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NOTE

Pigmentiphaga soli sp. nov., a Bacterium Isolated from Soil[§]

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Strain BS12^T, a Gram-negative motile bacterium, was isolated from soil in South Korea and characterized to determine its taxonomic position. Phylogenetic analyses based on the 16S rRNA gene sequence revealed that the strain belonged to the family *Alcaligenaceae* in the class *Betaproteobacteria*. The highest degree of sequence similarities of strain BS12^T were found with *Pigmentiphaga litoralis* JSM 061001^T (98.3%), *Pigmentiphaga daeguensis* K110^T (98.2%), and *Pigmentiphaga kullae* K24^T (98.1%). Chemotaxonomic data revealed that strain BS12^T possessed ubiquinone-8, which is common in the family *Alcaligenaceae*, and the predominant fatty acids were $C_{16:0}$, $C_{17:0}$ cyclo, summed feature 3 ($C_{16:1}$ *ω6c/ω7c*), and summed feature 8 ($C_{18:1}$ *ω6c/ω7c*). The major polar lipids of strain BS12^T were phosphatidylethanolamine and phosphatidylglycerol. Based on these data, BS12^T (=KCTC 23577^T =JCM 17666^T =KEMB 9004-082^T) should be classified as a type strain of a novel species, for which the name *Pigmentiphaga soli* sp. nov. is proposed.

***Keywords*:** *Alcaligenaceae*, *Betaproteobacteria*, *Pigmentiphaga*, taxonomy

The genus *Pigmentiphaga* was first proposed by Blümel *et al*. (2001), and *Pigmentiphaga kullae* was the type species. The genus *Pigmentiphaga* is a Gram-negative, catalase- and oxidase-positive, motile or non-motile rod containing ubiquinone Q-8 and phosphatidylethanolamine (PE). After proposing the genus *Pigmentiphaga*, *Pigmentiphaga daeguensis* was isolated from wastewater (Yoon *et al.*, 2007), and *Pigmentiphaga litoralis* was isolated from a tidal flat (Chen *et al*., 2009). At present, the genus *Pigmentiphaga* contains three validated species.

During the course of collecting microorganisms from soil samples a Gram-negative, motile, aerobic non-spore forming rod shaped bacterial strain BS12^T was isolated. Based on phylogenetic clustering of 16S rRNA gene sequences, strain BS12^T was assumed to be a new member of the genus *Pigmentiphaga* in the family *Alcaligenaceae*. Strain BS12^T was characterized using a polyphasic approach, including phylogenetic analysis of the 16S rRNA gene sequence, genomic relatedness, and chemotaxonomic and phenotypic properties. Results obtained in this study indicated that strain BS12^T should be assigned as a new species in the genus *Pigmentiphaga* of the family *Alcaligenaceae*.

Strain BS12^T was originally isolated from soil by direct plating onto ten-fold diluted R2A agar (Difco, USA) and incubated at 30°C. The purified colonies were identified tentatively by partial 16S rRNA gene sequences and preserved in a glycerol solution (20%, w/v) at -70°C. Strain BS12^T was deposited at the Korean Collection for Type Cultures (KCTC), Japan Collection of Microorganisms (JCM) and the Korea National Environmental Microorganism Bank (KEMB).

Gram reactions were conducted according to the non-staining method described by Buck (1982). Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy (Call Zeiss LEO912AB) after the cells had grown for 3 days at 30°C on R2A agar. Oxidase activity was evaluated by the oxidation of 1% (w/v) tetramethyl-p-phenylene diamine. Catalase activity was determined by measuring bubble production after applying 3% (v/v) hydrogen peroxide solution. Growth on different media was assessed on trypticase soy agar (TSA), Luria-Bertani agar (LB), nutrient agar (NA), and R2A agar. The API 20NE, API ID32GN, API 50CH, and API ZYM microtest systems were employed according to the recommendations of the manufacturer (bioMérieux, France) to study carbon source utilization and enzyme activities of the strains. Growth at different temperatures (4, 20, 30, 37, 40, and 42°C) was assessed on NA agar for 5 days. Growth at various pH levels (4, 5, 6, 7, 8, 9, and 10) was assessed in NA broth at 30°C.

The 16S rRNA gene of strain BS12^T was amplified from chromosomal DNA using the 9F and 1512R universal bacterial primer set (Weisburg *et al*., 1991). Purified PCR product was sequenced by Genotech (Korea) (Kim *et al*., 2005). The full sequence of the 16S rRNA gene was compiled with SeqMan software (DNASTAR Inc., USA). The 16S rRNA gene sequences of related taxa were obtained from GenBank and edited with the BioEdit program (Hall, 1999). Multiple alignments were performed with the CLUSTAL X program (Thompson *et al*., 1997). Pairwise distances for the neighbor-joining algorithm (Saitou and Nei, 1987) were calculated according to the Kimura two-parameter model (Kimura, 1983), and a phylogenetic tree was constructed using the MEGA

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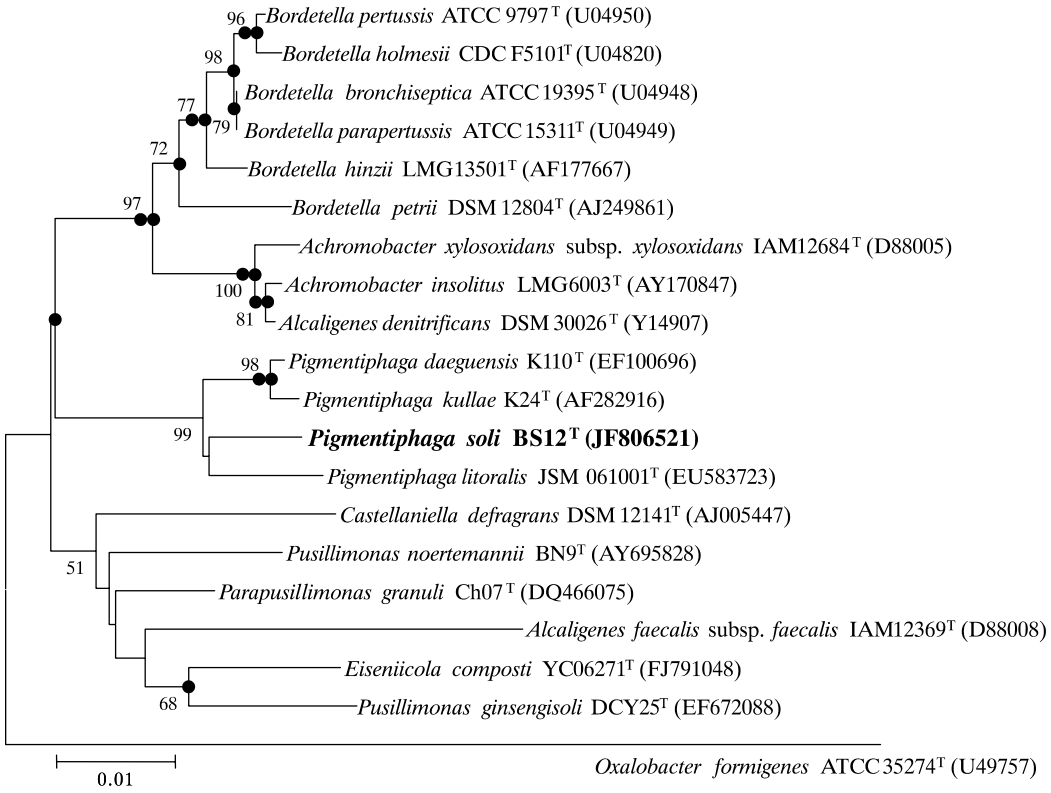

Fig. 1. A phylogenetic tree based on the 16S rRNA gene sequences of strain BS12^T and representatives of related taxa. The neighbor-joining method was used. Bar represents 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at branch points. Black circles indicate the common nodes recovered from either the maximum-parsimony algorithm or the maximum-likelihood tree. Black double-circles indicate that the corresponding nodes were recovered in both the maximum-parsimony tree and the maximum likelihood tree.

3 program (Kumar *et al.*, 2004). A bootstrap analysis with 1,000 replicates was also conducted to obtain confidence levels for the branches (Felsenstein, 1985). The min-mini heuristic method with a search factor of one was applied in a maximum-parsimony analysis using the MEGA 3 Program, and a maximum-likelihood analysis was performed with Phylip 3.69.

To determine G + C content, genomic DNA was extracted and purified with the QIAGEN Genomic-Tip System 100/G (Japan) and enzymatically degraded into nucleosides. The nucleosides were then analyzed using reverse-phase high performance liquid chromatography (HPLC), as previously described (Tamaoka and Komagata, 1984; Mesbah *et al*., 1989).

Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and identified using two-dimensional thin-layer chromatography (TLC) followed by spraying with the appropriate detection reagents (Minnikin *et al*., 1984; Komagata and Suzuki, 1987). The mobile phase for TLC development was chloroform/methanol/water (65:25:4, v/v/v), and the second mobile phase was chloroform/methanol/acetic acid/water (80:12:15:4, v/v/v/v). The total lipid profile was detected by spraying with molybdophosphoric acid solution (Sigma-Aldrich, USA) followed by heating at 150°C; aminolipids were detected by spraying with 0.2% (w/v) ninhydrin solution followed by heating at 105°C for 10 min; glycolipids were detected with 0.5% 1-naphthol in methanol/water (1:1, v/v) and sulfuric acid/ethanol (1:1, v/v) followed by heating at 120°C for 5-10 min; phospholipids were detected by spraying with Zinzadze reagent; and phosphatidylcholine was detected by spraying with Dragendorff reagent (Sigma-Aldrich).

Isoprenoid quinone was extracted with chloroform/methanol (2:1, v/v), purified via TLC, evaporated under vacuum conditions, and re-extracted with n-hexane:water (1:1, v/v). The crude n-hexane quinone solution was purified and subsequently analyzed by HPLC, as previously described (Collins and Jones, 1981). Cells were allowed to grow on TSA for 2 days at 30°C to perform the fatty acid methyl ester analysis, and then two loops of well-grown cells were harvested. Fatty acid methyl esters were prepared, separated, and identified with the Sherlock Microbial Identification System (Sherlock version 6.01; data base TSBA6; MIDI, Inc., USA) (Sasser, 1990).

DNA-DNA hybridization was performed fluorometrically, according to the method developed by Ezaki *et al*. (1989), using photobiotin-labeled DNA probes and micro-dilution wells. Hybridization was performed with five replications per sample. The highest and lowest values obtained for each sample were excluded, and the remaining three values were utilized to calculate hybridization values. DNA relatedness was expressed as the means of these three values.

Strain BS12^T was pale-yellow colored when routinely cul-

Table 1. Differential characteristics between strain BS12^T and all *Pigmentiphaga* species
Strains: 1, BS12^T; 2, *P. litoralis* KCTC 22165^T; 3, *P. daeguensis* KCTC 12838^T; 4, *P. kullae* KACC 11572^T.
All data were obtained in this study unless otherwise specified. All strains grew at 30 and 37°C but not at 4°C.
All strains produced leucine arylamidase, naphtol-AS-BI-phosphohydrolase, and valine arylamidase. All strains assimilated adipate and L-malate.
The strains did not produce *N*-acetyl-β-glucosaminidase, arginine dihydrolase, *α*-fucosidase, *α*-galactosidase, *β*-galactosidase (ONPG), *β*-galactosidase (PNPG), *α*-glucosidase, *β*-glucosidase (Esculin hydrolysis), *β*-glucosidase, *β*-glucuronidase, lipase (C14), *α*-mannosidase, or urease. They did not assimilate *N*-acetyl-D-glucosamine, gluconate, D-maltose, D-mannitol, D-mannose, or phenyl acetate.

Characteristic	1	2	3	4
Motility	+	-	-	+
Shape	short-rod	curved rod	rod	rod
Colony color (on LB agar)	PY	BY	PY	PY
Size (μm)				
Length	0.7-1.0	0.5-2.5	0.6-2.5	1.3-4
Width	0.5-0.6	0.5-0.8	0.3-0.6	0.7-1.2
Growth at				
42°C	+	–	+	+
pH 5	+	–	w	+
5% NaCl	w	w	w	+
Growth on R2A	w	+	+	+
Nitrate reduction to NO_2	–	–	–	+
Enzyme activity				
Acid phosphatase	+	–(+)	+(–)	w
Alkaline phosphatase	w	+	+(–)	+
α-Chymotrypsin	–	–	++	++
Cystine arylamidase	–	–(+)	–	w
Esterase (C4)	+	+(–)	+	w
Esterase (C8)	+	+	+	w
Trypsin	–	–	–	+
Assimilation				
L-Arabinose	–	–(+)	–	–
Caparate	w	–	–	–
Citrate	+	w	+	+
D-Glucose	+	–(+)	–	+(–)

+, positive; –, negative; w, weakly positive; ++, strongly positive; PY, pale-yellow, BY, bright-yellow.
Discrepancies between the original species descriptions and current data are included in parentheses. (Blumel *et al*., 2001; Yoon *et al*., 2007; and Chen *et al*., 2009).

tured on LB agar at 30°C. It was a Gram-negative, aerobic, motile, and short rod-shaped cell (Supplementary data Fig. 1) and was able to grow at a temperatures of 15-42°C, but not at 4°C. Optimal growth occurred at 30°C. Strain BS12^T grew well at pHs of 5-8. Other physiological characteristics of strain BS12^T are summarized in the species description.

The 16S rRNA gene sequence of strain BS12^T was a continuous stretch of 1453 nucleotides. Strain BS12^T belonged to the class *Betaproteobacteria*, order *Burkholderiales*, and family *Alcaligenaceae*. The highest degrees of sequence similarity of strain BS12^T was found with three *Pigmentiphaga* species: *P. litoralis* JSM 061001^T (98.3%) (Chen *et al*., 2009), *P. daeguensis* K110^T (98.2%) (Yoon *et al*., 2007), and *P. kullae* K24^T (98.1%) (Blümel *et al*., 2001). The closest species in another genus, *Parapusillimonas granuli* Ch07^T (Kim *et al*., 2010), showed a lower degree of sequence similarity (96.5%). In the phylogenetic tree (Fig. 1), strain BS12^T clearly belonged to the *Pigmentiphaga* linage (ML, MP, and NJ trees) in the family *Alcaligenaceae* as evidenced by the high bootstrap value of 99%. The phylogenetic position of strain BS12^T among members of the genus *Pigmentiphaga* was unique and distinct.

The predominant cellular fatty acids of strain BS12^T were $C_{16:0}$ (30.3±1.8%), $C_{17:0}$ cyclo (15.8±1.0%), summed feature 3 ($C_{16:1}$ *ω6c*/*ω7c*) (11.8±0.4%), and summed feature 8 ($C_{18:1}$ *ω6c*/*ω7c*) (11.6±0.8%). Minor fatty acids of strain BS12^T were summed feature 2 ($C_{14:0}$ 3OH/$C_{16:1}$ iso I) (4.8±4.3%), $C_{12:0}$ 2OH (4.8±1.2%), $C_{18:1}$ 2OH (4.1±0.3%), $C_{10:0}$ 3OH (4.0±1.4%), $C_{16:1}$ 2OH (3.3±0.7%), $C_{18:0}$ (2.7±0.2%), $C_{19:0}$ cyclo *ω8c* (2.7±0.5%), $C_{16:0}$ 2OH (1.4±0.2%), $C_{16:0}$ 3OH (0.9%), $C_{17:0}$ (0.5%), $C_{17:0}$ iso 3OH (0.5±0.9%), $C_{19:0}$ iso (0.2±0.4%), and $C_{14:1}$ *ω5c* (0.1±0.2%). The fatty acid profile of strain BS12^T was similar to that of other closely related *Pigmentiphaga* species, but there were some qualitative and quantitative differences. Strain BS12^T contained higher amounts of $C_{12:0}$ 2OH (4.8±1.2%), whereas it was mostly absent in other *Pigmentiphaga* species, and $C_{14:0}$ 2OH was absent in strain BS12^T but was present in other *Pigmentiphaga* species (Table 2).

Strain BS12^T contained ubiquinone-8 (Q-8) as the predominant respiratory quinone, which is common to species in the

Table 2. Cellular fatty acid profiles of strain BS12^T and all *Pigmentiphaga* species
Strains: 1, BS12^T; 2, *P. litoralis* KCTC 22165^T; 3, *P. daeguensis* KCTC 12838^T; 4, *P. kullae* KACC 11572^T.
All strains were grown on TSA at 30°C for 2 days. The position of the double bond was located by counting from the methyl (ω) end of the carbon chain for unsaturated fatty acids.

Fatty acids	1	2	3	4
Saturated				
12:0	-	tr	-	tr
14:0	-	2.4	1.0	1.0
16:0	30.3	27.6	28.8	31.4
17:0	tr	-	-	-
17:0 cyclo	15.8	12.0	9.0	15.7
18:0	2.7	tr	tr	1.6
19:0 iso	tr	-	-	-
19:0 cyclo ω8*c*	2.7	-	1.2	4.6
Unsaturated				
14:1 ω5*c*	tr	-	-	-
18:1 ω9*c*	-	-	-	tr
2-Hydroxy				
12:0 2OH	4.8	tr	-	-
14:0 2OH	-	2.0	4.5	3.5
16:0 2OH	1.4	-	2.3	2.5
16:1 2OH	3.3	-	2.4	1.0
18:1 2OH	4.1	-	2.4	tr
18:1 2OH	-	-	-	1.2
3-Hydroxy				
10:0 3OH	4.0	3.0	4.3	2.9
12:0 3OH	-	tr	-	-
16:0 3OH	tr	-	-	tr
17:0 iso 3OH	tr	-	-	-
Summed feature 2† (14:0 3OH/16:1 iso I)	4.8	4.7	7.2	5.5
Summed feature 3† (16:1 ω6*c*/ω7*c*)	11.8	30.4	15.1	8.4
Summed feature 8† (18:1 ω6*c*/ω7*c*)	11.6	15.9	20.2	18.7

† Summed feature contained fatty acids that could not be separated by GLC with the Microbial Identification System. -, not detected; tr, trace (<1.0%)

class *Betaproteobacteria.* Major polar lipids found in strain BS12^T were PE and phosphatidylglycerol (PG). Minor amounts of unknown phospholipid (PL1-PL3) were also found (Supplementary data Fig. 2). Strain BS12^T showed a polar lipid profile similar to that of other *Pigmentiphaga* species (Chen *et al.*, 2009).

The G+C content of genomic DNA from strain BS12^T was 67.3±1.2 mol%. Strain BS12^T exhibited low DNA-DNA relatedness with the closely related strains *P. kullae* K24^T (12±7%), *P. daeguensis* K110^T (10±5%), and *P. litoralis* JSM 061001^T (9±7%). DNA-DNA hybridization levels between strain BS12^T and other type strains were determined to be less than 70%, which is the threshold for delineating a genomic species (Wayne *et al*., 1987; Stackebrandt and Goebel, 1994). Thus, our results support the placement of strain BS12^T as a representative of a separate and previously unrecognized genomic species.

Strain BS12^T and closely related type strains are distinguished from other members of the genus *Pigmentiphaga* by exhibiting a different fatty acid profile (Table 2) and by having short-rod shaped cells. Strain BS12^T revealed pale-yellow colored colonies and motile short-rod shaped cells but the phylogenetically closest type strain, *P. litoralis*, showed bright-yellow colored colonies and non-motile and slightly curved rod shaped cells. Additionally, strain BS12^T was distinguished from *P. kullae* by the absence of denitrification and trypsin production. The differential characters that distinguished strain BS12^T from other members of the genus *Pigmentiphaga* are shown in Table 1.

In Table 1, enzyme activities and assimilation tests were conducted using API kits, and some data showed discrepancies from the original species descriptions (Blumel *et al*., 200l; Yoon *et al*., 2007; Chen *et al*., 2009).

Based on the phylogenetic, chemotaxonomic, and phenotypic data, we conclude that strain BS12^T is a representative novel species, for which the name *Pigmentiphaga soli* sp. nov. is proposed.

Description of *Pigmentiphaga soli* sp. nov.

Pigmentiphaga soli (so'li. L. neut. gen. n. *soli* of soil, the source of the organism).

Strain BS12^T is 0.5-0.7 μm wide and 0.7-1.0 μm long, Gram-negative, aerobic, motile, and short rod shaped when grown on LB agar (Difco) at 30°C for 3 days. Colonies grown on LB agar for 3 days are circular, convex, smooth, and pale-yellow in color. Growth occurs on TSA, LB, and NA, but weak growth was observed on R2A agar. Single subterminal flagella are present. Growth occurred at temperatures of 15-42°C but not at 4°C. The optimum growth occurred at 30°C. Strain BS12^T grew well at pHs of 5-8 and can tolerate up to 3% NaCl (w/v). No reduction of nitrate to nitrite or nitrogen was evident. The bacteria are oxidase-positive and catalase positive. Acid is not produced from D-glucose, and indole is not produced.

Growth is observed with acetate, adipate, caprate, citrate, D-glucose, glycogen, D,L-3-hydroxybutyrate, 4-hydroxybenzoate, L-maltate, and suberate.

Growth is not observed with *N*-acetyl-D-glucosamine, L-alanine, L-arabinose, gluconate, L-fucose, L-histidine, 3-hydroxybenzoate, itaconate, 2-ketogluconate, 5-ketogluconate, D,L-lactate, D-maltose, D-melibiose, malonate, D-mannitol, D-mannose, myo-inositol, propionate, L-rhamnose, D-ribose, salicin, L-serine, D-sorbitol, D-sucrose, phenyl acetate, L-proline, or n-valerate.

Acid is produced with D-adonitol, amygdalin, erythritol, esculin ferric citrate, glycerol, 2-ketogluconate, 5-ketogluconate, D-lyxose, α-methyl-D-glucopyranoside, β-methyl-D-xylose, salicin, and L-xylose.

Acid is not produced with *N*-acetyl-glucosamine, amidon, D-arabinose, L-arabinose, D-arabitol, L-arabitol, arbutin, D-cellobiose, dulicitol, D-fructose, D-fucose, L-fucose, D-galactose, gentiobiose, gluconate, D-glucose, glycogen, inositol, inulin, D-lactose, D-maltose, D-mannitol, D-mannose, D-melobiose, α-methyl-D-mannopyranoside, D-melezitose, D-raffinose, L-rhamnose, D-ribose, D-sucrose, D-sorbitol, L-sorbose, D-tagatose, D-trehalose, D-turanose, xylitol, or D-xylose.

In tests with the API Zym system, the bacteria is positive for acid phosphatase, alkaline phosphatase, esterase (C4), esterase (C8), leucine arylamidase, naphtol-AS-BI-phosphohy-

drolase, and valine arylamidase.

The bacteria is negative for *N*-acetyl-β-glucosaminidase, arginine dihydrolase, α-chymotrypsin, cysteine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase (OPNG), β-galactosidase (PNPG), α-glucosidase, β-glucosidase, β-glucuronidase, lipase (C14), protease (gelatin), α-mannosidase, trypsin, and urease.

Q-8 is the major quinone. The predominant cellular fatty acids of strain BS12^T are $C_{16:0}$, $C_{17:0}$ cyclo, summed feature 3 ($C_{16:1}$ $\omega6c/\omega7c$), and summed feature 8 ($C_{18:1}$ $\omega6c/\omega7c$). The major polar lipids are PE and PG. The G+C content of genomic DNA from strain BS12^T was 67.3 mol%.

The type strain, BS12^T (=KCTC 23577^T =JCM 17666^T =KEMB 9004-082^T), was isolated from soil in South Korea.

The NCBI GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BS12^T (=KCTC 23577^T =JCM 17666^T =KEMB 9004-082^T) is JF806521.

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