Pseudomonas sabulinigri sp. nov., isolated from black beach sand

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A novel Gram-negative, aerobic, motile, short rod-shaped bacterium, designated J64^T, was isolated from black sand collected from Soesoggak, Jeju Island, Korea. Cells grew at 4–37 °C, at pH 5.5–10.0 and with 0–10 % NaCl. The strain was found to be oxidase- and catalase-positive. Phylogenetic analyses showed that strain J64^T belongs to the genus *Pseudomonas*, forming a monophyletic group with *Pseudomonas pachastrellae*, *Pseudomonas pertucinogena* and '*Pseudomonas denitrificans*'. The 16S rRNA gene sequence similarity between strain J64^T and type strains of all *Pseudomonas* species with validly published names was below 96.6 %. Low levels of DNA–DNA relatedness were found with respect to type strains of *P. pachastrellae* and *P. pertucinogena*, supporting the classification of strain J64^T within a novel species of the genus *Pseudomonas*. Strain J64^T contained $C_{18:1}\omega7c$ (37.2 %), $C_{16:0}$ (20.4 %), summed feature 3 (17.4 %; comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$) and $C_{12:0}$ (7.6 %) as major cellular fatty acids. On the basis of the phenotypic and phylogenetic data, strain J64^T represents a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas sabulinigri* sp. nov. is proposed. The type strain is J64^T (=KCTC 22137^T =JCM 14963^T).

The genus *Pseudomonas* was defined and established by Migula (1894). Since then, the genus has undergone various taxonomic revisions (Anzai *et al.*, 2000; De Ley, 1992; Palleroni *et al.*, 1973; Stanier *et al.*, 1966). At the time of writing, more than 100 species names have been validly published for the genus *Pseudomonas* (http://www.bacterio.cict.fr). An investigation of the 16S rRNA gene sequences of pseudomonads with and without validly published names indicated a classification based on two clusters containing seven groups (Anzai *et al.*, 2000). One cluster contained six groups and the other cluster included only one, namely the *Pseudomonas pertucinogena* group, with just two species, *P. pertucinogena* and '*Pseudomonas denitrificans*' (Anzai *et al.*, 2000). During a study designed to investigate the bacterial community present in black beach sand, a bacterium was isolated and subjected to taxonomic characterization. On the basis of this characterization, we propose a novel species of the genus *Pseudomonas* belonging to the *P. pertucinogena* group.

The novel bacterium, designated strain J64^T, was isolated from a sample of black sand collected from Soesoggak, Jeju Island, Korea, by using a standard dilution-plating method. Jeju Island is a volcanic island and the black sand originates from the black volcanic basalt rock. Colonies of strain J64^T were creamy, smooth, circular, convex and measured 0.5–1.5 mm in diameter after incubation at 30 °C on marine broth agar (MA; Difco) for 3 days. Cells grew on R2A (Difco) and trypticase soy broth agar (TSA; Difco).

DNA was extracted using a genomic DNA extraction kit (G-spin; iNtRON Biotechnology). PCR-mediated amplification (PCR Pre-Mix; Solgent) of the 16S rRNA gene and sequencing of the purified PCR product with a PCR purification kit (Cosmo Genetech) were carried out. Database searches showed that strain J64^T was related to the genus *Pseudomonas*. Reference 16S rRNA gene sequences were collected from GenBank and those of related strains were aligned using the CLUSTAL_X program (Thompson *et al.*, 1997). The MEGA3 program (Kumar *et al.*, 2004) was used for the editing and construction of a neighbour-joining phylogenetic tree (Saitou & Nei, 1987). Distance matrices were calculated using Kimura's two-parameter model (Kimura, 1980). A bootstrap analysis was performed, using 1000 replications (Felsenstein, 1985). A maximum-likelihood tree (Felsenstein, 1981) was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain J64^T is EU143352.

constructed using PAUP 4.0b10 (Swofford, 2003). As shown in the neighbour-joining tree (Fig. 1), strain J64^T formed a monophyletic cluster with *Pseudomonas pachastrellae* KMM 330^T, *P. pertucinogena* IFO 14163^T (Kawai & Yabuuchi, 1975) and '*P. denitrificans*' IAM 12023, with a high level of bootstrap support (80 %). The monophyletic group was also demonstrated in the maximum-likelihood tree. Strain J64^T showed low pairwise similarities with respect to *P. pachastrellae* KMM 330^T (96.6 %), '*P. denitrificans*' IAM 12023 (96.4 %), *P. pertucinogena* IFO 14163^T (95.6 %) and other recognized species of the genus *Pseudomonas* (<96.0 %). These low similarities demonstrated that strain J64^T was distinct from any of the previously described *Pseudomonas* species. Further confirmation was obtained using DNA–DNA hybridization, which was performed as described previously (Ezaki *et al.*, 1989). The relatedness values with respect to *P. pachastrellae* DSM 17577^T and *P. pertucinogena* DSM 18268^T were 15.0 and 5.6 %, respectively.

The Gram reaction was investigated using the 3 % KOH method (Buck, 1982). Cell morphology and motility were investigated using a Nikon phase-contrast microscope at ×1000 magnification, with cells grown for 3 days on MA at 30 °C. Growth was tested at different temperatures (4, 10, 15, 25, 30, 37, 41 and 45 °C) and at pH 4.0–13.0 on MA and in marine broth (MB; Difco). Salt tolerance was determined using MB made up to contain NaCl at concentrations in the range 0–30 % (w/v). Catalase and oxidase tests were performed using 3 % (v/v) H_2O_2 and 1 % (w/v) tetramethyl-*p*-phenylenediamine, respectively. The production of pyocyanin and of fluorescent pigments were tested using King A medium and King B medium, respectively (King *et al.*, 1954). API 20NE (bioMérieux), API ZYM (bioMérieux) and Biolog GN2 galleries were used (according to the instructions of the manufacturers) to determine the physiological characteristics of strain J64^T, *P. pachastrellae* DSM 17577^T and *P. pertucinogena* DSM 18268^T. For the Biolog GN2 test, the NaCl concentration of the medium was adjusted to 2.0 % and the cells were incubated for 6 days at 30 °C.

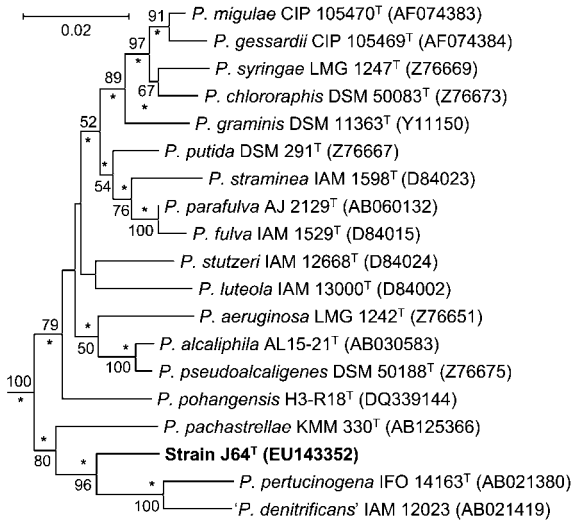

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain J64^T and representatives of the genus *Pseudomonas*. Asterisks indicate that the branches are also found in the maximum-likelihood tree. Sequences from *Halomonas elongata* ATCC 33173^T (GenBank accession no. M93355) and an unknown strain of *Escherichia coli* (V00348) were used as an outgroup (not shown). Numbers indicate bootstrap percentages (based on 1000 repetitions). GenBank accession numbers are shown in parentheses. Bar, 0.02 nucleotide substitutions per site.

Strain J64^T is a Gram-negative, short-rod-shaped bacterium. Cells were found to be motile and single polar flagella were observed under transmission electron microscopy. Cells were positive for catalase and oxidase activity. Pyocyanin and fluorescent pigments were not detected. Strain J64^T grew at 4–37 °C, at pH 5.5–10.0 and with 0–10 % NaCl. Strain J64^T, *P. pachastrellae* DSM 17577^T and *P. pertucinogena* DSM 18268^T showed relatively similar patterns of carbon assimilation and enzyme activity. The enzyme activities tested differed only with respect to acid phosphatase, which was detected strongly in strain J64^T, not detected in *P. pachastrellae* DSM 17577^T and detected weakly in *P. pertucinogena* DSM 18268^T. A large number of the carbon sources tested were not used as sole carbon and energy sources. This limited carbon utilization differentiates this *P. pertucinogena*-related group from other type species of the genus *Pseudomonas* (refer to Table 1 in Romanenko *et al.*, 2005). Some physiological characteristics serve to differentiate them from each other, as shown in Table 1.

The DNA G+C content was determined thermodynamically using a fluorimetric method (Gonzalez & Saiz-Jimenez, 2002). The DNA G+C content of strain J64^T was 58.1 mol%, which is consistent with values reported for members of the genus *Pseudomonas*. The cellular fatty acids of J64^T and its two closest neighbours were analysed using cells grown on TSA for 3 days at 30 °C. The cellular fatty acids were saponified, methylated, extracted and analysed using gas chromatography, according to the protocol of the Sherlock Microbial Identification System (Sasser, 1990). The results of this fatty acid analysis are shown in Table 2. The fatty acid profile of J64^T was similar to that of *P. pachastrellae* DSM 17577^T. Fatty acids $C_{18:1}\omega7c$, $C_{16:0}$, summed feature 3 and $C_{12:0}$ were major components for these two strains. However, *P. pertucinogena* DSM 18268^T had a different fatty acid profile: $C_{19:0}$ cyclo $\omega8c$ was present and there were relatively small amounts of $C_{18:1}\omega7c$ and summed feature 3 and a large amount of $C_{17:0}$ cyclo.

Taken together, these physiological, chemotaxonomic and phylogenetic distinctions demonstrated that strain J64^T was not affiliated with any recognized species of the genus *Pseudomonas*. Therefore, on the basis of the data presented,

Table 1. Physiological characteristics of strain J64^T and type strains of its closest relatives in the genus *Pseudomonas*

Data are from this study. Pyocyanin and fluorescent pigments were not detected (on King B medium) in any of the type strains. In the API 20NE test, all type strains were negative for nitrate reduction, indole production, glucose fermentation, hydrolysis of aesculin and gelatin, β-galactosidase and assimilation of *N*-acetylglucosamine, L-arabinose, gluconate, D-glucose, maltose, D-mannitol, D-mannose and phenylacetate. In the Biolog GN2 test, all type strains were positive for assimilation of L-alanine, sebacate and Tweens 40 and 80, but were negative for assimilation of acetate, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, alaninamide, L-alanyl glycine, γ-aminobutyrate, 2-aminoethanol, L-arabinose, D-arabitol, L-aspartate, 2,3-butanediol, DL-carnitine, cellobiose, α-cyclodextrin, dextrin, L-erythritol, formate, D-fructose, L-fucose, D-galactonate lactone, D-galacturonate, D-galactose, gentiobiose, D-gluconate, D-glucosaminate, α-D-glucose, D-glucose 1-phosphate, D-glucose 6-phosphate, glucuronamide, D-glucuronate, glycerol, DL-α-glycerol phosphate, glycogen, glycyl L-aspartate, glycyl L-glutamate, L-histidine, α-hydroxybutyric acid, *p*-hydroxyphenylacetate, hydroxy-L-proline, inosine, *myo*-inositol, itaconate, α-ketobutyrate, α-ketovalerate, α-D-lactose, lactulose, L-leucine, malonate, maltose, D-mannitol, D-mannose, melibiose, methyl β-D-glucoside, L-ornithine, DL-phenylalanine, phenylethylamine, psicose, putrescine, L-pyroglutamate, quinate, raffinose, L-rhamnose, D-saccharate, D-serine, L-serine, D-sorbitol, succininamate, sucrose, L-threonine, trehalose, turanose, thymidine, uridine, urocanate and xylitol. In the API ZYM test, all strains were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14) and naphthol-AS-BI-phosphohydrolase, but negative for *N*-acetyl-β-glucosaminidase, α-chymotrypsin, cystine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, α-mannosidase, trypsin and valine arylamidase. ++, Strongly positive; +, positive; −, negative; W, weakly positive.

Characteristic	Strain J64^T	*P. pachastrellae* DSM 17577^T	*P. pertucinogena* DSM 18268^T
Acid phosphatase (API ZYM)	++	−	W
Growth at:			
4 °C	+	−	−
41 °C	−	+	+
Tolerance of 8 % NaCl	+	+	−
Assimilation of (Biolog GN2):			
D-Alanine	−	+	+
L-Asparagine	+	+	−
Bromosuccinate	−	W	−
cis-Aconitate	+	+	−
Citrate*	+	+	−
L-Glutamate	+	−	−
β-Hydroxybutyrate	−	−	+
γ-Hydroxybutyrate	+	−	−
α-Ketoglutarate	−	+	−
DL-Lactate	+	+	−
Methyl pyruvate	+	+	−
Monomethyl succinate	−	+	−
L-Proline	+	−	−
Propionate	+	+	−
Succinate	−	+	+
Adipate*	+	−	+
Caprate*	−	+	+
Malate*	−	+	−

*Same results obtained using API 20NE kit.

strain J64^T represents a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas sabulinigri* sp. nov. is proposed.

Description of *Pseudomonas sabulinigri* sp. nov.

Pseudomonas sabulinigri (sa.bu.li.ni′gri. L. n. *sabulum* sand; L. adj. *niger -gra -grum* black; N.L. gen. n. *sabulinigri* of black sand).

Cells are Gram-negative, aerobic, short rods (0.7–1.0 μm wide and 1.5–2.0 μm long). Cells are motile with single polar flagella. Colonies are circular, smooth, creamy, convex and measure 0.5–1.5 mm in diameter after 3 days incubation on MA at 30 °C. Growth occurs on R2A and TSA. Cells grow at 4–37 °C, at pH 5.5–10.0 and with 0–10 % NaCl. Cells are positive for catalase and oxidase. The DNA G+C content of the type strain is 58.1 mol%. The cellular fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$, summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$), $C_{12:0}$, $C_{12:0}$ 3-OH, $C_{17:0}$ cyclo, $C_{18:0}$, $C_{10:0}$ 3-OH, iso-$C_{17:0}$ and anteiso-$C_{17:0}$; trace amounts (<1 %) of $C_{10:0}$, iso-$C_{11:0}$ 3-OH, $C_{11:0}$ 2-OH, iso-$C_{13:0}$, $C_{13:0}$, $C_{14:0}$, $C_{15:0}$,

Table 2. Fatty acid compositions of strain J64^T and type strains of related species of the genus *Pseudomonas*

Data are from this study. Cells were grown on TSA for 3 days at 30 °C. Values are percentages of total fatty acids; −, not detected or <1 % of the total fatty acid content.

Fatty acid	Strain J64^T	*P. pachastrellae* DSM 17577^T	*P. pertucinogena* DSM 18268^T
Saturated fatty acids			
$C_{12:0}$	7.6	10.2	4.5
$C_{15:0}$	−	1.8	2.4
$C_{16:0}$	20.4	16.9	11.4
$C_{17:0}$	−	1.3	2.4
$C_{18:0}$	1.7	1.1	−
Unsaturated fatty acids			
$C_{16:1}\omega5c$	−	−	1.5
$C_{17:1}\omega8c$	−	1.1	−
$C_{18:1}\omega7c$	37.2	35.2	8.8
$C_{18:1}\omega5c$	−	−	1.3
Branched fatty acids			
iso-$C_{17:0}$	1.1	−	3.7
anteiso-$C_{17:0}$	1.0	−	−
Hydroxy fatty acids			
$C_{10:0}$ 3-OH	1.7	2.7	2.0
$C_{12:0}$ 3-OH	3.5	4.2	2.8
Cyclopropane acids			
$C_{17:0}$ cyclo	2.5	1.4	26.4
$C_{19:0}$ cyclo $\omega8c$	−	−	23.1
Summed feature 3*	17.4	21.6	2.4

*Summed feature 3 comprises iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$.

iso-$C_{16:0}$, $C_{17:1}\omega8c$, $C_{17:0}$, iso-$C_{18:0}$ and $C_{19:0}$ cyclo $\omega8c$ were also detected in the type strain. Additional physiological characteristics are shown in Table 1.

The type strain, J64^T (=KCTC 22137^T =JCM 14963^T), was isolated from black sand collected from Soesoggak, Jeju Island, Korea.

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