Detection and sequence analysis of the *alkM* gene in *Acinetobacter baumannii* strain OS1 from oil sludge sample

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This study was conducted as an initial approach to come up with biotechnological solutions to address problems associated with oil spill and waste oil contamination. Oil sludge sample from an oil refinery in Manila (Philippines) was used to inoculate minimal broth supplemented with bunker oil in order to isolate putative bunker oil-degrading bacteria. A bacterial isolate identified as a strain of *Acinetobacter baumannii* based on 16s rRNA gene sequence exhibited significant bunker oil utilization in minimal broth and minimal agar plate with bunker oil and was designated as the oil sludge *Acinetobacter baumannii* strain OS1. Six primers *alkMF1*, *alkMF2*, *alkMF3*, *alkMR1*, *alkMR2*, *alkMR3* were designed and used in PCR in order to detect and amplify the alkane-1 monooxygenase gene (*alkM*) that codes for an enzyme involved in hydrocarbon degradation. Primer sets *alkMF1*/*alkMR1*, *alkMF2*/*alkMR2*, *alkMF1*/*alkMR2*, and *alkMF3*/*alkMR3* amplified the expected 715-bp, 807-bp, 1,340-bp, and 506-bp fragments, respectively. These primers can be used in screening procedures (including a metagenomic approach) to identify bacteria that possess *alkM*. Sequence analysis of the different amplicons resulted in the elucidation of the complete sequence of strain OS1 *alkM* gene (GenBank: accession no. KC888016). BLAST analysis revealed 99% sequence similarity of *A. baumannii* strain OS1 *alkM* gene to *alkM* reported for *A. baumannii* AB307-0294 (GenBank: accession no. CP001172). A fourteen-nucleotide variation expected to result in 3 amino acid differences was observed. The amplification and isolation of the complete *alkM* gene from strain OS1 will pave the way to the cloning and expression of the gene in an appropriate host cell.

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Received: November 18, 2013
Revised: January 7, 2014
Accepted: January 8, 2014
Published: January 18, 2014
Editor-in-charge: Eduardo A. Padlan
Reviewers: Esperanza C. Cabrera and Windell L. Rivera

INTRODUCTION

Cases of bunker oil spills over the years, including those which occurred in the Philippines in the Guimaras Strait off the coast of Guimaras and Negros Occidental on August 11, 2006 and in Sarangani Bay on August 2011 (Toms 2008), have caused serious concerns because this pollutant is chemically inert, difficult to degrade (Labinger and Bercaw 2002), and poses threat to living organisms in affected areas. Bunker oil is a type of liquid fuel obtained through fractional distillation of crude oil.

KEYWORDS

Acinetobacter sp., alkane-1-monooxogynase, *alkM*, bioremediation, bunker oil, hydrocarbon degradation

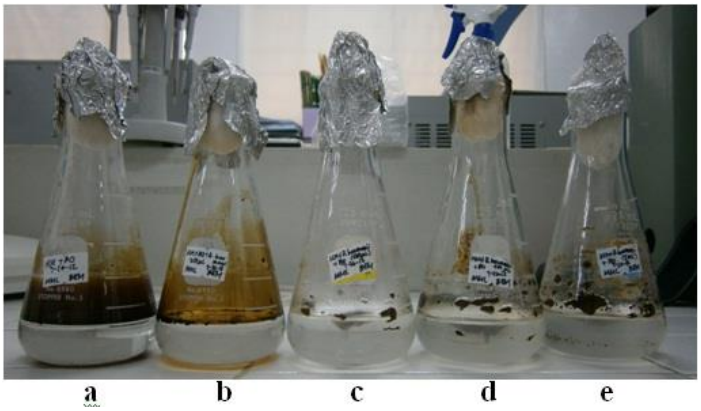

Figure 1. Growth of *Acinetobacter baumannii* strain OS1 in minimal broth with bunker oil after four days incubation at 37°C. a, minimal broth plus bunker oil; **b-e**, minimal broth with bunker oil, plus 250 µL, 500 µl, 750 µL, and 1000 µL OS1 inoculum, respectively. One µL inoculum has approximately 90 bacterial cells.

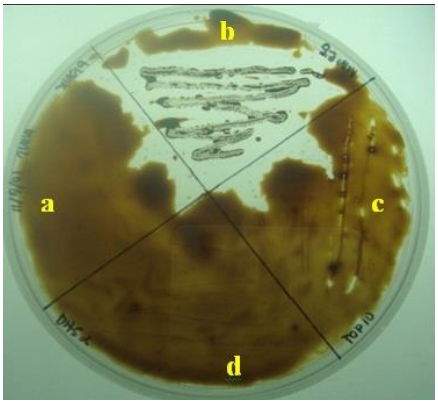

Figure 2. Bunker oil degradation of isolate *Acinetobacter baumannii* strain OS1 in minimal agar with bunker oil overlay after 3 days incubation at 37°C. a, with no inoculum; **b-d**, inoculated with isolate OS1, *E. coli* DH5α, and *E. coli* Top 10 strain, respectively.

It contains aromatic, aliphatic and napthenic hydrocarbons that typically have high carbon numbers from C_{20} to C_{70} (Concawe 2007). Alkanes (saturated hydrocarbons) are among the major components of bunker oil and fortunately there are bacteria that produce enzymes for converting this pollutant to useful form, in order to use the alkanes as carbon sources (Rojo 2009). *Acinetobacter baumannii* is among the many bacterial species reported to produce enzymes that can contribute to alkane degradation.

In this study and consistent with reports, an isolate that belongs to *A. baumannii* (referred to as strain OS1) was observed to exhibit significant bunker oil clearing in both liquid and agar media supplemented with bunker oil. This bacterium could be used directly in bioremediation where the live bacteria help convert oil pollutants to products that are less toxic, or more easily metabolized. Genes for enzymes implicated in hydrocarbon degradation from this isolate could also be amplified, cloned, and expressed for possible commercial enzyme production. The enzyme product is collected and used to address problems of waste oil contamination. Cloning and expression of genes for enzymes implicated in hydrocarbon degradation, however, is more advisable for genes isolated from pathogenic bacteria like *A. baumannii* because of health concerns in dealing with live pathogens for bioremediation.

Among the specific objectives addressed by this study are the design and use of PCR primers to detect different regions of the *A. baumannii* strain OS1 *alkM* gene, a gene for alkane-1-monooxygenase enzyme that introduces oxygen to alkanes, which is necessary for degradation. These primers are expected to be useful in routine screening for bacteria that possess an *alkM* homologue, indicating the possibility of isolating an oil-degrading strain. In this study, *alkM* amplicons (produced in PCR using *alkM*-targeted primers) were subjected to sequence analysis, generating the complete *alkM* gene sequence of *A. baumannii* strain OS1. Availability of the complete *alkM* gene from OS1 will pave the way to the cloning of this gene from this pathogenic hydrocarbon-degrading bacteria and its possible expression in a non-pathogenic host cell. Recombinant bacteria that produce this enzyme may be used in commercial production of the enzyme, or in in-situ bioremediation in combination with other bacteria that produce other enzymes for alkane degradation.

MATERIALS AND METHODS

Sample collection and selection for hydrocarbon-utilizing bacteria

Oil sludge samples were obtained from a refinery of Petron Corporation in Manila, Philippines. These samples were used as sources of inoculum to grow bacteria in 40 mL of liquid minimal media (LMM, contains 0.5 g KH_2PO_4, 2.0 g KNO_3, 1.0 g $MgSO_4$ $7H_2O$, 0.0012 g $CaCl_2$•$2H_2O$, 0.002 g $FeSO_4$•$7H_2O$ / L adjusted to pH 7.4) supplemented with 0.1% vol/vol bunker oil. The flasks were incubated at 37°C with shaking in a Vision Shaking Incubator Model VS-8580SF (Vision Scientific Co. Ltd., Bucheon, South Korea). Turbidity of the inoculated cultures were checked against two control flasks, one containing 40 ml minimal media with 50 µL bunker oil, and the other containing 40 ml minimal media (without bunker oil) with sludge sample inoculum.

Screening for putative bunker oil-degrading bacteria

Turbid minimal broth culture supplemented with bunker oil and inoculated with oil sludge samples was used as source of inoculum for streak plating in minimal agar plates with bunker oil overlay. Bacteria that grew on the solid minimal media with

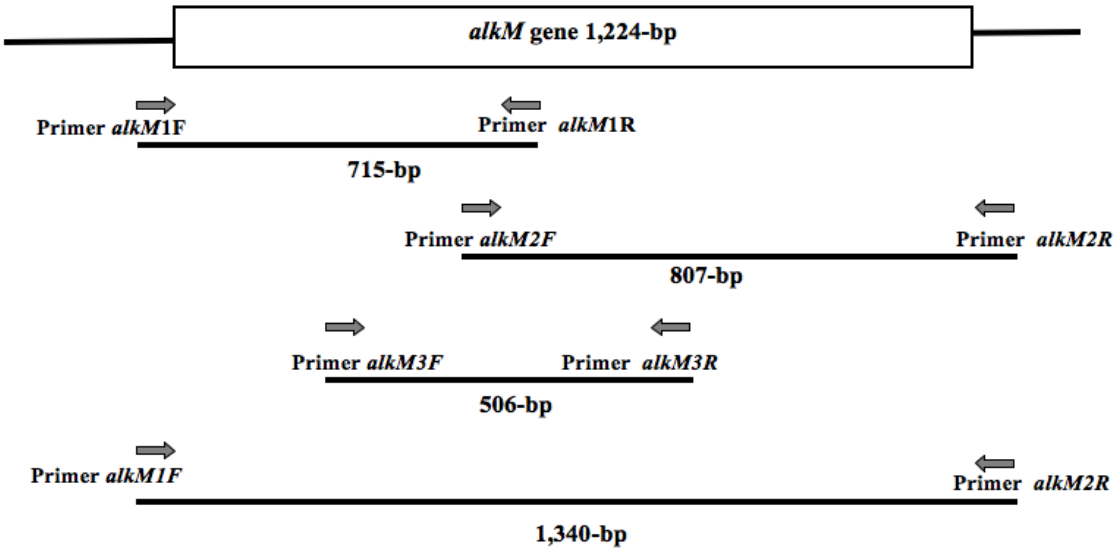

Figure 3. Schematic diagram of the relative annealing sites of *alkM*-targeted primers designed in this study.

bunker oil were streak plated to enrichment Luria-Bertani agar in order to obtain single colonies of pure bacterial isolates. Each bacterial colony was streak plated onto fresh minimal agar plates with bunker oil overlay to confirm bunker oil degradation. Control streak plates without inoculum, or with non-hydrocarbon-degrading strains of *Escherichia coli* (DH5α and Top 10) as inocula, were also prepared. Oil-degradation was confirmed by observing bunker oil clearing zone in minimal broth and minimal agar plates with bunker oil after 3 to 4 days incubation at 37°C.

DNA extraction and bacterial identification by 16s rRNA gene sequence analysis

Genomic DNA of the bacterial isolate was prepared from an overnight culture in Luria-Bertani broth using the Nucleospin Tissue kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) following manufacturer's protocol. Extracted DNA was used as template for PCR with universal primer for bacterial 16s rRNA gene (8FPL 5'-AGTTTGATCCTGGCTCAG-3' and 806R 5'-GGACTACCAGGGTATCTAAT-3'; Eden et al. 1991), and using the following PCR profiles: 5 minutes at 94ºC; 30 cycles of 1 minute at 94ºC, 1 minute at 55ºC, and 1 minute at 72ºC; 10 minutes at 72ºC, and 4ºC. The 16s rRNA gene sequences were analyzed through the Basic Local Alignment and Search Tool (BLAST; Altschul et al. 1997).

Design of PCR primers for the detection and amplification of the *alkM* gene

Three sets of *alkM* gene-targeted forward and reverse primers were designed based on reported 3' and 5' *alkM* sequences of *Acinetobacter baumannii* strain AB307-0294 (GenBank accession no. CP001172) in the database. Another set of primers was designed in order to amplify the middle fragment of OS1 *alkM* and based on 3' and 5' sequences from *A. baumannii* strain OS1 in this study. Conditions for PCR using all primer sets and DNA template from strain OS1 were optimized (Table 1) with 94°C initial denaturation (5 min) followed by 30 cycles of denaturation (94°C at 0.5 min), annealing (0.5 min at 56, 49, 59.6, and 58°C for primers pairs *alkMF1*/*alkMR1*, *alkMF2*/*alkMR2*, *alkMF1*/*alkMR2*, and *alkMF3*/*alkMR3*, respectively), and extension at 72°C (1 min). Final extension at 72°C for 10 minutes was also used. The resulting PCR amplicons were submitted to 1st BASE (BASE Life Sciences Holdings, Singapore) for sequencing, generating the complete *alkM* gene sequence of *A. baumannii* strain OS1. The strain OS1 *alkM* sequence was compared to sequences of the gene

Table 1. Optimized PCR conditions for amplification of *alkM* gene fragments using primers designed in this study.

PCR step		Primer pairs			
		alkMF1 / *alkMR1*	*alkMF2* / *alkMR2*	*alkMF1* / *alkMR2*	*alkMF3* / *alkMR3*
Initial denaturation		94°C (5 min)	94°C (5 min)	94°C (5 min)	94°C (5 min)
30 cycles	denaturation	94°C (0.5 min)	94°C (0.5 min)	94°C (0.5 min)	94°C (0.5 min)
	annealing	56°C (0.5 min)	49°C (0.5 min)	59.6°C (0.5 min)	58°C (0.5 min)
	extension	72°C (1 min)	72°C (1 min)	72°C (1 min)	72°C (1 min)
Final extension		72°C (10 min)	72°C (10 min)	72°C (10 min)	72°C (10 min)

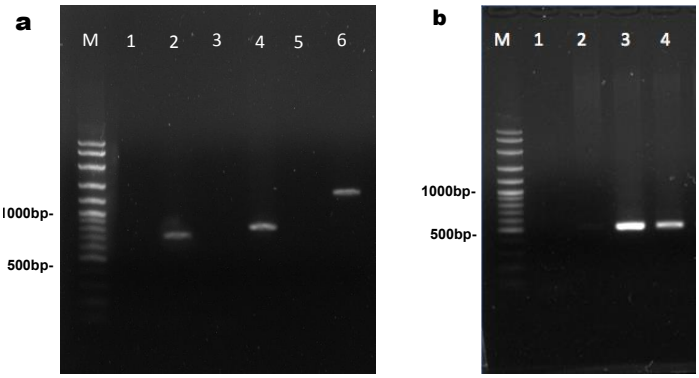

Figure 4. Amplicons of PCR using *alkM*-targeted primers and *Acinetobacter baumannii* strain OS1 DNA template. **a**, Lanes 1, 3 and 5, result of PCR with no template DNA; lanes 2,4, and 6, result of PCR using primer pairs *alkMF1*/*alkMR1*, *alkMF2*/*alkMR2*, and *alkMF1*/*alkMR2*, respectively. **b**, Lane 1 shows result of PCR with no template DNA; lanes 2,3, and 4 show results of a temperature gradient from 57, 58 to 59°C, respectively, during PCR using primer pair *alkMF3*/*alkMR3*. M is the 100-bp Vivantis DNA ladder (CA, USA).

reported in the database using Multalin (http://multalin.toulouse.inra.fr/multalin/). The amino acid sequence based on nucleotide sequence was analyzed using CLC Sequence Viewer.

Agarose gel electrophoresis and visualization of DNA

To visualize DNA after DNA extraction and PCR, the extracted genomic DNA and the PCR reaction mixture after PCR were subjected to agarose gel electrophoresis (1% agarose) for 30 minutes at 100V. Genomic DNA or PCR amplicons were visualized under UV after ethidium bromide staining and photographed using Alpha DigiDoc Pro (Alpha Innotech, USA).

RESULTS

Significant turbidity was observed in the minimal liquid medium supplemented with bunker oil after inoculation with oil sludge sample and incubation at 37°C for at least 5 days. No change in turbidity was observed in the control medium without inoculum (data not shown). Streak plating using turbid cultures as inoculum resulted in the growth of bacteria in minimal agar with bunker oil overlay. From among the pure bacterial colonies isolated, strain OS1 exhibited extensive growth and bunker oil clearing in both liquid and solid minimal media with bunker oil and was used in further experiments (Fig. 1).

Confirmation of bunker oil degradation

Bunker oil degradation by isolate OS1 was confirmed, resulting in significantly higher bunker oil clearing in minimal broth than the control without inoculum. In minimal agar plates, no bunker oil clearing zone was observed in agar plates with no inoculum, nor in the plates inoculated with non hydrocarbon-degrading control strains of *E. coli*, DH5α and *E. coli* Top 10 (Fig. 2). The bunker oil overlay was almost completely cleared in agar plates inoculated with isolate OS1.

Identification of the isolate OS1

BLAST analysis of the 16s rRNA gene sequence of isolate OS1 revealed 99% similarity with *Acinetobacter baumannii* AB307-0294 (GenBank accession no. CP001172). This identification is consistent with the observed colonial and cellular morphology of strain OS1 cells which are Gram-negative short rods, characteristics of *A. baumannii*.

Design of PCR primers and amplification of the *alkM* gene

Two forward PCR primers (*alkMF1*: 5'-CCACATGATTA-GAGGGTTCGG-3'and*alkMF2*:5'-AGCACTCATACCCCAGC-3') and two reverse primers (alkMR1: 5'-CAGCTACTCCTGAA-GATCCG-3' and *alkMR2*: 5'-GCCCATTCACCTTGTGC-3') were designed based on *alkM* sequence reported for *A. baumannii* strain AB307-0294 (GenBank accession no. CP001172). The schematic diagram (Fig. 3) illustrates the relative annealing sites of four primers and the size of expected amplicons if the *alkM* gene is present in the DNA template used. PCR using optimized conditions resulted in a 715-bp *alkM* gene fragment using primer pair *alkMF1*/*alkMR1*, 807-bp amplicon with primer pair *alkMF2*/*alkMR2* and 1,340-bp amplicon (containing the complete *alkM* gene) using primer pair *alkMF1*/*alkMR2* (Fig. 4A). When another set of primers *alkMF3*/*alkMR3* (*alkMF3*: 5'-AAC-GCTTGGAATGGACGAGT-3' *alkMR3*: 5'-CCTACAGGTTA-CAACCATTTCCG-3') designed based on OS1 sequences from the 715-bp and 807-bp amplicons was used in PCR, the expected 506-bp amplicon was produced (Fig. 4B). Sequence analysis of the 506-bp amplicon confirmed nucleotide sequences in the middle region of the gene. Four primer pairs from this study could be used in detecting the presence of *alkM* gene homologues from other bacteria or samples collected for metagenomics studies. Amplicon sequencing revealed that the target gene fragments were amplified.

Complete *alkM* gene sequences from *Acinetobacter baumannii* strain OS1

The complete *alkM* gene sequence of *A. baumannii* strain OS1 was obtained from the sequence data of amplicons from four primer pairs (GenBank accession no. KC888016). BLAST and Multalin programs revealed 99% sequence similarity of strain OS1 *alkM* gene fragments with the alkane-1-monooxygenase gene of *Acinetobacter baumannii* strain ATCC 17978. Nucleotide variation was observed in *alkM* between the reference strain *Acinetobacter baumannii* AB307-0294 (GenBank accession no. CP001172) and strain OS1, with 14 nucleotide difference in different positions within the gene (Fig. 5). Corresponding

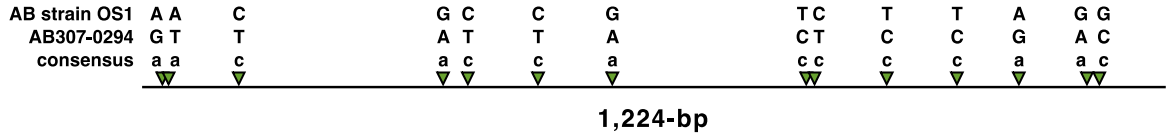

Figure 5. *AlkM* sequence variation of *Acinetobacter baumannii* strain OS1 with a reference strain *Acinetobacter baumannii* AB307-0294. Green arrows point to the 14 nucleotide variations between strain OS1 and the reference strain.

Figure 6. Relative positions of expected amino acid sequence variation in AlkM protein of strain OS1 versus the reference strain *Acinetobacter baumannii* AB307-0294. Green arrows highlight 3 amino acid variations at positions 23, 256, and 401 with a proline to arginine, glycine to aspartic acid and serine to threonine in OS1 versus the reference, respectively.

amino acid sequences in these strains of *A. baumannii* (based on nucleotide variations) are expected to result in only 3 amino acid changes (Fig. 6) at positions 23 (proline in OS1 and arginine in the reference strain), 256 (glycine in OS1 and aspartic acid in the reference strain), and 401 (serine in OS1 and threonine in the reference strain).

DISCUSSION

The study used minimal broth with bunker oil as a selective medium for the isolation of hydrocarbon-utilizing bacteria, since the liquid medium contains only high carbon numbers (from C_{20} to C_{70}) as sole carbon source for microorganisms. Increased turbidity of the culture medium after inoculation with oil sludge sample from an oil refinery is interpreted to indicate the ability of bacteria from the oil sludge to synthesize enzymes that could convert the hydrocarbon to products that could serve as carbon source and support bacterial growth.

This paper reports for the first time, a test for bacterial bunker oil degradation using a minimal agar plate with bunker oil overlay. The simple protocol was able to clearly distinguish bunker oil degrading bacteria from non-degrading control strains of *Escherichia coli*, without the need to perform expensive procedures such as gas chromatography. This simple and cheap protocol was valuable in this study and is expected to be useful in future screening activities for waste oil-degrading bacteria.

The bacterium that exhibited visually evident bunker oil utilization in a minimal agar plate with bunker oil overlay was identified to be a strain of *Acinetobacter baumannii* referred to as strain OS1. This identification is consistent with previous reports that this species of bacterium exhibits the capability to degrade alkanes, which are major components of bunker oil (Funhoff et al. 2006). Because *A. baumannii* is a human pathogen that exhibits antibiotic multi-resistance (Cunha 2013), and is associated with infections including pneumonia, urinary tract infection, and wound infection (Maragakis and Peri 2008), the use of this species in in-situ bioremediation to address oil pollution is not advisable. The alternative approach is to isolate genes for enzymes involved in oil degradation from strains that exhibit efficient degradation. Genes will be cloned into expression vectors and consequently, gene constructs will be introduced into non-pathogenic expression host bacteria. It is therefore necessary to conduct initial experiments to detect the presence of genes implicated in oil degradation in the putative oil degrading strains. One of the genes of interest is *alkM* for alkane-1-monooxygenase, an enzyme needed to initiate biodegradation by introducing oxygen to the alkane substrate.

In this study, four sets of PCR primers targeting different fragments of the alkane-1-monoxygenase gene were designed and used in PCR with *A. baumannii* OS1 DNA template. Four sets of PCR primers targeting the 5', middle, 3' and complete regions of *alkM* are reported (Table 1) and will be valuable in future screening experiments for bacteria that contain an *alkM* gene homologue. These primers will also be valuable in detecting the presence of *alkM*-containing bacteria in samples collected for metagenomic bacterial identification and enumeration.

Amplification of the *alkM* gene fragments in *A. baumannii* strain OS1 paved the way to obtaining the complete sequence of the alkane-1-monooxygenase gene in this bacterium (Genbank: accession no. KC888016). Sequence analysis of the amplicons from PCR using the *alkM* gene-targeted primer pairs revealed 99% sequence similarity with the alkane-1-monooxygenase gene of *Acinetobacter baumannii* strain AB307-0294 (GenBank accession no. CP001172). The complete *alkM* gene of strain OS1 consists of 1,224 nucleotides that correspond to 407 amino acids

if translated. Alignment using Multalin program revealed that 14 nucleotide (Fig. 5) and 3 amino acid (Fig. 6) variations exist between the reference *A. baumannii* strain AB307-0294 and the strain OS1 in this study. The effect of these amino acid changes in the enzyme structure and function is not known at this time and could be a subject of further studies.

CONCLUSION

Waste oil sludge proved to be a good source of oil-degrading bacteria that could be used to address problems of waste oil-contaminated environments. A putative oil-degrading strain of *Acinetobacter baumannii* was isolated using minimal broth supplemented with bunker oil. Approximate degree of bunker oil utilization of a bacterial isolate could be evaluated by observing bunker oil clearing zone in a minimal agar plate with bunker oil overlay as evidenced by results of this study. Enzyme gene-targeted PCR primers are not only useful for generating complete gene sequences but could also be valuable for screening of putative oil-degrading bacteria. Nucleotide and corresponding amino acid variations were observed between *alkM* of *A. baumannii* strain OS1 and *A. baumannii* AB307-0294. To determine if these variations affected enzyme structure and function, further studies are suggested.

ACKNOWLEDGEMENTS

The authors would like to thank the National Institute of Molecular Biology and Biotechnology for funding the study, Ms. Jalea L. Coralde and Ms. Fatima Mae M. Hermo for their assistance in performing experiments for bacterial isolation and gene-targeted PCR, and Petron, Philippines for providing the oil sludge sample.

NO CONFLICT OF INTEREST STATEMENT

There is no conflict of interest among authors, institutions, and individuals mentioned above in the conduct of this study and the preparation and submission of this manuscript.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Ms. A. Mittsu G. Sarmago conducted experiments under the guidance of Dr. Cynthia T. Hedreyda and assisted in writing the manuscript. Dr. Hedreyda conceptualized the study and wrote the manuscript.

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