Molecular Identification of *Methylorubrum extorquens* using PCR-Amplified MxaF Gene Fragments as A Molecular Marker

Anwar A. Maki1, Asaad M. R. Al-Taee1,2* Zeenah Weheed Atwan1,2

1Department of biological Development, Marine Science Center, Basra University, Basra, Iraq.  
2Department of Microbiology, College of Medicine, University of Basra, Basra, Iraq.  
*Corresponding author: amraltaee@yahoo.com  
E-mail addresses: anwar.maki@uobasrah.edu.iq, zeenah.atwan@uobasrah.edu.iq

Received 8/9/2022, Revised 6/12/2022, Accepted 8/12/2022, Published Online First 20/5/2023,  
Published 01/1/2024

Abstract:  
Methylotrophs bacteria are ubiquitous, and they have the ability to consume single carbon (C1) which makes them biological conversion machines. It is the first study to find facultative methylotrophic bacteria in contaminated soils in Iraq. Conventional PCR was employed to amplify MxaF that encodes methanol dehydrogenase enzyme. DNA templates were extracted from bacteria isolated from five contaminated sites in Basra. The gene specific PCR detected *Methylorubrum extorquens* as the most dominant species in these environments. The ability of *M. extorquens* to degrade aliphatic hydrocarbons compound was tested at the laboratory. Within 7 days, gas chromatographic (GC) studies of remaining utilized crude oil revealed that 61.14% of the initial content had been degraded, and GC fingerprinting of the utilized aliphatic compounds revealed significant reductions in C_{12}, C_{13}, C_{14}, and C_{15}. Globally this is the first time found a new strain of *M. extorquens* has the ability to degrade aliphatic hydrocarbons compound. Conventional PCR and gene sequencing revealed the presence of the facultative methylotrophic bacteria in polluted areas in Basra. *M. extorquens* was dominant and showed a substantial ability to degrade crude oil which makes them an important tool to be employed in bioremediation.

Keywords: gas chromatography, Methanol dehydrogenase, *Methylorubrum extorquens*, Methylotrophs bacteria, MxaF gene.

Introduction:  
Various species of methylotrophic bacteria are distributed in nearly all natural environments. Methylotrophs use reduced single-carbon (C1) molecules like methanol as carbon sources for growth, making them methylal biological convertors. More than 50 methylotrophic taxa have been identified, including Alpha, Beta, and Gamma-proteobacteria, Verrucomicrobia, Firmibacteria, Actinobacteria, and Flavobacteria. Pink-pigmented facultative methylotrophs (PPFMs), are present in the rhizosphere or exist in soil, air, or water. PPFMs belong to methyl bacteriaceae family, they are gram negative and utilize C1 molecules as their only source of energy and carbon, methanol, methylamine, formate, and formaldehyde are among the examples.

*Methylobacterium* has more species than any other within the family methylobacteriaceae, order rhizobiales, and class alphaproteobacteria. *Methylobacterium* includes facultative methylotrophs that may live on carbon and energy sources other than organic acids and sugars, such as methane or methanol. All other previously PPFMs bacteria were classified within *Methylobacterium* in a taxonomic analysis. Following that, according to 16S rRNA gene sequences, multi-locus sequence analysis (MLSA), phenotypic data, and genomics, eleven *Methylobacterium* species were reclassified as *Methylorubrum*, a new genus.

Methanol dehydrogenase (MDH) converts the methanol to formaldehyde, which is the second crucial enzyme in methane metabolism. The MDH is a pyrroloquinoline quinone (PQQ)-containing soluble periplasmic enzyme with a α2β2 structure, consisting of two large subunits MxaF and two tiny subunits MxaI, the active site contains a Ca^{2+} ion. MxaF encodes for the large alpha-subunit of MDH and other functional molecular marker genes are highly conserved across methylotrophs and have been utilized in environmental studies to identify methylotrophs in different environments.
One of the most significant environmental pollutants, with bad impacts on both people and the environment is hydrocarbons. It is a crucial global environmental pollutant because it spills and leaks often throughout the exploration, transport, refining, and storage of petroleum and petroleum products. The fundamental approach for reducing biodegradable contaminants is biodegradation, which is a cost-effective alternative. It is one of the most effective and promising methods for cleaning up soil that has been contaminated with diesel. This choice has the potential to remove harmful contaminants through biological activity.

The aim of this study is to isolate methylotrophic bacteria from hydrocarbon-contaminated soils. Identification of these bacteria according to their morphological features and 16S rRNA gene sequencing. Determination of their capability to degrade aliphatic compounds in vitro.

Material and Methods:

Sampling
A total of 10 g of oil-polluted soil was collected aseptically in sterile plastic bags from five oil sites in Basra city, southern Iraq in December 2020, Fig. 1 and Table 1.

Table 1. Sampling stations coordination.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30.36449</td>
<td>47.63551</td>
</tr>
<tr>
<td>2</td>
<td>30.36475</td>
<td>47.63612</td>
</tr>
<tr>
<td>3</td>
<td>30.36587</td>
<td>47.63696</td>
</tr>
<tr>
<td>4</td>
<td>30.51714</td>
<td>47.60423</td>
</tr>
<tr>
<td>5</td>
<td>30.45591</td>
<td>47.39529</td>
</tr>
</tbody>
</table>

Preparation of media for isolation and purification
The isolation basal salt medium (BSM) has been modified according to Fujii et al. by increasing thiamine and biotin, Table 2. The medium components were dissolved in 1000 ml distilled water pH 6.8-7.0 and after sterilization, thiamine, biotin, and 20 ml of methanol was added aseptically to the medium in addition to that fluconazole was added as an antifungal. A 0.5 g of soil was placed in Erlenmeyer flasks containing 100 ml of BSM and incubated for 7 days at 30°C in a shaking incubator (Sartorius, Stidem, Germany) at 180 rpm. Methanol-utilizers were cultured for several replicates on a basal salt medium and were picked up after 5-7 days of incubation at 30°C.
Methanol-salt medium (MSM) of 13 was modified also by increasing the weights of some ingredients and adding fluconazole as an antifungal, Table 3. The medium was dissolved in 1000 ml distilled water pH 6.8. Fluconazole and 10 ml of methanol were added after sterilization.

### Table 2. Composition of medium used for cultivating Methylotrophic bacteria.

<table>
<thead>
<tr>
<th>Component</th>
<th>Kouno et al13</th>
<th>Current study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nitrogen and Sulfur source</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.1 g</td>
<td>1.0 g</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>3.0 g</td>
<td>3.0 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5 g</td>
<td>0.5 g</td>
</tr>
<tr>
<td>MnSO₄·5H₂O</td>
<td>2.0 mg</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>2.0 mg</td>
<td>2.0 mg</td>
</tr>
<tr>
<td><strong>Phosphorous source and pH buffering</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KHP₂O₄</td>
<td>2.0 g</td>
<td>2.0 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>7.0 g</td>
<td>7.0 g</td>
</tr>
<tr>
<td><strong>Amino acid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thiamine.HCl</td>
<td>100 µg</td>
<td>0.2 mg</td>
</tr>
<tr>
<td><strong>Vitamin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>10 µg</td>
<td>0.1 mg</td>
</tr>
<tr>
<td><strong>Antifungal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluconazole</td>
<td>--</td>
<td>0.05 mg</td>
</tr>
</tbody>
</table>

Methanol-salt medium (MSM) of 13 was modified also by increasing the weights of some ingredients and adding fluconazole as an antifungal, Table 3. The medium was dissolved in 1000 ml distilled water pH 6.8. Fluconazole and 10 ml of methanol were added after sterilization.

### Table 3. Composition of methanol salt medium.

<table>
<thead>
<tr>
<th>Component</th>
<th>Kouno et al13</th>
<th>Current study</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>2.0 g</td>
<td>-</td>
</tr>
<tr>
<td>KNO₃</td>
<td>-</td>
<td>2.0 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.2 g</td>
<td>0.2 g</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>1.0 mg</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.21 g</td>
<td>0.21 g</td>
</tr>
<tr>
<td>KHP₂O₄</td>
<td>0.09 g</td>
<td>0.09 g</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>50.0 µg</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>H₂BO₃</td>
<td>10.0 µg</td>
<td>30.0 mg</td>
</tr>
<tr>
<td>MnSO₄·5H₂O</td>
<td>10.0 µg</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>70.0 µg</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>10.0 µg</td>
<td>3.0 mg</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>-</td>
<td>0.05 mg</td>
</tr>
</tbody>
</table>

### Morphological and Biochemical Tests

The morphological and biochemical tests were used to identify the isolates including to their cell shape, colony morphology, pigment production, Gram staining, catalase and oxidase 15.

### Extraction of Genomic DNA and Identification of bacteria using 16S rDNA

Bacterial genomic DNA was isolated using the Geneaid PrestoTM Mini gDNA Kit (Korea) according to the manufacturer’s instructions. A 0.5% agarose gel electrophoresis was used to determine the purity of the DNA. Eluted DNA concentrations were measured using Nano-Drop (Optizen/Korea). 16S rRNA gene sequencing was used to identify consuming methanol bacteria grown on MSM plates, using the following primers: 27F AGAGTTTGATCCTGGCTCAG, 1492R GGTTACCTTGTGACTCAG 16. A master mix of 25 µl from Go Taq Green master mix (Promega, USA), was mixed with 19 µl of Nuclease Free water, 2 µl (10-20 ng) of DNA template, and 100 Pmol (2 µl) of each primer to a total volume of 50 µl PCR reaction. In order to perform the PCR reaction, the thermal cycler (Eppendorf, Germany) was programmed with the following parameters: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 Sec, 55 °C for 30 Sec, and 72 °C for 60 Sec, with a final extension at 72 °C for 5 min.

### Gel Electrophoresis

Using a 100bp DNA ladder (Promega, USA) and a UV transilluminator (ATTA, Korea), agarose gel was prepared by dissolving 0.25 g agarose powder in 25 ml TBE buffer with 0.2 g of Ethidium bromide as visualizing dye was used to detect 16S rDNA bands. PCR amplicons were sent to Macrogen for further purification and sequencing. The National Center for Biotechnology Information's BLAST was employed to align sequences in order to identify the isolated methylotrophs through 16S rRNA gene.
Identification of the isolates through *MxaF* gene

The methylotrophic isolates were identified using *MxaF* specific primers which encodes for gene methanol dehydrogenase enzyme, producing an amplicon of ~ 550bp. The primers sequences were:

F1003degen 5'-GGNCANACYTGGGNTGGT-3',
R1561degen 5'-GGGARCCNTTYATGCTNCCN-3'.

Each PCR reaction mixture included 12.5 µl of Go Taq Green master mix (Promega, USA), 7.5µl of Nuclease Free water, 3 µl (15-30 ng) of DNA template, and 1 µl (100 Pmol) of each primer make up a 25µl PCR reaction mixture. The thermal cycle was set (Eppendorf, Germany) as the following conditions were used for the PCR reaction: 94 °C for 45 Sec, 59 °C for 1 min, and 72 °C for 1.5 min, for 30 cycles, with a final extension at 72 °C for 10 min.

Biodegradation of crude oil

Pure cultures of the isolated bacteria were prepared by adding 1 ml of the liquid pure culture in a conical flask containing 100 ml of MSM and 0.5% (v/v) crude oil supplied from Al-Shua’aba Refinery-Basra city. The flasks were incubated in a shaking incubator for 7 days at 30 °C with 120 rpm.

Extracting residual crude oil

A liquid-liquid extraction technique was used to extract the leftover crude oil by a separating funnel. The aqueous phase was discarded, and the remaining oil was dried in the oven at 40 °C to remove the chloroform. The aliphatic fraction was separated, and the residual oil was diluted in 25 ml of n-hexane. The aliphatic fraction was collected and sent to be analyzed by Gas Chromatography to estimate aliphatic compounds (Agilent Chem Station).

Results:

Isolation of Methanol utilizers

Little pink spherical colonies began to form after 3–5 days of incubation at 30 °C. Gram-negative bacteria might be seen alone, in pairs, or in large numbers. There was no spore formation, and tests for catalase and oxidase were positive, Fig. 2.

Identification of the isolates through 16S rRNA gene

The sequencing of six nominated isolates revealed the presence of methylotrophic bacteria using16S rRNA amplicon size of 1500bp on a 1% agarose gel. *Methylorubrum extorquens* were identified at the species level based on a 99% similarity of 16S rRNA sequences to the intended type in GenBank Fig. 3.
Identification of bacteria using MxaF gene

Using the MxaF- specific primers, MxaF was detected in four isolates at the conservative region of methylotrophic bacteria, the isolates were genetically identified. On a 2 % agarose gel, the MxaF amplicon names and affiliations of Methylorubrum genomic DNA were visualized at the expected size of 550bp, Fig. 4.

Biodegradation of crude oil

Significant increases in cell density were seen after 7 days of incubation when crude oil was used as the sole carbon and energy source, with simultaneous decreases in several components of the used crude oil, Fig. 5. During the incubation time, there were significant changes in numerous components of the used crude oil. After 7 days, most of the peaks had significantly shrunk. The short-chain alkanes nC_{12}-nC_{15} were efficiently destroyed by presumptive M. extorquens which the best-growing isolate was used on crude oil, while long-chain alkanes nC_{16}-nC_{37} degraded at a slower rate. The total degradation ratio was 61.14% (0.5% v/v) of crude oil, Fig. 6.
Figure 5. Crude oil degradation by *M. extorquens* after incubated at 30 °C with 120 rpm shaking. A/ control has crude oil only. B/ bacteria with crude oil after 7 days’ of incubation.

Figure 6. GC chromatograms of aliphatic compound following crude oil degradation (a) crude oil in the control group; (b) aliphatic compound following biodegradation.
Discussion:

The study aimed to isolate and identify facultative methylotrophs from oil contaminated sites in Basra, Iraq and test their ability to naturally degrade the crude oil at the laboratory. In this study, a new modified medium has been introduced according to the ability of bacteria to grow. The isolates grew very poorly on the methanol-salt medium and did not grow if the inoculum was small. Also, the isolates could not grow in the medium of Kouno et al. These may be due to the harsh environment from which they were isolated. So, the increase of biotin and thiamine and the addition of antifungals led to the flourishing of these bacteria and increased their ability to consume methanol, which reached 6% (unpublished data) and reduced the incubation period.

The results of morphological and biochemical tests of Methylobacterium according to Bergey's Manual of Determinative Microbiology's description of phenotypic identification of Methylobacterium species. These bacteria were genetically identified using the 16S rRNA gene as Methylorubrum extorquens. These results come in consistent with findings published by Rojas-Gätjens et al. who detected an abundance of methylotrophic bacteria in oil contaminated areas, including Paracoccus communis and Methylorubrum rhodesianum. Furthermore, Godini et al. isolated nine bacterial species from oil-polluted locations from Iran's Kharg Island; Methylobacterium persicinum was one of them.

In the current study, we found that methanol can be consumed during colonization by Methylorubrum in addition to using carbon sources other than methanol, such as crude oil. This is consistent with the study conducted by Hu and Lidstrom, which found that methylotrophs can consume single-carbon compounds or multiple-carbon compounds without a carbon-carbon bond.

The MxaF gene has been used to detect methylotrophs in the environment because it is highly conserved among the methylotrophs that have been studied. A pair of specific primers was chosen from the common conserved area of the MxaF gene for the identification of methylotrophic bacteria, to validate the identification of the genus Methylorubrum and to detect the MxaF gene. The size was amplified by these primers, resulting in a 550-bp amplicon. The MxaF gene was employed by Lau et al. as a biomarker for methanotrophic proteobacteria found in the Methylocystaceae and Methylococcaceae families.

In contaminated environments, petroleum hydrocarbons are digested by bacteria and used as their sole source of carbon and energy. Genetics determines a microbe's ability to incorporate molecular oxygen into a hydrocarbon and generate intermediates that enter the cell's overall energy-producing metabolic pathway. A new strain of Methylorubrum extorquens has been isolated and with the ability to exploit the aliphatic hydrocarbon compounds as a source of energy in addition to methanol, in spite of carbon-carbon bonds. This is a globally new finding. So, after 7 days of incubation, the rate of degradation was 61.14% and short-chain alkanes nC₁₂-nC₁₅ were broken down, whereas long-chain alkanes nC₁₆-nC₇ degraded at a slower rate. Moreover, these bacteria have the ability to degrade aromatic hydrocarbon compounds (unpublished data). Perhaps the reason behind that, is these bacteria have been isolated from harsh environments and exposed to many types of oil and chemical pollutants, in addition to high temperatures that may sometimes reach more than 65°C, forcing them to adapt to these conditions and exploit what is available to them from nutrients.

This degradation rate is less than the 83.8 and 81.63% at 0.5% crude oil reported for Vibrio vulnificus and Brevundimonas diminuta respectively and higher than 51.64% and 58.31% at 7 days reported for Ochrobactrum anthropic and Sphingomonas paucimobilis isolated from contaminated soils in the Khor Al-Zubair channel, southern Iraq. As a source of carbon and energy, the bacteria used the hydrocarbon substrate, as evidenced by a considerable reduction in peaks between 0 to 7 days which coincided with the exponential development of bacteria. Salam et al. also found that Methylobacterium mesophilicum strain RD1 destroyed 61.2% and 89.5% of the starting concentration of the used motor oil during 12–21 days, respectively. They explain that this is because strain RD1 contains numerous degradative genes.

Conclusion:

Due to the high oil pollution at the locations of M. extorquens isolation, these sites are of significant interest for isolating novel hydrocarbon-degrading bacteria with high catabolic abilities enhanced by living in a highly polluted site. Without no doubt, this elevated bioremediation ability is attributed to the high and continuous exposure to hydrocarbon chemicals over time. In the present study, conventional PCR was used to identify methylotrophic bacteria and the methanol dehydrogenase enzyme. The analysis of GC showed about 61.14% of crude oil was degraded, and the GC fingerprinting appears to show that C₁₂, C₁₃, C₁₄, and C₁₅ had decreased significantly. Our
findings of the ability of *M. extorquens* to successfully metabolize aliphatic compounds can be employed and genetically upregulated to bioremediate aromatic compounds in other studies. *M. extorquens'* ability to break down crude oil is a good start for genetically modifying these strains to make them twice as good using advanced methods like CRISPR Cas9.

**Authors' declaration:**
- Conflicts of Interest: None.
- We hereby confirm that all Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Basra.

**Authors’ Contribution Statement:**
The authorship have participated in different roles as follows:
- The research plan was developed by A A-T and Z A as supervisors for the PhD student A A. M. The research was conducted and written by the student, and it was reviewed and revised by her supervisors.

**Reference:**
التشخيص الجزيئي لبكتريا مxaF باستخدام PCR

كعلامة جزيئية

أورد عدوان ميكي

أقسم التطور الاحيائي، مركز علوم البحار، جامعة البصرة، العراق.

أقسم الاحياء المحجية، كلية الطب، جامعة البصرة، العراق.


الخلاصة:

واسعة الاستخدام ولها القدرة على استهلاك كربون واحد (C1) methyloptroph بكتريا الدراسة الأولى التي تبحث عن بكتريا methyloptroph الالتهابية في النسب المطلقة في العراق. تم استخدام PCR التقييدي لتنصيب مxaF Instantiate حاجي اليماربي مازلاي وحذف الهيدروجينase الذي ينجم على الالتحام المحاوري من المهاجم المسئول من الحمض النووي مxaF من البداية. استخدمت PCAة في الخ.take in التحالل من المركبات الهيدروكربونات النفطية في المختبر. في غضون 7 أيام، كشفت دراسة ما مxaF. تم اختبار قدرة M. extorquens على تحلل مركبات الهيدروكربونات النفطية في المختبر. GC للمركبات النفطية Chromatography M. extorquens المستخدمة ناقصات كبيرة في الهيدروكربونات النفطية. أوضح أيضاً وجود بكتريا مxaF في المنطقة المطلقة في البصرة. كانت الحالة المطلقة M. extorquens الغاز، ام الПিটер commitments to methylotroph MxaF عبد المحسن، وحذف الهيدروجينase الذي تبحث عن المهاجمن_Verifying the expression of MxaF gene in M. extorquens using real-time PCR and the three days older cultures were selected. PCR analysis was done to identify new strains of crude oil degrading bacteria from Kharg Island, Iran. Pet Sci Technol. 2018 Mar; 36(12): 869-874.


