Pigmentiphaga litoralis sp. nov., a facultatively anaerobic bacterium isolated from a tidal flat sediment

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A novel Gram-negative, facultatively anaerobic, non-sporulating, non-motile, catalase- and oxidase-positive, rod-shaped bacterium (strain JSM 061001^T) was isolated from a tidal flat in the South China Sea, China. Growth occurred with 0–5 % (w/v) NaCl [optimum, 0.5–1 % (w/v) NaCl], at pH 5.0–10.0 (optimum, pH 7.0) and at 4–35 °C (optimum, 25–30 °C). The major cellular fatty acids were C_{16:0}, cyclo C_{17:0}, C_{18:1}ω7c and C_{16:1}. Strain JSM 061001^T contained ubiquinone Q-8 as the predominant respiratory quinone, and phosphatidylglycerol, phosphatidylethanolamine and an unidentified phospholipid as the polar lipids. The genomic DNA G+C content was 65.5 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain JSM 061001^T belongs to the family *Alcaligenaceae* and was related most closely to the type strains of the two recognized species of the genus *Pigmentiphaga*. The three strains formed a robust cluster in the neighbour-joining, maximum-parsimony and maximum-likelihood phylogenetic trees. Levels of DNA–DNA relatedness between strain JSM 061001^T and the type strains of *Pigmentiphaga daeguensis* and *Pigmentiphaga kullae* were 15.8 and 10.5 %, respectively. The combination of phylogenetic analysis, DNA–DNA hybridization data, phenotypic characteristics and chemotaxonomic differences supported the view that strain JSM 061001^T represents a novel species of the genus *Pigmentiphaga*, for which the name *Pigmentiphaga litoralis* sp. nov. is proposed. The type strain is JSM 061001^T (=CCTCC AA207034^T=KCTC 22165^T).

The genus *Pigmentiphaga*, belonging to the family *Alcaligenaceae* (De Ley *et al.*, 1986), was first proposed by Blümel *et al.* (2001) and was defined as comprising Gram-negative,

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JSM 061001^T is EU583723.

Maximum-parsimony and maximum-likelihood phylogenetic trees showing the position of strain JSM 061001^T among other related taxa inferred from 16S rRNA gene sequences, and a table detailing the fatty acid compositions of strain JSM 061001^T and members of the genus *Pigmentiphaga* are available as supplementary material with the online version of this paper.

motile or non-motile, non-sporulating, catalase- and oxidase-positive rods, with ubiquinone Q-8 as the predominant respiratory quinone and phosphatidylethanolamine as the major polar lipid. At the time of writing, the genus comprised two recognized species, *Pigmentiphaga kullae* and *Pigmentiphaga daeguensis*. During investigation of the microbial diversity of Naozhou Island, Leizhou Bay, South China Sea, China, a Gram-negative bacterium, designated strain JSM 061001^T, was isolated from a tidal flat sediment sample. Based on the results of a polyphasic taxonomic study, this strain is proposed to represent a novel species of the genus *Pigmentiphaga*.

Strain JSM 061001^T was isolated from a tidal flat sediment sample by plating 1:10 serial dilutions of the sample on trypticase soy agar (TSA; Difco) prepared with 50 % (v/v) seawater at 28 °C for 14 days. After primary isolation and purification, the isolate was preserved both on TSA slants at 4 °C and in trypticase soy broth (TSB; Difco) supplemented with 20 % (v/v) glycerol at –80 °C. Reference strains *P. kullae* DSM 13608^T and *P. daeguensis* KCTC 12838^T were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and the KCTC (Korean Collection for Type Cultures), respectively. Unless otherwise indicated, morphological and physiological studies were performed with cells grown on TSA at 28 °C. Cell morphology was examined by using light microscopy (model BH 2; Olympus). Gram staining was carried out by using the standard Gram reaction combined with the KOH lysis test method (Gegersen, 1978). Growth was tested at various temperatures [0, 4 and 5–45 °C (at increments of 5 °C)] on TSA and at different pH values [5.0–11.0 (at increments of 0.5 pH units)] in TSB. Growth in the absence of NaCl and in the presence of various NaCl concentrations [0.5 % (w/v), and 1.0–10.0 % (w/v, at increments of 1.0 %)] was investigated by using TSB prepared according to the formula of the Difco medium except that no NaCl was used, and on some other media as controls, i.e. marine agar 2216 (MA; Difco), nutrient broth (NB; Difco) and ISP medium 2 agar (Shirling & Gottlieb, 1966). Hydrolysis of aesculin and nitrate reduction were determined as described by Lányi (1987). Urease activity and hydrolysis of casein, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, urea and xanthine were determined as described by Cowan & Steel (1965). Anaerobic growth was determined on TSA at 28 °C for 2 weeks by using the GasPak Anaerobic System (BBL) according to the manufacturer's instructions, and reference strains *P. kullae* DSM 13608^T and *P. daeguensis* KCTC 12838^T were incubated under the same conditions as controls. Motility, and catalase and oxidase activities were detected as described by Chen *et al.* (2007). Other enzyme activities, acid production from carbohydrates, utilization of various substrates, and other physiological and biochemical properties were tested by using the API 20E, API 20NE, API 50 CH and API ZYM systems (bioMérieux) according to the manufacturer's instructions. Morphological, cultural, physiological and biochemical characteristics of strain JSM 061001^T are given in the species description below and in Table 1.

DNA was isolated according to Hopwood *et al.* (1985) and the G+C content was determined by using the HPLC method (Mesbah *et al.*, 1989). Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of PCR products were performed as described by Cui *et al.* (2001). The resulting 16S rRNA gene sequence of strain JSM 061001^T was compared with sequences obtained from public databases (GenBank/EMBL/DBJ) to find the most closely related species. Phylogenetic analysis was performed by using the software package MEGA version 3.1 (Kumar *et al.*, 2004) after multiple alignment of the sequence data by CLUSTAL_X (Thompson *et al.*, 1997).

Table 1. Differential characteristics between strain JSM 061001^T and recognized *Pigmentiphaga* species

Strains: 1, JSM 061001^T (*Pigmentiphaga litoralis* sp. nov.; data from the present study); 2, *P. daeguensis* KCTC 12838^T (Yoon *et al.*, 2007); 3, *P. kullae* DSM 13608^T (Blümel *et al.*, 2001). +, Positive; –, negative.

Characteristic	1	2	3
Motility	–	–	+
Facultatively anaerobic growth	+	–	–*
Growth at:			
4 °C	+	–	–
40 °C	–	+	+
Production of:†			
Alkaline phosphatase	+	–	–
α-Chymotrypsin	–	+	+
Cystine arylamidase	+	–	–
Esterase (C4)	–	+	+
α-Glucosidase	+	–	–
β-Glucosidase	+	–	–
Major polar lipids‡	PG, PE	PG, PE, AL	PE
DNA G+C content (mol%)	65.5	67.4	68.5

*Data from this study under identical conditions.

†Data for *P. kullae* DSM 13608^T were taken from Yoon *et al.* (2007).

‡AL, unidentified aminolipids; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

Distances (corrected according to Kimura's two-parameter model; Kimura, 1980) were calculated and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) trees were generated by using the treeing algorithms contained in the PHYLIP software package (Felsenstein, 1993). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by means of 1000 resamplings (Felsenstein, 1985). DNA–DNA hybridization was carried out at 45 °C by using photobiotin-labelled DNA probes and microdilution wells as described by Ezaki *et al.* (1989). A microplate spectrofluorimeter (GeminiXPS; Molecular Devices) was employed for fluorescence measurements.

The DNA G+C content of strain JSM 061001^T was 65.5 mol%. A 1475-bp fragment of the 16S rRNA gene was amplified. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the novel isolate was closely related to the type strains of the two recognized species of the genus *Pigmentiphaga* (Blümel *et al.*, 2001), and these three strains formed a robust cluster in the neighbour-joining, maximum-likelihood and maximum-parsimony phylogenetic trees, in which strain JSM 061001^T occupied a distinct subline branching at the periphery of the cluster and supported by a significant bootstrap resampling value (100 %) (see Fig. 1 and Supplementary Fig. S1 available in IJSEM Online). Levels of 16S rRNA gene sequence similarity between strain JSM 061001^T and the type strains of *P. daeguensis* and *P. kullae*

Fig. 1. Phylogenetic dendrogram based on 16S rRNA gene sequence analysis constructed by using the neighbour-joining method showing the position of strain JSM 061001^T among related taxa. Numbers at nodes indicate bootstrap values (>50%) based on a neighbour-joining analysis of 1000 resampled datasets. Bar, 1 substitution per 100 nt.

were 97.9 and 97.8%, respectively. By contrast, levels of DNA–DNA relatedness between strain JSM 061001^T and the type strains of *P. daeguensis* and *P. kullae* were 15.8 and 10.5%, respectively. It has been suggested that bacterial strains showing a level of DNA–DNA relatedness of less than 70% are not members of the same species (Wayne *et al.*, 1987). Thus, the results of the phylogenetic analysis and DNA–DNA hybridization suggested that strain JSM 061001^T was a member of the genus *Pigmentiphaga* distinct from *P. daeguensis* and *P. kullae*.

Isoprenoid quinones were analysed by HPLC as described by Groth *et al.* (1996). Polar lipids were extracted according to the method of Minnikin *et al.* (1984) and were then identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). Fatty acid compositions were determined as described by Sasser (1990) by using the Microbial Identification System (MIDI; Microbial ID) with cells grown on TSA after incubation for 3 days at 28 °C. Chemotaxonomic data for strain JSM 061001^T were compatible with its assignment to the genus *Pigmentiphaga* (Blümel *et al.*, 2001). The predominant respiratory quinone of strain JSM 061001^T was ubiquinone Q-8 (peak area ratio of approximately 96.9%). The polar lipids of this strain consisted of phosphatidylglycerol, phosphatidylethanolamine and an unidentified phospholipid. The major fatty acids (>10% of the total) were C_{16:0}, cyclo C_{17:0}, C_{18:1}ω7c and C_{16:1} (see Supplementary Table S1, available in IJSEM Online).

The results of phylogenetic analysis and the chemotaxonomic studies presented above supported the affiliation of strain JSM 061001^T to the genus *Pigmentiphaga* (Blümel *et al.*, 2001). However, it could be distinguished from recognized species of the genus based on a number of phenotypic properties (Table 1) and by its discriminative

fatty acid profile (Supplementary Table S1), although the latter may have resulted partly from the different culture conditions used here and by Blümel *et al.* (2001) and Yoon *et al.* (2007). The presence of noticeable amounts of unbranched monounsaturated fatty acids (making up 34.8% of the total) and the low concentration of cyclo C_{19:0}ω8c in the fatty acid profile of strain JSM 061001^T (Supplementary Table S1) distinguished it from *P. daeguensis* and *P. kullae*. Strain JSM 061001^T differed markedly from the two recognized *Pigmentiphaga* species based on its ability to grow under anaerobic conditions and at a comparatively low temperature range (down to 4 °C), and by its distinct activity profile of constitutive enzymes (Table 1). Together with the relatively low DNA G+C content of strain JSM 061001^T (Table 1) and DNA–DNA hybridization data, the results of this polyphasic taxonomic study support the suggestion that strain JSM 061001^T represents a novel species of the genus *Pigmentiphaga*, for which the name *Pigmentiphaga litoralis* sp. nov. is proposed.

Description of *Pigmentiphaga litoralis* sp. nov.

Pigmentiphaga litoralis (li.to.ra'lis. L. fem. adj. *litoralis* of or belonging to the seashore).

Cells are Gram-negative, non-sporulating, non-motile, catalase- and oxidase-positive, facultatively anaerobic, straight to slightly curved rods, approximately 0.5–0.8 µm wide and 1.0–2.5 µm long. Colonies are yellow-pigmented, somewhat convex and non-translucent, with glistening surfaces and circular/slightly irregular margins, and are 1–2 mm in diameter after incubation for 3 days at 28 °C on TSA. No diffusible pigments are produced. Growth occurs with 0–5% (w/v) NaCl [optimum, 0.5–1% (w/v) NaCl], at pH 5.0–10.0 (optimum, pH 7.0) and at 4–35 °C (optimum, 25–30 °C). Aesculin is hydrolysed, but casein, gelatin, hypoxanthine, starch, L-tyrosine, Tweens

20, 40, 60 and 80, urea and xanthine are not. Voges–Proskauer test and citrate utilization are positive. Nitrate is not reduced to nitrite. H₂S and indole are not produced. Positive for activity of acid phosphatase, alkaline phosphatase, arginine dihydrolase, cystine arylamidase, esterase lipase (C8), α -glucosidase, β -glucosidase, lysine decarboxylase, ornithine decarboxylase and naphthol-AS-BI-phosphohydrolase (API ZYM and API 20E). No activity is observed for α -chymotrypsin, esterase (C4), α -fucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, lipase (C14), leucine arylamidase, α -mannosidase, trypsin, urease or valine arylamidase (API ZYM and API 20E). Arabinose, glucose, inositol, malate, maltose, mannitol, mannose, rhamnose, sorbitol and sucrose are utilized, but *N*-acetylglucosamine, adipic acid, amygdalin, capric acid, gluconate, melibiose and potassium phenylacetic acid are not (API 20E and API 20NE). Acids are produced from D-fructose, D-glucose, glycerol, D-lactose, maltose, D-mannitol, D-ribose, sucrose, trehalose and D-xylose (API 50 CH). Major fatty acids (>10 % of the total) are C_{16:0}, cyclo C_{17:0}, C_{18:1} ω 7c and C_{16:1}. The predominant respiratory quinone is ubiquinone Q-8, and the polar lipids are phosphatidylglycerol, phosphatidylethanolamine and an unidentified phospholipid. The DNA G + C content of the type strain is 65.5 mol% (HPLC method).

The type strain, JSM 061001^T (=CCTCC AA207034^T=KCTC 22165^T), was isolated from a tidal flat sediment sample collected from the shore of Naozhou Island, Leizhou Bay, South China Sea, China.

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