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Isolation and characterization of petroleum product emulsifying *Pseudomonas* strains from a generating set fuel tank

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This study characterized microbial strains isolated from diesel fuel samples collected from the tank of a generating set at the Institute of Research for Development in Pointe-Noire (Congo). Two bacterial isolates (G2 and G3) were distinguished by their color on agar plates and were characterized by their API 20E biochemical profiles and by 16S rRNA gene sequencing. The phenotypic properties of these isolates were consistent with their assignment to the genus *Pseudomonas*. Comparative 16S rRNA gene sequence analysis demonstrated that the G2 and G3 isolates were close relatives of *P. aeruginosa* strain GIM 32 and *P. aeruginosa* strain NV2, respectively, with 97% sequence identity. These two *P. aeruginosa* strains were able to grow in a mineral salt medium supplemented with 2% diesel fuel or SAE 90 gear oil as the only source of carbon. *P. aeruginosa* G3 showed faster growth and was able to emulsify diesel fuel (53%), gasoline (90%) and hexane (95%) more strongly than *P. aeruginosa* G2.

Key words: *Pseudomonas*, emulsification index, diesel fuel, gasoline, hexane.

INTRODUCTION

Oil spills are of increasing environmental concern, including physical damage that has a direct impact on wildlife and habitats. They affect a wide range of organisms that are linked in a food chain that includes human food resources (Pennings et al., 2014; Silliman et al., 2012). Traditional measures such as containment using floating devices so that the oil can be recovered or absorption by natural or synthetic materials, do not include breaking down the petroleum products (Pirôllo et al., 2008). Therefore, the use of biological surface-active compounds such as biosurfactants is promising owing to their biodegradability, low toxicity and effectiveness in increasing the biodegradability of low solubility organic compounds (Desai and Banat, 1997; Khire, 2010).

Biosurfactants are amphiphilic compounds with both hydrophobic and hydrophilic moieties that partition

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preferentially at the interfaces between non-polar and polar fluids, such as oil/water and air/water (Desai and Banat, 1997). These properties enable biosurfactants to reduce surface and interfacial tension and thus increase the surface area of the immiscible phases, increasing mobility, bioavailability and subsequent biodegradation (Leahy and Colwell, 1990). Biosurfactants are produced by a large number of microorganisms which utilize a wide range of carbon sources, including oil, resulting in the breakdown of the pollutants into low molecular weight compounds (Banat et al., 2000, 2014; Abdel-mawgoud et al., 2010). The breakdown and solubilization of hydrocarbons form an important stage in the bioremediation of oil spills. Hydrocarbon-degrading microorganisms produce biosurfactants, with a wide range of chemical compounds and molecular weights that increase the surface area of the hydrophobic substrates and their bioavailability, thereby increasing bacterial growth and the bioremediation rate (Ron and Rosenberg, 2002).

Microorganisms thriving on oil are found in a wide variety of genera and are able to degrade various types of hydrocarbon, including polycyclic aromatic hydrocarbons (Atlas, 1981; Liang et al., 2014; Dhamodharan and Jayapriya, 2016). Microbial contamination of stored hydrocarbon fuels has been reported in the literature (Gaylarde et al., 1999; Yoshida et al., 2005; Klofutar and Golob, 2007). Much attention has been paid to screening for potential biosurfactant-producing microorganisms in recent years (Desai and Banat, 1997; Dieter et al., 2005). Microorganisms growing on oil usually produce potent emulsifiers. The high emulsification index (E_{24}) is generally a result of the production of a biosurfactant that is able to emulsify the hydrocarbons and render them more accessible for biological breakdown (Perfumo et al., 2006; Qazi et al., 2013). Emulsification is, therefore, one of the criteria used to select potential biosurfactant producers. Most previous screening studies have been carried out on microorganisms isolated from oil-polluted sites.

This study set aimed (i) to culture and isolate bacteria in the diesel fuel tank of a generating set, (ii) to characterize the phenotypes and identify the isolates and (iii) to estimate the emulsification index (E_{24}) of the isolates.

MATERIALS AND METHODS

Sample collection, culture and isolation of bacteria

Diesel oil samples were collected from the tank of an SDMO generating set (Products, Leroy Somer, France) at the Institute of Research for Development (IRD) in Pointe Noire (Congo) and stored at 4°C until use. For the bacterial cultures, 100 µL aliquots of the diesel fuel samples were plated onto a plate count agar (PCA) medium (Fluka, Spain) with 5 g tryptone, 2.5 g yeast extract, 1 g dextrose and 9 g agar per L of distilled water. The pH was adjusted to 7.0±0.1. The cultures were incubated at 37°C for 24 to 96 h under aerobic conditions. Single colonies with a distinct color were isolated by repeated streaking onto PCA medium until pure. The isolates were preserved on PCA slants kept at 4°C.

Phenotypic characterization of isolates

Phenotypic characterization was performed by combining analysis of the morphological traits and the biochemical properties of the isolates. Cell morphology, motility and the presence of spores were assessed using wet mounts with a Paralux optical microscope (L1100 S2 Trino-1600x, France). Gram staining was performed as described in the literature (Gregersen, 1978). Catalase and oxidase tests were performed using standard methods (Seeley et al., 1995; Smibert and Krieg, 1994). The range of growth-permissive temperatures was determined by assessing the growth of isolates on PCA incubated at 25, 37, 44 and 55°C.

Biochemical characterization was performed by a set of 20 enterobacteria identification tests using Analytical Profile Index test strips (API 20E, BioMerieux, France) following the manufacturer's instructions. The twenty microtubes containing dehydrated substrates were inoculated with a bacterial suspension. Each bacterial inoculum was prepared by homogenizing a bacterial colony from the PCA plates in a medium provided with the API 20E strips following the manufacturer's instructions. The inoculated API 20E strips were incubated at 37°C. During incubation, the microbial metabolism produces color changes that are either immediate or revealed later by the addition of reagents. After incubation, the positive and negative reactions in each strip were interpreted using the Reading Table provided. On the results sheets, the tests were separated into groups of 3 and a value of 1, 2 or 4 was assigned for each positive reaction, in accordance with the manufacturer's instructions. Adding together the values for a positive reaction for each test within each group gives a 7-digit profile number for the 20 tests in the API 20E strip. The bacteria were identified by referring the profile number in the Analytical Profile Index (p/n 20 190, BioMerieux-France).

Identification of strains using 16S rRNA gene sequencing

A 1.5 mL subsample of the bacterial culture was transferred to an sterile Eppendorf tube and centrifuged at 10,000g for 10 min. DNA was extracted from the pellets using Fast DNA® SPIN Kit (MP Biomedical, Santa Ana, CA, USA), following the manufacturer's instructions. Extractions were made in triplicate for each sample. The DNA extracted was quantified using a spectrophotometer (NanoDrop, France). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) in a 25 µL reaction mixture (total volume) using Taq polymerase Ready-To-Go (Amersham Pharmacia Biotech, USA), 0.25 mM of primer 27F (5'AGAGTTTGATCCTGGCTCAG3'), 0.25 mM of primer 1492R (5'GAC GGG CGG TGT GTA CAA-3') and 20 ng of the template DNA. The PCR was carried out using a thermocycler (VWR, France) with an initial denaturation for 8 min at 94°C; 30 cycles of 30 s at 94°C, 1 min at 56°C and 30 s at 72°C, and a final extension for 15 min at 72°C. The PCR products were run on 1.5% (wt/vol) agarose- 0.5X TAE (Tris, Acetate, EDTA) buffer gel stained with SYBR Safe stain (Invitrogen, France) at 80 V for 30 min and the migration patterns of the PCR products were captured using a GelDoc 2000 transilluminator system (BioRad, Richmond, California, USA). The PCR products were sequenced on both strands using the ABI 3730XL Sanger sequencing platform at the Beckman Coulter Genomics (Essex, United Kingdom). 16S rRNA gene sequences were aligned using Mega 6 and compared with those available in the GenBank database using the gapped sequence BLASTN 2.0.5 program on the National Center for Biotechnology Information server (Altschul et al., 1997). Comparisons were performed using the BLOSUM 62 matrix with default parameters. Each sequence was aligned with the first 10

Table 1. Characteristics of *Pseudomonas* isolated from diesel tank and grown on BH medium supplemented with 2% gasoil as sole carbon source.

Strain	16S rRNA gene identity[Ŧ]	Morphology	Motility	Catalase	Oxydase	Gram staining	Growth temperature			
							25°C	37°C	44°C	55°C
Isolate G2	Pseudomonas aeruginosa strain GIM 32 (97%) (HM067869.1)	Rodlike	+	+	+	-	+	+	-	-
Isolate G3	Pseudomonas aeruginosa strain NV2 (97%) (KF574080.1)	Rodlike	+	+	-	-	+	+	-	-

[Ŧ]First blast hit on the query sequence; positive reaction; -, negative reaction.

database sequences giving the highest scores of sequence similarity and the quality of the database sequences was assessed. Bacterial classification and identification were performed at 97% identity of the 16S rRNA gene sequence with that of the closest sequence in GenBank.

Cell growth on diesel-supplemented medium

Colonies of both bacterial isolates were transferred into 20 mL nutrient broth (Liofilchen, Italy) and incubated at 30°C for 24 h. Bacterial cells were harvested by centrifugation at 44,000 rpm for 10 min (Eppendorf 5709R centrifuge) and washed three times with 0.85% NaCl. Pellets were then homogenized in 5 mL of 0.85% NaCl and used as an inoculum. Five milliliters of Bushnell Hass (BH) mineral salt broth (Difco, France) consisting of 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1.0 g K_3PO_4, 1.0 g $(NH_4)_2HPO_4$, 1.0 g KNO_3 and 0.05 g $FeCl_3$ per liter was inoculated with 1% inoculum. The pH was adjusted to pH 7.0 ± 0.2. The medium was supplied with 2% (v/v) autoclaved diesel fuel as the sole source of carbon. The cultures were incubated at room temperature for 20 days and shaken manually every day. Samples were taken every 10 days to determine the cell density. The specific growth rate was calculated using the following formula:

$$\mu = \ln X - \ln X_0 / t - t_0$$

where, X_0 is the initial number of cells; X is the final number of cells; t_0 is the initial time; t is the final time.

Emulsification activity assay

The isolates were cultivated in Bushnell Haas (BH) broth with 2% v/v diesel fuel for five days. Two milliliters of the culture were poured into a test tube containing 2 mL of petroleum product (diesel fuel or gasoline) or pure hydrocarbon (hexane). After vigorous stirring for 2 min using a standard vortex mixer (VELP Scientifica, Italy), the test tubes were allowed to stand for 24 h at room temperature. The height of the emulsion layer and the total height of the mixture were then measured using a digital slide caliper (DCA150, Velleman, China). The emulsification index (E_{24} %) was calculated using the standard formula (Ferhat et al., 2011; Gudina et al., 2012):

$$E_{24}\% = \frac{He}{Ht} \times 100$$

Where He is the height of the emulsion and Ht is the total height of the mixture.

RESULTS

Characterization and identification of isolates

The isolates G2 and G3 obtained from culture on PCA supplemented with gas oil as sole carbon source (called in the text isolate G2 and isolate G3) were selected based on the color of their colonies. Isolates G2 and G3 were motile, rod-shaped, Gram-negative, non-spore forming bacteria, with catalase and oxidase activity (Table 1). Growth was observed in cultures incubated at 25 and 37°C but not at 44 and 55°C.

The reactions of the two isolates to 17 API 20E biochemical assays were negative while the assimilation of citrate (CIT) and melibiose (MEL) was positive in both cases (Table 2). For the three remaining biochemical assays, arginine dihydrolase (ADH), gelatinase (GEL) and glucose (GLU) reactions were positive for isolate G2 and negative for isolate G3. Both isolates were identified as members of the genus *Pseudomonas* by checking the profile number, determined using the Reading Table, in the Analytical Profile Index. The PCR products of targeted 16S rRNA genes were about 1200 bp long. Sequencing of the PCR products and analysis of the distribution of 10 Blast hits on the query sequence resulted in assignments of the isolates G2 and G3 as closest to *Pseudomonas aeruginosa* strain GIM 32 and *P. aeruginosa* strain NV2, respectively with 97% sequence identity.

Growth of the *Pseudomonas* strains G2 and G3 on BH medium supplemented with either diesel fuel or SAE 90 gear oil as the sole source of carbon

Monitoring the cell concentration showed that both *Pseudomonas* strains G2 and G3 were able to grow on mineral BH medium supplemented with either diesel fuel or SAE 90 gear oil as the sole source of carbon (Figure 1). For the *Pseudomonas* strain G2 cultured with SAE 90 gear oil, the cell concentration increased to the maximum of 65×10^7

Table 2. API 20E biochemical test results for the Pseudomonas strains isolated from diesel tank.

Test	Isolate G2	Isolate G3
B-galactosidase (ONPG)	-	-
Arginine dihydrolase (ADH)	-	-
Lysine decarboxylase (LDC)	-	-
Ornithine decarboxylase (ODC)	+	-
Production of H_2S	-	-
Urease (URE)	-	-
Tryptophan deaminase (TDA)	-	-
Production of indole (IND)	-	-
Acetyl methylcarbinol (VP)	-	-
Gelatinase (GEL)	-	-
Assimilation of:		
Citrate (CIT)	+	-
Glucose (GLU)	+	+
Mannose (MAN)	-	-
Inositol (INO)	-	-
Sorbitol (SOR)	-	-
Rhamnose (RHA)	-	-
Sucrose (SAC)	-	-
Melibiose (MEL)	+	-
Amygdalin (AMY)	-	+
Arabinose (ARA)	-	-
NO_2 production (NO_2)	-	-
Reduction to N_2 gas	-	-
Motility (MOB)	-	-

cells mL^{-1} over the first 10 day, corresponding to a specific growth rate (μ) of 0.32 d^{-1}, whereas the specific growth rate on diesel fuel was lower at 0.17 d^{-1}. In contrast, for the *Pseudomonas* strain G3, the growth on SAE 90 gear oil was very slow during the first 10 days of incubation, corresponding to a lag phase while the bacteria adapted to the substrate. After this period, the specific growth rate increased to 0.36 d^{-1} which was similar to the specific growth rate of *Pseudomonas* strain G2 under the same culture conditions. The highest specific growth rate of 0.51 d^{-1} was observed in cultures of *Pseudomonas* strain G3 supplemented with diesel fuel as the sole source of carbon. No lag phase was observed in cultures of the Pseudomonas strain G3 supplemented with diesel fuel as the sole source of carbon.

Emulsification activity of the *Pseudomonas* strains G2 and G3

The *Pseudomonas* strains G2 and G3 were tested for emulsification activity in diesel fuel, gasoline and hexane (Figure 2). The E_{24} for the *Pseudomonas* strain G3 was 90% in gasoline and 95% in hexane but only 53% in diesel fuel. The E_{24} activities for the *Pseudomonas* strain G2 were 2 to 7 times lower in gasoline/diesel fuel and hexane than those for the strain G3. The strain G2 had no emulsification activity in gasoline/diesel fuel.

DISCUSSION

Certain microorganisms are better adapted to particular environments, such as oil tanks. This study set out to isolate and determine the phenotypic and molecular characteristics of bacteria in a generating set fuel tank, in particular, their ability to emulsify diesel fuel, gasoline and hexane. The isolates were identified by their API 20E biochemical profiles and by 16S rDNA sequencing since the latter has the highest concordance with conventional methods and is superior to identification by analysis of carbon source utilization (Tang et al., 1998).

Although, the isolate G2 was positive for arginine dihydrolase (ADH) and for the assimilation of glucose (GLU) and melibiose (MEL), unlike the isolate G3, the phenotypic and molecular characteristics of the two isolates in this study were consistent with their assignment to the genus *Pseudomonas*. Comparison of the 16S rRNA gene sequences of isolates G2 and G3 with the GenBank database allowed their assignment to *P. aeruginosa* strain GIM 32 and *P. aeruginosa* strain NV2, respectively. These findings are in agreement with previous studies that reported the presence of the strains of *P. aeruginosa* in hydrocarbon-polluted sites (Widada et al., 2002; Ismail et al., 2015; Das et al., 2014; Coutinho et al., 2013; Sharma et al., 2014) and in stored fuels (Gaylarde et al., 1999; Hamed et al., 2013).

The growth of the two *Pseudomonas* strains isolated in this study was investigated in BH medium which is widely used for the examination of fuels for microbial contamination and for studying hydrocarbon degradation by microorganisms (Silva et al., 2015; Gudiña et al., 2012). Both strains G2 and G3 were able to grow in BH mineral medium with either diesel fuel or SAE 90 gear oil as the sole source of carbon. The increase in cell concentration provides indirect evidence of the utilization of the substrates by the two *Pseudomonas* strains although the degradation of hydrocarbons was not directly investigated in this study. These observations are in line with previous studies that reported the degradation of gas oil and gear oils by *P. aeruginosa* strains (Pirôllo et al., 2008; Perfumo et al., 2006; Pacwa-Płociniczak et al., 2014; Obayori et al., 2014). It has been demonstrated that bacterial specific growth rates varied with the hydrocarbons or products derived from crude oil (petroleum) used as the source of carbon (Wongsa et al., 2004; Nie et al., 2010; Obayori et al., 2014). Results from the present study indicate that strains belonging to the same genus may show different specific growth rates in culture media supplemented with the same source of

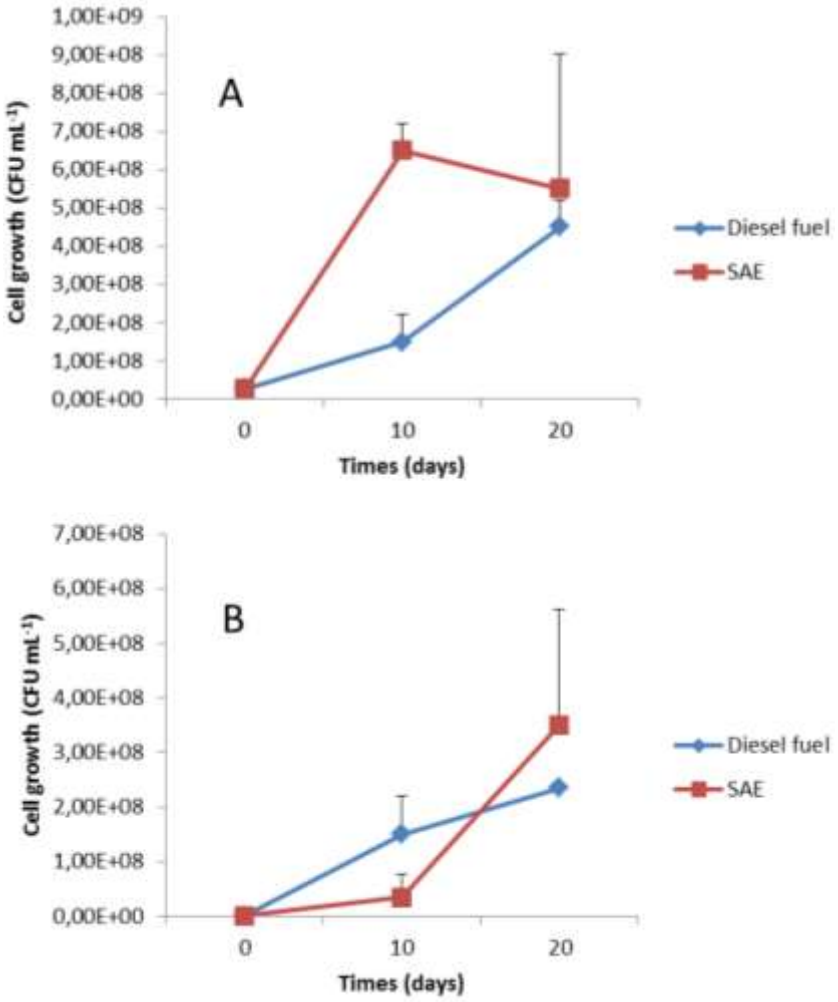

Figure 1. Growth curves of strain G2 (A) and strain G3 (B) on diesel fuel and SAE 90 as sole source of carbon.

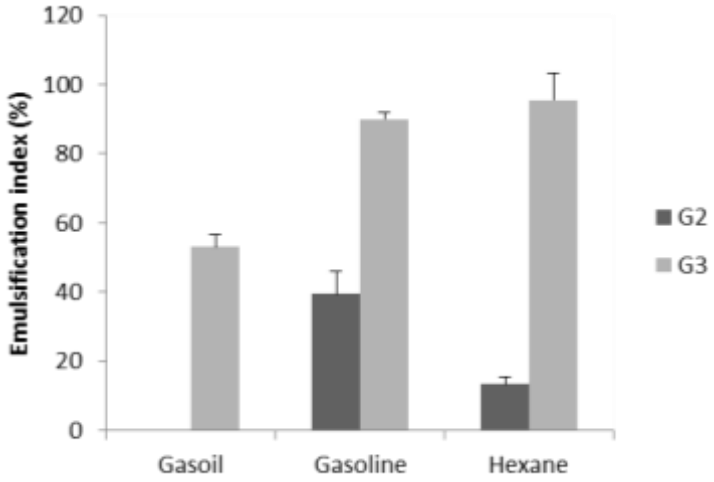

Figure 2. Emulsification activity (E_{24}) of suspension of strain G2 and strain G3. Each value is the mean of three replicates.

carbon. The isolate G3, for which *P. aeruginosa* strain NV2 was the closest relative, showed higher specific growth rates than the isolate G2, for which *P. aeruginosa* GIM 32 was the closest relative, on both 2% SAE 90 gear oil and 2% diesel fuel. These differences may be attributed to the unusually versatile genome of *Pseudomonas* spp. (Stover et al., 2000). Although, there are clusters of closely related strains whose sequence signatures are conserved in the gene pool (Kiewitz and Tümmler, 2000), the *P. aeruginosa* genome exhibits great plasticity (Klockgether et al., 2011; Dötsch, 2015; Shen et al., 2006; Liu et al., 2012). The highest cell concentrations in both strains isolated in this study were observed, over the 20 days incubation period, when diesel fuel was used as the sole source of carbon rather than SAE 90 gear oil. This could be explained by the differences in chemical composition between diesel fuel and gear oils. Diesel fuel gas oil is a hydrocarbon mixture with chain lengths of approximately 8 to 21 carbon atoms per molecule obtained by the crude oil distillation whereas gear oils consists of hydrocarbons with chain lengths of between 18 to and 34 carbon atoms per molecule (Collins, 2007). Furthermore, mild extreme pressure (EP) additives for lubricants and functional fluids in gear oils include zinc dialkyldithiophosphate and molybdenum disulfide which may provide metal ions acting as cofactors in essential enzymatic reactions in the *P. aeruginosa* strain NV2, thus stimulating its growth on SAE 90 gear oil as the sole source of carbon. It has been reported that the emulsification activity of *P. aeruginosa* is associated with the production of rhamnolipid-type biosurfactants which are among the most effective surfactants known today (Patel and Desai, 1997; Soberon-Chavez et al., 2005; Arutchelvi and Doble, 2010; Marchant and Banat, 2012). Itoh and Suzuky (1972) established a correlation between biosurfactant production and the cell growth of the *P. aeruginosa* PU-1 mutant strain in water-insoluble substrates. Similar results were also found in the biosurfactant-negative mutant, *P. aeruginosa* PG-201 (Koch et al., 1997). It is, therefore, likely that biosurfactants were produced by the two *Pseudomonas* strains isolated in the present study, as suggested by their ability to emulsify gas oil, gasoline and hexane. Of the two strains isolated in the present study, that closer to *P. aeruginosa* strain NV2 had a higher emulsification index in gasoline (90%) and hexane (95%) and diesel fuel (53%). This confirms that the emulsification activity depends on the hydrocarbon. Similar observations have been reported by Qazi et al. (2013) who demonstrated that *Pseudomonas putida* strain SOL-10 had different emulsification activities for a variety of hydrocarbons including n-hexane, N-hexadecane, kerosene, xylene. The differences in emulsification activity in the hydrocarbons tested in this study might be attributed to the production of rhamnolipid congeners which depend on *Pseudomonas* strains and carbon substrates as shown by Bharali and Konwar (2011). Rhamnolipid 1 and rhamnolipid 2 are the main glycolipids produced by *P. aeruginosa* (Desai and Banat, 1997). However, although the emulsification index for *P. aeruginosa* strain NV2 was of the same order of magnitude as that reported by Das et al. (2014) in diesel fuel, in gasoline and hexane the indices were higher than in previous studies (Gudiña et al., 2012; Rocha et al., 2014). Although, further work will be required to gain a better understanding of the physiology of the strains isolated in this study, the results indicated that the isolate whose closest relative was *P. aeruginosa* strain NV2 may have great potential for the remediation of sites contaminated by petroleum products.

Conflict of interests

The authors did not declare any conflict of interest.

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