

# Two strains of *Gordonia terrae* isolated from used engine oil-contaminated soil utilize short- to long-chain n-alkanes

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This study aimed to isolate bacteria that are able to degrade a wide range of hydrocarbon (HC) compounds. Samples were taken from soil contaminated with engine oil using mineral salt medium (MSM). Taxonomic classification of two isolates, using the Biolog Microbial Identification System, revealed that they are actinomycetes belonging to the suborder Corynebacterineae. Nucleotide sequences from amplified 16S ribosomal RNA (rRNA) gene and phylogenetic analysis showed that the isolates (designated as G1 and G2) are two different strains of *Gordonia terrae*. The isolates are capable of degrading HCs specifically n-alkanes as demonstrated by HC utilization fingerprints and gas chromatography-mass spectrometry (GC-

MS) experiments. Their capability to grow on a wide range of saturated HCs is attributed to the presence of the alkane hydroxylase (*alkB*) gene. The isolation of these bacteria has added the limited number of species that are able to degrade short- to long-chain saturated alkanes.

## KEYWORDS

n-alkanes; hydroxylase (*alk*) gene; engine-oil contaminated soil; *Gordonia terrae*; hydrocarbon utilization; polyaromatic hydrocarbon

## INTRODUCTION

The increasing environmental threats brought about by the utilization of fossil fuel or petroleum oil albeit beneficial have led researchers to come up with various methods of control, one of which is bioremediation enhanced by bioaugmentation and biostimulation (Ueno et al. 2007, Mishra et al. 2001, Kanaly et al. 1997). Bioremediation is the management or utilization of organisms such as microbial community (microbial remediation) to remove, detoxify, or mineralize organic contaminants in the ecosystem (Atlas and Bartha 1998). Microbial remediation occurs naturally through the action of microbial enzymes (Churchill et al. 1999). It can be enhanced through the addition

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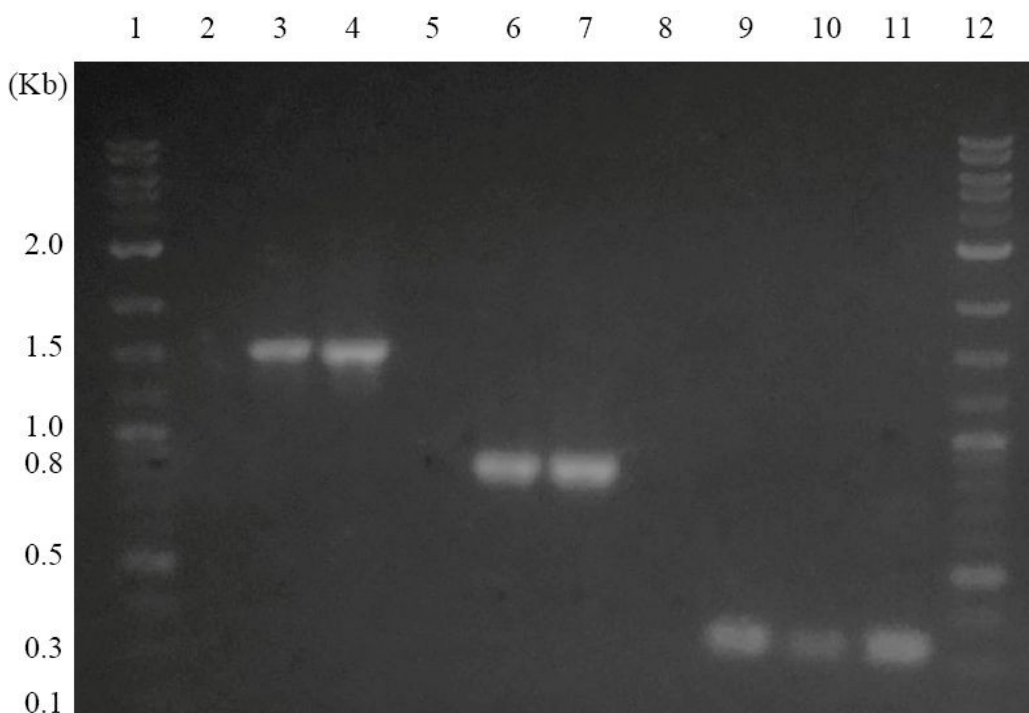
of factors that increase the activity of the autochthonous/indigenous microorganisms (Atlas and Bartha 1998, Leahy and Colwell 1990) or by in vitro enrichment of these microorganisms, and subsequent reintroduction to the contaminated area (Mishra et al. 2001, Hinchee et al. 1994). Such enhancements are known as biostimulation or environmental modification. In addition, application to the soil of extrinsic bacterial strains with biodegrading abilities (Leahy and Colwell 1990) along with indigenous bacteria or nutrients would improve the bioremediation process (Ueno et al. 2006, Atlas and Bartha 1998). This, on the other hand, is microbial seeding or bioaugmentation (Leahy and Colwell 1990).

Numerous studies on bioremediation have established a long list of aerobic and anaerobic microorganisms that have biodegradation potentials. Among these microorganisms, the bacteria and fungi are involved primarily in such processes (Mishra et al. 2001, Leahy and Cowell 1990). Other types of microorganisms, however, have limited available evidences suggesting their role in the degradation of hydrocarbons (HC) in the environment.

The HC-degrading potential of these microorganisms is attributed mainly to the enzymes encoded by HC-degrading genes (Van Hamme et al. 2003, Iwabuchi et al. 2002). The most studied genes are the *alk* gene found in the OCT plasmid of *Pseudomonas oleovorans* GPo1 and the *nah* metabolic gene in the NAH7 naphthalene catabolic plasmid of *P. putida* strain G7 (Van Hamme et al. 2003). Other plasmid-encoded catabolic pathways have also been partially characterized (Van Hamme et al. 2003, Hinchee et al. 1994, Takizawa et al. 1994). Studies have also shown the high diversity among HC-degrading genes (Smits et al. 2002, Vomberg and Klinner 2000) and their functionality in the degradation of specific HC compounds (Heiss-Blanquet et al. 2005). Hence, degenerate primer pairs are used in the detection of these genes (Quatrini et al. 2008, Heiss-Blanquet et al. 2005, Margesin et al. 2003) or primers designed from

the gene sequence of an individual species that are able to detect the same genes in other genera (Duarte et al. 2001, Vomberg and Klinner 2000).

Researchers have also found that some microorganisms can degrade both alkanes and aromatic compounds and possess both the alkane and polyaromatic hydrocarbon (PAH) catabolic pathways (Van Hamme et al. 2003). Some of these microorganisms are *Pseudomonas* sp. strains B17 and B18 which degrade C<sub>5</sub> to C<sub>12</sub> n-alkanes, toluene and naphthalene (Whyte et al. 1997) and *Mycobacterium* sp. strain CH1 which is capable of mineralizing phenanthrene, pyrene, fluoranthene as well as short- to long-chain aliphatic compounds, dodecane (C<sub>12</sub>) to octacosane (C<sub>28</sub>) (Churchill et al. 1999). Although most microorganisms degrade only a specific length of aliphatic HCs, such as *P. aeruginosa* PAO 1 and *Acinetobacter calcoaceticus* DSM 30007 which degrade only medium-length HC compounds (Vomberg and Klinner 2000), there are several species of bacteria that are able to degrade short- to long-chain n-alkanes. Some of these species are *Nocardia cyriacigeorgica* (*Nocardia* sp. SoB), *Rhodococcus ruber* (*Rhodococcus* sp. SoD) and *Gordonia amicalis* (*Gordonia* sp. SoCg) (Quatrini et al. 2008). The existence of these biodegrading microorganisms



**Figure 1.** 1.5% agarose gel electrophoresis pattern of amplified 16s rRNA and *alkB* genes of the two isolates and a control organism. Lanes 1 and 12, DNA ladder; 2, 5 and 8, negative control; 3 and 6, 16S rRNA gene of isolate G1; 4 and 7, 16S rRNA gene of isolate G2; and 9 to 11, *alkB* genes of *Mycobacterium ratisbonense*, isolates G1 and G2.

could significantly improve the bioremediation of polluted areas.

In this study, biodegrading indigenous bacteria from used engine oil-contaminated soil were isolated and characterized. Their ability to utilize crude oil, diesel oil, petrol oil, and different HC compounds as the sole source of carbon was assessed. The type of HC fraction being degraded from crude oil was determined. The gene responsible for the HC degrading potential of the isolates was also detected and compared with known biodegraders.

## MATERIALS AND METHODS

### Collection of soil samples

The collection site is located in Magalang, in the western portion of Pampanga, Philippines. It is a motor pool with an area of approximately 40 m<sup>2</sup>. Twenty-five percent or approximately 10m<sup>2</sup> of the site is contaminated with used engine oil. Three soil samples approximately 50 grams each were randomly collected from the surface to 5 centimeters deep in the highly contaminated area. Samples were kept in a sterile container at room temperature and analyzed within 36 hours from collection.

### Culture media

Mineral salt medium (MSM) was used in the isolation of HC-degrading bacteria from the soil samples. Composition of the medium includes 4.0 g of Na<sub>2</sub>HPO<sub>4</sub>, 1.5 g of KH<sub>2</sub>PO<sub>4</sub>, 1.0 g of NH<sub>4</sub>Cl, 0.2 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.0 mg of ferric ammonium citrate and 1.0 mL of modified Hoagland trace elements for every 1.0 liter aqueous solution (Atlas 1993). Modified Hoagland in 3.6 liter solution contains 11.0 g of H<sub>3</sub>BO<sub>3</sub>, 7.0 g of MnCl<sub>2</sub>·4H<sub>2</sub>O, 1.0 g each of AlCl<sub>3</sub>, CoCl<sub>2</sub>, CuCl<sub>2</sub>, KI, NiCl<sub>2</sub>, ZnCl<sub>2</sub>, 0.5 g each of BaCl<sub>2</sub>, KBr, LiCl, Na<sub>2</sub>MoO<sub>4</sub>, FeCl<sub>3</sub>, SeCl<sub>4</sub>, SnCl<sub>2</sub>·2H<sub>2</sub>O and 0.1 g of NaSO<sub>3</sub>·H<sub>2</sub>O. The pH of the medium is 7.0 ± 0.2, maintained at 25 °C (Ueno et al. 2007, Atlas 1993). MSM broth was used for enrichment.

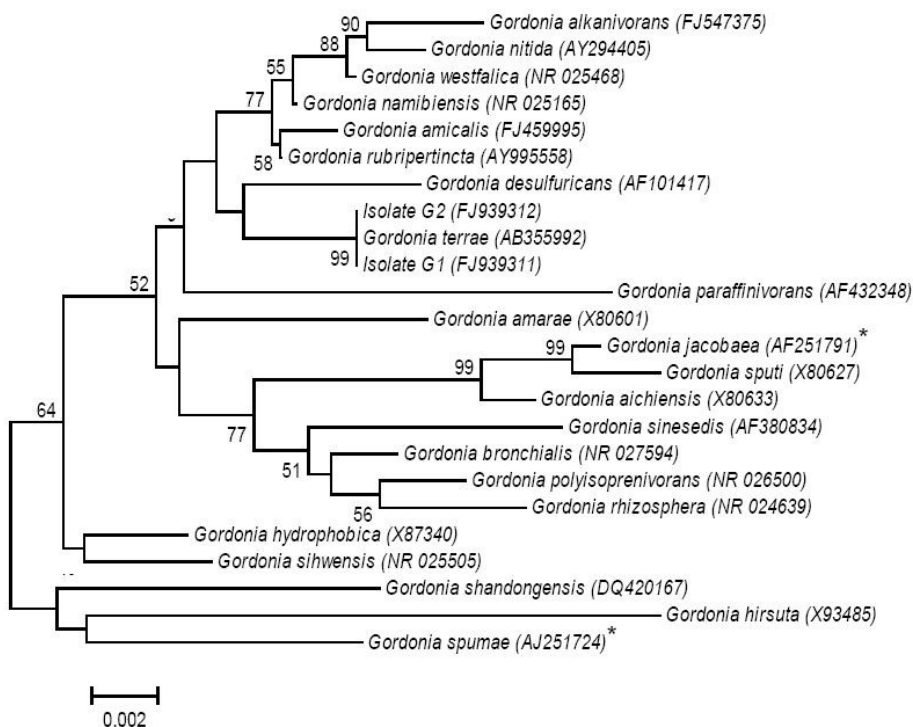
Three formulations of solidified MSM agar were used for isolation, purification and assessment of engine-oil utilization: MSM plus engine oil overlay (MSM<sub>1</sub>); MSM with engine oil as the substitute carbon and energy sources for ferric ammonium citrate (MSM<sub>2</sub>); and MSM without ferric ammonium citrate or any source of carbon (MSM-C).

### Isolation and purification of culture

Soil sample was inoculated into MSM and incubated at room temperature for one week using a rotary shaker. A loopful from the MSM culture was transferred onto MSM<sub>1</sub> agar plates and incubated at 32°C. Observation of colonies was done five days after incubation. Growth curve analysis of the microorganisms was also performed to determine their logarithmic phase of growth. with varying morphology were selected and subcultured for purification using MSM<sub>1</sub> plates.

### Determination of putative biodegrading bacteria

The pure cultures were evaluated for the ability to grow in the presence of used engine oil. Isolates were grown on MSM<sub>2</sub> and MSM-C agar plates and incubated at 32°C for five days. This was performed in three subsequent trials or subculture. Five days of incubation revealed sufficient growth and day 7 was the best time to subculture as indicated by initial growth curve analysis. Subsequent subculturing of 7-day cultures prevented microorganisms from utilizing left-over engine oil since the cells started to undergo stationary phase



**Figure 2.** Neighbor-joining tree of the 16S rDNA sequences showing the position of isolates G1 and G2 in the family *Gordoniaceae*. Bootstrap values are shown on branch points. Bar indicates nucleotide substitutions per position. [\*not validly described species as stated by Arenskötter and his colleagues (2004)].

and the amount of nutrients began to decline on day 7. Growth until the third trial on MSM<sub>2</sub> but inability to grow on MSM-C indicated their capability to utilize engine oil as the sole source of carbon and energy.

### Identification and characterization of the putative biodegrading indigenous bacteria

The putative biodegrading isolates were phenotypically characterized using biochemical procedures. In addition, Biolog Microbial Identification/ Characterization System - Microlog<sup>TM</sup> (Biolog Inc., Hayward, California) was used for the initial identification of the isolates.

Identification was confirmed using molecular techniques. The genomic DNA was extracted using organic solvent extraction technique (Persing et al. 1993) and the 16S rRNA genes of genomic DNA were amplified by polymerase chain reaction (PCR) using primers 27F and 1541R (Ueno et al. 2007). Internal primers named 244IP-F (5'-CTTGTGGTGGGGTAATG -3') and 1086IP-R (5'-TCGTTGCGGGACTTAACC -3') which amplify the 16S rRNA gene from nucleotide 244 to 1086 were designed and used to determine the complete sequence of the 1.5-kb PCR product. PCR was performed according to the standard conditions as indicated in the study of Ueno et al. (2007). PCR products were determined by agarose gel electrophoresis and visualized using a UV transilluminator after staining with ethidium bromide.

PCR products were sent to Macrogen, Inc. (Seoul, Korea) for purification and sequence analysis. DNA sequences were subjected to homology analysis to database sequences found in the NCBI GenBank online services. The full sequences of the 16S rRNA genes of isolates G1 and G2 were submitted to GenBank under accession numbers, FJ939311 and FJ393312, respectively. Confirmation of the identities was performed through phylogenetic analyses of the obtained sequences and the 16S rRNA gene sequences of all known *Gordonia* species using 1000 datasets of neighbor-joining, maximum parsimony, maximum likelihood and minimum evolution trees.

Results of the biochemical tests, 16S rRNA gene amplification and phylogenetic analysis of all known *Gordonia* species were compared to identify the two isolates. Quality

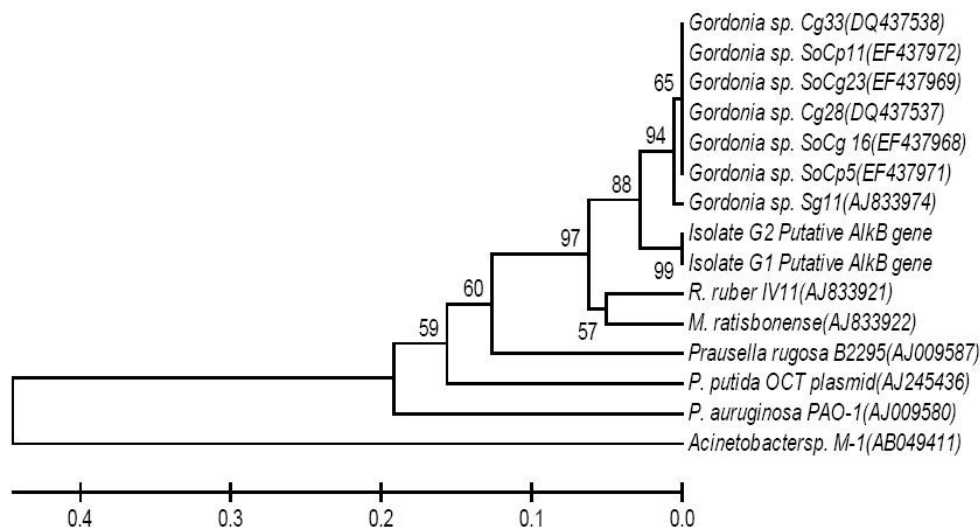
assurance was performed in all tests using *Escherichia coli* as the control organism.

### HC utilization fingerprints and degradation abilities

Candidate isolates were inoculated onto MSM-C plates supplemented with benzene, toluene, dichloromethane, carbon tetrachloride, n-hexane, cyclohexane, n-heptane, n-octane, n-decane, n-hexadecane, diesel fuel oil, unleaded petrol, n-eicosane (C<sub>20</sub>), n-octacosane (C<sub>28</sub>), anthracene, naphthalene, fluoranthene, pyrene, or dibenzothiophenes (DBT) (Quatrini et al. 2008, Duarte et al. 2001, Churchill et al. 1999, Kanaly et al. 1997). Growth was evaluated after one week of incubation at 32°C by comparison with positive controls on the maintenance medium (MSM<sub>1</sub>) and negative controls, same sets of MSM plates without inoculum (Control A) and inoculated with *E. coli* (Control B).

### Total petroleum hydrocarbon (TPH) and HC fraction measurement by gas chromatography coupled with mass spectroscopy (GC-MS)

Two hundred fifty (250) ml of MSM-C broth in Erlenmeyer flask was added with 2.5 ml membrane-filtered crude oil (Meintanis et al. 2006, Ueno et al. 2006, Iwabuchi et al. 2002) inoculated with the isolates (Treatments G1 and G2) and incubated at room temperature for 8 days with continuous aeration (Meintanis et al. 2006). Negative control was left uninoculated and incubated under the same conditions to



**Figure 3.** Neighbor-joining tree based on *alkB* genes of *Gordonia* species and other genera. *Acinetobacter* sp. M-1 was used as an outgroup. Bootstrap values are indicated at the nodes/ branches. Bar indicates substitutions per nucleotide position (*alkB* gene has not been studied in *Gordonia terrae*).

monitor abiotic losses of HC.

Total petroleum hydrocarbon (TPH) was extracted from the 8-day culture with one volume of 10% dichloromethane in n-hexane using a separatory funnel, likewise removing residual cells (Meintanis et al. 2006, De Domenico et al. 2004, Iwabuchi et al. 2002, Mishra et al. 2001, Whyte et al. 1997, US EPA 1996). Extraction procedure was repeated (Quatrini et al. 2008, De Domenico et al. 2004) three times until the crude oil was completely dissolved and extracted (Iwabuchi et al. 2002). All extracts were pooled making a volume of approximately 75ml. The extracts were subsequently filtered using a glass funnel with glass wool loaded with anhydrous sodium sulfate (Na<sub>2</sub>SO<sub>4</sub>). The filtrates were then added with the solvent to fill the remaining volume to 100ml. One milliliter of the extract was

passed through 20 grams of alumina equilibrated in hexane in a glass column. The saturated HCs were eluted with 50 ml hexane while the aromatic fraction in 50 ml of 25% dichloromethane in hexane. The two fractions were dried to 1 ml in a rotary evaporator and were transferred into calibrated centrifuge tubes. The internal standards (chlorooctadecane for the TPH fraction and mixture of phenanthrene-d<sub>10</sub>, pyrene- d<sub>10</sub>, and chrysene-d<sub>12</sub> for PAH fractions) were added to the extracts prior to GC-MS determination (De Domenico et al. 2004, Iwabuchi et al. 2002, Mishra et al. 2001, US EPA 1996).

Gas chromatography analyses were carried out in a 30-cm-long and 0.25 mm wide DB5 column (0.25 um-film thick) with the following GC conditions: injector temperature, 280°C; oven

temperature, kept at 55°C for 2 minutes and then increased to 300°C at a rate of 20°C/min until it reached 130°C, then reprogrammed to rise to 240°C at 5°C/min increase rate and maintained for 20 minutes and finally to 300°C at a rate of 10°C/min and a final hold of 15 minutes. The mass spectrometry analyses were performed with an ion source temperature of 200°C and interface temperature of 280°C (US EPA 1996).

The negative control or uninoculated media was also analyzed and served as the basal point for the determination of degradation and to monitor biotic and abiotic losses of HC (et al. 1994).

#### Detection of the HC-degrading gene, *alkB*

The detection of alkane hydroxylase (*alk*) gene fragment for utilization of a wide range of n-from C<sub>6</sub> to above C<sub>30</sub> was performed using primers named as Rhose and Rhoas (Heiss-Blanquet et al. 2005). The 343-bp gene fragment was amplified at annealing temperature of 52°. This primer pair was designed from the

**Table 1.** HC utilization fingerprints of the isolates on Mineral Salts Medium (MSM) supplemented with different carbon sources after the third subsequent subculture/trial.

Substrate	Ctrl A	Ctrl B	G1	G2
Diesel	(-)	(-)	+	+
Biodiesel	(-)	(-)	+	+
Premium	(-)	(-)	(-)	(-)
Benzene	(-)	(-)	(-)	(-)
Toluene	(-)	(-)	(-)	(-)
DCM	(-)	(-)	(-)	(-)
CCl <sub>4</sub>	(-)	(-)	(-)	(-)
Hexane	(-)	(-)	+	+
Cyclohexane	(-)	(-)	+	+
Heptane	(-)	(-)	+	+
Octane	(-)	(-)	+	+
Decane	(-)	(-)	+	+
Hexadecane	(-)	(-)	+	+
Eicosane	(-)	(-)	+	+
Octacosane	(-)	(-)	+	+
Anthracene	(-)	(-)	(-)	P
Naphthalene	(-)	(-)	P	P
Fluoranthene	(-)	(-)	P	P
Pyrene	(-)	(-)	(-)	(-)
DBT	(-)	(-)	P	(-)
Engine Oil	(-)	(-)	+	+
Crude oil	(-)	(-)	+	+
Acetone	(-)	(-)	P	P
MSM-C	(-)	(-)	P	P

Ctrl A: medium without inoculum

Ctrl B: negative control, *Escherichia coli*

G1 and G2: treatments inoculated with isolates G1 and G2

(-): Negative growth

+ : Positive growth -- growth and colony size comparable to those grown on nutrient agar medium

P : Poor growth -- decreased number of colonies on streak lines and small colony size

sequences of 40 bacterial species in a dendrogram which were grouped according to the chain length of alkanes they utilized (Heiss-Blanquet et al. 2005). Control bacteria namely *Mycobacterium ratisbonense*, used to assess the efficiency of the above-mentioned primers. This was provided by Dr. Senta Heiss-Blanquet of the Institut Francais du Pétrole, Rueil-Malmaison in France.

Verification of the existence of the gene was performed through the alignment of the resultant *alkB* gene sequences of isolates G1 and G2 with the corresponding *alkB* sequences of several *Gordonia* strains and other genera retrieved from EMBL and GenBank using Clustal W multiple alignment of the Bioedit program (Hall 1999). Neighbor-joining trees were constructed using MEGA3 program (Kumar et al. 2004). Confidence levels were estimated using bootstrap analysis of 1000 datasets to determine the stability of every branching point.

## RESULTS

### Isolation and identification of putative biodegrading indigenous bacteria from used engine oil-contaminated soil

The soil samples when inoculated on MSM<sub>1</sub> plates using streak plate technique, yielded 23 isolated colonies. These were selected, subcultured, purified and maintained on the same culture medium. Upon inoculation in MSM<sub>2</sub> plates for three consecutive trials, only seven isolates were able to survive and showed excellent growth. These were characterized and found to belong to two distinct phenotypes. The first type of isolate (designated as G1) showed orange, glistening, circular, dome-shaped colonies with regular margins and a diameter ranging from 1 mm to 3 mm on nutrient agar. The second type of isolate (designated as G2) showed colonies with sizes from 4 mm to 7 mm that were orange, dry, rough and circular with crenated/irregular margins.

Two representative isolates from each type were randomly selected and subjected to Gram stain. They were characterized as Gram variable bacteria. They

produced very short branching hyphae which disintegrated into rod- and cocci-like elements. Hence, they appeared as cocci in moderate pairs and clusters, and their rods were like short chains. Both were catalase-positive and oxidase-negative. These two representative isolates were used in the succeeding experiments and tests.

The two isolates were initially classified using the Biolog Microlog Identification System-GP2. They were found to belong to the suborder Corynebacterineae with carbon profiles similar to genus *Gordonia* or *Rhodococcus*. The universal primers amplified the 16S rDNA of the two isolates and generated approximately 1.5 kb gene segments (Figure 1, lanes 3 and 4). The internal primers, on the other hand, amplified approximately 850 bp of the gene (Figure 1, lanes 6 and 7). The 16S rRNA gene sequences analyzed by MacroGen, Inc. were compared with database sequences and were 99% identical to several strains of *Gordonia terrae* and unnamed *Gordonia* spp. with 97 to 99% query coverage.

**Table 2.** Saturated HC average peak area ration (n=2; mean of two trials) of duplicated treatments after growth of the organisms for 8 days.

HC	Retention times, minutes	Ctrl	G1	Percent Decrease	G2	Percent Decrease
C <sub>13</sub>	15.84	1.520	0.609*	59.93%	0.630*	58.55%
C <sub>14</sub>	17.45	0.915	0.000*	100.00%	0.000*	100.00%
C <sub>15</sub>	18.85	1.420	0.099*	93.03%	0.139*	90.21%
C <sub>16</sub>	20.11	1.080	0.070*	93.51%	0.077*	92.87%
C <sub>17</sub>	21.28	0.842	0.037*	95.60%	0.029*	96.56%
C <sub>18</sub>	22.46	0.247	0.000*	100.00%	0.278 <sup>ns</sup>	0.00%
C <sub>19</sub>	23.25	0.579	0.044*	92.40%	0.055*	90.50%
C <sub>20</sub>	23.99	0.500	0.019*	96.20%	0.040*	92.00%
C <sub>21</sub>	24.63	0.444	0.428 <sup>ns</sup>	3.60%	0.006*	98.65%
C <sub>22</sub>	25.2	0.378	0.019*	94.97%	0.013*	96.56%
C <sub>23</sub>	25.71	0.349	0.016*	95.42%	0.020*	94.27%
C <sub>24</sub>	26.18	0.344	0.021*	93.90%	0.000*	100.00%
C <sub>25</sub>	26.62	0.298	0.032*	89.26%	0.037*	87.58%
C <sub>26</sub>	27.03	0.293	0.040*	86.35%	0.047*	83.96%
C <sub>27</sub>	27.44	0.123	0.044*	64.23%	0.033*	73.17%
C <sub>28</sub>	27.89	0.199	0.010*	94.97%	0.010*	94.97%
C <sub>29</sub>	28.38	0.189	0.031*	83.60%	0.019*	89.95%
C <sub>30</sub>	28.94	0.237	0.040*	83.12%	0.010*	95.78%

Ctrl (Control): MSM with 1% crude oil and no inoculum

G1 and G2: inoculated with isolates G1 and G2

\* The mean difference from Control is significant at 0.05 level.

<sup>ns</sup> The mean difference from Control is not significant at 0.05 level.

The 16S rDNA sequence of isolate G1 is 1418 nucleotide long while isolate G2 contains 1421 nucleotides after the primer sequence has been removed. Both sequences contained all signature nucleotides for the family Gordoniaceae (Stackebrandt et al. 1988). However, the isolates had a sequence difference of four nucleotide substitutions and seven nucleotide insertions/deletions identifying them as two distinct strains. Phylogenetic analyses of the 16S rRNA gene sequences of the two isolates and all the species of Gordoniaceae family showed that the two isolates formed a monophyletic clade with *G. terrae* with a bootstrap value of 99%. A neighbor-joining tree (Figure 2) representing the different phylogenetic trees demonstrates the described phylogenetic clustering.

### HC utilization fingerprints

Different HC substrates were added to MSM-C to determine the HC utilization fingerprints of the two isolates (Table 1). Both bacteria exhibited excellent growth on short- to long-chain n-alkanes, cyclohexane, crude oil, engine oil and diesel. These were exhibited by colonies spreading on the streak lines and isolated colonies of sizes comparable to those grown on nutrient agar medium. However, they grew poorly on some PAH compounds such as naphthalene and fluoranthene and showed no growth on other HC compounds. In addition, isolate G2 was found to grow poorly on anthracene while isolate G1 on DBT. Unexpectedly, the two isolates grew on MSM without any source of carbon (MSM-C) and on acetone (solvent for the HC substrates) although growth was poor. The control microorganism, *E. coli* (Control B), was unable to proliferate on any media. Uninoculated media (Control A) were also incubated at the same condition and microbial contamination from the added substrate was not observed.

### TPH and HC fraction measurement by GC-MS

Crude oil was separated from the MSM-C broth inoculated with the isolated bacteria (Treatment G1 and G2) and was prepared for GC-MS analyses. Average peak area ratio based on specific retention times (normalized using chlorooctadecane internal standard) from the GC-MS analyses detected saturated alkanes from C<sub>13</sub> to C<sub>30</sub> and concentrations of PAHs (internal standard: mixture of phenanthrene-d<sub>10</sub>, pyrene-d<sub>10</sub>, and chrysene-d<sub>12</sub> for PAH fractions) in nanograms per milliliter of extract (ng/ml), namely acenaphthylene, fluorene, phenanthrene, fluoranthene, pyrene, chrysene, benzo (b) fluoranthene, 7, 12 dimethyl benzanthracene, benzo (e) pyrene, benzo (a) pyrene, and benzo (ghi) perylene in two repeated measurements of duplicated samples (n=2) were observed. Shorter-chained saturated alkanes, such as decane (C<sub>10</sub>) or hexane (C<sub>6</sub>), and longer-chained alkanes, like hexatriacontane (C<sub>36</sub>) and other PAHs were not identified. Reduction of the average peak ratio showed that the isolates could degrade saturated HCs ranging from C<sub>13</sub> to C<sub>30</sub>. HCs from C<sub>14</sub> to C<sub>23</sub> and C<sub>28</sub> were degraded by 90 to 100%. Other HCs, such as C<sub>13</sub>, C<sub>27</sub>,

C<sub>29</sub> and C<sub>30</sub>, were degraded by 59 to 89%. However, isolate G2 did not degrade C<sub>18</sub> (0.00%) while isolate G1 was not able to utilize C<sub>21</sub> (3.60%) (Table 2). Statistical analyses showed that most of the saturated alkanes were degraded significantly by the two isolates as indicated by a 59% to 100% decrease in the peak ratios of the treatments compared to the control treatment. With regard to the PAHs, the two isolates did not utilize or reduce any of the eleven detected compounds.

### Detection of HC-degrading gene, *alkB*

The primer pair Rhose and Rhoas was able to amplify a fragment of the *alkB* gene necessary for degradation of short- to long-chain n-alkanes (Figure 1, lanes 9 to 11). The results were verified by amplifying the same gene fragment from a control organism (Figure 1, lane 9) and generating the expected fragment size approximately 300 bp.

The detected gene was confirmed by determining its phylogenetic relationship to the *alkB* genes of *Gordonia* species and other genera based on neighbor joining as shown in Figure 3. The tree was rooted with the *alkB* gene of *Acinetobacter* sp. M-1 to compare divergence from the *alkB* genes of other genera creating two major lineages. Only bootstrap values more than 50% were observed on every node based on neighbor-joining analysis of 1000 resampled datasets. The scale bar indicates the nucleotide substitution per position. Comparison of *alkB* gene sequences of several bacterial strains showed that the putative alkane hydroxylase genes of the two isolates formed a phylogenetic clade with *Gordonia* species as supported by high bootstrap value (88%). Although a single lineage was formed by the *Gordonia* species, isolates G1 and G2, *Mycobacterium ratisbonense* and *Rhodococcus ruber* IV11, the last two species were distant from the *Gordonia* isolates cluster at about 6 to 7 substitution per 100 nucleotides (indicated by the bar below the neighbor-joining tree). The genes of *Prausella rugosa*, *P. putida* and *P. aeruginosa* were apparently distant to the *alkB* genes from the isolates as demonstrated by the tree.

### DISCUSSION

The morphologic characteristics of isolates G1 and G2 were consistent with the observed characteristics of the genus *Gordonia* as investigated in several studies (Nishioka et al. 2006, Arenskötter et al. 2004, Kim et al. 1999, Linos et al. 1999). The Gram variability and the rod-coccus growth cycle are typical of actinomycetes. The genus *Gordonia* belong to Order *Actinomycetales*, hence exhibited this pleomorphic characteristic (Arenskötter et al. 2004, Linos et al. 1999). The positive result in the catalase test indicates the presence of the enzyme catalase which is a consistent marker among the species of *Gordonia* (Kim et al. 1999). However, the oxidase test detects the presence of cytochrome C which produces a dark-blue end product when reacted with tetramethyl-*p*-phenylenediamine. This is found commonly among *Micrococcus* organisms and is usually not

performed in the characterization of actinomycetes (Gerhardt et al. 1994, Delost 1997) but is a protocol in the selection of the correct type of Biolog ID plate and the database to which the unknown isolate is evaluated (Biolog Inc. Hayward, California).

Initial classification using Biolog ID System-GP2 revealed that the isolates belong to the suborder Corynebacterineae. The system also points out that the isolates have higher similarity to the genus *Rhodococcus* and *Gordonia* than the other members of the suborder. Although the system was not able to produce high similarity scores to indicate the exact genus and identities of the isolates, it narrowed the list of probable identities of the two isolates giving a baseline data for the molecular identification in addition to their phenotypic characteristics. Amplification of their 16S rDNA and sequence analysis showed the isolates having 99% homology to several strains of unnamed *Gordonia* spp. and *G. terrae*. Phylogenetic analysis of the 16S rRNA gene sequences of the isolates and all known *Gordonia* species indicated that the two isolates formed a monophyletic clade with *G. terrae*.

In addition, among the nineteen validly published species of *Gordonia*, only five of which were isolated from soil. *G. terrae* and *G. sinesedis* are the most common (Arenskötter et al. 2004). *G. terrae* is often isolated in polluted areas specifically those contaminated with HC compounds (Li et al. 2006). With all the data elucidated, there is strong evidence that the isolates are strains of *G. terrae*.

As indicated by their growth after several transfers on MSM plates with engine oil, the isolates were observed to utilize engine oil. In the absence of a carbon source (MSM-C), bacterial growth was still observed until the third inoculation. Although cells were inoculated with engine oil prior to their growth on MSM-C, subculturing was done on the 7<sup>th</sup> day of incubation, during which, as indicated by growth curve analysis, nutrients started to decline; hence, there were no sufficient left-over nutrients from the previous medium that could be utilized by the cells in the subsequent culturing. However, it was not significant enough to be considered as positive. Very few colonies were observed on the streak lines and colony size was very small as compared to those grown on nutrient agar medium. The HC utilization fingerprint experiments showed that the isolates were able to utilize saturated alkanes of wide chain length but not PAHs. Bacterial isolates with HC-degrading potentials commonly metabolize saturated alkanes but only of limited chain lengths. There is smaller number of species that are able to degrade wide-range saturated alkanes and isolates G1 and G2 are now among them. Owing to this ability, the isolates could also grow on crude oil, engine-oil and diesel substrates. These petroleum products all contain paraffins which are saturated alkanes (De Dominico et al. 2004, Van Hamme et al. 2003, Mishra et al. 2001, Hinchee et al. 1994, Leahy and Colwell 1990). Their poor growth on some PAH substrates may be attributed to the scavenging of the bacteria on low

concentrations of available complex polysaccharides and variable amounts of vitamins and ions present in the agar (Isaac and David 1995) and not a result of substrate utilization. This was also observed on MSM-C plates. However, in the presence of alkanes such as those observed in the HC utilization fingerprint experiment, the isolates grew excellently. This indicates that the microorganisms prefer the HCs as their primary carbon source and may only use the other nutrients found in the agar if the main source of carbon is present. The isolates were unable to grow on carbon substrates such as toluene, benzene, pyrene and others, probably because these inhibited their growth instead of acting as their carbon and energy sources. The possibility that these organisms use alkanes as their source of energy was confirmed by the GC experiments and internal standards (chlorooctadecane for the TPH fraction and mixture of phenanthrene-d<sub>10</sub>, pyrene- d<sub>10</sub>, and chrysene- d<sub>12</sub> for PAH fractions) were used to normalize the peak areas for quality assurance. As shown by the GC-MS measurements, the isolates were able to degrade aliphatic HCs ranging from C<sub>13</sub> to C<sub>30</sub> by more than 50% to as much as 100%. However, the absence of PAH reduction in the GC-MS analysis suggests that the two isolates were not able to utilize PAH compounds as their carbon sources. This supports the negative or poor growth of the isolates on several PAH substrates in the HC fingerprinting experiments.

The ability of the isolates to degrade a wide range of aliphatic HCs was primarily attributed to the alkane hydroxylase gene found in the isolates. The primer pair used in the study amplifies only a fragment of the gene that is commonly observed among bacteria which are able to utilize short- to long-chain n-alkanes such as *M. ratisbonense* and *R. ruber* IV11. Moreover, phylogenetic analysis revealed that the sequences of the amplicons of the isolates have similarities to the *alkB* gene sequences of the *Gordonia* species, and form a monophyletic clade with *M. ratisbonense* and *R. ruber* IV11. This explains that the PCR products detected were amplified *alkB* genes and are homologous to *Gordonia* species and other bacterial strains that were able to degrade the same type of saturated alkanes. Although their genes appeared to be similar, sequence diversity was high among genera and species and even within strains (Heiss-Blanquet et al. 2005). The detection of these genes validates the data from the HC utilization fingerprinting and GC-MS analyses.

The growth of the isolates on different HC substrates, detection of *alkB* gene, and reduction of peak ratios of HC compounds in the GC-MS analyses suggest that the two isolates have potential application in bioremediation. Their isolation has added the limited number of bacterial species that are able to degrade short- to long-chain saturated alkanes since other strains of *G. terrae*, such as strain IEGM AC148, can only metabolize a limited range of saturated alkanes, C<sub>10</sub> and C<sub>16</sub> (Vomberg and Klinner, 2000). There are also a few studies indicating the potential of this species in bioremediation but their significance



is observed in opportunistic infections on humans, such as skin infection, brain abscess and bacteremia (Arenskötter et al. 2004). Furthermore, either of the two isolates may be used in the reduction of saturated HC, since both are able to degrade wide chain length saturated alkanes. The use of one bacterial species to degrade a wide range of saturated alkanes may also decrease the number of bacterial species to be utilized in microbial remediation, especially if most known species can only degrade limited chain length saturated alkanes, such as other strains of *G. terrae*. On the other hand, remediation of oil-contaminated areas may be performed using these isolates in combination with other species that degrade other HC compounds, such as PAH. For these reasons, it is imperative to perform field tests to fully elucidate the commercial significance of *Gordonia* species in pollution control. The safety of utilizing these isolates should also be established through the detection of virulence genes since studies have shown that some strains of *G. terrae* may cause opportunistic infection.

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## CONFLICT OF INTEREST

There is no conflict of interest relating to this work.

## CONTRIBUTIONS OF INDIVIDUAL AUTHORS

M.A.C. Nicdao organized the study, performed the experiments and wrote the manuscript; W.L. Rivera organized the study, provided laboratory support and guidance in the conduct of experiments and edited the manuscript.

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