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The characteristics and comparative analysis of methanotrophs reveal genomic insights into *Methylomicrobium* sp. enriched from marine sediments

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ABSTRACT

Methanotrophic bacteria are widespread and use methane as a sole carbon and energy source. They also play a crucial role in marine ecosystems by preventing the escape of methane into the atmosphere from diverse methane sources, such as methane seeps and hydrothermal vents. Despite their importance for methane carbon cycling, relatively few marine methanotrophic bacteria have been isolated and studied at the genomic level. Herein, we report the genome of a marine methanotrophic member of the genus *Methylomicrobium*, metagenome-assembled genome (MAG) wino1, which was obtained through enrichment using methane as the sole carbon source. Phylogenetic analysis based on 16S rRNA sequences and comparison of *pmoA* genes supported the close relationship of MAG-wino1 to the genus *Methylomicrobium* and it possessed a genome of 5.06 Mb encoding many specialized methanotrophic genes. A comparison of MAG-wino1 with the genomes of other strains (*Methylomicrobium alcaliphilum* 20Z^T and *Methylomicrobium buryatense* 5G) showed that genes (e.g. *ectABC*, *ask*, and *mscLS*) involved in the accumulation of compatible solutes required for survival in marine environments might be conserved. Methane utilization genes, including methanol dehydrogenase, and key enzymes related to ribulose monophosphate (RuMP) metabolism were identified. The wino1 genome harbored nitrogen fixation, urease, urea and nitrate transporter genes involved in the exploitation of nitrogen sources. Poly-β-hydroxybutyrate degradation and glycogen synthesis-related genes may facilitate survival under nutrient-limiting conditions. Additionally, genome analysis revealed three dominant taxa in the enrichment culture, methanotroph *Methylomicrobium* sp., methylotroph *Methyloceanibacter* sp., and non-methylotroph *Labrenzia* sp., which provided insights into microbial associations in marine sediments.

Introduction

Methane is one of the most important greenhouse gases and a key component in the global carbon cycle. Natural methane emissions from hydrothermal vents, volcanoes and other terrestrial and marine seeps total ~80 Tg per year [1]. In addition, methane has contributed approximately 40% to global warming over the last two decades [2]. Numerous studies have focused on methane utilization by methanotrophic bacteria because of the importance this process has in reducing the contribution of methane to global warming and in biotechnological applications [3].

Most proteobacterial methanotrophs (methane-oxidizing bacteria; MOB) oxidize and utilize methane as a sole carbon and energy source [4]. MOB play an important role in the oxidation of methane in marine environments with diverse methane sources, including methane seeps, mud volcanoes and hydrothermal vents, but oceanic methane production still remains poorly understood. Methane production is widespread in both the oxic ocean surface mixed layer and in anoxic sites [5,6], and it is produced by microorganisms as well as non-microbial sources (i.e. thermogenic methane) [7]. In the absence of electron acceptors, such as O_2, NO_3^-, Fe_3^+ and SO_4^-, reduction of CO_2 or methyl groups to methane, coupled with oxidation of molecules, including acetate and hydrogen, occurs as the final step in the degradation of organic matter by methanogenic archaea (i.e. methanogens). Methanogens play an important role in the global carbon cycle under anoxic conditions [8]. Some archaea (i.e. anaerobic methanotrophs; ANME) oxidize methane under anoxic conditions by coupling with sulfate-reducing bacteria [9], and the emitted methane is consumed by

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MOB and other lithotrophs as a major energy source both on the seafloor and in the main water column [10].

Although MOB have been isolated and characterized for over 100 years [4], anaerobic methane oxidation was only discovered during the last decade, and pure isolates have still not been obtained [11,12]. *Candidatus* Methylomirabilis oxyfera, belonging to the phylum NC10, employs nitrite reduction as an electron acceptor, and oxidizes methane under anoxic conditions by producing oxygen from nitric oxide [12]. Thus, MOB also make a contribution to global carbon and nitrogen cycles in anoxic environments.

Most aerobic methanotrophic bacteria identified to date belong to the phylum *Proteobacteria*, which includes the *Alpha-* and *Gammaproteobacteria* classes. Two distinct families, *Methylocystaceae* and *Beijerinckiaceae*, belong to *Alphaproteobacteria*, whereas *Gammaproteobacteria* contains the family *Methylococcaceae* and the *Candidatus* family Crenothrichaceae [13,14]. Interestingly, non-proteobacterial methanotrophs (i.e. *Methylacidiphilum* spp.) belonging to the phylum *Verrucomicrobia* have been reported [15]. They are extremely acidophilic and possess a Calvin-Benson-Bassham cycle as a carbon fixation cycle, as well as carboxysome-like structures or vesicular membranes [8], even though they are obligate aerobic methanotrophs.

Aerobic methanotrophs harbor the key enzyme methane monooxygenase (MMO) that oxidizes methane to methanol, and it exists in both soluble/cytoplasmic (sMMO) and particulate/membrane-bound (pMMO) forms [16]. Two different pathways participate in carbon assimilation; the ribulose monophosphate (RuMP) pathway in type I methanotrophs (*Methylococcaceae* family of *Gammaproteobacteria*), and the serine pathway in type II methanotrophs (*Methylocystaceae* and *Beijerinckiaceae* families of *Alphaproteobacteria*).

Although methane oxidizers are important for the global carbon cycle (e.g. they reduce global warming by consuming methane), relatively few methanotrophic bacteria from marine environments have been isolated and studied. Therefore, genome analysis could expand our understanding of the ecological niches occupied by marine methanotrophs and their roles in marine methane cycles. In the present study, we investigated the genomic traits of wino1, which was newly binned from a marine sediment through an enrichment strategy. Major gene categories were identified using comparative analysis of genomes from the genus *Methylomicrobium*, and possible associations between methanotrophs and other organisms were hypothesized.

Materials and methods

Sampling, cultivation and methanotroph identification

The methanotrophic community was enriched from surface marine sediments (sampling depth, <1 cm) located in Muchangpo, Republic of Korea (36°14′ N, 126°32′ E). The environmental properties of the sampling site (bottom water depth, 0.5m) were a pH of 8.3, a temperature of 1.5 °C and a total salinity of 3.2% [data from Ref. [17]]. For enrichment, a 1 g marine sediment sample was suspended in 10 mL of sterile artificial seawater medium (ASW) containing (per L) 24.6 g NaCl, 0.67 g KCl, 1.36 g $CaCl_2{\cdot}2H_2O$, 6.29 g $MgSO_4{\cdot}7H_2O$, and 4.66 g $MgCl_2{\cdot}6H_2O$, supplemented with sodium nitrate (final concentration, 0.5 mM) as a nitrogen source, sodium bicarbonate (3 mM), potassium phosphate (0.1 mM), 1 × trace element mixture and 1 × vitamin solution [18]. The pH of the medium was adjusted to 7.5 with NaOH or HCl. The suspended sample in ASW was vortexed and serially diluted. Methane (10%; v/v) was added to the headspace as a sole carbon and energy source. Test tubes were sealed with butyl stoppers, capped with aluminum caps, and incubated on a rotary shaker at 100 rpm at 30 °C for 4 weeks. After methane oxidation (typically after 3 weeks), 5% (v/v) of the total culture volume was transferred to fresh medium at 30 °C under dark conditions for a period of one year. Unless otherwise stated, each starting batch culture was supplemented with 10% (v/v) methane as the sole energy source. Methane consumption in the headspace above the liquid medium was measured by gas chromatography using a GC-2010 Plus (SHIMADZU, Kyoto, Japan) every 3 days. In addition, to investigate the correlation between copy numbers of bacterial 16S rRNA and *pmoA* genes [19] and methane consumption, genomic DNA (gDNA) was extracted from the cultures. For methanotroph identification, massive PCR sequencing and bioinformatics analysis were employed, as described previously [20,21]. Massive sequencing of the bacterial 16S rRNA gene was performed using the MiSeq system (Illumina, San Diego, CA). For estimation of 16S rRNA and *pmoA* gene copy numbers, quantitative real-time PCR (qPCR) experiments using the CFX Connect™ Real-Time System (Bio-Rad Laboratories, Hercules, CA) and built-in CFX manager software (version 3.0, Bio-Rad Laboratories, Hercules, CA) were used. For the amplification of all genes, the thermal cycling parameters were 15 min at 95 °C for initial denaturation, 40 cycles of 20 s at 95 °C, 20 s at 55 °C, and 30 s at 72 °C, with fluorescence readings taken between each cycle. Standard curves, generated for each run by using reference gene standards with gene copies ranging from 0 to 10^9 per reaction, were used to estimate gene abundance in the enrichment sample. Standard curves showed the relationship between a known gene copy number and the cycle threshold (C_T) value, as described previously [18,22]. Specificity of qPCRs was tested by analyzing melting curves, checking the sizes of reaction products by gel electrophoresis, and sequencing the reaction products. A *coxL* gene coding for the large subunit of carbon monoxide dehydrogenase was amplified as described in a previous study [23].

Genome sequencing, assembly, annotation, and comparative analysis

For metagenome sequencing, gDNA was extracted from replicate samples of two enrichment cultures showing methane consumption using a gDNA extraction kit (GeneAll Biotechnology Co. Ltd, Republic of Korea) according to the manufacturer's instructions. A gDNA library was constructed following the protocol provided by Pacific Biosciences (Menlo Park, CA). Briefly, gDNA was sheared to an average fragment length of 20 kb using SAGE ELF (Sage Science, Beverly, MA), end-repaired, and blunt-end ligated with single-molecule real-time (SMRT) bell oligonucleotide adaptors to construct a DNA fragment library for sequencing on the Pacific Biosciences RSII instrument. One SMRT cell produced 1.27 Gb (184-fold depth) read lengths in 165,285 polymerase reads that passed filtering. Then, *de novo* genome assembly was performed by FALCON (version 0.2.1) with default settings. After assembly, 29 contigs (average length, ~502 kb) were identified. Assembled contigs with lengths <5 kb and those with fewer than three predicted genes were discarded. For taxonomic classification, putative coding sequences (CDSs) from each contig were predicted using Prodigal [24]. Protein sequences were annotated using the best BLAST hit against the NCBI NR database, and tRNAs were identified using tRNAscan-SE [25]. Only contigs that yielded consistent hits to a single high-level taxon (e.g. *Alphaproteobacteria* and *Gammaproteobacteria*) were retained. Strict assembly requirements combined with taxonomic uniformity were imposed on assembled contigs with lengths >5 kb. The contigs had a consistent phylogenetic profile and probably originated from a single organism (e.g. *Methylomicrobium*). To test if the assembly strategy produced contigs that were "real," the predicted taxonomy for all putative coding sequences (CDSs) of all contigs was manually determined. The criterion for assigning contigs to the clades *Methylomicrobium*,

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