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ORIGINAL ARTICLE

Description of a methanotrophic strain BOH1, isolated from Al-Bohyriya well, Al-Ahsa City, Saudi Arabia

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KEYWORDS

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Abstract Methanotrophic bacteria have a unique ability to utilize methane as their carbon and energy sources. Therefore, methanotrophs play a key role in suppressing methane emissions from different ecosystems and hence in alleviating the global climate change. Despite methanotrophs having many ecological, economical and biotechnological applications, little is known about this group of bacteria in Al-Ahsa. Therefore, the main objective of the current work was to expand our understanding of methane oxidizing bacteria in Al-Ahsa region. The specific aim was to describe a methanotrophic strain isolated from Al-Bohyriya well, Al-Ahsa using phenotypic, genotypic (such as 16S rRNA and *pmoA* gene sequencing) and phylogenetic characterization. The results indicated that the strain belongs to the genus *Methylomonas* that belongs to *Gammaproteobacteria* as revealed by the comparative sequence analysis of the 16S rRNA and *pmoA* genes. There is a general agreement in the profile of the phylogenetic trees based on the sequences of 16srRNA and *pmoA* genes of the strain BOH1 indicating that both genes are efficient taxonomic marker in methanotrophic phylogeny. The strain possesses the particulate but not the soluble methane monooxygenase as a key enzyme for methane metabolism. Further investigation such as DNA: DNA hybridization is needed to assign the strain as a novel species of the genus *Methyomonas* and this will open the door to explore the talents of the strain for its potential role in alleviating global warming and biotechnological applications in Saudi Arabia such as bioremediation of toxic by-products released in oil industry. In addition, the strain enhances our knowledge of methanotrophic bacteria and their adaptation to desert ecosystems.

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1. Introduction

Methane is a potent greenhouse gas that occurs in the atmosphere at the highest concentration of any of the trace gases. One important characteristic of methane is its efficient capacity as a greenhouse (heat-trapping) gas in comparison to carbon dioxide. This is due to its strong infrared absorbance, with the re-emitted radiation being a major contributor to the destruction of the ozone layer, leading to global warming (Crutzen, 1994; Lelieveld et al., 1998; Christiansen et al., 2015).

Methanotrophs are group of Gram-negative bacteria that utilize methane as their sole source of carbon and energy, thereby reducing the level of methane from the environment significantly and contributing to the mitigation of global warming (Hanson and Hanson, 1996; Trotsenko and Murrell, 2008).

The ability of methanotrophs to consume methane as the sole source of carbon and energy is due to their possession of methane monooxygenase (MMO), a subclass of oxidoreductase enzymes. There are two well-studied forms of MMO: the cytoplasmic soluble form (sMMO) and the membrane-bound, particulate form (pMMO). All methanotrophs contain either sMMO or pMMO while only some of them contain both forms. Two marker genes are essential in detecting MMO: *mmoX* (a gene encoding a subunit of soluble methane monooxygenase) and *pmoA* (a gene encoding a subunit of particulate methane monooxygenase) (Dong et al., 2015). The sMMO and pMMO play a major role in biotechnological application via their ability to oxidize many halogenated hydrocarbon.

Recently, several novel methanotrophic genera and species have been described by many researchers in different laboratories worldwide. *Methyloparacoccus murrellii* (Hoefman et al., 2014a); *Methylogaea oryzae* (Geymonat et al., 2011); *Methyloprofundus sedimenti* (Tavormina et al., 2015) *Methylocapsa palsarum* (Dedysh et al., 2015); *Methylomarinovum caldicuralii* (Hirayama et al., 2014). *Methyloglobulus morosus* (Deutzmann et al., 2014); *Methylomonas lenta* (Hoefman et al., 2014b) and *Methylocaldum marinum* (Takeuchi et al., 2014) are representatives of novel strains. The increase in the number of the novel strains could be attributed to the recent advances in molecular techniques used for identification and obtaining isolates from unexplored environmental sites.

Methanotrophs oxidize about 80% of methane before it reaches atmosphere. Therefore, methanotrophs are major players in the removal of methane from the atmosphere, thereby mitigating global warming. Methanotrophs have a potential role in bioremediation as they have the ability to co-oxidize trichloroethylene, a toxic compound, in addition to a variety of hydrocarbons including alkanes, alkenes, heterocyclic compounds and aromatic ethers (Smith and Dalton, 2004; Smith and Murrell, 2011). In addition, methanotrophs can be used to produce a biofuel, methanol under ambient conditions, (Xin et al., 2004). Methanotrophs can be used to produce single-cell protein, as an alternative source for proteins for consumption as feedstocks by animals and fish (Winder, 2004).

Due to the above-mentioned ecological, economical and biotechnological applications, it was of interest to explore the methane-consuming bacteria in different environments in Al-Ahsa and expand our knowledge about this interesting group of bacteria. The specific aim of the project was to characterize and identify isolated strain by using state-of the- art-molecular techniques.

2. Materials and methods

2.1. Isolation of the strain BOH1

Water samples were collected from Al-Bohyriya well, Al-Ahsa, Saudi Arabia, early March, 21, 2015, in sterilized plastic bottles. BOH1 was isolated from the water samples in Nitrate Mineral Salts (NMS) liquid medium (Whittenbury et al., 1970) enriched with methane according to the method described previously (Dianou et al., 2012). The flasks were sealed with suba-seals to avoid any gas leak, and gassed with 20% (v/v) methane and incubated at 30 °C on a shaking incubator (150 r.p.m min^{-1}) for 5–7 days. Bacterial strain was streaked on NMS agar plates and incubated in a methane/air atmosphere in a gas-tight container for one week. The strain was maintained by streaking on NMS plates every 2–3 weeks, in a routine basis. Purity check and morphological features of the cell and motility were carried out by examining the strain under oil immersion lens using the phase contrast microscopy with an (Olympus BX50, Japan).

2.2. Testing the ability to utilize different carbon sources

The ability of the strain BOH1 to utilize different carbon sources was tested in a microtitre plate containing liquid NMS medium without adding methane. The plate was supplemented with 0.1% (w/v) of filter-sterilized glucose, sucrose, fructose, starch, acetate or pyruvate.

2.3. Effect of different concentration of the NaCl on BOH1

To check the salt tolerance, the strain BOH1 was grown in plates containing NMS medium supplemented with 0.1, 0.15, 0.2, 0.3, 0.5, 1, 2 and 5%, w/v of NaCl. The plates were incubated in gas-tight containers in the presence of methane for 7 days at 30C. The growth was checked visually and the experiment was done in triplicate.

2.4. Extraction and purification of genomic DNA

Extraction and purification of genomic DNA was carried out as mentioned by Marmur (1961). Briefly, about 0.5 gram wet weight obtained from the strain that was growing at late exponential phase cultures. After resuspension of the pellet in 0.75 ml TE buffer, 20 µl of freshly prepared lysozyme (100 mg ml^{-1}, Sigma) was added and the tubes were incubated at room temperature for 20 min. Then, 40 µl of 10% (w/v) SDS and 8 µl of proteinase K (10 mg ml^{-1}) were added and mixed gently and tubes were incubated at 37 °C for 1 h. Following incubation, 100 µl of 5 M NaCl and 100 µl of warm cetyl-trimethylammonium bromide (10% in 0.7 M NaCl) were added and tubes incubated at 65 °C for 10 min. Then, 0.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) was added, the contents of tubes were mixed gently before centrifugation at 4000*g* for 10 min at room temperature, to remove proteins. The top aqueous layer was carefully transferred to new tubes

and 0.5 ml chloroform/isoamyl alcohol (24:1 v/v) treated as the previous step. Precipitation of DNA was carried out by adding 0.6 volumes of isopropanol and 0.3 volumes of sodium acetate then the DNA spooled out using sterilized glass rod 30 min incubation at room. The DNA was washed in 200 µl of 70% v/v ethanol, air-dried and suspended in 200 µl of nuclease-free water (Ambion).

2.5. Genotypic identification of the strain BHO1

A comparative sequence analysis of the 16s rDNA gene and *pmoA* was used to identify the strain BHO1.

2.6. PCR amplification of the 16S rDNA

Amplification of the 16S rRNA gene was carried out using the universal primers; 27F 5′- and 1492R (Weisburg et al., 1991) (Table 1), in a total 50 µl of PCR reaction, using the standard PCR programme. PCR water and Genomic DNA from *Methylosinus trichosporium* were used as negative and positive controls, respectively. The PCR amplification and size were checked on agarose gel using 1 K DNA ladder. The PCR was then cleaned up to remove primer dimers using a Clean-up kit (Millipore), according to the manufacturer's instruction.

2.7. Amplification of the pmoA gene

The *pmoA* gene encoding a polypeptide of pMMO was amplified by PCR with primers A189f/mb661r using the recommended PCR conditions (Holmes et al., 1995; Costello and Lidstrom, 1999) (Table 1). Attempts to amplify the *mmoX* gene were carried out using the primer set mmoX206f/mmox886r (Hutchens et al., 2004) (Table1).

2.8. Sequencing of the 16S rDNA and the pmoA genes

Sequencing of the 16S rRNA and the *pmoA* genes was performed using the Big Dye terminator cycle sequencing kit and resolving of the sequencing products was determined with on an ABI 3130 genetic analyser automated DNA sequencing system (Applied BioSystems, USA).

2.9. Phylogenetic analysis

A neighbour-joining phylogenetic tree based on 16S rDNA or *pmoA* gene sequences showing the relationships between the strain BOH1 and other related species, top-hits in BLASTN, was constructed based on the Tamura–Nei model (Tamura and Nei, 1993). Evolutionary analyses were inferred in MEGA5.02 (Tamura et al., 2011). The 16S rRNA and *pmoA* gene sequences of BOH1were sent for deposition in the NCBI database.

3. Results and discussion

The current study aimed at characterizing of a methanotrophic bacterial strain isolated from Al-Bohyriya well, Al-Ahsa. The strain is designated as BOH1 refereed to the place of isolation. The colony morphology of the strain BOH1 growing on NMA plates was investigated visually. The strain exhibited a white, rounded, entire colony with 3–4 mm in diameter after 7 days of incubation at 30 °C (Fig. 1). The cells were rounded and negative towards Gram staining. BOH1 couldn't grow on glucose, sucrose, fructose and starch as a sole energy and carbon source. Moreover, the strain tolerated up to 0.15% of NaCl (Table 2).

BOH1 was further investigated using more sophisticated molecular biology techniques such as 16S rRNA and *pmoA* gene sequences. The sequence results of both genes were in common agreement and showed that BOH1 is related to the genus *Methylomonas*. The strain clustered with the *Methylomonas* clade in the phylogenetic trees based on the 16s rRNA and *pmoA* genes. Unlike different species of *Methylomonas*, *mmoX* gene couldn't be detected using PCR amplification with the common primers. This distinguished feature provides an indication that this strain is likely to be a novel species.

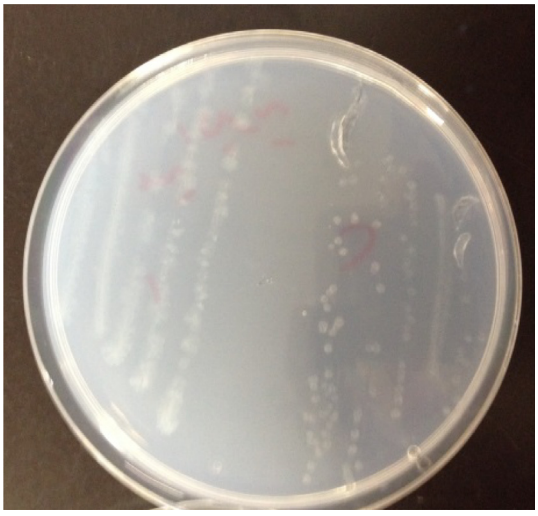

Figure 1 The strain BOH1 growing on NMS medium after one week of incubation at 30 °C.

Table 1 A list of the primer used in the current study.

Primer	Sequence	References
A189f	5′-GGNGACTGGGACTTCTGG-3′	Holmes et al. (1995)
mb661r	5′-CCGGMGCAACGTCYTTACC-3′	Costello and Lidstrom (1999)
27F	5′-AGAGTTTGATCM TGG CTC AG-3′	Weisburg et al. (1991)
1492R	5′-TACGGYTACCTTGTTACGACTT-3′	
mmoX206F	5′-ATCGCBAARGAATAYGCSCG-3′	Hutchens et al. (2004)
mmoX886r	5′-ACCCANGGCTCGACYTTGAA3′	

Table 2 Characteristics of strain BOH1.

Characteristic	Result
Colony morphology	Circular colony – entire edge
Pigmentation	White
Gram staining	Negative
NaCl tolerance (0.15)%	+
Glucose	–
Sucrose	–
Fructose	–
Starch	–
Acetate	–
Pyruvate	–

3.1. Genotypic identification of the strain BHO1

3.1.1. PCR-amplification of the 16srRNA gene of the strain BOH1

The 16S ribosomal RNA of the strain BOH1 was amplified using PCR with universal primers 27F/1492R (Fig. 2). The size of the PCR product was about 1465 bp as indicated by DNA ladder. Analysis of the 16S rRNA gene sequence is an efficient tool that is commonly used for identifying bacterial genera and species for many reasons. Firstly, it has regions with unique sequences varied with various species; these species-specific "barcode" sequences are the basis for bacterial identification (Pereira et al., 2010; Kolbert and Persing, 1999). Secondly, 16S rRNA genes exist in all prokaryotic organisms. Thirdly, the gene's length is quite sufficient for bioinformatics purposes. Lastly, the gene shows a very slow evolution rate and therefore, providing insights into phylogenetic relationships among bacterial taxa (Woese, 1987; Stackebrandt, 2002). The rationale of selecting the primer set, 27F and 1492R, for PCR amplification of the BOH1 strain was both of them bind to highly conserved regions and provide almost full length coverage of the 16srRNA gene. Consequently, accurate identification of the bacterial strain can be easily done by BLAST alignment against recognized data bases such as NCBI, http://www.ncbi.nlm.nih.gov/.

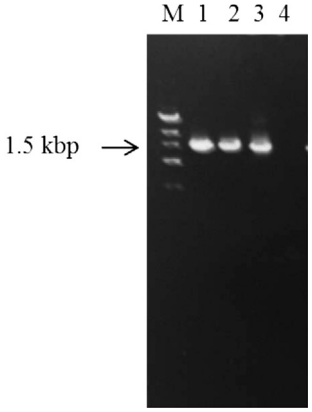

Figure 2 Agarose gel electrophoresis of PCR of 16SrRNA. Lanes 1 and 2 positive controls genomic DNA and DNA from a colony of *Methylosinus trichosporium*; lane 3, genomic DNA of strain BOH1. Lane 4, negative control (no template DNA). The expected size is approximately 1, 492 bp. M, size marker (bp), 1 kb DNA ladder (Invitrogen).

3.1.2. 16S rRNA gene sequence analysis and phylogenetic tree

Taking advantage of the freely available DNA and protein sequences on the NCBI data bases, top hits of strains that showed high similarity with the 16SrRNA of the strain BOH1 were retrieved, aligned and used to construct a phylogenetic tree (Fig. 3).

A comparative sequence analysis of the 16S rRNA gene of BOH1 showed that this strain was closely related to the genus *Methylomonas* which belongs to *Gammaproteobacteria*. *Methylomonas methanica* was the closest species to BOH1 with 97% homology of the 16s rRNA sequence analysis. The strain BOH1 formed a monophyletic group with species of the *Methylomonas* group (Fig. 3), *Methylomonas rubra* (accession No. AF150807), *Methylomonas_koyamae* (accession No. NR113033), *Methylomonas scandinavica* (accession No. NR041958), *Methylomonas* sp. (accession No. HE801216), (*M. methanica* accession No. AF304196). Representatives of other *Gammaproteobacteria* methanotrophs; *Methylococcus mobilis* (accession No. NR104922), *Methylobacter tundripaludum* (accession No. NR042107), *Methylococcus capsulatus* (accession No. NR042183), *Mg. oryzae* (accession No. EU672873), *Methylocaldum szegediense* (accession No. NR026064.), *Methylocaldum gracile* (accession No. NR026063) formed a recognized clade within *Gammaproteobacteria*. *Alphaproteobacteria* exemplified by *Ms. trichosporium* (accession No. DQ149124) formed distinct outgroup providing a significant robustness of the constructed phylogenetic tree. Similar results have been achieved by Islam et al. (2015), who reported that novel methanotrophic strains were recovered from different geographic habitat in Bangladesh, based on the 16S rRNA gene sequences. It is worth mentioning that although analyses of 16S rRNA sequences can be used to define species (Wu et al., 2006), this is not always the case because 16S rRNA genes might contain highly conserved regions that do not allow the discrimination among some species and subspecies within this group (Shaver et al., 2002; Zhang et al., 2014). In general, strains that show sequence similarity level of $<97\%$ to the closest strains are candidates to be a novel species whereas those with a similarity score of $>97\%$ most probably, but not necessarily, belong to the same species (Stackebrandt and Goebel, 1994).

3.1.3. PCR-amplification of the pmoA gene of the strain BOH1

Methanotrophs consume methane as their sole source of carbon and energy with the aid of methane monooxygenase (MMO). There are two common forms of MMO: the cytoplasmic soluble form (sMMO) and the membrane-bound, particulate form (pMMO). It was of interest to investigate whether or not the BHO1 strain contains which form of MMO. To answer this question, PCR-amplification of two marker genes which are essential in detecting MMO: *mmoX* (a gene encoding a subunit of soluble methane monooxygenase) and *pmoA* (a gene encoding a subunit of particulate methane monooxygenase) (Dong et al., 2015) was carried out using the specific primers. Amplification of *pmoA* with primers A189f/mb661r using the recommended PCR conditions (Holmes et al., 1995; Costello

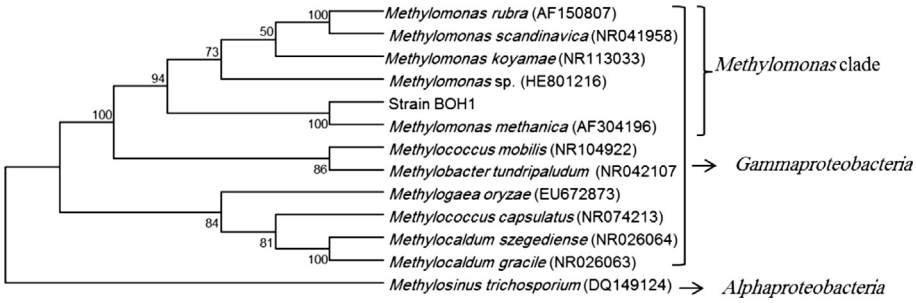

Figure 3 A neighbour-joining phylogenetic tree based on 16SrDNA gene sequences showing the relationships between the strain BOH1 and other related species.

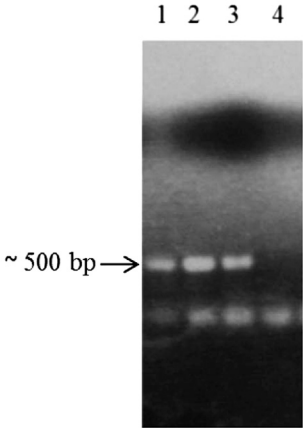

Figure 4 Agarose gel electrophoresis of PCR of *pmoA*. Lanes 1 and 2 positive controls genomic DNA and DNA from a colony of *Methylosinus trichosporium*; lane 3, genomic DNA of strain BOH1. Lane 4, negative control (no template DNA). The expected size is approximately 500 bp. M, size marker (bp), 1 kb DNA ladder (Invitrogen).

and Lidstrom, 1999) resulted in a product of about 500 bp length as expected (Fig. 4). The identity of the PCR product was further determined by sequencing to be *pmoA*. This result indicates that the strain BOH1possessed pMMO for consuming methane as its sole carbon and energy source. However, several trails of PCR amplification of *mmoX* failed indicating that the strain doesn't contain sMMO.

3.1.4. pmoA gene sequence analysis and phylogenetic tree

The *pmoA* gene sequences of the strain BOH1 revealed that this strain was closely related to the genus *Methylomonas* with a 97% identity. These results were in general agreement with those obtained by the 16S rRNA sequence analysis of the strain under consideration.

Similarly, the strain BOH1 grouped with the *Methylomonas* clade (Fig. 5), within *Gammaproteobacteria: Methylomonas* sp. (accession No. HG915726, *M. koyamae* (accession No. AB538965), and *M. methanica* (accession No. EU722434). *Mg. oryzae* (accession No. EU359002), *Mc. capsulatus* (accession No. MCU94337), *Mm. caldicuralii* (accession No. AB302948), *Methylohalobius crimeensis* (accession No. AB687535), *Mca. gracile* (accession No. MGU89301)m *Methylocaldum* sp (accession No. AB275418)m *Mca. szegediense* (accession No. MSU89303) are other related *Gammaproteobacteria* formed distinct group from *Methylomonas*. *Ms. trichosporium* (accession No. AJ868409) a representative of *Aphaproteobacteria* was clearly out grouped from the rest of the other methanotrophic species (Fig. 5). There is a general agreement in the profile of the phylogenetic trees based on the sequences of 16srRNA and *pmoA* genes of the strain BOH1 indicating that both genes are efficient taxonomic markers in methanotrophic phylogeny. Consistency of the *pmoA* phylogeny with that of 16S rRNA gene has been reported previously in methanotrophs (Islam et al., 2015; Horz et al., 2001; Vigliotta et al., 2007).

The Saudi habitats are vital biological resources for description of novel methanotrophic bacteria as they are not previously explored. Further investigations are highly recommended to be conducted to the strain BOH1 using for example

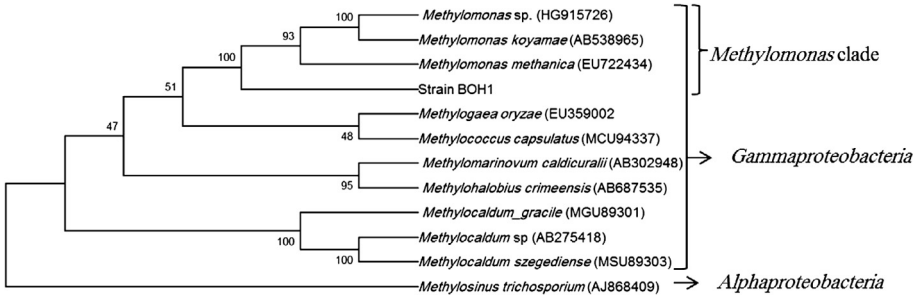

Figure 5 A neighbour-joining phylogenetic tree based on *pmoA* gene sequences showing the relationships between the strain BOH1 and other related methanotrophic species.

DNA:DNA hybridization with related *Methylomonas* species. Additionally, the ability of the strain BOH1 to bioremediate many environmentally-unfriendly compounds released from oil industry is promising future research of this strain.

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