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Acinetobacter oleivorans sp. nov. Is Capable of Adhering to and Growing on Diesel-Oil[§]

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A diesel-oil and *n*-hexadecane-degrading novel bacterial strain, designated DR1[T], was isolated from a rice paddy in Deok-So, South Korea. The strain DR1[T] cells were Gram-negative, aerobic coccobacilli, and grew at 20-37°C with the optimal temperature of 30°C, and an optimal pH of 6-8. Interestingly, strain DR1[T] was highly motile (swimming and swarming motility) using its fimbriae, and generated *N*-acyl homoserine lactones as quorum-sensing signals. The predominant respiratory quinone as identified as ubiquinone-9 (Q-9) and DNA G+C content was 41.4 mol%. Comparative 16S rRNA gene sequence-based phylogenetic analysis placed the strain in a clade with the species *A. calcoaceticus*, *A. haemolyticus*, *A. baumannii*, *A. baylyi*, and *A. beijerinckii*, with which it evidenced sequence similarities of 98.2%, 97.4%, 97.2%, 97.1%, and 97.0%, respectively. DNA-DNA hybridization values between strain DR1[T] and other *Acinetobacter* spp. were all less than 20%. The physiological and taxonomic characteristics with the DNA-DNA hybridization data supported the identification of strain DR1[T] in the genus *Acinetobacter* as a novel species, for which the name *Acinetobacter oleivorans* sp. nov. is proposed. The type strain is DR1[T] (=KCTC 23045[T] =JCM 16667[T]).

***Keywords*:** bacteria, biodegradation, genome, diesel, soil, biofilm

The genus *Acinetobacter* was originally described by Brisou and Prevot (1954). The genus *Acinetobacter* presently comprises 22 species with validly published names including most recently described species *A. berezinae* and *A. guillouiae* (Nemec *et al.*, 2009a). Additionally, the *Acinetobacter* species are distributed widely throughout many environments, including soil, water, sewage, and activated sludge, and are known as effective degraders of alkanes and aromatic hydrocarbons (Throne-Holst *et al.*, 2006, 2007; Fischer *et al.*, 2008). Some *Acinetobacter* species including *A. baumannii* are pathogenic and they have proteolytic or haemolytic features on mammalian erythrocytes (Vaneechoutte *et al.*, 1999; Nemec *et al.*, 2009b). A recent environmental isolate, *Acinetobacter* sp. strain DR1 was identified as an aliphatic hydrocarbon and diesel oil-degrading bacterium (Kang and Park, 2010a). Because of its biotechnological potential, genome sequences of *Acinetobacter* sp. DR1 has been recently completed (Jung and Park, 2010). Here, we have conducted a detailed taxonomic characterization of a novel bacterial strain, DR1[T], which was isolated from a soil environment.

Materials and Methods

Isolation and culture of strain DR1[T]

The DR1[T] strain was originally isolated from a rice paddy in Deok-So, South Korea by enrichment with corn oil as the sole carbon. After enrichment, the cells were cultivated on nutrient agar plate (NA) or minimal salts medium (MSB, Stanier *et al.*, 1966) supplemented with 2% diesel-oil or 2% *n*-hexadecane at 30°C (Kang and Park, 2010a).

Phenotypic and enzymatic characterization

Growth conditions were tested at various temperatures (20, 25, 30, 37°C) and pH (6-9) in nutrient media with 220 rpm agitation. Oxidase activity was tested using 1% (w/v) *N,N,N',N'*-tetramethyl-p-phenylenediamine dihydrochloride. A positive reaction is shown by a color change to violet or purple within 20 sec when 10 μl reagent was dropped onto culture on a glass slide. Catalase activity was investigated by checking for the production of bubbles in a 3% (v/v) hydrogen peroxide solution. Starch hydrolysis was tested on NA supplemented with 2% soluble starch. After culture for 2-4 days, Gram's iodine was flooded on agar plates and clear zone around streaked cells was determined as a positive result. For gelatin hydrolysis assay, cells grown for 24-48 h in nutrient broth containing 12% gelatin and liquefaction of culture medium at 4°C was gelatinase positive. Lipase activity was tested on tributyrin agar plate and clear zone around colony indicated lipase positive (Lawrence *et al.*, 1967). For motility test, cells grown in nutrient broth (NB) were spotted on NA containing 0.2% agar for swimming test and 0.5% agar for swarming test. Motility agar plates were incubated at 30°C. Motility was observed after 24 h for swimming and 72 h for swarming. Enzymatic features were determined using VITEK 2 (bioMérieux, France) system in accordance with the manufacturer's instructions. Strain DR1[T] was grown on NA plates, suspended in 3 ml of autoclaved distilled water or 0.45% NaCl solution, and adjusted to an optical density equivalent of a 0.6-0.7 McFarland standard using a colorimeter. The prepared solutions were loaded onto VITEK2 GN (Gram-negative) cards and the data were analyzed with the AES parameter version of VITEK2 (03.01). HPLC analysis identified DNA G+C content (Mesbah *et al.*, 1989).

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Detection of quorum sensing signal

The presence of quorum sensing (QS) signals in strain DR1^T and other related *Acinetobacter* strains was evaluated using the QS indicator strain. *Acinetobacter* strains including strain DR1^T and the *A. tumefaciens* C58 (pZLR4) indicator strain were co-cultured for 48 h in a T-shape on a modified M9 agar plate containing 1% glucose, 0.3% casamino acids and X-gal at 30°C. The colony of indicator changed to blue by strains producing acyl homoserine lactone (AHL).

Microbial adhesion to hexadecane (MATH)

MATH assays were conducted as described previously (Rosenberg *et al.*, 1980). Stationary phase cells were washed twice with phosphate-buffered saline (PBS, pH 7.5) and the bacterial cells were resuspended in PBS to an OD of 0.3-0.5. Subsequently, 10% volumes of hexadecane (0.2 ml) were added to the washed cells. After 30 min of shaking, the mixtures were vortexed for 1 min. The mixture was stored for 30 min in static conditions. The aqueous layer including the cells was then measured. The percentage of cells attached to the hydrocarbons was expressed as the MATH value.

Phylogenetic analysis

The 16S rRNA gene was amplified from the DNA extracts using universal 27f and 1492r primers, and was purified as described previously (Kim *et al.*, 2005). The resultant 16S rRNA gene sequence (1,460 nucleotides) of strain DR1^T was compared with the available 16S rRNA gene sequences using Eztaxon server (Chun *et al.*, 2007) to generate phylogenetic affiliation and sequence similarities. The 16S rRNA sequences were then aligned with those of closely related species and the phylogenetic trees were constructed by neighbor joining, maximum parsimony, and maximum likelihood methods as previously described (Kang *et al.*, 2007).

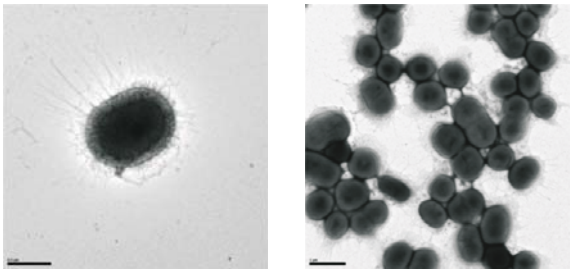

Fig. 1. Transmission electron microscopy (TEM) images of strain DR1^T. Fimbriae protruding from the negative-stained cell surface (A) and aggregated cells (B). Black bar; 0.5 μm (A), 1.0 μm (B).

Table 1. Physiological characterization of strain DR1^T and other related *Acinetobacter* taxa. 1) Enzyme presence, 2) acidification, 3) alkalinisation, 4) assimilation, and 5) other characteristics were determined by VITEK2 system. *Acinetobacter* strains; 1, strain DR1^T; 2, *A. calcoaceticus* DSM 30006^T; 3, *A. haemolyticus* ATCC 17906^T; 4, *A. baumannii* ATCC 19606^T; 5, *A. baylyi* DSM 14961^T; 6, *A. beijerinckii* CCUG 51249^T. All results including other related *Acinetobac*ter taxa were obtained in this study

	1	2	3	4	5	6
Oxidase	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-	-
Gelatin hydrolysis	-	-	+	-	-	-
Swimming	+	-	-	-	+	-
Swarming	+	-	-	-	+	-
n-Hexadecane utilization	+	+	+	-	+	+
MATH (%)	93.2	81.5	88.3	54.1	12.1	47.5
Quorum sensing signal	+	+	-	+	-	-
Enzyme activities						
Glutamyl arylamidase pNA	-	+	+	+	+	-
Lipase[a]	+[a]	+[a]	+[a]	+[a]	+	+
Tyrosine arylamidase	+	+	+	-	-	+
Urease	+	-	-	-	+	-
L-Proline arylamidase	-	-	+	-	-	-
Acidification						
D-Cellobiose	+	-	-	+	-	-
D-Glucose	+	+	+	+	+	-
D-Mannose	+	+	-	+	-	-
Citrate (sodium)	+	-	+	+	+	+
Malonate	+	-	-	-	-	-
Coumarate	+	+	+	+	-	-
Assimilation						
L-Lactate	+	-	-	+	+	-
Succinate	+	-	+	+	+	+
L-Histidine	+	-	-	-	-	+
L-Malate	+	-	-	-	+	-

[a] Lipase activity determined by VITEK2 system was negative. However we confirmed positive lipase activity on tributyrin agar plates. Lipase may not be induced in NA we used for VITEK analysis because NA does not contain substrates of lipase.

DNA-DNA hybridization

In order to evaluate the relatedness of strain DR1^T to its closest 5 strains, DNA-DNA hybridizations were conducted using the methods described by Kang *et al.* (2007). The genomic DNA of strain DR1^T was extracted with a genomic DNA purification kit (Promega, USA) and used as a probe. Genomic DNA solutions (300 ng) were transferred onto membranes (NytranN; Schleicher and Schuell) using a dot-blot apparatus (Bio-Rad). Purified genomic DNA (2 mg) was labeled with ^{32}P-labeled dCTP (Perkin Elmer) and a random primer labeling kit (Invitrogen). Image analyzer (ProXPRESS 2D, Perkin Elmer) was used to calculate DNA-DNA hybridization values.

Fatty acid methyl esters analysis

In order to analyze cellular fatty acids, strain DR1^T and its related 5 stains were cultivated for 1 day at 30°C in nutrient broth with shaking at 220 rpm. Fatty acid methyl ethers were prepared in accordance with MIDI technical note #101 (Sasser, 1990) and analyzed by GC-FID (Agilent 7890GC), Sherlock Microbial Identification System (version 6.0B) and TSBA6 database.

Results and Discussion

Phenotypic and enzymatic characterization

Growth on nutrient medium at various temperatures (20, 25, 30, 37°C) with different pH values (6-9) was measured for 24 h. Strain DR1^T grew at all tested temperature and pH range. The optimum temperature range is 30-37°C. The optimum pH for growth was between 6.0 and 8.0. Growth condition was also tested with NaCl between 0-3% in nutrient media. Growth occurred 0-3% NaCl concentration and optimal range is between 0% and 1% NaCl. Bacterial morphology was determined electron-microscopically (Fig. 1). Strain DR1^T was a Gram-negative, aerobic coccobacillus, which occurred singly and tended to generate exopolysaccharides (EPS) in the presence of 1% NaCl or hydrocarbons, such as diesel-oil or *n*-hexadecane (Kang and Park, 2010a). Studies on the *Acinetobacter* genus have reported that *Acinetobacter* spp. evidenced no swimming or swarming motility on semi-solid surfaces (Baker and Maxted, 1975). However, we demonstrated that strain DR1^T and *A. baylyi* DSM 14961^T evidenced active movement on motility agar plates (Kang and Park, 2010b). Transmission electron microscopy (TEM) images showed that the DR1^T strain has broadly dispersed fimbriae on the cell surface (Fig. 1). VITEK2 systems were utilized for the enzymatic characterization of the strain DR1^T, in accordance with the manufacturer's instructions (Table 1).

Quorum sensing and cell hydrophobicity

Additionally, we evaluated the various characteristics via tests of hydrocarbon utilization, the production of quorum sensing (QS) signals, and microbial adhesion to hydrocarbons (MATH). Previous reports have shown that some *Acinetobacter* spp. generated QS signals that responded to the increases in cell density and functioned in bacterial pathogenesis and biofilm formation (Gonzalez *et al.*, 2001; Niu *et al.*, 2008; Sarkar and Chakraborty, 2008). The presence of QS signals in strain DR1^T and other related *Acinetobacter* strains was evaluated using the QS indicator strain *Agrobacterium tumefaciens* C58

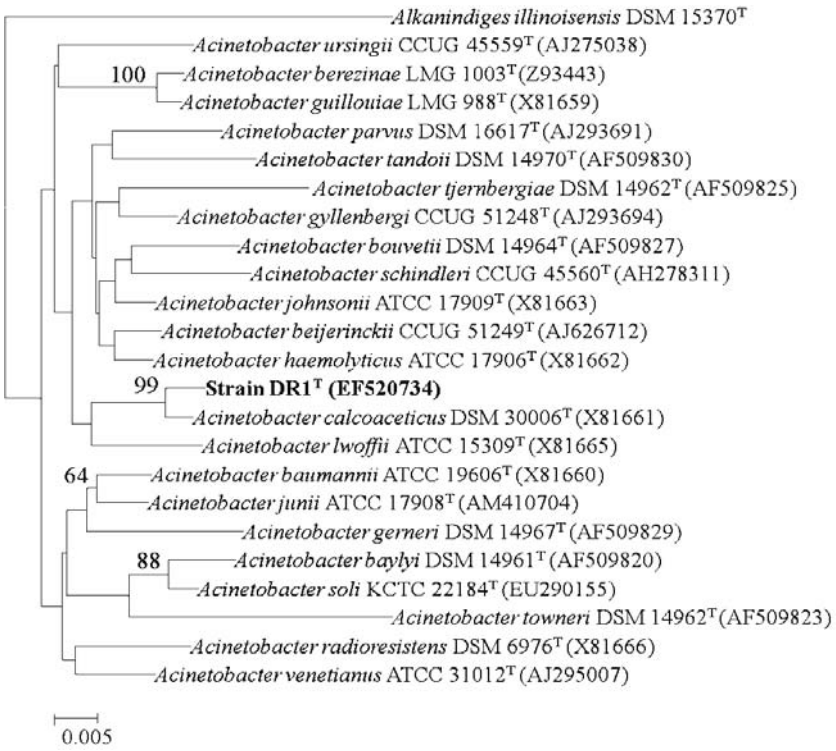

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain DR1^T and other related species. Bootstrap values (expressed as percentages of 1,000 replicates) greater than 50% are shown at branch points. *Alkanindiges illinoisensis* DSM 15370^T was used as an out-group. Bar, 0.005 changes per nucleotide position.

(pZLR4). A summary of the characteristics of strain DR1^T as compared with other closely related *Acinetobacter* species is provided in Table 1.

Phylogenetic analysis

In order to evaluate the phylogenetic relationship between strain DR1^T and other *Acinetobacter* spp., a comprehensive phylogenetic study of genus *Acinetobacter* was conducted using 16S rRNA gene analysis. Usually, bacterial strains with 16S rRNA gene sequence identity of less than 97% are considered novel species (Stackebrandt and Goebel, 1994). Comparative 16S rRNA gene sequence analyses showed that the DR1^T strain was affiliated with the genus *Acinetobacter*. The isolate was found to be most closely related to *A. calcoaceticus* DSM 30006^T, *A. haemolytics* ATCC 17906^T, *A. baumannii* ATCC 19606^T, *A. baylyi* DSM 14961^T, and *A. beijerinckii* CCUG 51249^T with 16S rRNA gene sequence similarities of 98.2%, 97.4%, 97.2%, 97.1%, and 97.0%, respectively. Based on 16S rRNA gene sequences, phylogenetic trees were constructed by neighbor joining (Fig. 2), maximum parsimony (Supplementary data Fig. 1), and maximum likelihood (Supplementary data Fig. 2).

DNA-DNA hybridization

The DNA-DNA hybridization data demonstrated that strain DR1^T evidenced DNA-DNA relatedness values of 18.0, 14.4, 19.7, 14.5, and 15.0%, with *A. calcoaceticus* DSM 30006^T, *A. haemolyticus* ATCC 17906^T, *A. baumannii* ATCC 19606^T, *A. baylyi* DSM 14961^T, and *A. beijerinckii* CCUG 51249^T, respectively (Fig. 3). A DNA-DNA hybridization value below 70% has been suggested as a possible phylogenetic definition of a novel species (Wayne *et al*., 1987). Therefore, the phylogenetic and DNA-DNA hybridization results showed that the DR1 strain was related phylogenetically with the genus *Acinetobacter* and strain DR1 represented *Acinetobacter* as a novel species.

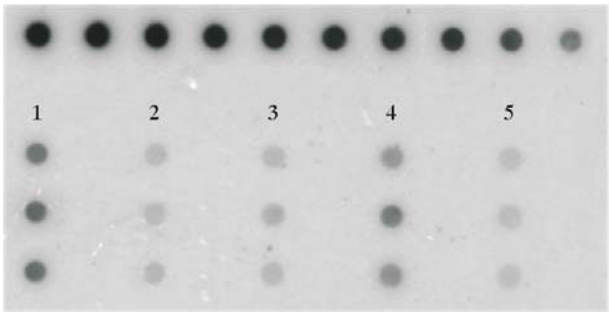

Fig. 3. DNA-DNA hybridization of strain DR1^T and related *Acinetobacter* spp. The genomic DNA of DR1^T was used as a probe. 300 μg (Left) to 30 μg (Right) of DNA concentrations was loaded on the membrane. Genomic DNA (each 300 μg) of related *Acinetobacter* spp. (1, *A. baumannii* ATCC 19606^T; 2, *A. baylyi* DSM 14961^T; 3, *A. beijerinckii* CCUG 51249^T; 4, *A. calcoaceticus* DSM 30006^T; and 5, *A. haemolyticus* ATCC 17906^T) was also loaded.

FAME analysis

The fatty acids of strain DR1^T were $C_{10:0}$ (14.90%), $C_{11:0}$2OH (4.66%), $C_{12:0}$3OH (2.78%), $C_{14:0}$iso (1.32%), C14:1 ω5c

Table 2. Cellular fatty acid compositions (%) of strain DR1^T and related species of the genus *Acinetobacter*. 1, Strain DR1^T; 2, *A. calcoaceticus* DSM 30006^T; 3, *A. haemolyticus* ATCC 17906^T; 4, *A. baumannii* ATCC 19606^T; 5, *A. baylyi* DSM 14961^T; 6, *A. beijerinckii* CCUG 51249^T. Values are percentage of total fatty acids. ND, not detected.

Fatty acid	1	2	3	4	5	6
$C_{8:0}$3OH	ND	1.08	1.61	1.25	ND	ND
$C_{10:0}$	14.90	9.74	20.20	14.80	16.23	11.10
$C_{11:0}$2OH	4.66	3.17	6.86	4.69	5.51	ND
$C_{11:0}$3OH	ND	ND	ND	ND	4.87	ND
$C_{11:0}$ iso3OH	ND	2.32	ND	ND	ND	ND
$C_{12:0}$ iso	ND	ND	ND	ND	3.33	ND
$C_{12:0}$3OH	2.78	3.17	4.33	2.42	2.37	2.54
$C_{13:0}$2OH	ND	ND	2.62	ND	ND	1.51
$C_{14:0}$iso	1.32	ND	2.21	1.80	ND	1.52
$C_{14:1}\omega$5c	1.38	1.37	2.23	1.81	ND	ND
$C_{15:0}$ anteiso	ND	ND	ND	ND	ND	17.71
$C_{15:1}$ ω8*c*	ND	ND	3.70	ND	ND	2.62
$C_{16:0}$	23.74	20.97	9.09	18.60	21.00	13.34
$C_{17:0}$ anteiso	ND	2.56	ND	ND	ND	8.58
$C_{18:0}$	7.92	10.27	ND	7.41	8.51	4.99
$C_{18:1}\omega$9*c*	7.08	5.78	7.97	10.46	4.96	3.44
$C_{18:3}\omega$6*c* (6, 9, 12)	18.81	19.37	19.27	19.36	22.95	16.96
$C_{19:0}$ anteiso	ND	ND	ND	ND	ND	7.03
$C_{20:2}$ ω6,9c	4.85	3.21	5.88	4.68	4.31	ND
Summed feature 1[a]	7.04	4.50	10.17	5.82	5.98	5.67
Summed feature 2[a]	ND	1.55	ND	2.24	ND	ND
Summed feature 3[a]	5.51	10.95	3.86	4.66	ND	2.98

[a] Summed feature 1 comprising $C_{15:1}$ iso H/$C_{13:0}$3OH; Summed feature 2 comprising $C_{14:0}$3OH/$C_{16:1}$ iso I; Summed feature 3 comprising $C_{16:1}\omega$7*c*/$C_{16:1}\omega$6*c*.

(1.38%), summed feature 1 (7.04%) comprising $C_{15:1}$iso H/$C_{13:0}$3OH, summed feature 3 (5.51%) comprising $C_{16:1}$ ω7c/$C_{16:1}$ ω6c, $C_{16:0}$ (23.74%), $C_{18:0}$ (7.92%), $C_{18:1}$ ω9c (7.08%), C18:3 ω6c (6, 9, 12) (18.81%), and $C_{20:2}$ ω6,9c (4.85%). The results of FAME analysis from Strain DR1^T closely related species are shown in the Table 2. On the basis of the above data, strain DR1^T and other strains within the same genus are considered to be distinguished.

Description of *Acinetobacter oleivorans* sp. nov.

Acinetobacter oleivorans (o.le.i.vo'rans. L. n. *oleum* oil; L. part. adj. *vorans* devouring; N.L. adj. *oleivorans* oil or hydrocarbon-utilizing).

Growth occurs at 20-37°C, pH 6-9, and 0-3% NaCl concentration. Optimum growth is observed at 30-37°C, pH 6.0-8.0, and 0-1% NaCl. This strain is capable of utilizing diesel oil or *n*-hexadecane as a sole carbon. Cells are Gram-negative, coccobacilli-shaped (0.5×1.0 μm), and motile by means of fimbriae distributed widely throughout the cell surface. Bacterial cells generally occur singly or are capable of aggregation via exopolysaccharide formation. Colonies on nutrient agar medium are round and smooth. Colonies are approximately 0.5-2.0 mm in diameter after 1 day of incubation. Acid is generated from: D-cellobiose, D-glucose, D-mannose, citrate (sodium), malonate, coumarate, and is not generated from adonitol, L-arabitol, D-maltose, D-mannitol, D-sorbitol, palatinose, sucrose, D-tagatose, D-trehalose, 5-keto-D-gluconate, and ellman. Lipase, tyrosin arylamidase, and urease are positive. Alkali is generated from L-lactate, succinate L-histidine, and L-malate. Negative for Ala-Phe-Pro-arylamidase, L-pyrrolidonyl-arylamidase, β-galactosidase, β-N-acetyl-glucosaminidase, glutamyl arylamidase, γ-glutamyl-transferase, β-glucosidase, α-glucosidase, β-N-acetyl-galactosaminidase, α-galactosidase, phosphatase, glycine arylamidase, ornithine decarboxylase, lysine decarboxylase, β-xylosidase, β-alanine aryamidase, L-proline arylamidase, β-glucoronidase, and Glu-Gly-Arg-arylamidase. H_2S is not generated from glucose. Resistance to vibriostatic agent O129 was positive. The major cellular fatty acids are $C_{10:0}$, $C_{16:0}$, and $C_{18:3}$ ω6c (6, 9, 12). The type strain is DR1^T (=KCTC 23045^T =JCM 16667^T), isolated from soil samples taken from rice fields in Korea. The DNA G+C content of the type strain is 41.4 mol%.

Acknowledgements

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