Rhizobium petrolearium sp. nov., isolated from oil-contaminated soil

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Two Gram-negative, aerobic, rod-shaped bacteria, designated strains SL-1^T and F11, which had the ability to decompose polycyclic aromatic hydrocarbons (PAHs), were isolated from soil samples contaminated by oil. The cells were motile by polar or lateral flagella. According to comparison of 16S rRNA gene sequences, strains SL-1^T and F11 were identical and showed the greatest degree of similarity (96.8%) to both *Rhizobium oryzae* Alt505^T and *Rhizobium mesosinicum* CCBAU 25010^T; however, only *Rhizobium oryzae* with SL-1^T and F11 formed a separate clade. There were low similarities (<90%) between the *atpD* and *recA* sequences of the two strains and those of the genus of *Rhizobium*. The bacteria grew at temperatures of 10–40 °C with an optimum of 30 °C. The pH range for growth was 6.0–10.0 and optimum pH was 7.0–8.0. Growth occurred at NaCl concentrations up to 3.0% (w/v). They were catalase- and oxidase-positive. The main cellular fatty acids were summed feature 8 (18:1 ω 7c and/or 18:1 ω 6c) and 16:0. The DNA G+C content was 62.2 mol%. Strain SL-1^T showed 29 and 0% DNA–DNA relatedness, respectively, with the most related strains *R. oryzae* Alt505^T and *R. mesosinicum* CCBAU 25010^T according to phylogenetic analysis of the 16S rRNA gene. According to physiological and biochemical characteristics and genotypic data obtained in this work, the bacteria represent a novel species of the genus *Rhizobium*, and the name *Rhizobium petrolearium* is proposed. The type strain is SL-1^T (=ACCC 11238^T=KCTC 23288^T) and it could nodulate *Medicago sativa* in nodulation tests.

Rhizobia have great environmental and agricultural importance because their symbioses with legumes are responsible for most of the atmospheric nitrogen fixed on earth. It is well established that in addition to symbiotic association with legumes, a rather large population of genetically unstable and non-symbiotic rhizobia are found in the bulk soil, the rhizospheres of legumes and other

plants (Sullivan *et al.*, 1996; Chaintreuil *et al.*, 2000; Peng *et al.*, 2008; Berge *et al.*, 2009; Zhang *et al.*, 2011). Apart from the capacity for nitrogen fixing, rhizobia species act as a kind of plant-growth-promoting rhizobacteria as endophytes or rhizobacteria of nonleguminous plants (Tan *et al.*, 2001; Yanni *et al.*, 1997). Rhizobia may also have potential in bioremediation of polycyclic aromatic hydrocarbons (PAHs) which lead to environmental pollution (Parshetti *et al.*, 2009). At the time of writing, the genus *Rhizobium* contains 48 species, including those published ahead of print in IJSEM: *Rhizobium skierniewicense*, *Rhizobium leucaenae*, *Rhizobium taibaishanense*, *Rhizobium pusense*, *Rhizobium pseudoryzae*, *Rhizobium herbae* and *Rhizobium vallis*. In this study, we describe two phenanthrene-degrading strains, SL-1^T and F11, isolated from petroleum-contaminated sludge samples in two different laboratories of the Agricultural

Abbreviations: PAH, polycyclic aromatic hydrocarbons; PAL, L-phenylalanine ammonia lyase.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of SL-1^T is EU556969, for the *nifH* sequence is FJ712276, for the *atpD* sequence of strain SL-1^T is EU556968 and for the *recA* sequence of strain SL-1^T is EU556970.

Six supplementary figures and a supplementary table are available with the online version of this paper.

Culture Collection China and the Key Laboratory of Microbiological Engineering of Agricultural Environment of Nanjing Agricultural University, respectively. The petroleum-contaminated sludge samples were taken from refineries located in Dongying and Liaocheng, respectively, in Shengli oilfield, Shandong Province, China.

The isolation methods for the two strains were similar. For oil-contaminated soil samples, 1.0 g was used as inoculum for enrichment cultivation and inoculated into mineral salts medium (MM), and 5.0 g crude oil was added. MM contained NaNO₃ 1.5 g, (NH₄)SO₄ 1.5 g, K₂HPO₄ 1.0 g, MgSO₄·7H₂O 0.15 g, KCl 0.15 g, FeSO₄·7H₂O 0.01 g, CaCl₂ 0.002 g, distilled water 1 l, and was at pH 7.0 (Yan *et al.*, 2010). The enrichment was carried out at 28 °C for 7 days in shaking flasks (about 150 r.p.m.). Subsequently, 5 ml enrichment culture broth was then subcultured into 100 ml fresh MM containing 30 mg phenanthrene l⁻¹ every 7 days. After five rounds of transferring, the enrichment culture was serially diluted and spread on LB/10 agar plates [LB/10 (per l); 1.0 g tryptone, 0.5 g yeast extract, 1.0 g NaCl and pH 7.0] (Sambrook, *et al.*, 1989) supplemented with 50 mg phenanthrene l⁻¹. All visible colonies were removed and their 16S rRNA genes were sequenced. On the basis of complete 16S rRNA gene sequences, strains SL-1^T and F11 were within the genus *Rhizobium* but unlike any previously described species. The cells were incubated at 30 °C on YMA medium (mannitol 10 g, K₂HPO₄ 0.25 g, KH₂PO₄ 0.25 g, MgSO₄·7H₂O 0.2 g, NaCl 0.1 g, yeast extract powder 0.8 g, agar 15 g) (Vincent, 1970) or LB medium. The strains were preserved in a 20 % (v/v) glycerol solution in distilled water at -80 °C and in freeze-drying ampoules at 4 °C.

The 16S rRNA gene was analysed as described by Zhang *et al.* (2008). Briefly, a loop of biomass was suspended in sterile double-distilled water and lysed by boiling followed by freezing. Following centrifugation, the supernatant was used as the template for a PCR with universal primers 27F and 1492R. Phylogenetic analysis of the sequence data was performed using the software package MEGA version 4.0 (Tamura *et al.*, 2007) after multiple alignments of the data using CLUSTAL_X 1.83 (Thompson *et al.*, 1997). A distance matrix method (distance options according to the Kimura two-parameter model), including clustering by neighbour-joining (Fig. 1), maximum-parsimony and minimum-evolution methods (Figs S1 and S2, respectively, available in IJSEM Online) were used. In each case, bootstrap values were calculated based on 1000 replications. Approximately 1440 bp of the 16S RNA gene was amplified and sequenced for each of the two strains. Sequence similarity calculations indicated that strains SL-1^T and F11 were identical and showed the greatest degree of similarity (96.8 %) to both *Rhizobium oryzae* Alt505^T and *Rhizobium mesosinicum* CCBAU 25010^T; however, only *R. oryzae* with SL-1^T and F11 formed a separate clade. Lower sequence similarities (<96.0 %) were found with all other species of the genus *Rhizobium*. Therefore, SL-1^T and F11 may represent a novel taxonomic group of PAH-degrading bacteria.

We also determined partial *recA* (about 530 bp) and *atpD* (about 520 bp) sequences for the novel isolates using methods reported previously (Martens *et al.*, 2007, 2008). Distances were calculated and clustering was performed with the neighbour-joining algorithm as described above. Bootstrap analysis was performed using 1000 replications. Sequence similarity between the two strains isolated from petroleum-contaminated soil was 100 % for *atpD* and *recA*. Strain SL-1^T and F11 had 90 % gene sequence similarity to *Rhizobium galegae* USDA 4128^T, which was the most closely related strain for the *recA* sequence (Fig. S3). The *atpD* gene sequence of strains showed the greatest degree of similarity to *Rhizobium alkalisoli* CCBAU 01393^T (89.6 %; Fig. S4). The low similarities found between the house-keeping gene sequences of the two strains and those of known sequences held in the GenBank database indicate that these strains represent a separate *Rhizobium* species. The position of these species relative to the other *Rhizobium* species varied in the three phylogenetic trees, indicating that the 16S rRNA gene and housekeeping genes might have a different phylogenetic history.

Box-PCR is a useful method for differentiating closely related rhizobia that can be accurate to strain level (Menna *et al.*, 2009). We used primers BOXAIR (5'-CTACGGCAA-GGCGACGCTGACG-3') (Versalovic *et al.*, 1994) for Box-PCR. PCR conditions were as follows: preheating at 94 °C for 2 min followed by 30 cycles of denaturing at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 65 °C for 8 min, with a final extension at 65 °C for 18 min. The Box-PCR profiles of SL-1^T and F11 showed similar genomic fingerprinting (Fig. S5), suggesting that they probably indicate the same genomic species. The profiles of novel strains were clearly different from the defined species *R. oryzae* Alt505^T, *R. pseudoryzae* J3-A127^T and *R. mesosinicum* CCBAU 25010^T.

DNA-DNA hybridization is considered to be a standard method for species definition and the recommended minimum value for strains of the same species is 70 % (Graham *et al.*, 1991; Wayne *et al.*, 1987). For the determination of the DNA base composition and DNA-DNA relatedness, total DNA was extracted from each strain using the method of Marmur (1961). DNA relatedness values were determined using the spectrophotometric method of De Ley (1970). The DNA-DNA relatedness value between SL-1^T and F11 was 85 %, which is above the species threshold of 70 %, indicating that they are the same genomic species. Strain SL-1^T showed 29 % and zero DNA-DNA relatedness values, respectively, with the most closely related strains *R. oryzae* Alt505^T and *R. mesosinicum* CCBAU 25010^T, which were much lower than the recommended threshold value. These results demonstrated that strains SL-1^T and F11 belonged to a different genomic species from the defined species. Using the thermal denaturation method (Marmur & Doty, 1962) and *Escherichia coli* K-12 as a standard, the DNA G + C content of strain SL-1^T and F11 was found to be 62.2 and 59.3 mol% (*T_m*) respectively. These values are within

Fig. 1. Phylogenetic tree resulting from the analysis of nearly complete 16S rRNA gene sequences of strains SL-1^T, F11 and closely related type strains. The branching pattern was generated by using the neighbour-joining method. The topology shown was obtained using 1000 bootstrap replications. Bootstrap values >70 % are shown at the nodes. *Mesorhizobium huakuii* IFO 15243^T was used as an outgroup. GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide position.

the range reported for members of the genus *Rhizobium* (57–66 mol%; Jordan, 1984).

Colony properties of the isolates were observed on YMA agar. Cell morphology was examined by light microscopy and transmission electron microscopy (TEM; Hitachi H-600). For visualization of flagella, cells were mounted on copper grids, negatively stained with 1 % (w/v) uranyl acetate for 15 min and then subjected to TEM at 100 kV (Fig. S6). The NaCl range for growth was tested in YMA containing 0–5 % (w/v) NaCl. The pH range for growth was determined in YMA medium in the range of 5.0 to 11.0 in steps of 1.0 pH units. The pH value was adjusted by addition of 1 M HCl or 1 M NaOH after YMA medium was sterilized. The temperature range for growth (4, 10, 15, 30, 40 or 60 °C) was examined by using the methods of Gao *et al.* (2004). Utilization of various carbon sources was

investigated by GN2 MicroPlate (Biolog) in comparison with reference *Rhizobium* species. Cultures grown for 48 h in YMA medium were used to inoculate the GN2 MicroPlates according to the manufacturer's instructions. Tests for the utilization of sole carbon and nitrogen sources, indole production, nitrate reduction, the Voges–Proskauer (VP) and methyl red (MR) reaction, hydrolysis of gelatin, starch, Tween 80 and aesculin, production of urease and L-phenylalanine ammonia lyase (PAL) were determined according to the methods of Dong & Cai (2001) and Gao *et al.* (2004). The reference strains used in this study were as follows: *R. oryzae* Alt505^T (Peng *et al.*, 2008), *R. pseudoryzae* J3-A127^T (Zhang *et al.*, 2011) and *R. mesosinicum* CCBAU 25010^T (Lin *et al.*, 2009). The discriminative features among the novel isolates and their closest relatives are shown in Table 1.

Table 1. Discriminative features of the novel isolates and related species of the genus *Rhizobium*

Strains: 1, SL-1^T; 2, F11; 3, *R. oryzae* Alt505^T; 4, *R. pseudoryzae* J3-A127^T; 5, *R. mesosinicum* CCBAU 25010^T. All data in the table were obtained in this study. +, Positive; –, negative.

Characteristic	1	2	3	4	5
DNA G+C content (mol%)*	62.2	59.3	64.1–64.4	59.5	68.1
pH range for growth	6–10	6–10	6–11	5–9	5–9
Growth at 3% NaCl	+	+	–	+	–
Utilization as sole carbon source of:					
Dextrin	+	+	+	+	–
Fructose	–	–	+	+	+
Inositol	–	–	+	+	+
Lactose	–	–	+	+	+
D-Mannitol	+	+	–	+	+
Fucose	–	–	–	+	+
Glycine	–	–	+	+	–
Utilization as sole nitrogen source of:					
DL-Alanine	+	+	+	+	–
L-Phenylalanine	+	+	–	+	+
D-Threonine	+	+	–	–	–
Glycine	+	+	+	–	–
L-Methionine	+	+	–	+	+
Biolog (GN) oxidation					
Adonitol	–	–	–	–	+
α-Lactose	–	–	–	+	+
Lactulose	–	–	–	+	+
β-Methyl-D-glucoside	–	–	–	+	+
cis-Aconitic acid	+	+	–	+	+
D-Glucosaminic acid	–	–	–	+	+
D-Glucuronic acid	+	+	–	–	–
α-Ketobutyric acid	+	+	+	+	–
Quinic acid	–	–	–	+	+
D-Saccharic acid	–	–	–	+	+
Succinic acid	–	–	+	+	+
Bromosuccinic acid	–	–	+	+	+
L-Leucine	–	–	+	–	–
L-Pyrogutamic acid	+	+	–	+	–
DL-Carnitine	–	–	–	–	+
Urocanic acid	+	+	–	+	+
Inosine	–	–	+	+	+
Nitrate reduction	+	+	–	–	–
Voges-Proskauer	–	–	+	–	–
H ₂ S production	+	+	–	+	–
Hydrolysis of gelatin	+	–	–	–	–

*The DNA G+C content data of *R. oryzae* Alt505^T (3) and *R. mesosinicum* CCBAU 25010^T (5) were obtained from Peng *et al.* (2008) and Lin *et al.* (2009), respectively.

Fatty acid profiling is a useful tool for the identification of *Rhizobium* and related genera (Tighe *et al.*, 2000). In this study, strains SL-1^T, F11 and all the reference strains were grown on YMA medium for 2 days at 30 °C. Fatty acid

methyl esters were prepared and separated using the previously described method (Sasser, 1990) and identified with the MIDI Sherlock microbial identification system (Library RTSA6 6.0, MIDI Sherlock software package, version 6.0). The cellular fatty acid profiles of the novel strains and related species of the genus *Rhizobium* are shown in Table S1. The main fatty acids of strains SL-1^T and F11 are summed feature 8 (18:1 ω 7c and/or 18:1 ω 6c, 61.5–63.5%) and 16:0 (8.8–11.5%). They had similar cellular fatty acid profiles with other reference strains of the genus *Rhizobium*. However, strains SL-1^T and F11 could be differentiated from other related species by the absence of summed feature 3.

Nodulation and nitrogen fixation abilities are important characteristics for *Rhizobium* species and the host range is an important feature for the description of novel rhizobial species (Graham *et al.*, 1991). In this study, partial sequences of the *nifH* gene of novel isolates were amplified using previously described primers (*nifH* 19F and *nifH* 407R) and PCR conditions as described by Ueda *et al.* (1995). The *nifH* gene sequence of SL-1^T showed sequence similarity of 97.8% to that of *Ensifer meliloti* CCBAU 65135 and RP 254. The *nifH* gene could not be amplified from F11. A nodulation test was carried out in a test tube (18 × 180 mm) containing a quarter-strength nitrogen-free plant nutrient solution (Vincent, 1970). Four legume plant species were selected: soybean (*Glycine max*), kidney bean (*Phaseolus vulgaris*), clover (*Trifolium repens*) and lucerne (*Medicago sativa*). The selected full bean seeds were immersed in 70% ethanol for 5 min, and in 0.1% HgCl₂ for 3 min, successively for surface sterilization followed by rinsing with sterile water several times. To verify the sterilization, the last rinsing water was spread on LB medium and incubated. The seeds were put in darkness (30 °C) for germination for 2 days until they were budding. Surface-sterilized germinated seedlings were planted aseptically in each tube after being immersed in the culture broth (YM broth, 1 ml) of strains with approximately 10⁴ cells ml⁻¹ for 1 h. The plants were grown at 25 °C for 6 weeks in an artificial climate chamber. The non-inoculated seedlings were used as controls and were cultured under the same conditions. The results showed that SL-1^T could form effective nodules on lucerne but not on the other three legume plants and F11 could not nodulate any of the tested plants. To the best of our knowledge, many well-defined nitrogen fixation (*nif*) genes are clustered on large plasmids or megaplasmids, but plasmids and megaplasmids are not very stable during long-term evolution, they are lost or horizontally transferred across species boundaries (Kuykendall *et al.*, 2005). This may be why F11 could not form nodules with legume plants and that the *nifH* gene could not be amplified from it.

Therefore, the novel group was differentiated genotypically and phenotypically from previously described species; based on the results obtained in this study and the current criteria of rhizobial species description (Graham *et al.*, 1991; Wayne *et al.*, 1987), we propose it as a novel

Rhizobium species and name it *Rhizobium petrolearium* sp. nov.

Description of *Rhizobium petrolearium* sp. nov.

Rhizobium petrolearium (pe.tro.le.a'ri.um. L. fem. n. *petra*, rock; L. neut. *olearium* related to oil, of or belonging to oil; N.L. neut. adj. *petrolearium* related to mineral oil).

Gram-negative aerobic, motile with polar or lateral flagella, non-endospore-forming rods (0.5–1.0 × 1.0–2.5 µm). Colonies are circular, pearl white on YMA at 30 °C. The pH range for growth is 6.0–10.0 and optimum pH is 7.0–8.0. The range temperature for growth is 10–40 °C and optimum temperature is 30 °C. Catalase- and oxidase-positive. NaCl concentrations up to 3% (w/v) are tolerated, but the optimal concentration for growth is 1% (w/v). Nitrate is reduced to nitrite. Urease and PAL are not produced. The reaction was negative for indole, VP and MR tests. This species hydrolyses asculin but not starch or Tween 80. The following compounds are used as sole carbon, nitrogen and energy sources: D-arabinose, glucose, dextrin, D-galactose, inulin, calcium malate, maltose, D-mannitol, melezitose, melibiose, sodium pyruvate, rhamnose, sodium acetate, sodium D-gluconate, sodium succinate, sorbitol, starch, sucrose, tartrate, xylose, DL-aspartic acid, L-methionine and L-threonine, DL-alanine, L-arginine, L-cystine, D-glutamic acid, L-lysine, L-phenylalanine, D-threonine, glycine, L-threonine and L-methionine. Positive in the Biolog system for dextrin, *myo*-inositol, D-psicose, L-rhamnose, pyruvic acid methyl ester, succinic acid monomethyl-ester, *cis*-aconitic acid, D-galacturonic acid, D-glucuronic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, α -keto butyric acid, propionic acid, glucuronamide, L-alaninamide, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, L-pyroglytamic acid, L-serine, urocanic acid, α -keto glutaric acid, *N*-acetyl-D-glucosamine, L-arabinose, D-arabitol, cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α -D-glucose, maltose, D-mannitol, D-mannose, D-sorbitol, sucrose, trehalose, turanose, xylitol, acetic acid, formic acid, DL-lactic acid, L-alanine, L-alanyl-glycine, L-glutamic acid, L-proline, uridine and glycerol.

The type strain, SL-1^T (=ACCC 11238^T=KCTC 23288^T), was isolated from petroleum-contaminated sludge samples in Shengli oilfield, Shandong Province, China. The DNA G + C content of type strain SL-1^T is 62.2 mol%. The main fatty acids are: summed feature 8 (18:1 ω 7c and/or 18:1 ω 6c) and 16:0.

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