Isolation and Characterization of a Restricted Facultatively Methylotrophic Bacterium *Methylovorus* sp. Strain SS1

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A restricted facultatively methanol-oxidizing bacterium, *Methylovorus* sp. strain SS1, was isolated from soil samples from Kuala Lumpur, Malaysia, through methanol-enrichment culture technique. The isolate was nonmotile Gram-negative rod and did not have complex internal membrane system. The colonies were small, pale-yellow, and raised convex with entire margin. The cell did not produce any spores and capsular materials. The cell was obligately aerobic and exhibited catalase, but no oxidase, activity. Plasmid, carotenoid pigment, and poly-β-hydroxybutyric acid were not found. The guanine plus cytosine content of the DNA was 55%. The isolate was found to grow only on methanol, methylamine, or glucose. Growth factors were not required. Cells growing on methanol was found to produce extracellular polysaccharides containing glucose, lactose, and fructose. Growth was optimal (t_d = 1.7) with 0.5% (v/v) methanol at 40°C and pH 6.5. No growth was observed at over 60°C. Cell-free extracts of the methanol-grown cells exhibited the phenazine methosulfate-linked methanol dehydrogenase activity. Methanol was found to be assimilated through the ribulose monophosphate pathway.

KEY WORDS [ ] **methanol, methylotroph, restricted facultative methylotroph, *Methylovorus* sp. strain SS1, ribulose monophosphate pathway, extracellular polysaccharide**

Methylotrophic bacteria are a very diverse group of bacteria sharing a common physiological ability to obtain energy from the oxidation of carbon compounds that are reduced more than carbon dioxide and contain no carbon-carbon bonds (C_1 compounds) and an ability to assimilate formaldehyde derived from the oxidation of the primary substrate for the synthesis of cell material (1, 2, 8, 30). Obligate methylotrophic bacteria utilize only C_1 compounds (7). Facultative methylotrophs, on the other hand, are also capable to grow on a variety of multicarbon compounds (2). The third group of methylotrophic bacteria, the restricted facultative methylotrophs, can only utilize a relatively narrow range of multicabon compounds in addition to C_1 compounds (16, 17).

Methylotrophic bacteria have received a great deal of attention since they have been found to have many possibilities in the commercial and biotechnological applications including the use of the bacteria as hosts for expression of recombinant genetic information and as sources for the production of single cell proteins, vitamins, amino acids, other metabolites, biopolymers, enzymes, and the products of biotransformation (21, 22). The proposed applications of methylotrophs to bioremediation of water and soils contaminated with toxic chemicals is a recent development (26). Because of these potential interests of methylotrophic bacteria, there have recently been increased efforts to isolate and characterize new methanol-oxdizing bacteria (4, 6, 13, 14, 19, 27, 29).

In this study, we have carried out a number of enrichments to isolate restricted facultatively methanol-oxidizing bacteria in order to understand better the mechanism of C_1-metabolism in bacteria and also to use the new isolate to produce biotechnological products in the future. We describe several properties of a bacterial isolate which grows only on methanol, methylamine, and glucose as sole carbon and energy sources. The isolate is proposed as a new methylotrophic bacterium of *Methylovorus*.

MATERIALS AND METHODS

Isolation and cultivation

The bacterium under study was isolated from soil samples from Kuala Lumpur, Malaysia by a modified method of Kim *et al.* (18) using liquid mineral medium and 1.0% (v/v) methanol. Selected colonies which were not pink-pigmented were then tested for restricted facultativity using nutrient agar plates and solid mineral medium

supplemented with yeast extracts (0.2%, w/v) or several other organic compounds. Utilization of organic substrates other than methanol was tested in liquid mineral medium supplemented with 0.2 % (w/v or v/v) of each substrate except for methane which was supplied after mixing with air (methane:air=3:7).

The isolate was grown on a modified mineral medium (19) of Kim and Hegeman (18) containing 1.0% (v/v) methanol at 30°C for standard assay. The growth conditions were modified for several specific tests. Growth was measured by turbidity determined at 600 nm using a Hitachi U-2000 spectrophotometer.

Identification methods

Several physiological and biochemical tests were performed by the methds of Gerhardt *et al.* (12). Sensitivity to antibiotics was tested with cells growing on 0.5% (v/v) methanol using antibiotic discs (BBL or Difco) according to the BBL instruction manual.

DNA was extracted by the method of Marmur (25), and the guanine-plus-cytosine (G+C) composition was determined by the method of thermal denaturation of DNA (11) using a Gilford 2600 spectrophotometer and thermoprogrammer 2527 (temperature change, 1.0°C/min).

Extracellular polysaccharides in the culture filtrates were identified by the presence of white precipitates after centrifugation (15.000×*g*/40 min/4°C) of a mixture of cell-free supernatant of liquid culture and cold absolute ethanol (supernatant: ethanol=1:4, v/v). Analysis in sugar composition of the polysaccharides was carried out by the phenol-sulfuric acid method (9).

Methanol dehydrogenase activity was assayed by the method of Anthony and Zatman (3) using crude cell-free extracts prepared in 100 mM Tris-HCl (pH 9.0). 3-Hexulose phosphate synthase was assayed by the method of Ferenci *et al.* (10) using crude cell extracts prepared in 50 mM phosphate buffer (pH 7.0). Proteins were determined by the method of Lowry *et al.* (24) using bovine serum albumin as a standard.

RESULTS

Isolation and morphological properties

The restricted facultatively methylotrophic isolate, designated SS1, was stabilized as indicated by morphology and color of colonies and cell shape. The isolate was grown well on solid media as well as in liquid media using methanol as the source of carbon and energy. Colonies were whitish-yellow, small (0.2~0.5 mm in diameter), smooth, and raised convex with entire margin. The bacterium was a Gram-negative, non-motile rod (0.4~0.6×1.5~2.0 μm) that multiplies by binary fission (Fig. 1). Electron microscopic observation revealed the presence of irregular

Fig. 1. *Electron micrographs of the restricted facultatively methylotrophic isolate, Methylovorus sp. SS1.*
Transmission electron micrograph of thin sectioned (A) and negative stained (B) SS1. Negative staining was done with phosphotungstic acid. The bars represent 0.1 μm.

structure on the cell surface and the absence of internal membrane structure. Spore, cyst, and capsular materials were not observed.

Physiological and biochemical properties

The isolate grew without aggregation and pigmentation in mineral liquid medium containing methanol. When cells growing at the stationary phase were harvested, slimy materials were found to present between supernatant and pink-colored cell pellets. The isolate was found to be strict aerobe with respiratory metabolism. Denitrification and nitrate/nitrite respiration were not observed. Catalase activity was present, but oxidase activity was not. Gelatin and starch were not hydrolyzed. The cell did not produce carotenoids, poly-β-hydroxybutyrate, indole, and hydrogen sulfide. Growth was inhibited by kanamycin (30 mcg), tetracycline (30 mcg), gentamicin (10 mcg), carbenicillin (10 mcg), and tobramycin (10 mcg), but was not inhibited by ampicillin (10 mcg), chloramphenicol (30 mcg), and penicillin G (10 IU). Of the various subatrates tested, the isolate was able to grow only on methanol, methylamine, and glucose. Methane, ethanol,

Table 1. *Effect of nitrogen sources on the growth of Methylovorus sp. SS1*

Nitrogen sources	Growth rate (μ)	Relative growth rate (%)
NH_4Cl	0.34	100
$(NH_4)_2NO_3$	0.31	91
$(NH_2)_2CO$	0.31	91
$NaNO_3$	0.29	84
KNO_3	0.26	76
$NH_4H_2PO_4$	0.32	95
$(NH_4)_2SO_4$	0.24	72
$CH_3NH_2 \cdot HCl$	0.21	61
Peptone	0.15	45
CH_3COONH_4	0	0

Growth rate of cells growing in the presence of ammonium chloride was set as 100%.

propanol, butanol, pentanol, octanol, isopropanol, isobutanol, formaldehyde, acetaldehyde, fructose, lactose, sucrose, mannose, mannitol, cellobiose, galactose, maltose, xylose, dimethylamine, trimethylamine, succinate, acetate, citrate, lactate, L-glycine, L-alanine, L-serine, L-glutamic acid, and L-valine were not utilized. The G+C content of the DNA was estimated to be 55%. Plasmids were not detected from cells grown on methanol, methylamine, or glucose by the methods of Birnboim and Doly (5) and Kim and Lidstrom (20). Cells grown on methanol was found to contain phenazine methosulfate-linked methanol dehydrogenase activity which requires ammonium ion as an activator. The cell also contained 3-hexulose-6-phosphate synthase activity, indicating that methanol is assimilated through the ribulose monophosphate pathway in this bacterium (1). The isolate was found to produce extracellular polysaccharides. The polymers were found to contain glucose, lactose, and fructose.

Cultural properties

Growth was optimal at 40°C and pH 6.5. The growth rates at 45°C and 50°C were 90% and 80% of that at 40°C, respectively. The cell, however, did not grow at temperatures over 60°C. The optimal concentration of methanol for growth was 0.5% (v/v). Ammonium chloride was found to be the best nitrogen source among 10 compounds tested (Table 1). Ammonium acetate did not serve as a nitrogen source. Vitamins and growth factors were not required. The optimal concentration of phosphate in mineral medium was found to be 35 mM. The doubling time and growth yield under the optimal condition were found to be 1.7 h and 0.48 g dried cells/g methanol. The methanol dehydrogenase activity was maximal in cells growing at the mid-exponential growth phase and then decreased thereafter (Fig. 2).

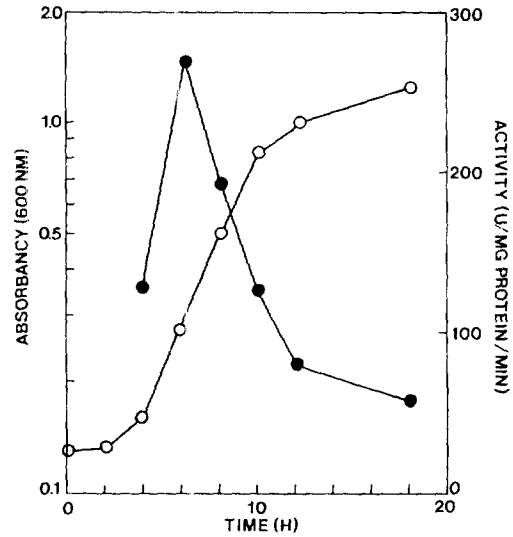

Fig. 2. *Methanol dehydrogenase activity during growth of Methylovorus sp. SS1.*
The isolate was cultivated at 40°C in a shaking flask containing liquid mineral medium supplemented with 0.5% (v/v) methanol. Growth was measured at 600 nm (-○-). Methanol dehydrogenase activity (-●-) in crude cell extract was assayed by measuring the methanol-dependent reduction of absorbancy of 2,4-dichlorophenol indophenol (3).

DISCUSSION

Up to date, three genera have been described for restricted facultatively methylotrophic bacteria with the ribulose monophosphate pathway for C_1 assimilation (14-17, 28). Terrestrial strains have been classified into the *Methylophilus* and *Methylovorus* genera (14, 16, 17). The genus *Methylophaga* has been proposed for the marine bacteria (15, 28).

Strains in *Methylovorus* are Gram-negative, motile rods with a polar flagellum (17). They don't have spores and capsule, but some produce slimy materials. No complex intracellular membranes are present. Colonies are smooth, round, and raised convex. No aggregation and pigmentation occurs in liquid medium. Nitrates, ammonium salts, methylated amines, and peptone served as nitrgen sourses. Indole and hydrogen sulfide are not produced. The strains hydrolyze starch, but not gelatin. They have catalase and oxidase. Optimal temperature and pH for growth are 35~37°C and 7.0~7.2, respectively. The G+C content of the strains ranged from 55.8 to 56.9 mol%. In addition to the methanol, they are able to use

glucose as the sole source of carbon and energy. Strains in *Methylophilus* share many properties with *Methylovorus* strains, except for the following characteristics (16). Some strains are immotile. Optimal temperature and pH for growth are 30~37°C and 6.5~7.2, respectively. No growth occurs at 45°C. Some strains use methylamine, formate, fructose, or glucose, in addition to methanol, as carbon and energy sources. Nitrates and ammonium salts are used as nitrogen sources. The G+C mol% of DNA is 50 to 53%.

The new restricted methylotrophic bacterium isolated in this study has many properties similar to those of the type species of *Methylophilus, M. methylotrophus,* and *Methylovorus, M. glucosotrophus.* The range of the optimal temperature for growth and that of the G+C content of the two type strains and the inability of *M. methylotrophus* to grow at temperatures over 45°C, however, imply that it may be better to classify the new restricted facultative methylotrophic isolate as a *Methylovorus* species than as a *Methylophilus* species. The isolate, however, cannot be treated as one of *M. glucosotrophus* strains since it is immotile and has no oxidase and starch hydrolyzing activity. The optimal temperature for growth and the G+C content of DNA also indicate that the new isolate is different from the type species. We, therefore, concluded that the SS1 is a new methylotrophic bacterium and named it *Methylovorus* sp. strain SS1.

The isolate grew very fast (t_d=1.7 h) in shaking flasks with a maximal yield of 0.48 g (dry weight)/g methanol. These are comparable to those (t_d= 1.2~1.8 h; Y_{MeOH}=0.4~0.5 g dried cell/g methanol) of *M. methylotrophus, Methylomonas methanolis, Methylomonas ceredia,* and *Methylomonas clara* (23) which have been used in the commercial production of single cell proteins and *Methylobacillus* sp. strain SK1 which was isolated in the authors' laboratory (19). Since the growth rate and cell yield of the isolate were estimated using cells growing in shaking flasks, not in fermentor, we believe that the rate and yield may be improved if cells are cultivated in a fermentor with sufficient supply of oxygen. These together with the facts that the isolate grows optimally at 40°C, shows comparable growth rate even at 50°C to that at optimal temperature, and produces extracellular heteropolysaccharides suggest the possibility to use the new isolate for the commercial production of biotechnological products such as single cell proteins and biopolymers.

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초 록: 제한통성 메탄올자화세균인 *Methylovorus* sp. Strain SS1의 분리 및 특성

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Malaysia의 Kuala Lumpur에서 수집한 토양으로부터 메탄올을 이용한 enrichment 배양법을 사용하여 제한통성 메탄올자화세균을 분리하고 *Methylovorus* sp. SS1이라 명명하였다. 분리된 균주는 비운동성 그람음성균으로 복잡한 내막구조가 없었다. 집락의 모양은 작고 둥글며 연황색을 띄었다. 이 세균은 절대 호기성세균으로 포자와 피막구조물이 없었으며, catalase 활성은 나타내었으나 oxidase 활성은 나타내지 않았다. 분리균주에는 plasmid와 carotenoid 및 poly-β-hydroxybutyrate가 없었다. DNA의 G + C 함량은 55 mol%였다. 분리균주는 메탄올과 메틸아민 및 포도당만 에너지 및 탄소원으로 이용하였으며 생육인자는 요구하지 않았다. 이세균은 메탄올을 이용하여 성장할 때 포도당과 과당 및 유당 등으로 구성된 세포외 다당류를 생산하였다. 이 세균은 0.5% 메탄올을 이용하여 40°C와 pH 6.5에서 가장 빨리 성장하였으며, 이때의 성장시간은 1.7시간이었고 60°C에서는 성장하지 않았다. 메탄올을 이용하여 성장한 세균의 세포추출액은 phenazine methosulfate와 연관된 methanol dehydrogenase 활성을 나타내었다. 분리균주는 ribulose monophosphate 경로를 이용하여 메탄올을 동화하였다.