A *Methylobacillus* Isolate Growing Only on Methanol

Kim, Si W.[1], Byung H. Kim[3] and Young M. Kim[2*]
[1] Department of Environmental Science, Chosun University, Kwangju
[2] Department of Biology, College of Science, Yonsei University, Seoul
[3] Process Reserch Division, Korea Institute of Science and Technology, Seoul

메탄올만 이용하여 성장하는 *Methylobacillus*의 분리 및 특성

김시욱[1] · 김병홍[3] · 김영민[2*]
[1] 조선대학교 자연과학대학 환경학과
[2] 연세대학교 이과대학 생물학과
[3] 한국과학기술연구원 공정연구부

ABSTRACT: An obligate methanol-oxidizing bacterium, *Methylobacillus* sp. strain SK1, which grows only on methanol was isolated from soil. The isolate was nonmotile Gram-negtive rod. It does not have internal membrane system. The colonies were small, whitish-yellow, and smooth. The guanine plus cytosine content of the DNA was 48 mol%. Cellular fatty acids consisted predominantly of large amounts of straight-chain saturated $C_{16:0}$ acid and unsaturated $C_{16:1}$ acid. The major ubiquinone was Q-8, and Q-10 was present as minor component. The cell was obligately aerobic and exhibited catalase, but no oxidase, activity. Poly-β-hydroxybutyrate, endospores, or cysts were not observed. The isolate could grow only on methanol in mineral medium. Growth factors were not required. The isolate was unable to use methane, formaldehyde, formate, methylamine, and several other organic compounds tested as a sole source of carbon and energy. Growth was optimal at 35°C and pH 7.5. It could not grow at 42°C. The doubling time was 1.2 h at 30°C when grown with 1.0%(v/v) methanol. The growth was not affected by antibiotics inhibiting cell wall synthesis and carbon monoxide but was completely suppressed by those inhibiting protein synthesis. Methanol was found to be assimilated through the ribulose monophosphate pathway. Cytochromes of *b*-, *c*-, and *o*- types were found. Cell-free extracts contained a phenazine methosulfate-linked methanol dehydrogenase activity, which required ammonium ions as an activator. Cells harvested after the late exponential phase seemed to contain blue protein.

KEY WORDS □ Obligate methanol-oxidizing bacterium; Methylotroph; Methanolotroph; *Methylobacillus* sp. strain SK1; Ribulose monophosphate pathway.

INTRODUCTION

Methylotrophic bacteria are a group of bacteria that can grow aerobically on compounds containing one or more carbon atoms but no carbon to carbon bonds. Obligate methylotrophs grow only on such compounds, whereas facultative methylotrophs are also able to grow on a variety of other multicarbon substrates (Anthony, 1982, 1986, and 1988; Colby and Zatman, 1972; Colby *et al.*, 1979; Dalton and Higgins, 1987; Hou, 1984a; de Vries, 1986).

Methylotrophic bacteria have received a great deal of attention due in part to their commercial and biotechnological potentials since these organisms are a good source for the production of single cell proteins from methanol (Dijkhuizen *et al.*, 1985; Litchfield, 1983; Lidstrom and Stirling, 1990; Powell and Rodgers, 1984; Tani, 1985) and are capable of converting C_1 compounds into commercially important compounds such as biopolymers, organic acids, amino acids, coenzymes, vitamins, and cytochrome *c* (Anthony, 1982; Lidstrom and Stirling, 1990; Tani, 1985;

Hou, 1984b; Morinaga and Hirose, 1984). In addition, methylotrophs contain unique oxidative enzymes which can be developed as biocatalysts (Anthony, 1982; Lidstrom and Stirling, 1990). The potential interests of the methylotrophs thus have resulted in an increase in the isolation and biochemical studies of new bacteria (Govorukhina and Trotsenko, 1991; Govorukhina *et al.*, 1988 and 1989; Reed and Duga, 1987; Tani *et al.*, 1985; Urakami and Komagata, 1987a and b; Yang and Kim, 1989).

We have tried a number of enrichment cultures to isolate pure cultures of obligate methanol-oxidizing bacteria which grow fast with methanol in order to understand better the mechanim of methanol oxidation in bacteria and also to use the new isolates for commerical and biotechnological purposes in the future. We, in this study, describe some properties of a methylotrophic isolate which grows only on methanol as the sole source of carbon and energy. The name of the genus proposed for this bacterium is *Methylobacillus*. We also propose a new term "obligate methanolotroph" for an aerobic methylotrophic bacterium which grows only on methanol.

MATERIALS AND METHODS

Enrichment and isolation

Five grams of soil sample from a decaying forest area in Kuala Lumpur, Malaysia, was suspended in a flask (250 m*l*) containing 50 m*l* of liquid mineral salt medium (MSM) and 1.0% (v/v) 1 methanol. The composition of MSM was the same as that of the standard mineral base medium of Kim and Hegeman (1981a) except that the pH and concentration of sodium phosphate buffer were 7.5 and 50 mM, respectively, and that the MSM contained an additional mineral salt, $NaNO_3$ (1.0 g/*l*). The flask was agitated at 150 rpm for 2 days at 30℃, and 0.5 ml of the turbid suspension was then transferred to a fresh liquid medium (50 m*l*) supplemented with 1.0% (v/v) methanol and incubated as before. After three serial transfers under the same condition, loopful suspensions were streaked onto solid methanol (1.0%, v/v)-MSM plates. The plates were incubated at 30℃ for two days, and fast-growing colonies without pink pigments were transferred to new methanol-MSM plates. The colony selection step was repeated three times. The selected colonies were then tested for obligate methanol-utilizer using nutrient agar plates and MSM plates supplemented with yeast extracts (0.2 g/*l*) 1 or several C_1 compounds (0.2% w/v or v/v).

Growth conditions

The isolate was cultivated in MSM supplemented with 1.0% (v/v) methanol at 30℃. For several specific tests, the growth conditions were modified for each experiment. Specific growth rate and cell yield were determined using a New Brunswick fermentor (MF-114, 10 *l*). The fermentor was stirred at 400 rpm with an aeration of 1 vvm. The actual amount of methanol consumed by growing cells was periodically analyzed with a Varian 3300 gas chromatograph equipped with a Porapak Q column (0.3×100 cm). The temperature for column, injector, and detector was 200℃. Growth was measured by turbidity determined at 650 nm in a Shimadzu UV-240 spectrophotometer (d=1 cm).

Biochemical and nutritional properties

Utilization of organic compounds other than methanol was tested at 30℃ in liquid MSM supplemented with 0.2% (w/v or v/v) of each substrate except that methane gas was supplied as a gas mixture of 30% methane-70% air and that formaldehyde was added at concentration from 0.001 to 0.04% (w/v). Several biochemical tests were carried out by the methods of Gerhardt *et al.* (1981). Sensitivity to various antibiotics was tested at 30℃ on methanol (1.0%, v/v)-MSM plates using various antibiotic discs (BBL).

DNA base composition

DNA was extracted by the method of Marmur (1961). The guanine plus cytosine (G+C) content of DNA was determined by the reversed phase high-performance liquid chromatography following the method of Tamaoka and Komagata (1984) except that Cosmosil packed column 5C18 (4.6×150 cm) (Waters) installed in L-3000 detector (Hitachi) and D-2000 integrator (Hitachi) were used. Reference DNAs were purchased as a kit (code No. 7160) from Yamasa Co., Japan.

Cellular fatty acid composition

Cells harvested during the late exponential phase were used for the cellular fatty acid analysis after treated by the method of Ikemoto *et al.* (1978). Fatty acid composition was determined using a gas chromatograph (Shimadzu GC-14A) equipped with a coated fused silica capillary column (Dura bond-1, 0.25 mm×3 m). Temperatures for column, injector, and detector were 200℃, 250℃, and 250℃, respectively.

Ubiquinone system

Cells growing at the late exponential phase were used for the quinone analysis. Quinone system was determined by the procedure described by Yamada *et al.* (1969).

Enzyme assay

3-Hexulose phosphate synthase (HPS) activity was assayed by the method of Ferenci *et al.* (1974). One unit of HPS activity was defined as the amount of enzyme that catalyzed the removal of 1 mM formaldehyde per min. Methylene tetrahydrofolate dehydrogenase and hydroxypyruvate reductase activities were assayed by the methods of Kato *et al.* (1977) and Heptinstall and Quayle (1970). The crude cell extracts were prepared from cells resuspended in 50 mM phosphate buffer (pH 7.0) by sonic disruption under the conditions described by Yang and Kim (1989). *Methylophilus methylotrophus* (NCIB 10515) and *Methylobacterium extorquens* (*Methylobacterium* sp. AM1, NCIB 9133) were used as controls. Methanol dehydrogenase (MDH) activity was assayed by the method of Anthony and Zatman (1967) using crude cell extracts prepared in 100 mM Tris hydrochloride buffe (pH 9.0). Proteins were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Analysis of cytochromes

Particulate fraction obtained by ultracentrifugation (100,000×g/90 min/4℃) of crude extracts was resuspended in 30% bovine serum albumin to analyze membrane-bound cytochromes (Kim and Hegeman, 1981b). A few crystals of potassium ferricyanide or sodium hydrosulfite were added to oxidize or to reduce the preparation, respectively. To examine the effect of CO, the suspension reduced with sodium hydrosulfite were flushed with CO for 30 sec. The absorption spectra of samples were recorded from 400 nm to 650 nm at room temperature using a Hitachi 200-20 spectrophotometer. The amount of cytochrome *c* in crude extracts was calculated from the difference in absorbancy at 551 nm and 535 nm of reduced crude cell extracts as described by Tani *et al.*, (1985).

Electrophoresis

Denaturing polyacrylamide gel electrophoresis (PAGE) of crude cell extracts in 12.5% acrylamide was performed by the procedure of Laemmli (1970) with several modifications as described by Kim *et al.* (1989). Proteins were stained with Coomassie brilliant blue R-250 (CBB)1 by the modified method (Kim and Hegeman, 1981a) of Weber and Osborn (1969).

RESULTS

Isolation and morphology

After several times of selection with methanol and organic materials, an obligate methanol-utilizing bacterium, designated SK1, was stabilized as indicated by morphology and color of the colony and by the cell shape. Growth was good on agar plates as well as in liquid medium. Colonies were whitish-yellow, small (0.3-0.5 mm in diameter), and raised convex with entire edge. The cell was nonmotile, Gram-negative, and rod-shape with dimensions of 0.4−0.6×1.5−2.0 μm (Fig. 1). It did not have flagella and internal membrane structure. Spore, cyst, and capsular materials were not observed. Electron microscopic

Fig. 1. *Electron micrograph of a methylotrophic isolate growing only on methanol. Negative staining was done with 2.0% phosphotungstic acid. The bar equals 0.5 μm.*

observation revealed that the isolate reproduces by binary fission. Cell aggregates were not observed during cultivation in liquid medium.

Biochemical and nutritional properties

Catalase was present in the isolate, but oxidase and nitrogenase were not. Denitrifiation and nitrate/nitrite respiration were not observed. It did not form carotenoids, H_2S, and poly-β-hydroxybutyrate. Gelatin and starch were not hydrolyzed. Growth was sensitive to chloramphenicol (30 mcg) and tetracycline (30 mcg) but was resistant to ampicillin (10 mcg), vancomycin (30 mcg), and methicillin (5 mcg). The isolate was able to grow only on methanol. Methane, nutrient broth, glucose, fructose, lactose, formate, succinate, L-alanine, L-valine, L-glycine, L-serine, L-glutamate, monomethylamine, dimethylamine, trimethylamine, ethanol, propanol, butanol, pentanol, octanol, nonanol, decanol, allylalcohol, formaldehyde, and acetaldehyde were not utilized. Crude cell extract was found to contain HPS but no methylene tetrahydrofolate dehydrogenase and hydroxypyruvate reductase. The HPS activity was 0.88 units/mg protein. The extract also contained a phenazine methosulfate-linked MDH which required ammonium ion as an activator. The G+C content of the DNA of SK1 was estimated to be 48.0 mol%. The isolate contained large amounts of straight-chain unsaturated $C_{16:1}$ acid (44.8% of the total fatty acids) and saturated $C_{16:0}$ (31.9% of the total fatty acids) and $C_{18:0}$ (17.4% of the total fatty acids) acids. The ubiquinone system of the isolate consisted of Q-8 as major component and Q-10 as minor component. Plasmids were not resolved from the isolate with the methods of Kim and Lidstrom (1989) and Birnboim and Doly (1979).

Nature of cytochromes

The reduced minus oxidized difference

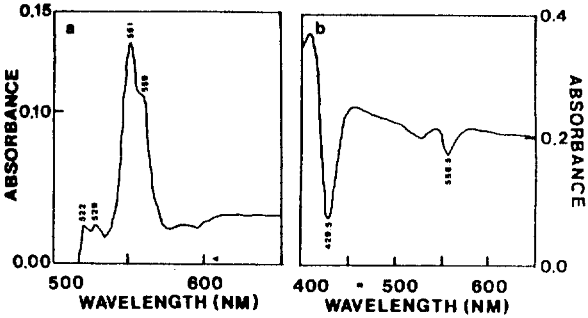

Fig. 2. *Absorption spectra of particulate fractions from the obligate methylotrophic isolate SK1. (1a) Reduced minus oxidized difference spectrum. Particulate fractions obtained from crude cell extracts were resuspended in 30% bovine serum albumin and then treated with a few crystals of potassium ferricyanide or sodium hydrosulfite for oxidation or reduction, respectively. (b) (Reduced plus CO) minus reduced difference spectrum. The reduced preparation was flushed with CO for 30 sec to examine the effect of CO on the spectrum.*

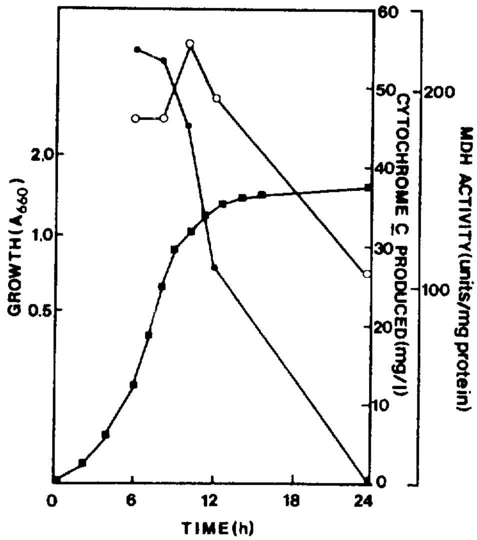

Fig. 3. *Changes in the MDH activity and the amount of cytochrome c during growth of SK1. The isolate was grown in MSM supplemented with 1.0% (v/v) methanol in a fermentor at 30℃. Growth was measured at 660 nm (■—■). MDH activity (●—■) in crude cell extract was assayed by measuring the methanol-dependent reduction of absorbancy of 2,4-dichlorophenol indophenol (Anthony and Zatman, 1967). The amount of cytochrome c (○—○) was estimated from the absorbancy difference, A_{551} $-A_{535}$, of the cell extracts as described in methods..*

spectrum of the particulate fraction clearly disclosed the presence of cytochromes of the *b* (peaks at 529 and 559 nm) and *c* types (peaks at 522 and 551 nm) (Anthony, 1975) (Fig. 2a). No peaks for *a* type cytochromes were observed. When the reduced particulate fraction was flushed with CO, two troughs at 429.5 nm and 556.5 nm which are an indicative of the presence of *b* type cytochrome that is able to react rapidly with CO appeared (Fig. 2b). No spectra characteristic of a CO-reacting cytochrome a_3 (trough at 440 nm)1 were observed.

Cultural properties

Growth with 1.0% (v/v) methanol was optimal at 35℃ and pH 7.5. No growth occurred at temperatures below 26℃ and above 42℃. Grwoth rate increased rapidly with a methanol concentration up to 1.0% (v/v) and then decreased thereafter; the specific growth rate with 5% (v/v) methanol was one sixth of that with 1.0% (v/v) methanol. Ammonium sulfate was found to be the best nitrogen source, while ammonium acetate and urea could not serve as a nitrogen source. Vitamins or organic growth factors were not required. Optimal initial concentration of phosphate ion in MSM was 50 mM. Grwoth was not inhibited by a gas mixture of 20% CO-80% air. Even though the isolate was not able to grow on formaldehyde and formate, formaldehyde and formate at concentrations below 0.01% (w/v) and 0.2% (w/v), respectively, were found to stimulate the growth of the isolate growing on 0.075% (v/v) methanol. When the isolate was batch-cultured on methanol (1.0%, v/v) in a fermentor for 12 h at 30℃, doubling time and growth yield were found to be 1.2 h and 0.4 g dried cells/g methanol. The amount of oxygen dissolved in the medium started to decrease at the mid-exponential phase and reached near zero by the end of the exponential phase, and increased soon after the cells stopped to grow.

Metabolic characteristics

The MDH activity was maximal in cells growing at the mid-exponential phase and then decreased thereafter (Fig. 3). The activity was not detected in cells harvested after 25 h of cultivation. Denaturing PAGE of crude cell extracts, however, showed that there is no difference in the amount of of MDH in cells growing at different growth stages (Fig. 4). The amount of cytochrome *c* was maximal in cells growing at the late exponential phase, and decreased during the stationary phase (Figs. 3 and 4). It was found that the crude cell extracts prepared from cells growing at the mid-exponential phase were red. The extracts prepared from cells growing at the early stationary phase were slightly greenish pink, and those from the

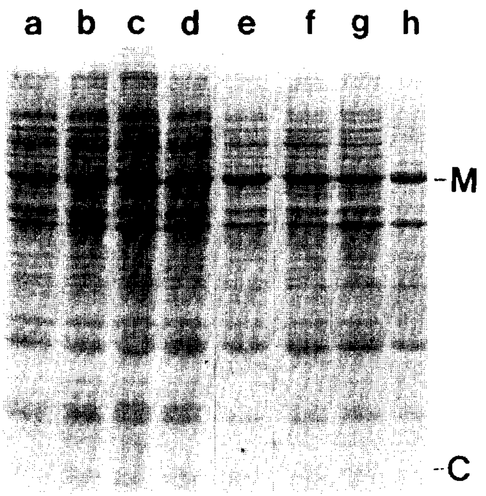

Fig. 4. *Electrophoretic comparison of total soluble proteins in cell extracts prepared from cells harvested at various growth stages. Gels containing 12.5% acrylamide were run according to Laemmli (1970) in the presence of 0.1% SDS with 70 μg protein of each sample and stained with CBB. Cells harvested after 6 h (a), 8 h (b), 10 h (c), 12 h (d), 24 h (e), 30 h (f), 40 h (g), and 96 h (h) of cultivation were used for cell extract preparation. M; methanol dehydrogenase, C; cytochrome c.*

late stationary phase were more greenish. When the cell extracts, especially those from cells harvested after the early stationary phase, were incubated at 4℃ under air, the color was changed rapidly to green from the top of the extracts in an hour; the extracts from cells growing at the mid-exponential phase turned to green very slowly. The green colored portions of those extracts were found to have no MDH activity but contained equal amount of MDH proteins to that present in the red extracts. The green-colored extracts showed a broad absorption peak around 624.6 nm. When the red extracts were incubated under nitrogen gas soon after preparation, no changes were observed in color and MDH activity for a week.

DISCUSSION

Several obligate methanol-oxidizing bacteria which have been studied to date are also known to utilize other C_1 compounds such as methylamine (Colby and Zatman, 1972; Dahl *et al.*, 1972; Govorukhina *et al.*, 1988; Yordy and Weaver, 1977) and formaldehyde (Chen *et al.*, 1977; Hirt *et al.*, 1978). The SK1 isolated in this study, however, did not grow on any C_1 compounds tested except methanol, implying that the isolate is the real obligate methanol-oxidizing bacterium, the obligate methanolotroph. Presence of HPS in the isolate indicates that methanol is assimilated through the ribulose monophosphate pathway. This and other properties such as the Gram reaction and inability to grow at 42℃ are similar to those of other obligate methanol-oxidizing bacteria (Anthony, 1982). Yordy and Weaver (1977) proposed the genus name *Methylobacillus* for Gram-negative rod, nonmotile obligate methylotrophic bacteria which do not grow with methane. Urakami and Komagta (19 86) later emeded the genus *Methylobacillus* and *Methylobacillus glycogenes*, in which polarly flagellated organisms and D-fructose-utilizing organisms are also included. The isolate shares many properties with the type strain of *Methylobacillus*, *M. glycogenes*, but it also has many properties different from those of the type strain; the G+C content of DNA in the isolated is lower than that of the type strain. The isolate is oxidase-negative and grows only on methanol. It cannot grow at below 26℃, and cannot use urea as a nitrogen source. It contains Q-10, not Q-7 or Q-9, as a minor component of ubiquinone system. We, therefore, concluded that the SK1 is a new and unique methylotrophic baterium and named it *Methylobacillus* sp. strain SK1.

It was found that the isolate is obligately aerobic since no fermentative metabolism and the processes for denitrification and nitrate/nitrite respiration were observed. Susceptibility of growth to antibiotics was interesting. Antibiotics which inhibit protein synthesis were inhibitory, but those which inhibit cell wall biosynthesis were not as in the case of methanogenic bacteria (Mathrani *et al.*, 1988).

It is well-known that cytochromes aa_3 and *o* function as a terminal electron acceptor in methylotrophic bacteria (Anthony, 1982, 1986, and 1988). The reduced minus oxidized spectrum revealed that the SK1 contains no *a* type cytochromes. This was further supported by the negative result obtained from the Nadi reaction (Marrs and Gest, 1973) using cells grown under methanol-excess and -limited conditions. The isolate, however, was found to use cytochrome *o* as a terminal oxidase since the (reduced plus CO) minus reduced difference spectrum revealed two troughs which are indicative of the presence of CO-reacting *b* type cytochrome (cytochrome *o*) (Vrdoljak and Froud, 1982). It is well-known that CO is inhibitory to virtually all aerobic respiratory organisms. The growth of the new isolate, however, was not affected by CO, further supporting that cytochrome *o* which has a lower affinity for CO than cytochrome aa_3 functions for the oxidation of methanol in the isolate (Broberg and Smith, 1967)].

In a batch culture system using a fermentor, the isolate grew very fast (t_d=1.2 h) with a maximum cell yield of 0.4 g dried cells/g methanol. These are comparable to those (t_d=1.3-1.8 h; Y_{MeOH}=0.47−0.5 g dried cells/g methanol) of *M. methylotrophus* (Gow *et al.*, 1975; MacLennan *et al.*, 1973). *Methylomonas clara* (Faust *et al.*, 1977), *Methylomonas methanolis* BNK-84 (Kuraishi *et al.*, 1979), and *Methylomonas ceredia* BU-1 (Kuraishi *et al.*, 1979) which are utilized commercially in the production of single cell proteins. Taken together with the fact that the isolate grows optimally at slightly high temperature, this suggests that the new isolate may become an excellent source for the production of single cell proteins.

Decrease in the activity, but not in the amount, of MDH after the mid-exponential phase suggests that the MDH activity may be inhibited by the changes in the redox state of the MDH prosthetic group resulted from modification by oxygen of the electron transport complex between MDH and cytochrome *c* as indicated by Duine *et al.* (1979) or by certain inhibitor(s) produced after the mid-log phase. Variations in the amount of dissolved oxygen in the medium, differences in the color and MDH activity of the extracts prepared from cells harvested at different growth phases, changes in the color and MDH activity of the extracts exposed to air, and constancies in the color and MDH activity of the extracts under nitrogen gas imply that oxygen is involved in the inactivation of MDH. The coincidence of the appearance of green color and the loss of MDH activity and the absorption peak of the green-colored extracts also suggest that certain kind of blue protein (Anthony, 1988; Auton and Anthony, 1989; Tobari, 1984; Tobari and Harada, 1981) which is produced in the isolate during growth at the stationary phase or is made upon exposure of the cell extracts to air may be involved in the inactivation process.

적 요

토양으로 부터 유일한 탄소 및 에너지원으로 메탄올만을 이용하여 성장하는 편성 메탄올자화세균을 분리하여 *Methylobacillus* sp. strain SK1이라 명명하였다. 분리균주는 비운동성 그람음성균으로 세포내막 구조가 없으며, 집락의 모양은 작고 둥근 연황색을 띄었다. DNA의 guanine plus cytosine함량은 48 mol%이고, 세포내 지방산은 대부분 직쇄구조의 포화지방산($C_{16:0}$)과 불포화지방산($C_{16:1}$)으로 구성되어 있으며 ubiquinone은 주로 Q-8이지만 Q-10도 소량 포함되어 있었다. 분리균주는 절대 호기성세균으로 catalase활성은 나타냈으나 oxidase활성은 없었고 poly-β-hydroxybutyrate, endospore, cyst 등이 관찰되지 않았다. 이 세균은 메탄올이외의 메탄, 포름알데히드, 포름산, 메틸아민 그리고 유기산 등을 탄소 및 에너지원으로 이용하지 못하였으며 특이한 생육인자를 요구하지 않았다. 이 세균은 35℃, pH 7.5에서 가장 좋은 성장을 보였으며 42℃에서는 자라지 않았다. 일산화탄소 및 세포벽합성 억제 항생제는 성장에 아무런 영향을 미치지 않은 반면 단백질합성 억제 항생제는 성장을 완전히 저해하였다. 이 세균은 1.0%(v/v)의 메탄올을 이용하여 30℃ 조건에서 성장할때 1.2 시간의 세대시간을 나타냈고, ribulose monophosphate pathway를 이용하여 메탄올을 동화하였다. 이 세균은 *b*-, *c*-, *o*- 형태의 cytochrome을 내포하고 있으며 암모늄이온을 활성촉진제로 필요로하는 phenazine methosulfate-linked methanol dehydrogenase의 활성을 나타내었다. 한편 지수 성장기 말기의 세포내에는 blue protein이 존재하는 것으로 추측된다.

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