IJSEM Papers in Press. Published January 11, 2013 as doi:10.1099/ijs.0.049551-0

Methylobacterium tarhaniae sp. nov., isolated from arid soil.

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Subject category: New Taxa: *Bacteria*

Running title: *Methylobacterium tarhaniae* sp. nov.

The GenBank accession number for the 16S rRNA gene sequence of *Methylobacterium tarhaniae* N4211^T is (= KCTC 23615^T = DSM 25844^T) **JQ864432**.

Key words: *Proteobacteria*, *Alphaproteobacteria*, *Rhizobiales*, *Methylobacterium tarhaniae*, Polyphasic taxonomy

Abstract

A reddish orange-pigmented, Gram negative, aerobic, facultatively methylotrophic strain, N4211^T, isolated from arid soil, collected from Abuja, Nigeria, was analysed by using a polyphasic approach. Phylogenetic analysis, based on 16S rRNA gene sequences, showed that strain N4211^T belonged to the genus *Methylobacterium*. Strain N4211^T was most closely related to *Methylobacterium aquaticum* DSM 16371^T (98.56 %), *Methylobacterium platani* KCTC 12901^T (97.95 %) and *Methylobacterium variabile* DSM 16961^T (97.2 %), and the phylogenetic similarities to all other *Methylobacterium* species with validly published names were less than 97.0%. The major ubiquinones detected were Q-10. The major fatty acids were summed feature 7 ($C_{18:1}$ *cis*11/t9/t6) 61.52 %. The DNA G+C content was 67.3 mol %. DNA relatedness of the strain N4211^T and its most closely related strains *M. aquaticum* DSM 16371^T and *M. platani* KCTC 12901^T were 60.0 and 48.2 %, respectively. On the basis of the phenotypic, phylogenetic and DNA-DNA hybridization data, strain N4211^T is assigned to a novel species of the genus *Methylobacterium* for which the name *Methylobacterium tarhaniae* sp. nov. is proposed (type strains N4211^T = KCTC 23615^T = DSM 25844^T)

Introduction

The genus *Methylobacterium* was proposed by Patt *et al*. (1976), and revised descriptions have been emended by Green & Bousfield (1983) belongs to the class *Alphaproteobacteria* and includes strictly aerobic, Gram-negative, rod-shaped, pink-pigmented, facultatively methylotrophic (PPFM) bacteria, which can grow on single carbon compounds such as formate, formaldehyde, methanol and methylamine as sole source of carbon and energy as well as on a wide range of multi-carbon growth subtrates (Green, 1992; Raja *et al*., 2008). The primary reservoir of methylotrophic bacteria are mainly soil and water but are also

present in variety of natural and man-made environments, including dust, lake sediments, freshwater, seawater, phyllosphere, tree tissues, root nodules, rice grains, air, face-creams, fermented products, water supplies, bathrooms, air-conditioning systems (Austin *et al*., 1978; Yoshimura, 1982; Green & Bousfield, 1983; Corpe & Rheem, 1989; Green, 1992; Hiraishi *et al*., 1995; Trotsenko *et al*., 2001; Lidstrom & Chistoserdova, 2002; Van Aken *et al*., 2004; Anesti *et al*., 2005; Kang *et al*., 2007; Kato *et al*., 2008; Madhaiyan *et al*., 2009; Madhaiyan *et al*., 2012; Tani *et al*., 2012). Members of the genus *Methylobacterium* species have been found to be a dominant component of bacterial phyllosphere communities (Delmotte *et al*., 2009). *Methylobacterium* species are known to produce phytohormones, which can stimulate plant growth (Ivanova *et al*,. 2001; Koenig *et al*., 2002), allow for fixation of atmospheric nitrogen (Sy *et al*., 2001) and help plants agains to pathogens (Holland & Polacco, 1994).

Strain N4211^T was isolated on *Streptomyces* isolation medium starch casein agar (Küster, 1959), supplemented with filter-sterilized cycloheximide (50 μg ml^{-1}), nystatin (50 μg ml^{-1}) and rifampicin (0.5 μg ml^{-1}), after 28^{o}C for 21 days following inoculation with a suspension of an arid soil collected from Abuja, Nigeria. Reddish orange-pigmented colonies were selected and studied in more detail. The isolate was maintained on glucose-yeast extract (Gordon & Mihm, 1962) and yeast extract-malt extract (ISP medium 2; Shirling & Gottlieb, 1966) agar slopes at room temperature and as glycerol suspensions (20 %, v/v) at -20 °C.

Genomic DNA e xtraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product were carried out following Chun & Goodfellow (1995). The almost complete (1417 bp) 16S rRNA gene sequence of strain N4211^T was determined using an ABI PRISM 3730 XL automatic sequencer. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim *et al*., 2012). Multiple alignment with sequences from closely related species was performed by using the program CLUSTAL W in

MEGA5 (Tamura *et al*., 2011). Phylogenetic trees were constructed with the neighbour-joining (Saitou & Nei, 1987), maximum parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA5 (Tamura *et al*., 2011). Evolutionary distances were calculated using model of Jukes & Cantor (1969). Topologies of the resultant trees were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings.

The phylogenetic tree based on the neighbour-joining algorithm showed that strain N4211^T forming a cluster with most related strains *Methylobacterium aquaticum* DSM 16371^T, *Methylobacterium platani* KCTC 12901^T and *Methylobacterium variabile* DSM 16961^T within members of the genus *Methylobacterium* (Fig. 1). The other two tree-making algoritms (maximum-likelihood and maximum-parsinomy) resulted in trees showing similar topologies (Supplementary Fig. S1 and S2). Strain N4211^T shares 16S rDNA similarity of 98.56 % (20 nt differences at 1392 locations), 97.95 % (29 nt differences at 1415 locations) and 97.2 % (39 nt differences at 1393 locations) respectively, with its nearest relatives, *M. Aquaticum* DSM 16371^T, *M. platani* KCTC 12901^T and *M. variabile* DSM 16961^T. Sequence similarities with all other members of the genus *Methylobacterium* were < 97.0 %.

DNA-DNA relatedness values between isolate N4211^T and its closes phylogenetic neighbors *Methylobacterium aquaticum* DSM 16371^T and *Methylobacterium platani* KCTC 12901^T, were performed by the Identification Service at the Deutsche Sammlung von Mikroorganismen und Zelkulturen Braunschweig, Germany. DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al*. (1977). DNA-DNA hybridization was carried out as described by De Ley *et al.* (1970) following the modifications described by Huss *et al*. (1983) using a model Cary 100 B io UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with *in situ* temperature probe (Varian).

The taxonomic position of the strain N4211^T was supported by DNA:DNA relatedness data. Strain N4211^T showed DNA relatedness values of 60.0 % to *M. aquaticum* DSM 16371^T and 48.2 % to *M. platani* KCTC 12901^T (based on a mean of duplicate determinations), the phylogenetically closest related species within the genus *Methylobacterium*, a result well below the 70 % threshold recommented for the delination of bacterial species by Wayne *et al*. (1987).

Biomass for chemotaxonomic studies was prepared by growing strain N4211^T in ISP 2 broth cultures, at 160 rpm for 10 days at 28 ºC; cells were harvested by centrifugation, washed twice in distilled water and re-centrifuged freeze-dried. Respiratory lipoquinones were extracted from 100 mg of freeze dried cells based on the two stage method described by Tindall (1990a; 1990b) and carried out by the Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany. Respiratory lipoquinones were separated into their different classes (menaquinones and ubiquinones) by thin layer chromatography on silica gel (Macherey-Nagel Art. NO. 805 023), using hexane: tert-butylmethylether (9:1 v/v) as solvent. UV absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed by HPLC. This step was carried out on a LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (Macherey-Nagel, 2 mm x 125 mm, 3 µm, RP18) using methanol as the eluant. Respiratory lipoquinones were detected at 269 nm.

A starter collection for the fatty acid analyses was prepared in a flask containing 20 ml Trypticase Soy Broth (Difco) which was shaken at 150 rpm at 28 ^oC for 5 days. Five ml of the resultant culture was used to inoculate 50 ml of TSB which was incubated under the same conditions, the biomass harvested by cellulose filtration (pore size 0.45 µm) and the wet cells (200 mg) placed in an extraction tube. Cellular fatty acids were extracted, methylated and separated by gas chromatography using an Agilent Technologies 6890 N instrument, fitted

with an autosampler and a 6,783 injector, according to the standard protocol of the Sherlock Microbial identification (MIDI) system (Saser 1990; Kampfer & Kroppenstedt, 1996), the fatty acid methyl ester peaks were quantified using TSBA 5.0 software. The DNA G+C content of the isolate was determined following the procedure of Gonzalez & Saiz-Jimenez (2005).

Predominant cellular fatty acids are summed feature 7 (61.5 %) comprising $C_{18:1}$ *cis*11 / t9 / t6, summed feature 3 (9.2 %) comprising $C_{16:1}$ *iso* I / 14:0 3OH, $C_{16:1}$ *cis* 9 (8.4 %), $C_{16:0}$ (6.9 %), $C_{15:0}$ 3OH (5.3 %), *iso*-$C_{18:0}$ 10-*methyl*, (3.8 %), $C_{18:0}$ 3OH (3.1 %) and $C_{12:0}$ (1.7 %) (see Supplementary Table S1). The predominant ubiqinone of strain N4211^T was Q-10 (72.0 %); an unknown component (28.0 %) was also detected. The G+C content of the DNA of the isolate was 67.3 %, which is within the range expected for members of the genus *Methylobacterium* (Green, 1992).

Phenotypic characteristics of strain N4211^T, *Methylobacterium aquaticum* DSM 16371^T and *Methylobacterium platani* KCTC 12901^T were determined after incubation at 28 ºC for 14 days on various media as described by Shirling & Gottlieb (1966): yeast extract-malt extract agar [International *Streptomyces* Project (ISP) 2], oatmeal agar (ISP 3), inorganic salt-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract-iron agar (ISP 6), tyrosine agar (ISP 7), modified Bennett's agar (MBA; Jones,1949), Czapek's and nutrient agar (NA; Difco). National Bureau of Standards (NBS) Colour Name Charts (Kelly, 1964) was used for determining colour designation and names. Growth was tested at different temperatures (4, 10, 20, 28, 37, 45, 50 and 55 ºC) and pH values 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0, and in the presence of sodium chloride (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 15 %; w/v) using GYM agar as the basal medium (DSMZ medium no: 65). Established methods were used to determine whether the strains degraded chitin (Hsu & Lockwood, 1975), RNA

149 (Goodfellow *et al*. 1979) and Tween 20, 40 and 80 (Nash & Krent, 1991); the remaining
150 degradation tests were carried out using the media and methods described by Williams *et al*.
151 (1983). Carbon source utilization was tested using carbon source utilization (ISP 9) medium
152 (Shirling & Gottlieb, 1966) supplemented with a final concentration of 1 % of the tested
153 carbon sources. Nitrogen source utilization was examined using the basal medium
154 recommended by Williams *et al.* (1983) supplemented with a final concentration of 0.1 % of
155 the tested nitrogen sources. Tests in the commercial system API-20E and API-ZYM
156 (Biomerieux) were performed according to the manufacturer's instructions.

157 The morphological characteristic and physiological properties of strain N4211^T were also
158 consistent with those of the genus *Methylobacterium* with cells being Gram-negative, aerobic,
159 rood-shaped and motile (Suppelementary figure S3). Cell of strain N4211^T are 1.0-1.6 x 2.6-
160 5.6 μm after 7 days culture on GYM agar (Suppelementary figure S4). It produced small,
161 smooth, dark reddish orange colonies grew well on modified Bennett's, Czapek's, nutrient,
162 ISP 2, ISP 3, ISP 6 and ISP 7 agar. Strain N4211^T differed from its most closest relatives, *M.*
163 *aquaticum* DSM 16371^T and *M. platanii* KCTC 12901^T, in several tests such as aesculin and
164 arbutin hydrolysis, nitrate reduction, growth on D (+) mannose, D-phenylalanine, L-
165 phenylalanine and the activity of the enzyme naphthol-AS-BI-phosphohydrolase. Strain
166 N4211^T grew on formaldehyde (0.01 % v/ v) and methanol (1.0 % v/v). The phenotypic
167 characteristics that differentiate the novel species from its phylogenetically closest relatives
168 are summarized in Table 1.

169 It is clear from the genotypic and phenotypic data described above that strain N4211^T
170 considered to be a novel species in the genus *Methylobacterium*, for which the name
171 *Methylobacterium tarhaniae* sp. nov. is proposed.
172
173

Description of *Methylobacterium tarhaniae* sp. nov.

Methylobacterium tarhaniae (tar.han'i.ae. N.L. gen. fem. n. *tarhaniae* of Tarhan, named in honour of Leman Tarhan for her contributions to microbial biotechnology).

Cells are Gram-negatif, aerobic, motile and rood shaped (1.0-1.6 x 2.6-5.6 μm). Colonies are dark reddish orange, smooth and translucett with undulate magrine. Growth occurs at 10-37 ºC (optimum 25-28 ºC) and at pH 4.0-9.0 (optimal pH 7.0). Nitrate reduction, urea hydrolysis tests and catalase are positive but not aesculin and arbutin hydrolysis, indole and H_2S production. Starch and Tween 20 are degraded but not elastin, guanine, L-tyrosine, Tween 80, xanthine and xylan. Adanitol, amygdalin, D(-)cellobiose, formaldehyde, D(+)galactose, D(-)sorbitol, glucose, inositol, D(+)mannose, D-mannitol, D(+)melezitose, dextrin, inuline, L(+)arabinose, lactose, maltose, methanol, rhamnose, saccharose and starch are utilized as sole carbon sources. D-L-phenyalanine, L-alanine, L-arginine, L-methionine, L-proline, L-serine and L-threonine are utilized as sole nitrogen sources. Does not utilize L-isoleucine, L-cysteine, glycine, L-hydroxyproline and L-valine as sole nitrogen sources. The organism is positive for acid phosphatase, citrate, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, alkaline phosphatase, trypsin and urease, and negative for arginine dihydrolase, gelatinase, lysine decarboxylase, ornithine decarboxylase, α-galactosidase, α-glucosidase, β-glucosidase, esterase-lipase, N-acetyl-β-glucosaminidase, chymotrypsin, cystine arylamidase, esterase, lipase, α-fucosidase, α-mannosidase, β–galactosidase, β-glucuronidase, tryptophane deaminase and valine arylamidase. The major isoprenoid quinone is Q-10. The major fatty acids are summed feature 7 contained $C_{18:1}$ *cis*11/t9/t6, $C_{18:1}$ *trans* 9/t6/c11 or $C_{18:1}$ *trans* 6/t9/c11 and summed feature 3 contained $C_{14:0}$ 3OH, $C_{16:1}$ *iso*-I or both. The DNA G+C content of the type strain is 67.3 %.

The type strain, N4211^T (= DSM 25844^T = KCTC 23615^T)**,** was isolated from arid soil collected from Abuja, Nigeria.

Acknowledgements

This research was supported by Ondokuz Mayis University (OMU), project no. PYO. FEN. 1901.09.003.

References

Anesti, V., Vohra, J., Goonetilleka, S., McDonald, I. R., Sträubler, B., Stackebrandt, E., Kelly, D. P. & Wood, A. P. (2004). Molecular detection and isolation of facultatively methylotrophic bacteria, including *Methylobacterium podarium* sp. nov., from the human foot microflora. *Environ Microbiol* **6**, 820–830.

Austin, B., Goodfellow, M. & Dickinson, C. H. (1978). Numerical taxonomy of phylloplane bacteria isolated from *Lolium perenne*. *J Gen Microbiol* **104**, 139–155.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

Chun, J. & Goodfellow, M. (1995). A phylogenetic analysis of the genus *Nocardia* with 16S rRNA gene sequences. *Int J Syst Bacteriol* **45**, 240–245.

Corpe, W. A. & Rheem, S. (1989). Ecology of the methylotrophic bacteria on living leaf surfaces. *FEMS Microbiol Ecol* **62**, 243–250.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 143–153.

Delmotte, N., Knief, C., Chaffron, S., Innerebner, G., Roschitzki, B., Schlapbach, R., von Mering, C. & Vorholt, J. A. (2009). Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proc Natl Acad Sci* **106**, 16428–16433.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.

Felsenstein, J. (1985). Confidence limits on ph ylogeny: an approach using the bootstrap. *Evolution* **39**, 783–791.

Goodfellow, M., Alderson, G. & Lacey, J. (1979). Numerical taxonomy of *Actinomadura* and related actinomycetes. *Journal of General Microbiology* **112**, 95-111.

Gonzalez, J. M. & Saiz-Jimenez, C. (2005). A simple fluorimetric method for the estimation of DNA-DNA relatedness between closely related microorganisms by thermal denaturation temperatures. *Extremophiles* **9**, 75-79.

Gordon R. E. & Mihm J. M. (1962). Identification of *Nocardia caviae*. (Erikson) nov. comb. *Ann NY Acad Soc* **98,** 628–636.

Green, P. N. & Bousfield, I. J. (1983). Emendation of *Methylobacterium* (Patt, Cole, and Hanson 1976); *Methylobacterium rhodinum* (Heumann 1962) comb. nov. corrig. *Methylobacterium radiotolerans* (Ito & Iizuka 1971) comb. nov., corrig.; and *Methylobacteriu mesophilicum* (Austin & Goodfellow 1979) comb. nov. *Int J Syst Bacteriol* **33**, 875–877.

Green, P. N. (1992). The genus *Methylobacterium*. In *The Prokaryotes*, 2nd edn, pp. 2342–2349. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Hiraishi, A., Furuhata, K., Matsumoto, A., Koike, K. A., Fukuyama, M. & Tabuchi, K. (1995). Phenotypic and genetic diversity of chlorine-resistant *Methylobacterium* strains isolated from various environments. *Appl Environ Microbiol* **61**, 2099–2107.

Holland, M. A. & Polacco, J. C. **(1994).** PPFMs and other covert contaminants: is there more to plant physiology than just plant? *Annu Rev Plant Physiol Plant Mol Biol* **45**, 197–209.

Hsu, S. C. & Lockwood, J. L. (1975). Powdered chitin agar as a s elective medium for enumeration of actinomycetes in water and soil. *Appl Microbiol* **29**, 422–426.

Huss, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrometric determination of DNA hybridisation from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

Ivanova, E. G., Doronina, N. V. & Trotsenko, Iu. A. (2001). [Aerobic methylobacteria are capable of synthesizing auxins]. *Mikrobiologia* **70**, 452–458.

Jones, K. L. (1949). Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic. *J Bacteriol* **57**, 141-145.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In Mammalian Protein Metabolism, *vol. 3*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Kämpfer, P. & Kroppenstedt, R. M. (1996). Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

Kang, Y.-S., Kim, J., Shin, H.-D., Nam, Y.-D., Bae, J.-W., Jeon, C. O. & Park, W. (2007). *Methylobacterium platani* sp. nov., isolated from a leaf of the tree *Platanus orientalis*. *Int J Syst Evol Microbiol* **57**, 2849–2853.

Kato, Y., Asahara, M., Goto, K., Kasai, H. & Yokota, A. (2008). *Methylobacterium persicinum* sp. nov., *Methylobacterium komagatae* sp. nov., *Methylobacterium brachiatum* sp. nov., *Methylobacterium tardum* sp. nov. and *Methylobacterium gregans* sp. nov., isolated from freshwater. *Int J Syst Evol Microbiol* **58**, 1134–1141.

Kelly, K. L. (1964). Inter-Society Color Council-National Bureau of Standards color-name charts illustrated with centroid colors published in US.

Kim, O.-S., Cho, Y.-J., Lee, K., Yoon, S.-H., Kim, M., Na, H., Park, S.-C.,Jeon, Y. S., Lee, J. H., Yi, H., Won, S. & Chun, J. (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716–721.

Kluge, A. G. & Farris, F. S. (1969). Quantitative phyletics and the evolution of anurans, *Syst Zool* **18**, 1–32.

Koenig, R. L., Morris, R. O. & Polacco, J. C. (2002). tRNA is the source of low-level trans-zeatin production in *Methylobacterium* spp. *J Bacteriol* **184**, 1832–1842.

Küster, E. (1959). Outline of a comparative study of criteria used in characterisation of the actinomycetes. *Int Bull Bacteriol Nomencl Taxon* **9**, 97–104.

Lidstrom, M. E. & Chistoserdova, L. (2002). Plants in the pink: cytokinin production by *Methylobacterium*. *J Bacteriol* **184**, 1818.

Madhaiyan M., Poonguzhali S., Kwon S.-W. & Sa T.-M. (2009). *Methylobacterium phyllosphaerae* sp. nov., a pink-pigmented, facultative methylotroph from the phyllosphere of rice. *Int J Syst Evol Microbiol* **59**, 22–27.

Madhaiyan, M., Poonguzhali, S., Senthilkumar M., Lee J. S. & Lee K. C. (2012) *Methylobacterium gossipiicola* sp. nov., a pink-pigmented, facultatively methylotrophic bacterium isolated from the cotton phyllosphere. *Int. J. Syst. Evol. Microbiol* **62**, 162-167.

Nash, P. & Krent, M. M. (1991). Culture media. *In Manual Of Clinical Microbiology*, 5th Edition, pp: 1268-1270, Edited by A. Ballows, W.J. Hauser, K.L. Herrmann, H.D. Isenberg & H.J. Shadomy. *American Society for Microbiology*, Washington DC.

Patt, T. E., Cole, G. C. & Hanson, R. S. (1976). *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. *Int J Syst Bacteriol* **26**, 226–229.

Raja, P., Balachandar, D. & Sundaram, S. P. (2008) Genetic diversity and phylogeny of pink-pigmented facultative methylotrophic bacteria isolated from the phyllosphere of tropical crop plants. *Biol. Fertil. Soils* **45**, 45–53.

Saitou, N. & Nei, M. (1987). The neighbor-joining method. A new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sasser, M. (1990). Identification of Bacteria By Gas Chromatography of Cellular Fatty Acids. Technical Note 101. Newark, DE: MIDI Inc.

Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species, *Int J Syst Bacteriol* **16**, 313–340.

Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., de Lajudie, P., Prin, Y., Neyra, M., Gillis, M. & other authors (2001). Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *J Bacteriol* **183**, 214–220.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsinomy Methods. *Mol Bio Evol* **28**, 2731–2739.

Tani A., Sahin, N. & Kimbara, K. (2012) *Methylobacterium gnaphalii* sp. nov., isolated from leaves of *Gnaphalium spicatum*. *Int. J. Syst. Evol. Microbiol* **62**, 2602–2607.

Tindall, B. J. (1990a). A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

Tindall, B. J. (1990b). Lipid composition *of Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

Trotsenko, Y. A., Ivanova, E. G. & Doronina, N. V. (2001). Aerobic methylotrophic bacteria as phytosymbionts. *Mikrobiologiya* **70**, 725–736 (in Russian).

Van Aken, B., Peres, C. M., Lafferty-Doty, S., Yoon, J. M. & Schnoor, J. L. (2004). ***Methylobacterium*** *populi* sp. nov., a novel aerobic,pink-pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees (*Populus deltoides* × *nigra* DN34). *Int J Syst Evol Microbiol* **54**, 1191–1196.

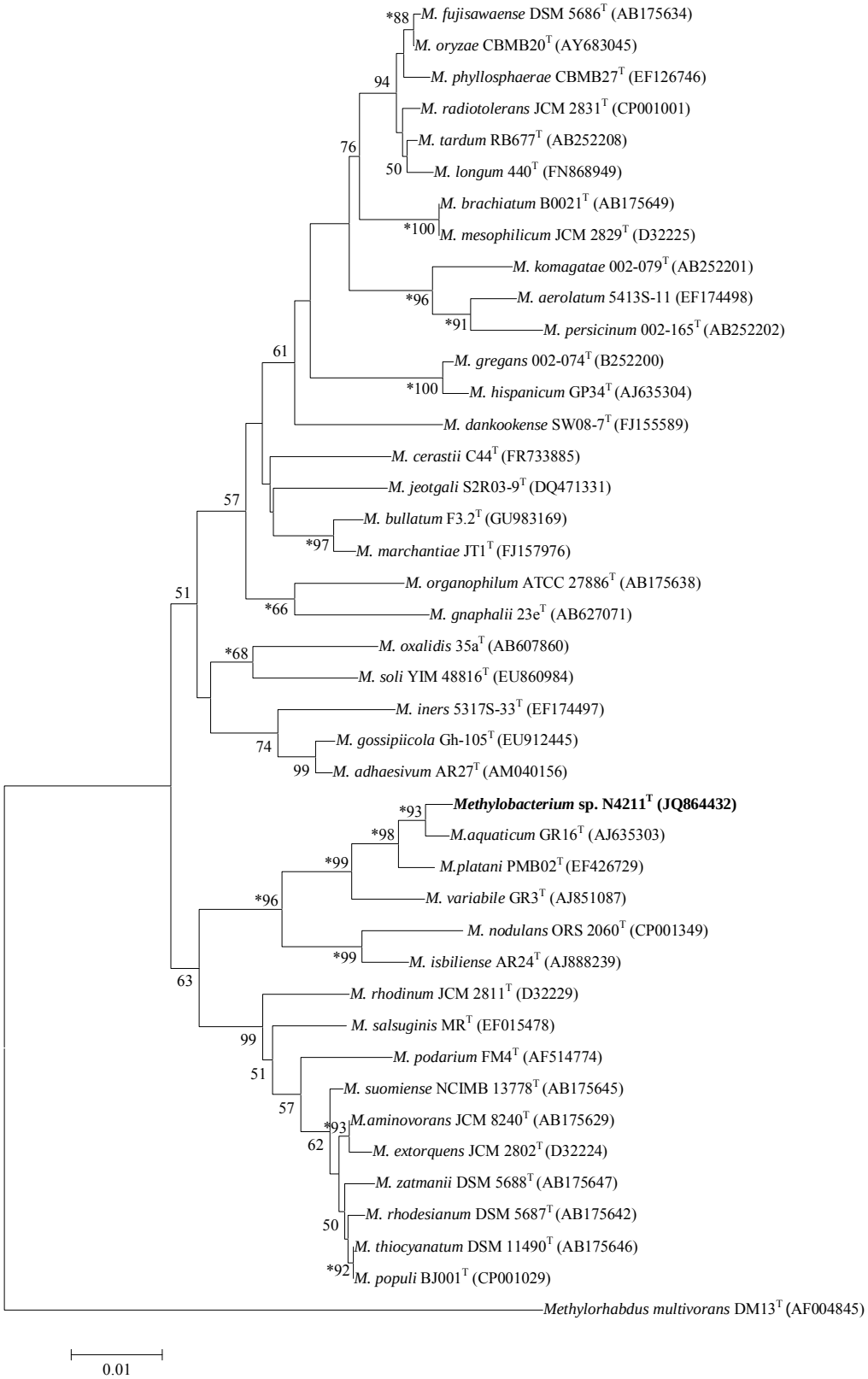
Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Williams, S. T., Goodfellow, M., Alderson, G., Wellington, E. M. H., Sneath, P. H. A. & Sackin, M. J. (1983). Numerical classification of *Streptomyces* and related genera. *J Gen Microbiol* **129**, 1743–1813.

Yoshimura, F. (1982). Phylloplane bacteria in a pine forest. *Can J Microbiol* **28**, 580–592.

Table 1. Phenotypic properties of strain N4211^T and closely related type species. Strains: 1, N4211^T; 2, *Methylobacterium aquaticum* DSM 16371^T; 3, *Methylobacterium platani* KCTC 12901^T. Strains were positive for citrate utilization, activity of catalase, ability of growth at D (-) sorbitol, D (-) mannitol, L (+) arabinose, glucose as sole carbon sources (1.0 %), L-methionine, L-serine, L-threonine as sole nitrogen sources (0.1 %), alkaline phosphatase, leucine arylamidase, acid phosphatase. But negative for hydrolysis of allantoin, H_2S production, degradation of elastin (0.3 %), guanine (0.05 %), L-tyrosine (0.5 %), tween 80 (1.0 %), xhantine (0.4 %), xylan (0.4 %), L (+) rhamnose as sole carbon sources (1.0 %), alpha-iso-leucine, glycine, L-cysteine, L-hydroxyproline, L-valine, as sole nitrogen sources (0.1 %), esterase lipase, lipase, cystine arylamidase, chymotrypsin, α-glucosidase, ß-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, α-galactosidase, ß-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase, indole production, voges proskauer and gelatinase. All data were obtain in this study.

	1	**2**	**3**
Cell length (µm)	2.6-5.6	3.9–6.5	2.5–6.1
Cell width (µm)	1.0-1.6	1.5–1.7	1.4–1.5
Pigmentation	Red	Pink, red	Pink
Colony diameter (mm)	0.3-1.2	0.7–1.6	0.2–1.5
Biochemical Tests			
Aesculin Hydrolysis	-	+	+
Arbutin Hydrolysis	-	+	+
Nitrate Reduction	+	-	-
Urea Hydrolysis	+	+	-
pH tolerence			
4.0	+	+	-
5.0	+	+	-
9.0	+	+	-
Temperature			
10°C	+	+	-
37 °C	+	+	-
NaCl (%)			
1.0	+	-	-
Degradation			
Starch (%1)	+	-	-
Tween 20 (%1)	+	+	-
Sole carbon sources (1.0 %)			
Adonitol	+	+	-
D(-)Cellobiose	+	+	-
D(+)Galactose	+	+	-
D(+)Mannose	+	-	-
D(+)Melezitose	+	+	-
Dextrin	+	+	-
Inuline	+	+	-
L(+)Rhamnose	+	+	-
Lactose	+	+	-
Maltose	+	+	-
Starch	+	+	-
Sucrose (Saccharose)	+	+	-
Sole nitrogen sources (0.1 %)			
D-L Phenylalanin	+	-	-
L-Alanine	+	+	-
L-Arginine	+	+	-
L-Proline	+	+	-
API-ZYM			
Esterase	-	+	-
Valine arylamidase	-	+ -	
Trypsin	+	+	-
Naphthol-AS-BI-phosphohydrolase	+	-	-
API-20E			
Inositol	+	+	-
Melibiose	-	+	-
Amygdalin	+	+	-


*88 M. fujisawaense DSM 5686T (AB175634)
M. oryzae CBMB20T (AY683045)
94 M. phyllosphaerae CBMB27T (EF126746)
M. radiotolerans JCM 2831T (CP001001)
76 M. tardum RB677T (AB252208)
50 M. longum 440T (FN868949)
M. brachiatum B0021T (AB175649)
*100 M. mesophilicum JCM 2829T (D32225)
M. komagatae 002-079T (AB252201)
*96 M. aerolatum 5413S-11 (EF174498)
*91 M. persicinum 002-165T (AB252202)
61 M. gregans 002-074T (B252200)
*100 M. hispanicum GP34T (AJ635304)
M. dankookense SW08-7T (FJ155589)
M. cerastii C44T (FR733885)
57 M. jeotgali S2R03-9T (DQ471331)
M. bullatum F3.2T (GU983169)
*97 M. marchantiae JT1T (FJ157976)
51 M. organophilum ATCC 27886T (AB175638)
*66 M. gnaphalii 23eT (AB627071)
*68 M. oxalidis 35aT (AB607860)
M. soli YIM 48816T (EU860984)
M. iners 5317S-33T (EF174497)
74 M. gossipiicola Gh-105T (EU912445)
99 M. adhaesivum AR27T (AM040156)
*93 Methylobacterium sp. N4211T (JQ864432)
*98 M.aquaticum GR16T (AJ635303)
*99 M.platani PMB02T (EF426729)
M. variabile GR3T (AJ851087)
*96 M. nodulans ORS 2060T (CP001349)
*99 M. isbiliense AR24T (AJ888239)
63 M. rhodinum JCM 2811T (D32229)
M. salsuginis MRT (EF015478)
99 M. podarium FM4T (AF514774)
51 M. suomiense NCIMB 13778T (AB175645)
57 M.aminovorans JCM 8240T (AB175629)
*93 M. extorquens JCM 2802T (D32224)
62 M. zatmanii DSM 5688T (AB175647)
M. rhodesianum DSM 5687T (AB175642)
50 M. thiocyanatum DSM 11490T (AB175646)
*92 M. populi BJ001T (CP001029)
Methylorhabdus multivorans DM13T (AF004845)
0.01

412

413 **Fig. 1.**

Legends for Figures

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on almost complete 16 rRNA gene sequences (1417 nt) showing the position of strain N4211^T amongst its phylogenetic neighbours. *Methylorhabdus multivorans* DM13^T (AF004845) was used as an outgroup. Asterisks indicate branches of the tree that were also recovered using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) tree-making algorithms. Numbers at the nodes indicate the levels of bootstrap support (%); only values ≥ 50 % are shown. GenBank accession numbers are given in parentheses. Bar, 0.01 substitutions per site.

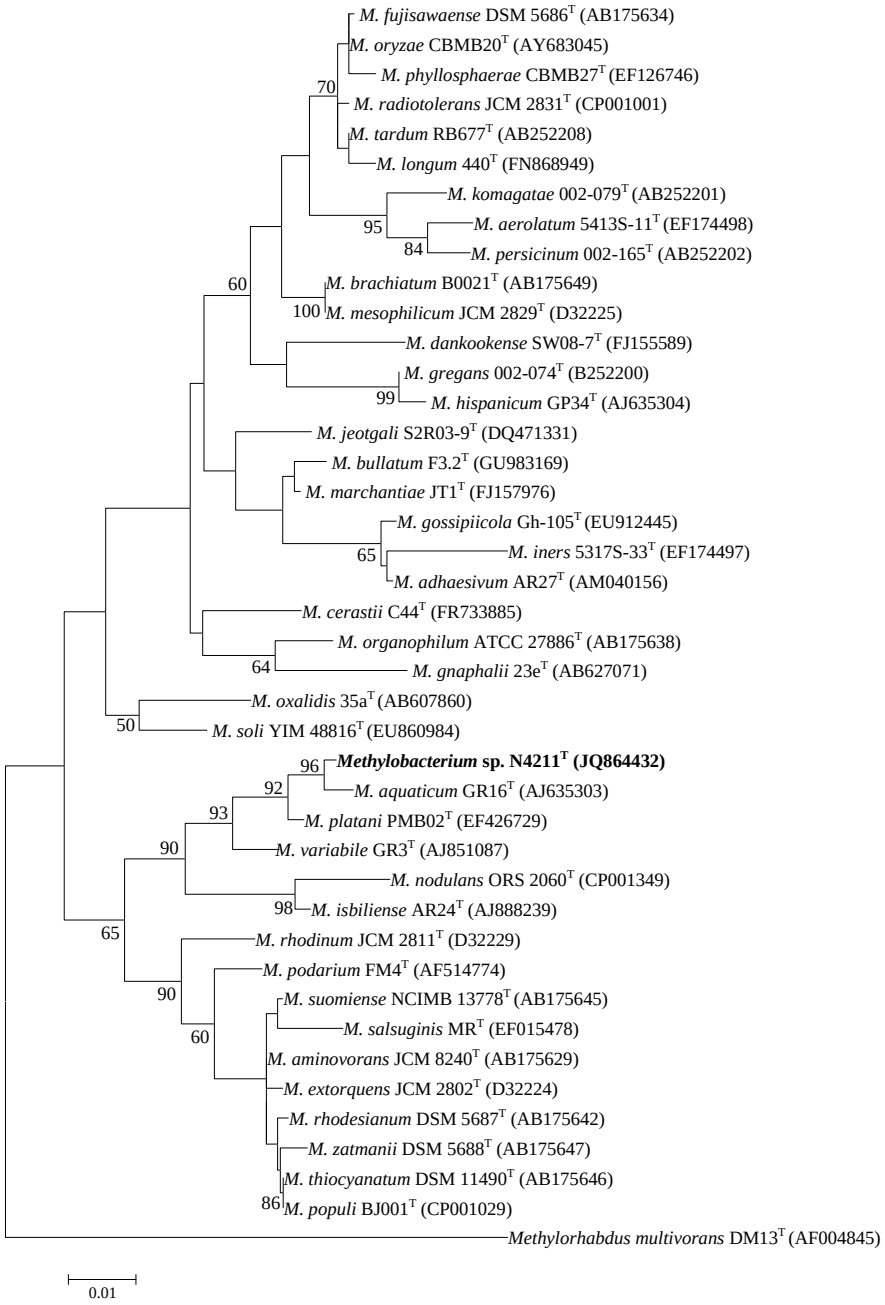
Supplementary Fig. S1. Maximum-likelihood (Felsenstein, 1981) tree of N4211^T amongst its phylogenetic neighbours.

Supplementary Fig. S2. Maximum-parsimony (Fitch, 1971) tree of N4211^T amongst its phylogenetic neighbours.

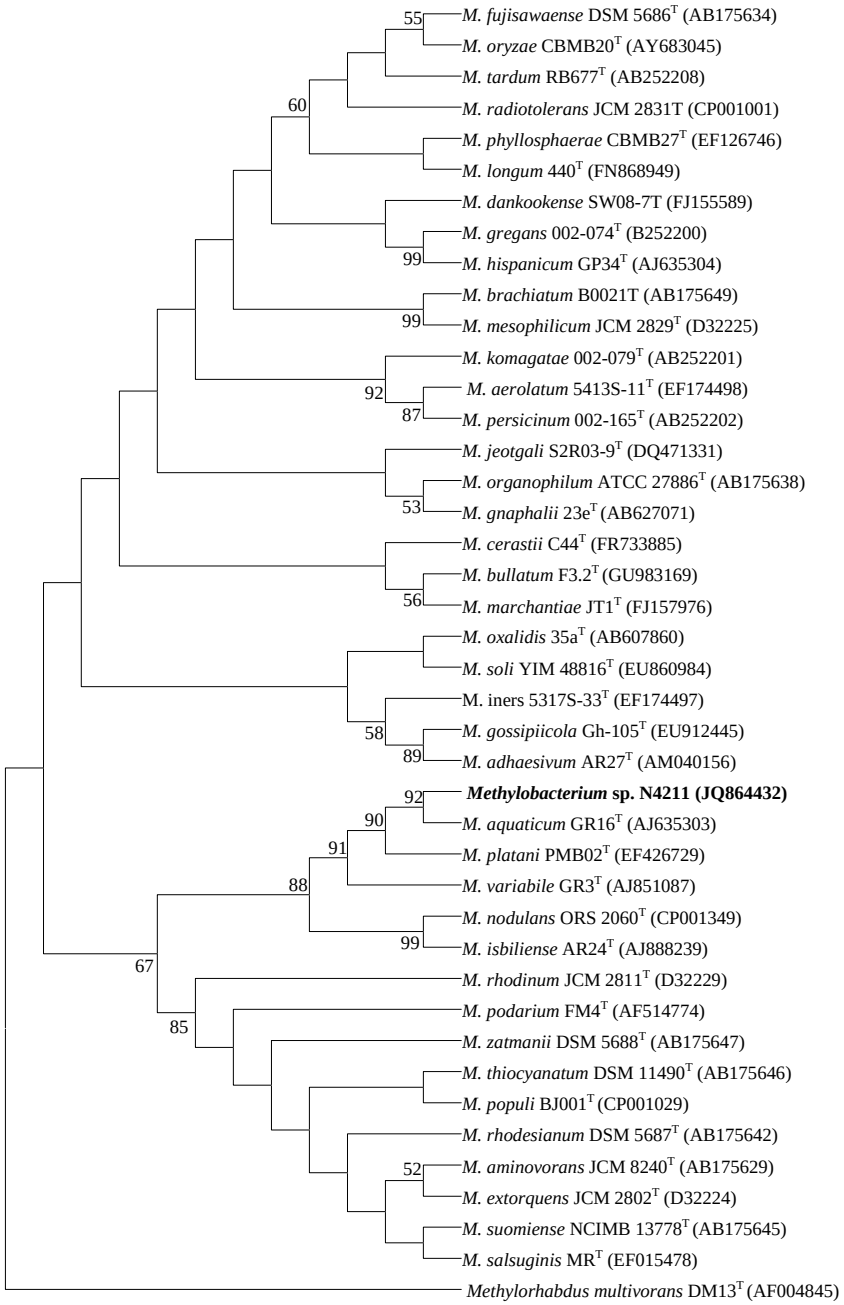
Supplementary Fig. S3. Light optic microphotograph of strain N4211^T methylene blue stained cells.

Supplementary Fig. S4. Apochromatic optic microphotograph of strain N4211^T colonies growth on ISP 2 medium for 7 days.

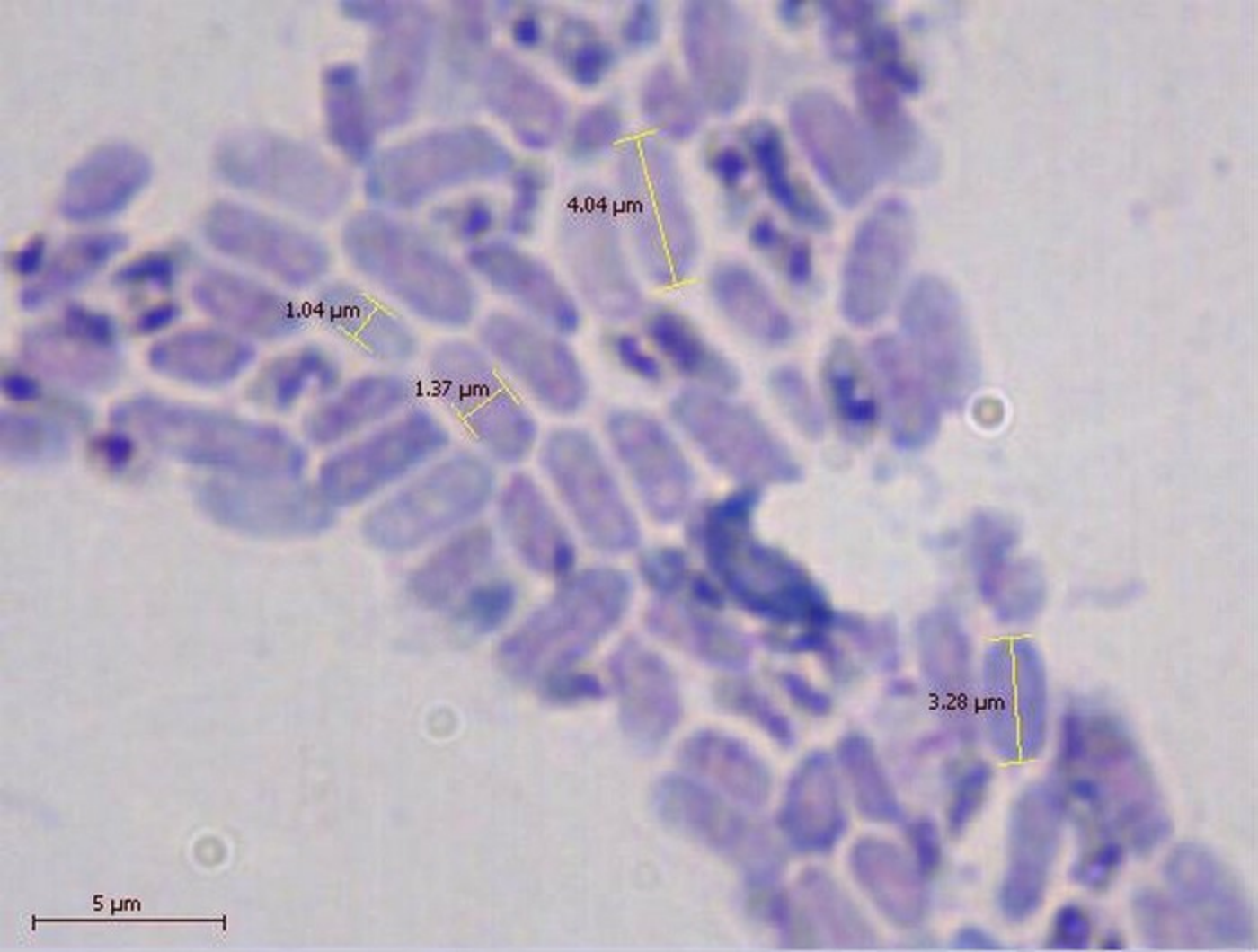
Supplementary Tab. S1. Fatty acids profiles of strain N4211^T and its closely related type species. Strains: **1**, N4211^T; **2**, *Methylobacterium aquaticum* DSM 16371^T; **3**, *Methylobacterium platani* KCTC 12901^T

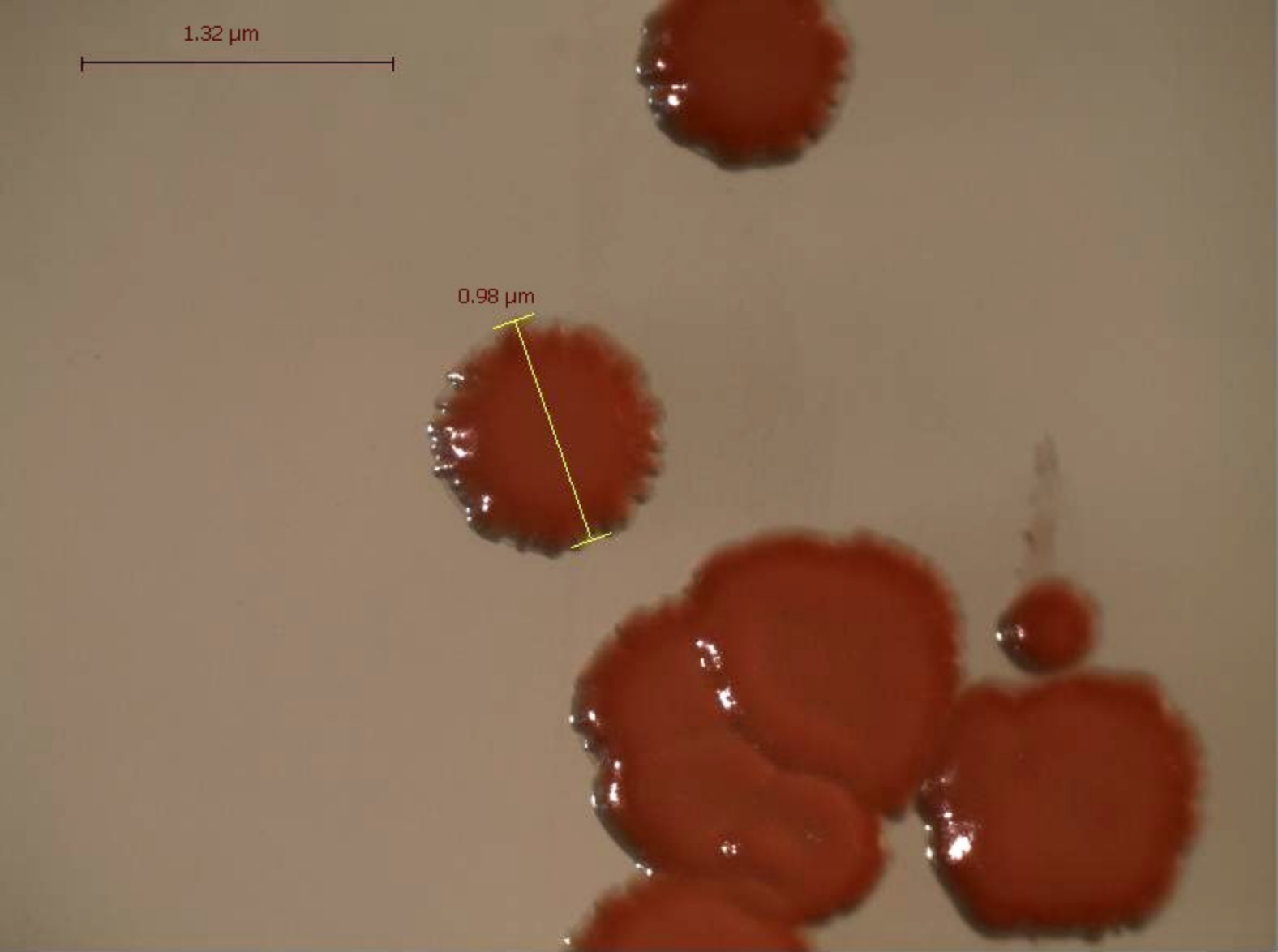


Supplementary Fig. S1



Supplementary Fig. S2


4.04 µm
1.04 µm
1.37 µm
3.28 µm
5 µm


1.32 µm
0.98 µm

Supplementary Table S1. Fatty acids profiles of strain N4211^T and closely related type species. Strains: 1, N4211^T; 2, *Methylobacterium aquaticum* DSM 16371^T; 3, *Methylobacterium platani* KCTC 12901^T.

Fatty acids	**1**	**2**	**3**
Saturated			
C $_{12:0}$	1.7	2.0	1.3
C $_{16:0}$	6.9	4.0	5.8
Unsaturated			
C $_{16:1}$ *cis* 9	8.4	4.7	4.3
Branched			
iso-C $_{18:0}$ 10-*methyl*	3.8	2.9	4.1
C $_{15:0}$ 3OH	5.3	4.7	4.7
C $_{18:0}$ 3OH	3.1	3.7	4.0
Summed Feature 3	9.2	11.1	8.8
Summed Feature 7	61.5	66.8	67.0

Summed feature 3 comprised 16:1 ISO I/14:0 3OH / 14:0 3OH/16:1 ISO I
Summed Feature 7 comprised 18:1 CIS 11/t 9/t 6 / 18:1 TRANS 9/t6/c11 / 18:1 TRANS 6/t9/c11