Methylocystis iwaonis sp. nov., a type II methane-oxidizing bacterium from surface soil of a rice paddy field in Japan, and emended description of the genus *Methylocystis* (*ex* Whittenbury *et al.* 1970) Bowman *et al.* 1993.

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22 Abbreviations: MDH, methanol dehydrogenase; MOB, methane-oxidizing bacteria;
23 pMMO, particulate methane monooxygenase; sMMO, soluble methane
24 monooxygenase.
25
26 The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, *pmoA*, *mmoX*
27 and *mxaF* sequences of strain SS37A-Re^T are AB669149, AB669161, LC583144 and
28 LC583145, respectively. The whole genome sequences are available in the DDBJ under
29 accession numbers AP027142, AP027143, AP027144, AP027145, AP027146,
30 AP027147 and AP027148 for strain SS37A-Re^T, AP027149 and AP027150 for
31 *Methylocystis bryophila* DSM21852^T and BSEC01000001, BSEC01000002,
32 BSEC01000003, BSEC01000004, BSEC01000005, BSEC01000006, BSEC01000007,
33 BSEC01000008, BSEC01000009, BSEC01000010 and BSEC01000011 for
34 *Methylocystis echinoides* LMG27198^T.
35

Abstract

A novel methane-oxidizing bacterial strain SS37A-Re^T was isolated from surface soil of a rice paddy field in Japan. Cells were Gram-negative, motile rods with single polar flagellum and type II intracytoplasmic membrane arrangement. The strain grew on methane or methanol as the sole carbon and energy source. It grew at 15–37 ºC (optimum 25–30 ºC), pH 6.0–9.0 (optimum 7.0–8.0) and with 0–0.1% (w/w) NaCl (no growth at 0.5% or above). Cells formed cysts, but not exospores. Sequence analysis of 16S rRNA gene showed that the strain SS37A-Re^T belonged to the family *Methylocystaceae*, with the highest similarity (98.9%) to *Methylocystis parva* corrig. OBBP^T. Phylogenetic analysis of *pmoA* and *mxaF* genes and core genes in genome indicated that the strain was closely related to the genus *Methylocystis*, while the analysis of *mmoX* gene showed the close relationships with the genus *Methylosinus*. The values of genome relatedness between strain SS37A-Re^T and *Methylocystis* and *Methylosinus* species were 78.6–82.5% and 21.7–24.9% estimated by the average nucleotide identity and digital DNA-DNA hybridization, respectively, showing the highest values with *Methylocystis echinoides* LMG 27198^T. The DNA G+C content was 63.2 mol% (genome). The major quinone and fatty acids were Q-8 and, $C_{18:1}$ ($C_{18:1}\omega 8t$ and $C_{18:1}\omega 8c$) and $C_{18:2}$, respectively. Based on the phenotypic and phylogenetic features, the strain represents a new species of the genus *Methylocystis*, for which the name *Methylocystis iwaonis* sp. nov. is proposed. The type strain is SS37A-Re^T (=JCM 34278^T =NBRC 114996^T =KCTC 82710^T).

DATA SUMMARY

Supplementary materials for this manuscript are available at:

INTRODUCTION

Paddy fields are an important source of methane emission [1] and a habitat for methane-oxidizing bacteria (MOB). MOB play important roles in regulating methane efflux from paddy fields because net emission of methane is the difference between production and oxidation by methanogens and MOB, respectively, and the oxidation is the only biological suppression of methane emission.

Aerobic MOB are a unique bacterial group that utilizes methane as a sole energy and carbon source and include *Gammaproteobacteria* (type I) and *Alphaproteobacteria* (type II) as major groups [2–4]. Various MOB have been isolated from the paddy field ecosystem and five species of type I MOB, *Methylogaea oryzae* [5], *Methylomonas koyamae* [6], *Methylomagnum ishizawai* [7], *Methyloterricola oryzae* [8] and *Methylocucumis oryzae* [9], have been described with the type strains isolated from the ecosystem. As for the type II MOB, however, no strains have been taxonomically characterized so far [10] though many studies reported several isolates of type II MOB belonging to the genus *Methylocystis* or *Methylosinus* from the paddy field ecosystem [e.g., 11–19]. Type II MOB include the family *Methylocystaceae* comprising the genera *Methylocystis* and *Methylosinus*. Major phenotypic characteristics differentiating the two genera are morphology and motility of cells, flagellation, and formation of cysts or exospores [20, 21]. The genera consist of seven and two species,

Methylocystis parva corrig. (*Methylocystis parvus* [sic]) [20], *Methylocystis echinoides* [20], *Methylocystis rosea* [22], *Methylocystis heyeri* [23], *Methylocystis hirsuta* [24], *Methylocystis bryophila* [25] and *Methylocystis silviterrae* [26], and *Methylosinus trichosporium* and *Methylosinus sporium* [20], respectively. During the cultivation of MOB from several compartments in paddy field ecosystem, strain SS37A-Re^T was isolated from surface soil of a rice paddy field in Japan [27]. In this communication, we characterized the strain and propose that strain SS37A-Re^T is a novel species of the genus *Methylocystis*.

ISOLATION AND ECOLOGY

Surface soil sample was collected from a rice paddy field in Crop Institute, Aichi Agricultural Research Center (formerly Anjo Agricultural and Extension Centre, Aichi-ken Agricultural Experiment Station), Anjo, Aichi, Japan (34º97' N, 137º07' E) on August 11, 2003. Details of the sampling and cultivation procedures were described elsewhere [27]. In brief, surface soil sample was taken from 0–0.5 cm depth from three sites in the paddy field plot. The soil samples were mixed to form a composite sample and ground with a sterile mortar and pestle. Ten grams of the sample were put into the mixture (50 ml) of 25 ml nitrate mineral salt (NMS) medium [28] and 25 ml ammonium mineral (AM) medium [29] at pH 6.8. The preparation was incubated with shaking (100 rpm) at 4 ºC for 2 h. The obtained suspension was serially 10-fold diluted (10^{-1} to 10^{-3}) and the dilution series were spread onto AM agar (7 ml) slants in 34 ml test tubes. The tube was sealed with a butyl rubber stopper and methane (6 ml) was injected into the

tube, giving about 18% (v/v) methane in the headspace. Then the tubes were incubated for 5 weeks at 30 ºC. Single colonies formed on the agar slants were transferred into liquid NMS medium (7 ml) and cultivated with shaking (150 rpm) at 30 ºC under 18% (v/v) methane for 2 weeks. Colony isolation from the agar slants and cultivation in the liquid medium were repeated by checking methane consumption in the headspace by gas chromatography. Finally, a pure culture was obtained by isolation of a single colony on 1a agar plates [30] with a slight modification ($NaNO_3$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $Na_2HPO_4 \cdot 12H_2O$, 0.56 g; KH_2PO_4, 0.22 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 2 mg; $ZnSO_4 \cdot 7H_2O$, 0.44 mg; $CuSO_4 \cdot 5H_2O$, 0.2 mg; $MnSO_4 \cdot 2H_2O$, 0.17 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.06 mg; H_3BO_3, 0.1 mg; $CoCl_2 \cdot 6H_2O$, 0.08 mg; agar, 14 g; per litre distilled water; pH 7.0) after several subcultures in liquid 1a medium. Culture purity was confirmed by phase-contrast microscopy (BX50; Olympus) and failure to grow in liquid media (nutrient broth medium, Luria-Bertani medium, 1a medium supplemented with 0.1% [w/v] sucrose, polypeptone medium and polypeptone medium supplemented with methanol 0.05% [v/v]) without addition of methane.

PHYLOGENY OF 16S rRNA, *pmoA, mmoX and mxaF*

Cell pellets (1.44 g wet weight) of strain SS37A-Re^T were suspended in 10 ml of TESS buffer (25 mM Tris HCl; 5 mM EDTA; 50 mM NaCl; 25% [w/v] sucrose) and treated with lysozyme (1 mg ml^{-1}) and SDS (1%) at 60 ºC for 1 h. Then genomic DNA was isolated and purified as described by Sowers [31]. Gene fragments of 16S rRNA, *pmoA* (particulate methane monooxygenase [pMMO] gene), *mmoX* (soluble methane

monooxygenase [sMMO] gene) and *mxaF* (methanol dehydrogenase [MDH] gene) were amplified by PCR using the following primers: 27f/1492r [32], A189f [33]/mb661r [34], mmoX206f/mmoX 886r [35] and 1003F [36]/1555r [37], respectively. The sequences were determined at the Eurofins Genomics K.K. (Tokyo, Japan). The sequences of 16S rRNA (1411 bp), *pmoA* (495 bp), *mmoX* (664 bp) and *mxaF* (514 bp) genes were subjected to the EzBioCloud (https://www.ezbiocloud.net/resources/16s_download) [38] or BLAST search for related sequences. Pairwise nucleotide sequence similarity values were calculated for the close relatives of the 16S rRNA gene at the EzBioCloud. The sequences were aligned using MUSCLE (ver. 3.8.1551) and the alignment curation was conducted using Noisy (ver. 1.5.12.1) through the online service of NGPhylogeny.fr (https://ngphylogeny.fr) [39]. Phylogenetic trees were constructed using the maximum likelihood (PhyML; ver. 3.0), parsimony (dnapars in the PHYLIP ver. 3.696) and distance (BioNJ algorithm; distance options according to the Kimura two-parameter model) methods with the SeaView (ver. 5.0.4) [40] program based on 1000 or 100 replications bootstrap analysis.

Sequence analysis of 16S rRNA gene showed that strain SS37A-Re^T was most closely related to the genus *Methylocystis* (Fig. 1; trees by the parsimony and distance methods are shown in Figs. S1a and S1b, respectively) with the highest pairwise similarity value (98.9%) to *Methylocystis parva* OBBP^T. Phylogenetic analysis of pMMO gene (*pmoA*) (Fig. 2; trees by the parsimony and distance methods are shown in Figs. S2a and S2b) and MDH gene (*mxaF*) (Fig. S3) of strain SS37A-Re^T also

indicated that the strain belonged to the genus *Methylocystis* with the highest similarity to *Methylocystis echinoides* IMET10491^{T}. sMMO gene (*mmoX*) of strain SS37A-Re^{T} was most closely related to the species *Methylosinus sporium* (Fig. S4), indicating that the strain represents some distinctiveness from the genus *Methylocystis* phylogenetically. In addition to the analyses above, phylogenetic analysis was conducted by including the sequences of 16S rRNA, *pmoA*, *mxaF* and *mmoX* genes of *Methylocystis* and *Methylosinus* strains isolated from various environments [41]. Constructed trees (Figs. S5–S8) show the similar arrays of closely related bacteria, *Methylocystis* strains for 16S rRNA, *pmoA* and *mxaF* genes and *Methylosinus* strains for *mmoX* gene, to those revealed by the trees using gene sequences of type strains (Figs. 1, 2 and S1–S4), confirming the results of phylogenetic analyses based on the sequences from type strains.

GENOME FEATURES

Genome sequencing and *De novo* assembly were outsourced to the Bioengineering Lab Ltd. (Sagamihara, Japan) and carried out by the hybrid analysis on DNBSEQ-G400RS (MGI Tech Co., Ltd., Shenzhen, China) with pair-end libraries (2 x 200 bp) and GridION with R4.9.1 flow cell (Oxford Nanopore Technologies, Oxford, UK). Low quality reads (Q score, < 20; length < 127 bp) from DNBSEQ were removed with Sickle ver.1.33 after trimming the adaptor sequence with Cutadapt ver. 2.7 and sampling 3458457 pair reads with Seqkit ver. 0.11.0. Short reads (< 1000 bp) from GridION were filtered out with Filtlong ver. 0.2.0 after trimming the adaptor sequence

168 with Porechop ver. 0.2.3. The quality-controlled reads were assembled on Unicycler
169 ver. 0.4.7 [42]. The quality of assemblies was assessed with Bandage ver. 0.8.1 and
170 CheckM ver. 1.1.2 [43]. Prokka ver. 1.13 [44] was used for the genome annotation.
171 Phylogenetic tree was inferred by the core genome identification using PIRATE ver.
172 1.0.5 [45] and subsequent phylogenetic inference using SeaView (ver. 5.0.4) [40]
173 program by the distance (BioNJ algorithm; distance options according to the Kimura
174 two-parameter model) methods with the based on 1000 replications bootstrap analysis.
175 A complete genome of 4483149 bp was obtained for strain SS37A-Re^T and
176 the genome sequence was submitted to DDBJ (accession numbers AP027142–
177 AP027148). Since genome sequences were not available for the type strains of
178 *Methylocystis bryophila* and *Methylocystis echinoides* in the genus *Methylocystis*,
179 genome sequencing analysis was also performed for *Methylocystis bryophila*
180 DSM21852^T (=H2s^T) and *Methylocystis echinoides* LMG27198^T (=IMET10491^T).
181 Genomic DNA of *Methylocystis bryophila* DSM21852^T was obtained from the DSMZ
182 (Braunschweig, Germany) and subjected to the sequencing. A complete genome of
183 4715351 bp was obtained and the genome sequence was submitted to DDBJ (accession
184 numbers AP027149 and AP027150). *Methylocystis echinoides* LMG27198^T was
185 obtained from the BCCM/LMG (Ghent, Belgium). The strain was cultivated in the
186 liquid DNMS (dilute NMS) medium [46] and genomic DNA was isolated from the
187 collected cells by the same method as that for strain SS37A-Re^T. A draft genome of
188 5326437 bp comprised 11 contigs and the sequence was submitted to DDBJ (accession
189 numbers BSEC01000001–BSEC01000011).

General genomic information of the strains is shown in Table S1. The genome of strain SS37A-Re^T possesses three sets of rRNA operon (5S–23S–16S rRNA), two copies of *pmoCAB1* operons and one *pmoCAB2* operon. Three and two singleton *pmoC* paralogs are present in the chromosome and plasmids, respectively. The genome contains a nitrogenase structural gene operon (*nifHDK*) and a complete set of genes for the formaldehyde assimilation by the serine pathway.

Average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values were calculated using ANI calculator (http://enve-omics.ce.gatech.edu/ani/) [47] and Genome-to-Genome Distance Calculator (http://ggdc.dsmz.de/ggdc.php#) [48], respectively, for the genome sequences of type strains of *Methylocystis* and *Methylosinus* species. Table 1 shows a matrix of the pairwise ANI and dDDH values. The pairwise ANI and dDDH values between strain SS37A-Re^T and type strains of *Methylocystis* and *Methylosinus* species were lower than the cut-off values of 95% and 70%, respectively, for species delineation [47, 48], showing that strain SS37A-Re^T is phylogenetically distant from *Methylocystis* and *Methylosinus* species and represents a novel species. The strain SS37A-Re^T showed the highest values 82.5% and 24.9% for ANI and dDDH, respectively, with *Methylocystis echinoides* LMG27198^T, indicating that strain SS37A-Re^T belongs to the genus *Methylocystis*. The genome-based phylogenetic tree also shows that strain SS37A-Re^T is most closely related to the genus *Methylocystis* (Fig. 3). DNA G+C content of strain SS37A-Re^T was 63.2 mol%.

PHYSIOLOGY AND CHEMOTAXONOMY

The strain was routinely cultured in 1a liquid medium at 30 ºC for 2–3 days under 18–50% (v/v) methane and maintained by subculturing every 5–6 months. For long-term preservation, cells were frozen with sterile glass beads (2–3 mm in diameter) [49] or with 5% (v/v) dimethylsulfoxide, slightly modified from the procedure by Bowman [3], at –80 ºC. Colony morphology was observed for cultures on 1a agar medium. Cell morphology and motility were observed by phase-contrast with an Olympus BX50 microscope. Gram-staining was performed by Hucker's modification method. Formation of 'lipid' cysts was tested as described by Whittenbury *et al.* [50]. Flagellation and size of cells were observed for negatively stained cells with 2% (w/v) uranyl acetate with a H-7500AMT Advantage HR transmission electron microscope (Hitachi). Intracytoplasmic structures were observed for ultra-thin sections of cells fixed with 2% (v/v) glutaraldehyde and 2% (w/v) osmium tetroxide and stained with 2% (w/v) uranyl acetate and lead stain solution with a H-7600 transmission electron microscope (Hitachi) at Hanaichi UltraStructure Research Institute (Okazaki, Japan).

Colonies formed by strain SS37A-Re^T on agar medium were round, entire, convex, smooth, white and opaque. The colonies reached 0.5–1 mm after 5 days of incubation at 30 ºC. Cells were Gram-stain-negative, motile rods (2.7 ± 0.4 μm long and 1.1 ± 0.1 μm wide; mean ± S.D., n = 24) with a polar flagellum (Fig. 4a). The cells occurred singly or in pairs (Fig. 4b) and occasionally formed rosettes (Fig. 4c). Cells were sensitive to heat (80 ºC, 20 min) and drying. 'Lipid' cysts were observed, but exospores were not observed. Electron microscopy of ultrathin sections of cells showed

234 a typical internal cytoplasmic membrane structure of type II MOB with membranes
235 parallel to the cell wall (Fig. 4d).
236 Effects of temperature (4, 10, 15, 20, 25, 30, 37, 40 and 45 ºC), pH (4.5, 6.0,
237 7.0, 8.0, 9.0, 10.0 and 11.0), concentrations of NaCl (0, 0.1, 0.5, 1.0, 2.0, 3.0, 5.0%
238 [w/w]) and methane (0, 10, 18, 30, 50, 70, 90, 100% [v/v]) on cell growth were tested in
239 the liquid 1a medium by measuring OD_{600} of cultures. The pH was adjusted with 0.2 M
240 KH_2PO_4/Na_2HPO_4 from pH 4.5 to 9.0 or 0.1 M Glycine/NaCl/NaOH from pH 9.0 to
241 11.0. Utilization of carbon sources for growth was investigated in test tubes containing
242 the liquid 1a medium supplemented with 0.1% (w/v) of the following filter-sterilized
243 compounds: methanol, formaldehyde, formate, formamide, methylamine,
244 dimethylamine, trimethylamine, tetramethylammonium chloride, trimethylamine N-
245 oxide, trimethyl sulfonium iodide, dimethyl carbonate, ethanol, acetate, pyruvate,
246 citrate, malate, succinate, D-arabinose, D-xylose, D-glucose, maltose, sucrose, mannitol
247 and glycerol. Utilization of nitrogen sources for growth was examined in test tubes
248 containing the nitrogen-free liquid 1a medium supplemented with 0.1% (w/v) of the
249 following filter-sterilized compounds: nitrite, urea, ammonium, glycine, alanine, serine,
250 glutamate, glutamine, aspartate, asparagine, tryptophane, cysteine, lysine and yeast
251 extract. Catalase and cytochrome c oxidase were tested as described by Cleenwerck *et*
252 *al*. [51]. The activity of sMMO was demonstrated for cells grown in the liquid 1a
253 medium without added copper by a naphthalene oxidation assay according to the
254 methods by Brusseau *et al.* [52] and Koh *et al.* [53].

255 The strain SS37A-Re^T grew on methane and methanol as sole carbon and
256 energy sources. Although growth was also observed on dimethyl carbonate, the strain
257 probably grew on methanol formed by hydrolyzation of dimethyl carbonate. Doubling
258 time in the 1a liquid medium with 20% (v/v) methane was 5.9 hours at 30 ºC. The strain
259 grew in the ranges of 15 to 37 ºC and pH 6.0–9.0 with the optimum at 25–30 ºC and pH
260 7.0–8.0. It showed good growth with 0–0.1% (w/w) NaCl, but no growth at 0.5% or
261 above. The strain grew under 10–90% (v/v) methane and the specific growth rate
262 showed almost similar values between 10–70%. The strain SS37A-Re^T grew with urea,
263 ammonium, alanine, glutamine, aspartate and asparagine besides nitrate as nitrogen
264 sources and was also capable of growth in nitrogen-free 1a media supplemented with
265 0.45% (w/v) gellan gum. Cells were positive for catalase and cytochrome c oxidase.
266 Production of naphthol from naphthalene was verified with the cells grown in the
267 medium without added copper, indicating expression of sMMO activity.
268 Cells for chemotaxonomic analyses were harvested from liquid cultures at late
269 exponential phase (3 days) grown at 30 ºC. Composition of total cellular fatty acids was
270 determined using the Sherlock Microbial Identification (MIDI) System version 6.0 at
271 the TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan) and the methyl-esterified
272 fatty acids were also analysed by GC-MS with a JMS-K9 (JEOL). To determine the
273 position of double bond in mono-unsaturated fatty acids, fatty acids were extracted and
274 methyl-esterified according to Wartiainen *et al.* [22], and then dimethyl disulphide
275 derivatives were prepared according to the method described by Nichols *et al.* [54] and
276 analysed by GC-MS with a JMS-T2000GC (JEOL). Quinones were isolated and

277 purified using a Sep-Pak plus silica column (Waters) from total lipids extracted from
278 lyophilized cells according to Bligh and Dyer [55] and analyzed by HPLC with a
279 ACQUITY UPLC H-Class system (Waters) at the TechnoSuruga Laboratory Co., Ltd.
280 (Shizuoka, Japan).

281 Table 2 shows the composition of cellular fatty acids of strain SS37A-Re^T.
282 Major two fatty acids, FA1 and FA2 showing 79% and 9%, respectively, were not
283 identified by the MIDI system. GC-MS fragment analysis of the methyl-esterified fatty
284 acids indicated that FA1 and FA2 were $C_{18:1}$ and $C_{18:2}$, respectively. Database search by
285 the NIST Mass Search Ver.2.0 showed that $C_{18:2}\omega 6c$/$C_{18:2}\omega 9c$ were the candidate $C_{18:2}$
286 fatty acids. Analysis of the DMDS derivatives by GC-MS revealed that $C_{18:1}\omega 8t$ and
287 $C_{18:1}\omega 8c$ (65% and 35%, respectively, of the $C_{18:1}$ isomers) were the $C_{18:1}$
288 monounsaturated fatty acids (Fig. S9). Fatty acids $C_{18:1}\omega 7$ were not detected. The major
289 lipoquinone was ubiquinone 8 (Q-8; 95.4%) with slight proportions of Q-7 (3.5%) and
290 Q-9 (1.1%).

291 The morphological, physiological and chemotaxonomic features of strain
292 SS37A-Re^T mentioned above are summarized in Table 3. These features indicated that
293 the strain belonged to the family *Methylocystaceae* [20, 56, 57]. The sequence analysis
294 of 16S rRNA gene showed that the strain SS37A-Re^T was most closely related to
295 *Methylocystis parva* OBBP^T (Figs. 1, S1 and S5) with 98.9% pairwise similarity value.
296 However, the pairwise ANI and dDDH values between strain SS37A-Re^T and type
297 strains of *Methylocystis* and *Methylosinus* species were lower than the cut-off values of
298 95% and 70%, respectively (Table 1), indicating that the strain SS37A-Re^T represents a

299 novel species. The highest values 82.5% and 24.9% for ANI and dDDH, respectively,
300 with *Methylocystis echinoides* LMG27198^T and the phylogenetic analysis of core genes
301 in the genome (Fig. 3) indicated that the strain SS37A-Re^T belongs to the genus
302 *Methylocystis*. Highest similarity values with *Methylocystis echinoides* LMG27198^T by
303 the phylogenetic analysis of *pmoA* and *mxaF* genes of strain SS37A-Re^T (Figs. S2, S3,
304 S6 and S7) and the slightly lower G+C content (63.2%) of DNA of the strain than that
305 of the genus *Methylosinus* (about 65%) also support the strain belongs to the genus
306 *Methylocystis* (Table 3). The strain SS37A-Re^T resembled members of the genus
307 *Methylocystis* in cysts formation and no exospores formation (Table 3).
308 However, the *mmoX* gene of strain SS37A-Re^T was most closely related to the
309 species *Methylosinus sporium* (Figs. 2, S4 and S8). In addition, the strain exhibited the
310 following distinctive phenotypic features in the genus *Methylocystis*: cells were motile
311 with single polar flagellum; cells formed rosettes; the major fatty acid was $C_{18:1}\omega 8t$
312 (Tables 2 and 3). The phenotypes of motility and flagellation have never been reported
313 so far for the genus *Methylocystis* [20, 21, 58] though the flagella-encoding genes were
314 found in some strains of *Methylocystis* [59]. Predominance of the fatty acid $C_{18:1}\omega 8t$ has
315 not been found in neither *Methylocystis* nor *Methylosinus* [20, 60, 61]. Based on these
316 features, we propose the name *Methylocystis iwaonis* sp. nov. for the strain SS37A-Re^T.
317
318 **Emended description of the genus *Methylocystis* (ex. Whittenbury *et al.* 1970)**
319 **Bowman, Sly, Nichols and Hayward 1993 emend. Belova *et al.* 2013**

320 Cells are Gram-negative rods or coccobacilli to reniform, 0.3–1.4 μm wide
321 and 0.5–4.0 μm long with type II intracytoplasmic membrane arrangement. Motility of
322 cells varies among species; if present, cells possess single polar flagellum. May produce
323 lipoidal cysts, but not exospores. The optimum temperature and pH are 25–30 ºC and
324 5.5–9.0. May form rosettes. Cells possess particulate methane monooxygenase (pMMO)
325 and some species may possess soluble methane monooxygenase (sMMO). Assimilate
326 C_1 compounds via the serine pathway; some strains utilize acetate or ethanol. Oxidase
327 and catalase are positive. Fix atmospheric nitrogen. Primary fatty acids are $C_{18:1}\omega 8c$,
328 $C_{18:1}\omega 8t$ and $C_{18:1}\omega 7c$. Some species possess $C_{18:2}\omega 7$, 12*c*, $C_{18:2}\omega 6$, 12*c* and $C_{18:2}\omega 6$, 9*c*.
329 Primary quinone is ubiquinone-8 (Q-8). The mol % G+C of the DNA is 62–64.
330 *Methylocystis parva* is the type species.
331
332 **Description of *Methylocystis iwaonis* sp. nov.**
333 *Methylocystis iwaonis* (i.wa.o'nis. N.L. masc. gen. n. *iwaonis* of Iwao, after
334 the Japanese soil microbiologist Iwao Watanabe, in honor of his valuable contribution
335 to soil microbiology in paddy fields).
336
337 Cells are Gram-negative, motile rods (1.1 μm wide and 2.7 μm long) with
338 single polar flagellum. Grow only on methane or methanol as the sole carbon and
339 energy source. Form rosettes occasionally. The optimum temperature, pH and NaCl
340 concentration are 25–30 ºC, 7.0–8.0 and 0–0.1% (w/w); cells are sensitive to NaCl
341 above 0.5%. Possess soluble methane monooxygenase (sMMO). Fix atmospheric

342 nitrogen. Major quinone is Q-8 and $C_{18:1}$ ($C_{18:1}\omega 8t$ and $C_{18:1}\omega 8c$) and $C_{18:2}$ are
343 predominant fatty acids.
344 The type strain is SS37A-Re^T (=JCM 34278^T =NBRC 114996^T =KCTC
345 82710^T), which was isolated from surface soil of a rice paddy field in Japan. The DNA
346 G+C content of the type strain is 63.2 mol%. The GenBank/EMBL/DDBJ accession
347 numbers for the 16S rRNA gene, *pmoA*, *mmoX* and *mxaF* sequences of strain SS37A-
348 Re^T are AB669149, AB669161, LC583144 and LC583145, respectively. The whole
349 genome sequences are available in the DDBJ under accession numbers AP027142–
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361
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Conflicts of interest

The authors declare that there is no conflict of interest.

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562 **Table 1.** Genome relatedness indexes between strain SS37A-Re^T and *Methylocystis* and *Methylosinus* species

563 ANI and dDDH values are shown in the upper and lower triangles, respectively. Accession number are presented in parentheses.

Strain	1	2	3	4	5	6	7	8	9	10
1. SS37A-Re^T (GCA_027925385.1)	100	82.3	82.5	80.0	79.2	80.0	78.6	80.0	79.2	79.4
2. *Methylocystis parva* OBBP^T (GCA_000283235.1)	24.8	100	82.2	79.7	78.9	79.8	78.7	79.8	78.7	78.9
3. *Methylocystis echinoides* LMG27198^T (BSEC01000001–BSEC01000011)	24.9	24.3	100	80.0	79.0	80.1	78.9	79.9	79.1	78.7
4. *Methylocystis rosea* SV97^T (GCA_000372845.1)	22.3	21.6	21.9	100	78.4	91.9	78.4	92.4	78.7	78.1
5. *Methylocystis heyeri* H2^T (GCA_004802635.2)	22.5	21.2	21.6	20.8	100	78.7	78.7	78.3	78.5	78.2
6. *Methylocystis hirsuta* CSC1^T (GCA_003722355.1)	22.0	21.7	22.2	47.2	21.9	100	78.3	94.1	78.6	78.3
7. *Methylocystis bryophila* H2s^T (GCA_027925445.1)	21.7	21.3	21.9	21.5	21.4	21.5	100	78.6	78.7	78.4
8. *Methylocystis silviterrae* FS^T (GCA_013350005.1)	21.8	21.7	21.8	49.1	21.1	56.0	21.0	100	78.7	78.5
9. *Methylosinus trichosporium* OB3b^T (GCA_002752655.1)	21.7	21.4	21.6	21.4	21.3	21.5	21.0	21.6	100	83.1
10. *Methylosinus sporium* 5^T (GCA_009811675.1)	22.0	21.3	21.2	20.6	21.2	20.6	20.9	20.5	25.4	100

564

Table 2. Cellular fatty acids composition of SS37A-Re^T and type strains of *Methylocystis* and *Methylosinus*

Strains: 1, strain SS37A-Re^T; 2, *Methylocystis parva* OBBP^T [25]; 3, *Methylocystis echinoides* IMET10491^T [60]; 4, *Methylocystis rosea* SV97^T [26]; 5, *Methylocystis heyeri* H2^T [23]; 6, *Methylocystis hirsuta* CSC1^T [26]; 7, *Methylocystis bryophila* H2s^T [25]; 8, *Methylocystis silviterrae* FS^T [26]; 9, *Methylosinus trichosporium* OB3b^T [61]; 10, *Methylosinus sporium* 5^T [61].Values are percentages of the total fatty acids. −, Not detected; tr, Trace component.

Fatty acid	1	2	3	4	5	6	7	8	9	10
iso-$C_{14:0}$	−	−	−	−	−	−	−	−	−	0.1
iso-$C_{15:0}$	−	−	−	−	−	−	0.2	−	−	0.5
anteiso-$C_{15:0}$	−	−	−	−	−	−	−	−	−	3.5
$C_{15:0}$	−	−	−	−	tr	−	−	−	−	0.7
iso-$C_{16:0}$	−	−	−	−	−	−	−	−	−	1.4
10-Methyl $C_{16:0}$	−	−	−	−	3.5	−	−	−	−	−
$C_{16:1}\omega 9c$	−	−	−	0.6	−	−	−	−	−	−
$C_{16:1}\omega 9t$	−	−	−	−	−	2.3	−	−	−	−
$C_{16:1}\omega 8c$	−	−	−	−	29.0	−	−	−	−	−
$C_{16:1}\omega 7c$	1.2*	0.5	0.8	−	3.4	−	15.4	−	14.2	9.3
$C_{16:1}\omega 6c$	−	−	−	−	0.5	−	−	−	−	−
$C_{16:1}\omega 5t$					2.8	−	−	−	−	−
$C_{16:0}$	4.7	0.2	0.3	0.9	1.2	0.3	2.4	−	0.7	2.2
iso-$C_{17:0}$	0.2	−	−	−	−	−	0.2	−	−	0.1
anteiso-$C_{17:0}$	−	−	−	−	−	−	−	−	−	0.6
$C_{17:1}\omega 8c$	0.2	−	−	−	−	−	0.2	−	−	−
$C_{17:0}$ cyclo	−	−	−	−	0.3	−	−	−	−	0.2
$C_{17:0}$	0.4	−	−	−	tr	−	−	−	−	0.3
iso-$C_{18:0}$	3.6	−	−	−	−	−	−	−	−	−
$C_{18:2}\omega 7$, 12*c*	−	8.0	22.7	−	−	−	7.8	−	−	−
$C_{18:2}\omega 6$, 12*c*	−	22.1	4.4	−	−	−	−	−	−	(tr)†
$C_{18:2}\omega 6$, 9*c*	9.0	−	−	−	−	−	−	−	−	(tr)†
$C_{18:1}\omega 9c$	−	−	−	−	−	−	−	−	−	0.2
$C_{18:1}\omega 9t$	−	−	1.5	−	14.7	−	−	−	−	−
$C_{18:1}\omega 8c$	27.3‡	45.5	51.9	74.8	32.0	71.1	53.1	74.5	67.5	−
$C_{18:1}\omega 8t$	51.8‡	−	−	−	−	−	0.8	−	−	−
$C_{18:1}\omega 7c$	−	23.7	18.1	23.7	10.9	26.1	19.3	24.7	13.1	78.7
$C_{18:1}\omega 7t$	−	−	−	−	−	−	−	−	4.6	−
$C_{18:1}\omega 5c$	−	−	0.3	−	−	−	−	−	−	−
$C_{18:0}$ 2-OH	0.3	−		−		−	−	−	−	−
$C_{18:0}$	1.4	−	0.2	−	0.7	0.3	−	0.8	−	1.2
$C_{19:0}$ branched	−	−	−	−	−	−	−	−	−	0.8
$C_{19:0}$ cyclo	−	−	−	−	0.2	−	−	−	−	−
$C_{20:0}$	−	−	−	−	0.1	−	−	−	−	−

* The fatty acid was assigned to $C_{16:1}\omega 7c$/ $C_{16:1}\omega 9t$ as "summed feature" in the report of the MIDI System.

† Bowman *et al.* [61] showed the fatty acid as "$C_{18:2}\omega 6$".

‡ The fatty acids were identified by the analysis of the DMDS derivatives (Fig. S9)

574 **Table 3.** Phenotypic characters of type strains of *Methylocystis* and *Methylosinus* species

575 Strains: 1, strain SS37A-Re^T; 2, *Methylocystis parva* OBBP^T [20, 57, 60]; 3, *Methylocystis echinoides* IMET10491^T [20, 60, 62, this study]; 4, *Methylocystis rosea* SV97^T [22, 26]; 5,
576 *Methylocystis heyeri* H2^T [23]; 6, *Methylocystis hirsuta* CSC1^T [24, 26]; 7, *Methylocystis bryophila* H2s^T [25, this study]; 8, *Methylocystis silviterrae* FS^T [26]; 9, *Methylosinus*
577 *trichosporium* OB3b^T [20, 57]; 10, *Methylosinus sporium* 5^T [20, 57, 61]. +, Positive; -, negative; NR, Not reported.

Characteristic	1	2	3	4	5	6	7	8	9	10
Cell shape	Rods	Reniform, coccobacilli	Reniform, coccobacilli, rods	Rods	Straight, polymorphic or regularly curved rods, ovoids	Dumbbell	Small curved coccoids, short rods	Small curved coccoids/rods	Pear (pyriform)	Vibrioids, rods
Cell width (μm)	1.1	0.3–0.5	0.6	0.8–1.1	0.8–1.2	0.3–0.6	0.9–1.4	0.5–0.7	0.5–1.5	0.5–1.0
Cell length (μm)	2.7	0.5–1.5	0.8–1.2	1.1–2.5	1.4–4.0	0.7–1.0	1.8–3.4	1.7–3.4	2.0–3.0	1.5–3.0
Motility	+	-	-	-	-	-	-	-	+	+
Flagellum	Single polar	-	-	NR	NR	-	NR	NR	Polar tuft	Polar tuft
Lipid cyst formation	+	+	-	-	-	+	NR	NR	-	-
Exospore formation	-	-	-	-	-	-	NR	NR	+	+
Rosette formation	+	-	-	-	-	-	-	NR	+	+
Optimum growth condition:										
Temperature (ºC)	25–30	30	30	27	25	30	25–30	25–30	30	30
pH	7.0–8.0	7.0	7.0	5.5–9.0	5.8–6.2	7.0	6.0–6.5	6.0–6.5	6.5–7.0	6.5–7.0
Growth at 37 ºC	+	+	-	+	-	-	+	+	+	+
sMMO	+	-	-	-	+	+	+	-	+	+
Predominant fatty acids	$C_{18:1}\omega8t$	$C_{18:1}\omega8c$	$C_{18:1}\omega8c$	$C_{18:1}\omega8c$	$C_{18:1}\omega8c$ $C_{16:1}\omega8c$	$C_{18:1}\omega8c$	$C_{18:1}\omega8c$	$C_{18:1}\omega8c$	$C_{18:1}\omega8c$	$C_{18:1}\omega7c$
G+C content (mol%)*	63.2	63.9	63.9	62.5	63.0	62.4	63.2	62.6	65.9	65.2

578 * Determined by genome analysis.

Figure legends

Fig. 1. Maximum-likelihood phylogenetic tree of 16S rRNA gene sequences showing the relationships between strain SS37A-Re^T and related bacteria. Bar represents 0.02 substitutions per nucleotide sequence position. Closed circles indicate internal nodes with at least 50% bootstrap support from 1000 data resampling. The tree was rooted using *Rhodoplanes elegans* AS130^T as the outgroup. GenBank accession numbers are given in parentheses.

Fig. 2. Maximum-likelihood phylogenetic tree of *pmoA* gene sequences showing the relationships between strain SS37A-Re^T and related bacteria. Bar represents 0.1 substitutions per nucleotide sequence position. Closed circles indicate internal nodes with at least 50% bootstrap support from 1000 data resampling. The tree was rooted using *Methylomonas koyamae* Fw12E-Y^T as the outgroup. GenBank accession numbers are given in parentheses.

Fig. 3. Phylogenomic tree based on the concatenated nucleotide sequence of core genes of *Methylocystis* and *Methylosinus* species by the distance method. The bar shows nucleotide substitutions per site. Closed circles indicate internal nodes with at least 70% bootstrap support from 1000 data resamplings. The tree was rooted using *Methylomonas koyamae* Fw12E-Y^T as the outgroup. GenBank accession numbers are given in parentheses.

Fig. 4. (a) Transmission electron micrograph of negatively stained cells of strain SS37A-Re^T. Bar, 1 μm. (b) Phase-contrast micrograph of strain SS37A-Re^T cells. Bar, 5 μm. (c) Phase-contrast micrograph of rosettes of strain SS37A-Re^T cells. Bar, 5 μm. (d) Transmission electron micrograph of ultrathin sections of strain SS37A-Re^T cells. Bar, 200 nm. Arrow shows the intracytoplasmic membrane structure.

606

607 **Supplementary figures**

608

609 **Fig. S1.** Phylogenetic trees of 16S rRNA gene sequences showing the relationships
610 between strain SS37A-Re^T and related bacteria by the parsimony (a) and distance (b)
611 methods. Bars represent 0.01 (a) and 0.005 (b) substitutions per nucleotide sequence
612 position. Closed circles indicate internal nodes with at least 50% bootstrap support from
613 1000 data resamplings. The tree was rooted using *Rhodoplanes elegans* AS130^T as the
614 outgroup. GenBank accession numbers are given in parentheses.

615

616 **Fig. S2.** Phylogenetic trees of *pmoA* gene sequences showing the relationships between
617 strain SS37A-Re^T and related bacteria by the parsimony (a) and distance (b) methods.
618 Bars represent 0.02 (a) and 0.01 (b) substitutions per nucleotide sequence position.
619 Closed circles indicate internal nodes with at least 50% bootstrap support from 1000
620 data resamplings. The tree was rooted using *Methylomonas koyamae* Fw12E-Y^T as the
621 outgroup. GenBank accession numbers are given in parentheses.

622

623 **Fig. S3.** Phylogenetic trees of *mxaF* gene sequences showing the relationships between
624 strain SS37A-Re^T and related bacteria by the maximum-likelihood (a), parsimony (b),
625 and distance (c) methods. Bars represent 0.05 (a) and 0.02 (b and c) substitutions per
626 nucleotide sequence position. Closed circles indicate internal nodes with at least 50%
627 bootstrap support from 1000 data resamplings. The tree was rooted using *Methylomonas*
628 *koyamae* Fw12E-Y^T as the outgroup. GenBank accession numbers are given in
629 parentheses.

630

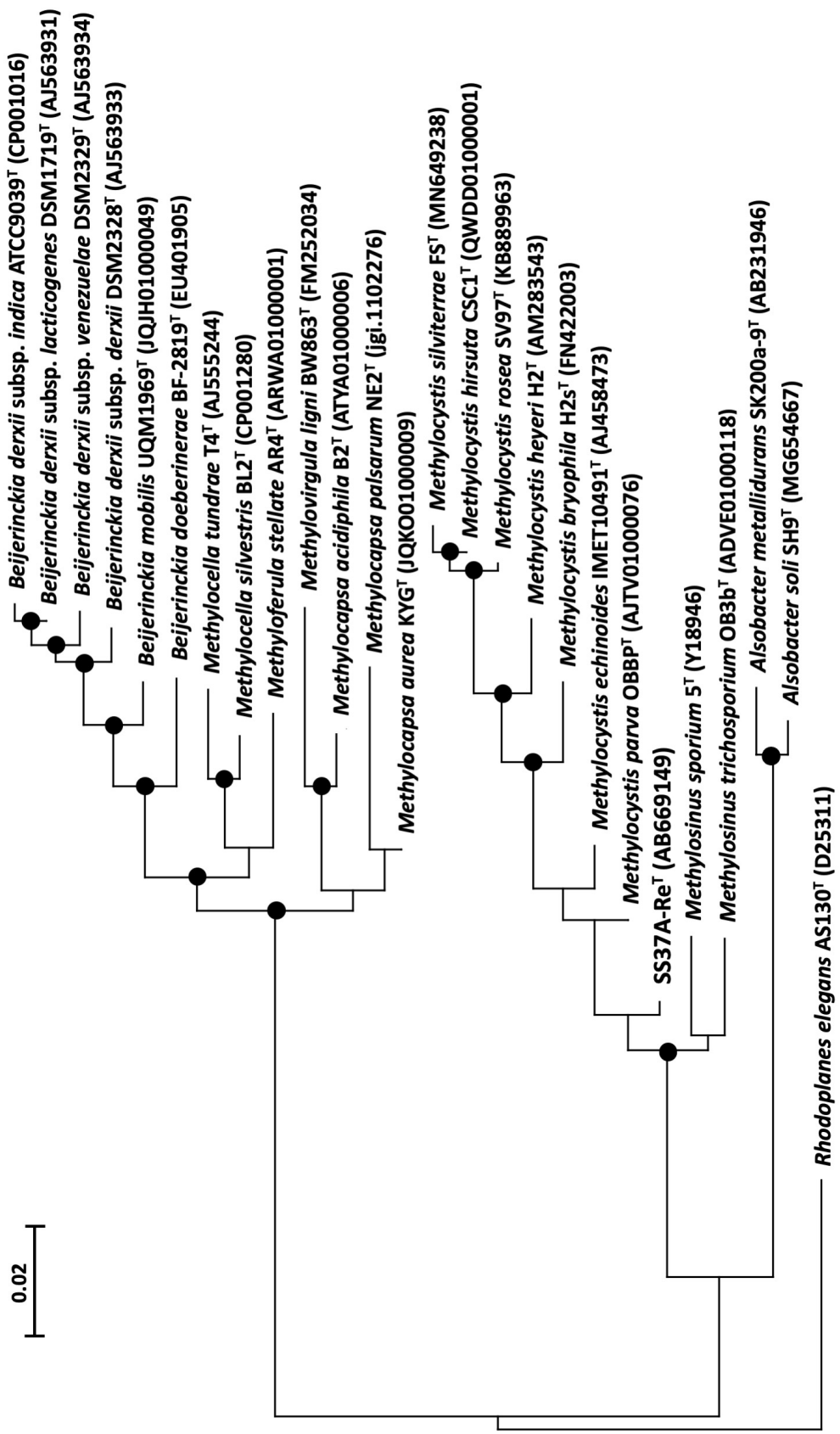
631 **Fig. S4.** Phylogenetic trees of *mmoX* gene sequences showing the relationships between
632 strain SS37A-Re^T and related bacteria by the maximum-likelihood (a), parsimony (b)

633 and distance (c) methods. Bars represent 0.1 (a) and 0.02 (b and c) substitutions per
634 nucleotide sequence position. Closed circles indicate internal nodes with at least 50%
635 bootstrap support from 1000 data resamplings. The tree was rooted using
636 *Methylomagnum ishizawai* RS11D-Pr^T as the outgroup. GenBank accession numbers
637 are given in parentheses.
638
639 **Fig. S5.** Phylogenetic trees of 16S rRNA gene sequences showing the relationships
640 between strain SS37A-Re^T and related *Methylocystis* and *Methylosinus* strains isolated
641 from various environments [41] by the maximum-likelihood (a), parsimony (b), and
642 distance (c) methods. Bars represent 0.02 (a), 0.01 (b) and 0.002 (c) substitutions per
643 nucleotide sequence position. Closed circles indicate internal nodes with at least 50%
644 bootstrap support from 1000 (a and c) or 100 (b) data resamplings. The tree was rooted
645 using *Methylocapsa palsarum* NE2^T as the outgroup. GenBank accession numbers are
646 given in parentheses.
647
648 **Fig. S6.** Phylogenetic trees of *pmoA* gene sequences showing the relationships between
649 strain SS37A-Re^T and related *Methylocystis* and *Methylosinus* strains isolated from
650 various environments [41] by the maximum-likelihood (a), parsimony (b), and distance
651 (c) methods. Bars represent 0.01 (a) and 0.02 (b and c) substitutions per nucleotide
652 sequence position. Closed circles indicate internal nodes with at least 50% bootstrap
653 support from 1000 (a and c) or 100 (b) data resamplings. The tree was rooted using
654 *Methylomonas koyamae* Fw12E-Y^T as the outgroup. GenBank accession numbers are
655 given in parentheses.
656
657 **Fig. S7.** Phylogenetic trees of *mxaF* gene sequences showing the relationships between
658 strain SS37A-Re^T and related *Methylocystis* and *Methylosinus* strains isolated from
659 various environments [41] by the maximum-likelihood (a), parsimony (b), and distance

(c) methods. Bars represent 0.05 (a and b) and 0.02 (c) substitutions per nucleotide sequence position. Closed circles indicate internal nodes with at least 50% bootstrap support from 1000 (a and c) or 100 (b) data resamplings. The tree was rooted using *Methylomonas koyamae* Fw12E-Y^T as the outgroup. GenBank accession numbers are given in parentheses.

Fig. S8. Phylogenetic trees of *mmoX* gene sequences showing the relationships between strain SS37A-Re^T and related *Methylocystis* and *Methylosinus* strains isolated from various environments [41] by the maximum-likelihood (a), parsimony (b), and distance (c) methods. Bars represent 0.1 (a), 0.02 (b) and 0.01 (c) substitutions per nucleotide sequence position. Closed circles indicate internal nodes with at least 50% bootstrap support from 1000 (a and c) or 100 (b) data resamplings. The tree was rooted using *Methylomagnum ishizawai* RS11D-Pr^T as the outgroup. GenBank accession numbers are given in parentheses.

Fig. S9. (a) Total ion current chromatogram of dimethyl disulphide adducts from strain SS37A-Re^T monounsaturated fatty acids. Mass spectra of dimethyl disulphide adducts of $C_{18:1}$ fatty acids eluted at the retention times 10.23 min (b) and 10.44 min (c), respectively, on the chromatogram (a). Ions at *m*/*z* 159, 231, and 390 correspond to ω-fragment, Δ-fragment, and M+ of the dimethyl disulphide adduct of $C_{18:1}\omega 8$ [54].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Beijerinckia doeberinerae BF-2819T (EU401905)
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Methyloferula stellate AR4T (ARWA01000001)
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Methylocapsa acidiphila B2T (ATYA01000006)
Methylocapsa palsarum NE2T (jgi.1102276)
Methylocapsa aurea KYGT (JQKO01000009)
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Methylocystis hirsuta CSC1T (QWDD01000001)
Methylocystis rosea SV97T (KB889963)
Methylocystis heyeri H2T (AM283543)
Methylocystis bryophila H2sT (FN422003)
Methylocystis echinoides IMET10491T (AJ458473)
Methylocystis parva OBBPT (AJTV01000076)
SS37A-ReT (AB669149)
Methylosinus sporium 5T (Y18946)
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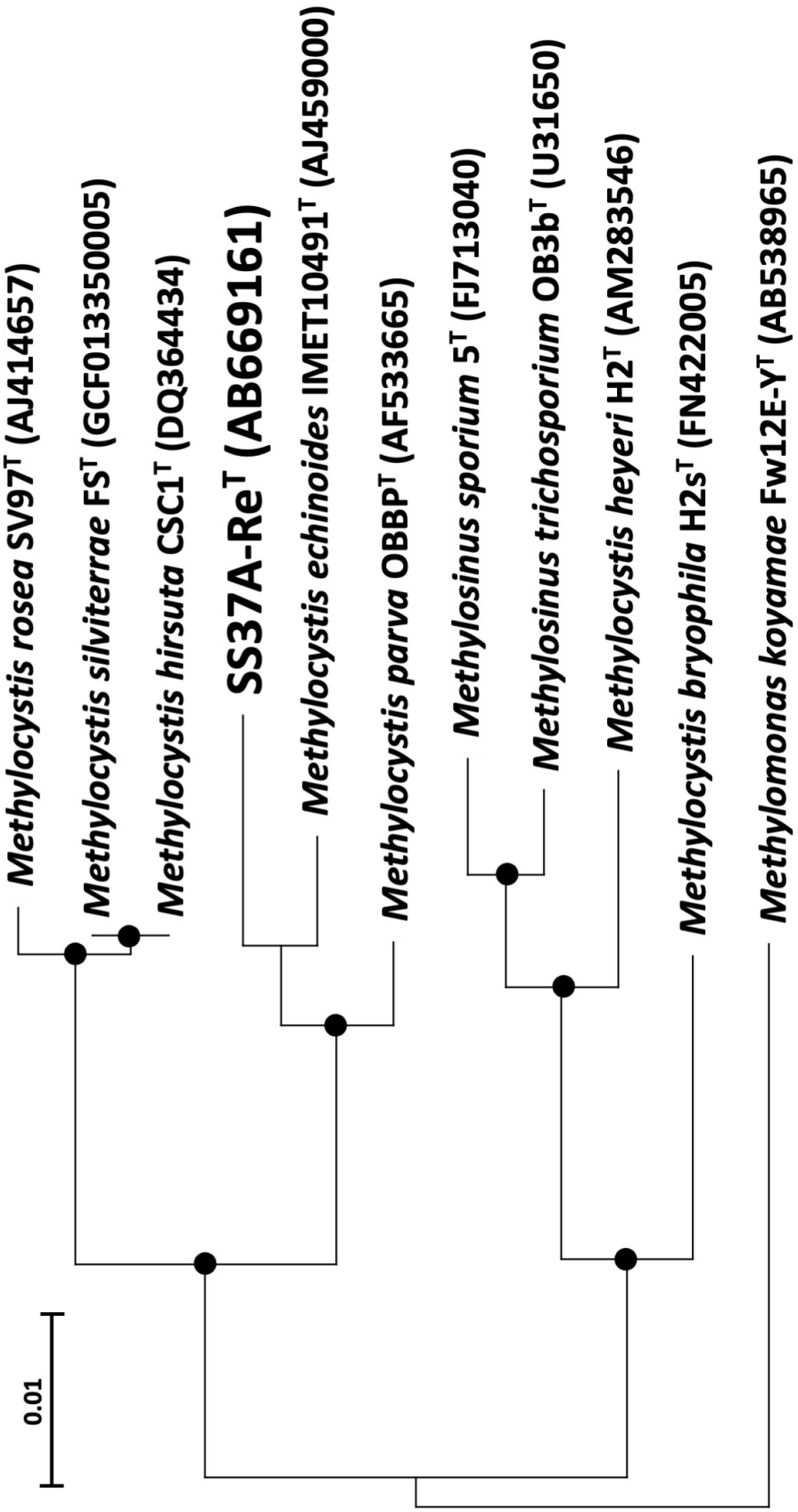
Figure 1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Methylocystis heyeri H2T (AM283546)
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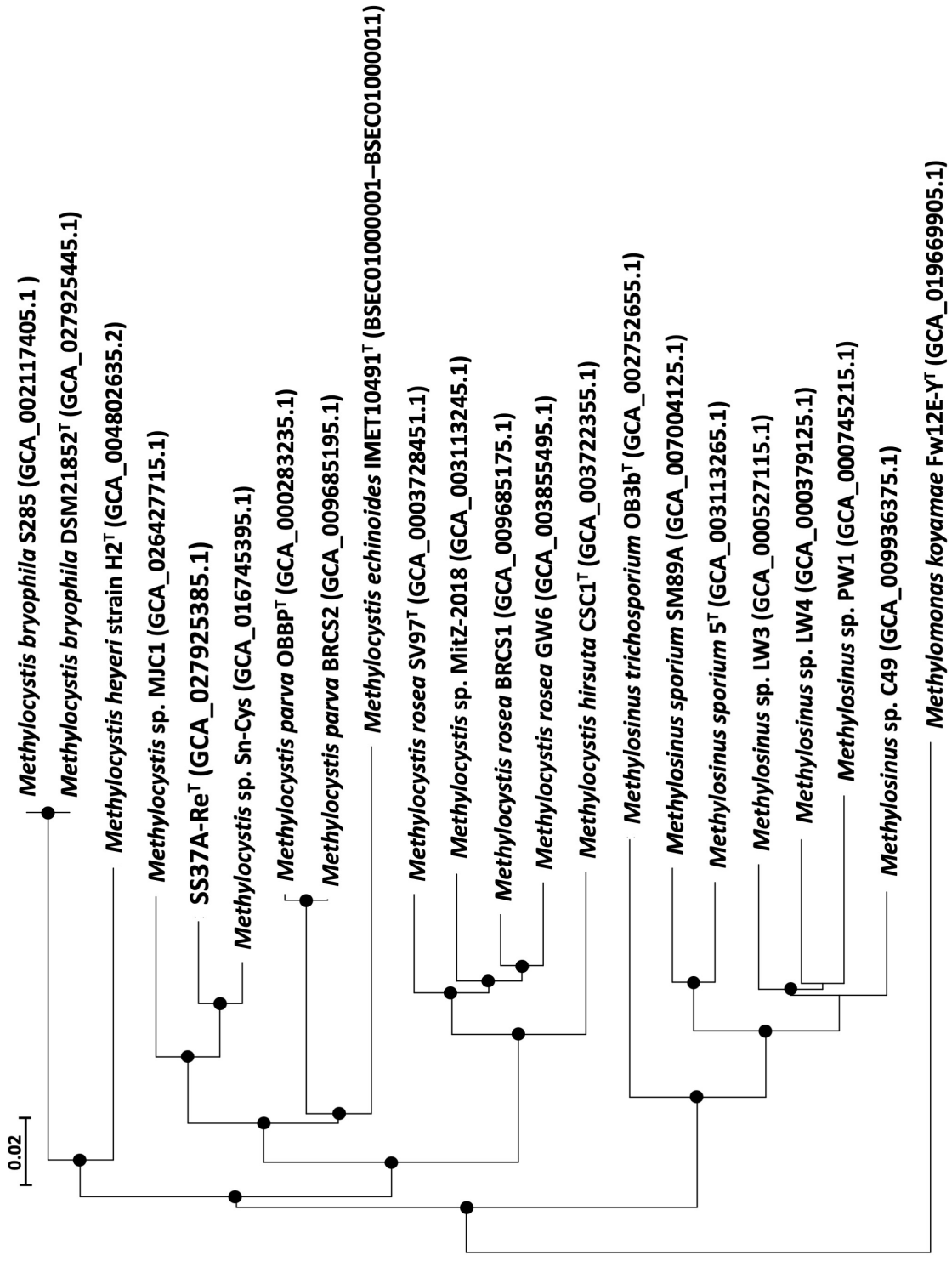
0.01

Figure 2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SS37A-ReT (GCA_027925385.1)
Methylocystis sp. Sn-Cys (GCA_016745395.1)
Methylocystis parva OBBPT (GCA_000283235.1)
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Methylocystis echinoides IMET10491T (BSEC01000001–BSEC01000011)
Methylocystis rosea SV97T (GCA_000372845 1.1)
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Methylosinus sp. LW4 (GCA_000379125.1)
Methylosinus sp. PW1 (GCA_000745215.1)
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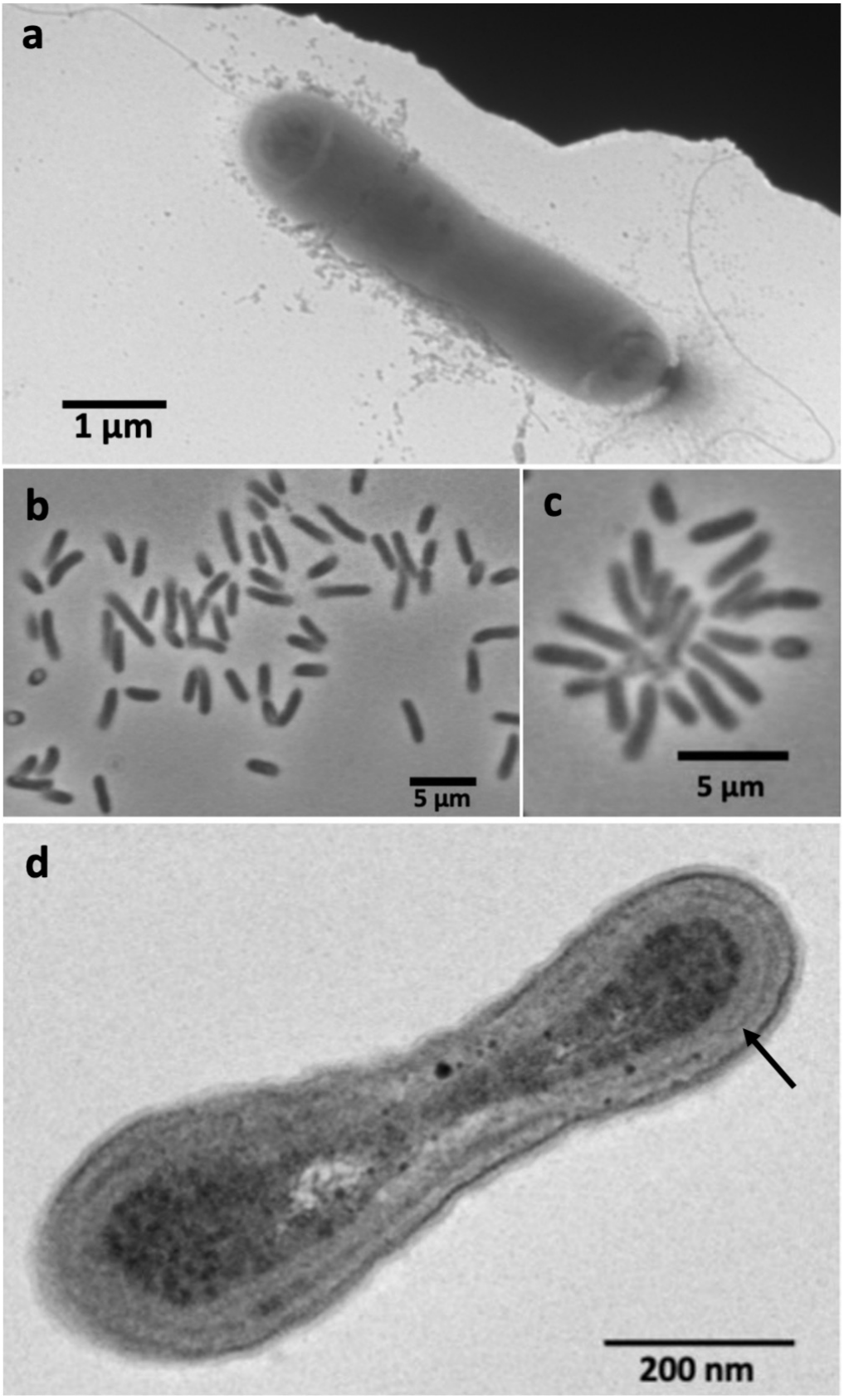
Figure 3



690

691 Figure 4