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Dyadobacter subterraneus sp. nov., isolated from hydrocarbon polluted groundwater from an oil refinery of Hungary

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The DDBJ/ENA/GenBank accession numbers for the 16S rRNA gene sequence and the whole genome sequence of strain UP-52^T are MN165456 and JACYGY000000000, respectively.

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4 **ABSTRACT**

Gram-stain-negative, aerobic, non-spore-forming, rod-shaped UP-52^T bacterial strain was isolated from hydrocarbon polluted groundwater located in the industrial zone of the oil refinery near to Tiszaujvaros, Hungary. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolate belongs to the genus *Dyadobacter* in the family *Cytophagaceae*. Its closely related species are *Dyadobacter frigoris* (98.00 %), *Dyadobacter koreensis* (97.64%), *Dyadobacter psychrophilus* (97.57%), *Dyadobacter ginsengisoli* (97.56%), and *Dyadobacter psychrotolerans* (97.20%). The predominant fatty acids are summed feature 3 ($C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω7c/ C16:1 w6c*), $C_{15:0}$ iso, $C_{16:1}$ *ω5c,* $C_{17:0}$ iso 3OH. The predominant respiratory quinone detected in strain UP-52^T is quinone MK7, dominant polar lipids are glycolipid, phosphoaminolipid, phospholipid and aminolipid. The DNA G+C content is 40.0 %, flexirubin-type pigment was present Based on the these phenotypic, chemotaxonomic, and phylogenetic analysis, UP-52^T represents a novel species of the genus, the name *Dyadobacter subterraneus.* sp. nov. is proposed. The type strain is UP-52^T (= NCAIM B.02653 = CCM 9030).

The genus *Dyadobacter* was first described by Chelius and Triplett, the type species is *Dyadobacter fermentans*, it belongs to family *Cytophagaceae* and order *Flavobacteriales* [1]. At the time of writing, 23 species were described in the genus *Dyadobacter* which all have valid published names except one (*Dyadobacter luteus)* https://www.namesforlife.com/10.1601/tx.8294).

Members of *Dyadobacter* have been isolated from various sources such as plant, seawater, soil, sediments, glacial samples, desert sands. Members of the *Dyadobacter* genus are aerobic, yellow pigmented, catalase and oxidase positive, Gram-stain-negative, rods and non motile, occur in pairs in young cultures and in chains in older cultures, The major respiratory quinone is MK-7, the major polar lipd is phosphatidylethanolamine, summed feature 3

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($C_{16:1}0\omega 7c$ and/or $C_{16:1}$ $\omega 6c$), iso-$C_{15:0}$, $C_{16:1}$ $\omega 5c$ and iso-$C_{17:0}$ 3-OH are the major fatty acids, *Dyadobacter* species have G+C content ranging from 44.0 to 51.3 mol%. Having the flexirubin-type pigment is the main characteristic feature of genus *Dyadobacter* [2].

Isolation and Ecology

In May 2018, hydrocarbon-polluted groundwater was sampled via a monitoring well, used for site remediation of a Hungarian oil refinery located near to Tiszaujvaros, Hungary (47°54' 20" N 21° 2' 29" E). The well had been sampled with sterile tools and the sample was kept in a sterile container and transferred to the laboratory. Strain UP-52^{T} was isolated by diluting the water sample up to 10^{-6} using dilution water. 1 ml from each of the initial water sample and the serial dilutions was plated on TGY-5 agar plates (pour plate method) [3]. After incubation at 28°C for 72h, selected colonies were purified again on TGY-5 and incubated under the same conditions.

16S RNA phylogeny

Genomic DNA was extracted by using the UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories, USA). Subsequently the 16S rRNA gene was amplified with 27F and 1492R primers [4]. Amplification was performed by using Eppendorf Mastercycler (Eppendorf, Germany). PCR products were purified with NucleoSpin® Gel and PCR Clean Up Kit by the manufacturer's (Macherey-Nagel GmbH, Germany) instructions. The almost complete 16S rRNA gene sequence of the strain was determined by using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing products were separated on a Model 3130 Genetic Analyzer (Applied Biosystems, USA). The 16S rRNA gene sequence of strain UP-52^{T} was compared to the type strains of closely related members of the genus *Dyadobacter* obtained from GenBank [5]. Multiple alignments of 16S rRNA gene sequences were made with CLUSTAL_X [6] . The 16S rRNA sequence based phylogenetic trees were constructed using the maximum-likelihood [7] and neighbour-joining [8] methods with Kimura's two-

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55 parameter calculation model and the maximum-parsimony algorithm [9] using MEGA X
56 10.0.5 [10] . Tree topologies and distances were evaluated by bootstrap analysis based on 1000
57 replicates.

58 The 16S rRNA gene sequence of strain UP-52^T determined in this study was a continuous
59 stretch of 1509 bp (positions 50–1458 with respect to the *Escherichia coli* numbering system).
60 Sequence similarity calculations using the EzTaxon server (http://www.eztaxon.org/) indicated
61 that strain UP-52^T was closely related to *D. frigoris* (98.04% sequence similarity), *D. koreensis*
62 (97.69%), *D. psychrophilus* (97.62%), *D. ginsengisoli* (97.62%), and *Dyadobacter*
63 *psychrotolerans* (97.20%) . Similar 16S rRNA gene sequences were isolated from soil in korea
64 (strain DD-d2, percent identity 99.71%) and from skin microbiome (strain ncd252c07c1,
65 percent identity 99.92%) Moreover, on the basis of the 16S rRNA gene sequence analysis,
66 the phylogenetic position of strain UP-52^T among the other members of genus *Dyadobacter* is
67 unique and distinct (Fig. 1.). The overall topology of the maximum-likelihood tree was similar
68 to that of the neighbour-joining and maximum parsimony trees. The whole genome-based
69 phylogeny has also shown that the strain UP-52^T is a potential new bacterial species which
70 doesn't belong to any species found in TYGS database, However, *D. frigoris* is the closest
71 relative (Fig.2.).

72 **Genome analysis**

73 Whole genome sequencing including G+C determination was carried out in our partner's
74 (SeqOmics Biotechnology Ltd.) molecular laboratory in Morahalom, Hungary. The whole
75 genome sequencing of UP-52^T was conducted based on procedure described by Borsodi et
76 al.[11], mate-paired libraries were generated using Nextera Mate Pair Sample Preparation Kit
77 (Illumina, USA) according to manufacturer protocol of gelplus version after minor
78 modifications for production of a robust smear within the 7-11 kbp region 13 µl of Mate-Paired

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Tagment Enzyme was used. Zymoclean Large Fragment DNA Recovery kit (Zymo Research, USA) was used to excise the 7-11 kbp DNA fraction from the gel, then the the circularized DNA was sheared using Covaris S2. The quality measurements were conducted using TapeStation 2200 instrument (Agilent, USA). Qubit (ThermoFisher, USA) was used to quantify the final libraries, which were sequenced on an Illumina MiSeq instrument using MiSeq Reagent Kit v2 (500 cycles) sequencing chemistry. De novo assembly and scaffolding were performed with CLC Genomics Workbench Tool v11 (Qiagen, Germany).

Automatic annotation of the genome was performed by the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAP) v4.5 [12], the genome was also annotated using Rapid Annotation using subsystem technology (RAST; https://rast.nmpdr.org) server [13]. For the identification of biosynthetic gene cluster (BGCs) encoding different secondary metabolites, anti-SMASH server was used [14]. For the whole genome-based phylogeny, type strain genome server (TYGS) was used [15]. Digital DNA–DNA hybridization values (dDDH) among strain UP-52^{T} and related species were determined using the Genome-to-Genome Distance Calculator (GGDC, https://ggdc.dsmz.de/) version 2.1. [16]. For the calculation of orthologous average nucleotide identity (OrthoANI) values between strain UP-52^{T} and its closest relatives, OAT software was used [17].

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Genomic characteristics

The whole genome sequence of strain UP-52^{T} comprised 20 scaffolds (N50 = 6 070 096 bp) and 21 contigs, total genome size is 7 787 995 bp, the total number of genes is 6 590 and 6 438 coding genes, a total of 56 RNA genes, 42 tRNA genes are found in the genome. Furthermore, the sequence coverage is 64.0-fold, the DNA G+C content of strain UP-52^{T} was 40.0%. The dDDH, OrthoANI values between strain UP-52^{T} and the closely related *Dyadobacter* species *D. frigoris* were 29.3, 84.97 respectively. The OrthoANI, dDDH values for other *Dyadobacter*

103 relatives are shown in Table 1. the ANI as well as dDDH values were much lower than the
104 threshold values of DDH (70%) and ANI (95%) to discriminate bacterial species. Rast analysis
105 showed that 263 subsystems are present, 14 metabolism of aromatic compounds, 10 secondary
106 metabolisms (lanthionine synthetases, auxin biosynthesis). Genome of strain UP-52^T also
107 contains number of antibiotic resistance genes such as Fluoroquinolone, Beta lactam antibiotics
108 (Penicillin).

109 Regarding hydrocarbon degradation, after thorough manual curation of the annotated genome
110 (by CLC Genomics Workbench) we could not identify any specific gene or gene cluster, which
111 could be linked to the degradation of aliphatic or aromatic hydrocarbons. Accordingly, strain
112 UP-52^T most probably does not play role in the degradation of petroleum hydrocarbons,
113 although it was isolated from a hydrocarbon containing environment. These results were also
114 confirmed phenotypically by gravimetric hydrocarbon degradation method; there was no
115 significant difference in hydrocarbon concentration between the uninoculated (control) sample
116 comparing to UP-52^T inoculated sample after shaking in room temperature for 10 days in liquid
117 media supplemented with no additives containing gasoline.

118 Antismash analysis revealed the presence of six BGCs (Ribosomally synthesized and post-
119 translationally modified peptides (RiPP-like), RiPP recognition element (RRE-containing),
120 Type III polyketide synthases (T3PKS) an enzyme complex that produce a class of secondary
121 metabolites called polyketides, many of polyketides are clinically important like antimicrobial
122 and anticancer polyketides [18], Terpenes which are large hydrocarbon groups that consist of
123 5-carbon isoprene (C5H8) units as their basic building block were also present in UP-52^T
124 genome, terpenes are phytochemical with a promising antimicrobial properties [19]. The
125 presence of aryl polyene genes was also revealed; a yellow pigment embedded in bacterial
126 membrane serving as protection against oxidative stress or reactive oxygen species, it is
127 abundant in in many multi-drug resistant pathogen [20], resorcinol gene cluster was also

present in UP-52^T genome, dialkylresorcinol (DAR) is needed for the production of flexirubin pigment that is produced by all *Dyadobacter* species including UP-52^T [21].

RAST results have shown the occurrence of antibiotic resistance genes, therefore, we have used the minimum inhibitory concentration (MiC) technique as per manufacturer instruction (Liofilchem strips) to confirm RAST results.

Physiology and Chemotaxonomy

Electron-microscopic morphology was made from 48-hours old cultures grown in tryptic soy broth (TSB) at 28°C. The cell morphology of strain UP-52^T was investigated during the exponential growth phase using transmission electron microscopy (H-7100; Hitachi) by applying the shadow-casting technique described by Ohad et al.[22]. Electron-microscopic morphology of strain UP-52^T is shown in Supplementary figure 1. The rod-shaped cells are about 2 µm long and 0.8 µm wide. The surface of the cells is totally smooth with no flagella. Colony morphology on R2A agar after 96h was large (3-4 mm), yellow, circular, raised, translucent, moist and smooth.

Carbon-source utilization and enzyme activities were tested by using API 20, API 20NE, and API ZYM test kits (bioMérieux, France) according to the manufacturer's instructions. All API tests were carried out for strain UP-52^T in parallel with *D. koreensis, D. psychrophilus,* and *D. ginsengisoli.* The data were retrieved from the literature in the case of *D. frigoris* only [2].

Examination of oxidase test, catalase activity were fulfilled by the methods from Barrow and Feltham (1993) [23] verifying the API tests, Flexirubin-type pigment was examined using 20% (w/v) KOH solution. Gram-reaction was performed by using the nonstaining method, as described by Buck (1982) [24]. Growth at different temperatures (4, 10, 20, 28, 30, 32, 37ºC) and pH (pH 4.0–10.0, in increments of 1 pH units at 28 °C) was assessed after 10-day incubation in TSB. After autoclaving, TSB pH was controlled (S220 SevenCompact, Mettler

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Toledo) and adjusted by adding sterile solutions of HCl or NaOH (1 M each), the following buffers were used depending on the tested pH; citrate buffer (pH4-5) MES (pH6), MOPS (pH7), Tris (pH8), CHES (pH9), and CAPS buffer (pH10). Salt tolerance was tested after 10 days incubation in TSB supplemented with 0.0–5.0 % (w/v) NaCl (at 28 ºC). Growth on nutrient agar, trypticase soy agar (TSA), TGY-5, and R2A agar was also evaluated at 28ºC. other phenotypic differential features of novel strain with other closely related reference strains are summarized in (Table 2).

The optimal circumstances for the growth of strain UP-52^T was observed at 28°C on R2A agar. Other physiological characteristics of strain UP-52^T are summarized in the species description. Analyses of respiratory quinones, fatty acids, and polar lipids were conducted by the Identification Service of Leibniz Institute - DSMZ German Collection of Microorganisms and Cell Cultures GmbH. Cellular fatty acids were analysed after conversion into fatty acid methyl esters by saponification, methtlation and extraction with minor modification of method described by Miller (1982) and Kuykendall et al., (1988). The fatty acid methyl esters mixtures were separated by gas chromatography and detected by a flame ionization detector using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.). Peaks are automatically integrated and fatty acid names and percentages calculated by the MIS Standard Software (Microbial ID), followed by identification by TSBA40 and TSBA6 methods [25-26]. 200 mg of freeze dried cell material were used to extract respiratory quinones and polar lipids using the two stage method described by Tindall [27-28].

The major respiratory quinone was MK 7, the polar lipid profile consist of glycolipids (GL), phospholipid (PL), aminolipid (AL), phosphoaminolipid (PAL), lipid (L), and phosphatidylethanolamine (PE) (Supplementary figure 2), whereas the polar lipids of the closely related D. frigoris consisted of a major polar lipid phosphatidylethanolamine (PE), one unknown phospholipid (PL) and two kind of unknown aminolipids (AL) [2]. The major cellular

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177 fatty acids (>5% of total fatty acids) of strain UP-52^T were summed feature 3 ($C_{16:1}$ $\omega 7c$/ iso-
178 $C_{15:0}$ 2-OH/ $C_{16:1}$ $\omega 6c$) (40.1%), iso-$C_{15:0}$ (19.8%), $C_{16:1}$ $\omega 5c$ (18.4%) and iso-$C_{17:0}$ 3-OH
179 (8.6%). Differences in the proportions of these major fatty acids were observed when compared
180 to the closest relatives [2], [29-30], fatty acid C15:1 G iso was detected in *D. frigoris* but not
181 in strain UP-52^T (Table 3). This finding also confirms that strain UP-52^T differs at the species
182 level from other *Dyadobacter* genus members.

183 Antibiotic resistance was determined with Liofilchem MTS (MIC Test Strips) according to the
184 instructions of the manufacturer. Minimal Inhibitory Concentrations (MIC values) of the
185 chosen antibiotics (ceftazidime, cefepime, ceftriaxone, imipenem, meropenem, piperacillin,
186 gentamicin, tigecycline, ciprofloxacin, colistin, levofloxacin) were detected on TGY-5 agar
187 plates with incubation at 28°C for 48h. According to the breakpoint interpretation of EUCAST
188 (www.eucast.org), UP-52^T is resistant to ceftazidime (>256 μg ml^{-1}), cefepime (>256 μg ml^{-1}),
189 ciprofloxacin (>32 μg ml^{-1}), colistin (8 μg ml^{-1}), gentamicin (12 μg ml ml^{-1}), levofloxacin (8
190 μg ml^{-1}) and sensitive to ceftriaxone (3 μg ml^{-1}), imipenem (0.094 μg ml^{-1}), meropenem (0.38
191 μg ml^{-1}) piperacillin (6.0 μg ml^{-1}) and tigecycline (0.25 μg ml^{-1}), in summery, it is resistance
192 to 6 of the examined 11 antibiotics. These results are in accordance with the genotypic findings
193 of RAST mentioned above that the genome of strain UP-52^T contains fluoroquinolone
194 resistance genes and Beta-lactamase coded genes. In addition to that the known genome of all
195 closest relatives of UP-52^T contain these genes.

196 Considering that 98.65% is the threshold for differentiating two species [31], and based on the
197 16S rRNA gene sequence similarities between strain UP-52^T and its closely related *D. frigoris*,
198 in addition to the results of the biochemical, genomic, physiological, and chemotaxonomic
199 analysis, strain UP-52^T is considered to represent a novel species within the genus *Dyadobacter*
200 for which the name *Dyadobacter subterraneus* sp. nov. is proposed.

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Description *of Dyadobacter subterraneus* sp. nov.

Dyadobacter subterraneus (sub.ter.ra'ne.us. L. masc. adj. *subterraneus* underground).

Cells are Gram-negative, obligate aerobe, non-spore-forming, non motile, rods and approximately 2 µm long and 0.8 µm wide in size. Colonies grown on R2A agar plates, after 96h and 28 ºC of incubation, were 3-4mm, yellow, circular, raised, translucent, moist, and smooth. Well performed growth of colonies was also observed on TSA, R2A, and TGY-5, on nutrient agar the growth was weak. Growth was observed at temperatures between 5-32 ºC with optimum at 28 ºC, growth was observed at pH 5-8 with optimum at pH 7, and at concentration of NaCl below 1% (w/v).

Strain UP-52^T is catalase and oxidase positive, The results of API 20E, 20NE, and API ZYM showed that strain UP-52^T is positive for, alkaline phosphatase, α-Chymotrypsin, acid phosphatase, trypsin, Leucine arylamidase, *β*-glucosidase, estrerase (C4), esterase lipase (C8) *β*-galactosidase, *β*-glucosidase, and esculine ferric citrate and negative reactions were observed for nitrate reduction, glucose fermentation, arabinose, mannitol, sorbitol, indol, urease, *β*-glucuronidase.

The major fatty acids are summed feature 3 ($C_{16:1}$ *ω*7*c*/ iso-$C_{15:0}$ 2-OH/ $C_{16:1}$ *ω*6*c*), iso-$C_{15:0}$, $C_{16:1}$ *ω*5*c*, and iso-$C_{17:0}$ 3-OH, Quinone (MK7) is the predominant (100 %) respiratory quinine. The polar lipids consist of glycolipids (GL), phospholipid (PL), aminolipid (AL), phosphoaminolipid (PAL), lipid (L), and phosphatidylethanolamine (PE). The DNA G+C content of the type strain is 40.0 %. Strain UP-52^T (= NCAIM B.02653 = CCM 9030) was isolated from hydrocarbon polluted ground water sample located near oil refinery in Tiszaujvaros, Hungary.

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223 This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the
224 accession JACYGY000000000. The version described in this paper is version
225 JACYGY000000000.1, the accession number for the 16S rRNA gene sequence is MN165456.

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227 # Author Statements

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228 **Conflicts of interest**

229 The authors declare that there are no conflicts of interest

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235 **ABBREVIATIONS**

236 ANI, averagenucleotide identity; dDDH, In silico DNA-DNA hybridization; NCAIM, National
237 Collection of Agricultural and Industrial Microorganisms (Hungary); CCM, Czech Collection of
238 Microorganisms.

239 # REFERENCES

240 1. **Chelius MK, Triplett EW.** *Dyadobacter fermentans* gen. nov., sp. nov., a novel
241 Gram-negative bacterium isolated from surface-sterilized Zea mays stems. *Int J Syst*
242 *Evol Microbiol.* 2000;50(2):751–8.

CONFIDENTIAL

243 2. **Chaudhary DK, Dahal RH, Kim J.** *Dyadobacter psychrotolerans* sp. Nov. and
244 dyadobacter frigoris sp. nov., two novel psychrotolerant members of the family
245 cytophagaceae isolated from arctic soil. *Int J Syst Evol Microbiol.* 2020;70(1):569–75.

246 3. **Szabó I, Szoboszlay S, Táncsics A, Szerdahelyi SG, Szűcs, Radó J, et al.** Taibaiella
247 coffeisoli sp. nov., isolated from the soil of a coffee plantation. *Int J Syst Evol*
248 *Microbiol.* 2016;66(4):1627–32.

249 4. **Lane D.** 16S/23S rRNA Sequencing. In: Stackebrandt, E. and Goodfellow, M., Eds.,
250 Nucleic Acid Techniques in Bacterial Systematics. new york: John Wiley and Sons;
251 1991. 115–175 p.

252 5. **Kim O-S, Cho Y-J, Lee K, Yoon S-H, Kim M, Na H, et al.** Introducing EzTaxon-e:
253 a prokaryotic 16S rRNA gene sequence database with phylotypes that represent
254 uncultured species. Int J Syst Evol Microbiol 2012;62:716–21.

255 6. **Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG.** The
256 CLUSTAL _ X windows interface : flexible strategies for multiple sequence alignment
257 aided by quality analysis tools. *Nucleic Acids Res.* 1997;25(24):4876–82.

258 7. **Felsenstein J.** Evolutionary Trees from DNA Sequences : A Maximum Likelihood
259 Approach. *J Mol Evol.* 1981;17:368–76.

260 8. **Saitou N, Nei M.** The Neighbor-joining Method : A New Method for Reconstructing
261 Phylogenetic Trees '. *Mol Biol Evol.* 1987;4(4):406–25.

262 9. **Kimura M.** A simple method for estimating evolutionary rates of base substitutions
263 through comparative studies of nucleotide sequences. *J Mol Evol* 1980 Jun 16(2):111–
264 20.

265 10. **Kumar S, Stecher G, Li M, Knyaz C, Tamura K.** MEGA X: Molecular evolutionary
266 genetics analysis across computing platforms. *Mol Biol Evol.* 2018;35(6):1547–9.

267 11. **Borsodi AK, Aszalós JM, Bihari P, Nagy I, Schumann P, Spröer C, et al.**
268 *Anaerobacillus alkaliphilus* sp. Nov., a novel alkaliphilic and moderately halophilic
269 bacterium. *Int J Syst Evol Microbiol.* 2019;69(3):631–7.

270 12. **Tatusova T, Dicuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L,**
271 **et al.** NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res* 2016
272 ;44(14):6614–24.

273 13. **Aziz RK, Bartels D, Best AA, Dejongh M, Disz T, Edwards RA, et al.** The RAST
274 Server: Rapid Annotations using Subsystems Technology. 2008 1471-2164/9/75

275 14. **Blin K, Shaw S, Steinke K, Villebro R, Ziemert N, Lee SY, et al.** AntiSMASH 5.0:
276 Updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Res.*
277 2019;47(W1):W81–7.

278 15. **Meier-Kolthoff JP, Göker M.** TYGS is an automated high-throughput platform for
279 state-of-the-art genome-based taxonomy. *Nat Commun.* 2019 Dec 1;10(1).

280 16. **Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M.** Genome sequence-based
281 species delimitation with confidence intervals and improved distance functions. *BMC*
282 *Bioinformatics*. 2013;14(1):60.

283 17. **Lee I, Ouk Kim Y, Park S-C, Chun J.** OrthoANI: An improved algorithm and
284 software for calculating average nucleotide identity. *Int J Syst Evol Microbiol*. 2016

CONFIDENTIAL

285 Feb;66(2):1100–3.

286 18. **Richardson M, Khosla C.** Structure, Function, and Engineering of Bacterial Aromatic
287 Polyketide Synthases. In: *Comprehensive Natural Products Chemistry*. Elsevier; 1999.
288 p. 473–94.

289 19. **Mahizan NA, Yang S, Moo C-L, Song AA-L.** Terpene Derivatives as a Potential
290 Agent against. Molecules. 2019;24(2631):1–21.

291 20. **Grammbitter GLC, Schmalhofer M, Karimi K, Shi YM, Schöner TA, Tobias NJ,**
292 **et al.** An Uncommon Type II PKS Catalyzes Biosynthesis of Aryl Polyene Pigments. *J*
293 *Am Chem Soc.* 2019 ;141(42):16615–23.

294 21. **Nowak-Thompson B, Hammer PE, Hill DS, Stafford J, Torkewitz N, Gaffney TD,**
295 **et al.** 2,5-Dialkylresorcinol biosynthesis in *Pseudomonas aurantiaca*: Novel head-to-
296 head condensation of two fatty acid-derived precursors. *J Bacteriol* . 2003 185(3)
297 PMC142816/

298 22. **Ohad I, Danon D.** The use of Shadow-Casting technique for measurement of the
299 width of elongated particles. *J Cell Biol.* 1963;17(7):321–6.

300 23. **Barrow, J.I & Feltham RKA.** Cowan and Steel's Manual for the Identification of
301 Medical Bacteria. *J Clin Pathol.* 1993;46(10).

302 24. **Buck JD.** Nonstaining (KOH) Method for Determination of Gram Reactions of
303 Marine Bacteriat. *Appl Environ Microbiol.* 1982;44(4):992–3.

304 25. **Kuykendall LD, Roy MA, O'Neill JJ, Devine TE.** Fatty acids, antibiotic resistance,
305 and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. *Int J Syst*
306 *Bacteriol.* 1988;38(4):358–61.

307 26. **Miller LT.** Single Derivatization Method for Routine Analysis of Bacterial Whole-
308 Cell Fatty Acid Methyl Esters, Including Hydroxy Acids. *J Clin Microbiol.*
309 1982;16(3):584–6.

310 27. **Tindall BJ.** Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol*
311 *Lett.* 1990;66(1–3):199–202.

312 28. **Brian J. Tindall, Johannes Sikorski, Robert A Smibert NRK.** Phenotypic
313 Characterization and the Principles of Comparative Systematics. In: *Methods for*
314 *General and Molecular Microbiology.* third edit. Washington DC: ASM Press; 2007.
315 p. 330–93.

316 29. **Baik KS, Kim MS, Kim EM, Kim HR, Seong CN.** *Dyadobacter koreensis* sp. nov.,
317 isolated from fresh water. *Int J Syst Evol Microbiol.* 2007;57(6):1227–31.

318 30. **Liu QM, Im WT, Lee M, Yang DC, Lee ST.** *Dyadobacter ginsengisoli* sp. nov.,
319 isolated from soil of a ginseng field. *Int J Syst Evol Microbiol.* 2006;56(8):1939–44.

320 31. **Kim M, Oh HS, Park SC, Chun J.** Towards a taxonomic coherence between average
321 nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of
322 prokaryotes. *Int J Syst Evol Microbiol.* 2014;64(PART 2):346–51.

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323 # FIGURES AND TABLES

324 **Fig.1** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the
325 phylogenetic positions of strain UP-52^T and related species.

326 **Fig.2** Genome- based phylogenetic tree showing the phylogenetic position of strain UP-52^T and related
327 species.

328 **Table 1.** Average nuclutide Identity (ANI) and digital DNA–DNA hybridisation (dDDH) values between strain
329 UP-52^T and closest *Dyadobacter* relatives.

330 **Table 2.** Characteristics that differentiate strain UP-52^T from the closest relatives of *Dyadobacter*

331 **Table 3.** Cellular fatty acids compositions by TSBA 40 method of strain UP-52^T and the closest relatives.

332 **Supplementary Figure 1:** Shadow casting electron microscopic images of strain UP-52^T

333 **Supplementary Figure 2:** Two-dimensional TLC polar lipid images of strain UP-52^T

334 **Supplementary Figure 3:** Two-dimensional TLC polar lipid images of strain *Dyadobacter frigoris* AR-3-
335 8^T

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337 **Table.1** Average nucleotide identity (ANI) and digital DNA–DNA hybridisation (dDDH) values between strain
338 UP-52^T and closest *Dyadobacter* relatives.

Strain	UP-52^T	
	ANI (%)	dDDH (%)
D. frigoris AR-3-8^T	85.20	29.3
D. koreensis KCTC 12537^T	77.60	21.7
D. psychrophilus BZ26^T	71.92	18.3
D. psychrotolerans AR-3-6^T	73.16	19.4

339 The whole genomes for *D. hamtensis* and *D. ginsengisoli* are not available

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340 **Table.2** Characteristics that differentiate strain UP-52^T from the closest relatives of *Dyadobacter* (n.a: not
341 available) Strains: 1, UP-52^T; 2, *D. frigoris* AR-3-8^T; 3, *D. koreensis* KCTC 12537^T ; 4, *D. psychrophilus* BZ26^T
342 ; 5, *D. ginsengisoli* Gsoil 043^T; 6, *D. psychrotolerans* AR-3-6^T. The results for all strains were taken from this
343 study except AR-3-8^T(2) and AR-3-6^T (2)

Characteristic	**Strain**					
	1	**2**	**3**	**4**	**5**	**6**
Growth temperature	4-32	0-30	4-30	1-30	4-30	0-30
Maximum tolerance in	1%	2%	1%	1%	1%	2%
pH range	5-8	6-10.5	5-11	6-8	5.5-8.5	6-10.5
β-Galactosidase	+	+	+	+	n.a	+
Gelatin hydrolysis	+	n.a	-	delayed	-	n.a
Nitrate reduction	-	-	-	-	-	-
Indole production	-	-	-	+	-	-
D-Glucose fermentation	-	-	-	-	-	-
Urease production	-	-	-	+	-	-
Aesculin hydrolysis	+	+	+	delayed	-	+
D-Mannitol utilization	-	+	-	-	-	-
α-Glucosidase activity	+	+	n.a	+	n.a	+
Lipase (C14) activity	-	+	-	-	-	+
L-Arabinose utilization	-	+	+	-	-	-
Alkaline phosphatase	+	+	+	+	n.a	+
Esterase (C4) activity	+	weak	n.a	+	n.a	weak
Esterase lipase (C8)	+	weak	n.a	+	n.a	weak
L-Rhamnose utilization	-	+	weak	-	+	-
DNA G+C content (%)	40	40.1	44.0	48.9	48	42.1

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344 **Table 3** Cellular fatty acids compositions by TSBA 40 method of strain UP-52^T and the closest relatives. **Strains: 1**, UP-52^T; **2**, *D. frigoris* AR-3-8^T; **3**, *D. koreensis* KCTC 12537^T; **4**, *D. psychrophilus* BZ26^T ; **5**, *D. ginsengisoli* Gsoil 043^T; 6, *D. psychrotolerans* AR-3-6^T .The results for all strains were taken from this study except AR-3-8^T (2) and AR-3-6^T (2)

Fatty acids	Strain					
	1	2	3	4	5	6
Summed feature 3	**40.1**	**50.5**	**42.8**	**37.3**	**42.1**	**50.2**
iso-$C_{15:0}$	**19.8**	**10.9**	**20.7**	**24.2**	**16.7**	**9.3**
$C_{16:1}$ *ω5c*	**18.4**	**15.2**	**12.9**	**9.8**	**12.8**	**20.2**
iso-$C_{17:0}$ 3-OH	**8.6**	**6.6**	**9.7**	**11.5**	**11.3**	4.9
$C_{16:0}$	3.4	1.7	3.1	**5.6**	4.5	1.1
$C_{16:0}$ 3-OH	2.8	3.0	2.3	2.5	3.1	4.1
iso-$C_{15:0}$ 3-OH	2.6	2.7	3.4	4.1	2.9	2.1
iso-$C_{15:1}$ G	**-**	4	**-**	**-**	**-**	2.0

345 Major fatty acids (>5 %) in each strain are shown in bold.

346 Summed feature 3: ($C_{16:1}$ *ω7c*/ iso-$C_{15:0}$ 2-OH/ $C_{16:1}$ *ω6c*)

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Figure 1

Click here to access/download;Figure - Phylogenetic tree in EPS format;Fig.1.eps

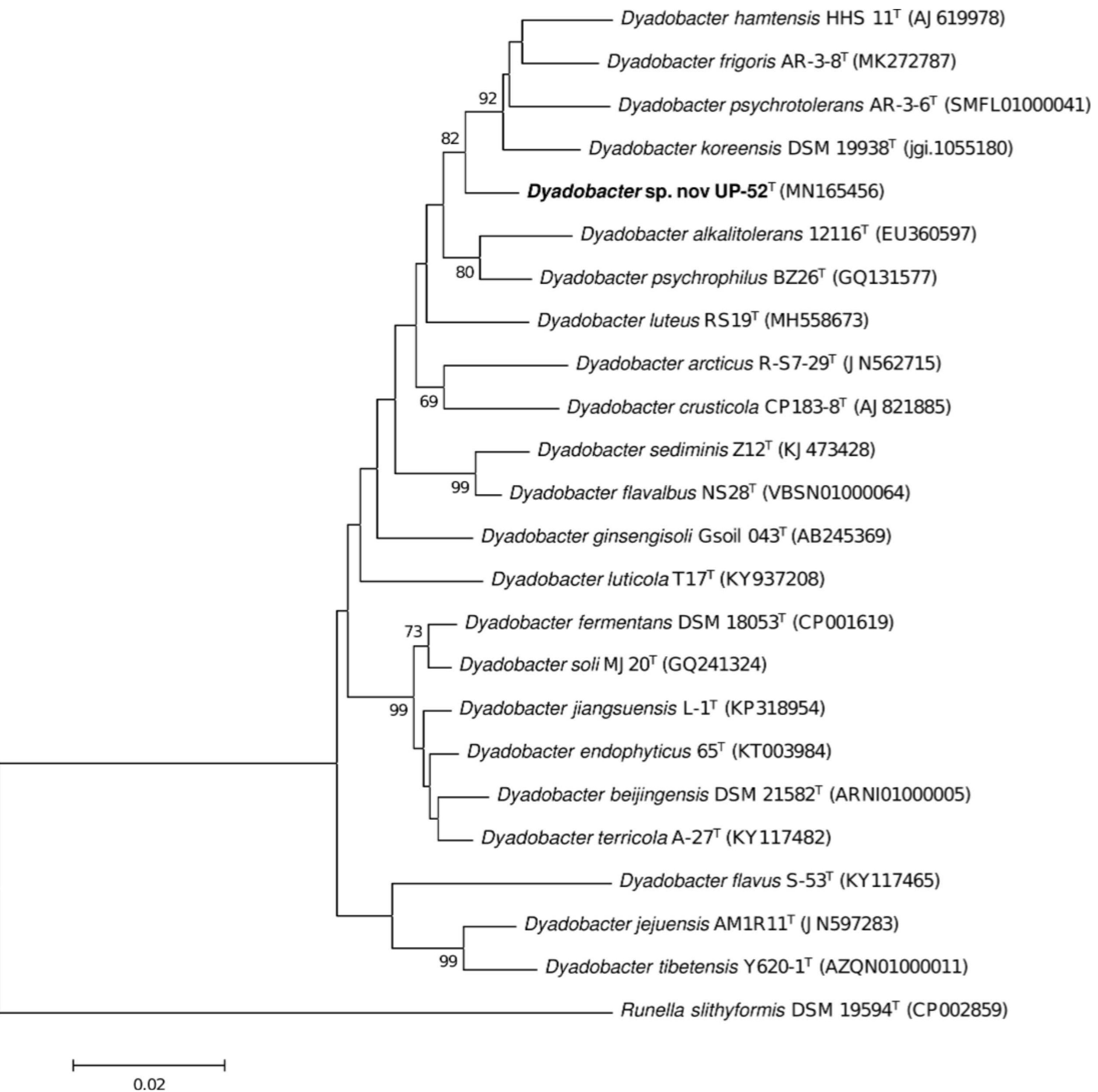
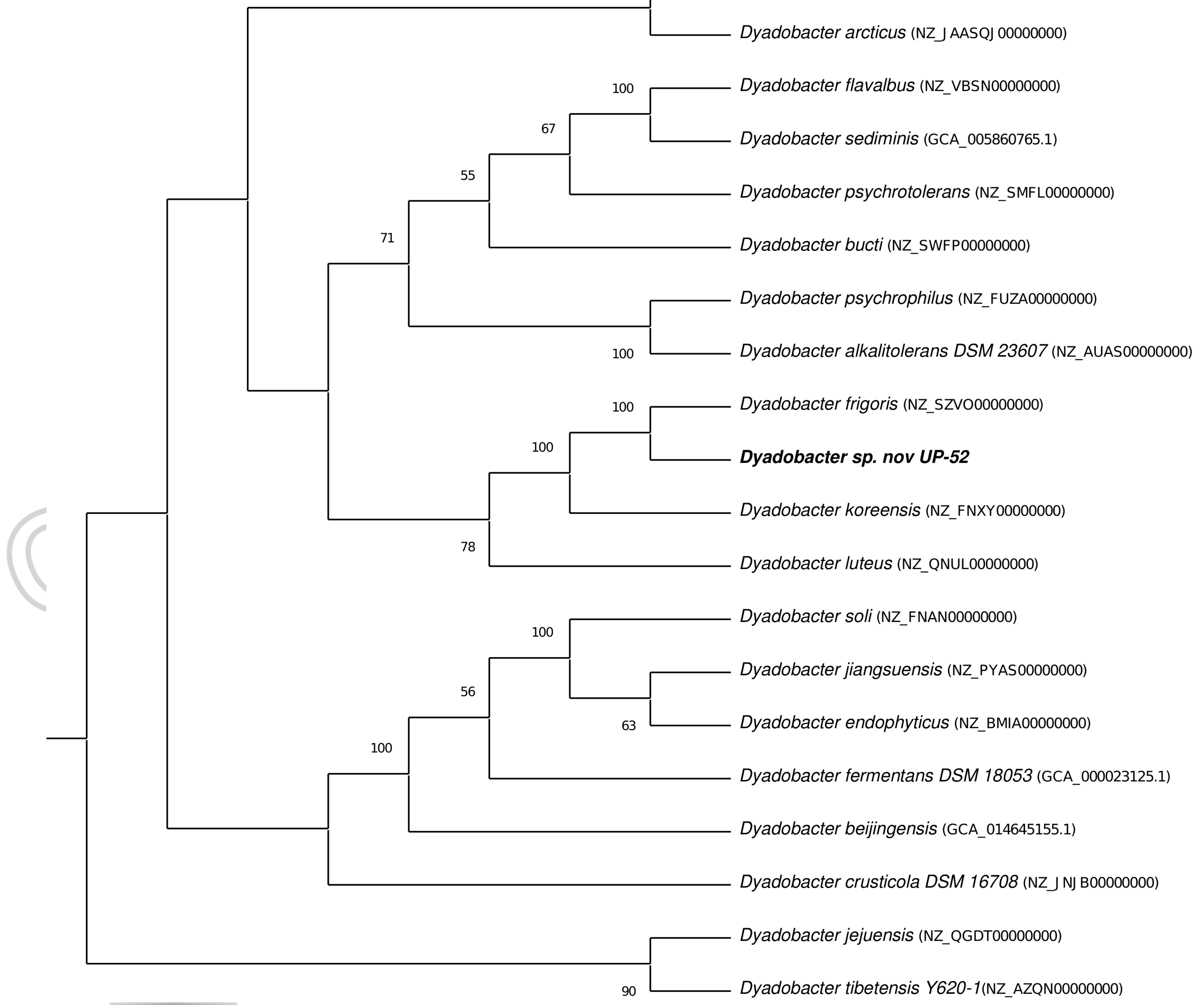


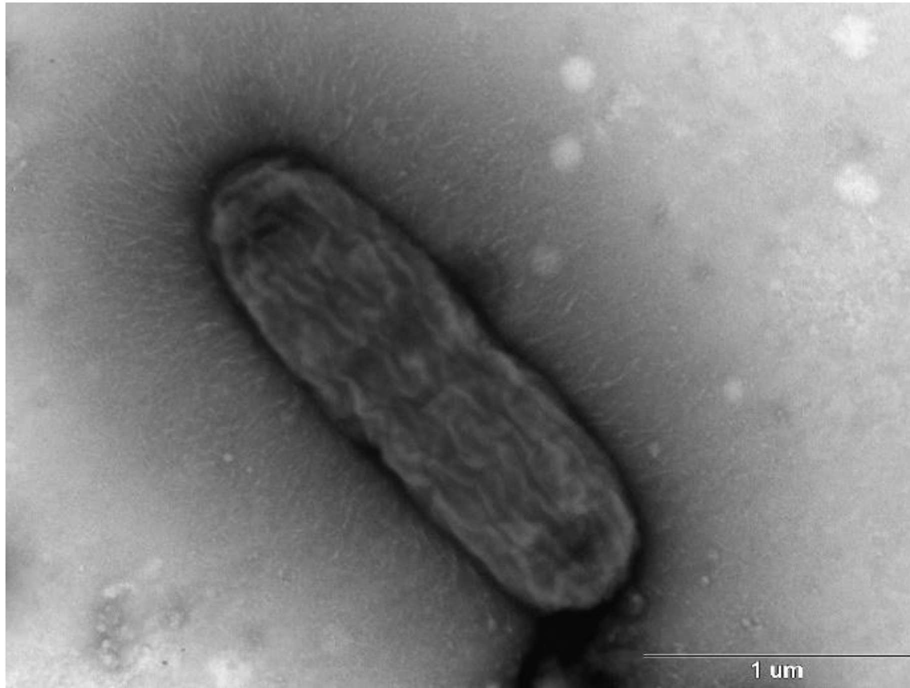
Figure 2

Click here to access/download;Figure - Phylogenetic tree in EPS format;Fig.2.eps



Supplementary Material
Click here to access/download;Supplementary Material;all-supplementary-figures-UP-52-new.pdf

Supplementary Fig 1: Shadow casting electron microscopic images of strain UP-52^{T}



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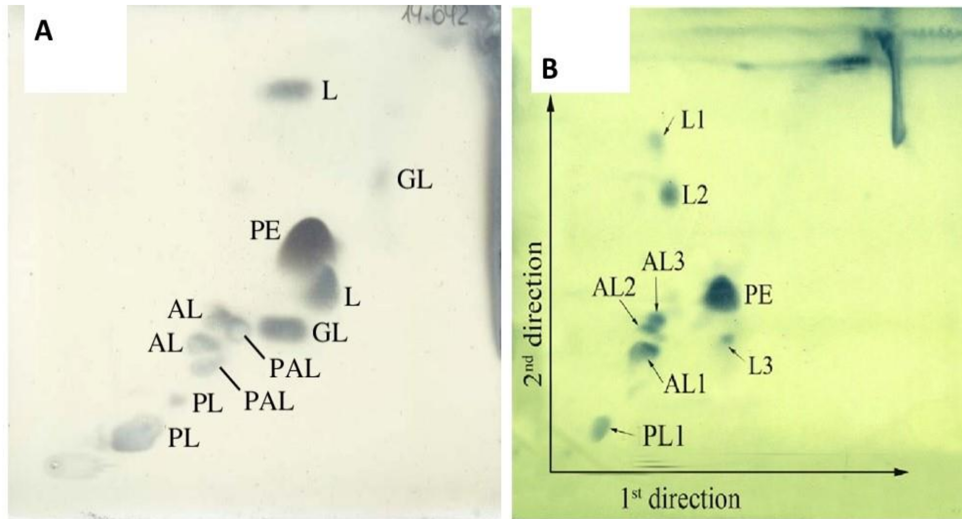
Supplementary Fig 2: Two-dimensional TLC polar lipid images of **A:** strain UP-52^T

B: strain *Dyadobacter* frigoris AR-3-8^T



L = Lipid

GL = Glycolipid

AL = Aminolipid

PL = Phospholipid

PE = Phosphatidylethanolamine

PAL = Phosphoaminolipid