Phenylobacterium koreense sp. nov., isolated from South Korea

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A Gram-negative, aerobic, rod-shaped, non-spore-forming bacterium, Slu-01^T, was isolated from activated sludge from a wastewater treatment plant in Daejeon, South Korea. After 3 days on R2A medium, strain Slu-01^T developed colourless colonies (0.7–1.2 mm). The non-motile rods (0.7–1.0 × 1.0–2.0 µm) were slightly curved and occurred singly and in pairs. No filamentous cells were found. On the basis of 16S rRNA gene sequence similarity, strain Slu-01^T was shown to belong to the family *Caulobacteraceae* and was most closely related to *Phenylobacterium immobile* (96.6%) and *Phenylobacterium lituiforme* (96.5%). The genomic DNA G + C content of strain Slu-01^T was 68.1 mol%, within the range of 67.3–68.4 mol% for the genus *Phenylobacterium*. Chemotaxonomic data (major ubiquinone, Q-10; major fatty acids C_{18:1ω7c}, C_{16:0}, C_{15:0} and C_{17:0}) and 16S rRNA gene sequence analysis supported the affiliation of strain Slu-01^T to the genus *Phenylobacterium*. However, DNA–DNA hybridization data and phenotypic properties showed that strain Slu-01^T could be distinguished from the two other *Phenylobacterium* species with validly published names. Thus, it is suggested that strain Slu-01^T represents a novel species of the genus *Phenylobacterium*, for which the name *Phenylobacterium koreense* is proposed. The type strain is Slu-01^T (=KCTC 12206^T = IAM 15119^T).

The genus *Phenylobacterium* was first described by Lingens *et al.* (1985) and at present comprises only two species, *Phenylobacterium immobile* DSM 1986^T (Lingens *et al.*, 1985) and *Phenylobacterium lituiforme* DSM 14363^T (Kanso & Patel, 2004). Kanso & Patel (2004) proposed an emendment to the description of the genus *Phenylobacterium* which states that members of the genus *Phenylobacterium* are Gram-negative, non-spore-forming, straight to slightly curved rods, coccobacilli or cocci measuring 0.7–1.0 × 1.0–2.0 µm and occur singly, in pairs or short chains. Species may be strict aerobes or facultative anaerobes and may be motile or non-motile. Members of the genus grow on L-phenylalanine. Based on the 16S rRNA gene sequence analysis, members of this genus form a monophyletic group within the order *Caulobacterales*, subclass *α-Proteobacteria*, class *Proteobacteria*.

Strain Slu-01^T was isolated from activated sludge from a wastewater treatment plant in Daejeon, South Korea. In this study, a polyphasic approach, including phylogenetic analysis based on 16S rRNA gene sequences and analysis of chemotaxonomic and phenotypic properties, was adopted to determine the precise taxonomic position of Slu-01^T. The results indicate that Slu-01^T can be assigned as a member of the genus *Phenylobacterium*, but the strain can be clearly distinguished from the existing *Phenylobacterium* species with validly published names. We propose Slu-01^T as the type strain of a novel species, namely *Phenylobacterium koreense* sp. nov.

The serial dilution method was used on R2A (Difco) agar plates to isolate aerobic bacteria from activated sludge. Slu-01^T was one of the dominant isolates appearing on the plates in aerobic conditions. Strain Slu-01^T was deposited in the Korean Collection for Type Cultures (KCTC 12206^T) and the IAM Culture Collection of the University of Tokyo (IAM 15119^T).

The Gram reaction was performed by the non-staining method as described by Buck (1982). The morphology of cells grown for 3 days at 30 °C on R2A agar was observed under a Nikon light microscope at ×1000 magnification. Catalase and oxidase tests were performed according to

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Slu-01^T is AB166881.

A table comparing the cellular fatty acid profiles of strain Slu-01^T, *Phenylobacterium immobile* and *Phenylobacterium lituiforme* is available as supplementary material in IJSEM Online.

Cappuccino & Sherman (2002). Substrate utilization as a sole carbon source and some physiological characteristics were determined using the API 32GN, API 20E and API 20NE galleries according to the manufacturer's instructions (bioMérieux). Degradation of DNA (using Difco DNA agar, supplemented with 0.01 % toluidine blue, Merck)

and degradation of casein, chitin, starch, L-tyrosine and skimmed milk (Atlas, 1993; Lewin & Lounsbury, 1969) reactions were read after 5 days.

Hydrolysis of carboxymethylcellulose was tested as described by Ten *et al.* (2004). Growth tests with L-phenylalanine

Table 1. Comparison of selected characteristics of *Phenylobacterium koreense* Slu-01^T with those of *Phenylobacterium immobile* DSM 1986^T and *Phenylobacterium lituiforme* DSM 14363^T

Strains: 1, *Phenylobacterium koreense* Slu-01^T (data from this study); 2, *P. immobile* DSM 1986^T, data from Lingens *et al.* (1985); 3, *P. lituiforme* DSM 14363^T, data from Kanso & Patel (2004). +, Positive; (+), positive, weak or delayed response; –, negative; ND, not defined. All species shown here are Gram-negative, grow on L-phenylalanine, do not form spores, do not grow on maltose or hydrolyse starch or aesculin. All species are catalase-positive, urease-negative and do not produce indole.

Characteristic	1*	2	3
Isolation source	Activated sludge	Soil	Water from subsurface aquifer
Pigmentation	Colourless	Colourless	Yellow to pale-yellow
Cell shape	Rods, coccid rods	Rods, coccid rods or cocci	Rods
Cell size	0.7–1.0 × 1.0–2.0 µm	0.7–1.0 × 1.0–2.0 µm	0.5 × 1–3 µm
Cell behaviour	Single, pairs	Single, pairs or short chains	Single, pairs
Motility	–	–	+
Flagella	None	None	Single, polar
Oxygen requirement	Obligate aerobes	Obligate aerobes	Facultative anaerobe
pH growth range	6.5–8, optimal at 7–8	6–9, optimal at 6–6.5	6.5–8, optimal at 6.8–7
Growth temperature (°C)	Optimum 28–30, growth at 37 °C	Optimum 28–30, no growth at 37 °C	Optimum 40–41, range 25–45, no growth at 50 °C
NaCl tolerance (% w/v)	Optimum at 0%, growth at 1–2 %	Optimum at 0%, no growth at 1 %	Optimum at 0%, no growth at 1 %
Oxidase	+	(+)	–
Nitrate reduction	+	–	+
Nitrogen gas from NO ₃ [–]	–	–	+
Methyl red	–	–	ND
Voges–Proskauer	–	–	ND
Litmus milk	–	–	ND
Gas from sugars	–	–	ND
Hydrolysis of gelatine	–	–	(+)
Production of H ₂ S	–	(+)	–
Growth on:			
Acetate	+	–	+
Antipyrin	–	+	–*
Chloridazon	–	+	–*
Citrate	–	–	+
Fumarate	–	(+)	+
Glutamate	–	(+)	ND
Glycerol	–	–	+
Malate	–	(+)	ND
Pyruvate	–	(+)	ND
Simple sugars, glucose	–	–	+
Succinate	–	(+)	+
Sucrose	–	–	+
Sucrose	–	–	+
Xylose	–	–	+
Ubiquinone	Q-10	Q-10	ND
DNA G + C content (mol%)	68.1	67.3–68.4	66.5 ± 0.5

*Data from this study.

(1.0 g l⁻¹), chloridazon (1.0 g l⁻¹) and antipyrin (1.0 g l⁻¹) as the sole carbon source were determined in minimal medium B (Lingens *et al.*, 1985) with vitamin B₁₂ (0.03 mg l⁻¹) at 30 °C. Growth at different temperatures and pH was assessed after 5 days incubation. Salt tolerance was tested in R2A broth medium supplemented with 1–10 % (w/v) NaCl after 7 days incubation. Duplicate antibiotic-sensitivity tests were performed using filter paper disks containing the following: streptomycin (5, 10 and 15 µg ml⁻¹) (Mast Diagnostics), tetracycline (5, 10 and 15 µg ml⁻¹), kanamycin (1.0, 1.5 and 2.0 mg ml⁻¹), ampicillin (30, 50 and 60 µg ml⁻¹) (Sigma), chloramphenicol (10, 20 and 30 µg ml⁻¹), carbenicillin (100, 200 and 300 µg ml⁻¹), rifampicin (50, 100 and 150 µg ml⁻¹) and hygromycin (100, 200 and 300 µg ml⁻¹). Disks were placed on R2A plates spread with Slu-01^T culture and incubated at 30 °C for 7 days. Higher antibiotic concentrations were tested if antibiotic resistance was noted. The physiological and biochemical characteristics of strain Slu-01^T and related type strains are summarized in Table 1.

Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Im *et al.* (2004). The 16S rRNA gene sequences of related taxa were obtained from GenBank. Multiple alignments were performed using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited using the BioEdit program (Hall, 1999). The Kimura two-parameter model (Kimura, 1983) was used to calculate evolutionary distances. A phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) with the MEGA2 program (Kumar *et al.*, 2001) with bootstrap values based on 1000 replications (Felsenstein, 1985).

The G + C content of chromosomal DNA was determined using a reverse-phase HPLC method (Mesbah *et al.*, 1989). Respiratory quinones were extracted from cells grown on R2A Broth (Difco) and analysed as described by Komagata & Suzuki (1987) using reverse-phase HPLC. Cellular fatty acids were determined from cultures grown on trypticase soy agar (TSA; Difco) for 2 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol for the Sherlock Microbial Identification System (MIDI). Fatty acids were analysed by GC (6890; Hewlett Packard) and identified by the Microbial Identification software package (Sasser, 1990).

Strain Slu-01^T was Gram-negative. Cells were rods and coccal rods (0.7–1.0 × 1.0–2.0 µm), slightly curved and occurred both singly and in pairs. No filaments were found, even in old cultures. No flagella movement was observed by light microscopy. Colonies grown on R2A agar plates for 3 days were smooth, circular, convex, colourless and 0.7–1.2 mm in diameter. When grown on medium C (Lingens *et al.*, 1985) supplemented with L-phenylalanine (0.1 %) and vitamin B₁₂ (0.03 mg l⁻¹), Slu-01^T formed a 2 mm colony after 4 days at 30 °C. Strain Slu-01^T could not grow

with chloridazon and antipyrin as the single carbon source. Spores were never observed and cells were heat-sensitive.

On R2A agar, the optimal growth temperature for Slu-01^T was 30 °C. Strain Slu-01^T was able to grow at 37 °C, but not at 4 or 45 °C. The pH growth range was between pH 6.5 and 8.5, with an optimum between pH 7 and 8. Growth occurred in the absence of NaCl and in the presence of 2.0 % (w/v) NaCl, but not 4 % (w/v) NaCl. In the presence of NaCl, most of the cells became long aberrant forms (Fig. 1). Strain Slu-01^T was resistant to 500 µg ml⁻¹ ampicillin, 50 µg ml⁻¹ tetracycline, 50 µg ml⁻¹ streptomycin, 2.0 mg ml⁻¹ kanamycin, 300 µg ml⁻¹ carbenicillin, 500 µg ml⁻¹ hygromycin and 100 µg ml⁻¹ rifampicin. It has been shown that it should be possible to identify species on the basis of susceptibility tests and that susceptibilities to certain antibiotics reflect adaptation to certain environmental conditions (Reva *et al.*, 1995). The fact that strain Slu-01^T exhibited resistance to antibiotics reflects its environmental habitat of activated sludge.

Physiological characteristics of strain Slu-01^T are summarized in the species description and a comparison of selective characteristics with related type strains is shown in Table 1.

The 16S rRNA gene sequence of strain Slu-01^T was a continuous stretch of 1416 bp. Sequence similarity calculations after a neighbour-joining analysis indicated that the closest relatives of strain Slu-01^T were *P. immobile* (96.6 %) and

Fig. 1. (a) Phase-contrast photomicrographs of cells of *Phenylobacterium koreense* Slu-01^T grown on R2A medium. (b) Cells grown with 1 % (w/v) NaCl. Bars, 8 µm.

Fig. 2. Phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of *Phenyllobacterium koreense* Slu-01^T with other related species. The tree was constructed using the neighbour-joining method and Jukes & Cantor evolutionary distance matrix data obtained from unambiguous aligned nucleotides. Bootstrap values (expressed as percentage of 1000 replications) greater than 70% are shown at the branch points. Bar, 1 substitution per 100 nucleotide position.

P. lituiforme (96.5%). A low level of similarity (94%) was found with the genera *Caulobacter* (Abraham *et al.*, 1999) and *Brevundimonas* (Fig. 2). In order to confirm whether Slu-01^T represented a novel species, DNA–DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989). An FL_X 800 Microplate Fluorescence Reader (Bio-Tek) was employed for fluorescence measurements. The hybridization temperature was 50 °C and reciprocal experiments were performed for *P. immobile* DSM 1986^T and Slu-01^T. The DNA–DNA relatedness level between *P. immobile* DSM 1986^T and Slu-01^T was 15%, which demonstrated that strain Slu-01^T belonged to a distinct genomic species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Thus, based on the 16S rRNA gene sequence analyses and DNA–DNA hybridization results, a novel taxon could be detected.

The genomic DNA G+C content of strain Slu-01^T was 68.1 mol%. Strain Slu-01^T had a ubiquinone of the Q-10 type. The major cellular fatty acid profile of strain Slu-01^T contained C_{18:1}ω7c (23.5%), C_{16:0} (10.4%), C_{15:0} (9.6%), C_{17:0} (8.7%), C_{18:0} (7.3%), summed feature 4 (C_{16:1}ω7c/C_{15:0} iso 2-OH, 5.7%), C_{18:1} 2-OH (4.9%), C_{16:1}ω11c (4.4%), C_{12:0} (4.0%), C_{17:1}ω6c (3.7%) and C_{12:1} 3-OH (3.4%). Although the major fatty acids of strain Slu-01^T were very similar to those of type strains of species from the genus *Phenyllobacterium*, some fatty acids, such as C_{11:0}, C_{16:1}ω11c, C_{17:1}ω9c and C_{18:1} 2-OH, were only present in strain Slu-01^T (see Supplementary Table S1 in IJSEM Online).

Strain Slu-01^T, being closely related to species of the genus *Phenyllobacterium*, was able to metabolize the aromatic amino acid L-phenylalanine for growth and had a DNA G+C content of 68.1 mol%, within the range of 67.3–68.4 mol% for the genus *Phenyllobacterium* (Lingens *et al.*, 1985). Other characteristics of strain Slu-01^T, such as temperature and pH requirements, colony colour and

size, cell size and shape and ubiquinone (Q-10) profiles (Table 1), showed that it belonged to the genus *Phenyllobacterium*. However, the low DNA–DNA relatedness with *P. immobile* DSM 1986^T and other characteristics, such as high resistance to antibiotics, no metabolism of chloridazon and antipyrin as the sole carbon source, tolerance of NaCl (2%) and fast growth on R2A agar and nutrient agar, support the recognition of a novel species within the genus *Phenyllobacterium*. It is concluded that strain Slu-01 represents a novel species for which the name *Phenyllobacterium koreense* sp. nov. is proposed.

Description of *Phenyllobacterium koreense* sp. nov.

Phenyllobacterium koreense (ko.re.en'se. N.L. neut. adj. *koreense* of Korea, from where the novel organism was isolated).

Cells are aerobic, Gram-negative, rod-shaped, non-spore-forming, non-motile, 0.7–1.0 µm wide × 1.0–2.0 µm long and slightly curved. Cells occur singly and in pairs. Cells do not make filaments. Colonies on R2A and complex medium are circular, convex, colourless and 0.7–1.2 mm after 3 days. The temperature range for growth is 20–37 °C, with an optimum temperature of 28–30 °C. No growth occurs at 4 or 45 °C. The pH growth range is between pH 6.5 and 8.5, with an optimum of between pH 7 and 8. Growth occurs in the absence of NaCl and in the presence of 2.0% (w/v) NaCl, but not at 4% (w/v) NaCl. In the presence of NaCl, most of the cells become long aberrant forms. Catalase- and oxidase-positive. H₂S is not produced. Nitrate is reduced. Tests for urease, β-glucosidase and β-galactosidase activity are negative. No utilization of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, gelatinase or citrate. Does not produce any acid or gas from glucose. Casein, xylan, chitin, tyrosine, CM-cellulose, gelatin and starch are not degraded.

DNA is degraded. The following compounds are utilized as sole carbon sources: L-phenylalanine, acetate, suberate, L-proline, adipate, 4-hydroxy-benzoate, 3-hydroxy-butyrate, valerate and caprate. The following compounds are not utilized as sole carbon sources: chloridazon, antipyrin, glucose, mannose, N-acetylglucosamine, maltose, propionate, L-arabinose, mannitol, gluconate, malate, citrate, phenylacetate, salicin, D-melibiose, L-fucose, D-sorbitol, histidine, 2-ketogluconate, 4-hydroxy-butyrate, rhamnose, D-ribose, inositol, D-sucrose, itaconate, malonate, lactate, L-alanine, 5-ketogluconate, glycogen and L-serine. Acetoin is produced. The species is resistant to 500 µg ml⁻¹ ampicillin, 50 µg ml⁻¹ tetracycline, 50 µg ml⁻¹ streptomycin, 2.0 mg ml⁻¹ kanamycin, 300 µg ml⁻¹ chloramphenicol, 300 µg ml⁻¹ carbenicillin, 500 µg ml⁻¹ hygromycin and 100 µg ml⁻¹ rifampicin. The major cellular fatty acids are C_{18:1}ω7c (23.5%), C_{16:0} (10.4%), C_{15:0} (9.6%), C_{17:0} (8.7%), C_{18:0} (7.3%), summed feature 4 (C_{16:1}ω7c/C_{15:0} iso 2-OH; 5.7%), C_{18:1} 2-OH (4.9%), C_{16:1}ω11c (4.4%), C_{12:0} (4.0%), C_{17:1}ω6c (3.7%) and C_{12:1} 3-OH (3.4%). The genomic DNA G+C content of the type strain is 68.1 mol% (as determined by HPLC).

The type strain, Slu-01^T (=KCTC 12206^T=IAM 15119^T), was isolated from activated sludge from a wastewater treatment plant in Daejeon, South Korea.

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