Characterization of novel diesel-degrading strains Acinetobacter haemolyticus *MJ01 and* Acinetobacter johnsonii *MJ4 isolated from oil-contaminated soil*

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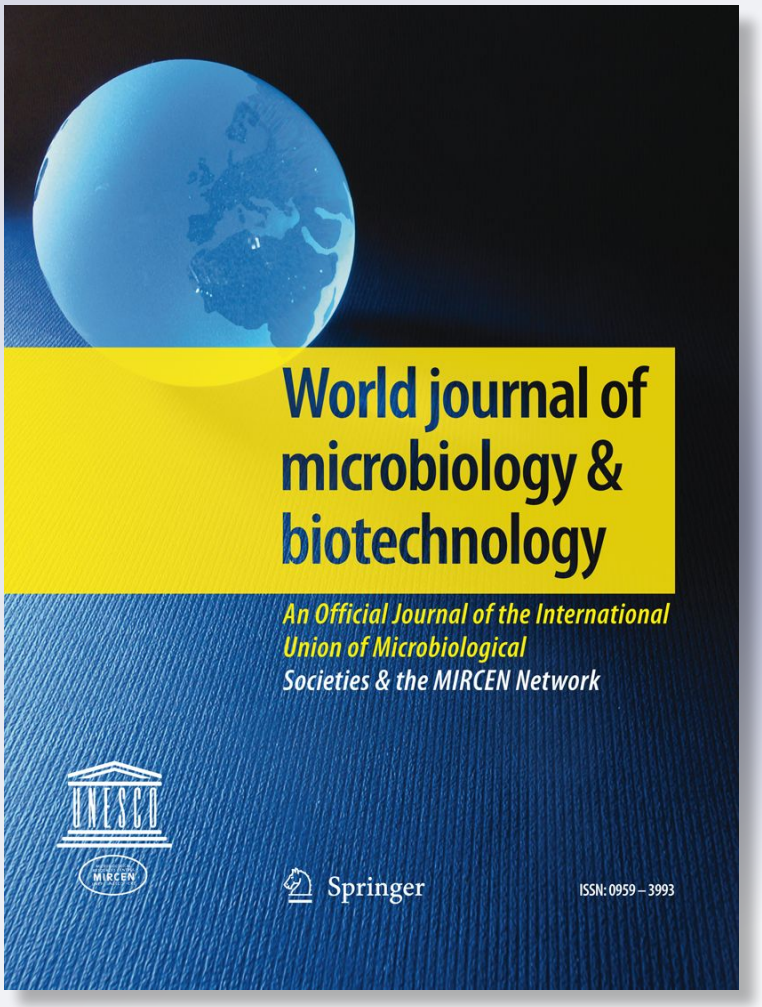

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ORIGINAL PAPER

Characterization of novel diesel-degrading strains *Acinetobacter haemolyticus* MJ01 and *Acinetobacter johnsonii* MJ4 isolated from oil-contaminated soil

Myungjin Lee · Sung-Geun Woo · Leonid N. Ten

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Abstract The diesel-degrading strains, designated as MJ01 and MJ4, were isolated from oil-contaminated soil in Daejeon (South Korea) and were taxonomically characterized using a polyphasic approach and their diesel oil degradation abilities were analyzed. The isolates MJ01 and MJ4 were identified as *Acinetobacter haemolyticus* and *Acinetobacter johnsonii*, respectively, based on their 16S rDNA gene sequences, DNA–DNA relatedness, fatty acid profiles and various physiological characteristics. Strains MJ01 and MJ4 were able to use diesel oil as the sole carbon and energy source. Both strains could degrade over 90% of diesel oil with an initial concentration of 20,000 mg/l after incubation for 7 days, the most significant degradation occurred during the first 3 days. To our knowledge, this is the first report on diesel oil-degrading microorganisms among bacterial strains belonging to *A. haemolyticus* and *A. johnsonii*.

Keywords *Acinetobacter haemolyticus* MJ01 · *Acinetobacter johnsonii* MJ4 · Biodegradation · Diesel oil

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Introduction

Soil and ground water contamination due to petroleum-derived products, in particular diesel oil, is an important environmental problem. Importantly, diesel oil is classified as hazardous waste (Bartha and Bossert 1984) and hydrocarbon-degrading microorganisms can potentially play a central role in addressing this problem. Thus, biodegradation of hydrocarbons by microorganisms represents one of the primary mechanisms by which those pollutants could be eliminated from the environment (Leahy and Colwell 1990; Van Hamme et al. 2003; Gouda et al. 2008; Cerqueira et al. 2011). The application of hydrocarbon-degrading bacteria in oil-contaminated sites does not guarantee that all oil components will be completely metabolized because some components, such as alkanes of shorter and longer chains ($<C_{10}$ and C_{20}–C_{40}), are not as readily biodegradable as are alkanes of intermediate lengths (Atlas and Cerniglia 1995; Yuste et al. 2000). It is therefore important to find a new bacterial strain that can metabolize a broad range of oil hydrocarbons, especially the highly persistent components. Many microorganisms have been reported to degrade fuel and diesel oils (Atlas and Cerniglia 1995; Hong et al. 2005; Cerqueira et al. 2011). Among them bacterial strains belonging to the genus *Acinetobacter* are known for their high ability to degrade a broad variety of hydrocarbons, including the *n*-alkanes (Espeche et al. 1994; Marin et al. 1996; Di Cello et al. 1997; Akinde and Obire 2008), aromatic compounds (Adebusoye et al. 2007; Fischer et al. 2008) and diesel oil (Su et al. 2008; Kang et al. 2011). At the time of writing, the genus *Acinetobacter* comprises 25 species with validly published names (Euzéby 2011), but the description of a twenty sixteenth member of the genus, *Acinetobacter rudis*, is available ahead of print (Vaz-Moreira et al. 2011).

Acinetobacter species are distributed widely throughout many environments, including soil (Prathibha and Sumathi 2008), seawater (Di Cello et al. 1997), wastewater (Pei et al. 2009; Vaz-Moreira et al. 2011), sewage (Lee and Lee 2010), oil (Marin et al. 1996) and human clinical specimens (Bouvet and Grimont 1986), suggesting the profound adaptability of the genus to various environments and its ubiquity and metabolic versatility.

Recently, *Acinetobacter* strain MJ01 was isolated and used in mixture with two other bacteria for biodegradation of diesel, but it has not yet been characterized in detail (Lee et al. 2010). At the same time, we isolated other diesel oil-degrading strain, designed as MJ4. The aim of the present study was taxonomic characterization of strains MJ01 and MJ4, that capable to use diesel oil as a sole carbon and energy source, and evaluation their diesel oil degradation potentials.

Materials and methods

Chemicals

Diesel oil was a gift from LG-Caltex Corporation (Daejeon, South Korea). All bacterial media components and organic solvents were at least analytical grade.

Isolation of diesel oil-degrading bacteria

Soil samples were collected from a diesel oil contaminated site in Daejeon, South Korea. The samples (2 g) were incubated in 50 ml minimal salts (MS) solution containing the following in g/l of distilled water; K_2HPO_4, 0.9; KH_2PO_4, 0.54; $MgSO_4{\cdot}7H_2O$, 0.25; KCl, 0.25; $CaCl_2{\cdot}2H_2O$, 0.01; plus trace amounts of micronutrients (Widdel and Bak 1992) supplemented with 0.5% (v/v) diesel oil. The final pH of the medium was 7.0. The medium was shaken at 150 rev/min at 30°C for 7 days, 5 ml of the suspension transferred to 45 ml of fresh medium and incubated for a further 7 days at 30°C. The resultant suspension (1 ml) was plated on solid media (1.5% w/v agar) supplemented with 0.5% (v/v) diesel oil, to obtain pure cultures. Fifty of single colonies were tested for their ability to degrade diesel oil in MS solution. Two strains, designed as MJ01 and MJ4, showed the highest degradation activity and were selected for further study.

Characterization of strains MJ01 and MJ4

Strains MJ01 and MJ4 were grown on trypticase soy agar medium (TSA; Difco) at 30°C for 3 days in order to determine their morphological and physiological characteristics. The Gram reaction was determined by using a Gram-stain kit (Difco) according to the manufacturer's instructions. Cell morphology and motility were observed under a Nikon light microscope at ×1000 magnification using cells exponentially and stationary growing cultures. Assimilation of single carbon sources, enzyme activities and other physiological characteristics were determined with the API ID 32 GN, API ZYM and API 20NE galleries according to the manufacturer's instructions (bioMérieux). The ability of the strains to grow at different temperatures (15, 25, 30, 37 and 41°C) was determined on TSA agar. Oxidase activity was tested using Bactident-Oxidase test strips (Merck) and catalase activity with 3% hydrogen peroxide. Cellular fatty acids were analyzed in organisms grown on TSA for 48 h at 28°C. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acid methyl esters were then analyzed by gas chromatography (model 6890; Hewlett Packard) using the Microbial Identification software package (Sasser 1990).

Determination of DNA G+C content and DNA–DNA hybridization

For the measurement of chromosomal DNA G+C content, the genomic DNA of the strains were extracted and purified as described by Moore and Dowhan (1995) and degraded enzymically into nucleosides; the DNA G+C contents were determined as described by Mesbah et al. (1989) by using reverse-phase HPLC. DNA–DNA hybridization to determine genomic relatedness was performed fluorometrically according to the method of Ezaki et al. (1989), by using photobiotin-labelled DNA probes (Sigma) and microdilution wells (Greiner), with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values are quoted as the DNA–DNA hybridization values.

Analysis of 16S rRNA gene sequence and phylogenetic analysis

Genomic DNA was extracted by using a commercial genomic DNA extraction kit (Solgent) and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Kim et al. (2005). Full sequences of the 16S rRNA gene were compiled by using SeqMan software (DNASTAR). The total 16S rRNA gene sequence of the test strains was edited using the BioEdit program (Hall 1999) and aligned using CLUSTAL_X software (Thompson et al. 1997). Related sequences were obtained from the GenBank database by using the BLAST search program. The distance matrix was

calculated by using the BioEdit program and the phylogenetic tree was constructed by using the neighbor-joining algorithm (Saitou and Nei 1987) and the MEGA4 program (Tamura et al. 2007). The stability of relationships was assessed by a bootstrap analysis of 1,000 trials.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of strains MJ01 and MJ4 determined in this study has been deposited in the GenBank database under the accession numbers GU991530 and HQ650820, respectively. Other accession numbers for reference 16S rRNA gene sequences used in the phylogenetic analysis are shown in Fig. 1.

Compositional analysis of diesel oil

Diesel oils supplied from LG-Caltex Corporation (Daejeon, South Korea) were used as target compounds for the degradation experiments. The diesel oil consisted of alkanes (42.7%), cycloalkanes (33.4%), and aromatics (23.9%) as described in the technical data sheets provided by LG-Caltex Corporation. The composition of the diesel oil was analyzed using a gas chromatograph (GC-17A; Shimadzu, Kyoto) equipped with a mass detector (GCMS-QP5050A; Shimadzu) and HP-1 column (30 m × 0.32 mm × 1 μm film thickness; J&W Scientific, Folsom, CA, USA). Sample volumes of 1 μl were injected into the column. The temperatures in the injector and detector were 250°C and 300°C, respectively. The column temperature was kept at

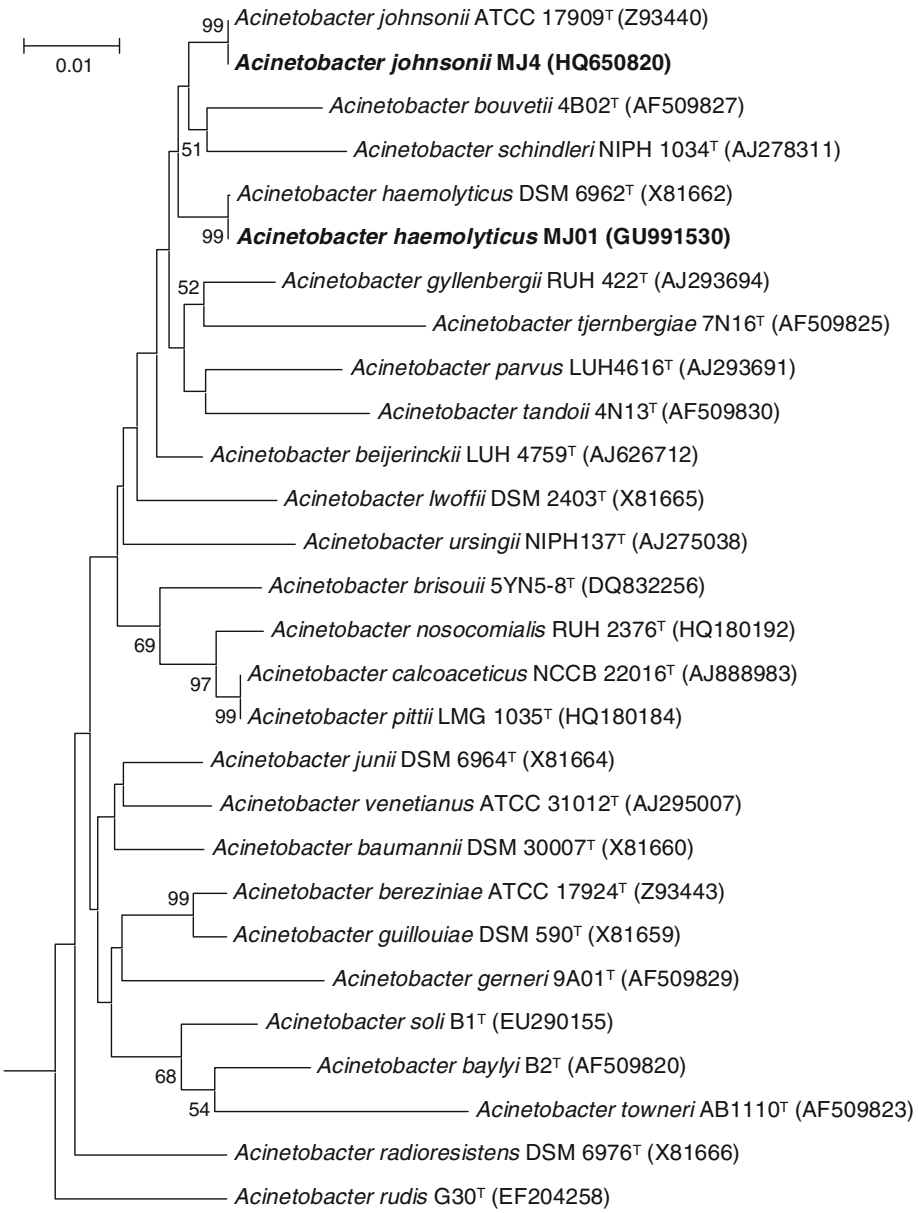

Fig. 1 Neighbor-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic position of strains MJ01 and MJ4 among recognized members of the genus *Acinetobacter*. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at branch points. *Moraxella lacunata* ATCC 17967^T (GenBank accession no. AF005160) was used as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position

70°C for 2 min, increased to 300°C at a ramp rate of 10°C/min and held at 300°C for 15 min. Only *n*-alkanes and a few branched hydrocarbons can be identified as separate compounds out of the 2,000 to 4,000 hydrocarbons that diesel oil contains. However, it is possible to quantify the main structural classes, namely *n*-alkanes, isoalkanes, cycloalkanes and aromatics which comprise diesel oil (Olson et al. 1999). A chromatogram profile of the diesel oil used in the study is shown in Fig. 2; it can be seen that all of the *n*-alkanes were clearly identified. Branched alkanes such as 2,6,10,14-tetramethylpentadecane (pristane) and 2,6,10,14-tetramethylhexadecane (phytane) were also detected. However, the major fractions of diesel oil were not identified because of the analytical complexity related to the large number of components. The same batch of diesel oil was used throughout the study.

Degradation of diesel oil

The bacteria were grown in triplicate in 50 ml MS solution (pH 7.0) with diesel oil as the sole carbon and energy source. Initial diesel concentrations were 1,000, 5,000, 10,000 and 20,000 mg/l. The resultant preparations were inoculated with strains MJ01 or MJ4 to give 6×10^6 c.f.u./ml while uninoculated control flasks were prepared to detect any decrease in diesel oil concentration due to factors other than microbial utilization. All flasks were closed with cotton-wool plugs that allow the passage of oxygen into the flasks. The cultures were incubated on a rotary shaker (300 rev/min) at 30°C for 7 days. Diesel oils are not homogeneously distributed in shake flasks which make representative sampling of broths virtually impossible; hence, sacrificial sampling of complete flask contents was carried out at each day. All samples were set up in triplicate. Thus the 50 ml samples were extracted in an equivalent volume of *n*-hexane.

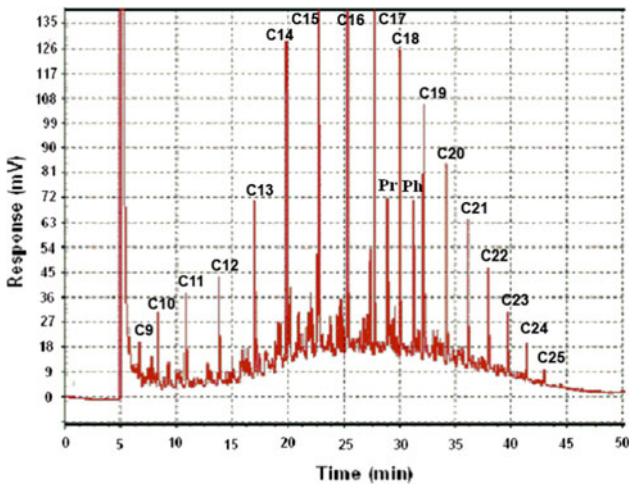

Fig. 2 Gas chromatogram of diesel oil. C numbers indicate *n*-alkanes; Pr, pristane (2,6,10,14-tetramethylpentadecane); Ph, phytane (2,6,10,14-tetramethylhexadecane)

Selected samples were analyzed by using a gas chromatograph fitted with an FID detector (HP 5890 series II; Hewlett-Packard, Palo Alto, CA, USA) and an HP-1 column (30 m × 0.32 mm × 1 μm; J&W Scientific) with helium as the carrier gas. The temperatures in the injector and detector were 280 and 300°C, respectively. The column temperature was kept at 40°C for 2 min, shifted to 300°C at 10°C/min and then held at 300°C for 15 min. Two samples were injected into the GC with the total petroleum hydrocarbon (TPH) measured as the sum of all of the peak areas on the chromatogram. The degree of degradation was calculated based on the following equation: degradation (%) = 100[(A−B)/A)], where A is the area of TPH from the control experiment without inoculation, and B is the area of TPH from the inoculated culture. The rate of diesel oil degradation was calculated using calibration curves with the internal standard (97% dotriacontane, Aldrich), and the TPH measurement and the FID response factors were equal for all compounds.

Oxygen uptake and growth of strain MJ01 and MJ4 on diesel oil

The measurements of oxygen uptake and viable cell concentrations were parallelly performed in separate vessels. Bacterial cell concentrations were determined by using the agar plate count method on TSA medium. Inoculated Petri dishes were incubated at 30°C for 48 h before cell counting. Growth of strains MJ01 and MJ4 was recorded as c.f.u./ml for 12 days. The oxygen uptake of strains MJ01 and MJ4 was measured by a respirometer (Challenge AER-200, Fayetteville, AR, USA). 10,000 mg/l diesel oil was added to a test respirometer vessel with 500 ml MS solution (pH 7.0). The resultant preparations were inoculated with strains MJ01 or MJ4 to give 6×10^6 c.f.u./ml while uninoculated control vessels were prepared to detect oxygen consumption due to factors other than microbial utilization. The preparation vessels were linked to the syringe of respirometer. The cultures were incubated on 30°C water bath with rotation using magnetic stir bar (300 rev/min) for 12 days. The oxygen consumption of strains MJ01 or MJ4 were measured while the diesel oil as the sole carbon and energy source was metabolized. All experiments were performed in triplicates.

Results and discussion

Morphological and biochemical characteristics of strains MJ01 and MJ4

Several bacterial strains that used diesel oil as sole carbon source were isolated using MS agar from diesel oil contaminated soil. Among the isolated single colonies, strains MJ01 and MJ4 were found to have the highest level of

Table 1 Comparison of phenotypic characteristics of strains MJ01 and MJ4 with phylogenetically closely related type strains in the genus *Acinetobacter*

Characteristic	1	2	3	4	5	6	7
Growth at 37 °C	−	−	+	+	+	+	+
Growth at 41 °C	−	−	−	−	−	−	+
Production of indole	−	−	−	−	−	−	+
Gelatin hydrolysis	+	+	+	+	−	−	−
Production of acid from glucose	+	−	+	+	+	−	−
Enzyme activities (API ZYM and API 20E)							
Acid phosphatase	+	+	−	−	−	−	+
Alkaline phosphatase	+	+	−	−	−	−	−
Cysteine arylamidase	+	+	+	+	−	−	−
Esterase (C4)	+	+	+	−	+	−	+
β-Glucosidase	+	+	−	−	−	−	−
Lipase (C14)	+	−	+	−	+	+	−
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	−	−	+
Valine arylamidase	−	−	−	−	+	−	−
Assimilation of (API ID 32GN and API 20 NE)							
Adipate	−	−	−	−	−	−	−
L-Alanine	+	+	+	+	−	−	+
Caprate	+	+	+	+	+	−	−
Citrate	−	−	+	+	+	+	+
L-Histidine	−	−	+	+	+	+	−
3-Hydroxybenzoate	−	−	−	−	−	−	+
4-Hydroxybenzoate	−	−	+	+	−	+	+
3-Hydroxybutyrate	+	+	−	−	+	−	−
Lactate	+	+	−	−	−	+	+
D-Malate	−	−	+	+	+	+	+
Phenylacetate	−	−	−	−	−	+	−
Propionate	+	+	+	−	−	−	+
L-Proline	+	−	+	+		+	+
L-Serine	−	−	+	+	−	−	−
Valerate	+	+	+	−	−	+	+
DNA G+C content (mol%)	44.3	44–45[a]	41.7	40–43[a]	42[b]	NA	NA

Taxa: 1, strain MJ4; 2, *A. johnsonii* KCTC 12405^T; 3, strain MJ01; 4, *A. haemolyticus* KCTC 12404^T; 5, *A. beijerinckii* CCUG 51249^T; 6, *A. gyllenbergii* DSM 22705^T; 7, *A. schindleri* LMG 19576^T

+, Positive reaction; −, negative reaction; NA, data are not available

[a] Data are taken from Bouvet and Grimont (1986)

[b] Data are taken from Lee and Lee (2010)

diesel oil-degrading activity and were selected for further study. The strains MJ01 and MJ4 were Gram-negative, non-motile cocci and their colonies were circular, convex, smooth, slightly opaque with entire margins and 1.1–1.9 mm in diameter after 24 h of growth. Optimal growth conditions of strains MJ01 and MJ4 were at 30°C and pH 7.0. No growth occurred at 41°C and under anaerobic conditions. Both strains, as well as their phylogenetically closest relatives of the genus *Acinetobacter* (Table 1) were positive for catalase, esterase lipase (C8), leucine arylamidase, and utilization of acetate, but negative for nitrate reduction, oxidase, *N*-acetyl-*β*-glucosaminidase, arginine dihydrolase, α-chymotrypsin, α-fucosidase, α-galactosidase, *β*-galactosidase, *β*-glucoronidase, α-glucosidase, α-mannosidase, trypsin, urease, and assimilation of *N*-acetyl-glucosamine, L-arabinose, L-fucose, gluconate, D-glucose, glycogen, inositol, itaconate, 2-ketogluconate, 5-ketogluconate, malonate, D-maltose, D-mannitol, D-mannose, D-melibiose, L-rhamnose, D-ribose, salicin, D-sorbitol, sucrose, and suberate. Other characteristics that support the affiliation of strain MJ01

Table 2 Fatty acid compositions of strains MJ01 and MJ4 and their phylogenetically closest relatives of the genus *Acinetobacter*

Fatty acid	1	2	3	4	5	6	7
$C_{10:0}$	ND	ND	2.2	1.4	1.3	1.8	ND
$C_{12:0}$	7.5	8.3	8.9	7.1	6.9	4.1	9.1
$C_{14:0}$	ND	ND	1.0	1.0	ND	ND	1.2
$C_{16:0}$	19.3	18.1	17.5	15.1	13.4	17.9	18.9
$C_{17:0}$	ND	ND	1.0	1.2	2.9	2.1	ND
$C_{18:0}$	1.9	1.3	1.1	1.2	1.2	1.0	1.2
$C_{12:0}$ 2-OH	1.2	1.7	2.8	3.3	2.5	4.7	ND
$C_{12:0}$ 3-OH	5.8	6.2	12.0	7.3	7.8	10.6	6.1
$C_{17:1}$ $\omega 8c$	ND	ND	1.1	1.0	3.0	4.1	ND
$C_{18:1}$ $\omega 9c$	21.7	23.4	21.0	29.7	40.8	35.3	21.9
Summed feature 3[a]	ND	ND	3.9	5.3	ND	ND	1.7
Summed feature 4[a]	38.7	36.1	23.9	24.5	19.1	16.1	37.8
Summed feature 7[a]	3.9	4.9	3.6	1.9	1.1	1.1	2.1

Taxa: 1, strain MJ4; 2, *A. johnsonii* KCTC 12405^T; 3, strain MJ01; 4, *A. haemolyticus* KCTC 12404^T; 5, *A. beijerinckii* CCUG 51249^T; 6, *A. gyllenbergii* DSM 22705^T; 7, *A. schindleri* LMG 19576^T

Values are percentages of total fatty acids. ND, not detected

[a] Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained iso-$C_{16:1}$ I and/or $C_{14:\ 0}$ 3-OH. Summed feature 4 contained $C_{16:1}$ $\omega 7c$ and/or iso-$C_{15:\ 0}$ 2-OH. Summed feature 7 contained $C_{18:1}$ $\omega 7c$ and/or $C_{18:1}$ $\omega 9t$ and/or $C_{18:1}$ $\omega 12t$

to *Acinetobacter haemolyticus* and strain MJ4 to *Acinetobacter johnsonii* and differentiate them from other closely related members of the genus *Acinetobacter* are shown in Table 1.

Chemotaxonomic characteristics and DNA base composition

The almost full-length 16S rRNA gene sequences of strains MJ01 (1430 bp) and MJ4 (1404) were obtained. In the neighbor-joining phylogenetic tree (Fig. 1), based on 16S rRNA gene sequence comparisons, both strains appeared within the genus *Acinetobacter* and MJ01 joined *A. haemolyticus* while MJ4 joined *A. johnsonii.* Pairwise comparisons of the16S rRNA gene sequences via the EzTaxon program (Chun et al. 2007) indicated that the closest relatives of strain MJ01 were *A. haemolyticus* DSM 6962^T (99.4%), *Acinetobacter beijerinckii* LUH 4759^T (98.7%), *A. johnsonii* ATCC 17909^T (98.4%) and *Acinetobacter gyllenbergii* RUH 422^T (97.7%). Strain MJ4 showed the highest 16S rRNA gene sequence similarity to the type strains of *A. johnsonii* (100%), *A. haemolyticus* (98.9%), *A. beijerinckii* (98.8%), *A. gyllenbergii* (98.6%) and *Acinetobacter schindleri* (97.8%). The generally accepted criteria for delineating bacterial species state that strains showing 16S rRNA gene sequence dissimilarity above 3% or showing a level of DNA–DNA relatedness below 70% (as measured by hybridization) are considered as belonging to separate species (Wayne et al. 1987; Stackebrandt and Goebel 1994). The recent recommendation proposed an increase from 97 to 98.7% in the 16S rRNA gene sequence similarity threshold used to determine the uniqueness of a new strain (Stackebrandt and Ebers 2006). In view of this definition, the above-mentioned data indicate that strains MJ01 and MJ4 can be clearly separated from other recognized members of the genus *Acinetobacter* with the exception of the strains given above. DNA–DNA hybridizations were performed to clarify the taxonomic position of isolated strains.

The cellular fatty acid profiles of strains MJ01, MJ4 and their phylogenetically closest members of the genus *Acinetobacter* are shown in Table 2. All strains contained $C_{18:1}$ $\omega 9c$, summed feature 4 ($C_{16:1}$ $\omega 7c$ and/or iso-$C_{15:\ 0}$ 2-OH) and $C_{16:0}$ as the common major fatty acids. Furthermore, the cellular fatty acid composition of strain MJ01 was very close to that of *A. haemolyticus* KCTC 12404^T but both microorganisms differed by the presence of $C_{10:0}$, $C_{17:0}$ and $C_{17:1}$ $\omega 8c$ from strain MJ4, *A. johnsonii* KCTC 12405^T and *A. schindleri* LMG 19576^T, and by the presence of $C_{14:0}$ and summed feature 3 (iso-$C_{16:1}$ I and/or $C_{14:\ 0}$ 3-OH) from *A. beijerinckii* CCUG 51249^T and *A. gyllenbergii* DSM 22705^T. Strain MJ4 and the type strain, *A. johnsonii* KCTC 12405^T, had very similar fatty acid compositions that differed them from other bacteria shown in Table 2.

The genomic DNA G+C content of strains MJ01 and MJ4 were 41.7 and 44.3 mol%, respectively, which lies within the range observed for recognized *Acinetobacter* species (40.0–46.0 mol%) (Bouvet and Grimont 1986; Lee and Lee 2010). As shown in Table 3, strain MJ01 exhibited a high level of DNA–DNA relatedness with respect to *A. haemolyticus* KCTC 12404^T (89.7%) while strain MJ4

Table 3 Levels of DNA–DNA relatedness (%) between strains MJ01 and MJ4 and the type strains of their phylogenetically closest neighbors in the genus *Acinetobacter*

Strain	MJ01	MJ4
MJ01	100[a]	19.2
MJ4	26.4	100
Acinetobacter haemolyticus KCTC 12404^T	89.7	18.3
Acinetobacter johnsonii KCTC 12405^T	27.1	92.8
Acinetobacter beijerinckii CCUG 51249^T	20.3	17.6
Acinetobacter gyllenbergii DSM 22705^T	18.5	16.7
Acinetobacter schindleri LMG 19576^T	15.2	13.5

[a] The standard deviation for levels of reassociation was ≤6%

showed a high level of DNA–DNA relatedness of 92.8% with *A. johnsonii* KCTC 12405^T. The DNA–DNA hybridization levels were determined to be more than 70% which is the threshold that has been suggested as delineating bacterial species (Wayne et al. 1987; Stackebrandt and Goebel 1994). Our results therefore support the affiliation of strain MJ01 to *A. haemolyticus* and strain MJ4 to *A. johnsonii*.

Diesel oil degradation by strains MJ01 and MJ4

The extent and rate of diesel oil degradation by strains MJ01 and MJ4 gave an indication of their intrinsic degradation capacity. The ability of strains MJ01 and MJ4 to degrade diesel oil at the added concentrations of 1,000, 5,000, 10,000 and 20,000 mg/l is shown in Figs. 3 and 4. The degradation of diesel oil over time in batch cultures was monitored, and the spontaneous decrease in total petroleum hydrocarbons (TPH) in uninoculated flasks was also measured. In our previous studies (Lee et al. 2005) it has been shown that the decrease of diesel concentration in uninoculated cultures was mainly caused by volatilization of low molecular weight components and this effect was taken into account in the calculation of diesel degradation. The strain MJ4 degraded 93.3% of 1,000 mg/l diesel oil after incubation for 3 days. The degrees of degradation of diesel oil at initial concentration of 5,000, 10,000 and 20,000 mg/l were 94.6, 94.5 and 93.6% degraded, respectively, after incubation for 7 days (Fig. 3). The strain MJ01 also degraded 92.9% of 1,000 g/l diesel oil after incubation for 3 days. The degrees of degradation of diesel oil at initial concentration of 5,000, 10,000 and 20,000 mg/l were 97.7, 91.8 and 91.4% degraded, respectively, after incubation for 7 days (Fig. 4). In general, the most significant degradation occurred during the first 3 days and degradation reached a plateau between days 4 and 7. Many other microorganisms have been tested for biodegradation using diesel concentrations ranging from 500 to 20,000 ppm (Hong et al. 2005; Kebria et al. 2009; Lee et al. 2006; Mohanty and Mukherji 2008; Ueno et al. 2007; Wongsa et al. 2004). It has been found that degradation is generally unfavorable at concentrations higher than 10,000 or

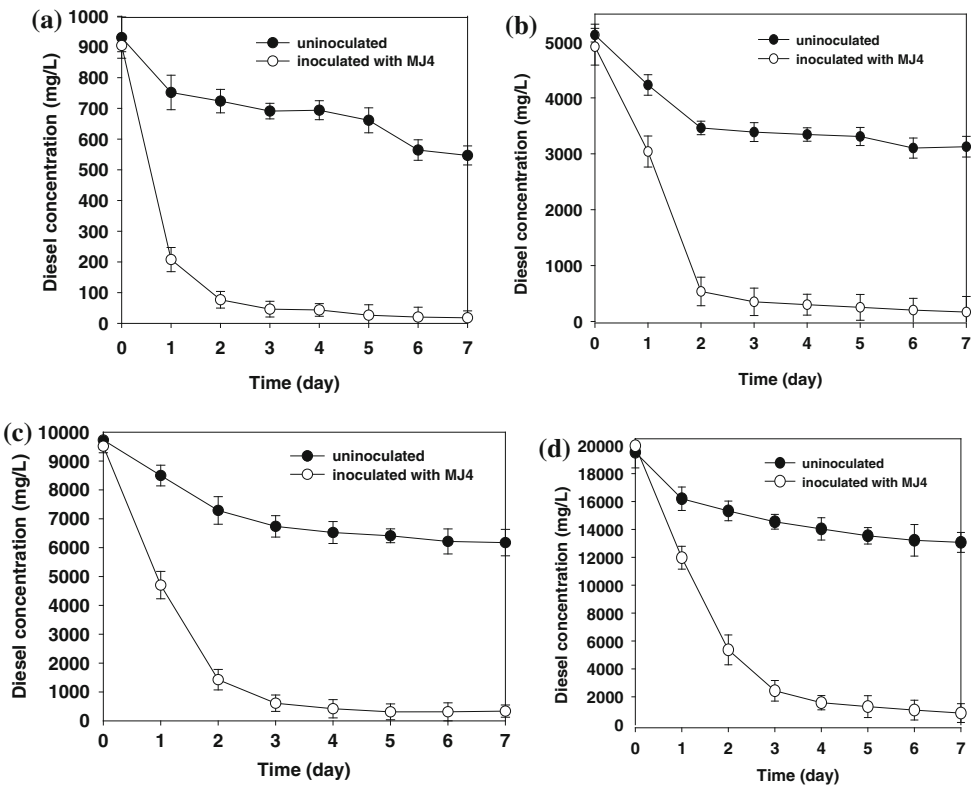

Fig. 3 Degradation of diesel oil by *Acinetobacter johnsonii* strain MJ4. Strain MJ4 was inoculated into MS solutions with diesel oil at different concentrations: **a** 1,000, **b** 5,000, **c** 10,000 and **d** 20,000 mg/l. Initial inoculated cell concentration was 6×10^6 c.f.u./ml

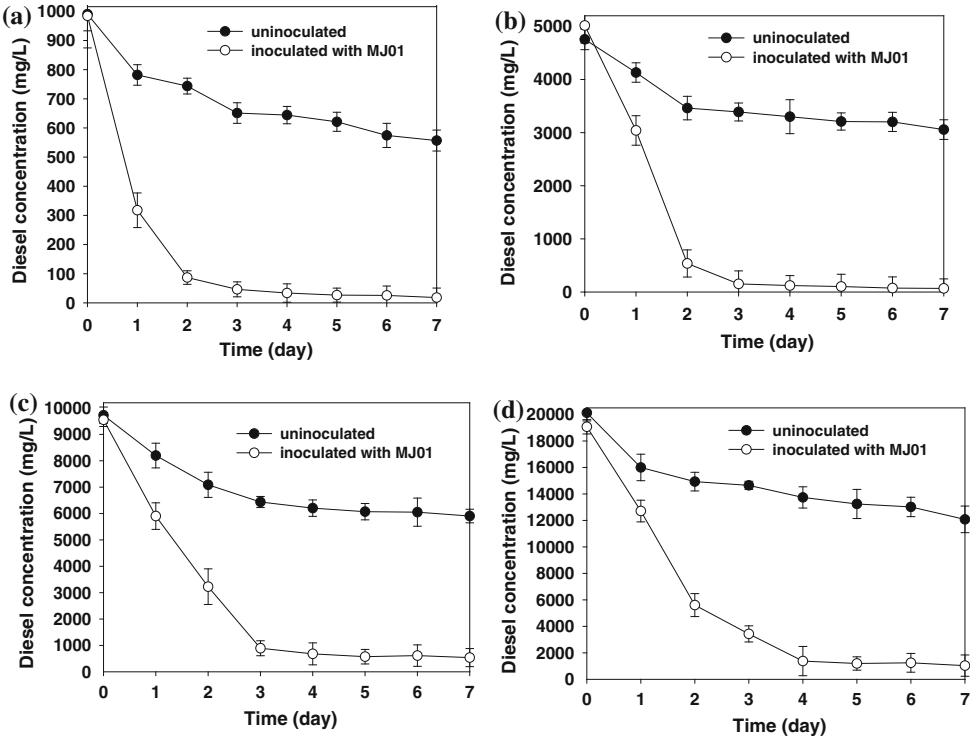

Fig. 4 Degradation of diesel oil by *Acinetobacter haemolyticus* strain MJ01. Strain MJ01 was inoculated into MS solutions with diesel oil at different concentrations: **a** 1,000, **b** 5,000, **c** 10,000 and **d** 20,000 mg/l. Initial inoculated cell concentration was 6×10^6 c.f.u./ml

15,000 ppm (Bicca et al. 1999; Espeche et al. 1994; Lee et al. 2006). Degradation at a more higher concentration (>30,000 ppm of diesel) has been reported for *Pseudomonas* sp. strain DRYJ3 (Shukor et al. 2009) and *Gordonia alkanivorans* S7 (Kwapisz et al. 2008) but, in last case, degradation requires glucose (0.2% w/v) and yeast extract (0.1% w/v). From this point of view, strains MJ01 and MJ4 demonstrated a comparative advantage in their ability to tolerate relatively high diesel concentrations. Biodegradation efficiency of various individual strains vary widely among different species of bacteria. *Pseudomonas aeruginosa* strain WatG was able to degrade diesel oil about 90% at concentration of 10,000 mg/l within 2 weeks (Wongsa et al. 2004). About 90% of 10,000 ppm diesel oil were removed by *Rhodococcus erythropolis* strain NTU-1 in 6 days of batch incubation but only 32–33% diesel removal was achieved by biodegradation (Liu and Liu 2010). Kwapisz et al. (2008) reported 48-60% degradation of diesel oil at concentration 60 ml/l by *G. alkanivorans* S7 for 7 days. The highest hydrocarbon consumption (81%) by this strain was detected in culture medium with nitrate after 14 days of incubation. Relatively high degradation efficiency were reported for other *Gordonia* species, *Gordonia nitida* strain LE31 (Lee et al. 2005) and *G. alkanivorans* strain CC-JG39 (Young et al. 2005). At high diesel concentrations (>15,000 mg/l) strains MJ01 and MJ4 degraded hydrocarbons more efficiently than many other diesel-degrading microorganisms and their degrading ability is comparable with that of most active bacterial diesel degraders.

Gas chromatograms of diesel oil degradation (10,000 mg/l) by strain MJ01 at the same time points are shown in Fig. 5. At the end of the incubation period, the *n*-alkanes were totally degraded by the inoculated microorganism, regardless of their chain lengths. Only small amounts of methylated alkanes, such as pristane and phytane, and some unidentified compounds remained and were not completely metabolized by strain MJ01 (Fig. 5). The same ability to degrade diesel oil was also observed for strain MJ4. Although previous reports mentioned that *Acinetobacter* species can use a large variety of carbon sources (Di Cello et al. 1997; Fischer et al. 2008; Su et al. 2008; Kang et al. 2011), not all oil-degrading strains of the genus *Acinetobacter* can grow on such a broad range of hydrocarbons. For example, *Acinetobacter* strains isolated by Kubota et al. (2008) degraded *n*-hexadecane (C_{16}) and *n*-eicosane (C_{20}) but did not degrade other *n*-alkanes or cyclic alkanes. Thus, the ability to use a broad range of diesel oil hydrocarbons would differentiate strains MJ01 and MJ4 as promising microorganisms within the genus *Acinetobacter* for bioremediation of diesel oil-contaminated sites.

In most cases described, aerobic bacterial degradation of n-alkanes starts by the oxidation of a terminal methyl group to generate a primary alcohol, which is further oxidized to the corresponding aldehyde, and finally converted into a fatty acid. The corresponding carboxylic acid

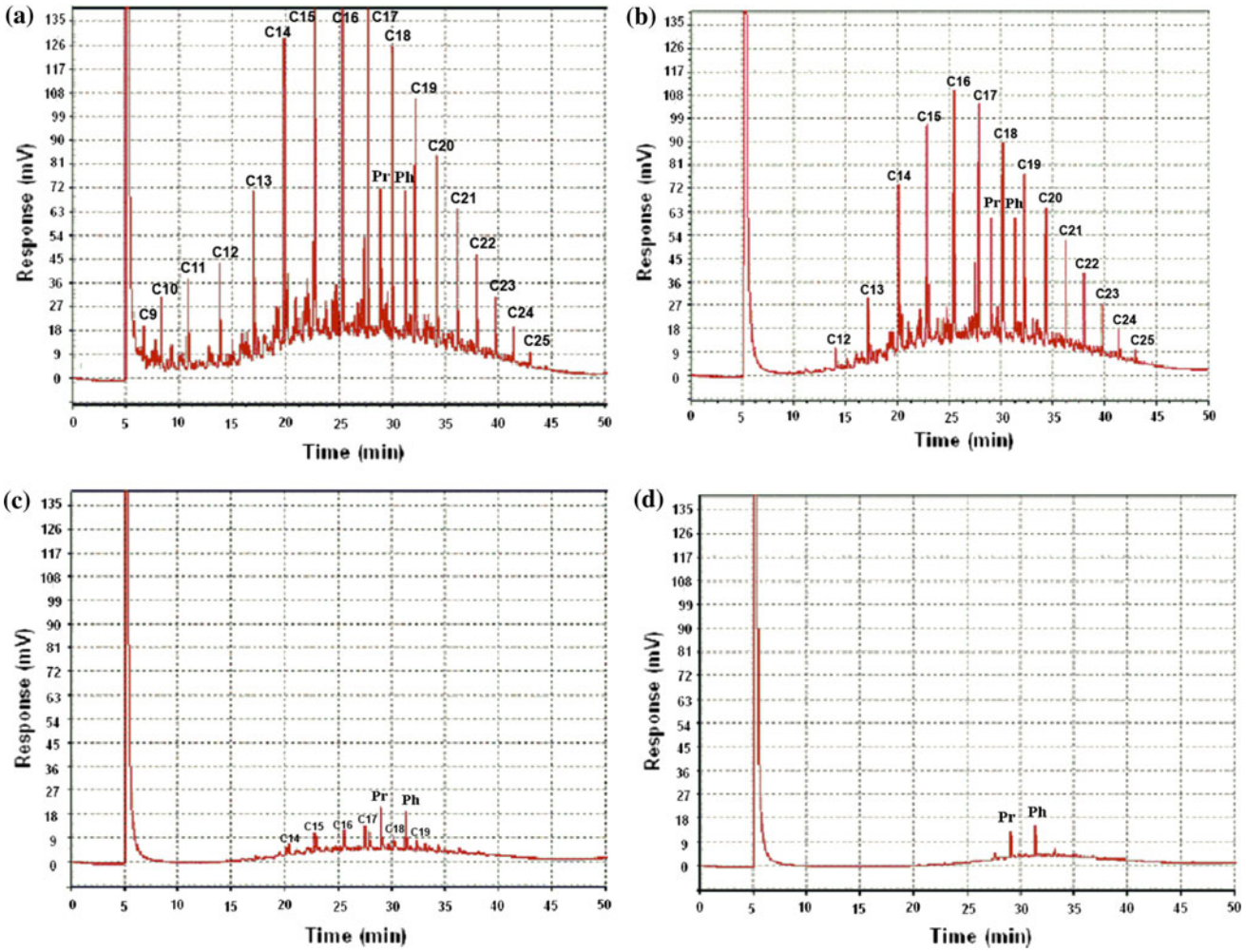

Fig. 5 Chromatograms of diesel oil degradation by *Acinetobacter haemolyticus* strain MJ01 (**a** 0 day, **b** second day, **c** fourth day and **d** seventh day). The added diesel oil concentration was 10,000 mg/l. Two of the residual peaks at the final day (d) were identified as the recalcitrant hydrocarbons pristane (Pr) and phytane (Ph)

is incorporated into β-oxidation cycle via acyl-CoA formation (Van Hamme et al. 2003; Rojo 2009). On the other hand, the Finnerty pathway, where a dioxygenase converts alkanes to aldehydes through *n*-alkyl hydroperoxides without an alcohol intermediate, has been described for some *Acinetobacter* spp. (Finnerty 1988; Sakai et al. 1996). Metabolism in *Acinetobacter* spp. seemed to be complicated due to the diversity of enzymes involved in n-alkane oxidation, further research is necessary to determine the metabolic pathways involved in aerobic diesel degradation in *A. haemolyticus* MJ01 and *A. johnsonii* MJ4.

Oxygen uptake and growth of strain MJ01 and MJ4 on diesel oil

The major degradation pathways for petroleum hydrocarbons involve oxygenases and molecular oxygen, indicating the importance of oxygen for oil degrading microorganisms (Leahy and Colwell 1990). The growth and the oxygen uptake pattern of the isolates on 10,000 mg/l diesel oil were shown in Fig. 6. Strains MJ01 and MJ4 showed over 90% degradation of diesel oil and reached a population size of about 9.1×10^8 and 7.8×10^8 c.f.u./ml at third day, respectively, while using 10,000 mg/l diesel oil as substrate. This showed that diesel oil was used as the sole carbon and energy source and that the oxygen uptake also correspondingly increased by the strains MJ01 and MJ4. Cell concentrations of both strains slowly decrease between the fourth and the seventh days although oxygen consumption was continued. It is to be noted that in this phase the cells had to degrade more and more recalcitrant hydrocarbons contained in diesel oil. After 7 days of incubation, diesel oil was almost completely degraded and this depletion of carbon source resulted in cell death due to unfavorable growth conditions. Metabolites of alkanes, such as alkanoates (Watkinson and Morgan 1990), could have accumulated in

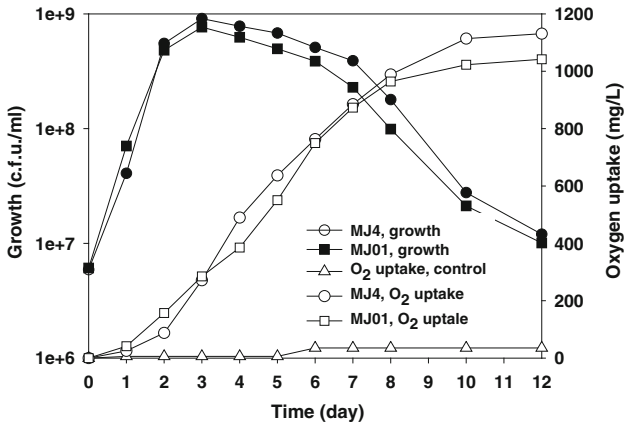

Fig. 6 Growth and oxygen uptake of *Acinetobacter* species strains MJ01 and MJ4. The strains were inoculated into MS solutions with diesel oil at 10,000 mg/l concentration. Initial inoculated cell concentration was 6×10^6 c.f.u./ml. Oxygen uptakes of uninoculated cultures were used as control. The standard deviation for all measurements did not exceed ±5%

the culture and low oxygen uptake after 7 days could be explained by oxidation of these metabolites. It is also possible that oxygen uptake during the later stage of incubation is the result of oxidation of a storage compounds (Ratledge 1978), formed from excess diesel during early stage of incubation.

Conclusions

This work deals with isolation and characterization of naturally occurring bacterial strains best suited to degrade diesel oil at very high concentrations. Two strains MJ01 and MJ4 were isolated from soil rich in diesel oil and were found to be capable of degrading diesel oil as high as 20,000 mg/l. The isolates were characterized as belonging to the genus *Acinetobacter* according to their 16S rDNA gene similarities, fatty acid profiles, as well as biochemical characteristics. DNA–DNA relatedness indicated that strain MJ01 and *A. haemolyticus* KCTC 12404^T are members of the same genomic species. Strain MJ4 exhibited high level of DNA–DNA relatedness with respect to *A. johnsonii* KCTC 12405^T, indicating that they are related to each other at the species level. Both isolated strains utilized diesel oil as a carbon and energy source and up to more than 90% of initial diesel oil was degraded during the 7-day tests. To our knowledge, this is the first report on diesel oil-degrading microorganisms among bacterial strains belonging to *A. haemolyticus* and *A. johnsonii*. Isolated microorganisms may prove to be promising microorganisms for bioremediation of diesel oil-contaminated sites. More research is needed to determine the mechanism of diesel biodegradation by strains *A. haemolyticus* MJ01 and *A. johnsonii* MJ4.

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