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Diversity of culturable aerobic bacteria colonizing four petroleum by-products storage reservoirs

Saïd Ben Hamed^{1,2}, Zouhaier Ben Ali Gam², Raja Rezgui¹, Abdeljelil Ghram¹, Abderrazak Maaroufi¹ and Marc Labat²

¹Laboratory of Microbiology, Bioprocess group, Institute Pasteur of Tunisia (IPT), Tunisia. Bp 74, 13 place Pasteur, Belvédère –1002, Tunis, Tunisia.

²Laboratoire de Microbiologie IRD, Biotechnologie Microbienne des Environnements, Chauds UMR D180, IRD ESIL, Marseille cedex 9, France.

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The petroleum reservoirs are commonly exposed to bacterial contamination. The proliferation of several bacterial species, could modify the product quality, change viscosity, help other sulfate-reducing, iron reducing bacteria to grow, and accelerate reservoir biocorrosion. Thus, companies of storage and transport of petroleum frequently use biocides to prohibit bacterial proliferation. However, the use of the appropriate biocide, need an obvious knowledge of bacterial population harbouring the reservoir. Thus, in this study, we intended to isolate and identify the aerobic bacterial population existing in different reservoirs of petroleum derivatives in the refinery of Bizerte, Tunisia. The isolation and identification of these microorganisms may be of great interest in bioremediation of polluted sites. Besides, it could inform about hydrocarbon specificity of these microorganisms and therefore lead to the use of suitable biocides to clean these storage reservoirs. 25 different bacterial strains were isolated from water collected from reservoirs of crude oil, gasoline, diesel oil and kerosene. The different strains were identified using morphological and biochemical characterization, as well as sequencing of 16S rRNA. The systematic study of these strains showed that they belong to six different genera. The highest strain number was affiliated to the genera of *Pseudomonas* and *Bacillus* followed by genera of *Staphylococcus*, *Enterobacter*, *Comamonas* and *Paenibacillus*. The results of this study are discussed in terms of the ecological significance of these microorganisms in relation with physico-chemical conditions of reservoirs and hydrocarbon composition.

Key words: Bacterial diversity, storage reservoirs, phylogenetic relationships.

INTRODUCTION

In natural fields, petroleum is generally found mixed with water that is frequently used in petroleum extraction process. Commonly, after extraction, the crude oil undergoes a process of decantation aimed at eliminating residual water. Although decantation steps are applied after extraction of crude oil and before storage of different crude oil derivatives (gasoline, kerosene, diesel oil) in petroleum refineries, storage reservoirs usually contain

variable quantities of water. These residual quantities of water can harbour indigenous microorganisms from the petroleum field or exogenous microorganisms, which uses mineral salts of water and petroleum and are able to use hydrocarbon as sole carbon source (Cunha et al., 2006). Consequently, these populations of microorganisms usually create serious problems because they modify the product quality (Cunha et al., 2006), help other

sulfate-reducing and iron reducing bacteria to grow, and accelerate reservoir biocorrosion (Magot et al., 2000). These strains, frequently known as biosurfactant producers, can emulsify hydrocarbons, changing viscosity and chemical properties.

The ability to degrade hydrocarbon compounds is exhibited by a wide variety of bacterial and fungal genera isolated from soil environment, seawater, saline sites, and even hot environments such as hot spring (Floodgate, 1984).

Based on bibliographic data, the most important hydrocarbon-degrading bacterial genera reported are *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Nocardia*, and *Pseudomonas* (Leahy and Colwell, 1990).

Rosenberg et al. (1975) were able to demonstrate removal of residual oil from the ballast of oil reservoirs by microbial seeding, although the removal appeared to result primarily from bioemulsification rather than biodegradation. Hydrocarbons differ in their susceptibility to microbial attack and they were generally ranked in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (Leahy and Colwell, 1990).

Isolation and identification of these microorganisms may be of great use in bioremediation of polluted sites. Knowledge about hydrocarbon specificity of these microorganisms could guide the use of suitable biocides to clean these storage reservoirs.

The present study was conducted to determine bacterial diversity in storage reservoirs of crude oil, gasoline, kerosene and diesel oil situated in the petroleum refinery of Bizerte (STIR), Tunisia. For this purpose, we isolated, characterized and phylogenetically classified the culturable bacterial population in the residual water collected from these four petroleum storage reservoirs. We also evaluated the isolates for their ability to grow in nutrient-poor media with phenol, catechol and benzoic acid as sole carbon sources.

MATERIALS AND METHODS

Sampling

Samples (3 L) were taken from water existing in the bottom part of storage reservoirs situated in the petroleum refinery of Bizerte, Tunisia. The reservoirs, with fixed roof and internal floating, were used to store crude oil, diesel oil, kerosene and gasoline. Samples were collected in pre-sterilised glass bottles and sterile conditions were maintained.

Enrichment and isolation of bacterial strains

Bacterial enrichment was conducted using liquid basal salt medium containing, per litre, 0.5 g KH_2PO_4 , 1.5 g K_2HPO_4 , 1.0 g NH_4Cl , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 g NaCl (SIGMA-ALDRICH) and 1.0 ml of trace metal solution. The trace metal solution contained, per liter, 1.5 g nitrilotriacetic acid, 5.0 g $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 0.01 g FeSO_4 , 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 g CoCl_2 ,

0.01 g $\text{Al}_2(\text{SO}_4)_3 \cdot 16\text{H}_2\text{O}$, 0.01 g H_3BO_3 and 0.01 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (SIGMA-ALDRICH) (Van Hamme and Ward, 2001). Crude oil at 1% (v/v) served as sole carbon source. Enrichment tests were conducted in 250 ml Erlenmeyer flasks with working volume of 100 ml on a rotary shaker (150 rpm, 30°C, and initial pH 7.2). After 48 h incubation, 0.1 ml of liquid culture suspension was spread on agar plate containing basal salt medium, amended with 18 g l⁻¹ agar and 1% (v/v) crude oil as a sole carbon source and then incubated for 48 h. Colonies were selected and purified several times to obtain pure cultures.

After purification of all strains on medium containing only crude oil as sole carbon sources, each strain were tested for growth on kerosene, gasoline and diesel oil separately. These growth tests were conducted in 250 ml Erlenmeyer flasks with working volume of 100 ml on a rotary shaker (150 rpm, 30°C, and initial pH 7.2). After 48 h of incubation, the increase in biomass was quantified by measuring the OD at a wavelength of 600 nm (BOECO S-22 UV/Vis. spectrophotometer, Germany). Growth tests were considered negative when the $\text{OD}_{600\text{nm}} \leq 0.1$. The reading of OD_{600} is frequently used in studies characterizing the physiology of hydrocarbon utilization (Ciric et al., 2010).

Characterization of isolates

Phenotypic characterization of isolates was based on phenotypic characteristics of colonies, microscopic analyses of cells, Gram staining, motility, catalase and oxidase tests. Further biochemical analysis was performed using commercially available miniaturized multitest identification systems: API (BioMerieux, France).

API 20E, API 20 NE, API Staph and API 50 CH kit system (BioMerieux) were inoculated and used according to the manufacturer's instructions. The API 20E, API 20NE, API Staph and API 50CH were designed for identifying members of the *Enterobacteriaceae*, Nonenteric Gram-negative rods; *Staphylococcus* and *Bacillus* respectively. APIWEB software was used for identification and was considered acceptable when given a probability of 80% or greater (BioMerieux, France) (Awong-Taylor et al., 2008; Logan and Berkeley, 1984).

DNA extraction

10 ml of bacterial culture were centrifuged (8000 g) for 10 min, the supernatant was removed and 480 µl of EDTA (50 mM), 140 µl of TE (10 mM Tris and 1 mM EDTA), and 40 µl of lysosyms (20 mg/µl) (SIGMA-ALDRICH) was added. After incubation at 37°C for 30 min, the mixture was centrifuged at 13000 g for 2 min and the supernatant was discarded. For cell lysis, 600 µl of nucleus lysis solution (SIGMA-ALDRICH) were added, and then the preparation was incubated at 80°C for 5 min then kept at ambient temperature for 30 min. 3 µl of Rnase solution were added to the preparation that was incubated at 37°C for 15 min. For protein precipitation, 200 µl of precipitation solution was added. After incubation on ice for 5 min, the preparation was centrifuged at (13000 g), and the supernatant was recuperated; the DNA was then precipitated with isopropanol and dehydrated with Tris before it was incubated for 1 h at 65°C.

PCR amplification of 16S rDNA

The 16S rRNA gene of isolated strains was amplified using PCR. 1 µl of cell culture was added to a thermocycler microtube containing 5 µl of 10 x Taq buffer, 0.5 µl of each 50 nM Fd1 and Rd1 primers, 5 µl of 25 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 µl of 25 mM dNTPs, 0.5 µl of Taq polymerase (5U µl⁻¹), and 38 µl of sterile distilled water. Universal primers Fd1 and Rd1 (Fd1, 5' AGAGTTTGATCCTGGCTCAG-3'

Table 1. Physico-chemical characteristics of different water samples collected from reservoirs of different petroleum by-products and numbers of bacterial strains isolated from each reservoir.

Parameter	Crude oil	Kerosene	Gasoline	Diesel oil
Temperature (°C)	19.8	33.5	29	32.8
pH	7.3	6.6	5	8
Salinity (mg/l)	68	41	28	30
Number of isolated strains	35	22	10	13

and Rd1, 5'-AAGGAGGTGATCCAGCC-3') (Abdelkefi et al., 2005) were used to obtain a PCR product varying between 1136 and 1500 kb. The sample was placed in a hybrid thermal reactor thermocycler (Perkin Elmer Gene Amp PCR system 2400, S/N N02529) denatured for 1 min at 95°C and subjected to 30 cycles for 20 s at 95°C, 30 s at 55°C, and 1 min and 50 s at 72°C. This was followed by a final elongation step for 5 min at 72°C. The PCR products were analysed on 1% (w/v) agarose gel added with 0.7 µl of BET and sent to GATC Biotech AG (Laborteam, Jakob-Stadler-Platz7, Germany) for sequencing. The sequences were aligned with Clustal X and Genedoc software and compared with available sequences in GenBank using the BLAST search. Phylogenetic analyses were performed using mega 3.1 software (Kumar et al., 2001). A neighbour-joining tree was constructed using the kimura 2-parameter model (Kimura, 1980) of evolution, and support for internal branches was assessed using 1000 bootstrap replications. Only bootstrap values above 50% are shown.

Enzymatic digestion with endonuclease

Strain variability was confirmed by Amplified ribosomal DNA restriction analysis (ARDRA) technique.

Enzymatic digestions were performed by incubating 5 µL of the PCR products with 10 U of each endonuclease and the corresponding enzyme buffer. Digestions were continued for 1 h at 37°C for *HaeIII* and *RsaI*. Digested products were analysed on 2% (w/v) agarose gels (Arturo et al., 1995).

In this study, two strains were considered similar when they have the same morphological, biochemical characteristics and the same ARDRA patterns.

Biodegradation of aromatic compounds

Isolated strains were tested for their abilities to aerobically metabolize three aromatic compounds: phenol, catechol and benzoic acid at 30°C and under agitation (150 rpm). 10 mM of aromatic compounds were added directly into flasks containing 50 ml basal salt medium, without glucose. Bacterial growth was confirmed microscopically as well as an increase in the OD₆₀₀ nm, and cultures containing aromatic compound was considered positive for growth. Two types of control cultures were included; without substrates with bacterial inoculation and with substrates without bacterial inoculation.

The degradation of the tested aromatic compounds was confirmed by HPLC analysis after 5 days incubation.

RESULTS AND DISCUSSION

Physico-chemical analysis

The physico-chemical characteristics of the studied sam-

ples are shown in Table 1. The inner temperature of reservoirs ranged between 19.8 and 33.5°C, which is comparable to ambient temperature in Bizerte. The pH of the residual water of gasoline reservoir was acidic (pH 5), whereas the pH of the residual water of diesel oil seemed to be alkaline (pH 8). For the crude oil and the kerosene, we measured neutral pH (7.3 and 6.6, respectively). Inversely, for the salinity, the residual water of kerosene and crude oil had the highest salinities (41 and 68 mg l⁻¹, respectively). The high salinity of crude oil reservoir was related to the salinity of petroleum fields. Indeed, water is usually added to the process of petroleum extraction to decrease its salinity that may obstruct pipes. Salinity may be a limiting factor in hydrocarbon biodegradation. In fact, Leahy and Colwell (1990) demonstrated that the rates of hydrocarbon metabolism decrease with increasing Salinity, (in the range of 3.3 to 28.4%). Other studies demonstrated that, even in high salinity environment, hydrocarbon can be subjected to biodegradation (Atlas, 1981). Besides, the pH influences hydrocarbon metabolism and favours selection of microbial population. Leahy and Colwell (1990) reported a nearly twofold increase in biodegradation rates of gasoline in an acidic soil (pH 4.5) by adjusting the pH to 7.4; these rates dropped significantly, however, when the pH was further raised to 8.5.

Microbial analysis

After several dilutions and subculturing in the same liquid medium, with 1% of crude oil as sole carbon source, under aerobic conditions, colonies were isolated in the agar medium. A total of 80 strains were isolated under aerobic conditions from all samples. The reservoir of crude oil contained the highest number of isolates (35 isolates), followed by the reservoir of kerosene (22 isolates), the reservoir of diesel oil (13 isolates) and the reservoir of gasoline (10 isolates) (Table 1).

For each strain, 32 biochemical tests were performed. The biochemical tests included enzymes production (urease, tryptophan desaminase, etc), fermentation/oxidation of different glucoses (D-glucose, L-rhamnose, L-arabinose, etc), and NO₃ and NO₂ reduction, etc. All biochemical tests were performed using commercially available miniaturized multitest identification systems: API (BioMerieux, France) (API 20E, API 20 NE, API Staph and API 50 CH).

In this study, we considered that two strains are similar when their phenotypic characteristics of the colonies (colony morphology, colony size, pigmentation etc.), the cells characteristics (microscopic analysis), the biochemical tests, and ARDRA patterns (digestion by two enzymes *HaeIII* and *RsaI*) are similar.

Thus, among all isolates (80 isolates), only 25 different isolates were selected and examined in greater detail. These strains were identified by analyzing sequences of genes encoding 16S rRNA (Figure 1).

Microbial analyses of different selected isolates (25 isolates) showed that 44% are Gram negative and 56% are Gram positive. A predominance of rod shaped (72%) microorganisms compared to cocci forms (28%) was registered. Bacterial population comprised motile or non motile cells, different types of colony (irregular, circular, spreading) with diameter ranges from 1 to 3 mm after 24 h of incubation (Table 2).

The 16S rRNA gene sequencing of these strains showed that they belong to six different genera. The greatest number of isolates were affiliated to the genera of *Pseudomonas* and *Bacillus* (7 strains for each genus), followed by *Staphylococcus* (6 strains) then *Enterobacter* (3 strains) and finally *Comamonas* and *Paenibacillus* genera (1 strain for each genus) (Figure 1).

Phylogenetic relationships among isolated strains, as compared to the 16S rRNA gene sequences in the GenBank and represented as a phylogenetic tree (Figure 1), showed that S29 and S17 strains are 99% similar to *Bacillus licheniformis* (JN180125.1 and AJ717380.1) isolated from underground gas storage and nonsaline alkaline environment, respectively. The isolates SS25, S212 and SSS13 were related to *Bacillus pumilus* (GQ152134.1 and EF173329.1) strains isolated from soil contaminated with chlorinated hydrocarbon, and characterised as alkali-philic. The six strains belonging to the genus of *Staphylococcus*, showed 99% similarity to the heterotrophic aerobic *Staphylococcus* strains, isolated from nonsaline alkaline environment (Tiago et al., 2004). The strains of the genus of *Enterobacter* showed homology with isolates characterized as chlorophenol and lignin degrading aerobic bacteria and 1,3-propanediol fermenting bacteria (GU193983.1 DQ185604.1). All strains identified in this study as *Pseudomonas*, presented homology with species isolated from specific environment, characterised by an alkaline pH (NR024734.1; AB109888.1), saline-alkali soil (JF727663.1) or found as indigenous microorganisms in oil reservoirs (HM030754.1).

Study of bacterial distribution in various reservoirs

The examination focused on the origin of the 25 selected isolates (Table 3) showed that all these strains were present in the crude oil reservoir. 13 isolates existed in both crude oil and kerosene reservoirs, eight strains existed in both crude oil and gasoline reservoirs and five isolates existed in both crude oil and diesel oil reservoirs.

Two strains (S23 and SS21) existed in crude oil, kerosene and diesel oil reservoirs. Only one strain (S212) existed in crude oil, kerosene and gasoline reservoirs. Further, we did not find any strains which exist in all reservoirs (Table 3).

Confirmative tests of growth of each strain on all studied petroleum derivatives were performed (in separate time after subculturing on liquid mineral medium with crude oil as sole carbon source for example strains isolated from kerosene reservoir were tested for kerosene, gasoline and diesel oil uses as sole carbon source) (Table 3).

The existence of all strains in the reservoir of crude oil indicated that salinity did not represent a limiting factor for these isolates and that all strains were tolerant to salt concentrations at 68 mg/l. Yoshida et al. (2005) reported the dominance of Gram-positive bacteria in the storage reservoir of crude oil; however, we did not find any clear dominance based on Gram-staining. The isolation of all strains from the reservoir of crude oil could be related to its heterogenic composition. Indeed, Leahy and Colwell (1990) reported that compositional heterogeneity, among different crude oils and refined products, influences the overall rate of biodegradation of the oil and of its component fractions.

The major constituents of Kerosene are alkanes and cycloalkanes (65 to 70%), benzene and substituted benzene (10 to 15%), naphthalene and substituted naphthalene (ASTM 2001) (Saratale et al., 2007; Wongsu et al., 2004). In this study, the greatest number of strains was isolated from the reservoir of Kerosene (13 strains); this can be related to pH that was almost neutral (pH 6.6). This confirms the results of Shabir et al. (2008) who showed that, generally, hydrocarbons in kerosene are not inhibitory to microbial activity and will biodegrade significantly, provided that sufficient amounts of essential nutrients are present. Moreover, Vieira et al. (2007) demonstrated that n-alkane compounds are the most susceptible to microbial attack, followed by branched alkanes, aromatics of low molecular weight. The isolated strains belonged to the genera *Bacillus*, *Staphylococcus* and *Pseudomonas*; all are well known to biodegrade a large spectrum of hydrocarbons, and are frequently isolated from polluted sites; the predominance of one genus is related to environmental conditions such as pH and salinity (AL-Saleh et al., 2009). In the current study, 23% of strains isolated from the reservoir of kerosene were able to degrade phenol, 15% were able to degrade catechol and 7.6% were able to degrade benzoic acid (Table 2).

The isolated *Bacillus* strains were affiliated to the species *B. licheniformis*, *B. firmus*, *B. pumilus*, and *B. subtilis*. In a previous study, we isolated two strains of *B. licheniformis* and *B. firmus* from a bioreactor designed for the biodegradation of the residual hydrocarbon issued from the refining process in the petroleum refinery of Bizerte. Biodegradation assays demonstrated that the

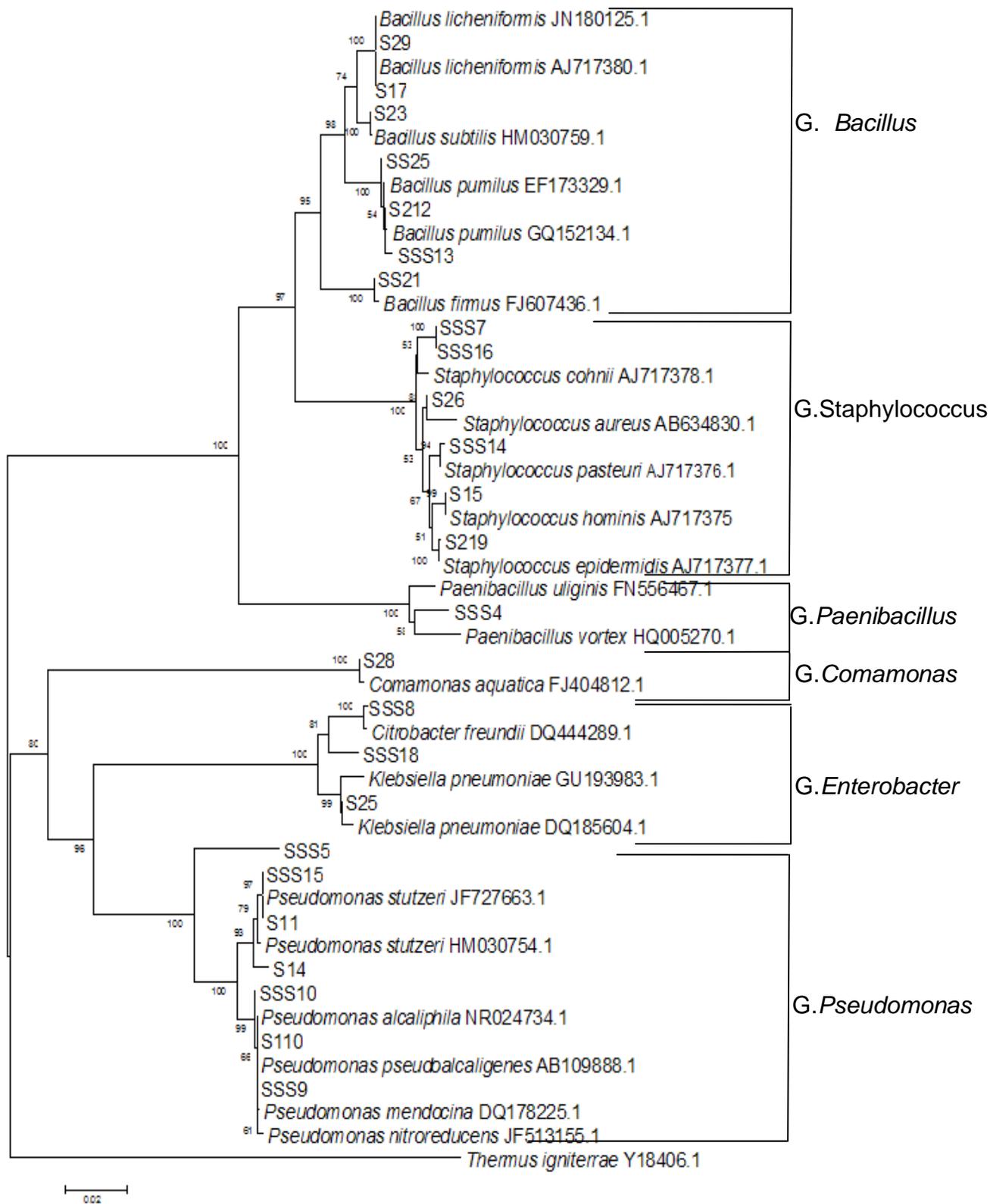


Figure 1. Phylogenetic dendrogram based on 16S rRNA gene sequences data, showing the position of bacterial population isolated from all petroleum derivatives reservoirs. The dendrogram was constructed using the kimura 2-parameter model (Kimura, 1980) of evolution. Bootstrap values (expressed as percentages of 100 replications) greater than 50 % are shown at branch points. Bar: 2 % sequence divergence. Numbers are Genbank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences.

Table 2. Morphological and biochemical characterization of bacterial isolates recovered from petroleum reservoirs in Bizerte, Tunisia.

Str	Res	Tx.S	C.m	Cs	Cd	Pg	Cel.ar	Cl.Sh	Grm	Mot	ox	Cat	Ph	Cte	Bnz
S11	K,C	<i>P.sp</i>	irr	2.3	T	W/Y	S,P	Rs	-	+	+	+	+	+	+
S14	K,C	<i>P.sp</i>	irr	2.3	T	W/Y	S,P	Rs	-	+	+	+	-	-	-
S15	G,C	<i>S.sp</i>	cir	1	T	W/Y	P,in4	Co	+	-	-	+	-	-	-
S17	K,C	<i>B.sp</i>	irr	3	om	Cr	S,P	Rs	+	+	+	+	-	-	-
S23	K,C,D	<i>B.sp</i>	cir	2	om	Cr	S,P	Rs	+	+	+	+	-	-	-
S25	G,C	<i>K.sp</i>	cir	2	M	NP	S,P	Rs	-	-	-	+	+	-	+
S26	G,C	<i>S.sp</i>	cir	1	T	W/Y	P,in4	Co	+	-	-	+	-	-	-
S28	C	<i>C.sp</i>	irr	2.3	o	Y/W	S,F	Rs	-	+	-	+	-	-	-
S29	K,C	<i>B.sp</i>	irr	3	om	Cr	S,P	Rs	+	+	+	+	-	-	-
S110	G,C	<i>P.sp</i>	irr	2.3	T	NP	SP	Rs	-	+	+	+	-	-	-
S212	G,K,C	<i>B.sp</i>	cir	1	om	Cr/W	S,P	Rs	+	+	-	+	+	-	-
S219	K,C	<i>S.sp</i>	cir	1	T	W/Y	P,in4	Co	+	-	-	+	-	-	-
SS21	K,C,D	<i>B.sp</i>	irr	2	Tm	W	S,P	Rs	+	+	+	+	-	-	-
SS25	C,D	<i>B.sp</i>	cir	1	om	Cr/W	S,P	Rs	+	+	-	+	-	-	-
SSS4	G,C	<i>Pa.sp</i>	irr	3	Tm	W	S,P	Co	+	+	+	-	+	-	-
SSS5	K,C	<i>P.sp</i>	irr	2.3	T	B/b	S,P	Rs	-	+	+	+	-	-	-
SSS7	K,C	<i>S.sp</i>	cir	1	o	Y	S,P	Co	+	-	-	+	-	-	-
SSS8	C	<i>Ci.sp</i>	cir	1	T	W	S,P	Rs	-	+	-	+	+	-	-
SSS9	D,C	<i>P.sp</i>	irr	2.3	T	Y	SP	Rs	-	+	+	+	+	+	-
SSS10	K,C	<i>P.sp</i>	irr	2.3	T	W	S,P	Rs	-	+	+	+	-	+	-
SSS13	G,C	<i>B.sp</i>	cir	1	om	Cr/W	S,P	Rs	+	+	-	+	+	-	-
SSS14	K,C	<i>S.sp</i>	cir	1	T	W/Y	P,in4	Co	+	-	-	+	+	-	-
SSS15	D,C	<i>P.sp</i>	irr	1	T	Y	S,P	Rs	-	+	+	+	+	-	+
SSS16	K,C	<i>S.sp</i>	cir	1	o	Y	P,in4	Co	+	-	-	+	-	-	-
SSS18	G,C	<i>Ci.sp</i>	cir	1.2	T	W	S,P	Rs	-	+	-	+	-	-	-

Str, Strain; Res, reservoir; K, kerosene; C, Crude oil; G, gasoline; D, diesel oil; Tx.S, Taxonomical status; *P.sp*, *Pseudomonas.sp*; *S.sp*, *Staphylococcus.sp*; *B.sp*, *Bacillus.sp*; *K.sp*, *Klebsiella.sp*; *C.sp*, *Comamonas.sp*; *Pa.sp*, *Paenibacillus.sp*; *Ci.sp*, *Citrobacter.sp*; C.m, Colonial morphology; irr, irregular; Cir, Circular; Cs, Colony size (mm); Cd, Colony density; T, Translucent; om, opaque matt; M, mucoid; o, opaque; Tm, Translucent matt; Pg, pigmentation; W, White; Y, Yellow; Cr, Cream; NP, No pigmentation; B, Bright; b, brown; Cel.ar, Cell arrangement; S, Single; P, Paired; Cl.Sh, Cell shape; RS, Rod shaped; Co, Cocci; Grm, Gram; Mot, Motile; ox, oxidase; Cat, Catalase; Ph, Phenol degradation; Cte, Catechol degradation; Bnz, Benzoic acid degradation.

strains of *B. licheniformis* and *B. firmus* are able to tolerate hydrocarbon concentrations of 800 and 6300 mg/l, respectively (Ben Hamed et al., 2010). *B. pumilus* was isolated from solid waste crude oil samples, collected from the clean up of oil storage containers (Calvo et al., 2004). This particular strain showed a high emulsion capacity of crude oil, an ability to use naphthalene as a sole carbon source, and demonstrated an important mineralisation rate of crude oil that can reach 23 µg/min (AL-Saleh et al., 2009). The two isolated strains SS21 and S23, related to *B. firmus* DQ 118015.1 and *B. subtilis* HM030759.1, respectively, existed in both kerosene and diesel oil reservoirs. The OD₆₀₀ of these strains, when they were cultured on kerosene and diesel oil separately (Table 3), ranged between 0.21 and 0.36. This recommends their ability to use heavy chain hydrocarbons.

The strains belonging to the genus *Staphylococcus*, isolated from the reservoir of kerosene (S219, SSS14, SSS16, SSS7) were affiliated to *S. pasteurii*, *S. succinus* and *S. epidermidis* with a percentage of similarity of 99%,

whereas, the two strains (S26 and S15) of *Staphylococcus* which were affiliated to *S. aureus* and *S. hominis*, were isolated from the reservoir of gasoline. These strains of *Staphylococcus*, isolated initially on crude oil as sole carbon source, were able to use kerosene or gasoline (0.11 ≤ OD_{600nm} ≤ 0.16) (Table 3).

In their study, Ijah and Antai (2003) showed that *S. aureus* isolated from sediment polluted with crude oil was unable to degrade crude oil. Isola-Kayode et al. (2008) demonstrated that *S. aureus* was sensitive to 5% of diesel oil. AL-Saleh et al. (2009) proved that *S. hominis*, isolated from the coast of Kuwait, was able to degrade hydrocarbon but only at low concentration (0.06%). The diversity in hydrocarbon uses for *Staphylococcus* was explicated by AL-Saleh et al. (2009) who suggested high degree of crude oil-degrading bacterial population at the strain level but low diversity at the genus level. In this study, all reservoirs contained, at least, one strain of *Pseudomonas*. Indeed, *Pseudomonas* strains have frequently been found in various sites polluted by petroleum

Table 3. Distribution of the 25 selected strains following to their origin of isolation, their taxonomic affiliation and the OD₆₀₀ of each isolates cultured on different petroleum by-products.

Cluster	Strain	Origin(s) of strain				Growth tests on different petroleum derivatives (OD ₆₀₀)		
		Crude oil	Kerosene	Gasoline	Diesel oil	Kerosene	Gasoline	Diesel oil
<i>Bacillus</i>	S17	+	+			0.28	0.03	0.00
	S23	+	+		+	0.36	0.00	0.23
	S29	+	+			0.28	0.01	0.00
	SS21	+	+		+	0.33	0.01	0.21
	SS25	+			+	0.02	0.01	0.14
	S212	+	+	+		0.19	0.28	0.03
	SSS13	+		+		0.00	0.16	0.00
<i>Pseudomonas</i>	SSS5	+	+			0.13	0.02	0.02
	SSS9	+			+	0.02	0.03	0.20
	SSS10	+	+			0.22	0.04	0.02
	S11	+	+			0.21	0.04	0.01
	S14	+	+			0.18	0.00	0.01
	S110	+		+		0.00	0.15	0.00
	SSS15	+			+	0.01	0.03	0.21
<i>Staphylococcus</i>	S26	+		+		0.03	0.16	0.02
	S219	+	+			0.16	0.01	0.00
	SSS14	+	+			0.12	0.00	0.01
	SSS16	+	+			0.16	0.05	0.03
	SSS7	+	+			0.11	0.03	0.03
	S15	+		+		0.02	0.14	0.01
<i>Enterobacter</i>	SSS8	+				0.00	0.01	0.00
	SSS18	+		+		0.06	0.16	0.02
	S25	+		+		0.04	0.12	0.00
<i>Paenibacillus</i>	SSS4	+		+		0.07	0.23	0.01
<i>Comamonas</i>	S28	+				0.00	0.00	0.00

Growth tests were considered negative when the OD_{600nm} ≤ 0.1

and petroleum derivatives (AL-Saleh et al., 2009; Floodgate, 1984; Richard and Vogel, 1999; Vieira et al., 2007), suggesting that this genus, effectively, metabolizes hydrocarbon molecules and may dominate bacterial populations in media where petroleum pollution has occurred.

Only five strains were isolated from diesel oil reservoir. These strains belonged to the genera of *Pseudomonas* and *Bacillus*. Considering that the pH is alkaline (pH 8), it was common to isolate alkalotolerant or alkalophile strains, such as SSS9 and SSS15, related to *P. alcaliphila* NR024734.1 and *P. stutzeri* CP002622.1, respectively. Saratale et al. (2007) reported that *Pseudomonas* strains have a mixed-functional oxidase system, inducible by n-alkanes as well as polyaromatic hydrocarbons. Furthermore, diesel oil is a complex fuel mixture, primarily

consisting of paraffinic, olefinic and aromatic hydrocarbons, and smaller quantities of substances containing sulfur, nitrogen, metals, and oxygen. The hydrocarbon molecules contain usually from 8 to 40 atoms of carbon (Vieira et al., 2009). In this study, 40% of strains isolated from diesel oil reservoir were able to degrade phenol, 20% were able to degrade catechol and 20% were able to degrade benzoic acid (Table 2).

Four out of the eight strains, isolated from the gasoline reservoir, were able to degrade phenol (S25, S212, SSS4, SSS13), whereas only one strain (S25) was able to degrade benzoic acid (Table 2). Gasoline is typically composed of 41 to 62% aliphatic hydrocarbons; the remainder being aromatic hydrocarbons including benzene, toluene, ethylbenzene and xylene (BTEX) fractions of 10 to 59%. The aromatic hydrocarbons are generally

more toxic than aliphatic compounds with similar number of carbon atoms and having more mobility in water related to their 3 to 5 times higher solubility (Vieira et al., 2009).

Conclusion

This investigation has explored the bacterial diversity of water in different reservoirs used for the storage of petroleum derivative compounds, mainly crude oil, gasoil, kerosene and diesel oil. The results suggest that the diversity of bacterial community is low in the water collected from these reservoirs and only six bacterial genera were described: *Bacillus*, *Staphylococcus*, *Paenibacillus*, *Comamonas*, *Enterobacter* and *Pseudomonas*. Microbial analysis of these isolates did not show dominance of specific bacteria based on Gram staining, but rod shaped strains predominated.

Biodegradation assays of mono-hydroxylated (phenol), di-hydroxylated (catechol) and carboxylated (benzoic acid) aromatic compounds showed that 36% of isolates were able to degrade phenol, but only 12% were able to degrade catechol and benzoic acid.

The taxonomic study of these isolates revealed that they are strongly related to species with high capacity and versatility to degrade many petroleum components. These isolates should be further investigated to determine their usefulness for bioremediation of polluted sites. Additionally, our data will assist the selection of suitable biocides for cleaning storage reservoirs.

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