

Zobellella aerophila sp. nov., isolated from seashore sand, and emended description of the genus *Zobellella*

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A strictly aerobic, nitrate-reducing, motile, rod-shaped member of the class *Gammaproteobacteria*, designated strain JC2671^T, was isolated from a seashore sand sample from Dokdo, Korea. The isolate reduced nitrate to nitrite, but not to nitrogen, and required NaCl for growth. 16S rRNA gene sequence analysis indicated that the isolate belonged to the genus *Zobellella* within the order *Alteromonadales* with sequence similarities of 96.0–97.6% to strains of *Zobellella* species with validly published names. However, in DNA–DNA hybridization studies, a low genomic relatedness (43%) between strain JC2671^T and the type strain of *Zobellella denitrificans* indicated that the isolate represented a novel genomic species. The polar lipid pattern (phosphatidylethanolamine and phosphatidylglycerol), predominant cellular fatty acids [C_{16:0}, summed feature 3 (C_{16:1ω6c} and/or C_{16:1ω7c}) and summed feature 8 (C_{18:1ω6c} and/or C_{18:1ω7c})] and the DNA G + C content (59 mol%) of the novel strain were consistent with its assignment to the genus *Zobellella*. In contrast, a number of phenotypic characteristics, namely a requirement of NaCl for growth, the inability to grow under facultatively anaerobic conditions, the absence of nitrite reduction and differences in carbohydrate utilization and enzymic activities, clearly distinguished the novel isolate from other species of the genus *Zobellella*. Data from this polyphasic study indicate that strain JC2671^T represents a novel species in the genus *Zobellella*, for which the name *Zobellella aerophila* sp. nov. is proposed. The type strain is JC2671^T (=KACC 15081^T =JCM 17110^T). The description of the genus *Zobellella* has been emended accordingly.

The genus *Zobellella* was proposed by Lin & Shieh (2006) to accommodate two novel Gram-negative, motile by a polar flagella, heterotrophic, denitrifying and facultatively anaerobic bacteria, namely *Zobellella denitrificans* and *Zobellella taiwanensis*. This genus belongs to the order *Alteromonadales* (Bowman & McMeekin, 2005), with members of the genera *Oceanimonas* and *Oceanisphaera* as its closest neighbours. During a recent study of the microbial diversity of Dokdo, Korea, a novel *Zobellella*-like

denitrifying bacterial strain was isolated and subjected to a comprehensive taxonomic study.

A Gram-reaction-negative strain, designated JC2671^T, was isolated from a seashore sand sample from Dokdo, Korea (37° 14' 28" N 131° 52' 9" E), using a standard dilution plating method on marine agar 2216 (MA; BD). The isolate was cultured routinely on MA and maintained as glycerol suspensions (20%, w/v) at –80 °C. The type strains of the two species of the genus *Zobellella* with validly published names, *Z. denitrificans* ZD1^T (=JCM 13380^T) and *Z. taiwanensis* ZT1^T (=JCM 13381^T), were also evaluated under identical experimental conditions to those used on strain JC2671^T.

The 16S rRNA gene was enzymically amplified from a single colony. Primers, PCR conditions and sequencing methods have been described previously (Chun &

Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JC2671^T is HM475140.

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

Goodfellow, 1995). Preliminary sequence identification against the 16S rRNA gene sequences held in the EzTaxon database of prokaryotic type strains with validly published names (Chun *et al.*, 2007) indicated that our isolate showed a close relationship with members of the genus *Zobellella*. The nearly complete 16S rRNA gene sequence (1409 bp) of strain JC2671^T was aligned manually against sequences of species of the genus *Zobellella* and representatives of phylogenetically related genera within the order *Alteromonadales* using the bacterial 16S rRNA secondary structure model. The regions available for all sequences (positions 93–820, 850–1126 and 1132–1388; based on the *Escherichia coli* numbering system) showed unambiguous alignment and were used to construct phylogenetic trees. Alignment and neighbour-joining (Saitou & Nei, 1987) analyses were carried out using the program jPHYDIT (Jeon *et al.*, 2005). An evolutionary distance matrix for the neighbour-joining tree was generated according to the model of Jukes & Cantor (1969) and the resultant tree topology was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. A Bayesian inference was performed with the program MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) with an evolutionary model of the General Time Reversible model with a gamma-distributed rate variation. The program was run for 1 000 000 generations with a sample frequency of 100 and a burn-in of 200. A maximum-likelihood (Felsenstein, 1993) tree was constructed using PAUP 4.0 (Swofford, 1998) with a heuristic search option and 1000 replicates. On the basis of 16S rRNA gene analyses, strain JC2671^T showed highest sequence similarity to *Z. denitrificans* ZD1^T (97.6%) and *Z. taiwanensis* ZT1^T (96.0%), followed by members of the genera *Oceanisphaera* (95.3–95.7%) and *Oceanimonas* (95.5–95.6%). No other taxon showed >93% sequence similarity to the novel isolate. This close relationship between strain JC2671^T and members of the above-mentioned genera was also evident in the phylogenetic tree (Fig. 1). Based on all three tree-inferring methods used in this study, the genera *Zobellella*, *Oceanimonas* and *Oceanisphaera*, including strain JC2671^T, formed a monophyletic clade with 100% bootstrap support. Within this suprageneric clade, strain JC2671^T and the type species of the genus *Zobellella*, *Z. denitrificans*, were consistently recovered as a monophyletic clade supporting the assignment of the novel isolate to the genus *Zobellella*. However, the monophyly of the genus *Zobellella* was hampered by *Z. taiwanensis*, the branching position of which varied depending on the tree inferring algorithm used. In the Bayesian tree, *Z. taiwanensis* branched as a sister group of the clade containing the genera *Oceanimonas* and *Oceanisphaera* rather than as a sister group of the clade containing strain JC2671^T and *Z. denitrificans*. Furthermore, in the maximum-likelihood tree, *Oceanimonas smirnovii* formed a monophyletic clade together with strain JC2671^T and with the two recognized species of the genus *Zobellella*. This indicated that the generic status of the genera *Zobellella* and *Oceanimonas* is questionable. Further study is needed to clarify the taxonomic position of

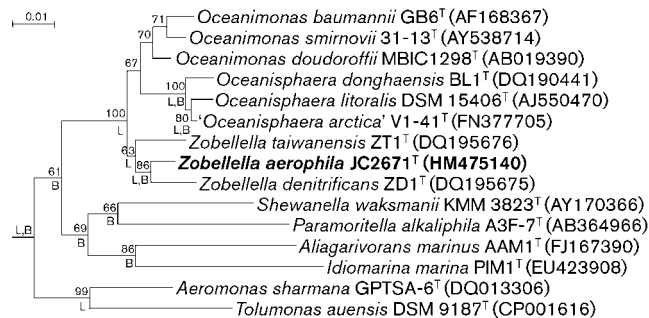


Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences. Numbers at the nodes are percentages of bootstrap support (>50%) from 1000 resampled datasets. Letters at the nodes indicate that the corresponding nodes (groupings) were also recovered in Bayesian (B) and/or in maximum-likelihood (L) tree-inferring methods. *Helicobacter pylori* NCTC 11637^T (Z25741) was used as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position.

these genera. The phylogenetic distinctiveness and robustness of the suprageneric clade containing the genera *Zobellella*, *Oceanimonas* and *Oceanisphaera* suggests the possibility that these three genera could be combined into a single genus in the future.

As the isolate shared a high 16S rRNA gene similarity of 97.6% with *Z. denitrificans* ZD1^T, genomic relatedness between the two strains was further examined using the method of Huß *et al.* (1983) with modifications (Yi & Chun, 2006). The two strains shared a low DNA–DNA relatedness value (43%), which is well below the threshold of 70% recommended for the delineation bacterial species (Wayne *et al.*, 1987). It was clear from DNA–DNA hybridization studies that strain JC2671^T represents a novel genomic species.

Cell morphology and motility were examined by using transmission electron (TEM) and phase-contrast microscopy, respectively, after growth on MA at 30 °C for 2 days. For TEM observation, negative staining with uranyl acetate was performed (Supplementary Fig. S1, available in IJSEM Online). Growth at 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 °C, at pH 5–10.5 (intervals of 0.5 pH units; adjusted with 10 M KOH or HCl) and in 0, 0.5, 1, 2, 3, 4, 5, 7, 10, 12, 15 and 20% (w/v) NaCl was assessed using synthetic ZoBell broth (ZoBell, 1941) to determine the ranges for growth. Growth under anaerobic conditions was assessed using anaerobically prepared MA in an anaerobic chamber (CO₂/H₂/N₂, 10:10:80, v/v/v) (Sheldon Manufacturing). Facultative anaerobic growth in the presence of KNO₃ was determined using anaerobically prepared MA. Although the two recognized species of the genus *Zobellella* were capable of reducing nitrate to nitrogen, strain JC2671^T reduced nitrate to nitrite only. In addition, the two recognized species of the genus *Zobellella* were capable of

facultative anaerobic growth in the presence of KNO_3 , whereas the novel isolate was not.

Standard physiological and biochemical tests were performed as described previously (Smibert & Krieg, 1994). Hydrolysis of alginic acids, casein, microcrystalline cellulose, chitin, egg yolk, elastin, pectin, starch, and Tweens 20, 40, 60 and 80 was tested using MA as the basal medium. Decomposition of adenine, hypoxanthine, L-tyrosine and xanthine was tested using MA according to Gordon *et al.* (1974). Other enzymic activities were determined using the API 20 NE, API 20 E and API ZYM kits (bioMérieux). Acid production by fermentation of carbon sources was tested by using the API 50 CH with CHB/E medium (bioMérieux) supplemented with half-strength seawater. The API kits were inoculated with a heavy bacterial suspension in half-strength artificial seawater and data were recorded for up to three consecutive days.

The DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962). For fatty acid analysis, strain JC2671^T and the two reference strains were grown on MA at 30 °C for 2 days. Extraction of fatty acid methyl esters and GC separation were performed by using the Instant FAME method of the Microbial Identification System (MIDI) version 6.1 and the RTSBA6 6.10 database. Polar lipids were extracted, purified and identified as described by Minnikin *et al.* (1984).

The results of morphological, cultural, biochemical, physiological and chemotaxonomic tests are presented in the species description.

The polar lipid compositions of the isolate and the two recognized species of the genus *Zobellella* were very similar with phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) as major components (Supplementary Fig. S2). However, this result was not congruent with the previous report of Lin & Shieh (2006) which described PE, PG and diphosphatidylglycerol as the major polar lipids of the two reference species. Thus, an emended description of the genus *Zobellella* is proposed.

Phylogenetic analyses based on 16S rRNA gene sequences showed that strain JC2671^T belonged to the genus *Zobellella*, representing a distinct phyletic line within the genus. Although the sequence similarity between the isolate and *Z. denitrificans* ZD1^T is relatively high (97.6%), the low DNA–DNA reassociation value (43%), which is well below the threshold of 70%, recommended for the delineation of bacterial species (Wayne *et al.*, 1987), indicated that the isolate represents a novel genomic species of the genus *Zobellella*. In addition, a number of differentiating phenotypic characteristics (Table 1), such as a requirement of NaCl for growth, an inability to grow under facultatively anaerobic conditions and an absence of nitrite reduction, as well as differences in utilization of fermentable carbohydrates, enzymic activities, fatty acid profiles and G+C contents, clearly supported assignment of the isolate to a novel species of the genus *Zobellella*, for which the name *Zobellella aerophila* sp. nov. is proposed.

Emended description of the genus *Zobellella*

The description of the genus is as given previously (Lin & Shieh, 2006) but with the following amendments. Facultative anaerobes or strict aerobes. Requires NaCl for growth or for growth stimulation. Major polar lipids are PE and PG. Major fatty acids are summed feature 3 ($\text{C}_{16:1\omega6c}$ and/or $\text{C}_{16:1\omega7c}$), summed feature 8 ($\text{C}_{18:1\omega6c}$ and/or $\text{C}_{18:1\omega7c}$) and $\text{C}_{16:0}$. The G+C content of the genomic DNA is 59–64 mol%.

Description of *Zobellella aerophila* sp. nov.

Zobellella aerophila (ae.ro'phi.la. Gr. masc. n. *aer* air; Gr. adj. *philos* loving; N.L. fem. adj. *aerophila* air-loving).

Cells are Gram-reaction-negative, motile rods, $0.5\text{--}1.1 \times 1.0\text{--}6.9 \mu\text{m}$, with a single polar flagellum. Catalase- and oxidase-positive, aerobic and nitrate-reducing. Reduces nitrate to nitrite. Facultative anaerobic growth in the presence of KNO_3 is not observed. Grows at 5–45 °C (optimum 30–35 °C), at pH 5–10.5 (optimum pH 7) and in 0.5–5% NaCl (optimum 1–3%). Does not grow in the absence of NaCl. Colonies on MA at 30 °C are cream-coloured, convex and circular with entire margins. Positive for arginine dihydrolyase, lysine decarboxylase, ornithine decarboxylase and urease activities. Utilizes citrate. Positive for Voges–Proskauer reaction. Does not show β -galactosidase or tryptophan deaminase activity. Does not produce hydrogen sulfide. Acid is not produced from glucose and indole is not produced from tryptophan. Decomposes Tween 20, adenine, hypoxanthine, L-tyrosine and xanthine. Decomposes gelatin in the API 20 E gallery but not in the API 20 NE gallery. Does not decompose agar, alginic acids, casein, cellulose, chitin, egg yolk, elastin, aesculin, pectin, starch, or Tweens 40, 60 or 80. In the API ZYM system, positive for alkaline phosphatase and leucine arylamidase activities and negative for esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α - and β -galactosidase, β -glucuronidase, α - and β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase activities. In the API 50 CH system, positive for fermentation of glycerol, D-fructose, inositol, D-mannitol and *N*-acetylglucosamine; negative for fermentation of erythritol, D-arabinose, L-arabinose, D-ribose, D- and L-xylose, D-adonitol, methyl β -D-xylopyranoside, D-galactose, D-glucose, D-mannose, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, amygdalin, arbutin, aesculin ferric citrate, salicin, cellobiose, maltose, lactose (bovine origin), melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. The major fatty acids are $\text{C}_{16:0}$, summed feature 3 ($\text{C}_{16:1\omega6c}$ and/or $\text{C}_{16:1\omega7c}$) and summed feature 8 ($\text{C}_{18:1\omega6c}$ and/or $\text{C}_{18:1\omega7c}$). The phospholipids are composed of PE, PG and three unidentified aminolipids.

Table 1. Phenotypic characteristics that differentiate strain JC2671^T from other species of the genus *Zobellella*

Strains: 1, JC2671^T; 2, *Z. denitrificans* ZD1^T; 3, *Z. taiwanensis* ZT1^T. All data are from this study unless indicated otherwise. All strains were positive for the following characteristics: catalase and oxidase activities, decomposition of adenine, hypoxanthine, L-tyrosine and xanthine, utilization of citrate, acetoin production, alkaline phosphatase and leucine arylamidase activities and utilization of D-fructose, inositol, D-mannitol and N-acetylglucosamine. All strains were negative for the following characteristics: Gram-reaction, decomposition of agar, alginate acid, casein, cellulose, chitin, egg yolk, elastin, aesculin, pectin, starch, and Tweens 40, 60 and 80, production of hydrogen sulfide and indole, tryptophan deaminase, esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α -chymotrypsin, α - and β -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase activities and utilization of D-arabinose, L-arabinose, D- and L-xylose, methyl β -D-xylopyranoside, D-mannose, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl α -D-mannopyranoside, amygdalin, arbutin, aesculin ferric citrate, cellobiose, lactose, inulin, glycogen, gentiobiose, D-lyxose, D-tagatose, D- and L-fucose, D-arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate. +, Positive; -, negative; w, weakly positive; v, variable.

Characteristic	1	2	3
Growth in 0% NaCl	-	+	+
Anaerobic growth on MA	-	w	w
Facultative anaerobic growth with KNO ₃	-	+	+
Reduction of nitrate to nitrite	+	-	-
Reduction of nitrate to nitrogen	-	+	+
Fermentation of glucose	-	+	+
Decomposition of Tween 20	+	-	-
Decomposition of gelatin	v	-	-
Activities of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease	+	-	-
API ZYM:			
Valine arylamidase, naphthol-AS-BI-phosphohydrolase	-	w	w
Acid phosphatase, α -glucosidase	-	+	w
Fermentation of carbohydrates (API 50CH):			
Glycerol	+	+	-
Erythritol, D-galactose, salicin, melibiose, trehalose, melezitose, raffinose, turanose, potassium gluconate	-	+	-
D-Ribose, D-glucose, maltose, methyl α -D-glucopyranoside, sucrose, starch	-	+	+
D-Adonitol, xylitol, L-arabitol	-	-	+
Fatty acids:*			
C _{12:0}	6.1	5.8	5.0
C _{16:0}	22.8	17.2	17.2
C _{17:0}	2.8	1.6	0.5
iso-C _{16:0}	2.0	1.7	0.5
C _{17:0} cyclo	0.0	5.0	0.0
iso-C _{17:0}	0.9	1.2	2.5
C _{17:1ω8c}	1.4	0.6	0.3
C _{19:0} cyclo ω 8c	0.0	1.7	0.0

Table 1. cont.

Characteristic	1	2	3
Summed features†			
2	6.7	6.1	5.9
3	29.7	23.8	28.9
8	22.7	30.0	33.5
9	0.3	0.8	1.5
DNA G+C content (mol%)	59	64‡	62‡

*Fatty acids amounting to <1% of the total fatty acids in all strains were not included.

†Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2 comprises C_{14:0} 3-OH and/or iso-C_{16:1} I; summed feature 3 comprises C_{16:1 ω 6c} and/or C_{16:1 ω 7c}; summed feature 8 comprises C_{18:1 ω 6c} and/or C_{18:1 ω 7c}; summed feature 9 comprises C_{16:0} 10-methyl and/or iso-C_{17:1 ω 9c}.

‡Data from Lin & Shieh (2006).

The type strain, JC2671^T (=KACC 15081^T =JCM 17110^T), was isolated from a seashore sand sample from Dokdo island, Korea. The DNA G+C content of the type strain is 59 mol%.

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