**Perspicuibacter marinus** gen. nov., sp. nov., a semi-transparent bacterium isolated from surface seawater, and description of **Arenicellaceae** fam. nov. and **Arenicellales** ord. nov.

Maki Teramoto,1 Ken-ichi Yagyu2 and Miyuki Nishijima3

1Oceanography Section, Kochi University, Kohasu, Oko, Nankoku, Kochi 783-8505, Japan
2The Facility for Research Instruments, Kochi University, Kohasu, Oko, Nankoku, Kochi 783-8505, Japan
3TechnoSuruga Laboratory Co. Ltd, 330 Nagasaki, Shimizu-ku, Shizuoka 424-0065, Japan

A Gram-stain-negative, non-motile, mesophilic, aerobic, rod-shaped bacterium, strain 2-9T, was isolated from surface seawater at Muroto city, Kochi prefecture, Japan. The strain was transparent on 1/5 strength marine broth plate but became easily visible when the plate was supplemented with pyruvate. Phylogenetic analyses based on the 16S rRNA gene sequence showed that the strain fell within the class **Gammaproteobacteria** and was most closely related to the genus **Arenicella** (92.7–93.0 % 16S rRNA gene sequence similarities to type strains of species of this genus) of an unclassified order within this class. The DNA G+C content of strain 2-9T was 41.7 mol%. The major fatty acids were C18 : 1ω7c (37.6 %), C16 : 1ω7c and/or iso-C15 : 02-OH (summed feature 3; 19.1 %), C18 : 0 (10.8 %), C16 : 0 (10.2 %) and an unidentified fatty acid with an equivalent chain-length value of 11.799 (9.5 %). The major polar lipids were phosphatidylglycerol, phosphatidylethanolamine and three unidentified lipids. Ubiquinone-8 (Q-8) was detected as the sole isoprenoid quinone. From these taxonomic data, it is proposed that strain 2-9T represents a novel species of a new genus, **Perspicuibacter marinus** gen. nov., sp. nov. The type strain of the type species is 2-9T (=NBRC 110144T=KCTC 42196T). A new family, **Arenicellaceae** fam. nov., and order, **Arenicellales** ord. nov., of the class **Gammaproteobacteria** are proposed to accommodate the novel taxon.

Muroto city, Kochi prefecture, Japan is one of the rare areas where deep-seawater is upwelling. Deep-seawater is relatively rich in nitrogen and phosphorus (DeLong et al., 2006; Hansman et al., 2009), which are the two main growth-limiting factors in marine environments. Therefore, surface seawater around Muroto could be richer in the nutrients than surface seawater in other areas, suggesting that rich microbial resources might be harboured in the Muroto surface seawater. The present study describes a novel bacterium, designated strain 2-9T, isolated from the Muroto surface seawater (33°18′ N 134°11′ E, water depth 0.5 m) in summer 2011. Marine broth 2216 (MB, BD) is generally used to isolate marine bacteria. However, some bacteria appear to be difficult to see and isolate on MB, including members of the genus **Alcanivorax**, because of the transparency of the colony. Strain 2-9T was one of such bacteria, but became easily visible when the medium was supplemented with pyruvate. Strain 2-9T was obtained from the seawater by direct plating on DSW2 medium, which comprised: 1 l deep seawater (from the coastal area of Muroto; 33°18′N 134°14′ E, depth of 320 m), 15 g agar, 0.1 g yeast extract (Bacto yeast extract; BD), 100 mg sodium acetate and 100 mg sodium pyruvate. Pyruvate was utilized by strain 2-9T as a single carbon and energy source but acetate was not, as tested on ONR7a medium (artificial seawater medium; Dyksterhouse et al., 1995) supplemented with (per litre) 15 g agar, and 100 mg sodium acetate or 100 mg sodium pyruvate, at 20 °C for 3 weeks. Strain 2-9T was cultivated at 28 °C for 48 h on a dMB1 plate, which contained 7.48 g MB, 15 g agar, 200 ml distilled water and 800 ml artificial seawater (AWS, Marine Art GF-1; Tomita Pharmaceutical). Phase-contrast microscopy (BX50F4; Olympus) showed that the strain was non-motile. Gram-staining tests (Favor G kit; Nissui) showed

**Abbreviations:** PE, phosphatidylethanolamine; PG, phosphatidylglycerol. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 2-9T is AB908276.

Three supplementary figures and a supplementary table are available with the online Supplementary Material.
that the strain was Gram-stain-negative. The strain was non-sporulating and formed smooth, lenticular, light yellow, semi-translucent, circular colonies with a waved edge that possessed a butyrous consistency on the dMB1 plate. The strain was oxidase- and catalase-positive (Barrow & Feltham, 1993), and negative for acid production from D-glucose on modified Hugh–Leifson’s O-F medium (Leifson, 1963). The API 20 NE kit (bioMérieux) was used for assessing biochemical characteristics according to the manufacturer’s instructions, with the exception that cells were suspended in ASW. In addition, API ZYM tests (bioMérieux) were conducted to examine enzyme activities as described in the manufacturer’s manual, with the exceptions that the inoculum was prepared using ASW and the incubation time was extended to 24 h at 28 °C. Strain 2-9ᵀ was negative for reduction of nitrate to nitrite, indole production from tryptophan, fermentation of glucose and activity of arginine dihydrolase, urease, aesculin hydrolysis, gelatinase and β-galactosidase. Other API test results are given in the species description.

Casein degradation was tested on a casein plate [1 % (w/v) skimmed milk in distilled water overlaid on an MB plate [1.5 % (w/v) agar]]. A clear zone was not observed on the casein plate around cells of strain 2-9ᵀ. Chitin degradation was tested on an MB plate [1.5 % (w/v) agar] supplemented with 2 % (v/v) chitin. Before plate preparation, chitin was dissolved in 30 % sulfuric acid and the solution was diluted with distilled water (1:5, v/v) and precipitated chitin was washed with distilled water until a neutral pH was obtained. A clear zone was not observed on the chitin-containing plate around cells of strain 2-9ᵀ. Starch degradation was tested on an MB plate [1.5 % (w/v) agar] containing 1 % (w/v) starch. A colourless area was not detected around cells of strain 2-9ᵀ after dripping Lugol’s iodine reagent on the surface of the starch-containing plate. Strain 2-9ᵀ was grown at 28 °C for 7 days on these degradation test plates.

For transmission electron microscopy (H7600; Hitachi), strain 2-9ᵀ was grown at 20–28 °C for 7 days on a dMB plate (composition as the dMB1 plate except that Muroto surface seawater was used instead of ASW), and negatively stained with 2 % (v/v) uranyl acetate. Cells of strain 2-9ᵀ were rod-shaped (0.3–0.8 x 0.8–3.0 μm) (Fig. S1, available in the online Supplementary Material).

The yellow pigment(s) was extracted with acetone from cells of strain 2-9ᵀ by shaking vigorously for 10 min. The extract was centrifuged at 15000 g for 3 min in order to remove cell debris, and the resulting supernatant was analysed using a spectrophotometer (DU 730; Beckman Coulter). The visible absorption spectrum of the pigment(s) had absorption maxima at 449 and 471 nm, which closely resembled that of authentic β-carotene reported in the literature (Fig. S2; Takaichi & Shimada, 1992), suggesting that strain 2-9ᵀ could contain β-carotene or a carotenoid structurally similar to β-carotene as the dominant pigment.

Anaerobic growth was tested on dMB plates at 20 °C in a GasPak anaerobic jar (BD) for 2 months. Strain 2-9ᵀ did not grow under anaerobic conditions. Growth temperature was tested on dMB plates at 4, 10, 15, 20, 22, 25, 28, 30, 31, 35, 37 and 40 °C for 2 months. Strain 2-9ᵀ grew at 15–31 °C (optimally at 22–30 °C). The NaCl requirement of strain 2-9ᵀ was tested at 28 °C for 1 week on modified MB medium (pH 7.6) supplemented with 0–7 % (w/v) NaCl (at intervals of 0.5 %). The modified MB medium contained (per litre distilled water): 5 g peptone (bacteriological neutralized; Oxoid), 1 g yeast extract (Oxoid), 0.1 g ferric citrate, 1.8 g CaCl₂, 2H₂O and 5.9 g MgCl₂. 6H₂O. Strain 2-9ᵀ grew with 2.0–3.5 % (w/v) NaCl (optimally with 3.0 %). Strain 2-9ᵀ was unable to grow on R2A agar (BD) with or without NaCl, while the strain grew on the same plate when seawater was used instead of distilled water, indicating that strain 2-9ᵀ requires sea salts other than NaCl for growth. The pH range for growth (tested at pH 5.5–10.5 at intervals of 0.5 pH unit) was determined at 28 °C for 1 week with shaking in MB supplemented with Good’s buffers (MES, Tris, MOPS, HEPES, EPPS, CHES and CAPS; Dojin) at a concentration of 0.1 M each. Strain 2-9ᵀ grew at pH 7.5–8.5 (optimally at pH 8.5).

The almost full-length 16S rRNA gene sequence of strain 2-9ᵀ was obtained as described previously (Teramoto et al., 2009). Among type strains, strain 2-9ᵀ was most similar to members of the genus Arenicella of an unclassified order, Arenicella xantha KMM 3895T (GenBank accession no. AB500096) and Arenicella chitinivorans KMM 6208T (KC136313), with 92.7–93.0 % similarities in the 16S rRNA gene sequence. Other than the genus Arenicella, strain 2-9ᵀ was most closely related to Methylophara hansoni AM6ᵀ (U67929) of the order Methyloccales with 90.4 % 16S rRNA gene sequence similarity. On the other hand, the closest relative to strain 2-9ᵀ in the GenBank database indicated by a BLAST search (Altschul et al., 1990) was an uncultured marine bacterium clone AP28 (JQ347320) with 95.5 % 16S rRNA gene sequence similarity. The 16S rRNA gene sequence of strain 2-9ᵀ was aligned with related sequences of members of the class Gammaproteobacteria available in public databases using the CLUSTAL X program (version 2.1) (Larkin et al., 2007). The alignments were manually modified where necessary, and trimming of gaps was performed. Phylogenetic trees were inferred from the aligned sequences of 1264 bp using the neighbour-joining algorithm (Saitou & Nei, 1987) in CLUSTAL X with default parameters (including Kimura’s correction) and the maximum-likelihood algorithm (Felsenstein, 1981) in the MEGA 6.06 software (Tamura et al., 2013) and analysed using bootstrapping (Felsenstein, 1985) based on 1000 resamplings. The neighbour-joining tree is shown in Fig. 1. The tree also showed that strain 2-9ᵀ was most closely related to the genus Arenicella, but did not cluster with the type strains of species of the genus Arenicella, which was supported by the high bootstrap values. Together with the low 16S rRNA gene sequence similarities to the type strains of species of the genera...
 Arenicella and Methylophaga, these results show that strain 2-9T could represent a novel genus (Yarza et al., 2008; Tindall et al., 2010). In addition, strain 2-9T and members of the genus Arenicella formed a distinct clade, which was supported by the high bootstrap values, and branched deeply relative to the representative type strains from all orders in the class Gammaproteobacteria (Fig. 1). The representative strain of the genus Arenicella, Arenicella xantha KMM 3895T, showed 89.0% similarity in the 16S rRNA gene sequence to the representative strain of the closest order Methylococcales, Methylococcus capsulatus ATCC 19069T (Fig. 1). Together with the 16S rRNA gene sequence similarity of strain 2-9T to the type strains of members of the genus Arenicella, these results indicate that strain 2-9T and the genus Arenicella could belong to the same family of a novel order in the class Gammaproteobacteria.

The cellular fatty acid composition of strain 2-9T was analysed by using cells grown at 28 °C for 3 days on marine agar 2216 (BD) when growth had reached its maximum. Cellular fatty acid methyl esters were prepared and analysed by GC (7890A GC system; Agilent Technologies) according to the instructions given for the Microbial Identification System version 6.0 (MIDI) and were compared using the database TSBA40. The results are given in Table S1. The major cellular fatty acids of strain 2-9T were C18:1ω7c (37.6% of the total), C16:1ω7c and/or iso-C15:0 2-OH (summed feature 3; 19.1%), C18:0 (10.8%), C16:0 (10.2%) and an unidentified fatty acid with an equivalent chain-length value of 11.799 (9.5%).

Isoprenoid quinones were extracted from cells of strain 2-9T grown in MB with shaking at 28 °C for 3 days (to early stationary phase) as described previously (Nishijima et al., 1997) and were analysed by using HPLC (Waters 600; Nihon Waters). Ubiquinone-8 (Q-8) was detected as the sole isoprenoid quinone.

For polar lipid analysis, cells of strain 2-9T were grown in MB with shaking at 28 °C for 2 days (to late exponential phase). Polar lipids were extracted, separated and revealed as described previously (Teramoto et al., 2011) except that total lipid content was revealed by spraying with 10 % w/v molybdophosphoric acid in 2-propanol and incubating at 180 °C for 20 min. The polar lipid profile is shown...
in Fig. S3. The major polar lipids of strain 2-9T were phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and three unidentified lipids; lysophosphatidylethanolamine (lysoPE; PE where one fatty acid chain is missing from the glycerol backbone), three unidentified phospholipids, one unidentified aminolipid and three unidentified lipids were found as minor components. Although diphosphatidylglycerol and aminophospholipid had been detected as major polar lipids from the type strains of members of the genus Arenicella (Nedashkovskaya et al., 2013; Romanenko et al., 2010), they were not found in strain 2-9T.

To determine the DNA G+C content, genomic DNA was extracted and purified from cells of strain 2-9T grown in MB with shaking at 28 °C for 3 days based on the protocol of Marmur (1961). During the protocol, DNA was first separated from protein by shaking with phenol as described by Saito & Miura (1963) before shaking with chloroform and isoamyl alcohol. Genomic DNA was digested to nucleotides with nuclease P1 using a DNA-GC kit (Seikagaku Kogyo) according to the procedures described by Katayama-Fujimura et al. (1984). The G + C content of the DNA was determined by HPLC (LC-10; Shimadzu) with an RP Aqueous column (4.6×250 mm; Nomura Chemical) and a UV-VIS spectrophotometric detector (SPD-10AV; Shimadzu) at 270 nm. The DNA G+C content of strain 2-9T was 41.7 mol%. This value was lower than those for the genus Arenicella (46.3–48.1 mol%; Romanenko et al., 2010; Nedashkovskaya et al., 2013).

Strain 2-9T showed low 16S rRNA gene sequence similarity to the type strain of the closest species of the genus Arenicella (Arenicella xantha KMM 3895T; 93.0%). In addition, strain 2-9T differed from the type strains of members of the genus Arenicella especially based on fatty acid compositions, major polar lipids and DNA G+C content (Table 1). On the basis of genotypic (Fig. 1) and chemotaxonomic (Table 1) data as well as phenotypic characteristics (Table 1), it is proposed that strain 2-9T represents a novel species of a new genus, for which the name Perspicuibacter marinus gen. nov., sp. nov. is proposed.

Strain 2-9T was most similar to the genus Arenicella with 92.7–93.0% 16S rRNA gene sequence similarities to type strains of members of this genus. At the time of writing, two species of this genus have been described and have not been classified into an existing order. The type strains from both of these species are of marine origin (Romanenko et al., 2010; Nedashkovskaya et al., 2013). Phylogenetic analysis indicated that strain 2-9T and species of the genus Arenicella formed a distinct clade, which was supported by the high bootstrap values, and branched deeply within the class Gammaproteobacteria (Fig. 1). The representative strain of the genus Arenicella (Arenicella xantha KMM 3895T) showed 89.0% 16S rRNA gene sequence similarity to the representative strain of the closest order Methylcoccales (Methyllococcus capsulatus ATCC 19069T). These results indicate that strain 2-9T and species of the genus Arenicella belong to a novel family of a novel order of the class Gammaproteobacteria, for which the names Arenicellaceae fam. nov. and Arenicellales ord. nov. are proposed.

**Description of Perspicuibacter gen. nov.**

*Perspicuibacter* (Per.spi.cu.i.bac’ter, L. adj. perspicuus transparent; N.L. masc. n. bacter rod; N.L. masc. n. *Perspicuibacter* a transparent rod).

According to 16S rRNA gene sequence analysis, belongs to the class Gammaproteobacteria. Cells are Gram-stain-negative, non-motile, aerobic rods. Catalase- and oxidase-positive. Negative for nitrate reduction. Predominant cellular fatty acids are C₁₈:₁ω7c, C₁₆:₁ω7c and/or isoc-C₁₅:₀ 2-ΟΗ (summed feature 3), C₁₈:₀, C₁₆:₀ and an unidentified fatty acid with an equivalent chain-length value of 11.799. Q-8 is detected as the sole isoprenoid quinone. Major polar lipids are PG, PE and three unidentified lipids.

The type species is *Perspicuibacter marinus*. The DNA G+C content of a known strain of the type species is 41.7 mol%.

**Description of Perspicuibacter marinus sp. nov.**

*Perspicuibacter marinus* (ma.ri’nus. L. masc. adj. marinus of the sea, marine).

Displays the following properties in addition to those given in the genus description. Smooth, lenticular, light yellow, semi-translucent, circular colonies with a waved edge are formed on dMB1 plates. Cells contain carotenoid(s), and the dominant carotenoid could be structurally similar to β-carotene. Cells are 0.3–0.8×0.8–3.0 μm. Growth occurs at 15–31 °C (optimally at 22–30 °C), at pH 7.5–8.5 (optimally at pH 8.5) and with 2.0–3.5% (w/v) NaCl (optimally with 3.0%). Pyruvate is utilized as a single carbon and energy source. Does not degrade casein, chitin or starch. In API 20 NE tests, negative for reduction of nitrate to nitrite, indole production from tryptophan, fermentation of glucose, activity of arginine dihydrolase, urease, aesculin hydrolase, gelatinase and β-galactosidase, and assimilation of glucose, l-arabinose, d-mannose, d-mannitol, N-acetyl-d-glucosamine, maltose, potassium gluconate, n-caprate, adiapat, DL-malate, sodium citrate and phenylacetate. In API ZYM tests, positive for naphthol-AS-BI-phosphohydrolase activity and negative for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trepsin, x-chymotrypsin, acid phosphatase, β-galactosidase, β-glucuronidase, β-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, x-mannosidase and x-fucosidase activities.

The type strain is 2-9T (=NBRC 110144T=KCTC 42196T), isolated from surface seawater at Muroto, Kochi, Japan. The DNA G+C content of the type strain is 41.7 mol%.
Table 1. Selected differential characteristics between strain 2-9T and type strains of species of the genus Arenicella

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
<td>0.3–0.8 × 0.8–3.0</td>
<td>0.5–0.6 × 3.0–4.0</td>
<td>0.5–0.6 × 2.1–3.3</td>
</tr>
<tr>
<td>Growth temperature (°C)</td>
<td>15–31</td>
<td>5–35</td>
<td>4–38</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein hydrolase</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chitin hydrolase</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Starch hydrolase</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Aesculin hydrolyase</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Esterase (C4)</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Esterase lipase (C8)</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Leucine arylamidase</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Valine arylamidase</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trypsin</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>41.7</td>
<td>48.1</td>
<td>46.3</td>
</tr>
<tr>
<td>Major polar lipids</td>
<td>PG, PE, UL</td>
<td>PG, PE, DPG, APL</td>
<td>PG, PE, DPG, APL</td>
</tr>
<tr>
<td>Major fatty acids</td>
<td>C_{18:1ω7c}, C_{16:1ω7c}iso-C_{15:0} 2-OH, C_{18:1ω7c} Unknown C_{11:799}</td>
<td>C_{16:1ω7c}, iso-C_{16:0}, iso-C_{18:0}, C_{18:1ω7c}</td>
<td>C_{16:1ω7c}, iso-C_{16:0}, iso-C_{18:0}, C_{18:1ω7c}</td>
</tr>
</tbody>
</table>

Description of Arenicellaceae fam. nov.

Arenicellaceae (A.re.ni.cell.ła.ce’a) N.L. fem. n. Arenicella the type genus of the family; suff. -aceae ending to denote a family; N.L. masc. pl. n. Arenicellaceae the family of the genus Arenicella.

Classification into a novel family together with members of the genus Arenicella is justified by their distinct phyletic lineage based on the 16S rRNA gene. Isolated from marine environments and requires NaCl. Contains Q-8 as the major isoprenoid quinone, PG and PE as the major polar lipids, and C_{18:1ω7c} and C_{16:1ω7c} and/or iso-C_{15:0} 2-OH (summed feature 3) as the major fatty acids. The DNA G+C content of known strains is 41.7–48.1 mol%.

The type genus is Arenicella.

Description of Arenicellales ord. nov.

Arenicellales (A.re.ni.cell.ła.les) N.L. fem. n. Arenicella type genus of the order; -ales ending to denote an order; N.L. fem. n. Arenicellales the order of Arenicella.

The description is the same as that for the family Arenicellaceae.

The type genus is Arenicella.

Acknowledgements

We thank Moriyuki Hamada of NITE (National Institute of Technology and Evaluation) for help with polar lipid analysis, Yasuyoshi Nakagawa of NITE for discussion on motility, Ayumi Komatsu of Kochi University for technical assistance, Satoru Ibuki & Takahiro Tsushima of Kochi Prefectural Deep Seawater Laboratory for providing Muroto seawater. This study was performed through Program to Disseminate Tenure Tracking System of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

References


