



Murdoch
UNIVERSITY

MURDOCH RESEARCH REPOSITORY

This is the author's final version of the work, as accepted for publication following peer review but without the publisher's layout or pagination.

The definitive version is available at

<http://dx.doi.org/10.1099/ijs.0.035097-0>

Ardley, J.K., Parker, M.A., De Meyer, S.E., Trengove, R.D., O'Hara, G.W., Reeve, W.G., Yates, R.J., Dilworth, M.J., Willems, A. and Howieson, J.G. (2012) *Microvirga lupini sp. nov.*, *Microvirga lotononidis sp. nov.*, and *Microvirga zambiensis sp. nov.* are Alphaproteobacterial root nodule bacteria that specifically nodulate and fix nitrogen with geographically and taxonomically separate legume hosts. *International Journal of Systematic and Evolutionary Microbiology*, 62 (11). pp. 2579-2588.

<http://researchrepository.murdoch.edu.au/8019/>

Copyright: © 2012 IUMS

It is posted here for your personal use. No further distribution is permitted.

Microvirga lupini sp. nov., *Microvirga lotononidis* sp. nov. and *Microvirga zambiensis* sp. nov. are Alphaproteobacterial root nodule bacteria that specifically nodulate and fix nitrogen with geographically and taxonomically separate legume hosts

Julie. K. Ardley^{a,+}, Matthew A. Parker^b, Sofie E. De Meyer^c, Robert D. Trengove^d, Graham W. O'Hara^a, Wayne G. Reeve^a, Ron J. Yates^{a,e}, Michael J. Dilworth^a, Anne Willems^c and John G. Howieson^{a,e}

^aCentre for *Rhizobium* Studies, Murdoch University, Murdoch W. A. 6150, Australia

^bDepartment of Biological Sciences, State University of New York, Binghamton, USA

^cMicrobiology Laboratory, University of Gent, Belgium

^dSeparation Science and Metabolomics Laboratory, Murdoch University, Murdoch W. A. 6150, Australia

^eDepartment of Agriculture Western Australia, Baron Hay Court, South Perth W.A. 6151, Australia

+ Corresponding author.

Telephone: + 61 8 9360 2372

Fax: + 61 8 9360 6303

E-mail address: J.Ardley@murdoch.edu.au

*Correspondence address for proofs:

Centre for *Rhizobium* Studies
School of Biological Sciences and Biotechnology
Murdoch University
Murdoch WA 6150
Australia

The GenBank accession numbers for the 16S rRNA gene sequences of strains WSM3557^T and WSM3693^T are HM362432 and HM362433, respectively. Accession

numbers for the *dnaK*, *gyrB*, *recA* and *rpoB* sequences of strains Llb5, Lut5, Lut6^T, WSM3557^T, WSM3693^T, *Microvirga flocculans* TFB^T and *Microvirga subterranea* DSM 14364^T; for the *nifD* sequences of strains Llb5, WSM3557^T and WSM3693^T and for the *nifH* sequences of strains Llb5, Lut5, WSM3557^T, WSM3693^T and *Mesorhizobium* sp. Lo5-9 are JF428144 - JF428179. Accession numbers for the *nodA* sequences of strains WSM3557^T and WSM3693^T are HQ435534 and HQ435535, respectively.

1 **Abstract**

2 Strains of Gram-negative, rod-shaped, non-spore-forming bacteria were isolated from
3 nitrogen-fixing nodules of the native legumes *Listia angolensis* (from Zambia) and
4 *Lupinus texensis* (from Texas, USA). Phylogenetic analysis of the 16S rRNA gene
5 showed that the novel strains belong to the genus *Microvirga*, with 96.1 % or greater
6 sequence similarity with type strains of this genus. The closest relative of the
7 representative strains Lut6^T and WSM3557^T was *M. flocculans* TFB^T, with 97.6-98.0
8 % similarity, while WSM3693^T was most closely related to *M. aerilata* 5420S-16^T,
9 with 98.8 % similarity. Analysis of the concatenated sequences of four housekeeping
10 gene loci (*dnaK*, *gyrB*, *recA*, *rpoB*) and cellular fatty acid profiles confirmed the
11 placement of Lut6^T, WSM3557^T and WSM3693^T within *Microvirga*. DNA:DNA
12 relatedness values and physiological and biochemical tests allowed genotypic and
13 phenotypic differentiation of Lut6^T, WSM3557^T and WSM3693^T from each other and
14 from other validly published *Microvirga* species. The *nodA* sequence of Lut6^T was
15 placed in a clade that contained strains of *Rhizobium*, *Mesorhizobium* and
16 *Sinorhizobium*, while the 100 % identical *nodA* sequences of WSM3557^T and
17 WSM3693^T clustered with *Bradyrhizobium*, *Burkholderia* and *Methylobacterium*
18 strains. Concatenated sequences for *nifD* and *nifH* show that Lut6^T, WSM3557^T and
19 WSM3693^T were most closely related to *Rhizobium etli* CFN42^T *nifDH*. On the basis
20 of genotypic, phenotypic and DNA relatedness data, three novel species of
21 *Microvirga* are proposed: *Microvirga lupini* (Lut6^T = LMG26460^T, = HAMBI 3236)
22 *Microvirga lotononidis* (WSM3557^T = LMG26455^T, = HAMBI 3237) and *Microvirga*
23 *zambiensis* (WSM3693^T = LMG26454^T, = HAMBI 3238).

24

25 Root nodule bacteria, collectively known as rhizobia, are soil bacteria that form
26 nitrogen-fixing symbioses with leguminous plants by eliciting nodules on the roots or
27 stems of their hosts. Within the nodule, the rhizobia differentiate into bacteroids that
28 convert atmospheric nitrogen (N₂) to ammonia. The microsymbiont's symbiotic
29 ability is conferred by nodulation and nitrogen fixation genes, which can be acquired
30 by horizontal gene transfer (Andam *et al.*, 2007; Barcellos *et al.*, 2007; Cummings *et*
31 *al.*, 2009; Nandasena *et al.*, 2007; Sullivan *et al.*, 1995). Rhizobia are a polyphyletic
32 group and genera capable of nodulating hosts are found in both the Alpha- and
33 Betaproteobacteria. Currently, 12 rhizobial genera and over 70 species have been
34 described (<http://www.rhizobia.co.nz/taxonomy/rhizobia.html>). Within the
35 Alphaproteobacteria, the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and
36 *Ensifer* (syn. *Sinorhizobium*) comprise the majority of described microsymbionts, but
37 novel rhizobial species of *Devosia* (Rivas *et al.*, 2002), *Methylobacterium* (Sy *et al.*,
38 2001), *Ochrobactrum* (Trujillo *et al.*, 2005) and *Shinella* (Lin *et al.*, 2008) have also
39 been described.

40

41 Recently, during the development of new perennial pasture legume symbioses for
42 southern Australian agriculture, light-pink-pigmented rhizobia isolated from nodules
43 of Zambian *Listia* (formerly *Lotononis*) *angolensis* (Boatwright *et al.*, 2011) were
44 identified as belonging to a novel lineage of root nodule bacteria (Yates *et al.*, 2007).
45 The 16S rRNA gene sequences of two of the *L. angolensis* isolates (strains WSM3674
46 and WSM3686) showed them to be closely related to rhizobia that specifically
47 nodulated *Lupinus texensis* plants growing in Texas, USA (Andam & Parker, 2007).
48 According to the 16S rRNA phylogenetic tree, the *L. angolensis* and *L. texensis*
49 strains were most closely related to *Microvirga flocculans* (previously *Balneimonas*

50 *flocculans* (Weon *et al.*, 2010)), a species described from a strain isolated from a
51 Japanese hot spring (Takeda *et al.*, 2004). Currently, four other *Microvirga* species
52 have been named and characterized: *M. subterranea* (Kanso & Patel, 2003), *M.*
53 *guangxiensis* (Zhang *et al.*, 2009), *M. aerophila* and *M. aerilata* (Weon *et al.*, 2010),
54 isolated from Australian geothermal waters, Chinese rice field soil and Korean
55 atmospheric samples (two strains), respectively. No *Microvirga* strain has previously
56 been characterized as a legume symbiont.

57

58 The availability of four other authenticated *L. angolensis* strains ((Eagles & Date,
59 1999), together with the 28 *L. texensis* isolates, allowed us to provide a polyphasic
60 description of these novel rhizobia. We present here an analysis of the phylogenetic
61 relationships of representative strains, via the sequencing of rRNA and housekeeping
62 genes. The symbiotic genes that code for nodulation and nitrogen fixation have also
63 been examined, and their phylogeny determined. Additional phenotypic data is
64 provided to further clarify the taxonomic positions and to validly name and describe
65 species within this novel group of root nodule bacteria.

66

67 The strains used in this study are shown in Table 1. Type strains have been deposited
68 in the BCCM/LMG and HAMBI Culture Collections. The *L. angolensis* strains are
69 derivatives of strains housed in the CSIRO CB strain collection (Eagles & Date,
70 1999), reisolated according to the methods of Yates *et al.* (2007) and confirmed to be
71 different strains by PCR fingerprinting, using ERIC primers (Versalovic *et al.*, 1991).
72 Isolation of the *L. texensis* strains has been described previously (Andam & Parker,
73 2007). All strains were routinely subcultured at 28°C on YMA (Vincent, 1970), TY

74 (Beringer, 1974), or modified ½ lupin agar (½ LA) (Yates *et al.*, 2007) plates. Broth
75 cultures were incubated on a gyratory shaker at 200 rpm.

76

77 Nearly full length amplicons were obtained for the 16S rRNA gene of WSM3557^T
78 and WSM3693^T, following PCR amplification with the universal eubacterial primers
79 FGPS6 and FGPS1509 (Normand *et al.*, 1992). Amplicons were purified and
80 sequenced according to the methods of Yates *et al.* (2007). Amplification and
81 sequencing of the 16S rRNA genes of the remaining strains was performed as
82 previously described (Andam & Parker, 2007; Yates *et al.*, 2007). 16S rDNA
83 sequence identity comparisons were performed against sequences deposited in the
84 National Centre for Biotechnology Information GenBank database, using the
85 BLASTN algorithm (Altschul *et al.*, 1990). A phylogenetic tree was constructed using
86 the MEGA version 4.0 (Tamura *et al.*, 2007) neighbour-joining (NJ) (Saitou & Nei,
87 1987) and maximum parsimony methods and the Maximum Composite Likelihood
88 model and bootstrapped with 1000 replicates.

89

90 Alignment of a 1396 bp internal fragment of the 16S rRNA gene showed that the *L.*
91 *angolensis* and *L. texensis* strains shared at least 96.1 % sequence identity with the
92 type strains of all *Microvirga* species. Based on the 95 % 16S rRNA gene similarity
93 that has been proposed as a ‘practicable border zone for genus definition’ (Ludwig *et*
94 *al.*, 1998), the *L. angolensis* and *L. texensis* strains therefore belong within the genus
95 *Microvirga*. The phylogenetic tree (Fig. 1) demonstrates that *Microvirga* species,
96 including the *L. angolensis* and *L. texensis* strains, form a clade that is clearly
97 separated from *Methylobacterium*, *Bosea* and *Chelatococcus* lineages and supported

98 by high (100 %) bootstrap values. The threshold for bacterial strains to be considered
99 for separate species status is cited as being 97 % 16S rRNA shared sequence
100 similarity (Tindall *et al.*, 2010). The sequences of WSM3674 and WSM3686 were
101 identical and shared 99.9 % identity with WSM3557^T. These three strains shared
102 98.2-98.3 % sequence identity with the 100 % identical Lut5 and Lut6^T strains. *M.*
103 *flocculans* TFB^T was the most closely related species to this group, with 97.6-98.0 %
104 sequence identity. In contrast, WSM3693^T shared only 96.9 % sequence identity with
105 the other *L. angolensis* strains and was most closely related to *M. aerilata* 5420S-16^T,
106 with 98.8 % sequence identity. The 16S rRNA gene sequence identity therefore shows
107 that the *L. angolensis* and *L. texensis* strains merit consideration as novel species
108 within the genus *Microvirga*.

109

110 Portions of four housekeeping loci (*dnaK* [746 bp], *gyrB* [652 bp], *recA* [487 bp] and
111 *rpoB* [542 bp]) were sequenced in five symbiotic *Microvirga* strains and in two non-
112 symbiotic *Microvirga* species (*M. flocculans* TFB^T and *M. subterranea* DSM 14364^T)
113 to further investigate the validity of relationships suggested by 16S rRNA sequence
114 variation. Primers for the four loci are shown in Supplementary Table S1 (available in
115 IJSEM Online). The GenBank accession numbers for these sequences and those from
116 eleven reference strains are provided in Supplementary Table S2 (available in IJSEM
117 Online). As preliminary phylogenetic analysis indicated that trees for the four loci
118 were largely congruent, a combined analysis of concatenated sequences was
119 performed. The tree was inferred by MrBayes (Ronquist & Huelsenbeck, 2003) with
120 nucleotide sites partitioned by codon position and a HKY substitution model. The
121 program was run for a 250,000 generation burn-in period and then results were
122 sampled every 250 generations for an additional 250,000 generations.

123

124 The Bayesian tree for the concatenated sequences (*dnaK*, *gyrB*, *recA*, *rpoB*) indicated
125 that the seven analyzed *Microvirga* strains formed a strongly supported clade
126 (Supplementary Fig. S1, available in IJSEM Online). Within the *Microvirga* group,
127 the two non-symbiotic taxa (*M. flocculans*, *M. subterranea*) were interspersed among
128 the rhizobial strains, implying either that the non-symbiotic taxa are derived from
129 symbiotic ancestors, or that there have been multiple independent origins of legume
130 nodule symbiosis in the genus *Microvirga*. It is also noteworthy that the two African
131 symbiotic strains (WSM3557^T and WSM3693^T) did not cluster as each other's closest
132 relatives. Instead, strain WSM3557^T was placed as a closer relative of the North
133 American symbiotic strains (Lut5, Lut6^T and Llb5).

134

135 High quality DNA was prepared by the method of Wilson (1989), with minor
136 modifications (Cleenwerck *et al.*, 2002). DNA-DNA hybridizations were performed
137 using a microplate method and biotinylated probe DNA (Ezaki *et al.*, 1989). The
138 hybridization temperature was 49°C ± 1°C. Reciprocal reactions (A x B and B x A)
139 were performed for each DNA pair and their variation was within the limits of this
140 method (Goris *et al.*, 1998). The values presented are the means of a minimum of
141 three replicates. The DNA G+C content was determined for the strains Lut5, Lut6^T,
142 WSM3557^T and WSM3693^T using the HPLC method (Mesbah *et al.*, 1989).
143 DNA:DNA hybridization data (Supplementary Table S3, available in IJSEM Online)
144 confirmed that WSM3557^T, WSM3693^T, Lut6^T and *M. flocculans* LMG 25472^T
145 represent four separate species with low hybridization values to each other. Lut5 and
146 Lut6^T, with 97% DNA:DNA hybridization, could be considered members of the same
147 species. The DNA G+C content of strains Lut5, Lut6^T, WSM3557^T and WSM3693^T

148 ranged from 61.9-62.9 % (Supplementary Table S3, available in IJSEM Online),
149 which is consistent with values reported for other *Microvirga* species.

150

151 Fatty acid analysis was performed on Lut5, Lut6^T, WSM3557^T and WSM3693^T from
152 cells grown at 28°C for three days on plates containing Trypticase Soy Broth (BBL,
153 Becton Dickinson, USA) (30 g l⁻¹ in distilled water), supplemented with Bacto Agar
154 (Difco Laboratories, USA) (15 g l⁻¹). Reference strains were cultured on the same
155 standard medium at 28°C for 24 hours, according to the MIDI protocol
156 (http://www.microbialid.com/PDF/TechNote_101.pdf). The FAME extraction and
157 analysis was performed using the MIDI protocol, including standardization of the
158 physiological age by harvesting the overlap area of the second and third quadrant
159 from a quadrant streak. The obtained profiles were subsequently identified and
160 clustered using the Microbial Identification System software and MIDI TSBA
161 database version 5.0. Additionally, an Agilent Technologies 6890N gas
162 chromatograph (Santa Clara, CA USA) was used to obtain the FAME profiles.
163 Analysis of polar lipids was performed on cell culture grown on YMA (Vincent,
164 1970) for three days at 28°C. Polar lipids were extracted and separated using two-
165 dimensional thin-layer chromatography according to Tindall (1990a; 1990b). The
166 total lipid profiles were visualized by spraying with molybdato-phosphoric acid and
167 further characterized by spraying with ninhydrin (specific for amino groups),
168 molybdenum blue (specific for phosphates) and α -naphthol (specific for sugars). Cell
169 biomass for respiratory lipoquinone analysis was obtained from late log phase culture
170 grown in ½ LA broth. Lipoquinones were extracted from lyophilized biomass by a
171 modified one-phase Bligh/Dyer extraction method (Bligh & Dyer, 1959). Organic
172 phase extracts were dried under a gentle nitrogen stream and resolved in methanol.

173 Lipoquinones were initially detected using an APCI source connected to a Varian 320
174 MS (Agilent Technologies) using the selected reaction monitoring mode transitions
175 given in Geyer *et al.* (2004) under conditions optimized for a ubiquinone Q-10
176 standard (Sigma-Aldrich). Lipoquinones were subsequently quantified by high
177 performance liquid chromatography/electrospray/tandem mass spectrometry using a
178 Varian 212-LC equipped with a Varian Pursuit XRs 3 μ m DP 50 mm x 20 mm
179 column and a Varian 325 MS (Agilent Technologies), with 20 mM ammonium acetate
180 buffer in both the aqueous and organic components of the mobile phase. Ubiquinone
181 Q-10 was used as a standard.

182

183 The major cellular fatty acids were 18:1 w7c (52.58-53%) and 19:0 CYCLO w8c
184 (17.25-17.65%) for WSM3557^T and WSM3693^T and 18:1 w7c (68.94-69.71%) and
185 SF2 (15.41-16.06%) for Lut5 and Lut6^T (Supplementary Table S4, available in IJSEM
186 Online). Cellular fatty acid composition was similar for all *Microvirga* species. Polar
187 lipids for Lut6^T, WSM3557^T and WSM3693^T were highly similar (Supplementary
188 Fig. S2, available in IJSEM Online), with phosphatidylethanolamine (PE) and
189 phosphatidylcholine (PC) as the major components. Diphosphatidylglycerol (DPG),
190 phosphatidylglycerol (PG), phosphatidylmethylethanolamine (PME) and an
191 unknown phospholipid (PL) were detected in moderate amounts. These results
192 correlate well with the polar lipid description for species of *Microvirga*, except for the
193 presence of an unknown phospholipid (PL) instead of
194 phosphatidylmonomethylethanolamine (PME), as indicated by Weon *et al.* (2010).
195 Lut6^T, WSM3557^T and WSM3693^T all had highly similar respiratory lipoquinones.
196 For all strains, ubiquinone Q-10 was the major respiratory lipoquinone (approximately

197 97%), with ubiquinone Q-9 (approximately 2.5%) and ubiquinone Q-8 (approximately
198 0.5%) also present.

199

200 Colony morphology was studied on ½ LA plates. Strains were assessed for growth on
201 nutrient agar and Gram stained (Vincent, 1970). Motility of overnight ½ LA broth
202 culture was observed using a light microscope and the hanging drop method. To try to
203 induce motility in the Lut5 and Lut6^T strains, they were also grown, using a method
204 modified from Bowra & Dilworth (1981), on JMM minimal media plates (O'Hara *et*
205 *al.*, 1989) containing 0.1 mM succinate as a carbon source, 0.05 % (w/v) yeast extract,
206 0.1 mM EDTA and 0.3 % agar. One drop of 0.3 mM MgSO₄ solution was applied to
207 the edge of the resulting two-day-old culture and the cells resuspended by gentle
208 pipetting, then examined for motility as previously described. For electron
209 microscopy, resuspended cells were collected from overnight ½ LA slopes to which
210 100 µl of sterile deionized water had been added. Strains were examined for spore
211 formation by light microscopy after staining stationary phase broth and plate cultures
212 with malachite green (Beveridge *et al.*, 2007). Stationary phase cultures were also
213 heated to 70°C for 10 min, and then reinoculated onto fresh media and observed for
214 growth. Growth range and growth optima for temperature (10-50 °C, at intervals of 5
215 °C and 33-46 °C, at 1 °C intervals) and salt (0.0-3.0 % (w/v) NaCl at 0.5 %
216 increments) were determined with ½ LA or TY plate and broth cultures. Tolerance of
217 pH was assessed over the range of pH 4.0-10.0 at 0.5 unit intervals, following the
218 method of Nandasena *et al.* (2007), but on TY medium buffered with 20 mM
219 Homopipes (pH 4.0-5.0), MES (pH 5.5-6.0), HEPES (pH 7.0-8.5) or CHES (pH 9.0-
220 10.0). Anaerobic growth was tested on plates of Hugh & Leifson's medium (Hugh &
221 Leifson, 1953) supplemented with yeast extract (0.05 % (w/v)) and either glucose or

222 pyruvate as a carbon source, and incubated in an anaerobic jar (BBL GasPac 100
223 Non-vented system) at 28 °C for 10 days. Intrinsic antibiotic resistance was
224 determined on ½ LA plates containing ampicillin (50 and 100 µg ml⁻¹),
225 chloramphenicol (10, 20 and 40 µg ml⁻¹), gentamicin (10, 20 and 40 µg ml⁻¹),
226 kanamycin (50 and 100 µg ml⁻¹), nalidixic acid (50 and 100 µg ml⁻¹), rifampicin (50
227 and 100 µg ml⁻¹), spectinomycin (50 and 100 µg ml⁻¹), streptomycin (50 and 100 µg
228 ml⁻¹) or tetracycline (10 and 20 µg ml⁻¹).

229

230 Growth factor requirements and tests for growth on carbon substrates were performed
231 in JMM broths with NH₄Cl (10 mM) replacing glutamate as a nitrogen source. The
232 growth factors tested included yeast extract (at 0.05, 0.01, 0.005 or 0.001 % (w/v));
233 the standard JMM vitamin mix (biotin, thiamine and pantothenic acid); a complex B
234 group vitamin mixture required for growth of *Chelatococcus asaccharovorans* in
235 minimal media (Egli & Auling, 2005) and the B group vitamin mixture plus casamino
236 acids (0.01 % w/v). Strains were examined for growth on L-arabinose, D-cellobiose,
237 β-D-fructose, α-D-glucose, glycerol, D-mannitol, acetate, succinate (all at 20 mM
238 concentration), benzoate, *p*-hydroxybenzoate (both 3 mM), glutamate (10 mM),
239 methanol (0.5 %, v/v) and ethanol (20 mM) as sole carbon sources. Stock solutions of
240 carbon substrates (adjusted to pH 7.0 where necessary) were filter sterilized (0.2 µm
241 filter) and added to the autoclaved JMM medium (devoid of carbon source) prior to
242 inoculation. Inocula were prepared by washing stationary cultures twice with 0.89 %
243 (w/v) saline then resuspending cells in JMM medium devoid of carbon source. The
244 resuspended cells were added to duplicate 5 ml broths containing one of the carbon
245 substrates to a final OD_{600nm} of 0.05. Inoculated culture media were incubated for 14
246 days at 28 °C on a gyratory shaker before a visual assessment was made. Glassware

247 used to grow cultures was soaked in a 10 % (v/v) hydrochloric acid solution for at
248 least 24 h and rinsed twice in reverse osmosis deionized (RODI) water prior to use.
249 Utilization of 95 sole carbon substrates was assessed using Biolog GN2 microplates
250 (Biolog Inc, CA, USA). Strains were grown on R2A agar (Reasoner & Geldreich,
251 1985) at 37 °C for 24 hours, then resuspended in GN/GP inoculation fluid to a
252 concentration of 85 % ± 2 % transmittance. Cells (150 µl) were inoculated into the
253 microplate wells, incubated for 96 hours at 35 °C and colour development determined
254 at 595 nm with a Biorad 680 microplate reader.

255

256 API –20E (bioMérieux) test strips were used to determine utilization of various
257 substrates and acid production from sugars. Inocula were prepared from fresh plate
258 culture resuspended in sterile RODI water containing either vitamin solution (Egli &
259 Auling, 2005) or yeast extract (0.005 % (w/v)) for the *L. angolensis* and *L. texensis*
260 strains, respectively. Strips were prepared in accordance with the manufacturer's
261 protocols and read after incubation at 28 °C for 40 hours. Oxidase activity was
262 detected by applying fresh plate culture to filter paper impregnated with a solution of
263 1% (w/v) tetramethyl-p-phenylenediamine HCl and 0.1% (w/v) ascorbic acid.
264 Catalase activity was determined on fresh plate culture using 3% (v/v) hydrogen
265 peroxide solution. Tests for nitrate reduction were performed on cell cultures grown
266 for 24 hours at 28 °C in shaking TY broths supplemented with KNO₃ (1 g l⁻¹), using a
267 method modified from Kohlerschmidt *et al.* (2009), in which 0.8 % (w/v) 8-
268 aminonaphthalene-2-sulphonic acid (Cleve's acid) replaced 0.5 % (w/v) *N,N*-
269 dimethyl-1-naphthylamine. Determination of starch hydrolysis was performed on TY
270 agar supplemented with 0.4 % (w/v) soluble starch. Oxidative or fermentative
271 catabolism was determined according to the method of Hugh & Leifson (1953), with

272 the basal medium supplemented with yeast extract (0.05 %) and L-arabinose, α -D-
273 glucose or pyruvate as a carbon source. Cultures were examined for growth and
274 colour change in the medium after incubation at 37 °C for 48 hours.

275

276 Electron micrographs of the *L. angolensis* and *L. texensis* strains showed rod shaped
277 cells, surrounded by a capsule (Supplementary Fig. S3, available in IJSEM Online).

278 Lut5 and Lut6^T did not possess flagella. On Biolog GN2 microplates, the carbon
279 sources oxidized by the *L. angolensis* and *L. texensis* strains spanned most of the 11
280 designated carbon source categories (Garland & Mills, 1991), with none of the
281 polymer, alcohol, phosphorylated chemical or amine substrates being oxidized. The
282 range of substrates oxidized within each category was, however, quite narrow. Only 9
283 of 28 carbohydrates and 7 of 24 carboxylic acids gave positive results. Oxidation of
284 amino acids varied according to strain, with 12 of the possible 20 amino acid sources
285 being utilized by at least one strain. Results for the full list of substrates are given in
286 Supplementary Table S5, available in IJSEM Online. Detailed phenotypic
287 characteristics are given in the species descriptions.

288

289 A nearly full-length portion of the *nodA* gene (562 bp) of WSM3557^T and
290 WSM3693^T was amplified using primers reported by Haukka *et al.* (1998). PCR
291 cycling conditions were as follows: four minutes at 94°C, followed by 35 cycles of 45
292 s at 94°C, 45 s at 55°C and 2 min at 68°C, and finally 1 cycle of 5 min at 70°C. The
293 resulting amplicon was sequenced using the *nodA* primers in a BigDye Terminator 3.1
294 (Applied Biosystems) PCR reaction, performed according to the manufacturer's
295 instructions. These sequences were aligned with *nodA* data from Lut6^T and from 29

296 other strains of nodule bacteria encompassing 20 species in eight genera. A
297 phylogenetic tree was inferred by MrBayes (Ronquist & Huelsenbeck, 2003),
298 according to the parameters described for the housekeeping loci. The Bayesian
299 phylogenetic analysis indicated that *Microvirga nodA* sequences were derived from
300 two different sources (Supplementary Fig. S4, available in IJSEM Online).
301 WSM3557^T and WSM3693^T had *nodA* genes that clustered in a strongly supported
302 clade (posterior probability of 1.0) with reference strains in the genera
303 *Bradyrhizobium*, *Burkholderia* and *Methylobacterium*. The *nodA* sequence from
304 Lut6^T was placed in an equally strongly supported clade with reference strains in the
305 genera *Rhizobium*, *Mesorhizobium* and *Sinorhizobium*. These results suggest that
306 *Microvirga nodA* genes were acquired in two separate horizontal gene transfer events
307 from distantly related donor lineages.

308

309 Portions of two genes encoding proteins involved with nitrogen fixation (*nifD* [491
310 bp] and *nifH* [388 bp]) were sequenced in five symbiotic *Microvirga* strains using
311 primers reported in Andam and Parker (2007). Fourteen Alphaproteobacterial taxa
312 with completed genome sequences, and three additional strains with both *nifD* and
313 *nifH* data in GenBank were used as references. A combined analysis of concatenated
314 *nifD* and *nifH* sequences was performed to provide an overview of relationships for
315 these nitrogen fixation genes. A phylogenetic tree was inferred by MrBayes,
316 according to the parameters described for the housekeeping loci. Bayesian analysis of
317 concatenated sequences for *nifD* and *nifH* showed a rather different pattern of
318 relationship from *nodA* (Supplementary Fig. S5, available in IJSEM Online).
319 Symbiotic *Microvirga* strains from both Africa and North America clustered into a
320 single well-supported group with affinities to *Rhizobium etli* CFN42^T. This group was

321 nested within a larger clade comprised of *Rhizobium*, *Mesorhizobium* and
322 *Sinorhizobium* strains. Because *Microvirga* is not a close relative of *Rhizobium*
323 according to the housekeeping gene loci (Supplementary Fig. S1), the close affinity of
324 *Microvirga nif* genes to those of *Rhizobium* (and related genera) suggests that these
325 genes were acquired through horizontal transfer.

326

327 Previous reports indicate that *L. angolensis* and *L. texensis* strains have a narrow host
328 range (Andam & Parker, 2007; Yates *et al.*, 2007). Inoculation of strains onto legume
329 hosts in a closed vial or open pot system was performed according to the methods of
330 Yates *et al.* (2007). Rhizobia were re-isolated from nodules and confirmed to be the
331 inoculant strain by PCR fingerprinting, using ERIC primers (Versalovic *et al.*, 1991).

332 WSM3557^T and WSM3693^T were unable to nodulate *Crotalaria juncea*, *Indigofera*
333 *patens*, *Lotus corniculatus*, *Lupinus angustifolius*, or *Macroptilium atropurpureum*.

334 WSM3693^T elicited and was reisolated from non-fixing nodules on the promiscuous
335 hosts *Acacia saligna*, *Phaseolus vulgaris* and *Vigna unguiculata* (Amrani *et al.*, 2010;

336 Broughton *et al.*, 2000) and on the South African *Indigofera frutescens*. WSM3557^T
337 was also able to form ineffective nodules on some *P. vulgaris* plants, but could not be

338 reisolated. Lut5 and Lut6^T were unable to nodulate *L. angolensis*, *Listia bainesii*,
339 *Listia heterophylla*, *Lotus corniculatus*, *Lupinus angustifolius* or *V. unguiculata*, but

340 formed ineffective nodules on *A. saligna* and *P. vulgaris*. Reisolates were obtained

341 only for Lut5 from an *A. saligna* nodule. Both WSM3557^T and WSM3693^T were able

342 to ineffectively nodulate *Lupinus texensis*. WSM3557^T was the most effective strain

343 for nitrogen fixation on *L. angolensis* (J. Ardley, unpublished data).

344

345 The ability to nodulate and fix nitrogen with legumes is a characteristic that
346 distinguishes Lut6^T, WSM3557^T and WSM3693^T from all previously described
347 *Microvirga* species. Additionally these strains can be clearly distinguished from other
348 *Microvirga* species by a number of phenotypic characteristics, in particular growth on
349 sole carbon substrates, mean generation time, weak production of acetoin and
350 antibiotic resistance (Table 2). Lut6^T can be differentiated from WSM3557^T and
351 WSM3693^T on the basis of motility and pigmentation and by means of its smaller
352 amounts of 16:00 and larger amounts of 18:1 w7c. WSM3693^T differs from
353 WSM3557^T in its lack of pigmentation, lower optimum growth temperature, higher
354 amounts of summed feature 2 and by its ability to grow on *p*-hydroxybenzoate.

355

356 In conclusion, the genotypic, phenotypic, and chemotaxonomic data presented here
357 support the classification of the *L. texensis* and *L. angolensis* strains as three novel
358 rhizobial species in the genus *Microvirga*. The names *M. lupini* sp. nov., *M*
359 *lotononidis* sp. nov. and *M. zambiensis* sp. nov. are proposed, with the isolates Lut6^T,
360 WSM3557^T and WSM3693^T representing the respective type strains.

361

362 **Emended description of *Microvirga* (Kanso & Patel, 2003 emend.**
363 **Zhang *et al.* 2009, emend. Weon *et al.* 2010)**

364 The description remains as given by Kanso & Patel (2003), Zhang *et al.* (2009) and
365 Weon *et al.* (2010), with the following modifications. Contains moderate amounts of
366 phosphatidylmethylethanolamine or phospholipid. Some strains are capable of
367 nodulation and symbiotic nitrogen fixation with legumes. The type species is
368 *Microvirga subterranea*.

369 **Description of *Microvirga lupini* sp. nov.**

370 *Microvirga lupini* (lu.pi'ni. L. n. lupinus, a lupine and also a botanical generic name
371 (*Lupinus*); L. gen. n. lupini, of *Lupinus*, isolated from *Lupinus texensis*.

372

373 Cells are strictly aerobic, asporogenous, Gram-negative non-motile rods (0.4-0.5 x
374 1.0-2.2 μ m). Grows well on YMA, ½ lupin agar, TY agar and nutrient agar. On ½ LA
375 after three days at 28 °C, colonies are pale orange, convex, smooth and circular, with
376 entire margins, 0.5-1.5 mm in diameter. Grows from 10-43 °C; optimum temperature
377 is 39 °C and mean generation time at this temperature is 1.8 hours. Best growth is at
378 pH 7.0-8.5 (range 5.5-9.5) and 0.0-0.5 % (w/v) NaCl (range 0-1.5 % (w/v)). Yeast
379 extract is an absolute requirement for growth in minimal media. The main cellular
380 fatty acids are 18:1 ω 7c and summed feature 2 (16:1 iso I / 14:0 3 OH / unknown
381 10.938). Ubiquinone Q-10 is the major respiratory lipoquinone. Positive for catalase
382 and urease and weakly positive for tryptophan deaminase and acetoin production.
383 Oxidase, β -galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine
384 decarboxylase, indole and hydrogen sulphide production are negative, as is utilization
385 of citrate. Gelatin and starch are not hydrolysed. Nitrite is not produced from nitrate.
386 Acid is produced from growth on L-arabinose but not from growth on α -D-glucose or
387 D-mannitol. Partially resistant to ampicillin, chloramphenicol, gentamicin and
388 streptomycin and sensitive to kanamycin, nalidixic acid, rifampicin, spectinomycin
389 and tetracycline. Assimilates L-arabinose, D-cellobiose, D-fructose, α -D-glucose, D-
390 mannitol, acetate, succinate, glutamate, ethanol and *p*-hydroxybenzoate. The G + C
391 content of the type strain is 61.9 %.

392

393 The type strain, Lut6^T (= LMG26460^T = HAMBI 3236) and other strains were
394 isolated from N₂-fixing nodules of *Lupinus texensis* collected in Texas, USA.

395 **Description of *Microvirga lotononidis* sp. nov.**

396 *Microvirga lotononidis* (lo.to.no'ni.dis. N.L. gen. n. lotononidis, of *Lotononis*, a taxon
397 of leguminous plants, referring to the isolation source of the first strains, nodules of
398 *Listia angolensis*, a species in the *Lotononis s. l.* clade.

399

400 Cells are strictly aerobic, asporogenous, Gram-negative rods (0.4-0.5 x 1.0-2.2 µm),
401 motile with one or more polar flagella. Grows well on YMA, ½ lupin agar, TY agar
402 and nutrient agar. On ½ LA after three days at 28 °C, colonies are light pink, convex,
403 smooth, mucilaginous and circular, with entire margins, 0.5-1.5 mm in diameter.
404 Grows from 15-44/45 °C; optimum temperature for the type strain is 41 °C and mean
405 generation time at this temperature is 1.6 hours. Best growth is at pH 7.0-8.5 (range
406 5.5-9.5), and 0.0-1.0 % (w/v) NaCl (range 0-2.0 % (w/v)). Yeast extract or the vitamin
407 mix detailed in Egli and Auling (2005) is an absolute requirement for growth in
408 minimal media. The main cellular fatty acids are 18:1 ω7c and 19:0 cyclo ω8c.
409 Ubiquinone Q-10 is the major respiratory lipoquinone. Positive for catalase and
410 urease and weakly positive for tryptophan deaminase and acetoin production.
411 Oxidase, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine
412 decarboxylase, indole and hydrogen sulphide production are negative, as is utilization
413 of citrate. Gelatin and starch are not hydrolysed. Nitrite is produced from nitrate. Acid
414 is produced from growth on L-arabinose but not from growth on α-D-glucose or D-
415 mannitol. Resistant to gentamicin and some strains are partially resistant to ampicillin,
416 chloramphenicol, kanamycin and spectinomycin. Sensitive to nalidixic acid,
417 rifampicin, streptomycin and tetracycline. Assimilates L-arabinose, D-cellobiose, D-

418 fructose, α -D-glucose, glycerol, D-mannitol, acetate, succinate and glutamate. The G
419 + C content of the type strain is 62.8-63.0 %.

420

421 The type strain, WSM3557^T (= LMG26455^T = HAMBI 3237) and other strains were
422 isolated from N₂-fixing nodules of *Listia angolensis* originally collected in Zambia.

423 **Description of *Microvirga zambiensis* sp. nov.**

424 *Microvirga zambiensis* (zam.bi.en'sis. N.L. fem. adj. zambiensis, of or belonging to
425 Zambia, from where the type strain was isolated).

426

427 Cells are strictly aerobic, asporogenous, Gram-negative rods (0.4-0.5 x 1.0-2.2 μ m),
428 motile with one or more polar flagella. Grows well on YMA, ½ lupin agar, TY agar
429 and nutrient agar. On ½ LA after three days at 28°C, colonies are cream coloured,
430 convex, smooth, mucilaginous and circular, with entire margins, 0.5-1.5 mm in
431 diameter. Grows from 15-38 °C; optimum temperature is 35 °C and mean generation
432 time at this temperature is 1.7 hours. Best growth is at pH 7.0-8.5 (range 6.0-9.5) and
433 0.0-0.5 % (w/v) NaCl (range 0-1.5 % (w/v)). Yeast extract or the vitamin mix detailed
434 in Egli and Auling (2005) is an absolute requirement for growth in minimal media.
435 The main cellular fatty acids are 18:1 ω 7c and 19:0 cyclo ω 8c. Ubiquinone Q-10 is
436 the major respiratory lipoquinone. Positive for catalase and urease and weakly
437 positive for acetoin production. Oxidase, β -galactosidase, arginine dihydrolase, lysine
438 decarboxylase, ornithine decarboxylase, tryptophan deaminase, indole and hydrogen
439 sulphide production are negative, as is utilization of citrate. Gelatin and starch are not
440 hydrolysed. Nitrite is produced from nitrate. Acid is produced from growth on L-
441 arabinose but not from growth on α -D-glucose or D-mannitol. Resistant to

442 gentamicin. Sensitive to ampicillin, chloramphenicol, kanamycin nalidixic acid,
443 rifampicin, spectinomycin, streptomycin and tetracycline. Assimilates L-arabinose, D-
444 cellobiose, D-fructose, α -D-glucose, glycerol, D-mannitol, acetate, succinate, *p*-
445 hydroxybenzoate and glutamate. The G + C content of the type strain is 62.6 %.

446

447 The type strain, WSM3693^T (= LMG26454^T = HAMBI 3238) was isolated from N₂-
448 fixing nodules of *Listia angolensis* originally collected in Zambia.

449 **Acknowledgements**

450 The authors would like to thank Regina Carr, Catherine Rawlinson and Gordon
451 Thompson (School of Biological Sciences and Biotechnology, Murdoch University)
452 for skilled technical assistance, Dr Bharat Patel for kindly providing *Microvirga*
453 *subterranea* strain FaiI4, Dr Alison McInnes for assistance in sourcing the *L.*
454 *angolensis* strains and Dr Judith Maitland (University of Western Australia) for help
455 with the Latin grammar. J.A. is the recipient of a Murdoch University Research
456 Scholarship.

References

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.
- Amrani, S., Noureddine, N.-E., Bhatnagar, T., Argandoña, M., Nieto, J. J. & Vargas, C. (2010). Phenotypic and genotypic characterization of rhizobia associated with *Acacia saligna* (Labill.) Wendl. in nurseries from Algeria. *Systematic and Applied Microbiology* **33**, 44-51.
- Andam, C. P., Mondo, S. J. & Parker, M. A. (2007). Monophyly of *nodA* and *nifH* genes across Texas and Costa Rican populations of *Cupriavidus* nodule symbionts. *Applied and Environmental Microbiology* **73**, 4686-4690.
- Andam, C. P. & Parker, M. A. (2007). Novel Alphaproteobacterial root nodule symbiont associated with *Lupinus texensis*. *Applied and Environmental Microbiology* **73**, 5687-5691.
- Auling, G., Busse, H.-J., Egli, T., El-Banna, T. & Stackebrandt, E. (1993). Description of the Gram-negative, obligately aerobic, nitritotriacetate (NTA)-utilizing bacteria as *Chelatobacter heintzii*, gen.nov., sp.nov., and *Chelatococcus asaccharovorans*, gen.nov., sp.nov. *Systematic and Applied Microbiology* **16**, 104-112.
- Barcellos, F. G., Menna, P., da Silva Batista, J. S. & Hungria, M. (2007). Evidence of horizontal transfer of symbiotic genes from a *Bradyrhizobium japonicum* inoculant strain to indigenous diazotrophs *Sinorhizobium (Ensifer) fredii* and *Bradyrhizobium elkanii* in a Brazilian savannah soil. *Applied and Environmental Microbiology* **73**, 2635-2643.
- Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology* **84**, 188-198.
- Beveridge, T. J., Lawrence, J. R. & Murray, R. G. E. (2007). Sampling and staining for light microscopy. In *Methods for General and Molecular Microbiology*, pp. 19-33. Edited by C. A. Reddy, T. J. Beveridge, J. A. Breznak, T. M. Marzluf, T. M. Schmidt & L. R. Snyder. Washington, D.C.: American Society for Microbiology Press.
- Bligh, E. H. & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911-917.
- Boatwright, J. S., Wink, M. & van Wyk, B.-E. (2011). The generic concept of *Lotononis* (Crotalariaeae, Fabaceae): Reinstatement of the genera *Euchlora*, *Leobordea* and *Listia* and the new genus *Ezoloba*. *Taxon* **60**, 161-177.
- Bowra, B. J. & Dilworth, M. J. (1981). Motility and chemotaxis towards sugars in *Rhizobium leguminosarum*. *Journal of General Microbiology* **126**, 231-235.
- Broughton, W. J., Jabbouri, S. & Perret, X. (2000). Keys to symbiotic harmony. *Journal of Bacteriology* **182**, 5641-5652.
- Cleenwerck, I., Vandemeulebroecke, K., Janssens, D. & Swings, J. (2002). Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **52**, 1551-1558.
- Cummings, S. P., Gyaneshwar, P., Vinuesa, P., Farruggia, F. T., Andrews, M., Humphry, D., Elliott, G. N., Nelson, A., Orr, C., Pettitt, D., Shah, G. R., Santos, S. R., Krishnan, H. B., Odee, D., Moreira, F. M. S., Sprent, J. I., Young, J. P. W. & James, E. K. (2009). Nodulation of *Sesbania* species by

- Rhizobium* (*Agrobacterium*) strain IRBG74 and other rhizobia. *Environmental Microbiology* **11**, 2510-2525.
- Eagles, D. A. & Date, R. A. (1999).** *The CB Rhizobium/Bradyrhizobium Strain Collection. Genetic Resources Communication No. 30.* St Lucia, Queensland, Australia: CSIRO Tropical Agriculture.
- Egli, T. W. & Auling, G. (2005).** Genus II. *Chelatococcus* In *Bergey's Manual of Systematic Bacteriology* pp. 433-437. Edited by D. J. Brenner, N. R. Krieg & J. T. Staley. New York: Springer.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989).** Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *International Journal of Systematic Bacteriology* **39**, 224-229.
- Garland, J. L. & Mills, A. L. (1991).** Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied and Environmental Microbiology* **57**, 2351-2359.
- Geyer, R., Peacock, A. D., White, D. C., Lytle, C. & Van Berkel, G. J. (2004).** Atmospheric pressure chemical ionization and atmospheric pressure photoionization for simultaneous mass spectrometric analysis of microbial respiratory ubiquinones and menaquinones. *Journal of Mass Spectrometry* **39**, 922-929.
- Goris, J., Suzuki, K., De Vos, P., Nakase, T. & Kersters, K. (1998).** Evaluation of a microplate DNA - DNA hybridization method compared with the initial renaturation method. *Canadian Journal of Microbiology* **44**, 1148-1153.
- Haukka, K., Lindström, K. & Young, J. P. W. (1998).** Three phylogenetic groups of *nodA* and *nifH* genes in *Sinorhizobium* and *Mesorhizobium* isolates from leguminous trees growing in Africa and Latin America. *Applied and Environmental Microbiology* **64**, 419-426.
- Hugh, R. & Leifson, E. (1953).** The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. *Journal of Bacteriology* **66**, 24-26.
- Kanso, S. & Patel, B. K. C. (2003).** *Microvirga subterranea* gen. nov., sp. nov., a moderate thermophile from a deep subsurface Australian thermal aquifer. *International Journal of Systematic and Evolutionary Microbiology* **53**, 401-406.
- Kohlerschmidt, D. J., Musser, K. A. & Dumas, N. B. (2009).** Identification of aerobic Gram-negative bacteria In *Practical handbook of microbiology* 2nd edition edn, pp. 67-79. Edited by E. Goldman & L. H. Green. Boca Raton CRC Press.
- Lin, D. X., Wang, E. T., Tang, H., Han, T. X., He, Y. R., Guan, S. H. & Chen, W. X. (2008).** *Shinella kummerowiae* sp nov., a symbiotic bacterium isolated from root nodules of the herbal legume *Kummerowia stipulacea*. *International Journal of Systematic and Evolutionary Microbiology* **58**, 1409-1413.
- Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. & Schleifer, K. H. (1998).** Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**, 554-568.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989).** Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid

- chromatography. *International Journal of Systematic Bacteriology* **39**, 159-167.
- Nandasena, K. G., O'Hara, G. W., Tiwari, R. P., Sezmiş, E. & Howieson, J. G. (2007).** *In situ* lateral transfer of symbiosis islands results in rapid evolution of diverse competitive strains of mesorhizobia suboptimal in symbiotic nitrogen fixation on the pasture legume *Biserrula pelecinus* L. *Environmental Microbiology* **9**, 2496-2511.
- Normand, P., Cournoyer, B., Simonet, P. & Nazaret, S. (1992).** Analysis of a ribosomal RNA operon in the actinomycete *Frankia*. *Gene* **111**, 119-124.
- O'Hara, G. W., Goss, T. J., Dilworth, M. J. & Glenn, A. R. (1989).** Maintenance of intracellular pH and acid-tolerance in *Rhizobium meliloti*. *Applied and Environmental Microbiology* **55**, 1870-1876.
- Reasoner, D. J. & Geldreich, E. E. (1985).** A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology* **49**, 1-7.
- Rivas, R., Velázquez, E., Willems, A., Vizcaíno, N., Subba-Rao, N. S., Mateos, P. F., Gillis, M., Dazzo, F. B. & Martínez-Molina, E. (2002).** A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (L.f.) Druce. *Applied and Environmental Microbiology* **68**, 5217-5222.
- Ronquist, F. & Huelsenbeck, J. P. (2003).** MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572-1574.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406-425.
- Sullivan, J. T., Patrick, H. N., Lowther, W. L., Scott, D. B. & Ronson, C. W. (1995).** Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 8985-8989.
- Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., de Lajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C. & Dreyfus, B. (2001).** Methylophilic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *Journal of Bacteriology* **183**, 214-220.
- Takeda, M., Suzuki, I. & Koizumi, J. I. (2004).** *Balneomonas flocculans* gen. nov., sp. nov., a new cellulose-producing member of the α -2 subclass of *Proteobacteria*. *Systematic and Applied Microbiology* **27**, 139-145.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007).** MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596-1599.
- Tindall, B. J. (1990a).** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Systematic and Applied Microbiology* **13**, 128-130.
- Tindall, B. J. (1990b).** Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiology Letters* **66**, 199-202.
- Tindall, B. J., Rosselló-Móra, R., Busse, H.-J., Ludwig, W. & Kämpfer, P. (2010).** Notes on the characterization of prokaryote strains for taxonomic purposes. *International Journal of Systematic and Evolutionary Microbiology* **60**, 249-266.
- Trujillo, M. E., Willems, A., Abril, A., Planchuelo, A.-M., Rivas, R., Ludeña, D., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2005).** Nodulation of

- Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. *Applied and Environmental Microbiology* **71**, 1318-1327.
- Versalovic, J., Koeuth, T. & Lupski, R. (1991).** Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19**, 6823-6831.
- Vincent, J. M. (1970).** *A Manual for the Practical Study of the Root-Nodule Bacteria*. Oxford, England: Blackwell Science Publications.
- Weon, H.-Y., Kwon, S.-W., Son, J.-A., Jo, E.-H., Kim, S.-J., Kim, Y.-S., Kim, B.-Y. & Ka, J.-O. (2010).** Description of *Microvirga aerophila* sp. nov. and *Microvirga aerilata* sp. nov., isolated from air, reclassification of *Balneimonas flocculans* Takeda et al. 2004 as *Microvirga flocculans* comb. nov. and emended description of the genus *Microvirga*. *International Journal of Systematic and Evolutionary Microbiology* **60**, 2596-2600.
- Wilson, K. (1989).** Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, pp. 2.4.1-2.4.2. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: John Wiley & Sons.
- Yates, R. J., Howieson, J. G., Reeve, W. G., Nandasena, K. G., Law, I. J., Bräu, L., Ardley, J. K., Nistelberger, H. M., Real, D. & O'Hara, G. W. (2007).** *Lotononis angolensis* forms nitrogen fixing, lupinoid nodules with phylogenetically unique, fast-growing, pink-pigmented bacteria, which do not nodulate *L. bainesii* or *L. listii*. *Soil Biology & Biochemistry* **39**, 1680-1688.
- Zhang, J., Song, F., Xin, Y. H., Zhang, J. & Fang, C. (2009).** *Microvirga guangxiensis* sp. nov., a novel Alphaproteobacterium from soil, and emended description of the genus *Microvirga*. *International Journal of Systematic and Evolutionary Microbiology* **59**, 1997-2001.

Table 1. List of strains.

Strain	Synonym (Derived from)	Host	Geographical source (Collector)	Reference or source
Llb5 <i>Microvirga lupini</i>		<i>Lupinus texensis</i>	Texas, USA	This study
Lut5 <i>Microvirga lupini</i>		<i>Lupinus texensis</i>	Texas, USA	Andam & Parker (2007)
Lut6 ^T <i>Microvirga lupini</i>		<i>Lupinus texensis</i>	Texas, USA	Andam & Parker (2007)
WSM3557 ^T <i>Microvirga lotononidis</i>	(CB1322)	<i>Listia angolensis</i>	Chibala/Fort Jameson Zambia (Verboom)	Eagles & Date (1999)
WSM3674 <i>Microvirga lotononidis</i>	(CB1323)	<i>Listia angolensis</i>	Chibala/Fort Jameson Zambia (Verboom)	Eagles & Date (1999) Yates <i>et al.</i> (2007)
WSM3686 <i>Microvirga lotononidis</i>	(CB1297)	<i>Listia angolensis</i>	Chibala/Fort Jameson Zambia (Verboom)	Eagles & Date (1999) Yates <i>et al.</i> (2007)
WSM3693 ^T <i>Microvirga zambiensis</i>	(CB1298)	<i>Listia angolensis</i>	Chibala/Fort Jameson Zambia (Verboom)	Eagles & Date (1999)
TFB ^T <i>Microvirga flocculans</i>	LMG 25472 ^T		Gunma Prefecture Japan	Takeda <i>et al.</i> (2004)
TE2 ^T <i>Chelatococcus asaccharovorans</i>	LMG 25503 ^T		Switzerland	Auling <i>et al.</i> (1993)
FaiI4 ^T <i>Microvirga subterranea</i>	LMG 25504 ^T DSM 14364 ^T		Great Artesian Basin Australia	Kanso & Patel (2003)

Table 2. Differentiating phenotypic characteristics of the novel strains Lut6^T, WSM3557^T and WSM3693^T and the type strains of closely related species of the genus *Microvirga*.

Strains: 1, *M. lupini* sp. nov. Lut6^T; 2, *M. lotononidis* sp. nov. WSM3557^T; 3, *M. zambiensis* sp. nov. WSM3693^T; 4, *M. flocculans* TFB^T (Takeda *et al.*, 2004); 5, *M. subterranea* FaiI4^T (Kanso & Patel, 2003); 6, *M. guangxiensis* 25B^T (Zhang *et al.*, 2009); 7, *M. aerophila* 5420S-12^T (Weon *et al.*, 2010); 8, *M. aerilata* 5420S-16^T (Weon *et al.*, 2010). All strains are rod-shaped, strictly aerobic and positive for catalase but negative for arginine dihydrolase and indole production. (+ = positive, w = weak, - = negative, ND = not determined)

Characteristic	1	2	3	4	5	6	7	8
Isolation source	Root nodule	Root nodule	Root nodule	Hot spring	Thermal aquifer	Soil	Air	Air
Colony	Pale orange	Light pink, mucilaginous	Cream, mucilaginous	White, rough	Light pink, smooth,	Light pink, smooth,	Light pink, smooth	Light pink, smooth
Flagella	Non-motile	Polar flagella	Polar flagella	Polar flagella	Non-motile	Non-motile	Non-motile	Non-motile
Cell size (µm)	0.4 - 0.5 x 1.0 - 2.2	0.4 - 0.5 x 1.0 - 2.2	0.4 - 0.5 x 1.0 - 2.2	0.5 - 0.7 x 1.5 - 3.5	1 x 1.5 - 4.0	0.6 - 0.8 x 1.3 - 2.1	0.8 - 1.1 x 1.6 - 4.2	1.2 - 1.5 x 1.6 - 3.3
Optimum temp (°C)	39	41	35	40 - 45	41	37	ND	ND
Growth range (°C)	10 - 43	15 - 44	15 - 38	20 - 45*	25 - 45	16 - 42	10 - 35	10 - 35
MGT	1.8 hrs	1.6 hrs	1.7 hrs	ND	4.5 hrs	230 min	ND	ND
Optimum pH	7.0 - 8.5	7.0 - 8.5	7.0 - 8.5	7.0	7.0	7.0	ND	ND
PH growth range	5.5 - 9.5	5.5 - 9.5	6.0 - 9.5	ND	6 - 9*	5.0 - 9.5	7.0 - 10.0	7.0 - 10.0
Optimum NaCl %	0.0 - 0.5	0.0 - 1.0	0.0 - 0.5	ND	0	ND	ND	ND
NaCl growth range (%)	0 - 1.5	0 - 2.0	0 - 2.0	0 - 1.5*	0 - 1%	0 - 2.0	0 - 2.0	0 - 3.0
Growth supplement required	Yeast extract	Vitamins or yeast extract	Vitamins or yeast extract	No	Yeast extract	No	ND	ND
Antibiotic sensitivity	Gm ^R	Gm ^R	Gm ^R	ND	Vm ^R	Azt ^R Ery ^R Km ^R	ND	ND
DNA G+C content (% mol)	61.9	62.9± 0.1	62.6	64	63.5 ± 0.5	64.3	62.2	61.5
Symbiotic nitrogen fixation	Yes	Yes	Yes	ND	ND	ND	ND	ND

Table 2 (cont.)

Characteristic	1	2	3	4	5	6	7	8
Carbon sources utilized								
L-Arabinose	+	+	+	ND	ND	ND	-	-
D-Cellobiose	+	+	+	ND	-	-	-	-
D-Fructose	+	+	+	-	-	-	ND	ND
α -D-Glucose	+	+	+	-	-	+	-	-
Succinate	+	+	+	-	-	ND	ND	ND
Ethanol	+	-	-	-	-	-	ND	ND
Glycerol	-	+	+	-	-	-	ND	ND
Mannitol	+	+	+	-	ND	+	-	-
<i>p</i> -Hydroxybenzoate	+	-	+	-	ND	ND	ND	ND
Hydrolysis of gelatin	-	-	-	+	+	-	-	W
Hydrolysis of starch	-	-	-	-	-	-	+	+
Acid production from α -D-Glucose	-	-	-	-	W	-	ND	ND
Oxidase	-	-	-	+	-	+	+	+
Urease	+	+	+	-	-	+	-	-
Tryptophan deaminase	W	W	-	ND	-	ND	ND	ND
Acetoin production	W	W	W	-	-	-	ND	ND
Nitrate reduction	-	+	+	-	+	+	-	-

Azt = aztreonam; Ery = erythromycin; Gm = gentamicin; Km = kanamycin; Vm = vancomycin

* Data taken from Weon *et al.* (2010)

Figure legends:

Fig. 1. NJ phylogenetic tree based on a comparative analysis of 16S rRNA gene sequences, showing the relationships between novel symbiotic *Microvirga* strains (indicated in bold) and closely related species. Numbers at the nodes of the tree indicate bootstrap values (expressed as percentages of 1000 replications). GenBank accession numbers are given in parentheses. *Bradyrhizobium japonicum* USDA 6^T was used as an outgroup. Scale bar for branch lengths shows 0.01 substitutions per site.

Supplementary Figure legends:

Supplementary Fig. S1. Bayesian tree for concatenated sequences of *dnaK*, *gyrB*, *recA*, *rpoB* (2427 bp) from seven *Microvirga* strains and eleven Alphaproteobacterial reference taxa. Posterior probabilities are listed above branches. Scale bar for branch lengths shows 0.05 substitutions per site.

Supplementary Fig. S2. Two-dimensional thin layer chromatography of polar lipids of strains Lut6^T (a), WSM3557^T (b) and WSM3693^T. DPG: diphosphatidylglycerol, PG: phosphatidylglycerol, PE: phosphatidylethanolamine, PDE: phosphatidylmethylethanolamine, PC: phosphatidylcholine, PL: unknown phospholipid.

Supplementary Fig. S3. Transmission electron micrograph of strain WSM3693^T grown overnight on a ½ LA slope

Supplementary Fig. S4. Bayesian tree for *nodA* sequences (594 bp) from three symbiotic *Microvirga* strains and 29 proteobacterial reference taxa. The posterior probability was 1.0 for 23 of the 29 internal branches of the tree; for the six other branches, the posterior probability is listed on the tree. Scale bar for branch lengths shows 0.05 substitutions per site.

Supplementary Fig. S5. Bayesian tree for concatenated sequences of *nifD* and *nifH* (879 bp) from five *Microvirga* strains and 17 Alphaproteobacterial reference taxa. Posterior probabilities are listed above branches. Scale bar for branch lengths shows 0.05 substitutions per site.

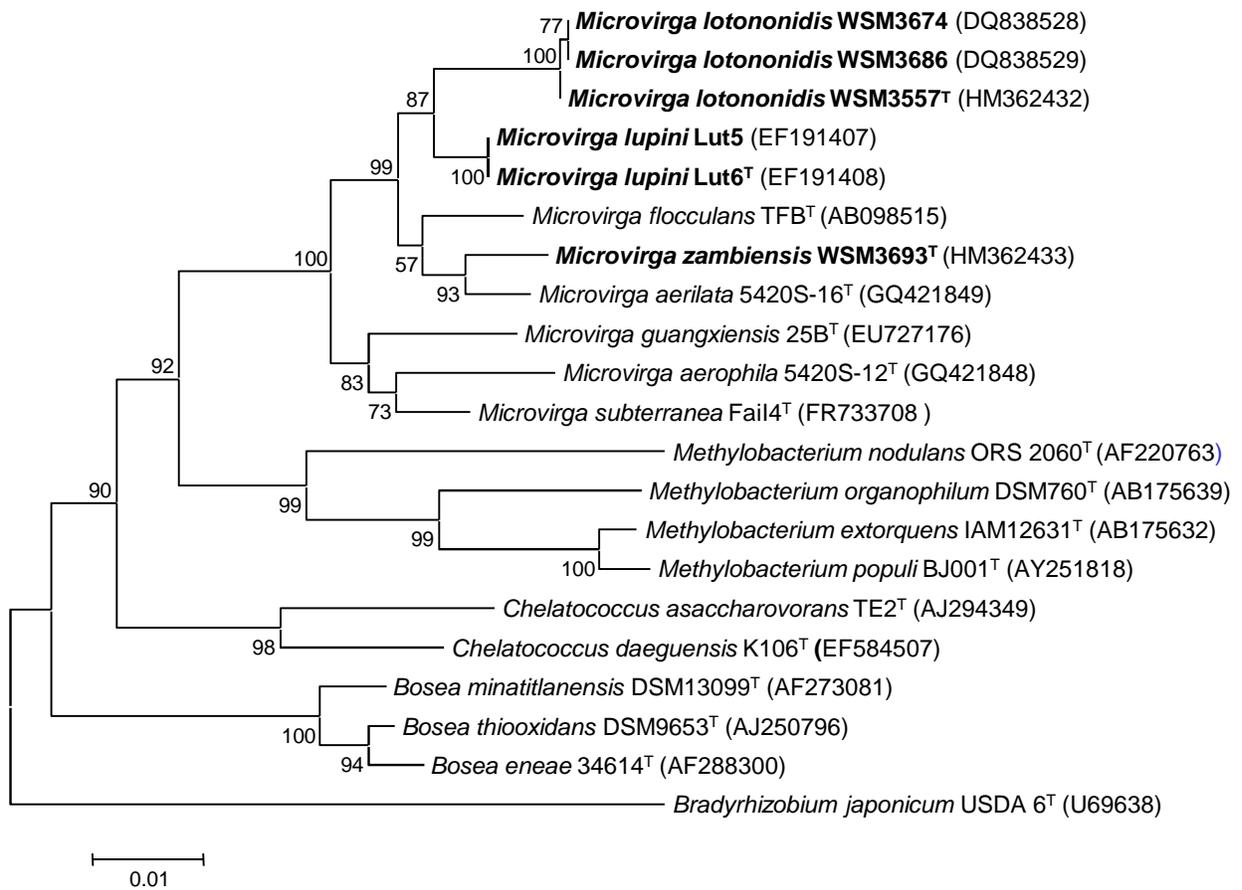


Fig. 1.