Molecular identification of methane oxidizing bacteria from paddy soils and detection methane monooxygenase gene

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Abstract. Methane uptake and production could take place in paddy soil. Methane oxidizing bacteria are the group of bacteria that play role in the methane uptake cycle and known as methanotroph. Methanotroph possess methane monooxygenase that could oxidize methane and short chain halalkane. Methanotroph in paddy soils play role in controlling methane emission. Identification conducted in order to identify methanotroph from paddy soils. In this study, 5 methane oxidizing bacteria from paddy soils have been identified based on 16S rRNA gene sequencing and pmoA gene encoding particulate methane monooxygenase sequence. Phylogenetic analysis deduced from 16S rRNA gene and pmoA gene showed that 4 bacteria were identified as Methylophilus leisingeri with sequence similarity 99% and pmoA like type II methanotoph, while 1 bacterium was identified as Methylomonas koyamae with sequence similarity 99% and pmoA like type I methanoroph.

1. Introduction
There are about 15 - 20% contribution of methane to greenhouse effect. Source of methane include, rice paddies, lake, wetlands, landfill, soils and sediment, ruminants, oil and gas recovery and gas transport [1, 2]. In 2010, methane from paddy rice cultivation were approximated about 493 - 723 MtCO₂eq/year [3]. Subadiyasa et al [4] reported the emission of methane during the growth period of paddy in Bali ranging from 3.9 - 10.7 g/m² which affected by type of soils. Setyanto et al [5] reported the emission of methane also affected by rice cultivars, start from 3.77 g/m² up to 9.48 g/m². Methane could be utilize by methane oxidizing bacteria, methanotroph, as a carbon and energy source [6]. There is about 10¹³ methanotrophic bacteria per gram paddy rhizosphere [7]. Methanotrophic bacteria assimilated carbon in the form of formaldehyde produced from oxidation of methane into methanol followed by oxidation of methanol into formaldehyde [8].

Methane monooxygenase (MMO) are important enzyme for methanotroph. It consist in two form, soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO) [6, 8]. Methane monooxygenase not only act on methane but also on chlorinated hydrocarbon [9, 10]. Expression of both enzyme are regulated by copper ion. pMMO will expressed more than sMMO when copper:biomass ratio is high, and vice versa [11]. Methane oxidizing bacteria are divided into two group, type I that assimilate carbon through ribulose monophosphate pathway (RMP) and dominated by γ-Proteobacteria from the genus of Methylomonas, Methylobacter, Methylocaldum, Methylococcus, Methylosporium and Methylomicrobium and type II that assimilate through serin metabolism pathway and dominated by α-Proteobacteria from the genus of Methylosinus and Methylocystis [2, 12].
Methane monooxygenase could oxidize alkane (C1 - C8) into primary and secondary alcohol and oxidize halogenated alkane include chloromethane, bromomethane and fluoromethane into formaldehyde [9]. Chlorinated hydrocarbon compound are used in bleach solution, pesticide, semiconductor manufacture, as extractants of caffeine from coffee and produced during water chlorination [2, 10]. Communities of methanotrophic bacteria could degrade trichloroethylene with rate 0.19 ppm h⁻¹ and it is also could reduce the concentration of trichloroethylene, tetrachloroethylene and vinylchloride ex situ [13-16]. In aerobic condition, chlorinated hydrocarbon could undergo co-metabolism by methanotrophic bacteria [15, 17]. The competition of both pollutants and obligate growth substrate become one of the challenge in the utilization of methanotrophic bacteria for bioremediation of chlorinated hydrocarbon [15].

Methanotrophic bacteria could reduce methane emmision and could become bioremediation agent for chlorinated hydrocarbon. Identification of methane oxidizing bacteria could give the information which bacteria that have role in oxidizing methane and its potency for utilizing chlorinated hydrocarbon based on pmoA gene, with the result that this bacteria could be applied as bioremediation agent.

2. Materials and Methods

2.1. Isolation of methane oxidizing bacteria
Soil samples are collected from two site ofpaddy soils, Site 1 (6°03’26.1"S 106°38’48.9"E) and Site 2 (6°04’41.6"S 106°38’32.7"E) at 5 cm depth from surface in Tangerang, Indonesia. Soil samples are enriched using 10 mL Nitrate Mineral Salts (NMS) [18] in 75 mL serum bottle supplemented with 15 mL methane and 100 µL methanol. The bottle was sealed with butyl rubber stopper and aluminum seal and incubated at 30°C, 150 rpm for 1 week in shaker incubator and subcultured in same medium for 5 times. The enrichment culture were diluted and streaked onto NMS agar plate. The plates are placed in Oxoid Anaerobic Jar, supplemented with methane up to 5 psi then incubated at 30°C for 2-3 weeks and subsequent subculture was done until pure distinguishable single colony are obtained.

2.2. PCR amplification and sequencing of 16S rRNA gene and pmoA gene
DNA extraction were carried out using boiling method [19] and used as template. Amplification of 16S rRNA gene were performed using universal bacterial primer pairs 27F (5’-AGAGTTTGTGATCMTGGCCTCAG-3’) and 1492R (5’-TACGGYTACCTTGTTACGAC-3’) [20] and amplification of pmoA gene were performed using primer pairs A189gc (5’-GGNGACTGGGACTTCTGG-3’) and mb661 (5’-CCGGMGCCACCGTCYTACC-3’) [21]. The PCR amplification cocktail consist of 1 µL DNA template, 0.5 µL of 10 pmol each primer pair, 12.5 µL GoTaq Green Master Mix and 10.5 µL nuclease-free water. The PCR amplification were conducted in Arktik Thermal Cycler (Thermoscientific) using following condition: initial denaturation at 98°C for 5 minutes followed by 30 amplification cycles (98°C for 60 s, 50°C for 30 s, 72°C for 90 s) and 10 min at 72°C for final extension. Sequencing of all PCR products was performed by1st Base (Singapore). All sequence were submitted into NCBI with accession number MH938148-MH938152 for 16S rRNA gene.

2.3. Phylogenetic construction of methane oxidizing bacteria
Sequence of 16S rRNA and pmoA gene obtained from sequencing were identified using BLAST (Basic local Alignment Search Tool) [22] to be used as reference in phylogenetic tree reconstruction. 16S rRNA and pmoA gene were aligned with type strain from GenBank/DDBJ/EMBL database using ClustalW [23]. Phylogenetic tree of 16S rRNA and pmoA gene were reconstructed using MEGA 7 software [24] with Neighbor-joining method [25]. Evolutionary distances of 16S rRNA gene were calculated with Tamura-Nei method [26] and pmoA gene were calculated using Poisson model [27]. Tree topology was evaluated by bootstrap analysis based on 1000 re-samplings [28].
3. Results and Discussion

3.1. Isolation of methane oxidizing bacteria

About five isolates were obtained, TP01, TP02, TP03, TP05 and TP06 from NMS medium supplemented with methane and methanol. Addition of methanol into medium instead only methane were to enrich the number of bacteria that could utilize methanol and methane. Selection of specific methane oxidizing bacteria are conducted in NMS agar supplemented with methane as sole carbon source. Distribution of oxygen during enrichment process were important during the cultivation, because methanotrophic bacteria use the one of oxygen atom as electron acceptor and the other is assimilated with methane into methanol [2]. Methanotrophic bacteria have been reported successfully isolated from paddy soil in Japan [29-32], Uruguay [33] and Indonesia [34].

![Figure 1](image-url)  
**Figure 1.** Neighbor-joining tree of 16S rRNA gene of methane oxidizing bacteria. The isolated bacteria shown in bold. Evolutionary distances were computed using Tamura-Nei method. Only bootstrap value >50% (n = 1000) shown in node. GenBank accession number are given in parentheses. Bar, 0.02 substitutions per nucleotide sequence position.
3.2. Identification of methane oxidizing bacteria based on 16S rRNA gene

Amplification of 16S RNA gene of five methane oxidizing bacteria have been conducted, resulting a DNA fragment with 1500 bp size. Isolate TP01, TP02, TP03 and TP05 have similarity >99% with Methylophilus luteus Mim\textsuperscript{T}, Methylophilus flavus Ship\textsuperscript{T}, Methylophilus leisingeri DM11\textsuperscript{T} and TP06 with Methylomonas koyamae Fw12E-Y\textsuperscript{T} (99%). Result from phylogenetic tree reconstruction of 16S rRNA gene (Figure 1), isolates TP01, TP02, TP03 and TP05 formed clade with Methylophilus, a methylo trophic bacteria, member of Methylophilaceae family from \( \beta \)-Proteobacteria sub-class which use methan e as carbon and energy source [35]. They formed monophyletic clade with M. leisingeri DM11\textsuperscript{T} and show adjacency with M. luteus Mim\textsuperscript{T}, M. flavus Ship\textsuperscript{T} and M. rhizosphaeracea CBMB127\textsuperscript{T}. Isolate TP06 formed a strong clade with Mm. koyamae Fw12E-Y\textsuperscript{T}, a member of Methylococccaceae family from \( \gamma \)-Proteobacteria sub-class, with bootstrap value 100%. Mm. koyamae Fw12E-Y\textsuperscript{T} could utilize methane and methanol as carbon source [36].

Methanotrophic bacteria were split into two big sub-class, from \( \gamma \)-Proteobacteria consist of Methylomonas, Methylobacter, Methylocaldum and from \( \alpha \)-Proteobacteria consist of Methylosinus and M ethylocystis [2, 37, 38]. There were also a member of Verrucomicrobia represented by Methylocaldum infernorum, an extremely acidophilic methanotroph that optimally growth at pH 2.0 - 2.5 [39, 40]. Several report about methanotroph isolated from paddy soil have been published inc luding from genus Methylosinus, Methylomonas, Methylocystis, Methylocaldum, Methylogaea and Methylobacterium [29-31, 33,34].

From the phylogenetic result, only isolate Mm. koyamae TP06 are considered as methanotrophic bacteria which can use methane as sole carbon source, while the rest are a member of methylo trophic methanol-oxidizing bacteria, which can use methanol as carbon source. In the culture enrichment, addition of methanol would enhance the growth of methylo trophic bacteria, but in the purification of isolates, methane are used as sole carbon source. Therefore, the isolates which grow on the medium are capable to utilize the methane catalyzed by MMO. Several methylo trophic bacteria are reported can grow on medium containing dichloromethane including M. rhizosphaeracea and M. Leisingeri [41, 42]. In order to utilize the dichloromethane, the bacteria must have MMO [43].

![Figure 2](image-url)  
**Figure 2.** Neighbor-joining tree of pmoA gene shows relationship between isolated strains with methanorophic bacteria. The isolated bacteria shown in bold. Only bootstrap value >50% (n = 1000) shown in node. GenBank accession number are given in parentheses. Bar, 0.05 substitutions per amino acid sequence position.
3.3. Identification of pmoA gene
Detection of pmoA gene have been conducted to confirm the ability of the isolates to utilize methane as carbon source. The PCR product from primer pair A189gc and mb661 is about 470 bp [21]. According to phylogenetic tree analysis of pmoA gene (Figure 2), isolate TP06 forms a clade with Mm. methanica and Mm. koyamae which are classified as methanotroph type I which assimilated carbon via RMP pathway. This result confirmed phylogenetic tree reconstruction of 16S rRNA gene of isolate TP06 as methanotrophic bacteria.

The amplification results of pmoA gene from isolates TP01, TP02, TP03 and TP05 give a positive results which have a similarity with pmoA gene from Methylosinus which are classified as methanotroph type II [12]. However, four of this isolates are identified as M. leisingeri according to 16S rRNA gene. This result supported the previous study conducted by Doronina (1994) [41] which found that M. leisingeri can grow on dichloromethane substrate.

4. Conclusion
Five methane oxidizing bacteria isolated from paddy soils have successfully identified as M. leisingeri TP01, M. leisingeri TP02, M. leisingeri TP03, M. Leisingeri TP05 and Mm. koyamae TP06. All of the isolates have pmoA gene which encoding methane monooxygenase which has the ability to oxidize methane.

5. References
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