Arenibacter hampyeongensis sp. nov., a marine bacterium isolated from a tidal flat

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A Gram-staining-negative, dark orange, strictly aerobic bacterium, designated strain HP12T, was isolated from a tidal flat at Hampyeong in South Korea. Cells were moderately halotolerant, catalase- and oxidase-positive, non-motile rods. Growth was observed at 5–35 °C (optimum, 25 °C), at pH 6.0–8.5 (optimum, pH 7.0–7.5), and in the presence of 1–6 % (w/v) NaCl (optimum, 1–2 %). The major cellular fatty acids were summed feature 3 (C16:1ω7c and/or iso-C15:0 2-OH), iso-C17:0 3-OH, C16:0, iso-C15:0 1ω7c and iso-C15:0. The polar lipid pattern indicated the presence of phosphatidylethanolamine and two unidentified lipids. The G+C content of the genomic DNA was 37.1 mol% and the predominant respiratory quinone was menaquinone-6. Phylogenetic analysis based on 16S rRNA gene sequences showed that the novel strain formed a tight phylogenetic lineage with members of the genus Arenibacter and was most closely related to Arenibacter palladensis KMM 3961T, Arenibacter troitsensis KMM 3674T and Arenibacter echinorum KMM 6032T, with 16S rRNA gene sequence similarities of 98.1 %, 98.0 % and 97.8 %, respectively. However, the DNA–DNA relatedness values between strain HP12T and A. palladensis JCM 13609T, A. troitsensis KCTC 12362T and A. echinorum KCTC 22013T were only 20.2±0.3 %, 22.6±0.6 % and 9.1±2.6 %, respectively. On the basis of phenotypic and molecular features, strain HP12T represents a novel species of the genus Arenibacter, for which the name Arenibacter hampyeongensis sp. nov. is proposed. The type strain is HP12T (=KACC 16193T=JCM 17788T).

The genus Arenibacter, a member of the family Flavobacteriaceae (Bernardet et al., 2002; Bernardet, 2011), was first proposed by Ivanova et al. (2001) to accommodate a Gram-negative, aerobic, heterotrophic and dark-orange-pigmented bacterium. At the time of writing, the genus Arenibacter comprised six species with validly published names, Arenibacter latericus, Arenibacter troitsensis, Arenibacter certesii, Arenibacter palladensis, Arenibacter echinorum and Arenibacter nanhaiticus (Ivanova et al., 2001; Nedashkovskaya et al., 2003, 2004, 2006, 2007; Sun et al., 2010), that have mainly been isolated from marine sediments. Tidal flats contain a wealth of valuable biological resources, such as micro-organisms and marine animals, and play crucial roles in the restoration of coastal ecosystems and nutrient cycling (Jin et al., 2011). In the course of recent studies investigating microbial communities inhabiting tidal flats, several novel species of bacteria have been characterized (Kim et al., 2010a, b; Lee et al., 2011; Park et al., 2011). Here, we describe the taxonomic characterization of a novel species of the genus Arenibacter isolated from a tidal flat on the coast of the Yellow Sea in South Korea.

In February 2011, a novel bacterium, designated strain HP12T, was isolated, at a depth of <5 cm, from a surface tidal flat on the Yellow Sea coast at Hampyeong, in South Korea (35° 05′ 42.35″ N 126° 27′ 41.06″ E). The isolation procedure was that previously described by Kim et al. (2010) and Jung et al. (2011), with some modifications. Briefly, a tidal flat sample was serially diluted with modified artificial seawater (ASW) containing (l−1) 20 g NaCl, 2.9 g MgSO4, 4.53 g MgCl2 . 6H2O, 0.64 g KCl and 1.75 g CaCl2 . 2H2O, spread on marine agar 2216 (MA; BD) and incubated at 25 °C for 5 days. PCR amplification from randomly selected colonies was performed, using the universal primers F1 and R13 (Lane, 1991), before the amplicons were double-digested with HaeIII and HhaI. PCR products with distinct restriction-fragment patterns were selected and sequenced. The resulting 16S rRNA gene sequences were analysed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in GenBank, and the search results were used as a guide to classify the colonies. From the analysis, a novel isolate that appeared to belong to the genus Arenibacter, designated strain HP12T, was selected for further phenotypic and phylogenetic analysis. Strain HP12T was routinely grown aerobically on MA at 25 °C for 3 days, except where indicated otherwise, and was stored at −80 °C in marine broth (MB; BD) supplemented with 10 % (v/v) glycerol. Arenibacter latericus KCTC...
12957\textsuperscript{T}, Arenibacter echinorum KCTC 22013\textsuperscript{T}, Arenibacter troitsensis KCTC 12362\textsuperscript{T} and Arenibacter palladensis JCM 13509\textsuperscript{T} were used as reference strains for phenotypic characterization and DNA–DNA hybridization.

The 16S rRNA gene amplicon of strain HP12\textsuperscript{T} was ligated for phylogenetic characterization and DNA–DNA hybridization. The resulting, almost-complete 16S rRNA gene sequence (1462 nt) of strain HP12\textsuperscript{T} was checked manually for the evaluation of quality and detection of gaps. Sequence similarity values between strain HP12\textsuperscript{T} and closely related taxa were evaluated using the nucleotide similarity search program (http://147.47.212.35:8080; Chun et al., 2007) and aligned using the Greengenes alignment program (http://greengenes.lbl.gov/). Phylogenetic trees based on the neighbour-joining (NJ) and maximum-parsimony (MP) algorithms were constructed within version 3.6 of the PHYLIP software package (Felsenstein, 2002). The topology of the resulting trees was evaluated within the PHYLIP package, using bootstrap analyses based on 1000 resampled datasets. Maximum-likelihood (ML) analysis was also performed, using version 7.2.8 of the RAxML-HPC BlackBox software package of the Cyber-Infrastructure Permutation package (CIPRES, www.phylo.org; Stamatakis et al., 2005) at the San Diego Supercomputer Center. Additional taxonomic assignment was performed using the Ribosomal Database Project (RDP) naïve Bayesian rRNA classifier tool (http://rdp.cme.msu.edu/classifier; Wang et al., 2007) with a 90 % confidence threshold.

Comparative analysis of the 16S rRNA gene sequences showed that strain HP12\textsuperscript{T} was most closely related to Arenibacter palladensis KMM 3961\textsuperscript{T}, Arenibacter troitsensis KMM 3674\textsuperscript{T} and Arenibacter echinorum KMM 6032\textsuperscript{2}, with sequence similarities of 98.1%, 98.0% and 97.8%, respectively. Phylogenetic analyses based on 16S rRNA gene sequences and the NJ, MP and ML algorithms, showed that strain HP12\textsuperscript{T} formed a tight phylogenetic cluster with members of the genus Arenibacter (Fig. 1), which was also confirmed by use of the RDP Classifier program.

DNA–DNA hybridizations were carried out, by the method of Lee et al. (2011), to evaluate the levels of DNA relatedness between strain HP12\textsuperscript{T} and reference strains that showed >97 % 16S rRNA gene sequence similarity. A. palladensis JCM 13509\textsuperscript{T}, A. troitsensis KCTC 12362\textsuperscript{T} and A. echinorum KCTC 22013\textsuperscript{T}. Briefly, different concentrations of extracted genomic DNA were blotted onto Hybond-N+ nylon membranes (Amersham Pharmacia Biotech) in three replicates, after denaturation using NaOH solution and heating (80 °C). The DNA from each strain (4 µg) was fragmented by HaeIII digestion and then used as a labelled probe for cross-hybridization. Random primed DNA labelling with digoxigenin (DIG)-deoxy uridine-5′-triphosphate and detection of the hybrids in enzyme immunoassays on nylon membrane were performed using the DIG High Prime DNA labelling kit (Roche Applied Science) according to the manufacturer's instructions and standard procedures (Sambrook & Russell, 2001). The hybridization signals were captured using a Scanjet 3770 scanner (Hewlett-Packard) and analysed within version 7.0 of the Photoshop software package (Adobe). The intensity of the signal produced by hybridization of the probe to the homologous target DNA was taken to be 100 %, and signal intensities by the self-hybridization of the series of dilutions were used for the calculation of the levels of DNA relatedness between strain HP12\textsuperscript{T} and reference strains. The results of each hybridization were compared with those of the corresponding reciprocal hybridization. The mean DNA–DNA relatedness values recorded between strain HP12\textsuperscript{T} and A. palladensis JCM 13509\textsuperscript{T}, A. troitsensis KCTC 12362\textsuperscript{T} and A. echinorum KCTC 22013\textsuperscript{T} were 20.2 ± 0.3 %, 22.6 ± 0.6 % and 9.1 ± 2.6 %, respectively, which are clearly below the 70 % threshold generally accepted for species delineation (Rosselló-Mora & Amann, 2001).

The temperature and pH ranges for growth of strain HP12\textsuperscript{T} were determined, respectively, on MA at 0–45 °C (at intervals of 5 °C) and in MB adjusted to pH 4.5–11.0 (at intervals of 0.5 pH unit). The MB with different pH values was prepared using appropriate biological buffers: Na2HPO4-NaH2PO4 buffer for values below pH 8.0,
Na₂CO₃-NaHCO₃ buffer (for pH 8.0–10.0) and Na₂HPO₄-NaOH buffer (for pH 11.0) (Gomori, 1955). The pH of the broth was adjusted prior to sterilization, by the addition of HCl or NaOH, and verified and, if necessary, corrected after sterilization. Growth in the presence of 0–10 % (w/v) NaCl (at 1 % intervals) was investigated using broth prepared in the laboratory from the formula for MB. Gram staining was performed using the bioMérieux Gram stain kit according to the instructions of the manufacturer. Growth under anaerobic conditions (with 4–10 % CO₂), created using the GasPak Plus system (BBL), was assessed on MA for 20 days at 25 °C. Cell morphology and the presence of flagella were studied using transmission electron microscopy (JEM-161 1010; JEOL) and cells from a culture in exponential growth on MA. Gliding motility was assessed by phase-contrast microscopy (Axio Lab.A1; Carl Zeiss), as described by Bowman (2000). The presence of flexirubin-type pigments was investigated as described by McCammon & Bowman (2000). Cellular pigments were extracted in acetone/methanol (7 : 2, v/v) before their absorption spectra were determined using a scanning UV/visible spectrophotometer (SynergyMx; BioTek) (Biebl et al., 2005). 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 H₂O₂ solution (Smibert & Krieg, 1994). Hydrolysis of agar, casein, Tween 80, Tween 20, tyrosine, starch and xylan was investigated on MA, according to the methods described previously (Lányi, 1987; Smibert & Krieg, 1994). Degradation of alginate was tested as described by Ivanova et al. (2001). Nitrate reduction was assessed according to the method of Lányi (1987) and the Voges–Proskauer reaction was determined as described by Cowan & Steel (1965). H₂S production was tested as described previously (Bruns et al., 2001). Acid production from carbohydrates was determined as described by Leifson (1963). Utilization of various substrates for growth was determined as described by Yurkov et al. (1994). Additional enzymic activities and biochemical features of strain HP12ᵀ and the reference strains were determined with API 20E kits (bioMérieux), according to the manufacturer’s instructions except for adjustments in the temperature and time of incubation and the use of cells suspended in ASW. Antibiotic susceptibility tests were performed using filter-paper discs (Whatman) containing the following antibiotics (µg per disc unless stated otherwise): ampicillin (10), polymyxin B (100 U), streptomycin (50), penicillin G (20 U), gentamicin (30), chloramphenicol (100), tetracycline (30), kanamycin (30), lincomycin (15), oleandomycin (15), carbenicillin (100), neomycin (30) and novobiocin (5).

Growth of strain HP12ᵀ was observed at temperatures between 5 and 35 °C but not at 0 °C or 40 °C, with optimum growth at 25 °C. Cells of strain HP12ᵀ were Gram-staining-negative, obligately aerobic and straight or slightly curved, non-motile rods 0.4–0.5 µm wide and 1.8–4.0 µm long. In MB at 25 °C, strain HP12ᵀ grew with pH 6.0–8.5, with an optimum between pH 7.0 and pH 7.5. Strain HP12ᵀ grew in MB containing 1.0–6.0 % (w/v) NaCl, with optimal growth occurring in the presence of 1.0–2.0 % NaCl. Anaerobic growth was not observed after 20 days at 25 °C on MA. Cells contained methanol-soluble, orange-coloured pigments that showed maximum absorption at 450 nm. More physiological and biochemical characteristics of strain HP12ᵀ are presented in Table 1 and in the species description. Some of these characteristics have also been reported for the closely related, established species in the genus Arenibacter but others allow the differentiation of strain HP12ᵀ from its closest phylogenetic neighbours (Table 1).

Cell mass for the analysis of isoprenoid quinones, fatty acids and polar lipids was obtained from cultures in at exponential growth phase. For analysis of cellular fatty acids, strain HP12ᵀ, A. latericius KCTC 12957ᵀ, A. echinorum KCTC 22013ᵀ, A. troitsensis KCTC 12362ᵀ and A. palladensis JCM 13509ᵀ were cultivated in MB at 25 °C and harvested at the same growth phase (as indicated by an optical density of 0.8, measured at 600 nm). Analysis of fatty acid methyl esters (FAME) was performed according to the protocol of the Sherlock Microbial Identification System (MIDI). The FAME peaks were automatically integrated and their fatty acid names and percentages were determined using version 4.0 of the Microbial Identification Software package (MIDI) and the TSBA 40 database. Isoprenoid quinones were analysed by HPLC (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). Polar lipids were determined by TLC as described by Minnikin et al. (1977). The genomic DNA G+C content of strain HP12ᵀ was determined by the fluorometric method (Gonzalez & Saiz-Jimenez, 2002) using SYBR Green I and a real-time PCR thermocycler (Bio-Rad).

The major cellular fatty acids (>5 % of the total fatty acids) of the novel strain were summed feature 3 (comprising C₁₆:1ω7c and/or iso-C₁₅:0 2-OH; 20.4 %), iso-C₁₇:0 3-OH (13.9 %), C₁₅:0 (13.5 %), iso-C₁₅:1 G (9.3 %), iso-C₁₅:0 (8.6 %) and iso-C₁₅:0 3-OH (5.2 %). Although the overall fatty acid profile of strain HP12ᵀ was similar to those of the reference strains, there were some differences in the respective proportions or presence/absence of some components (Table 2). The only respiratory lipoquinone detected in strain HP12ᵀ was menaquinone-6 (MK-6), which was in line with all recognised members of the genus Arenibacter (Ivanova et al., 2001; Sun et al., 2010). The major polar lipids were phosphatidylethanolamine and two unidentified lipids (Fig. S1, available in IJSEM Online). The genomic DNA G+C content of strain HP12ᵀ was 37.1 mol%, which was similar to those of established members of the genus Arenibacter (Sun et al., 2010). Therefore, the phenotypic features and the results of the phylogenetic as analyses and DNA–DNA hybridizations indicate that strain HP12ᵀ represents a novel species within Arenibacter hampyeongensis sp. nov.
Table 1. Differential phenotypic characteristics of strain HP12<sup>T</sup> and closely related members of the genus Arenibacter

Table 2. Cellular fatty acid contents (%) of strain HP12<sup>T</sup> and closely related members of the genus Arenibacter

*Data from this study using strain HP12<sup>T</sup>, *A. latericius* KCTC 12957<sup>T</sup>, *A. echinorum* KMM 12362<sup>T</sup> and *A. troitsensis* KMM 6032<sup>T</sup>. Summed feature 4 contained C<sub>16</sub>:1<sub>ω4</sub>c and/or anteiso-C<sub>17</sub>:1<sub>B</sub>. Summed feature 3 contained iso-C<sub>17</sub>:1<sub>I</sub> and/or anteiso-C<sub>17</sub>:1<sub>B</sub>.
0.4–0.5 μm wide and 1.8-4.0 μm long. Colonies on MA are dark-orange, convex and round. Growth occurs at 5-35 °C (optimum, 25 °C), at pH 6.0–8.5 (optimum, pH 7.0–7.5), and in the presence of 1–6 % (w/v) NaCl (optimum, 1–2 %). Cells contain methanol-soluble orange-coloured pigments but flexirubin-like pigments are absent. Oxidase- and catalase-positive. Tween 20 is hydrolysed but agar, casein, Tween 80, starch, tyrosine, alginate and xylan are not. H₂S and acetoin are not produced. Nitrate is not reduced. In API 20E tests, there are positive results for α-nitrophenyl-β-D-galactopyranosidase activity but negative results for urease, gelatinase, arginine dihydrolase, lysine decarboxylase, urease, galactosidase, β-glucosidase, β-glucuronidase, N-acetyl-β-glucosaminidase and x-mannosidase activities and weakly positive results for cystine arylamidase activity but negative results for lipase (C14), α-chymotrypsin, β-galactosidase and z-fucosidase activities. D-Glucose, D-galactose, D-fructose, melibiose, lactose, D-mannose, raffinose, sucrose and D-mannitol are utilized but L-rhamnose, DL-malic acid, inositol, citrate, glycerol and D-sorbitol are not utilized. Acid is produced from D-glucose, D-galactose, lactose, melibiose, raffinose, maltose, cellobiose and sucrose but not from L-rhamnose, L-arabinose, L-sorbose, adonitol, dulcitol, inositol, D-mannitol, fumarate, glycerol or citrate. Phosphatidylethanolamine and two unidentified lipids are detected. The type strain, HP12 T (KACC 16193 T), was isolated from a tidal flat on the Yellow Sea coast at Hampyeong, South Korea. The DNA G+C content of the type strain is 37.1 mol%.

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References


