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THE DIVERSITY OF CULTIVABLE HYDROCARBON-DEGRADING BACTERIA ISOLATED FROM CRUDE OIL CONTAMINATED SOIL AND SLUDGE FROM ARZEW REFINERY IN ALGERIA

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ABSTRACT

The use of autochtonious bacterial strains is a valuable bioremediation strategy for cleaning the environment from hydrocarbon pollutants. The isolation, selection and identification of hydrocarbon-degrading bacteria is therefore crucial for obtaining the most promising strains for decontaminate a specific site. In this study, two different media, a minimal medium supplemented with petroleum and with oil refinery sludge as sole carbon source, were used for the isolation of native hydrocarbon-degrading bacterial strains from crude oil contaminated soils and oil refinery sludges which allowed isolation of fifty-eight strain**s.**The evalution of diversity of twenty-two bacterials isolates reveled a dominance of the phylum *Proteobacteria* (20/22 strains), with a unique class of *Alphaproteobacteria*, the two remaining strains belong to the phylum *Actinobacteria*. Partial 16S rRNA gene sequencing performed on isolates showed high level of identity with known sequences. Strains were affiliated to *Sinorhizobium*, *Promicromonospora*, *Novosphingobium*, *Georgenia*, *Ancylobacter*, *Roseomonas*, *Hansschlegelia* and *Tistrella* genera. Research for the genes that encoding for degradation enzymes in isolated genera genome data deposited in Genbank reveled the presence of degradation gene in three species *Sinorhizobium meliloti*, *Novosphingobium panipatense*and *Tistrella mobilis*.

Keywords: crude oil polluted soil, oil refinery sludge, hydrocarbon-degrading bacteria, diversity, 16S rRNA gene sequencing, hydrocarbon-degradation genes

INTRODUCTION

Petroleum hydrocarbons are the most common environmental pollutants, their release into the environnement, whether accidentally or due to human activities poses a great hazard to terrestrial and marine ecosystems (**Holliger *et al.*, 1997**). The oil industry including, transport, storage, processing or use generates a significant quantity of sludge during the petroleum-refining process and as the installations are cleaned (**Cerqueira *et al.*, 2011**). The sludges that result from this treatment process have a high content of petroleum derived hydrocarbons, mainly alkanes and paraffins of 1–40 carbon atoms, along with cycloalkanes and aromatic compounds (**Overcash and Pal, 1979**). The soil contamination by petroleum hydrocarbons which are known to belong to the family of carcinogens and neurotoxic organic pollutants (**Abioye, 2011**) causes a significant decline in its quality and such soils become unusable (**Gojgic-Cvijovic *et al.*, 2012**). Algeria, whose national economy depends on petroleum exploitation, is not an exception to this pollution problem. The Arzew refinery, considered as one of the biggest and most profitable in the North Africa, the third of the country, possesses a high crude oil processing capacity, the various by-products stemming from the refining allow to satisfy fuel, lubricants, asphalt needs of the local population and also to export the surplus products (Naphta and Fuel). However, it is also accompanied by the annual discharge of an important quantity of sludge during the storage tank cleaning operations, which engenders hydrocarbon soil pollution. This is why, the need to clean up crude oil contaminated site is necessary. Bioremediation is an economically and environmentally-friendly procedure based on the metabolic capacities of microorganisms to degrade contaminants biochemically (**Huang *et al.*, 2013**). Bacteria in particular are considered to be the dominant agents of hydrocarbon degradation in the environment (**Röling *et al.*, 2002**). Biodegradation using autochthonous bacterial strains is a valuable bioremediation strategy, these indigenous microbes are more likely to survive and proliferate when reintroduced into the site (**Silva *et al.*, 2009; Madueño *et al.*, 2011; Fukuhara *et al.*, 2013; Shankar *et al.*, 2014**). The aim of the present work is to isolate autochnonous bacterial strains from polluted soil and oil sludge samples in Arzew refinery. The distribution of genes coding for twenty three enzymes degrading hydrocarbon will be also analyzed from hydrocarbon degrading genera genome data deposited in GenBank and compared to the isolated genera in this study.

MATERIALS AND METHODS

Site description and sampling

Oil polluted soil and oil sludge samples were collected from the Arzew refinery (35°49'21.29"N 0°18'37.58"O) in Northern Algeria (Sonatrach, Algerian Petroleum Company). Two soil samples were taken from the surrounding of the storage tank of crude oil at different period (soil A and soil B on 12.02.2015 and 26.02.2015 respectively). Each sample was a mixture of five sub-samples. A scoop was used to remove organic particles from the soil surface. Soil was collected at 0 to 20cm of depth randomly, air dried and sieved (2mm) and stored in a dry place at room temperature (~28°C). Two oil sludge samples were taken from the quagmire of the Arzew refinery, sampling were performed at two different dates (sludge C and sludge D on 12.02.2015 and 26.02.2015 respectively) and from 0 to 20cm depth. The physicochemical parameters of these soil and sludge samples were analyzed including water content (WC), total nitrogen (N_{tot}) was determined by Kjeldahl digestion, while total phosphorus (P_{tot}) using phosphor molybdic acid, total organic carbon (OC_{tot}) was determined according to Walkley-Black and total petroleum hydrocarbons (TPH) were determined by extracting the samples in methylene chloride, then analyzing the extracts by capillary gas chromatography with flame ionization detection. Oil used in this experiment was provided by the staff of the Arzew refinery and stored in the dark at ambient temperature throughout the study. Before use, oil was sterilized using 0.2µm membrane filter.

Count and isolation of hydrocarbon-degrading bacteria

Hydrocarbon-degrading bacteria were isolated from oil sludge and oil contaminated soil samples by using the Bushnell Hass (BH) Mineral Salts (BHMS) medium (**Bushnell and Hass, 1941**). This medium contains all nutrients except the carbon source. The medium composition is as follows: $MgSO_4.7H_2O$ (0.2 g/L), K2HPO4 (1.0 g/L), KH_2PO_4 (1.0 g/L), $FeCl_3$ (0.05 g/L), NH_4NO_3 (1.0 g/L) and $CaCl_2$ (0.02g/L) at pH 7.2, agar (20 g/L) was added for BH agar. The source of carbon and energy was provided either by the addition of sludge in BHMS medium before autoclaving or by adding sterilized oil in the BHMS medium after autoclaving.The hydrocarbon degrading-bacteria cultured from the contaminated soil (A and B) and oil sludge (C and D) samples were quantified by mixing 1g of sample with 9mL of sterile physiological serum (9‰ NaCl) for 2min at high speed. From this soil suspension, successive 1/10 dilutions were made (10^{-3}, 10^{-4}, 10^{-5}). An aliquot (0.1mL) of each diluted soil suspension was spread on BHMS agar plate with the addition of sludge as carbon source and on BHMS agar plate with 100µl of oil. The plates were incubated at 29˚C for 1 week. Bacterial hydrocarbon-degrading likely grown over incubation period. Colonies were then counted and the number was expressed as UFC / g of sample, bacterial counts were carried out in triplicate. Colonies presenting different morphological appearance were then sub-cultured, purified and conserved for molecular identification.Three mediums were used for strains subculture: Nutrient agar, Tryptic Soy Agar (TSA) medium and diluted 1/10 TSA medium (Biomeriu, France). The isolated strains were then conserved in their appropriated growth medium supplemented with 50% v/v glycerol solution at -80°C. For day-to-day experimentation strains were maintained on their growth medium slants at 4°C in refrigerator and sub-cultured at an interval of 30 days.

DNA Extraction

DNA from bacterial cells was extracted using NucleoSpin® 96 tissue-vacuum processing (Genomic DNA from tissue and cells, MACHEREY-NAGEL, Hoerdt, France), the strains were cultured for 72h at 29°C in 150µl of Nutrient Broth (NB) or TSA medium depending on their growth requirements, then 50 ml of bacteria cultures were transferred in 150ml of medium (Nutrient Broth or TSA) and were incubated until the appearance of a bacterial disorder, the cultures were then centrifuged at 4000rpm/min ; 4 °C for 30min, the obtained bacterial pellets were frozen at -80°C. Lysis was achieved with the NucleoSpin® 96 tissue method by incubation of the bacterial pellets in a solution containing Sodium Dodecyl Sulfate (SDS) and proteinase K. Appropriate conditions for binding of DNA to the silica membrane in the NucleoSpin® 96 tissue columns were created by addition of large amounts of chaotropic salt and ethanol to the lysate. The binding process was reversible and specific to nucleic acids. Contaminations were removed by washing with two different buffers. Pure genomic DNA was finally eluted under low ionic strength conditions in a slightly alkaline elution buffer and wasstored at −20 °C until 16S ribosomal RNA (rRNA) gene amplification.

PCR amplification of bacterial 16S rRNA genes

Bacterial 16S rRNA genes were amplified from all DNA samples using the bacterial specific primers pA (5'AGA GTT TGA TCC TGG CTC AG3') and pH (5'AAG GAG GTG ATC CAG CCG CA3') (Edwards and *al.* 1989). Amplification reaction was performed using Platinum® PCR SuperMix 96 (Invitrogen, France), each plate well contains anti-Taq DNA polymerase antibody, Mg++, deoxyribonucleotide triphosphates (dNTP), and recombinant Taq DNA Polymerase and each 22.5µl volume of Platinum® PCR SuperMix well was supplied at 1.1X concentration to allow approximately 10% of the final reaction volume to be used for the addition of 1µl of each primer and 2µl of DNA solution (10 to 50ng). The PCR amplification program was conducted as follows: Incubate at 94°C for 5min to activate the enzyme, and then perform 30 cycles of PCR. Denature 94°C for 15s, Anneal 55°C for 30s, Extend 72°C for 1min 30s then hold at 10°C. Amplified DNA was verified in 1% agarose gel electrophoresis.

Sequencing and phylogenetic analysis

PCR products were sequenced by GATC Biotech AG (Constance, Allemagne); 20 µl of PCR products were sent to sequence. The sequences were corrected by the SeqMan Pro softwwere (**http://www.dnastar.com**) before alignment and analysis of the 16S rRNA gene sequences using CLUSTAL X software (version 2.0) (**http://www.clustal.org**). Taxonomic affiliation was done by analyzing similarities using Blast on NCBI (**Altschul *et al.*, 1990**). A phylogenetic dendrogram was constructed by the Neighbor Joining Method and tree topology as evaluated by performing boot-strap using Mega 6.06.

Distribution of genes encoding for hydrocarbon degrading enzymes

Twenty-seven proteins sequences listed in Table 4 were selected as reference sequence of hydrocarbon dergradation and investigated for their different encoding genes. The conserved proteomic domains were checked in total sequenced gonome database from GenBank for nine genera (*Sinorhizobium, Promicromonospora, Novosphingobium, Georgenia, Starkeya, Roseomonas, Ancylobacter* and *Hansschlegelia*). These specific genes were highlited for genera level.

RESULTS AND DISCUSSION

Soils and sludges characteristics, counts of culturable hydrocarbon-degrading bacteria

The physico-chemical analysis of crude oil polluted soil and oil refinery sludge samples (Table 1) revealed the following C: N: P ratio: Polluted soil A (1 :1.2 : 2.5), Polluted soil B (1 : 1.25 : 3.35), Oil refinery sludge C (3.17 : 1 : 1.23), Oil refinery D (1.3 : 1 : 2.6), while for bioremediation operations, US EPA recommended a ratio of 100:10:1 in soil for appropriate nutrients to stimulate microorganism growth (**U.S. EPA, 2002**). This study reported that biostimulation (i.e., addition of nutrients N and P) strategies are essential to enhance bioremediation of the crude oil contaminated soil and oil refinery sludge collected from Arzew rafinery. Enumeration of culturable bacteria in soil and sludge samples (Table 2) demonstrated that significant hydrocarbon degrading bacteria populations were present in all samples. A number from $0.74x10^5$ to $1.8x10^5$ CFU/g are counted in the sludge medium and from $0.82x10^5$ to $2.02x10^5$ CFU/g in the petroleum medium. The same concentration was found in the soil contaminated with crude petroleum oil in Jordan (**Saadoun, 2002**).

Table 1 Physicochemical features of soil and sludge samples

Sample	WC (%)	N_{tot} (%)	P_{tot} (%)	OC_{tot} (%)	TPH ($mg.kg^{-1}$)
Crude oil polluted soil A	2.58	4.16	9.20	13.69	353
Crude oil polluted soil B	1.54	2.28	6.13	19.83	183
Oil refinery sludge C	14.95	13.08	16.87	55.10	4153
Oil refinery sludge D	18.03	10.58	13.80	57.06	2776

WC water content, $\mathbf{N_{tot}}$total nitrogen, $\mathbf{P_{tot}}$ total phosphorus, $\mathbf{OC_{tot}}$ total organic carbon, **TPH** total petroleum hydrocarbon

Table 2 Counts of hydrocarbons-degrading bacteria on oil refinery sludge and petroleum medium

Sample	bacterial count on oil refinery sludge medium	bacterial count on petroleum medium
Crud oil polluted soil A	$0.79\ X10^5$±0.17	$2.02X10^5$±0.38
Crud oil polluted soil B	$1.8X10^5$±0.51	$1.53X10^5$±0.46
Oil refinery sludge C	$1.06X10^5$±0.09	$0.91X10^5$±0.21
Oil refinery sludge D	$0.74X10^5$±0.17	$0.82X10^5$±0.21

Identification of hydrocarbon-degrading bacterial isolates

Fifty-eight bacterial strains were isolated from polluted soil and oil refinery sludge on petroleum and oil refinery sludge medium, cultures were maintained at 29°C for 1 week. Based on phenotypic characteristics (colony morphology, pigmentation, growth properties), 22 strains were selected for 16S rRNA gene sequencing. The comparison of obtained sequences by BLAST in Genbank showed that the majority of these strains (20/22) belonged to the phylum *Proteobacteria* (Table 3), with a unique class of *Alphaproteobacteria;* the two remaining strains belonged to the phylum *Actinobacteria*. Many studies on the

bacterial diversity in a hydrocarbon-contaminated soil, whether by culture-based methods or molecular methods, proved the dominance of *Proteobacteria* in hydrocarbonscontaminated soils (**Saul *et al.*, 2005; Uhlik *et al.*, 2012; Zhang *et al.*, 2012; Mao *et al.*, 2012**). The genus *Rhizobium* (9 strains) was dominating among *Alphaproteobacteria*; with a singlespecies *Sinorhizobium meliloti* (showed 100% 16S rRNA of gene sequence similarity). Other representatives of this class were related to the genera *Novosphingobium* (4 strains) with two species *Novosphingobium naphthalenivorans* (99% of similarity) and *Novosphingobium panipatense* (99% of similarity), *Tistrella* (4 strains), *Roseomonas* (1), *Hansschlegelia* (1) and *Ancylobacter* (1). The *Actinobacteria* comprised two genera *Promicromonospora* (1) and *Georgenia* (1). **Zhang *et al.*, (2012)** revealed a bacterial diversity close to the identified strains in this study, dominated by the phylum *Proteobacteria* with a majority of strains belonging to the genus *Rhizobium* in addition to both genera *Roseomonas* and *Tistrella*. The construction of a phylogenetic tree by the neighbor-joining method permitted grouping the isolates into 8 clusters (Figure 1).

The first cluster was close to *Sinorhizobium meliloti*, including SA1, SA12, SA13, SA14, SA15 and SA17 strains isolated from oil polluted soil A and SB4, SB5 and SB6 strains isolated from oil polluted soil B. Rhizobia survival under hydrocarbon contamination and the interaction of these bacteria with pollutant was reported by several authors (**Andreoni *et al.*,2004 ; Keum *et al.*, 2006; Poonthrigpun *et al.*, 2006 ; Wang *et al.*, 2007**), *Sinorhizobium meliloti* species had been isolated from hydrocarbon contaminated soil (**Ahmad*et al.*, 1997;Muratova, 2014 ; Abou-Shanab, 2016**) and had shown their ability to degrade various forms of hydrocarbon such as aromatic compounds (**Frassinetti *et al.*, 1998**) and PCB (Poly Chlororinated Biphenyl) (**Wang *et al.*, 2016**). Interestingly, the ability for N-fixation of the genus *Rhizobium* and their presence in the rhizosphere of oil-contaminated soils could play a major role in phytoremediation (**Jurelevicius *et al.*, 2010; Xu *et al.*, 2010**)thanks to their legumessymbiotic interaction (**Johnson *et al.*, 2004; Suominen *et al.*, 2000**). The second cluster comprised one strain SB15 isolated from oil polluted soil B and it was close to *Hansschlegelia zhihuaiae*. The genus *Hansschlegelia* is a member of family *Methylocystaceae* (**Ivanova *et al.*, 2010**) that belonged to the order *Rhizobiales*, within the class *Alphaproteobacteria*. This family was classified as type II methanotrophs, which was able to utilize methane and its derivatives as carbon sources through the serine pathway. Many of these methanotrophic bacteria performed important environmental functions as they were a link in the global carbon cycle, acted as nitrogen fixers, and have the ability to degrade a variety of organic contaminants (**Gulledge *et al.*, 2001**). *Hansschlegelia zhihuaiae* was isolated from a polluted-soil sample (**Wen *et al.*, 2011**), and known for its degradation ability of a variety of sulfonylurea herbicides (**Hang *et al.*, 2012**), whereas there are no reports for their ability to survive in hydrocarbon polluted soils. This is the first time where *Hansschlegelia zhihuaiae* shows ability to survive in oil polluted soil. The third cluster included one strain SB7 isolated from oil polluted soil B and it was close to *Starkeya koreensi* (Blast in NCBI), this species was isolated from rice straw (**Im *et al.*, 2006**), however another species *Starkeya novella* was isolated from agricultural soil and employed a mixotrophic growth strategy, *Starkeya novella* was a facultative sulfur oxidizing chemolithoautotroph, but is also capable of utilizing various carbon compounds, including methanol, for growth as well as atleast 39 reduced carbon sources including sugars, amino sugars, amino acids, and organic acids (**Starkey, 1935; Chandra and Shethna, 1977; Kelly et *al.*, 2000; Kappler *et al.*, 2012**). Since *Starkeya novella* used a wide range of carbon compounds for its metabolism, this could explain its presence in soils polluted by hydrocarbons. Phylogenetically, strain SB7 was related with *Ancylobacter dichloromethanicus* species, this strain was isolated from the polluted soil and was utilized dichloromethane, methanol, formate and formaldehyde along with a variety of polycarbon compounds (**Firsova *et al.*, 2009**). The fourth cluster grouped four strains ORSA6, ORSA7, SA9 and ORSA5 isolated from oil contaminated soil A and oil refinery sludge A and were close to two species *Novosphingobium naphthalenivorans* and *Novosphingobium panipatense*, respectively. Several aromatic-degrading strains of *Novosphingobium* such as *Novosphingobium aromaticivorans*, *Novosphingobium naphthalenivorans*, *Novosphingobium pentaromaticivorans*, *Novosphingobium panipatense*and *Novosphingobium naphthae*had been reported (**Kertesz and Kawasaki, 2010; Gupta, 2009; Suzuki and Hiraishi, 2007; Lyu, 2014; Chaudhary and Kim, 2016**).

Table 3 Origin, isolation medium and genotypic characteristics of isolated strains

Strain	Origin	Isolation medium	Acc. Num.	Nearest phylogenetic neighbor (% Similarity)	Phylum
SA1	Pollued soil A	petroleum	MG786631	*Sinorhizobium meliloti* strain WSM1022 100%	*Alphaproteobacteria*
SA3	Pollued soil A	petroleum	MG786632	*Promicromonospora aerolata* strain 12623 99%	*Actinobacteria*
SA9	Pollued soil A	petroleum	MG786633	*Novosphingobium naphthalenivorans* strain VIT-DD2 99%	*Alphaproteobacteria*
SA12	Pollued soil A	petroleum	MG786634	*Sinorhizobium meliloti* strain LMTR32 100%	*Alphaproteobacteria*
SA13	Pollued soil A	petroleum	MG786635	*Sinorhizobium meliloti* strain LLAN18 100%	*Alphaproteobacteria*
SA14	Pollued soil A	petroleum	MG786636	*Sinorhizobium meliloti* strain LMTR32 100%	*Alphaproteobacteria*
SA15	Pollued soil A	oil refinery sludge	MG786637	*Sinorhizobium meliloti* strain LLAN18 100%	*Alphaproteobacteria*
SA16	Pollued soil A	oil refinery sludge	MG786638	*Georgenia ferrireducens* strain F64 99%	*Actinobacteria*
SA17	Pollued soil A	oil refinery sludge	MG786639	*Sinorhizobium meliloti* strain LLAN18 100%	*Alphaproteobacteria*
SB4	Pollued soil B	oil refinery sludge	MG786640	*Sinorhizobium meliloti* strain LLAN18 100%	*Alphaproteobacteria*
SB5	Pollued soil B	oil refinery sludge	MG786641	*Sinorhizobium meliloti* strain LLAN18 100%	*Alphaproteobacteria*
SB6	Pollued soil B	oil refinery sludge	MG786642	*Sinorhizobium meliloti* strain LLAN18 100%	*Alphaproteobacteria*
SB7	Pollued soil B	oil refinery sludge	MG786643	*Starkeya koreensis* strain NBRC 100963 97%	*Alphaproteobacteria*
SB11	Pollued soil B	petroleum	MG786644	*Roseomonas ludipueritiae* 97%	*Alphaproteobacteria*
SB15	Pollued soil B	petroleum	MG786645	*Hansschlegelia zhihuaiae* strain S 113 99%	*Alphaproteobacteria*
ORSA5	oil refinery sludge C	oil refinery sludge	MG786624	*Novosphingobium panipatense* strain SM16 99%	*Alphaproteobacteria*
ORSA7	oil refinery sludge C	oil refinery sludge	MG786625	*Novosphingobium naphthalenivorans* strain VIT-DD2 99%	*Alphaproteobacteria*
ORSA6	oil refinery sludge C	petroleum	MG786626	*Novosphingobium naphthalenivorans* strain VIT-DD2 99%	*Alphaproteobacteria*
ORSB2	oil refinery sludge D	oil refinery sludge	MG786627	*Tistrella mobilis* strain DSD-PW4-OH23 100%	*Alphaproteobacteria*
ORSB4	oil refinery sludge D	oil refinery sludge	MG786628	*Tistrella mobilis* strain DSD-PW4-OH23 100%	*Alphaproteobacteria*
ORSB5	oil refinery sludge D	petroleum	MG786629	*Tistrella mobilis* strain DSD-PW4-OH23 100%	*Alphaproteobacteria*
ORSB6	oil refinery sludge D	petroleum	MG786630	*Tistrella mobilis* strain DSD-PW4-OH23 100%	*Alphaproteobacteria*

Table 4 Distribution of genes coding for degradation enzymes in isolated genera genome data deposited in Genbank

Degadation enzymes	Genes	*Sinorhizobium* genera	*Promicromonospora* genera	*Novosphingobium* genera	*Georgenia* genera	*Starkeya* genera	*Ancylobacter* genera	*Roseomonas* genera	*Methylocystaceae* family	*Tistrella* genera
benzoate dioxygenase subunit alpha	benA	*S.americanum*		*N.aromaticivorans* *N.naphthalenivorans*				*R.stagni* *R.gilardii*		
benzoate/naphthalene dioxygenase large alpha subunit	XylX/nahAc			*N.aromaticivorans* *N.panipatense*				*R.aerilata*	*Methylosinus sp*	
Benzene/toluene/chlorobenzene/ethylbenzene/naphtalene/cumen dioxygenase subunit alpha	nagAc/pahAc/phnAc/etbA1/narAa/nidA/pdoA2/cumA1/todC1/bphA/bphA1/phdA	*S.saheli*		*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*						*T.mobilis*
salicylate-5-hydroxylase large oxygenase component	nagG			*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*						*T.mobilis*
benzoate/cumen/ethylbenzene dioxygenase small beta subunit	benB/XylY/pdoB2/phnAd/phdB/nidB/bphA2/cumA2 /etbA2	*S.americanum* *S.saheli*		*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*				*R.stagni* *R.gilardii* *R.aerilata*		*T.mobilis*
naphthalene dioxygenase small beta subunit	nahAd/nagAd/pahAd			*N.aromaticivorans* *N.naphthalenivorans*						
salicylate-5-hydroxylase small oxygenase component	nagH			*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*				*R.stagni* *R.gilardii*		*T.mobilis*
dioxygenase small alpha subunit	nidB (Rhodo)			*N.aromaticivorans* *N.naphthalenivorans*						
catechol 1,2-dioxygenase	catA	*S.americanum*	*P. kroppenstedtii*	*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*				*R.stagni* *R.gilardii*		
biphenyl-2,3-diol 1,2-dioxygenase /1,2-dihydroxynaphthalene dioxygenase	bphC /nahC/doxG/etbC/pahC			*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*				*R.aerilata*		
catechol-2,3-dioxygenase	nahH			*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*				*R.stagni* *R.gilardii* *R.aerilata*		
1 4-hydroxybenzoate 3-monooxygenase	HBM	*S.americanum* *S.saheli* *S.meliloti*	*P. kroppenstedtii*	*N.aromaticivorans* *N.panipatense*	*Georgenia sp.*			*R.stagni* *R.gilardii* *R.aerilata*		*T.mobilis*
alkane-1 monooxygenase	alkB			*N.aromaticivorans* *N.panipatense*						
salicylate hydroxylase	nahG/nahW	*S.americanum* *S.saheli*	*P. kroppenstedtii*	*N.aromaticivorans* *N.naphthalenivorans*	*Georgenia sp.*	*S.novella*	*Ancylobacter sp.* *A.rudongensis*	*R.stagni* *R.gilardii*	*M.trichosporium* *Methylopila sp.*	*T.mobilis*

		S.meliloti		*N.panipatense*				*R.aerilata*		
1 2-carboxybenzaldehyde dehydrogenase	phdK	*S.meliloti*		*N.naphthalenivorans*				*R.aerilata*	*Methylopila sp*	
naphthalene dioxygenase/ferredoxin	doxA/NahAb/nagAb/pahAb	*S.americanum* *S.saheli* *S.meliloti*	*P. kroppenstedtii*	*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*	*Georgenia sp.*			*R.stagni* *R.gilardii* *R.aerilata*		*T.mobilis*
reductase naphthalene dioxygenase/ ferredoxin reductase	nahAa/nagAa/pahAa	*S.saheli*		*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*						*T.mobilis*
benzoate 1,2-dioxygenase ferredoxin	benC	*S.americanum*								
salicylaldehyde dehydrogenase	PhnF/nahF/doxF/nagF/pahF	*S.saheli*	*P. kroppenstedtii*	*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*				*R.gilardii* *R.aerilata*		
2-hydroxymuconic semialdehyde dehydrogenase	nahI			*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*						*T.mobilis*
Cis-naphthalene dihydrodiol dehydrogenase	nahB/doxE/nagB/phnB/pahB/nidC/bphB	*S.saheli*		*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*						*T.mobilis*
hydratase-aldolase	nahE/phnE/pahE/nidD			*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*						
2-hydroxychromene-2-carboxylate isomerase	nahD/phnD/pahD	*S.americanum*		*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*		*S.novella*	*Ancylobacter sp.* *A.rudongensis*	*R.stagni* *R.aerilata*	*Methylopila sp*	*T.mobilis*
2-hydroxymuconic semialdehyde hydrolase	nahN	*S.saheli*		*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*						*T.mobilis*
Ferredoxin	phdC		*P. kroppenstedtii*							
Ferredoxin reductase	phdD	*S.americanum* *S.saheli* *S.meliloti*	*P. kroppenstedtii*	*N.aromaticivorans* *N.naphthalenivorans* *N.panipatense*	*Georgenia sp.*	*S.novella*	*Ancylobacter sp.* *A.rudongensis*	*R.stagni* *R.aerilata*	*M.trichosporium* *Methylopila sp.*	*T.mobilis*
extradiol dioxygenase	phnC	*S.saheli*								*T.mobilis*

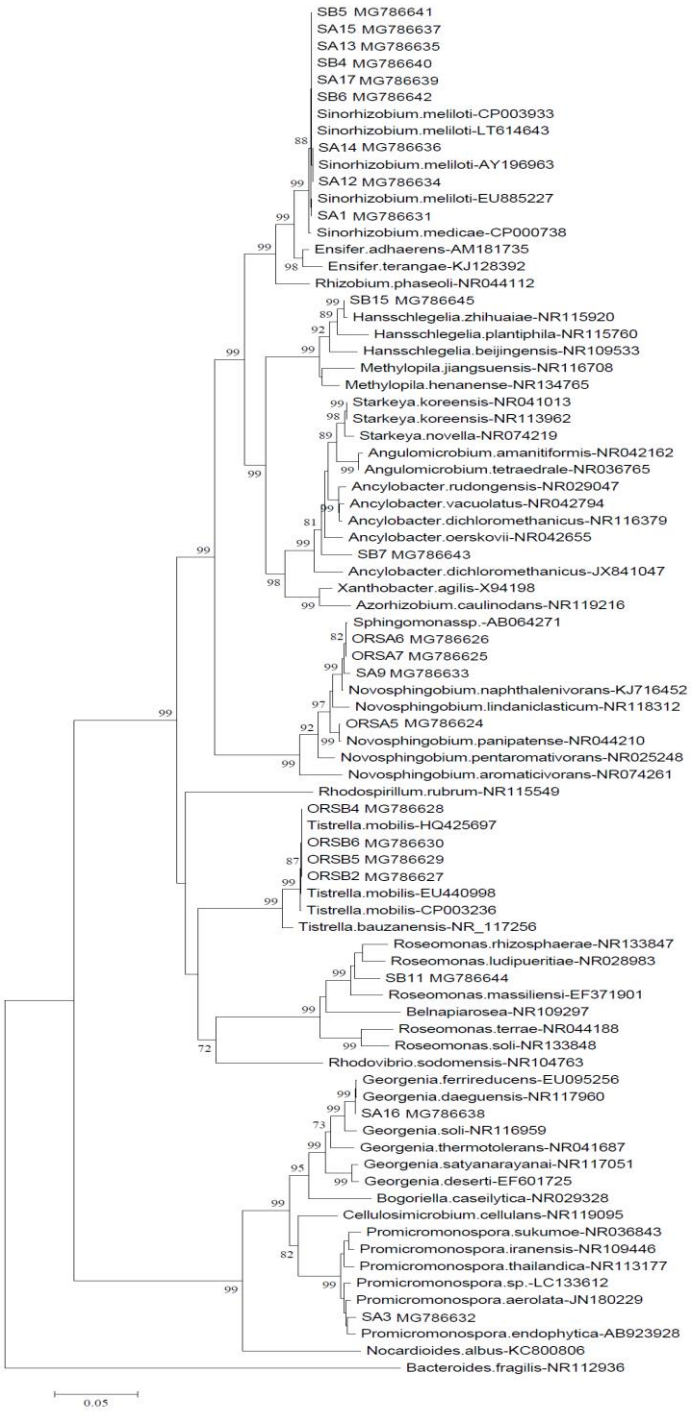

Figure 1 Phylogenetic tree based on 16SrRNA gene sequence (>900nt) analysis of new and reference strains. Neighbor-joinin method integrating Kimura-2distance was used. Data are bootstrap values issued from 1000 repetitions

The fifth cluster was close to *Tistrella mobilis* and included four strains, ORSB4, ORSB6, ORSB5 and ORSB2 isolated from oil refinery sludge B. The genus *Tistrella* was isolated from soil samples contaminated with polycyclic aromatic hydrocarbon (PAH)-containing waste from oil refinery field in Shanghai, China and can increase the speed of phenanthrene degradation (**Zhao *et al.*, 2008**), the species *Tistrella bauzanensis* was isolated from soil containing high levels of heavy oil (**Zhang *et al.*, 2011**)howeverthe species *Tistrella mobilis* was isolated only from wastewater, sea water and surface sediment collected from deep sea contaminated by hydrocarbons and proved its efficiency in this pollutants degradation (**Shi *et al.*, 2002 ; Cuiand Shao, 2009**). The sixth cluster comprised one strain SB11 isolated from oil polluted soil B and it was close to *Roseomonas ludipueritiae*. The genus *Roseomonas* of the familly *Acetobacteraceae* belonging to phylum *Proteobacteria*, was first described by **Rihs *et al.* (1993)** associated with bacteraemia and other humain infections. Recently, a new strain *Roseomonas nepalensis*was isolated from oil-contaminated soil of Nepal, during a study of oil-utilizing bacteria (**Chaudhary and Kim, 2017**). The seventh cluster included one strain SA16 isolated from oil polluted soil A and it was close to *Georgenia ferrireducens*.**Franzetti *et al.*(2011)** isolated *Georgenia ferrireducens* as bacteria able to produce biological emulsifiers from a polycyclic aromatic hydrocarbons (PAHs) contaminated soil. The eighth and last cluster comprised one strain SA3 isolated from oil polluted soil A and it was close to *Promicromonospora aerolata*.it was reported that *Promicromonospora aerolata*s pecies was isolated from air (**Busse *et al.*, 2003**). However, several species belonging to the genus *Promicromonospora* were isolated from soil; recently, two genera isolated from soil were added: *Promicromonospora soli* (**Zhang *et al.*, 2017**) and *Promicromonospora kermanensis* (**Mohammadipanah *et al.*, 2017**), however, there was no reports in their ability to survive in hydrocarbon polluted soils.

Distribution of genes coding for dgradation enzymes in isolated genera

Different genes encoding for hydrocarbons degradation were investigated through Genbank (tBLASTn) databases. Many studies dealing with total genome sequencing revealed the presence of these genes of interest (Table 4), but without proving their real ability to degrade hydrocarbons *in vitro* (*Sinorhizobium americanum, S. saheli, Promicromonospora kroppenstedtii, Starkeya novella, Ancylobacter* sp., *Methylosinus trichosporium, , Methylopila* sp., *Ancylobacter rudongensis, Roseomonas stagnii, R. gilardi,* and *R. aerilata*); Only seven species did degrade hydrocarbons and possessed these genes of interest : *Sinorhizobium meliloti* which was reported as PAH-degrading (**Ahmad *et al.*, 1997**) and harbored several putative genes of degradation (**Galibert *et al.*, 2001**); *Novosphingobium aromaticivorans* (**Fredrickson *et al.*, 1991 , 1995; Romine *et al.*, 1999**) ; *N. naphthalenivorans* (**Suzuki and Hiraishi, 2007**); *N. panipatense* (**Gupta *et al.*, 2009**) ; *Methylosinus sp*. (**Bowman *et al.*, 1993**); *Georgenia sp*. (**Al-Awadhi *et al.*,2007**); *Tistrella mobilis* (**Cui *et al.*, 2009**). Finally ; *Hansschlegelia zhihuaiae*did degrade hydrocarbon in this study but no work did reveal the gens of degradation.

CONCLUSION

In conclusion a high diversity of hydrocarbon-degrading bacteria were found in crude oil polluted soil and oil refinery sludge from Arzew refinery, cultured-method allowed to obtain different species known for their degradative capability and to demonstrate species never known for hydrocarbon degradation. A limited number of genes coding for degradation enzymes were revealed in these genera *Promicromonospora*, *Georgenia*, *Starkeya*, *Ancylobacter* and *Methylocystaceae* family. However, as a perspective it is interesting to look for these genes of interest in the isolated species belonging in these genera or complete sequencing of the genome.

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