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TAXONOMIC DESCRIPTION
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Azoarcus pumilus sp. nov., isolated from seawater in Sanya, China

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Abstract

A novel Gram-stain-negative, motile, rod-shaped (0.4–0.5×1.0–2.0 µm) strain with one polar flagellum, designated SY39^T, was isolated from seawater in Sanya, China. Strain SY39^T was able to grow at 15–40 °C (optimum, 35–37 °C), pH 6.5–8.5 (pH 8.0) and 0.5–6.0 % (w/v) NaCl (3.5 %). Chemotaxonomic analysis showed that the isoprenoid quinones were Q-8 (88.6 %) and Q-7 (11.4 %). The dominant fatty acids were $C_{16:0}$ and summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$). The polar lipids of strain SY39^T consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, one unknown phosphoglycolipid, one unknown glycolipid and two unknown aminophosphoglycolipids. The DNA G+C content of the genomic DNA was 66.5 mol %. The phylogenetic analysis of 16S rRNA gene sequences showed that strain SY39^T belongs to the genus *Azoarcus* with similarity ranging from 92.3 to 95.2 %. Based on the phenotypic, chemotaxonomic and phylogenetic features, strain SY39^T is concluded to represent a novel species of the genus *Azoarcus*, for which the name *Azoarcus pumilus* sp. nov. is proposed. The type strain is SY39^T (=KCTC 62157^T=MCCC 1K03430^T).

The genus *Azoarcus*, belonging to family *Rhodocyclaceae*, was first proposed by Reinhold-Hurek *et al.* [1] with the type species *Azoarcus indigens*, which was isolated from Kallar grass from Pakistan. At the time of writing, the genus *Azoarcus* is composed of nine validly published species [2] (www.bacterio.net/azoarcus.html). Members of *Azoarcus* are widely distributed in various environments, including stems, roots, aquifer sediment, oil-contaminated soil, petroleum-contaminated freshwater, sewage sludge and oxic soil [1, 3–7]. '*Azoarcus taiwanensis*' DSM 24109 was isolated from a hot sulfur spring on Yang-Ming Mountain and has the ability of sulfide oxidization under denitrifying conditions. All *Azoarcus* strains share characteristics such as staining Gram-negative, being oxidase-positive and having DNA G+C contents between 62 and 68 mol% [8]. In this study, strain SY39^T was isolated from surface seawater in Sanya. It is the first reported marine strain in the genus *Azoarcus*. The aim of the present investigation is to clarify the taxonomic position of strain SY39^T based on the analysis of phenotypic, phylogenetic, genomic and chemotaxonomic characteristics.

The surface seawater was collected in September 2012 from the South China Sea in Sanya (18° 15′ N 109° 47′ E) and stored at 4 °C in the laboratory for several weeks. Then a light yellowish pigmented strain (SY39^T) was isolated via modified marine agar 2216 (MA; Difco) [9] at 30 °C and conserved by freeze-drying (re-cultivating every 6 months) until revived for identification. The strain was maintained and sub-cultivated on MA or marine broth 2216 (MB; BD Difco) at 30 °C and subsequently characterized based on its 16S rRNA gene sequence, fatty acid composition and further phenotypic features. Reference strains used in this study ('*Azoarcus. taiwanensis*' DSM 24109 and *A. indigens* DSM 12121^T) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

Phenotypic tests were performed with cells grown on MB at 30 °C, unless otherwise indicated. The Gram reaction was tested by using the Gram-staining method as described previously[10]. After incubation on MA at 30 °C for 4 days, cell morphology and the presence of flagella were observed using transmission electron microscopy (JEM-1230, JEOL) after uranyl acetate staining. The motility test was

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Keywords: *Azoarcus pumilus*; polyphasic taxonomy; seawater; South China Sea.
Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PL, unknown phospholipid; GL, unknown glycolipid; PGL, unknown phosphoglycolipid; APL, unknown aminophospholipid; APGL, unknown aminophosphoglycolipid; MA, marine agar 2216; MB, marine broth 2216; MP, maximum-parsimony; ML, maximum-likelihood; NJ, neighbour-joining; Q-8, ubiquinone-8; Q-7, ubiquinone-7.
The GenBank accession numbers for the 16S rRNA gene sequence and the genome sequence of strain SY39^T are KY861745 and CP025682, respectively.

Table 1. Differential phenotypic characteristics of strain SY39^T and the type strains of related species

Strains: 1, SY39^T; 2, '*A. taiwanensis*' DSM 24109; 3, *A. indigens* DSM 12121^T. All data are obtained from this study unless otherwise indicated. +, Positive; −, negative; W, weakly positive; NA, no data available.

Characteristic	1	2	3
Relationship with O_2	Facultative	Facultative[a]	Aerobic[b]
Habitat	Seawater	Soil sample from a hot spring[a]	Kallar grass[b]
Motility	+	−	−
Optimum temperature for growth (°C)	35–37	35–37[a]	40[b]
Optimum growth pH	8.0	9.0[a]	NA
NaCl concentration for growth	0.5–6.0	1–3[a]	0–2[b]
Nitrite reduced to N_2	−	+	+
Voges–Proskauer reaction	−	−	+
Glucose fermentation	−	+	−
Enzyme activity:			
Acid phosphatase	−	−	+
Alkaline phosphatase	−	W	+
Arginine dihydrolase	−	−	+
Esterase lipase (C8)	+	+	W
Lipase (C14)	W	−	−
Hydrolysis of:			
Gelatin	+	−	−
Tween 20	+	+	−
Tween 40	−	+	−
Tween 60	−	+	−
Urea	+	−	+
Utilization of:			
Acetate	−	+	+
Adipic acid	−	+	−
Citrate	−	+	−
Capiric acid	+	−	+
DL-Malic acid	+	+	+
L-Arabinose	−	+	−
L-Serine	−	+	−
Sucrose	−	+	−
Major quinones	Q-8, Q-7	Q-8	Q-8, Q-7
DNA G+C content (mol%)	66.5	63.7[a]	66.6[b]

a, Data from Lee *et al.* [30]; *b*, data from Reinhold-Hurek *et al.* [1].

performed by inoculation in semi-solid marine broth 2216 [MB with 0.5 % agar (w/v)]. The temperature range for growth was determined in MB at 4–50 °C (4, 15, 20, 25, 28, 30, 35, 37, 40, 45 and 50 °C). The pH range for growth in MB was measured from pH 5.0 to 10.0 (at 0.5 pH unit intervals) by supplementing 40 mM buffering agents, including MES (pH 4.5–6.0), PIPES for (pH 6.5–7.5), Tricine (pH 8.0–8.5) and CAPSO (pH 9.0–10.0). Tolerance to NaCl (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8 and 10 %, w/v) was investigated in NaCl-free MB [11]. The temperature, pH and NaCl ranges for growth was monitored by measuring OD_{600} in a UV/visible spectrophotometer (Ultrospec 6300 pro, Amersham Biosciences). Anaerobic growth was detected by using the Hungate roll-tube technique with modified MA supplemented with sodium thiosulfate (20 mM), sodium sulfite (5 mM), sodium sulfate (20 mM), sodium nitrite (5 mM), sodium nitrate (20 mM) and L-arginine (5.0 gl^{-1}) as potential electron acceptors for over 15 days under pure N_2 [9].

For normal cultivation, all reference strains used in this study and strain SY39^T were cultured on Luria–Bertani (LB) agar at 35 °C. Catalase and oxidase activity were tested by the method as described by Sun *et al.* [12]. Hydrolysis of starch, gelatin and Tweens (20, 40, 60 and 80) were examined as described by Zhang *et al.* [13]. Nitrate reduction was tested according to Dong and Cai [14]. The methyl red and Voges–Proskauer tests were examined as described by Lányi [15]. Other biochemical properties and enzyme activities were tested using API ZYM and API 20NE kits

Table 2. Cellular fatty acid profile of strain SY39^T and the type strains of related species

Strains: 1, SY39^T; 2, '*A. taiwanensis*' DSM 24109; 3, *A. indigens* DSM 12121^T. Fatty acids that represented <0.5 % in all strains are not shown, major fatty acids (>10%) in all strains are shown in bold. TR, Trace (<0.5 %); –, not detected. All data are from this study.

Fatty acids	1	2	3
Straight chain			
$C_{10:0}$	0.6	TR	0.5
$C_{11:0}$	1.3	–	–
$C_{12:0}$	4.8	2.7	6.6
$C_{13:0}$	TR	–	–
$C_{14:0}$	0.6	TR	0.5
iso-$C_{16:0}$	–	–	TR
$C_{16:0}$	**28.0**	**21.5**	**41.7**
$C_{17:0}$ cyclo	**14.7**	0.7	**12.7**
$C_{17:0}$	5.0	TR	TR
$C_{18:0}$	1.7	0.6	0.7
$C_{19:0}$	1.7	–	1.4
$C_{20:0}$	TR	–	–
Unsaturated			
$C_{15:1}\omega 6c$	2.7	–	–
$C_{16:1}\omega 5c$	–	0.7	–
iso-$C_{17:0}$	0.7	–	–
$C_{17:1}\omega 8c$	2.0	–	–
$C_{18:1}\omega 5c$	–	TR	–
Hydroxy			
$C_{8:0}$ 3-OH	0.5	TR	TR
$C_{10:0}$ 3-OH	5.4	4.3	5.2
$C_{11:0}$ 3-OH	TR	–	–
Summed features			
3*	**21.3**	**38.5**	**21.4**
5†	1.9	–	1.5
8‡	6.3	**29.7**	7.3

*Summed feature 3 comprised $C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$.

†Summed feature 5 comprised $C_{18:0}$ ante and/or $C_{18:2}\omega 6,9c$.

‡Summed feature 8 comprised $C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$.

(bioMérieux) following the manufacturer's instructions. GN2 MicroPlates (Biolog) were used to detect the utilization of organic substrates according to the manufacturer's instructions.

Cells used for the analysis of fatty acids were harvested from the third quadrants of LB agar plates [16]. Fatty acid methyl esters were extracted as described by Kuykendall *et al.* [17] and analysed according to the instructions of the Sherlock Microbial Identification System (MIDI; Microbial ID). After incubation in LB broth at 35 °C for 4 days, cells of strain SY39^T and reference strains were collected for extraction of isoprenoid quinones which were subsequently purified by TLC and identified by using an HPLC-MS system (Agilent) [18, 19].

Genomic DNA of the strain SY39^T was extracted by using the Quick Bacteria Genomic DNA Extraction kit (Dong-Sheng Biotech). The 16S rRNA gene was amplified by PCR using the universal primer pair 27F (5′-GAGAGTTT-GATCCTGGCTCAG-3′)/1492R (5′-GTCGTAACAAGG-TAGCCGTA-3′). The purified PCR products were cloned into the vector pMD19-T (TaKaRa) and then sequenced. The almost-complete 16S rRNA gene sequence of strain SY39^T (1425 nt) was identified using the EzTaxon-e service [20] and the NCBI nr database. Multiple sequence alignment was performed with the CLUSTAL_X program in the MEGA 5 package [21]. Phylogenetic trees were reconstructed by using the neighbour-joining [22], maximum-likelihood [23] and maximum-parsimony [24]methods with the MEGA5 package. Bootstrap analysis was based on 1000 replications. To testify the phylogenetic tree reconstructed by MEGA5, the All-Species Living Tree LTPs123 and arb-6.0.6 databases were used as references. The SINA webserver [25] and ARB software [26] were used for alignment of 16S rRNA gene sequences into LTPs123 and for generation of a new maximum-likelihood phylogenetic tree, respectively. The complete genome was sequenced using the PacBio RS II platform and the Illumina HiSeq 4000 platform at the Beijing Genome Institute (BGI, Shenzhen, PR China) and assembled using Celera Assembler version 8.3 [27] against a high quality corrected circular consensus sequence subreads set. To improve the accuracy of the genome sequences, the GATK (www.broadinstitute.org/gatk/) and SOAP tool packages (SOAP2, SOAPsnp, SOAPindel) were used to make single-base corrections. For the measurement of G+C content, genomic DNA was treated with P1 nuclease (Sigma) and calf intestine alkaline phosphatase (TaKaRa) respectively. The G+C content of these dephosphorylated deoxyribonucleosides was determined by reversed-phase HPLC and calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) [28].

Cells of strain SY39^T were Gram-stain-negative, motile with one polar flagellum (Fig. S1, available in the online version of this article), facultative anaerobic, and rod-shaped (0.4–0.5 µm wide and 1.0–2.0 µm long). Colonies were 0.1–0.2 mm in diameter, circular, smooth, convex, transparent after growth on MA at 30 °C for 6 days. Strain SY39^T grew at 15–40 °C (optimum, 35–37 °C), pH 6.5–8.5 (pH 8.0) and with 0.5–6.0 % (w/v) NaCl (3.5 %). Detailed physiological and biochemical characteristics are displayed in Table 1 and the species description.

The predominant isoprenoid quinone detected in strain SY39^T was Q-8 (88.6 %), which was also detected in '*A. taiwanensis*' DSM 24109 and other species of *Azoarcus* [29], while Q-7 (11.4 %) could also be found. This result was in line with that in *A. indigens* DSM 12121^T (Q-8 and Q-7, 95.8 and 4.2 % respectively). The fatty acid profiles of strain SY39^T and the reference strains are shown in Table 2. Major fatty acids (>10 %) of strain SY39^T were $C_{16:0}$ (28.0 %), summed feature 3 ($C_{16:1}\omega 7c$/$C_{16:1}\omega 6c$, 21.3 %) and $C_{17:0}$ cyclo (14.7 %), which was similar to that of *A. indigens* DSM

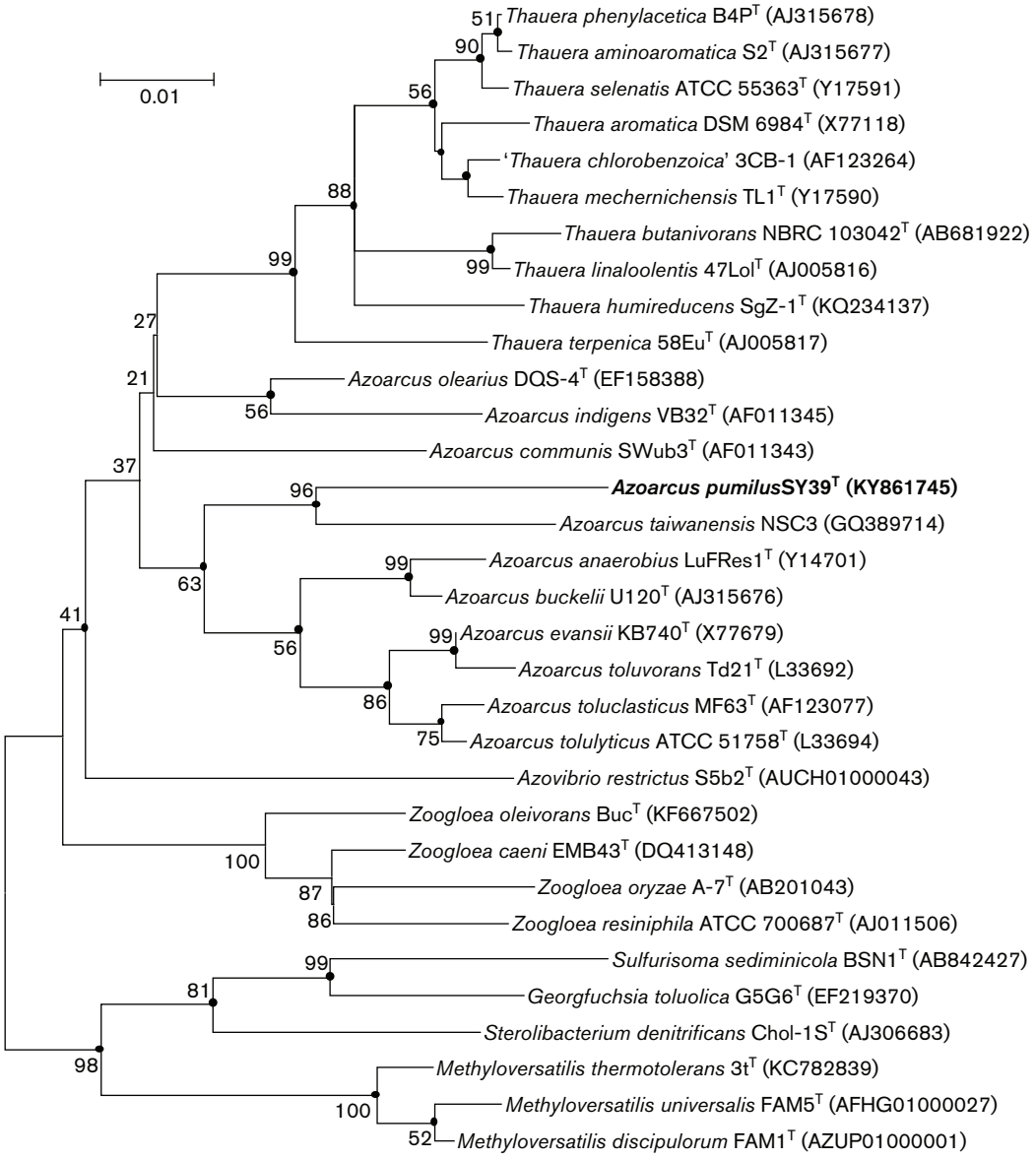

Fig. 1. Maximum-likelihood tree using the Kimura two-parameter model based on the 16S rRNA gene sequences, showing the phylogenetic relationships of the novel isolate and members of the genus *Azoarcus* and other relative genera. Bootstrap values are based on 1000 replicates; values ≥50 % are shown. Filled circles indicate nodes also obtained in the neighbour-joining and maximum-parsimony trees. Bar, 0.01 substitutions per nucleotide position.

12121^T (41.7 %, 21.4 %, 12.7 %, respectively), while the major fatty acids of '*A. taiwanensis*' DSM 24109 were summed feature 3 ($C_{16:1}\omega7c$/$C_{16:1}\omega6c$, 38.5 %), summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$, 29.7 %) and $C_{16:0}$ (21.5 %). All strains possess $C_{16:0}$ as major fatty acids (strain SY39^T, 28.0 %; '*A. taiwanensis*' DSM 24109, 21.5 %; *A. indigens* DSM 12121^T, 41.7 %) though some proportional differences existed. Strain SY39^{T}could be differentiated from '*A. taiwanensis*' DSM 24109 by the lower proportions of summed feature 3 ($C_{16:1}\omega7c$/$C_{16:1}\omega6c$) and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), and from *A. indigens* DSM 12121^T by the lower proportion of $C_{16:0}$. The polar lipids of strain SY39^T and the reference strains are shown in Fig. S2. The polar lipids of strain SY39^T were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), one unknown phosphoglycolipid (PGL1), one unknown glycolipid (GL1) and two unknown aminophosphoglycolipids (APGL1 and APGL2). DPG, PG and PE were also detected in the two reference strains and other species of genus *Azoarcus* [4], which would be a significant feature to support that strain SY39^T belongs to genus *Azoarcus*. The existence of GL1 in strain SY39^T was

consistent with that in *A. indigens* DSM 12121^T. Meanwhile the compositions of APGL1 and APGL2 in strain SY39^T could be used differentiate the isolate from the reference strains.

Based on the 16S rRNA gene sequence analysis with the EzTaxon-e tool, strain SY39^T exhibited the highest similarity value (95.2 %) to '*A. taiwanensis*' DSM 24109 and all *Azoarcus* species (≤93.9 %). As shown in Fig. 1, phylogenetic trees reconstructed by using the ML, NJ and MP algorithms indicated that strain SY39^T clustered with the members of genus *Azoarcus* and formed a distinct linkage with '*A. taiwanensis*' DSM 24109, meanwhile the phylogenetic trees based on 16S rRNA gene sequences using the ARB program also showed that strain SY39^T fell into the clade which comprises species of the genus *Azoarcus* (Fig. S3), supporting the conclusion that strain SY39^T represents a novel species of the genus *Azoarcus*. The complete genome sequences of strain SY39^{T}was constituted of 1 contig. The genome size was 3 225 512 bp. The GenBank accession number for the whole genome sequence of strain SY39^T is CP025682.

The DNA G+C content of strain SY39^T was 66.5 mol% (as determined by HPLC and counting G+C in full genome sequences), which was similar to those of the type strains of the validly described species of genus *Azoarcus* (ranging from 62 to 68 mol%).

A comparison of the physiological and biochemical characteristics between strain SY39^T and the related type strains of genus *Azoarcus* is shown in Table 1. Many similarities, such as rod-shape, activities of catalase and oxidase, ability to reduce nitrate to nitrite, and assimilation of malate, can be found. However, strain SY39^T also has distinct characteristics which could distinguish it from the reference strains, such as the absence of alkaline phosphatase and inability of reducing nitrite to N_2. Meanwhile, strain SY39^T cannot hydrolyse Tween 40 and Tween 60, and cannot assimilate acetate, adipic acid, citrate, L-arabinose, L-serine and sucrose, which is the same as found in *A. indigens* DSM 12121^T, but different from '*A. taiwanensis*' DSM 24109 (Table 1).

Based on the phylogenetic, genotypic, chemotaxonomic and phenotypic characteristics, it is proposed that strain SY39^T represents a novel species of the genus *Azoarcus*, for which the name *Azoarcus pumilus* sp. nov. is proposed.

DESCRIPTION OF *AZOARCUS PUMILUS* SP. NOV.

Azoarcus pumilus (pu′mi.lum. L. masc. adj. *pumilum* small or tiny, referring to the tiny colonies formed by this organism).

Cells are Gram-stain-negative, facultative anaerobic, motile with one polar flagellum and rod-shaped (0.4–0.5×1.0–2.0 μm). Colonies on LB agar after 4 days of incubation at 35 °C are circular, smooth, transparent, light yellow, convex and 1.0–2.0 mm in diameter. Growth occurs at 15–40 °C (optimum, 35–37 °C), pH 6.5–8.5 (pH 8.0) and with 0.5–6.0 % (w/v) NaCl (3.5 %). Oxidase- and catalase-positive. Nitrate is reduced to nitrite. Nitrite is not reduced to N_2. Tween 20, gelatin and urea are hydrolysed, while starch, Tweens (40, 60 and 80) are not. Negative for indole production, Voges–Proskauer test and methyl red test. Fermentation of glucose is negative. Utilization of 2-aminoethanol, arabinose, α-cyclodextrin, dextrin, glycerol, turanose, n-capiric acid, formic acid, malate, phenylacetic acid, propionic acid, D-gluconic acid, D-glucosaminic acid, glycyl L-glutamic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, succinic acid, succinic acid monomethyl ester and D-alanine are positive; utilization of D-galactonic acid lactone, γ-hydroxybutyric acid, α-ketovaleric acid and glucuronamide are weak; utilization of L-arabinose, D-arabitol, cellobiose, erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, *N*-acetyl-glucosamine, *N*-acetyl-D-galactosamine, D-glucose, D-glucose 1-phosphate, D-glucose 6-phosphate, D,L-α-glycerol phosphate, glycogen, inositol, maltose, lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl β-D-glucoside, potassium gluconate, D-psicose, L-rhamnose, raffinose, D-sorbitol, sucrose, trehalose, xylitol, acetic acid, adipic acid, bromosuccinic acid, cis-aconitic acid, citrate, D-galacturonic acid, D-glucuronic acid, L-glutamic acid, glycyl L-aspartic acid, inosine, itaconic acid, α-ketobuyric acid, α-ketoglutaric acid, L-lactic acid, malonic acid, L-pyroglutamic acid, p-hydroxyphenylacetic acid, quinic acid, D-saccharic acid, sebacic acid, succinamic acid, thymidine, urocanic acid, alaninamide, L-aspartate, L-alanine, L-alanyl glycine, L-asparagine, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, hydroxy L-proline, D-serine, L-serine, L-threonine, D,L-carnitine, phenyl ethylamine and putrescine are negative. Production of leucine arylamidase, esterase (C4) and esterase lipase (C8) are positive; production of lipase (C14), naphthol AS-BI phosphohydrolase and valin arylamidase are weakly positive; and production of acid phosphatase, alkaline phosphatase, arginine dihydrolase, chymotrypsin, cystine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, α-mannosidase, trypsin and *N*-acetyl-β-glucosaminidase are negative. The isoprenoid quinones are Q-7 (11.4 %) and Q-8 (88.6 %). Major fatty acids (>10 %) are $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$) and $C_{17:0}$ cyclo. The polar lipids consist of diphosphatidyglycerol, phosphatidylglycerol, phosphatidylethanolamine, one unknown phosphoglycolipid, one unknown glycolipid and two unknown aminophosphoglycolipids. The DNA G+C content of genomic DNA is 66.5
mol%. The type strain is SY39^T (=KCTC 62157^T=MCCC 1K03430^T), isolated from surface seawater in Sanya, PR China.

EMENDED DESCRIPTION OF *AZOARCUS* REINHOLD-HUREK *ET AL.* 1993

The major quinone is Q-8. The major polar lipids consist of diphosphatidyglycerol, phosphatidylglycerol and

phosphatidylethanolamine. The rest of the description is identical to that mentioned by Reinhold-Hurek and Hurek [8]. The type species is *Azoarcus indigens.*

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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