Current Topics in Toxicology
Vol. 18, 2022

Original Article

# Isolation and identification of cadmium-resistant bacteria that degrade crude oil

**Efendy Chew, Wong Rui Rui, Ong Ghim Hock and Wong Kok Kee***

Faculty of Health and Life Sciences, INTI International University, Persiaran Perdana BBN, Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia.

## ABSTRACT

Three bacterial isolates were successfully screened and shown to have the ability to biodegrade crude oil and are resistant to cadmium at 0.1 mg/L. However, when the Cd concentration was increased from 0.1 mg/L to 1.0 mg/L, only one isolate, isolate C, was able grow. Gram staining results showed the isolate C to be Gram-negative. The DNA extracted from isolate C was amplified using a pair of 16s rDNA primers. The amplified DNA sequences were used to construct a phylogenetic tree. Analysis of the phylogenetic tree showed that 16s rRNA sequences from isolate C have the highest similarity to the 16s rRNA sequence of *Pseudomonas aeruginosa*.

**KEYWORDS:** molecular characterisation, phylogenetic, *Pseudomonas*, 16s rRNA.

## INTRODUCTION

Crude oil is a major source of fuel for transportation and also for generation of electricity. Because of this, frequent exportation and importation of this commodity leads to accidental spills. An estimated volume of 41,000 to 119,000 $m^3$ of crude oil spill from Exxon Valdez polluted the sea in Prince William Sound, Alaska in 1989 [1]. Crude oil is composed of various hydrocarbons that are known carcinogens and neurotoxins that have deleterious effects on the biota [2], which is why removing pollutant hydrocarbons from the environment is important. Malaysia, a major crude oil producer is susceptible to oil spills during extraction and refinery processes.

According to the United States Environmental Protection Agency (EPA), bioremediation is the process of breaking down pollutants into less-toxic or non-toxic substances using microorganisms [3]. In the case of crude oil hydrocarbons, many bacteria have been reported to degrade hydrocarbons into smaller and less harmful constituents such as carbon dioxide, water and also minerals [4]. However, the effectiveness of bacteria in degrading hydrocarbons can be compromised when there are other pollutants present in the contaminated area. One of the major co-contaminants found in the places polluted by hydrocarbons is heavy metals [5].

Heavy metals are commonly reported to co-contaminate industrial effluents and oil rigs [6]. The concentration and type of heavy metals determine the level of bacterial inhibition in the environment [7]. Of the heavy metals, cadmium (Cd) is reported to inhibit hydrocarbon-degrading enzymes of bacteria, and this will impede the biodegradation process [5]. Thus, the objective of this study is to identify bacteria that can biodegrade hydrocarbons in the presence of Cd.

## MATERIALS AND METHODS

### Source of samples

Wastewater samples (pH 6.63 ± 0.12; 30.8°C ± 1.4°C) were collected from a crude-oil refinery plant in Terengganu, Malaysia. Enrichment and

*Corresponding author: kokkee.wong@newinti.edu.my

isolation of oil-degrading cultures were performed in mineral salt medium (MSM) supplemented with crude oil and cadmium (0.1 mg/L and 10 mg/L). A single isolate obtained by the method of serial dilution of the enriched culture in MSM was plated on nutrient agar (Oxoid, UK), and incubated at 30°C [8].

**Identification of bacterial species**

Gram staining of the bacterial isolate was performed according to the method described by Cappuccino and Sherman [9]. In brief, the bacteria culture was smeared onto a microscope slide and the slide was passed a few times over a flame to fix the smear. The smear was then stained with crystal violet, followed by iodine and counterstained with safranin and left to dry. The smear was rinsed with distilled water and then viewed under a bright-field microscope at 1000x magnification.

The bacterial DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The PCR reactions were carried out in a Biometra T-Gradient thermocycler (Gottingen, Germany). Amplification was performed in a 50-µl reaction mixture using universal F:5'-AGAGTTTGATCCTGGCTCAG-3, universal R: 3'-GGTTACCTTGTTACGACTT-5' and 1 U Taq polymerase (Promega Master Mix; Wisconsin, USA). The PCR product was sequenced with an ABI PRISM 377 sequencer and compared against the National Center for Biotechnology Information (NCBI) non-redundant protein database using the Basic Local Alignment Search Tool (BLAST) [10]. Multiple sequence alignment was performed using ClustalW [11] on the European Bioinformatics Institute (EBI) server (www.ebi.ac.uk/clustalw/index.html). A phylogenetic tree was constructed using SEQBOOT (for bootstrap analysis) and NEIGHBOR (for neighbour joining analysis) programs from MEGA4 version 4.0.2 [12].

**Statistical analysis**

Data obtained from the growth of bacteria exposed to Cd was analyzed using *T*-test with 95% confidence level. Results were reported as mean ± standard deviation ($n = 3$).

## RESULTS

Table 1 shows the number of single isolates obtained using the two Cd concentrations (mg/L) tested and the Gram staining results. From the enrichment cultures, a total of three isolates were obtained in cultures added with 0.1 mg/L of Cd. All three isolates were Gram-negative. The three isolates were temporary labelled as isolate A, isolate B and isolate C. When the three isolates were incubated in MSM media using crude oil as substrate and with Cd at 10 mg/L, only two Gram-negative isolates were able to survive.

When the Cd concentration was increased to 1.0 mg/L, which exceeded the safety limit recommended by US EPA [3], both isolates A and C showed significant growth ($p<0.05$), after 24 hours of incubation (Figure 1). Isolate B, however failed to survive. Bacterial isolate C showed higher growth ($p<0.05$) compared to isolate A. This result showed that isolate C was the most Cd-resistant bacteria compared to isolate A and B. Thus, isolate C was chosen for further identification.

**Phylogenetic tree construction**

The DNA from isolate C was successfully isolated and subjected to PCR. Forward and reverse sequences of the PCR-amplified 16S rDNA obtained from isolate C were used to generate a consensus sequence using Sequencher 5.4.5 software. The consensus sequence generated was subsequently used to find similar sequences within the NCBI database library using BLAST in order to determine the identity of the bacteria. From the BLAST result, isolate C was determined to belong to the

**Table 1.** Number of bacterial isolates screened and gram staining results.

| | Cadmium (0.1 mg/L) | | Cadmium (1.0 mg/L) | |
|---|---|---|---|---|
| | **Gram positive** | **Gram negative** | **Gram positive** | **Gram negative** |
| No. of isolates | 0 | 3 | 0 | 2 |

*Pseudomonas* species. A phylogenetic tree was constructed using 16s rDNA sequences from other known Gram-negative bacteria. Analysis of the phylogenetic tree showed that the 16s rRNA sequences from isolate C have the highest similarity to the 16s rRNA sequence of *Pseudomonas aeruginos* (Figure 2).

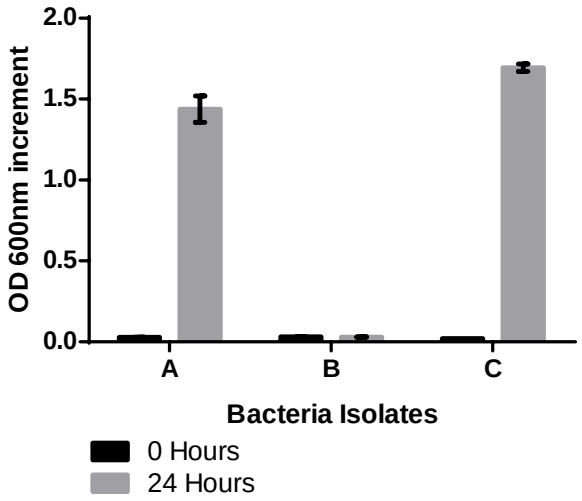


**Figure 1.** Growth of bacterial isolates exposed to 1.0 mg/L Cd.

## DISCUSSION

Initial screening of the wastewater sample with 0.1 mg/L of Cd yielded three Gram-negative isolates (isolate A, B and C) that survived in the MSM media supplemented with crude oil as substrate. However, when the Cd concentration was increased to 1.0 mg/L, only isolates A and C survived. This suggests that only isolates A and C were resistant to toxicity caused by Cd at the elevated concentration of 1.0 mg/L. Of the two isolates, isolate C showed the highest resistance to the toxic effects of Cd. Phylogenetic tree analysis identified isolate C as *Pseudomonas aeruginosa*.

The toxic effect of Cd at excess concentration that leads to cell death has been previously observed in most bacteria. At elevated levels, $Cd^{2+}$ displaced $Ca^{2+}$ in the proteins' transport system and entered the bacterial cells [13, 14, 15]. Once inside the cell, Cd can bind to various molecular binding sites of enzymes and inhibits protein function [16], or interacting with the nucleic acid and causing oxidative damage [17, 18] and breakage of DNA strands [19].

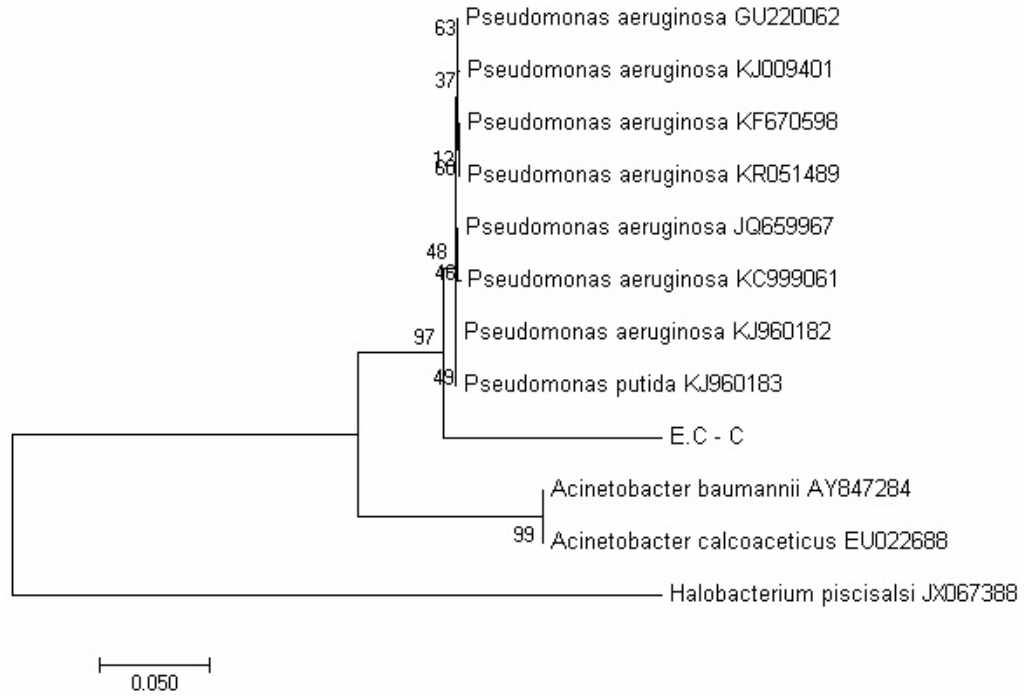


**Figure 2.** Neighbour-joining tree constructed using MEGA7. Bootstrap values in percentage are indicated at each node.

Since increasing the Cd concentration from 0.1 mg/L to 1.0 mg/L did not inhibit the growth of *P. aeruginosa*, the bacteria have certain mechanisms or alterations on the membrane structure to counter the toxicity presented by Cd.

One of the alterations on the membrane structure is the development of a thin peptidoglycan cell wall and an outer membrane composed mainly of lipopolysaccharides (LPS) and proteins [20]. The LPS consists of anionic phosphate groups [21] that can bind to $Cd^{2+}$ and prevents the Cd cations from penetrating into the bacterial cell. Furthermore, *P. aeruginosa* has been reported to secrete biosurfactants which acts as a Cd-chelating agent [5].

Another role of the LPS layer in Gram-negative bacteria is to prevent hydrocarbon molecules from entering the cell. The large amphiphilic molecules in the LPS layers can limit the transfer of hydrocarbons into the cell [20]. The LPS layer found in the Gram-negative *P. aeruginosa* in this study showed resistance to both $Cd^{2+}$ and crude oil contaminants in this study. Thus, the Gram-negative *P. aeruginosa* isolated in this study shows potential to be used in bioremediating wastewater samples that contain both crude oil and cadmium.

## CONCLUSION

Three isolates were successfully screened from the wastewater of an oil refinery in Kuala Terengganu and were found to be Cd-resistant. Of the three isolates, isolate C showed resistance to toxicity of Cd even at the relatively high concentration of 1.0 mg/L and had the highest growth ($p<0.05$). Gram staining and phylogenetic tree analysis using sequences from the amplified region of 16S rDNA identified isolate C as Gram-negative *Pseudomonas aeruginosa*. The ability of Gram-negative *Pseudomonas aeruginosa* to show resistance to Cd at elevated concentrations can be attributed to biosurfactants secreted by the Gram-negative bacteria and also the presence of a thick LPS layer enveloping the cell.

## ACKNOWLEDGEMENTS

This project was supported by the INTI International University Research Grant Scheme (INTI-FHLS-12-03-2021) and funded by the Biotechnology Programme.

## CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

## REFERENCES

1. Atlas, R. M. and Bartha, R. 1997, Microbial Ecology: Fundamentals and Applications (4th ed., 523-530). San Francisco, Calif, USA: Benjamin Cummings.
2. Das, N. and Chandran, P. 2011, Biotechnol. Res. Int., 1-13.
3. US EPA. 2016, Technologies for Cleaning Up Contaminated Sites.
4. Salleh, A. B., Ghazali, F. M., Rahman, R. N. Z. A. and Basri, M. 2003, Indian J. Biotechnol., 2(3), 411-425.
5. Wong, K. K., Quilty, B. and Surif, S. 2013, Adv. Environ. Biol., 577-586.
6. Abioye, O. P. 2011, Soil Contam., 7, 127-142.
7. Amor, L., Kennes, C. and Veiga, M. 2001, Bioresour. Technol., 78(2), 181-185.
8. Wong, K. K., Hazaimeh, H., Mutalib, S. A., Abdullah, P. S. and Surif, S. 2015, Int. J. Environ. Sci. Technol., 12(7), 2253-2262.
9. Cappuccino, J. G. and Sherman, N. 2013, Microbiology: A Laboratory Manual, 11, 1-560.
10. Altschul, S. 1997, Nucleic Acids Res., 25(17), 3389-3402.
11. Thompson, J., Higgins, D. and Gibson, T. 1994, Nucleic Acids Res., 22(22), 4673-4680.
12. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011, Mol. Biol. Evol., 28(10), 2731-2739.
13. Markovac, J. and Goldstein, G. 1988, Nature, 334(6177), 71-73.
14. Bouton, C., Frelin, L., Forde, C., Godwin, H. and Pevsner, J. 2001, J. Neurochem, 76(6), 1724-1735.
15. Hynninen, A. 2010, Zinc, cadmium and lead resistance mechanisms in bacteria and their contribution to biosensing.
16. Gaballa, A. and Helmann, J. D. 2003, Biometals, 16(4), 497-505.

17. Vallee, B. and Ulmer, D. 1972, Annu. Rev. Biochem., 41(1), 91-128.
18. Stohs, S. J. and Bagchi, D. 1995, Free Radic. Biol. Med., 18(2), 321-336.
19. Shapiro, N. and Keasling, J. D. 1995, Microbios, 86(346), 23-26.
20. Sikkema, J., De Bont, J. A. and Poolman, B. 1995, Microbiol. Rev., 59(2), 201-222.
21. Clifton, L., Skoda, M., Le Brun, A., Ciesielski, F., Kuzmenko, I., Holt, S. and Lakey, J. 2015, Langmuir, 31(1), 404-412.