Isolation and Characterization of a Pink-Pigmented Facultative Methylotrophic Bacterium

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분홍색 통성 메탄올 자화세균의 분리 및 특성

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ABSTRACT: A pink-pigmented facultative methylotrophic bacterium, *Methylobacterium* sp. strain SY1, was isolated from soil through methanol-enrichment culture technique. The isolate was gram-negative, slightly curved rod, and motile by a single polarly inserted flagellum. The colony was smooth, bright pink, and slimy. The guanine plus cytosine content of the DNA was 66%. The cell was obligately aerobic and exhibited both catalase and oxidase activities. Carotenoid pigment and poly-β-hydroxybutyrate were present. It was found to have three kinds of plasmid with molecular weights 45,000, 38,500, and 23,000. Growth with methanol (0.5%) was fast (t_d = 6.5 h) and was optimal at 30°C and at pH 7.0. The isolate could grow on several sugars, organic acids, amino acids, amines, and alcohols in addition to the methanol. Methanol was found to be assimilated through the serine pathway.

KEY WORDS□Pink-pigmented facultative methylotroph-*Methylobacterium* sp. strain SY1-Isolation of a PPFM

Methylotrophic bacteria are characterized by their ability to utilize reduced carbon compounds containing one or more carbons but no carbon-carbon bonds. Obligate methylotrophs grow only on such compounds, whereas facultative methylotrophs are also able to grow on a variety of other multicarbon substrates (Colby and Zatman, 1972; Anthony, 1982 and 1986).

Methylotrophs have received a great deal of attention recently, due in part to their commercial potential, since these organisms are capable of converting simple one-carbon compounds into commercially interesting multicarbon compounds such as amino acids and cytochrome *c* (Izumi *et al.*, 1977; Ogata *et al.*, 1977; Tani *et al.*, 1985). In addition, methylotrophs contain unique oxidative enzymes that have low substrate specificities and have potential interest for use in biocatalyst (Anthony, 1982).

Although these organisms have several interesting properties, the development of economically feasible processes will depend in most cases on the development of genetic technique. Recently genetic studies on the methylotrophic bacteria have mostly been done with facultative methylotrophs, especially with pink-pigmented facultative methylotrophic bacteria (PPFMs) (Anderson and Lidstrom, 1988; Lyon *et al.*, 1988; Machlin and Hanson, 1988; Machlin *et al.*, 1988; Nunn and Lidstrom, 1986; Tatra and Goodwin, 1985).

PPFMs are common inhabitants of a variety of terrestrial and aquatic environments (Green *et al.*, 1988). Over 150 strains of PPFMs have been studied taxonomically, and have been placed in the genus *Methylobacterium* (Green and Bousfield, 1982 and 1983; Hood *et al.*, 1987 and 1988). Eight

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species are recognized recently, with most known strains falling into two groups, *Methylobacterium extorquens* and *Methylobacterium organophilum* (Green *et al.*, 1988).

In this study, we describe some properties of a PPFM which is isolated for comparative genetic studies on the methylotrophs in the future to understand better the mechanism of methylotrophy at the molecular level. We propose the genus name *Methylobacterium* for the new PPFM isolate.

MATERIALS AND METHODS

Enrichment and isolation

1 g of soil sample from Seoul, Korea was suspended in 10 m*l* of liquid standard mineral base (SMB) medium (Kim and Hegeman, 1981) and the suspension was left for a couple of minutes to settle insoluble materials down. A loopful supernatant was then streaked onto solid SMB medium containing 0.5% (v/v) methanol. The plates were incubated at 30°C. After three days of incubation, fast growing colonies with pink color were transferred to new methanol (0.5%, v/v)-SMB plates and grown for three days at 30°C. This step was repeated for several times to select the most rapidly growing colony. The selected colony was then tested for homogeneity and facultative methylotrophy using nutrient agar plates.

Culture conditions

PPFM isolate was grown methylotrophically in SMB medium containing 0.5% (v/v) methanol at 30°C. For several specific tests, methanol was substituted by other organic compounds.

Biochemical and nutritional properties

Several biochemical tests were carried out by the methods of Gerhardt *et al.* (1981) except for the following. Carotenoid was examined using the methods of Anthony and Zatman (1964) and Starr and Stephens (1964). Nitrogenase was examined indirectly using C_2H_2 as described by Postgate (1972). Sensitivity to various antibiotics was tested on methanol (0.5%, v/v)-SMB plates using various antibiotics discs (Difco or BBL). Utilization of organic compounds other than methanol was tested on SMB plates supplemented with 0.2% (w/v) of each substrate. Several bacteria were used as positive and negative controls for the above tests.

DNA base composition

DNA was extracted by the method of Rodriguez and Tait (1983). The guanine and cytosine content of DNA was determined by the perchloric acid chemical hydrolysis and spectrophotometric procedure of Skidmore and Duggan (1971). Adenine, guanine, cytosine, and thymine (Sigma) were used as standards.

Enzyme assay

Methylenetetrahydrofolate dehydrogenase and hydroxypyruvate reductase activities were assayed by the methods of Kato *et al.* (1977) and Heptinstall and Quayle (1970) using crude cell extracts. The crude cell extracts were prepared from cells grown with methanol (0.5%, v/v) or sodium succinate (0.2%, w/v). Cells resuspended in 50 mM phosphate buffer (pH 7.0) were disrupted by sonic treatment (10 s/m*l*) at 0°C. The suspension was centrifuged at 15,000 × *g* for 15 min at 4 °C, and the supernatant fluid was referred to as crude cell extract. *Methylobacterium* sp. strain AM1 (NCIB 9133) and *Methylophilus methylotrophus* (NCIB 10515) were used as positive and negative controls, respectively, for serine type methylotrophs. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Plasmid DNA isolation

Plasmid DNA from PPFM isolate was prepared by acetone-alkaline hydrolysis method (Kim and Lidstrom, 1989) which is a modification of the alkaline hydrolysis procedure described by Maniatis *et al.* (1982), and then was analyzed on 0.5% agarose gels.

Effect of temperature on the growth with methanol

Cells were grown in liquid SMB medium (pH 7.0) supplemented with 0.5% (v/v) methanol at various temperatures to examine the effect of temperature on the growth with methanol of PPFM isolate. Growth was determined by turbidity measured at 430 nm.

Effect of initial pH on the growth with methanol

Cells were grown in liquid SMB media of various pHs at 30°C with 0.5% (v/v) added methanol to select the most favorable pH for the growth with methanol of PPFM isolate.

Optimal concentration of methanol for cell growth

Cells were grown in liquid SMB media (pH 7.0) containing various amounts of added methanol at 30°C to determine the optimal concentration of methanol for the growth of PPFM isolate.

RESULTS AND DISCUSSION

Isolation and morphology

After several times of selection with methanol, a PPFM isolate was stabilized as indicated by morphology and color of the colony and by the cell shape. The isolate, designated SY1, was able to grow actively with methanol as a sole source of carbon and energy aerobically. Growth was good on agar plates as well as in liquid medium. Growth factors were not required. Colonies were bright pink, slimy, glistening, raised convex with entire margin. The cell was gram-negative, slightly curved rod with dimension of 1.0-1.2 × 3.0-4.0 μm (Fig. 1). It was motile, and had a single polarly inserted flagellum (Fig. 2). It does not form spore, but has capsular materials around it. Electron microscopic examination revealed that the isolate reproduce by binary fission. The cell forms large amounts of sediments during cultivation in liquid medium, and adsorbes to the surface of flasks in the form of pellicle.

Fig. 1. *Phase contrast micrograph of a facultative methylotrophic isolate.*
Bar (lower right side of the micrograph) represents 1 μm.

Fig. 2. *Electron micrograph of a facultative methylotrophic isolate.*
Bar represents 1 μm.

Biochemical and nutritional properties

The isolate has no nitrogenase activity. Indole and H_2S were not formed. Starch and gelatin were not hydrolyzed. Glucose and lactose were not fermented, implying that it obtains energy through respiratory metabolism. It was found that the cell is obligate aerobic since denitrification and nitrate/nitrite respiration processes were absent in the cell. Oxidase and catalase were present. It had poly-β-hydroxybutyrate as a reserve material. Spectrum of methanol extracts of methanol-grown cell showed a peak at 492 nm and three shoulders at 314, 464, and 520 nm, implying presence of unsaturated carotenoid pigments in the cell (Karrer and Foster, 1950). The cell was negative for methyl red, Voges-Proskauer, and citrate utilization tests. The cell was very sensitive to 30 μg of tetracycline and slightly sensitive to gentamycin (10 μg) and kanamycin (30 μg), but was resistant to ampicillin (10 μg), chloramphenicol (30 μg), penicillin (10 IU), and bacitracin (15 μg).

The isolate was able to grow, in addition to the methanol, with various organic materials such as sugars, acids, amino acids, amines, and alcohols. Of 34 compounds tested, the following could serve as growth substrates: glucose, fructose, galactose, maltose, lactose, cellobiose, sucrose, mannose, arabinose, formate, lactate, succinate, acetate, tartarate, oxalate, glycolate, glutamic acid,

Table 1. *Enzyme activities of serine pathway in methanol- or succinate-grown methylotrophs*

Bacteria	Specific activity[a]	
	Methylene-THF dehydrogenase[b]	Hydroxypyruvate reductase
PPFM isolate		
Methanol-grown	0.12 (0.01)	0.20
Succinate-grown	0.03 (<0.001)	0.03
Methylobacterium AM1 (methanol-grown)	0.16 (0.02)	0.34
M. methylotrophus (methanol-grown)	<0.001 (<0.001)	<0.001

[a]Micromoles of NADH oxidized per milligram of protein per minute for methylene-THF dehydrogenase and ΔO.D. per milligram of protein per minute for hydroxypyruvate reductase.
[b]Methylenetetrahydrofolate dehydrogenase. The enzyme activity was measured using two kinds of substrate, serine (parenthesis) and formaldehyde.

asparagine, methylamine, ethylamine, triethylamine, ethanolamine, ethanol, glycerol, isopropanol, isobutanol, and mannitol, implying that the isolate is a facultative methylotrophic bacterium. The cell, however, could not grow on cellulose, propionic acid, maleate, methionine, glycine, triethanolamine, and formaldehyde.

It was found that the isolate has two key enzymes, methylenetetrahydrofolate dehydrogenase and hydroxypyruvate reductase, for serine pathway (Table 1), indicating that the bacterium assimilates C_1 compounds through serine pathway.

The G + C content of the isolate was estimated to be 66%. Three kinds of large plasmid with molecular weights 45,000, 38,500, and 23,000 were found from the cell (Fig. 3).

Taxonomic considerations

On the basis of gram reaction, cell morphology, color of colony, and several biochemical and nutritional properties, it was concluded that the new isolate is a PPFM. Since all those PPFMs studied to data were found to be closely related, and have been placed in the genus *Methylobacterium* (Green and Bousfield, 1982 and 1983; Hood *et al.*, 1987 and 1988; Green *et al.*, 1988). It may be able to assign the new PPFM isolate to the genus *Methylobacterium*. The new isolate, however, was found

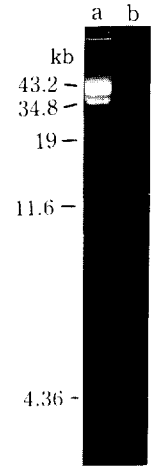

Fig. 3. *Agarose gel electrophoresis of plasmid DNA in the facultative methylotrophic isolate.*
Plasmid DNA from PPFM isolate was prepared and analyzed by the methods described in the text. Size markers in lane (a) are pDA4629 (43.2 kb), pDA4628 (34.8 kb), pRK310 (19 kb), pPstA 322 (11.6 kb), and pBR322 (4.36 kb). Plasmid DNA from PPFM isolate (lane b).

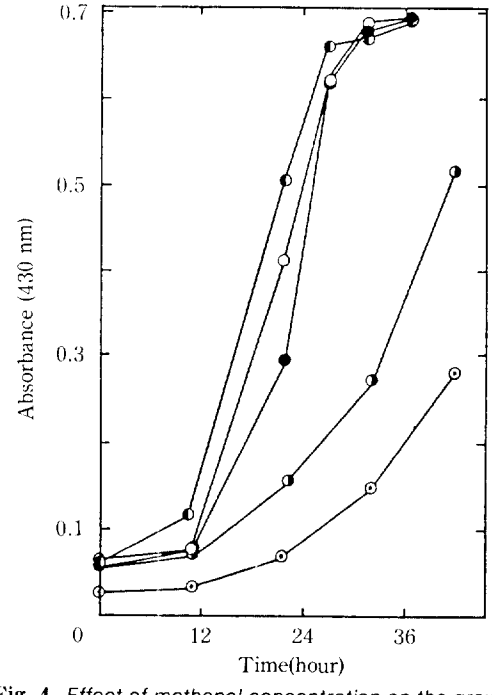

Fig. 4. *Effect of methanol concentration on the growth of PPFM isolate.*
The isolate was grown on various concentrations of methanol under the conditions described in the text. Symbols: 0.5% (v/v) (-●-), 1.0% (-○-), 1.5% (-◐-), 2.0% (-◑-), and 3.0% (-⊙-).

to be different in some nutritional and biochemical characteristics from the recognized eight species and some unassigned *Methylobacterium* strains (Gälli and Leisinger, 1988; Green *et al.*, 1988; Warner and Higgins, 1977). We suggest the name *Methylobacterium* sp. strain SY1 for the new PPFM isolate.

Effect of temperature and pH on the growth

When the isolate was grown on methanol at 20°C, 30°C, 35°C, and 37°C, cells grew most actively at 30°C (t_d = 6.5 h). The growth was slow at 35°C (t_d = 16 h), and was not observed at 20°C and 37°C.

The PPFM isolate was grown optimally with methanol at pH 7.0. The growth, however, was not affected significantly even when the cell was cultivated at pHs 6.5 and 8.0. The cell grew very slowly at pH 6.0 (t_d = 40 h), and did not show increase in turbidity for 3 days at pH 8.5.

Optimal concentration of methanol for growth

As shown in Fig. 4 the PPFM isolate grew actively in SMB liquid medium supplemented with 0.5-1.5% methanol (v/v) among those methanol concentrations tested. The growth was slow with 2.0% and 3.0% methanol. No growth was observed with over 5.0% methanol (data not shown), implying that methanol is still inhibitory for this bacterium even though the cell is able to grow on methanol as a sole source of carbon and energy. The doubling time with 0.5% methanol at pH 7.0 and 30°C was found to be 6.5 h.

Since the new PPFM isolate grows optimally in liquid SMB medium of pH 7.0 supplemented with 0.5% (v/v) methanol at 30°C, subsequent cultivation of this bacterium with methanol was carried out under these conditions.

적 요

흙으로부터 메탄올을 이용하여 성장하는 분홍색 통성 메탄올 자화세균을 분리하여 *Methylobacterium* sp. strain SY1이라 명명하였다. 이 세균은 그람음성세균으로 약간 굽은 간균이고 한쪽 끝에 달린 한개의 편모로 운동하였다. 세균의 군락은 매끈하고 밝은 분홍빛을 띄우며 점성이 높았다. 이 세균이 지니는 DNA의 G+C 함량은 약 66%로 나타났다. 이 세균은 절대호기성으로 catalase와 oxidase 활성을 나타내었고, carotenoid 색소와 poly-β-hydroxybutyrate를 가지고 있었다. 이 세균은 또한 세가지 종류의 큰 plasmid DNA (45,000, 38,500, 23,000)를 가졌으며, 30℃와 pH 7.0에서 0.5% (v/v)의 메탄올을 이용하여 빠른 성장을 하였다 (t_d=6.5 시간). 이 세균은 메탄올은 물론 여러가지 종류의 당, 유기산, 아미노산, 아민 및 알코올을 이용하여 성장할 수 있었고, 메탄올은 serine pathway를 통하여 이 세균의 세포 구성물질로 전환됨을 알 수 있었다.

ACKNOWLEDGEMENT

This work was supported by a research grant (881-0407-015-2) from the Korea Science and Engineering Foundation to YMK.

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(Received Jan. 20, 1989)