Pusillimonas harenae sp. nov., isolated from a sandy beach, and emended description of the genus *Pusillimonas*

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A Gram-stain-negative, motile bacterium with two lateral flagella, designated strain B201^T, was isolated from beach sand from the Taean coast in South Korea. Cells were ovoid rods and positive for catalase and oxidase. Growth of strain B201^T was observed between 15 and 45 °C (optimum, 30 °C) and between pH 5.0 and 9.0 (optimum, pH 6.0–7.5). Strain B201^T contained ubiquinone Q-8 as the major isoprenoid quinone, but MK-6 was also present as a minor quinone. The major fatty acids of strain B201^T were $C_{17:0}$ cyclo, $C_{16:0}$, summed feature 2 (iso-$C_{16:1}$ I/$C_{14:0}$ 3-OH and/or $C_{12:0}$ ALDE), $C_{12:0}$ and $C_{19:0}$ cyclo ω8c. The major cellular polar lipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, an unidentified phospholipid and three aminolipids. The genomic DNA G+C content was 53.1 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that the strain formed a phyletic lineage with *Pusillimonas ginsengisoli* DCY25^T within the genus *Pusillimonas*. Strain B201^T was most closely related to *P. ginsengisoli* DCY25^T and *Pusillimonas soli* MJ07^T with similarities of 98.6 and 97.5 %, respectively. However, DNA–DNA relatedness values of strain B201^T with *P. ginsengisoli* DCY25^T and *P. soli* MJ07^T were 30.2 ± 5.4 and 4.9 ± 1.8 %, respectively. On the basis of chemotaxonomic data and molecular properties, strain B201^T represents a novel species of the genus *Pusillimonas*, for which the name *Pusillimonas harenae* sp. nov. is proposed; the type strain is B201^T (=KACC 14927^T=JCM 16917^T). An emended description of the genus *Pusillimonas* is given.

The genus *Pusillimonas*, a member of the family *Alcaligenaceae* within the class *Betaproteobacteria*, was first proposed by Stolz et *al.* (2005). At the time of writing, the genus comprised only three species with validly published names: *Pusillimonas ginsengisoli*, *Pusillimonas soli* and *Pusillimonas noertemannii*. *P. noertemannii* BN9^T was isolated from the River Elbe in Germany (Stolz *et al.*, 2005), and *P. ginsengisoli* DCY25^T and *P. soli* MJ07^T were isolated from soil from a ginseng field and a farm, respectively, in South Korea (Lee *et al.*, 2010; Srinivasan *et al.*, 2010), indicating that members of the genus *Pusillimonas* may have a diverse habitat range. Typical features of the genus *Pusillimonas* are: the presence of ubiquinone Q-8; $C_{17:0}$ cyclo, $C_{16:0}$ and $C_{19:0}$ cyclo ω8*c* as major fatty acids; and phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) as major polar lipids. Coastal seashores are marine habitats that possess valuable biological resources such as micro-organisms and marine animals that play very important roles in the functioning of ecosystems. During a course of studies investigating microbial communities that inhabit coastal seashores, several bacterial community members have been characterized (Kim *et al.*, 2010a, b; Jin *et al.*, 2011; Jung *et al.*, 2011; Lee *et al.*, 2011). Here, the taxonomic characterization of a novel *Pusillimonas* species isolated from beach sand of the Yellow Sea in South Korea is described.

Strain B201^T was isolated from beach sand of the Taean coast (36° 50′ 5 N″ 126° 09′ 9″ E) in the Yellow Sea, South Korea, using a previously described procedure with some

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Abbreviations: ASW, artificial seawater; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B201^T is GQ232740.

Three supplementary figures are available with the online version of this paper.

modifications (Kim *et al.*, 2008). Briefly, a soil sample was serially diluted in 0.9 % (w/v) saline and spread on marine agar 2216 (MA; Difco) plates and incubated under aerobic conditions at 25 °C for 5 days. Colonies were randomly selected and crude genomic DNA from respective colonies was prepared as described previously (Lu *et al.*, 2006). Amplicons were double-digested with *Hae*III and *Hha*I. RFLP patterns were analysed on 2.5 % MetaPhore agarose (BioWhittaker) gels; colonies were grouped according to their RFLP patterns and representative PCR products containing distinct RFLP patterns were sequenced. The resulting 16S rRNA gene sequences were analysed using the program BLAST (http://www.ncbi.nlm.nih.gov/Blast.cgi/) in GenBank. From the analysis, a novel strain belonging to the genus *Pusillimonas*, designated strain B201^T, was selected for further phenotypic and phylogenetic analysis. The strain was grown routinely on R2A agar (Difco) aerobically at 30 °C for 3 days, except where indicated otherwise. The strain was stored at −80 °C in R2A broth supplemented with 10 % (v/v) glycerol. *P. ginsengisoli* KCTC 22046^T, *P. soli* MJ07^T and *P. noertemannii* KACC 13183^T were used as reference strains for DNA–DNA hybridization, phenotypic characterization and fatty acid analysis; *P. ginsengisoli* KCTC 22046^T was purchased from KCTC (Republic of Korea), and *P. soli* MJ07^T and *P. noertemannii* KACC 13183^T were gifts from M. Lee (H-Plus Eco, Daejeon, Republic of Korea; Lee *et al.*, 2010) and KACC (Republic of Korea), respectively.

The 16S rRNA gene sequence (1400 nt) of strain B201^T was compared with available 16S rRNA gene sequences from GenBank using the program BLAST to determine an approximate phylogenetic affiliation. Sequence similarity values between the isolate and related bacteria were evaluated using the EzTaxon nucleotide similarity search program (http://147.47.212.35:8080/; Chun *et al.*, 2007) and aligned by using CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic trees were constructed using the neighbour-joining and maximum-parsimony algorithms in the PHYLIP software (version 3.6; Felsenstein, 2002). The resulting tree topologies were evaluated using bootstrap analysis based on 1000 resampled datasets with the PHYLIP package. Maximum-likelihood analysis with bootstrap values was performed using RAxML-HPC on Abe (version 7.2.6) of the Cyberinfrastructure for Phylogenetic Research project (CIPRES; www.phylo.org) at the San Diego Supercomputer Center.

Comparative analysis of 16S rRNA gene sequences showed that strain B201^T was most closely related to *P. ginsengisoli* DCY25^T, *P. soli* MJ07^T and *P. noertemannii* BN9^T with similarities of 98.6, 97.5 and 96.9 %, respectively. Neighbour-joining phylogenetic analysis based on 16S rRNA gene sequences indicated that strain B201^T formed a phyletic lineage with *P. ginsengisoli* DCY25^T within the genus *Pusillimonas* with a bootstrap value of 62 % (Fig. 1). Analysis with the maximum-likelihood and maximum-parsimony algorithms also indicated that strain B201^T formed a phyletic lineage within the genus *Pusillimonas* (Supplementary Fig. S1, available in IJSEM Online).

DNA–DNA hybridization was carried out to evaluate the level of DNA relatedness of strain B201^T with *P. ginsengisoli*

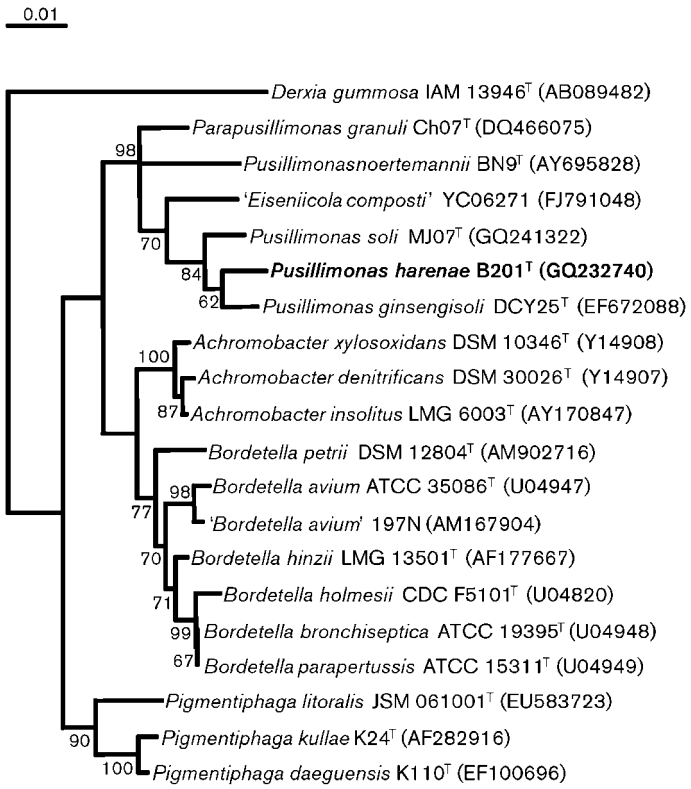

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain B201^T and related taxa. Bootstrap values (percentages of 1000 replicates) are shown at nodes; only values >50 % are shown. *Derxia gummosa* IAM 13946^T (AB089482) was used as the outgroup. Bar, 0.01 changes per nucleotide position. Phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms are also available as Supplementary Fig. S1 in IJSEM Online.

KCTC 22046^T and *P. soli* MJ07^T, as described previously (Lee *et al.*, 2011). Briefly, extracted genomic DNAs were fragmented with *Hae*III and, after denaturation using NaOH solution and heating (80 °C), various amounts of the fragmented DNA were blotted onto Hybond-N+ nylon membranes (Amersham Pharmacia Biotech) (three replicates). Each DNA sample (4 μg) was used individually as a labelled DNA probe for cross-hybridization (Park *et al.*, 2007). Random-primed DNA labelling with digoxigenin (DIG)-dUTP and detection of hybrids by enzyme immunoassay on a nylon membrane were performed using the DIG High Prime DNA labelling kit (Roche Applied Science) according to the manufacturer's instructions. Hybridization signals were captured and analysed with Bio-Rad Quantity One software (version 4.62). The signal produced by hybridization of the probes to the homologous target DNAs was taken to be 100 % and signal intensities by self-hybridization of the series of dilutions were used for calculation of the levels of DNA relatedness of strain B201^T with *P. ginsengisoli* KCTC 22046^T and *P. soli* MJ07^T. Hybridization experiments were always confirmed by cross-hybridization. DNA–DNA relatedness values of strain B201^T with *P. ginsengisoli* KCTC 22046^T and *P. soli* MJ07^T were 30.2 ± 5.4 and 4.9 ± 1.8 %, respectively, which are clearly below the 70 % threshold generally accepted for species delineation (Rosselló-Mora & Amann, 2001).

Growth was tested at 30 °C on several bacteriological media: R2A agar (Difco), laboratory-prepared Luria–Bertani (LB) agar, tryptic soy agar (TSA; Difco) and MA (Difco). Cultivation conditions for strain B201^T were examined by growing the isolate on R2A agar at 5–55 °C (at 5 °C intervals) and in R2A broth adjusted to pH 4.5–11.0 (at 0.5 pH unit intervals). Media with different pH values were prepared using the appropriate biological buffers (Gomori, 1955): pH values below 8.0, Na_2HPO_4/NaH_2PO_4 buffer; pH 8.0–10.0, Na_2CO_3/$NaHCO_3$ buffer; pH 11.0, Na_2HPO_4/NaOH buffer. The pH values were re-checked after sterilization. Gram staining was performed using the bioMérieux Gram Stain kit according to the instructions of the manufacturer. Cell morphology, motility and the presence of flagella were studied using phase-contrast microscopy (Axio Lab.A1; Carl Zeiss) and transmission electron microscopy (JEM-1010; JEOL) with 2-day-old cells grown on R2A agar as described previously (Jeon *et al.*, 2004). NaCl requirements and tolerance were determined in nutrient broth (NB: 3.0 g beef extract and 5.0 g peptone per litre) supplemented with modified artificial seawater (ASW) containing various amounts of NaCl [0–10 % (w/v) NaCl at 1 % intervals, 5.94 g $MgSO_4 . 7H_2O$, 4.53 g $MgCl_2 . 6H_2O$, 0.64 g KCl and 1.3 g $CaCl_2$ per litre] (Kahng *et al.*, 2009). Oxidase activity was tested by oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated by the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution (Smibert & Krieg, 1994). Additional enzymic activities and biochemical features were determined using API ZYM and API 20NE kits (bioMérieux) according to the instructions of the manufacturer except that inocula were prepared by suspending cells in 0.85 % (w/v) NaCl solution. Anaerobic growth was assessed on MA under anaerobic (with 4–10 % CO_2) conditions using the GasPak Plus system (BBL) at 30 °C for 20 days.

Strain B201^T grew well on R2A agar, LB agar, TSA and MA at 30 °C. Growth on R2A agar was slightly faster than on other agar media. When tested on R2A, growth of strain B201^T was observed at 15–45 °C and pH 5.0–9.0, with optimum growth at 30 °C and pH 6.0–7.5. Strain B201^T grew in NB supplemented with modified ASW containing 0–6 % (w/v) NaCl; optimal growth occurred in 0–3 % NaCl. Bacterial cells were Gram-negative, strictly aerobic, ovoid rods, 0.5–0.7 μm wide and 0.6–0.9 μm long, and motile by means of two lateral flagella on one side (Supplementary Fig. S2, available in IJSEM Online); motility clearly differentiated strain B201^T from *P. ginsengisoli* DCY25^T. Physiological and biochemical characteristics of strain B201^T are shown in Table 1 and the species description. Some characteristics are in accordance with those of members of the genus *Pusillimonas*, whereas others allow strain B201^T to be differentiated from other *Pusillimonas* species.

Isoprenoid quinones were analysed using HPLC (model LC-20A; Shimadzu) equipped with a diode array detector (SPD-M20A; Shimadzu) and a reversed-phase column (250 × 4.6 mm, Kromasil; Akzo Nobel) as described previously (Komagata & Suzuki, 1987). For analysis of fatty acid methyl esters, cells of strain B201^T and reference strains (*P. ginsengisoli* KCTC 22046^T and *P. noertemannii* KACC 13183^T) were harvested from R2A agar plates after incubation at 30 °C for 3 days. Analysis of fatty acid methyl esters was carried out according to the instructions of the Sherlock Microbial Identification System (MIDI; Microbial ID, Inc.). Polar lipids were determined by TLC as described by Minnikin *et al.* (1977). The DNA G+C content of strain B201^T was determined using HPLC fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE; GROM) according to the method of Tamaoka & Komagata (1984). The major respiratory lipoquinone detected in strain B201^T was ubiquinone Q-8, at a peak area ratio of approximately 85–87 %; a minor amount of menaquinone MK-6 was also present (peak area ratio of approximately 13–15 %). The major cellular fatty acids (>5 % of the total fatty acids) were $C_{17:0}$ cyclo (35.65 %), $C_{16:0}$ (32.12 %), summed feature 2 (iso-$C_{16:1}$ I/$C_{14:0}$ 3-OH and/or $C_{12:0}$ ALDE; 8.77 %), $C_{12:0}$ (7.87 %) and $C_{19:0}$ cyclo $\omega 8c$ (5.98 %). Although the overall fatty acid profile of strain B201^T was similar to those of other *Pusillimonas* species, differences in the amounts of various fatty acids distinguished the novel isolate from other *Pusillimonas* species (Table 2). The major polar lipids were PG, DPG, PE, three aminolipids and an unidentified phospholipid (Supplementary Fig. S3, available in IJSEM Online); this polar lipid profile was generally in accordance with those of other *Pusillimonas* species (Stolz *et al.*, 2005; Srinivasan *et al.*, 2010). The DNA G+C content of strain

Table 1. Phenotypic characteristics of strain B201^T and related *Pusillimonas* species

Strains: 1, strain B201^T; 2, *P. ginsengisoli* KCTC 22046^T (Srinivasan *et al.*, 2010); 3, *P. noertemannii* KACC 13183^T (Stolz *et al.*, 2005); 4, *P. soli* MJ07^T (Lee *et al.*, 2010). All strains are positive for the following: esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase and acid phosphatase (API ZYM); oxidase; and growth in 0 % (w/v) NaCl. All strains are negative for: α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase (API ZYM); Gram staining; growth at 4 °C; and hydrolysis of gelatin. +, Positive; −, negative; (+), weakly positive.

Characteristic	1*	2	3	4
Morphology	Ovoid rods	Short rods	Rods	Rods
Colony colour	Ivory	Pale yellow	Brownish	Yellow
Motility	+	−	+	+
Growth at/in:				
42 °C	+	−	+	−
5 % NaCl*	+	+	−	+
Hydrolysis of urea*	+	−	−	−
Enzyme activity (API ZYM)*				
Alkaline phosphatase	(+)	+	+	+
Lipase (C14)	−	(+)	+	−
Cystine arylamidase	+	+	(+)	−
Trypsin	(+)	+	(+)	−
α-Chymotrypsin	(+)	(+)	+	+
Naphthol-AS-BI-phosphohydrolase	(+)	(+)	+	+
API 20NE system*				
Nitrate reduction	−	+	−	+
Caprate	−	−	+	−
Adipate	+	+	−	+
Malate	+	+	−	+
Citrate	−	+	−	+
Phenylacetate	−	+	−	+
Polar lipids†	PE, PG, DPG, PL, AL1, AL2, AL3	PE, PG, DPG, AL1, AL2, APL	PE, PG, DPG, AL1, AL2, APL	PE, PG, DPG, AL1, AL2, APL1, L1
DNA G+C content (mol%)	53.1	57.3	61.8	59.4

*Data from this study.

†PE, Phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; AL, unidentified aminolipid; PL, unidentified phospholipid; APL, aminophospholipid; L, unidentified lipid.

B201^T was 53.1 mol%, which was a little lower than those of *Pusillimonas* species (Table 1). Based on physiological, biochemical and phylogenetic properties, strain B201^T represents a novel species within the genus *Pusillimonas*, for which the name *Pusillimonas harenae* sp. nov. is proposed.

Emended description of the genus *Pusillimonas* Stolz *et al.* 2005

The description of the genus *Pusillimonas* is as given by Stolz *et al.* (2005), with the following amendments. DNA G+C content is in the range 53.1–61.8 mol% and major non-polar fatty acids are $C_{17:0}$ cyclo, $C_{16:0}$ and summed feature 2 (iso-$C_{16:1}$ I/$C_{14:0}$ 3-OH and/or $C_{12:0}$ ALDE).

Description of *Pusillimonas harenae* sp. nov.

Pusillimonas harenae (ha.re′nae. L. gen. n. *harenae* of sand, from where the organism was isolated).

Cells are Gram-negative, strictly aerobic, motile rods (0.5–0.7 × 0.6–0.9 μm) with two lateral flagella. Colonies on R2A agar are ivory, convex and round with entire margins. Growth occurs at 15–45 °C (optimum, 30 °C), at pH 5.0–9.0 (optimum, 6.0–7.5) and in the presence of 0–6 % (w/v) NaCl (optimum, 0–3 %). Catalase- and oxidase-positive. Negative for nitrate reduction, indole production, glucose acidification and arginine dihydrolase activities. Positive for assimilation of malic acid and adipic acid, but negative for assimilation of D-glucose, L-arabinose, D-mannose, maltose, D-mannitol, potassium gluconate, *N*-acetylglucosamine, capric acid, trisodium citrate and phenylacetic acid. Urea is hydrolysed. Aesculin and gelatin are not hydrolysed. Esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase and acid phosphatase activities are present, but not lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. Weak enzymic activities

Table 2. Cellular fatty acid compositions (%) of strain B201^T and related *Pusillimonas* species

Strains: 1, strain B201^T; 2, *P. ginsengisoli* KCTC 22046^T; 3, *P. noertemannii* KACC 13183^T; 4, *P. soli* MJ07^T. All data are from this study. Data are expressed as percentages of total fatty acids. Fatty acids amounting to less than 0.5 % in all strains are not shown. Major components (>5.0 %) are highlighted in bold. tr, Trace amount (<0.5 %); ECL, equivalent chain-length; −, not detected.

Fatty acid	1	2	3	4
Saturated				
$C_{12:0}$	**7.87**	0.56	4.93	**9.83**
$C_{14:0}$	1.73	4.42	tr	0.51
$C_{15:0}$	0.68	0.57	−	0.55
$C_{16:0}$	**32.12**	**36.11**	**20.71**	**40.54**
$C_{17:0}$	0.36	0.66	0.31	0.73
$C_{17:0}$ cyclo	**35.65**	**31.03**	**22.51**	**31.26**
$C_{18:0}$	0.6	0.97	2.54	2.45
$C_{19:0}$ cyclo $\omega 8c$	**5.98**	2.77	**20.74**	2.39
$C_{19:0}$ 10-methyl	−	−	1.05	−
Hydroxy				
$C_{12:0}$ 2-OH	−	−	3.26	−
$C_{18:1}$ 2-OH	−	−	1.64	−
Unsaturated				
$C_{18:1}\omega 7c$	2.22	**6.71**	2.40	0.50
$C_{20:2}\omega 6,9c$	tr	tr	0.58	−
Branched				
anteiso-$C_{17:1}$ A	−	−	0.84	−
Summed features*				
2	**8.77**	**8.45**	**11.16**	**8.04**
3	1.79	**6.68**	3.68	2.11

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2 contains $C_{14:0}$ 3-OH, iso-$C_{16:1}$ I, an unidentified fatty acid with an equivalent chain-length of 10.928 and/or $C_{12:0}$ ALDE. Summed feature 3 contains a mixture of $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH.

are observed for alkaline phosphatase, trypsin, α-chymotrypsin and naphthol-AS-BI-phosphohydrolase. The major cellular polar lipids are PG, DPG, PE, an unidentified phospholipid and three aminolipids. The major cellular fatty acids (>5 % of the total fatty acids) are $C_{17:0}$ cyclo, $C_{16:0}$, summed feature 2 (iso-$C_{16:1}$ I/$C_{14:0}$ 3-OH and/or $C_{12:0}$ ALDE), $C_{12:0}$ and $C_{19:0}$ cyclo $\omega 8c$.

The type strain is B201^T (=KACC 14927^T=JCM 16917^T), isolated from beach sand from the Taean coast in South Korea. The DNA G+C content of the type strain is 53.1 mol% (HPLC).

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