Isolation and Taxonomic Characterization of a Novel Type I Methanotrophic Bacterium

Hee Gon Kim¹, Gui Hwan Han², Chi-Yong Eom³, and Si Wouk Kim¹, ²*

¹Department of Biomaterials Engineering, ²Department of Environmental Engineering, BK 21 Team for Biotechnology Production, Chosun University, Gwangju 501-759, Republic of Korea ³Metabolome Analysis Team, Korea Basic Science Institute, Seoul 136-713, Republic of Korea

(Received January 15, 2008 / Accepted January 31, 2008)

A methane-oxidizing bacterium was isolated from the effluent of manure and its molecular and biochemical properties were characterized. The isolate was aerobic, Gram-negative, and non-motile. The organism had a type I intracytoplasmic membrane structure and granular inclusion bodies. The outer cell wall surface (S-layers) was tightly packed with cup-shaped structures. Colonies were light yellow on nitrate mineral salt agar medium. In addition, the organism was catalase and oxidase positive. The isolate used the ribulose monophosphate (RuMP) pathway for carbon assimilation, and was able to utilize methane and methanol as a sole carbon and energy source; however, it could not utilize any other organic compounds that were tested. The cells grew well in a mixture of methane and air (methane:air=1:1, v/v) in a compulsory circulation diffusion system, and when grown under those conditions, the optimum pH was approximately 7.0 and the optimal temperature was 30°C. In addition, the specific growth rate and generation time were 0.13 per h and 5.43 h, respectively, when grown under the optimum conditions. The major ubiquinone was Q-8, and the G+C mol% of the DNA was 55.3. Phylogenetic analyses based on the 16S rRNA gene sequence comparisons showed that this bacterium belongs to a group of type I methanotrophs, and that it is most closely related to Methylomicrobium, with a sequence similarity of 99%. Therefore, the isolate was named Methylomicrobium sp. HG-1.

Keywords: Methanotrophic bacteria, intracytoplasmic membrane structure, ribulose monophosphate (RuMP) pathway, Methylomicrobium

Methane-oxidizing bacteria (methanotrophs) are widespread in nature and play an indispensable role in the global carbon cycling of methane. The atmospheric concentration of methane, which is an important global warming gas, has been increasing for many decades (Whittenbury et al., 1970; Hanson and Hanson, 1996). Methanotrophs are a ubiquitous group of microorganisms that possess the unique ability to utilize methane as the sole source of carbon and energy, and are therefore considered to be important regulators of atmospheric methane fluxes in nature (Reebergh et al., 1993; Mancinelli, 1995; Fjellbirkeland et al., 2001). Methanotrophs are aerobic bacteria that convert methane to methanol in the first step of their metabolic pathway using methane monoxygenase (MMO). In addition, methanol dehydrogenase (MDH), which is an enzyme responsible for the oxidation of methanol to formaldehyde, is assimilated into cellular biomass or oxidized to CO₂, thereby providing reducing power for biosynthesis. In vitro, MDH is coupled to the electron transport chain at the level of cytochrome c (Ro et al., 2000; Brantier et al., 2002; Koh et al., 2002; Kim et al., 2005, 2006).

Methanotrophs have been difficult to identify because in formation regarding their phenotypic and chemotaxonomic properties is limited. This has led to nomenclatural problems, especially concerning the assignment of species to genera. However, several studies have evaluated the species and genus organization of the methanotroph groups using a more thorough polyphasic taxonomic approach, which has led to redefinition of several species and genera (Bowman et al., 1995).

The current classification separates all known methanotrophs into three groups (Types I, II, and X) based on multiple criteria, including cell morphology, the arrangement of intracytoplasmic membranes (ICM), the pathway for formaldehyde assimilation, the DNA G+C content and the major cellular fatty acid profiles (Whittenbury et al., 1970; Higgins et al., 1981; Bowman et al., 1993, 1995; Kaluzhnaya et al., 2001). Type I methanotrophs include three broadly homologous clusters of species, referred to as Methyllobacter, Methylomicrobium, and Methylomonas (Bowman et al., 1995). The type II methanotrophs contain closely related groups that belong to the genera Methylophilus and Methylobacter. In addition, a new group, type X, was added to accommodate methanotrophs similar to Methyllococcus capsulatus that, like type I methanotrophs, utilize ribulose monophosphate (RuMP) as the primary pathway for formaldehyde assimilation. Type X
methanotrophs are also distinguished from type I methanotrophs because they possess low levels of ribulose-bisphosphate carboxylase, which is involved in the serine pathway by which carbon assimilation occurs (Hanson et al., 1996). Due to the ability of methanotrophs to catalyze a large number of biotransformations, they have attracted the interest of scientists studying the development of biological methods for degradation of toxic chemicals and the use of bacteria containing MMO for the production of chemicals with commercial values (Han et al., 1999; Han and Semrau, 2000).

In this study, we isolated, characterized, and phylogenetically positioned a novel strain of type I methanotrophic bacterium that grows rapidly in the presence of methane.

**Materials and Methods**

**Isolation and culture conditions**

A novel strain of bacteria was isolated from the effluent of manure in Goksung, Republic of Korea using the modified nitrate-mineral-salt (NMS) medium described by Higgins et al. (1981). The bacterium was cultured using medium in which the pH had been adjusted to 7.0 at an agitation speed of 150 rpm at 30°C.

An appropriate dilution of the liquid culture samples was spread onto a NMS plate that contained 1.5% (w/v) noble agar (Difco), and then placed in an airtight box. A mixture of methane and air (1:1, v/v) was then passed through a 0.2 µm pore air filter and injected into the box. Control plates were also incubated in the absence of methane to determine if the samples were contaminated with non-methane oxidizing colonies. The plates were incubated at 30°C in the dark and then observed at 3 day or 1 week intervals over 3~4 weeks. In addition, the gas mixture was exchanged every 3 days. Single colonies that formed on the NMS agar plates were transferred onto fresh NMS agar plates and then re-incubated for another week. Isolates were considered to be pure if they were morphologically similar in appearance. Methane-oxidizing colonies generally appeared after 5~7 days, and small, non-methane-oxidizing colonies also appeared on plates that were incubated with and without methane. The purity of the methanotrophic bacteria was ascertained by phase-contrast microscopic observation. For long-term storage, 500 µl of 50% (v/v) glycerol was added to 1 ml of late exponential phase liquid culture, and the mixed suspension was then frozen at -70°C.

The pH range at which growth could occur was determined to range from 4.0 to 10.0 and the growth temperature range was determined to range from 10 to 45°C. In addition, the tolerance to concentrations of NaCl ranging from 1 to 5% (w/v) was also evaluated.

**Morphology and electron microscopy**

Cell morphology was determined using 5-day-old liquid culture medium by Gram-staining, with detailed morphological examinations being made using electron microscopy. Briefly, the cells in the NMS medium were initially fixed with glutaraldehyde, which was added to the medium at a final concentration 2% (v/v), during the late-exponential phase. The cells were then subjected to an additional fixation using 1% (w/v) OsO₄ in 0.1 M cacodylate buffer, followed by two water washes. After dehydration in a series of alcohols, the cells were embedded with Epon resin and then polymerized at 60°C for 18 h. Sections of 70~80 nm were then cut using an ultramicrotome (LKB 2128 Ultratome; LKB) and viewed on a transmission electron microscope (JEOL JEM-2000F X2) operating at 80 kV.

**Utilization of carbon and nitrogen sources**

The ability of the isolate to utilize the following carbon sources at a concentration of 0.1% (w/v or v/v) was tested in the presence of potassium nitrate, which was used as a nitrogen source: ethanol, formate, formamide, urea, sodium succinate, malate, yeast extract, D-glucose, D-fructose, methylene, casitone, peptone, tryptone, and methanol. In addition, the ability of the isolate to grow on methanol was tested in a liquid medium that contained concentrations of methanol ranging from 0.1~5.0% (v/v). Volatile liquid carbon sources were filter sterilized and then added to the basal medium after autoclaving. This solution was then dispensed in 3 ml volumes into 20 ml glass vials. Nitrogen sources were tested similarly in the presence of methane using NMS medium in which the potassium nitrate was replaced with one of the following compounds at a concentration of 0.1% (w/v): ammonium molybdate, ammonium amidosulfate, ammonium vanadate, ammonium dihydrogen phosphate, potassium nitrate, formamide, glycine, urea, yeast extract, ammonium oxalate, L-alanine, L-glutamine, L-glutamic acid, L-tryptophan, L-asparagine, and casitone. All test preparations were incubated for 1 week in duplicate. For carbon and nitrogen source tests, growth was confirmed by comparison with negative controls. Sensitivity to antibiotics was examined by plating the cells onto agar-solidified medium and then placing BBL sensi-discs containing the following antibiotics (µg/ml) on the medium: Penicillin (10), Amoxicillin (30), Piperacillin (100), Aztreonam (30), Imipenem (10), Cefamandole (30), Cefazolin (30), Cefotaxime (30), Gentamicin (10), Amikacin (30), Netilimicin (30), Tobramycin (10), Tetracycline (30), Erythromycin (15), Chloramphenicol (30), Vancomycin (30), Ciprofloxacin (5), Clindamycin (2), and Sulfamethoxazole (23.75). Growth under methane was assessed after 5 days.

**Chemotaxonomic characterization**

Fatty acid methyl esters were prepared from a biomass harvested from the NMS medium after 7 days of incubation at 30°C, and then analyzed using a gas chromatograph (GC-14A, Shimadzu) according to the instructions provided by the Microbial Identification System (MIDI). Ubiquinones were extracted and purified according to the method described by Collins (1985). Analysis of the ubiquinones was conducted using a HPLC apparatus (Agilent 1200, USA) equipped with a C-18 reverse-phase column. DNA was extracted using the method described by Marmur (1961), and the DNA G+C content was determined following the method described Tamaoka and Komagata (1984) using a HPLC apparatus (Agilent 1200, USA) equipped with a C-18 reverse-phase column.

**Enzyme assay**

The activity of hexulose-6-phosphate synthase (HSP), the