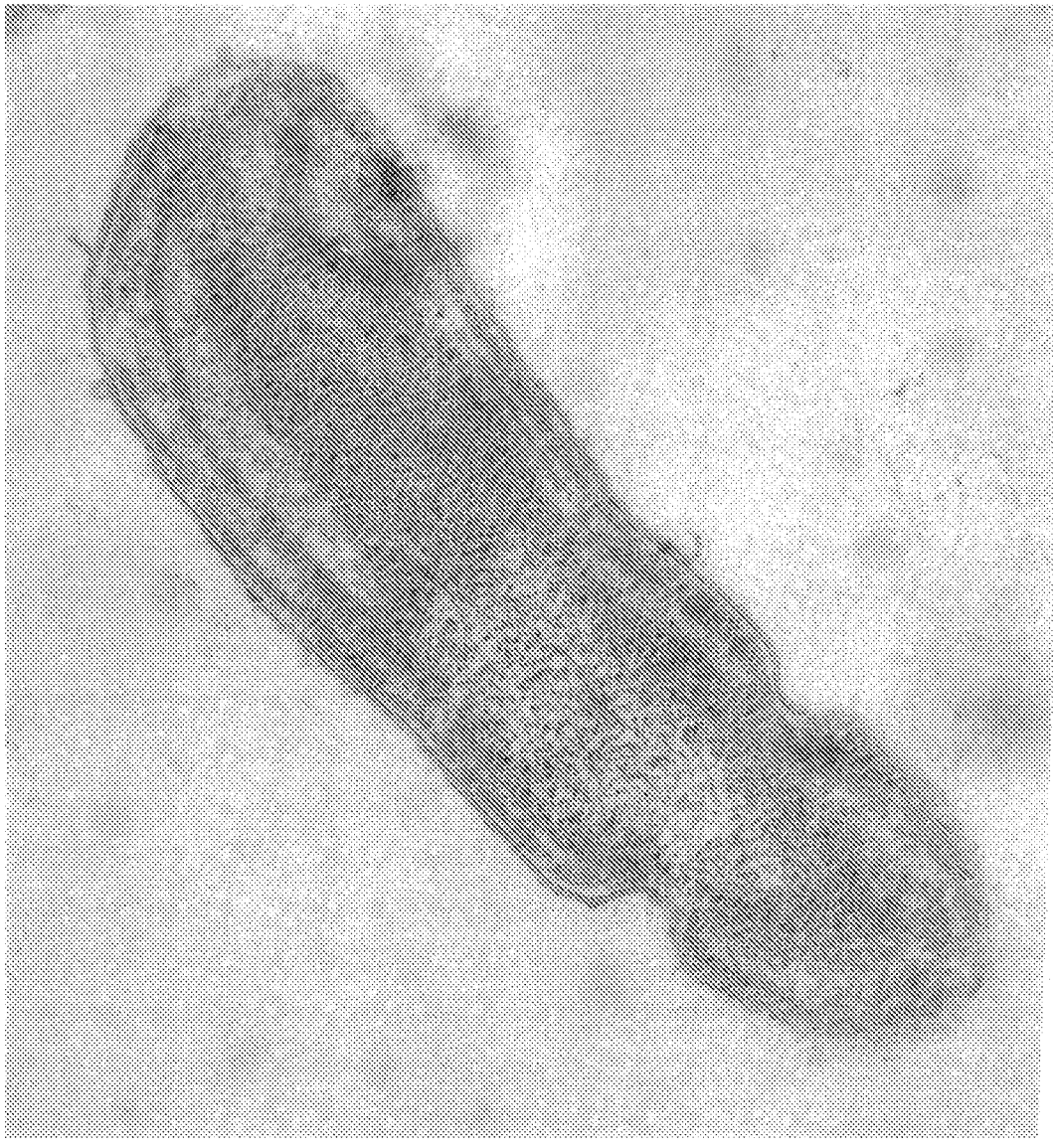

FIG. 1. *Electron micrograph of isolate R6 grown on methane. Magnification, ×60,000.*

indicating that the enzymes of C-1 oxidation are induced only during growth on C-1 compounds.

Enzymes of C-1 assimilation. Enzymes that play a key role in C-1 assimilation by the serine or hexulose pathway of formaldehyde fixation were examined in extracts of methane- and succinate-grown cells. Hydroxypyruvate reductase activity was found in high levels in the methane-grown cells, but could not be detected in succinate-grown cells. A specific activity (nanomoles of NADH oxidized per minute per milligram of protein) of 241 was obtained with extracts of methane-grown cells. Hexulose phosphate synthase activity could not be detected in the soluble or particulate fraction. This indicates that the organism utilized the serine pathway for cellular synthesis during growth on C-1 compounds. Isocitrate lysase activity was not detected in methane-grown cells; however, malate lyase activity was detected. A specific activity (nanomoles of glyoxylate formed per minute per milligram of protein) of 21 was obtained with extracts of methane-grown cells.

Enzymes of the tricarboxylic acid cycle. The levels of a number of enzymes associated with the tricarboxylic acid cycle in cells grown

TABLE 2. *Oxidation of various compounds by cell-suspensions of isolate R6 grown on different carbon sources*

Substrate	Rate of oxidation (nmol of O_2 consumed per min per mg of protein)		
	Methane-grown cells	Methanol-grown cells	Succinate-grown cells
Methane	106	0	0
Methanol	114	104	0
Formaldehyde	102	96	0
Formate	20	15	0
Ethane	38	0	0
Ethanol	49	48	0
Acetaldehyde	65	42	0
Acetate	10	8	12
Propane	40	0	0
Propanol	49	42	0
Propionaldehyde	61	48	0
Ethylene	32	0	0
Propylene	36	0	0
Succinate	10	10	87
Malate	9	10	270
Fumarate	8	9	60
Carbon monoxide	50	0	0
Dimethylether	60	0	0
Isocitrate	12	10	57
α-Keto glutarate	10	10	55

TABLE 3. *Enzymes of C-1 oxidation in extract of isolate R6 grown on different carbon sources*[a]

Enzyme	Sp act (nmol/min per mg of protein)	
	Methane-grown cells	Methanol-grown cells
Methane monooxygenase	16	0
Methanol dehydrogenase	85	110
Aldehyde dehydrogenase	250	280
Formate dehydrogenase	13	11

[a] Methane monooxygenase activity was estimated by using NADH as an electron carrier as described (10, 28). Methanol dehydrogenase activity was estimated by using phenazine methosulfate and 2,6-dichlorophenol indophenol as electron acceptors. Specific activity was expressed as nanomoles of 2,6-dichlorophenol indophenol reduced per minute per milligram of protein with methanol as substrate (21, 22). Aldehyde dehydrogenase was estimated by using phenazine methosulfate and 2,6-dichlorophenol indophenol as electron acceptors. Specific activity was expressed as nanomoles of 2,6-dichlorophenol indophenol reduced per minute per milligram of protein with propionaldehyde as a substrate. Formate dehydrogenase was estimated by using NAD as an electron acceptor (23).

TABLE 4. *Rate of oxidation of alcohols by methanol dehydrogenase*

Substrate	Rate of Oxidation[a] (%)
Formaldehyde	82
Methanol	100
Ethanol	75
Propan-1-ol	75
Butan-1-ol	87
Pentan-1-ol	85
Hexan-1-ol	87
Heptan-1-ol	75
Octan-1-ol	75
Nonan-1-ol	75
Decan-1-ol	70
3-Chloro-1-propanol	62
2-Methoxyethanol	75
2-Phenoxyethanol	80
Octan-2-ol	0
Butan-2-ol	0
Isobutanol	0
Cyclohexanol	0
Benzyl alcohol	0
Iso-propanol	0
4-Chloro-1-butanol	85

[a] Methanol dehydrogenase activity was estimated by using phenazine methosulfate and 2,6-dichlorophenol indophenol as electron acceptor as described previously (21, 22). 100% rate of oxidation represents 110 nmol of 2,6-dichlorophenol indophenol reduced per min per mg of protein.

TABLE 5. *Rate of oxidation of aldehydes by aldehyde dehydrogenase*

Substrate	Rate of oxidation[a] (%)
Formaldehyde	100
Acetaldehyde	67
Propionaldehyde	72
Butyraldehyde	130
Pentaldehyde	91
Hexaldehyde	84
Heptaldehyde	85
Octaldehyde	45
Nonaldehyde	15
Decaldehyde	10
Benzaldehyde	20
Salicylaldehyde	20
Glyoxylate	12
Glyceraldehyde	13

[a] Aldehyde dehydrogenase activity was estimated by using phenazine methosulfate and 2,6-dichlorophenol indophenol as electron acceptor as described in the text. 100% rate of oxidation represents 275 nmol of 2,6-dichlorophenol indophenol reduced per min per mg of protein.

on either methane or succinate are shown in Table 6. Extracts of methane-grown cells contained low levels of enzymes of the tricarboxylic acid cycle, including α-ketoglutarate dehydrogenase. The specific activity of enzymes of the tricarboxylic acid cycle during growth on succinate was several-fold higher than that on methane-grown cells.

Glucose-6-phosphate dehydrogenase was induced only during growth on glucose. Activity

TABLE 6. *Enzymes of tricarboxylic acid cycle in isolate R6 grown on methane and succinate*

Enzyme	Sp act (nmol/min per mg of protein)	
	Methane-grown cells	Succinate-grown cells
Citrate synthetase	120	254
Aconitase	24	63
Isocitrate dehydrogenase	16	475
α-Ketoglutarate dehydrogenase	10	30
Succinate dehydrogenase	17	40
Malate dehydrogenase	14	167

of glucose-6-phosphate dehydrogenase was not detected in methane-grown cells. NAD or NADP were equally effective as electron acceptors for glucose-6-phosphate dehydrogenase activity. A specific activity (nanomoles of NAD^+ or $NADP^+$ reduced per minute per milligram of protein) of 76 and 56 was obtained with NAD^+ and $NADP^+$, respectively, as electron acceptor in extracts of glucose-grown cells.

DISCUSSION

This paper describes the isolation and characterization of a facultative methylotrophic organism, isolate R6, which in addition to methane and methanol utilizes many common organic carbon and energy sources.

Methylotrophs have been classified into two distinct divisions. This division was initially made on the basis of the organization of intracytoplasmic membranes (8, 30), but subsequently it was found that the pathway of reduced C-1 compound assimilation follows a similar division (18). Type I methylotrophs possess closely packed bundles of membranes consisting of a number of disk-shaped vesicles found throughout the cell and use the hexulose phosphate pathway of formaldehyde fixation. Type II methylotrophs possess a system of paired membranes running throughout the organism or aggregated at the periphery and use the serine pathway to incorporate C-1 unit. Furthermore, type I methylotrophs have an incomplete tricarboxylic acid cycle with no detectable α-ketoglutarate dehydrogenase enzyme, whereas type II methylotrophs have a complete tricarboxylic acid cycle (6, 7, 17, 24, 27, 29, 33).

The enzymatic data and electron microscopic studies indicate that isolate R6 belongs in the type II group. Isolate R6 has a complete tricarboxylic acid cycle, including a low level of α-ketoglutarate dehydrogenase and the key enzymes of the serine pathway, hydroxy pyruvate reductase and malate lyase. Hexulose phosphate synthetase, the key enzyme of the hexulose phosphate pathway of formaldehyde fixation, was not detected in the particulate or soluble fraction.

Methylotrophs that utilize the serine pathway are further divisible into two groups: the obligate methylotrophs which can only use C-1 compounds and the facultative methylotrophs which use methanol, methylamine, formate, and complex organic compounds, but are unable to use methane (6, 27). Recently, Patt et al. (25) isolated and described facultative methane-utilizing organisms and characterized one, the isolate xx (ATCC 27886), in detail. Isolate xx was placed in the type II group on the basis of its organization of intracytoplasmic membranes and its C-1 assimilation pathway. They proposed *Methylobacterium* as a new genus name for facultative methane utilizers and *Methylobacterium organophilum* as a name for the type species, due to its preference for organic carbon and energy sources more complex than methane (26).

We propose that isolate R6 be included in genus *Methylobacterium* on the basis of its ability to utilize methane and complex organic carbon sources. Isolate R6 differs in some morphological and biochemical properties from *M. organophilum* strain xx. Isolate R6 does not produce pink-pigmented colonies and is unable to utilize lactose, galactose, or sucrose. Glucose-6-phosphate dehydrogenase activity in glucose-grown cells of *M. organophilum* strain xx was found to be $NADP^+$-specific; activity with NAD^+ as an electron acceptor was fivefold lower than with $NADP^+$. However, glucose-6-phosphate dehydrogenase in glucose-grown cells of isolate R6 was nonspecific; NAD^+ or $NADP^+$ was equally effective.

Oxidation of primary alcohols in isolate R6 is catalyzed by an ammonium ion-requiring, phenazine methosulfate-dependent methanol dehydrogenase. A similar enzyme has been reported from other obligate and facultative methylotrophs (14). The enzyme catalyzed the oxidation of primary alcohols, substituted primary alcohols and formaldehyde. Secondary alcohols, isoalcohols, and aromatic alcohols were not oxidized. In contrast, Wolf and Hanson (H. J. Wolf and R. S. Hanson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, K231, p. 224) purified methanol dehydrogenase from *M. organophilum* strain xx and reported that this enzyme also catalyzed the oxidation of secondary, tertiary, and cyclic alcohols at a reduced rate. Ammonium ion was not required for activity until the enzyme had been stored for several weeks.

Oxidation of primary and aromatic aldehydes in isolate R6 was catalyzed by a phenazine methosulfate-linked aldehyde dehydrogenase. A non-

specific 2,6-dichlorophenol indophenol-linked aldehyde dehydrogenase has been reported in the obligate methane-utilizing organism, *Pseudomonas methanica* and the facultative methanol-utilizing organism, *Pseudomonas* AM_1 (14) which catalyzed the oxidation of primary aldehydes. Aromatic aldehydes were not oxidized.

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