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Research Article

Isolation and Molecular Identification of New Benzene Degrading *Lysinibacillus* Strains from Gasoline Contaminated Soil

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Abstract: The aim of this study was to isolate and identify aerobic benzene-degrading bacteria from soil sample contaminated with gasoline. Soil samples were collected from gasoline-contaminated sites located around gas stations in the Al-Mafraq city of Jordan. Ten grams of soil were transferred to Stanier's medium supplemented with 1% benzene and aerobically incubate at 30°C for 72 h. Five morphologically different bacterial isolates were recovered from soil samples and designated as 1J1, 2J2, DJ2, 3J4 and 1J1-2. These isolates were subjected to analyses for identification and characterization. All isolates were Gram positive, endospore-forming, motile, mesophilic and rod-shaped aerobic bacteria. Biochemically, all were catalase and oxidase positive except DJ2, which was negative for oxidase. Based on their 16S rDNA sequences, they appear to be closely related to the genus *Lysinibacillus* (*L)*. Isolate 1J1 revealed 91% homology with *L. fusiformis*, suggesting a member of a new genus with some similarity to the genus *Lysinibacillus*. Isolates 2J2, DJ2, 3J4 and 1J1-2 exhibited more than 97% sequence identity to *L. xylanilyticus*, *L. odeysseyi*, *L. macrolides* and *L. boronitolerans*, respectively. The 16S rDNA sequences of these isolates were deposited in GenBank database. All isolates grew best in Stanier's medium when the optimal growth conditions were 1% benzene, 30°C and pH 6.8. In conclusion, five novel bacterial isolates with similarity to the genus *Lysinibacillus* that utilize benzene as the sole carbon and energy source were isolated and characterized from soil samples contaminated by gasoline. These five isolates can be considered as ideal candidates for bioremediation of benzene from soils.

Keywords: Aerobic bacteria, bioremediation, environment, polluted soil, 16S rDNA sequences

INTRODUCTION

Benzene occurs naturally in crude petroleum at level up to 4 g/L and represents one of the major components of gasoline (Snyder, 1987; Environmental Protection Agency (EPA), 1998; Otenio *et al*., 2005). It is a volatile aromatic hydrocarbon compound with limited water solubility (1.84 g/L at 30°C) and is characterized by long-term stability in the atmosphere (EPA, 1998; WHO, 2010; Yakout, 2014). Because of its low solubility in water, minor amounts of benzene may be removed by rain to contaminate soil and surface waters (WHO, 2010). In addition, benzene has a soil partition coefficient (Log K_{oc}) of 1.8 (EPA, 1998). This value makes benzene more likely to leach in solution rather than be adsorbed to soil particles.

Benzene can be released into the environment from different sources such as petroleum refining operations, petrochemical manufacturing and underground gasoline leaks (International Agency for Research on Cancer (IARC), 1982; Snyder, 1987; EPA, 1998; Otenio *et al*., 2005). Human exposure to benzene can occur due to widespread industrial use, poor working conditions, accidents and misuse of benzene. Exposure to benzene can have negative impacts on human health and the environment. The adverse health effects of human's exposure to benzene had been well established (IARC, 1982; Dean, 1985; Yin *et al*., 1987; Snyder, 1987; EPA, 1998). According to the EPA, inhalation or absorption rates of 800-1600 mg/m^3 benzene can lead to vertigo, drowsiness, headache, nausea and acute toxic effects on the central nervous system, whereas inhalation of 4800 mg/m^3 benzene might cause euphoria, giddiness and staggering gait. Inhalation or absorption of 64000 mg/m^3 benzene or higher can be lethal within a few minutes (EPA, 1998). Benzene has been classified as a potential human carcinogen and mutagenic agent as well as a fetotoxic agent (IARC, 1982; EPA, 1998; Duarte-Davidso *et al*., 2001). Furthermore, there is a growing body of evidence that continuous exposure to

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relatively low concentration of benzene may cause leukemia, in particular acute non-lymphocytic leukemia (Yin *et al*., 1987; Duarte-Davidso *et al*., 2001; Khalade *et al*., 2010). Recently, it has been reported that high concentrations of benzene in soil or atmosphere can be hazardous for both human and animals when inhaled or ingested as fine particles in dust or consumed in contaminated water (Yakout, 2014; Zhou *et al*., 2014). Accordingly, removal of benzene is very essential for human health and environmental well-being over the long-term.

Aerobic benzene degradation by microorganisms has been frequently described in the literature (Vogt *et al*., 2011; Xie *et al*., 2011). Benzene degradation under aerobic conditions is an energy-yielding process that proceeds according to the following exergonic reaction: $C_6H_6 + O_2 \rightarrow CO_2+H_2O$, ΔG = -6399 KJ/mol. Commonly identified aerobic benzene-degrading bacteria appear to fall within two phyla, the *Proteobacetria* (γ and β) and *Actinobacteria* (Xie *et al*., 2011). Examples of the first phylum include *Pseudomonas*, *Ralstonia* and *Acinetobacter* (Alagappan and Cowan, 2003; Kim and Jeon, 2009). Examples of the second phyluminclude *Rhodococcus* (Deeb and Alvarez-Cohen, 1999) and *Arthrobacter* (Fahy *et al*., 2008).

The upper layers of soil have been shown to harbor various bacterial strains with capability to grow on aromatic hydrocarbon compounds such as toluene, xylene, benzene and others (Deeb and Alvarez-Cohen, 1999; Van Hamme *et al*., 2003; Tarawneh *et al*., 2010; Xie *et al*., 2011; Ben Hamed *et al*., 2013; Olapade and Ronk, 2015; Irshaid and Jacob, 2015; Jacob and Irshaid, 2015). Only a handful of bacterial genera have been documented to possess the capacity to utilize benzene as the sole source of carbon and energy for growth (Fahy *et al*., 2008; Kim and Jeon, 2009; Vogt *et al*., 2011; Xie *et al*., 2011). None have been reported in or around Jordan. The purpose of this study was, therefore, to screen for aerobic benzene-degrading bacteria from soil samples taken from gasoline-contaminated sites surrounding gas stations in the northern part of Jordan.

MATERIALS AND METHODS

Sample collection: Soil samples were obtained from gasoline-contaminated sites located around gas stations in the Al-Mafraq city of Jordan. The soil samples were collected from a depth of about 1 to 10 cm in April, 2014. Soil samples of about 100 grams each were placed in labeled sterile plastic bags. The soil samples were cleaned of animal debris and other substances. The collected soil samples were immediately taken to the laboratory for further experimental analysis.

Media preparation and growth conditions: Vitamin-free sterile Stanier's Mineral Medium (SMM) was used for cultivation and isolation of benzene-degrading bacteria. This SMM was prepared as described previously (Jacob and Irshaid, 2015). For preparation of Stanier's agar medium, 2% agar was added to the medium. Then, the medium was boiled to dissolve the agar. The pH of the SMM was adjusted using either NaOH or HCIas needed. The SMM was sterilized by autoclaving. For all experiments, deionized water was used in all working solutions under aseptic conditions.

Benzene with 99% purity was purchased from Sigma-Aldrich Co. (USA). It was supplemented into the SMM as the sole energy and carbon source to a final concentration of 1 or 2%. Benzene was sterilized separately by filtration using nitrocellulose membrane with 0.2μm pore size and then added aseptically to the autoclaved SMM to obtain the appropriate final concentration.

Isolation of benzene-degrading bacteria: Ten grams of soil were added to 90 mL of sterile SMM supplemented with 1% benzene as enrichment medium in Erlenmeyer flasks with the capacity of 250 mL. The inoculated flasks were aerobically incubated in orbital shaking incubator (JS Research Inc., Korea) at 180 rpm, 30°C (close to *in situ* temperature) for 72 h. The inoculated flasks were crimp-sealed with Teflon-coated stoppers to prevent any losses from volatilization.

After 72 h of incubation, the culture medium was allowed to settle and then 1 mL from the supernatant was transferred to new fresh enrichment medium flasks and aerobically incubated again at 30°C while being shaken at 180 rpm for 48 h. This procedure was repeated for three successive times. After that, one ml of the culture was spread onto Stanier's agar plate containing 1% benzene by streak plate method and incubated at 30°C until colonies appeared. Five separate colonies were isolated from these plates based on their colony appearance on enrichment medium agar plates (shape, surface, color, texture, margin and elevation) using a single colony isolation procedure.

The selected colonies were designated as strains 1J1, 2J2, DJ2, 3J4 and 1J1-2. To confirm their ability to grow on benzene as the sole source of carbon and energy, the selected colonies were reinoculated into fresh enrichment medium. After that, these colonies were transferred to new liquid media and incubated at 30°C to obtain substantial growth and subjected to analyses for identifications and characterizations. Finally, 30% glycerol stock was prepared for each pure culture by mixing 0.7 mL of pure culture with 0.3 mL glycerol in small Eppendorf tubes and stored at -20°C.

Morphological and biochemical characterization of isolated bacteria: Generally, newly isolated bacteria were identified according to the standard morphological and biochemical characterization as described by Bergey's manual of determinative bacteriology (Garrity

et al., 2002). For the purpose of identification of the selected isolates, the Gram-stained technique was carried out as previously described (Cappuccino and Sherman, 2008). Briefly, fresh pure isolates were prepared by growing in agar plates aerobically at 30°C for 48 h. Fresh cells of the five isolates were then stained and observed under the light microscope. Motility was also examined by Phase contrast microscopy (Trinocular microscope (86.399-LED), EuromexMicroscopen BV, Arnhem, The Netherlnad).

The oxidase and catalase activities were assessed for the five isolates according to a previously described protocol (Irshaid and Jacob, 2015). The qualitative RapIDCB plus system (Remel, Thermo Scientific Inc., USA) was also employed to identify the five unknown isolates to the species level with the help of ERIC^{TM} software. This identification system is designed mainly to identify Gram positive bacillus bacteria. Briefly, the identification was performed by transferring some colonies of pure bacterial culture into a test tube containing 2 mL sterile normal saline as described by the manufacturer's instruction. After mixing the pure culture, the entire volumes of bacterial dilution was placed into wells of the identification kit and then incubated for 4 h at 37^{o}C. This test system is mainly based on the principle of pH change following substrate utilization. The data were entered into ERIC software and then analyzed.

Molecular identification and classification of isolated bacterial strains: For the purpose of molecular identification and classification, DNA extraction, 16S rDNA PCR and 16S rRNA gene sequencing techniques were performed to classify the five isolates to species levels as previously described (Jacob and Irshaid, 2012). Briefly, genomic DNA from each pure isolate was extracted and purified using EZ-10 Spin Column Genomic DNA Isolation Kit (Biobasic, Ontario, Canada) according to the manufacturer's instruction. For all obtained pure DNA samples from the five isolates, 16S rRNA gene was PCR amplified and then sequenced by GENEWIZ, Inc., USA.

To determine the closest cultured relatives of the five isolates on the basis of 16S rDNA sequence, the resulting DNA sequences of the isolates were probed against the nucleotide collection in NCBI (National Center for Biotechnology Information) genetic sequence database by BLASTN 2.2.31+Program (blast.ncbi.nlm.nih.gov).

Nucleotide sequence accession number: To obtained DNA accession numbers for the five isolates, the resulting 16S rDNA sequences of the five isolates were deposited in GenBank database (NIH database) [www.ncbi.nlm.nih.gov/genbank]. For the isolates 2J2, 1J1, DJ2, 3J4 and 1J1-2, the following accession numbers KP297817, KP297818, KP297820, KP297821 and KP297822 were given, respectively.

Phylogenetic analysis: A phylogenetic tree for the five isolates, 1J1, 2J2, DJ2, 3J4 and 1J1-2 was constructed according to the protocol described by Tamura *et al*. (2013). Briefly, sequences of closely related type strains used for constructing the phylogenetic tree were retrieved from the NCBI database by BLAST searches for bacteria. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969).The alignment and editing were carried out using MEGA software, version 6 (Tamura *et al*., 2013). The plotted with MEGA6 software were used to build the phylogenetic tree for the isolated bacteria.

Estimation of growth rates and generation times: The growth rates of the five isolates under all tested conditions were estimated by monitoring the change in Optical Density (OD) at 600 nm against a blank using a UV-Vis Spectrophotometer (JENWAY, UK). Abiotic enrichment medium was served as a blank in the absorbance measurements.

To calculate the generation times for the five isolates, a spread plate technique was employed for counting bacterial colonies. For each strain, an aliquot of 0.1 mL of fresh liquid culture was spread on agar plates containing enrichment medium. After incubation for 48 h at 30°C, the appeared colonies were manually counted on agar plates using a colony counter and reported as colony-forming units per ml (CFU/mL). All counts were carried out in triplicate. Finally, the generation time for each isolate was estimated by using the following formula: $g = t/n$, $n = 3.3 (\log N - \log N_o)$ (Solomon and Viswalingam, 2013); where g is the generation time, t is the time interval in hour, n is the number of generations during the period of exponential growth, N is the final cell number and N_o is the initial cell number.

Physiochemical analyses of the benzene-degrading bacteria: The effects of three physicochemical environmental factors (benzene concentration, pH and temperature) on the generation times and growth rates of the five strains were investigated. For all physicochemical experiments, 15 mL aerobic culture tubes with the capacity of 50 mL were prepared by inoculation with freshly prepared culture pre-grown in enrichment medium. These culture tubes were crimp-sealed with Teflon-coated stoppers to prevent loss from volatilization. In addition, inoculated tubes with SMM containing no benzene (0% benzene) were carried out as controls for all physicochemical experiments.

To determining the optimal benzene concentrations for the five isolates, three different benzene concentrations (0% as a control, 1% and 2%; v: v, benzene: SMM) were prepared. During this experiment, pure fresh cells from each isolate were inoculated in 15 mL of SMM supplemented with 0, 1 or 2% benzene in 50 mL tube. The pH of the three growth media were adjusted to 6.8. The tubes, in triplicate, were aerobically incubated for 120 h at 30°C with shaking at 180 rpm.

At periodic intervals after inoculation (time zero), aliquot samples of one milliliter were drawn aseptically from inoculated tubes. The growth rates and generation times of the five isolates were calculated under these conditions as mentioned above.

The effect of pH on growth was examined at pH 5.5, 6.8 and 8.5. Three enrichment media and with different pH values were made in crimp-sealed tubes. Pure fresh cells from each isolate were cultivated for each culture tube of the three enrichment media. The culture tubes were incubated, in triplicate, at 30°C with shaking at 180 rpm for 120 h. An aliquot of one milliliter was obtained from each tube at various time points after inoculation. Then, the growth rates and generation times for the five isolates were estimated as described above.

To assess the effect of temperature on the growth rates of the five isolates, three growth temperatures (25°C (considered as room temperature), 30°C (close *to in situ* temperature) and 45°C (as relatively high temperature)) were selected. Pure fresh cells from each strain were inoculated in tubes containing enrichment medium and pH 6.8, in triplicate and incubated at the three selected temperatures with shaking at 180 rpm for 120 h. An aliquot of one milliliter was collected from each tube at various time points after inoculation and the growth rates and generation times were determined under these conditions for all isolates as mentioned above.

RESULTS

To screen for benzene-degrading bacteria, soil samples were collected from gasoline-contaminated sites surrounding gas stations. Ten grams of soil sample were incubated for 72 h at 30°C in liquid SMM containing 1% benzene. Our enrichment technique and isolation procedure resulted in isolation of five bacterial isolates with the capacity of utilizing benzene as the sole source of carbon and energy under aerobic condition. The five isolates were designated as 1J1, 2J2, DJ2, 3J4 and 1J1-2. The ability of these five isolates to utilize benzene for growth was monitored by measurement of turbidity and formation of colonies on agar plate containing enrichment medium. Cell density changes were measured in all inoculated and control tubes at 600 nm.

The morphological characteristics of the five isolates were tabulated in Table 1. It can be seen that the morphologies of the five selected colonies slightly differed from each other. Morphological examination and Gram staining of the cells of the five isolates revealed that all isolates were Gram-positive, rod-shaped, spore-forming and motile aerobic bacteria.

Biochemical test results are summarized in Table 2. The five isolates were biochemically uniform with few exceptions. All isolates were positive for oxidase and catalase activities, except DJ2 was negative for oxidase activity. Several negative results for hydrolysis of various carbon sources were obtained with the RapIDCB plus characterization system. All isolates exhibited positive results for utilization of glucose, sucrose and maltose. None of the isolates utilized ribose.

The closest relative species, sequence identity values and given accession numbers for the five bacterial isolates (1J1, 2J2, DJ2, 3J4 and 1J1-2) were presented in Table 3. To place these five isolates to the closest relative strains, the resulting 16S rRNA gene sequences for these isolates were compared with sequences of strains retrieved from the public GenBank Database (http://blast.ncbi. nlm.nih. gov/blast /Blast.cgi). Analyses of the 16S rRNA gene sequences revealed that the five isolates were closely similar to the genus *Lysinibacillus* (Family *Bacillaceae* in the phylum Firmcutes of the Domain Bacteria). Isolates 2J2, DJ2, 3J4 and 1J1-2 exhibited very high homology with *L. xylanilyticus* (98% identity, accession number: LK391645), *L. odeysseyi* (98% identity, accession number: KP297820), *L. macrolides* (99% identity, accession number: JX502177) and *L. boronitolerans* (99% identity, accession number: FJ237498), respectively. The 16S rDNA sequence obtained for isolate 1J1 showed 91% similarity with *L. fusiformis* (accession number: KP192024) and less than

Table 1: Morphological characteristics of the five bacterial isolates obtained from soil samples contaminated by gasoline in Stanier's medium supplemented with 1% benzene

Parameter	1J1	2J2	DJ2	3J4	1J1-2
Colony shape	Rough	Circular	Circular	Circular	Circular
Colony surface	Dull surface	Dull surface	Smooth	Smooth	Dull surface
Colony elevation	Mucous	Butyrous	Mucous	Mucous	Butyrous
Colony texture	Flat	Flat	Convex	Raised	Flat
Colony color	White	Off white	Cream	Cream	White
Colony margin	Entire	Lobate	Lobate	Entire	Entire
Gram positive	Positive	Positive	Positive	Positive	Positive
Cell shape	Rod	Rod	Rod	Rod	Rod
Spore formation	Yes	Yes	Yes	Yes	Yes
Motility	Yes	Yes	Yes	Yes	Yes

Table 2: Biochemical characteristics of the five bacterial isolates recovered from soil samples contaminated by gasoline from the area surrounding gas stations

	Isolate				
Biochemical test	1J1	2J2	DJ2	3J4	1J1-2
CAT	+	+	+	+	+
OXD	+	+	-	+	+
GLU	+	+	+	+	+
SUC	+	+	+	+	+
RIB	-	-	-	-	-
MAL	+	+	+	+	+
αGLU	-	-	-	-	-
βGLU	+	+	+	+	+
NAG	-	-	-	-	-
GLY1	-	-	-	-	-
ONPG	-	-	-	-	-
PHS	-	-	-	-	-
EST	+	-	+	+	-
PRO	-	-	-	-	-
TRY	-	-	-	-	-
PYR	-	-	-	-	-
LGLY	-	-	-	-	-
LEU	-	-	-	-	-
URE	+	-	+	+	+
NIT	-	-	-	-	-
PIG	-	-	-	-	-

Legend (Abbreviations of chemical tests): +: Positive reaction, -: Negative reaction, CAT= Catalase Test, OXD = Oxidase Test, GLU: Utilization of Glucose, SUC: Utilization of Sucrose, RIB: Utilization of Ribose, MAL: Utilization of Maltose, αGLU: Hydrolysis of p-Nitrophenyl-α, D-glucoside, βGLU: Hydrolysis of p-Nitrophenyl-β, D-glucoside, NAG: Hydrolysis of p-Nitrophenyl-n-acetyl-β, D-glucosaminide, GLY1: Hydrolysis of p-Nitrophenyl-glycoside, ONPG: Hydrolysis of o-Nitrophenyl-β, D-galactoside, PHS: Hydrolysis of p-Nitrophenyl phosphate, EST: Hydrolysis of the fatty acid ester , PRO: Hydrolysis of Proline-β-naphthylamide, TRY: Hydrolysis of Tryptophan-β-naphthylamide, PYR: Hydrolysis of Pyrrolidine-β-naphthylamide, LGLY: Hydrolysis of Leucyl-glycine-β-naphthylamide, LEU: Hydrolysis of Leucine-β-naphthylamide, URE: Hydrolysis of Urea, NIT: Utilization of Potassium Nitrate, and PIG: Yellow Pigmentation

Table 3: The closest relative species, sequence identity values, and given accession numbers for the five bacterial isolates recovered from soil samples taken from gasoline-contaminated sites located around gas stations

Isolate	Closest Relative	Identity (%)	GBAN
1J1	*Lysinibacillus fusiformis*	91%	KP297818
2J2	*Lysinibacillus xylanilyticus*	98%	KP297817
DJ2	*Lysinibacillus odeysseyi*	98%	KP297820
3J4	*Lysinibacillus macrolides*	99%	KP297821
1J1-2	*Lysinibacillus bronitolerans*	99%	KP297822

GBAN: GenBank Accession Number

90% with other closely related species. The 16S rDNA sequences of isolates 2J2, 1J1, DJ2, 3J4 and 1J1-2 were deposited in GenBank database under the following accession numbers, KP297817, KP297818, KP297820, KP297821 and KP297822, respectively (Table 3). To determine the phylogenetic relationships among these five isolates with closely related strains of *Lysinibacillus*, the phylogenetic tree was constructed using sequences of *closely related* species. In Fig. 1, the phylogenetic tree indicated that these five isolates were clearly separated from each other and from their closely related relatives.

The growth curves of the five isolates at different growth conditions were constructed (Fig. 2). To assess the effect of benzene concentration on growth rate of the five isolates, three different concentrations were selected (0, 1 and 2%). The cells of the five isolates were grown in SMM supplemented with 0, 1 or 2 % and pH 6.8 at 30°C for 120 h. Results of these experiments revealed that the maximum bacterial densities or OD values were observed at 1% benzene concentration and reduced bacterial densities were obtained at 2% benzene concentration (Fig. 2A and B). It can also be seen that only DJ2 isolate showed a slight increase in OD in tubes containing the 2% benzene concentration as compared to other isolates incubated at the same conditions (Fig. 2B). Over the course of the experiment, all isolates failed to grow in SMM containing no benzene.

To determine the optimal growth temperature for the five isolates, three different growth temperatures (25, 30 and 45°C) were tested. All isolates were grown in SMM supplemented with 1% benzene and pH 6.8. The results of these experiments indicated that the growth of the five isolates proceeded at a faster rate at 30°C but less efficiently at 25°C (Fig. 2A versus 2C). Slight growth was obtained at 45°C, especially for isolates DJ2 and 1J1 (Fig. 2D). Among the three examined temperatures, 30°C was found to be the optimal growth temperature for the tested five isolates.

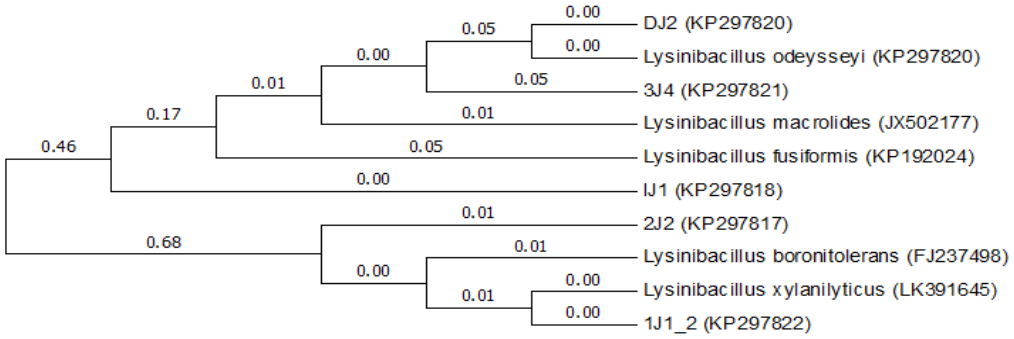

Fig. 1: Molecular Phylogenetic analysis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969). Evolutionary analyses and tree construction were conducted in MEGA6 (Tamura *et al*., 2013). This tree is based on 16S rRNA gene sequences, showing the position of the five isolates 1J1, 2J2, DJ2, 3J4 and 1J1-2recovered from soil sample surrounding gas stations and their closely related species of bacteria. All positions containing gaps and missing data were eliminated. Accession numbers of these species are placed in parentheses. The numbers at the branching points are the branch lengths measured in the number of substitutions per site

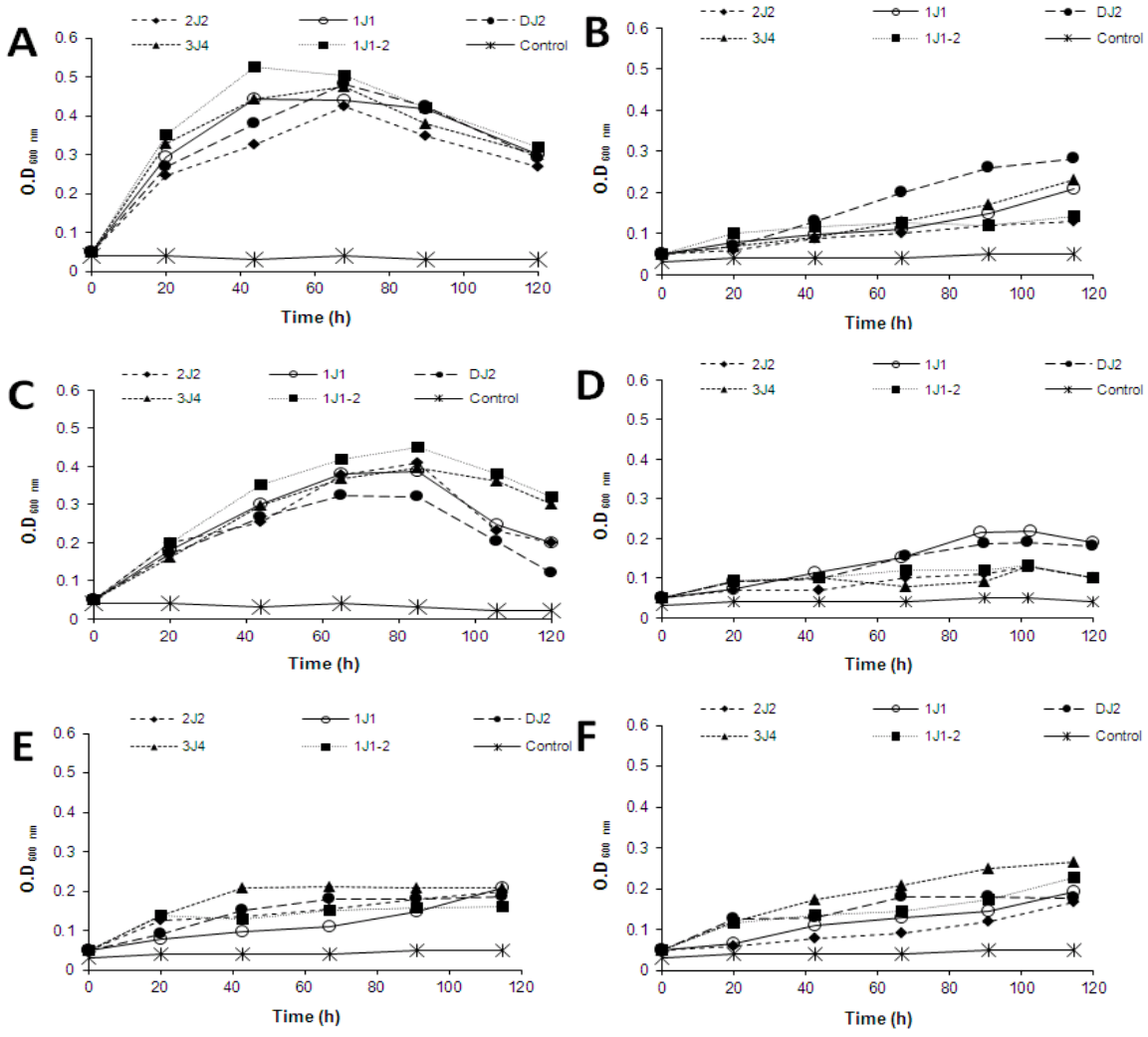

Fig. 2: Growth behavior of the five bacterial isolates 1J1, 2J2, DJ2, 3J4 and 1J1-2 obtained from soil sample contaminated by gasoline in Stanier's mineral medium under different growth conditions: (A) 1% benzene, pH 6.8, 30°C; (B) 2% benzene pH 6.8, 30°C; (C) 25°C, 1% benzene, pH 6.8; (D) 45°C, 1% benzene, pH 6.8; (E) pH 5.5, 1% benzene, 30°C; (F) pH 8.5, 1% benzene, 30°C. Each point in the graphs represents the mean of three independent experiments. Error bars were removed for clearness

Table 4: The mean generation times in hours for the five bacterial isolates recovered from gasoline contaminated soil samplein Stanier's medium supplemented with benzene under different growth conditions

	BC*		Temp (°C)			pH value		
Isolate	1%	2%	25	30	45	5.5	6.8	8.5
L. fusiformis 1J1	7.6	23.7	10.9	7.6	36.4	19.7	7.6	50.3
L. Xylanilyticus 2J2	8.7	24.7	10.1	8.7	41.5	15.2	8.7	24.3
L. odeysseyi DJ2	8.3	41.5	11.4	8.3	23.7	10.9	8.3	15.1
L. macrolides 3J4	7.4	40.4	12.0	7.4	23.7	10.8	7.4	13.4
L. boronitolerans 1J1-2	7.2	20.1	10.1	7.2	21.7	13.6	7.2	16.4

*Benzene concentration (benzene: medium; volume: volume). Each value represents the mean of three independent experiments

To determine the best pH value for the five isolates, three enrichment media with different pH values (pH 5.5, pH 6.8 and pH 8.5) were examined. The resulting data revealed that maximum bacterial densities were observed at pH 6.8 and reduced bacterial densities was shown at pH 5.5 or pH 8.5 for all isolates (Fig. 2A versus E and F). Also, isolate 3J4 showed a slight increase in growth in SMM supplemented with 1% at pH 5.5 or 8.5 as compared to the remaining four isolates incubated under the same condition (Fig. 2E and F). The pH 6.8 was found to be the optimal pH value among the tested pH values.

All five isolates reached their maximum cell density between 40 to 60 h in SMM supplemented with 1% benzene and incubated at 30°C and pH 6.8 (Fig. 2A). The data also showed that the density of cells started to decrease after 70 h and reached about 50% of the maximum cell density after 105 h. In addition, there was no noticeable lag phase within the growth curve for tested isolates. At 25°C and pH 6.8 in SMM supplemented with 1% benzene, all examined isolates showed maximum cell density between 65 and 85 h (Fig. 2C). At these conditions, the growth of the cells appeared slightly delayed. On other hand, minimum exponential growth rate and minimum cell density were observed when the cells of the five isolates incubated at 45°C and pH 6.8 in SMM supplemented with 1% benzene (Fig. 2D). Similar patterns of growth were observed when the cells of the five isolates were grown in SMM supplemented with 1% benzene and incubated at 30°C and pH 5.5, or at 30°C and pH 8.5 (Fig. 2E and F).

The mean generation times of the five isolates under different growth conditions were given in Table 4. These data indicated that the mean generation times of the isolates varied from 7.2 to 50.3 h under various conditions. The lowest generation times of the five isolates ranged from 7.2 h to 8.7 h, when the cells of all isolates were incubated at 30°C in SMM supplemented with 1% benzene and pH 6.8 during the first 60 h of the experiment. Isolate 1J1-2 gave the best growth results and lowest generation time with 7.2 h under 1% benzene concentration, 30°C and pH 6.8. Similarly, for isolate 3J4, the shortest generation time was estimated to be about 7.4 h at pH 6.8, when cells of this isolate were incubated at 30°C in SMM supplemented with 1% benzene. On other hand, the longest generation time (50.3 h) was recorded for isolate 1J1, when this isolate was incubated at 30°C and pH 8.5 in SMM supplemented with 1% benzene.

DISCUSSION

In the present study, our procedure for screening of benzene-degrading bacteria from soil contaminated by gasoline resulted in isolation of five bacterial isolates with the ability of utilizing benzene as the sole carbon and energy source under aerobic conditions. These five isolates were designated as 1J1, 2J2, DJ2, 3J4 and 1J1-2. These isolates were found to grow very well with the SMM supplemented with 1% benzene. The growth of these isolates was confirmed through the change in the turbidity and the increases in cell densities in tubes containing benzene. Additionally, none of these isolates were able to grow on SMM without benzene, indicating the importance of the benzene as the growth substrate for the isolated bacteria.

The new five isolates showed many morphological features and characteristics that were similar to each other and to some strains of the genus *Lysinibacillus* (Ahmed *et al*., 2007; Nam *et al*., 2012). In addition, these isolates gave closely similar results in biochemical tests, with few exceptions. 16S rDNA sequence analysis also confirmed that isolates 1J1, 2J2, DJ2, 3J4 and 1J1-2 were closely related to the genus *Lysinibacillus*, with their closest relatives being *L. fusiformis*, *L. xylanilyticus*, *L. odeysseyi*, *L. macrolides* and *L. boronitolerans*, respectively. The resulting 16 rDNA sequences for these isolates were deposited in the public GenBank database. The phylogenetic analysis of 16S rRNA gene sequences of the five isolates and their closest relatives also confirmed that the five isolates were separated from each other and represented species closely related to but separate from the genus *Lysinibacillus*. In addition, these isolates also exhibited differences in some morphological, biochemical and physiological characteristics from their closely related species and other type strains of the genus *Lysinibacillus*.

For the purpose of bacterial identifications at the genus and species levels and based on literature data derived from analysis of hundreds of species, it has been suggested that a strain exhibiting a sequence similarity score of lower than 99% may represent a prototype strain of a different genospecies and a strain with sequence similarity score of less than 97% could

represent a new genus (Drancourt *et al*., 2000; Stackebrandt and Ebers, 2006). On the basis of results of morphological, biochemical, physiological characteristics and phylogenetic and 16S rDNA sequence analyses, we concluded that isolates 2J2, DJ2, 3J4 and 1J1-2 represented separate strains of the genus *Lysinibacillus*. Isolate 1J1 showed less than 92% similarity level with closely related strains of the genus *Lysinibacillus*, indicating that this isolate could represent member of a new genus with some similarity to the genus *Lysinibacillus*.

It is important to note that the genus *Lysinibacillus* is a Gram-positive, spore-forming bacteria in the family *Bacillaceae* (Ahmed *et al*., 2007; Nam *et al*., 2012). Most members of this genus are naturally occurring soil bacteria. Members of this genus were previously considered as members of the genus *Bacillus* until the rRNA group-2 of the genus *Bacillus* was changed to the genus *Lysinibacillus* in 2007 (Ahmed *et al*., 2007; Nam *et al*., 2012). The cell wall-peptidoglycan contains lysine and aspartic acid as the diagnostic amino acids (Ahmed *et al*., 2007). The lysine-aspartic acid type of peptidoglycan was the reason for giving the genus this name. Based on these facts, it is possible to suggest that there is a need to establish a new genus or subgenus in *bacillus* RNA group 2.

It has been well documented that the genus *Bacillus* isolated from oil-contaminated sites was found to possess the ability to degrade crude oil (Olapade and Ronk, 2015). More importantly, some members of the genus *Lysinibacillus* with the ability to degrade hydrocarbons, xenobiotics, crude oil or other environmental pollutants have been previously described elsewhere. For instance, *Lysinibacillus* sp. strain ZB-1 was found to degrade the herbicide fomesafen as the sole carbon source of growth (Liang *et al*., 2009). *Lysinibacillus cresolivorans* was able to degrade *m*-cresol (Yao *et al*., 2011a). Recently, two studies showed that *Lysinibacillus* can degrade crude oil (Mnif *et al*., 2011) and hydrocarbon effluent (Ben Hamed *et al*., 2013). Another *Lysinibacillus* species was also isolated from landfill soils and proved to have a remarkable ability to degrade low density polyethylene (Esmaeili *et al*., 2013). Very recently, *Lysinibacillus sphaericus* was isolated and found to degrade various types of textile dyes (Rajeswari *et al*., 2014). At the same time, Bedekar *et al*. (2014) reported that *Lysinibacillus* sp. RGS have the capability to degrade sulfonated azo dye reductive Orange 16 efficiently. The present study is the first to show *Lysinibacillus* strains with the capability to utilize benzene as the sole source of carbon and energy. Moreover, these findings clearly indicated that *Lysinibacillus* strains are the most ideal for bioremediation of environmental pollutants because they are generally able to grow in contaminated soils and utilize various compounds as substrate growth.

Furthermore, it is surprising that only five bacterial strains belonging to the genus *Lysinibacillus* were isolated and no other bacterial genus was found upon culturing in benzene-enriched media. This finding may simply reflect that *Lysinibacillus* is unique in its ability to grow in medium containing benzene as the sole source of carbon and energy. This was consistent with the previously known facts that *Lysinibacillus* strains were spore-forming bacteria with the ability to grow in soil contaminated by various pollutants including crude oil (Ahmed *et al*., 2007; Liang *et al*., 2009; Mnif *et al*., 2011; Yao *et al*., 2011b; Nam *et al*., 2012; Rajeswari *et al*., 2014; Bedekar *et al*., 2014). It may also due to the operating conditions and procedure used during cultivation and isolation of the bacteria. Operating conditions such as aeration, agitation, pH temperature and medium constituents may affect the type and quantity of the isolated microorganisms (Deeb and Alvarez-Cohen, 1999; Fahy *et al*., 2008; Ben Hamed *et al*., 2013). Also, we cannot exclude the possibility that other unknown factors might be involved.

In terms of the effects of initial concentration, temperature and pH of the growth medium on growth rates, it was found that better growth rates of these five strains were observed during first two days of cultures at 1% benzene concentration with optimum temperature of 30^{o}C and pH 6.8, which means that these cells grow with almost no inhibition. These findings agree with a previous study done by Bam forth and Singleton (2005) that showed that the solubility and bioavailability of growth substrate decrease in parallel with decreasing temperatures, thus adversely affecting growth rates. Similar finding was also obtained by Liang *et al*. (2009) who reported that *Lysninbacillus* sp. strain ZB-1 could utilize the herbicide fomesafen as the sole carbon source of growth with degradation rate of 81% after 7 days and optimum temperature of 30^{o}C. Recently, Wan *et al*. (2010) demonstrated that the factors controlling the degradation efficiency of ethanethiol by *L. sphaericus* were the initial concentration, temperature and pH of the solution. Moreover, our results were practically similar to those obtained by Yao *et al*. (2011b) who isolated *L. cresolivorans* from aerobic sludge from a coking wastewater plant and also reported that the optimal conditions for degradation of m-cresol by *L. cresolivorans* were at 35°C and pH 6.8-7.3. These data clearly indicated that the growth rates of these strains were influenced by the benzene concentrations, pH and temperature levels. Therefore, for efficient degradation of benzene as growth substrate under *in situ* or *ex situ* conditions, the effects of these three variables should be considered jointly.

CONCLUSION

In conclusion, we reported for the first time the isolation, identification and characterization of five bacterial strains designated as 1J1, 2J2, DJ2, 3J4 and 1J1-2 from gasoline-contaminated soil sites

surrounding gas stations in the Al-Mafraq city of Jordan. Based on morphological, biochemical and molecular characterization and identification, isolates 1J1, 2J2, DJ2, 3J4 and 1J1-2 were found to be similar to the *L. fusiformis, L. xylanilyticus*, *L. odeysseyi*, *L. macrolides* and *L. boronitolerans*, respectively. Consequently, based on these findings, it seems reasonable to suggest that the five isolates may represent new species in the family *Bacillaceae* and could be placed in separate genera.

These five bacterial strains grew optimally at 30°C and pH 6.8 in SMM supplemented with 1% benzene when compared to other examined conditions and utilized benzene as the sole source of carbon for growth. The ability of the isolated strains to biodegrade benzene identifies them as promising candidates for the treatment of soil contaminated by benzene at low concentrations. Further studies to investigate the mechanism of utilization of benzene by these strains are warranted.

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