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

Ardley, J.K.; Parker, M.A.; De Meyer, S.E.; et.al.

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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]

[a]Centre for *Rhizobium* Studies, Murdoch University, Murdoch W. A. 6150, Australia

[b]Department of Biological Sciences, State University of New York, Binghamton, USA

[c]Microbiology Laboratory, University of Gent, Belgium

[d]Separation Science and Metabolomics Laboratory, Murdoch University, Murdoch W. A. 6150, Australia

[e]Department 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.

Abstract

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

Root nodule bacteria, collectively known as rhizobia, are soil bacteria that form nitrogen-fixing symbioses with leguminous plants by eliciting nodules on the roots or stems of their hosts. Within the nodule, the rhizobia differentiate into bacteroids that convert atmospheric nitrogen (N_2) to ammonia. The microsymbiont's symbiotic ability is conferred by nodulation and nitrogen fixation genes, which can be acquired by horizontal gene transfer (Andam *et al.*, 2007; Barcellos *et al.*, 2007; Cummings *et al.*, 2009; Nandasena *et al.*, 2007; Sullivan *et al.*, 1995). Rhizobia are a polyphyletic group and genera capable of nodulating hosts are found in both the Alpha- and Betaproteobacteria. Currently, 12 rhizobial genera and over 70 species have been described (http://www.rhizobia.co.nz/taxonomy/rhizobia.html). Within the Alphaproteobacteria, the genera *Rhizobium, Bradyrhizobium, Mesorhizobium* and *Ensifer* (syn. *Sinorhizobium*) comprise the majority of described microsymbionts, but novel rhizobial species of *Devosia* (Rivas *et al.*, 2002), *Methylobacterium* (Sy *et al.*, 2001), *Ochrobactrum* (Trujillo *et al.*, 2005) and *Shinella* (Lin *et al.*, 2008) have also been described.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Colony morphology was studied on ½ LA plates. Strains were assessed for growth on nutrient agar and Gram stained (Vincent, 1970). Motility of overnight ½ LA broth culture was observed using a light microscope and the hanging drop method. To try to induce motility in the Lut5 and Lut6^T strains, they were also grown, using a method modified from Bowra & Dilworth (1981), on JMM minimal media plates (O'Hara *et al.*, 1989) containing 0.1 mM succinate as a carbon source, 0.05 % (w/v) yeast extract, 0.1 mM EDTA and 0.3 % agar. One drop of 0.3 mM $MgSO_4$ solution was applied to the edge of the resulting two-day-old culture and the cells resuspended by gentle pipetting, then examined for motility as previously described. For electron microscopy, resuspended cells were collected from overnight ½ LA slopes to which 100 µl of sterile deionized water had been added. Strains were examined for spore formation by light microscopy after staining stationary phase broth and plate cultures with malachite green (Beveridge *et al.*, 2007). Stationary phase cultures were also heated to 70ºC for 10 min, and then reinoculated onto fresh media and observed for growth. Growth range and growth optima for temperature (10-50 ºC, at intervals of 5 ºC and 33-46 ºC, at 1 ºC intervals) and salt (0.0-3.0 % (w/v) NaCl at 0.5 % increments) were determined with ½ LA or TY plate and broth cultures. Tolerance of pH was assessed over the range of pH 4.0-10.0 at 0.5 unit intervals, following the method of Nandasena *et al.* (2007), but on TY medium buffered with 20 mM Homopipes (pH 4.0-5.0), MES (pH 5.5-6.0), HEPES (pH 7.0-8.5) or CHES (pH 9.0-10.0). Anaerobic growth was tested on plates of Hugh & Leifson's medium (Hugh & Leifson, 1953) supplemented with yeast extract (0.05 % (w/v)) and either glucose or

pyruvate as a carbon source, and incubated in an anaerobic jar (BBL GasPac 100 Non-vented system) at 28 ºC for 10 days. Intrinsic antibiotic resistance was determined on ½ LA plates containing ampicillin (50 and 100 μg ml^{-1}), chloramphenicol (10, 20 and 40 μg ml^{-1}), gentamicin (10, 20 and 40 μg ml^{-1}), kanamycin (50 and 100 μg ml^{-1}), nalidixic acid (50 and 100 μg ml^{-1}), rifampicin (50 and 100 μg ml^{-1}), spectinomycin (50 and 100 μg ml^{-1}), streptomycin (50 and 100 μg ml^{-1}) or tetracycline (10 and 20 μg ml^{-1}).

Growth factor requirements and tests for growth on carbon substrates were performed in JMM broths with NH_4Cl (10 mM) replacing glutamate as a nitrogen source. The growth factors tested included yeast extract (at 0.05, 0.01, 0.005 or 0.001 % (w/v)); the standard JMM vitamin mix (biotin, thiamine and pantothenic acid); a complex B group vitamin mixture required for growth of *Chelatococcus asaccharovorans* in minimal media (Egli & Auling, 2005) and the B group vitamin mixture plus casamino acids (0.01 % w/v). Strains were examined for growth on L-arabinose, D-cellobiose, β-D-fructose, α-D-glucose, glycerol, D-mannitol, acetate, succinate (all at 20 mM concentration), benzoate, *p*-hydroxybenzoate (both 3 mM), glutamate (10 mM), methanol (0.5 %, v/v) and ethanol (20 mM) as sole carbon sources. Stock solutions of carbon substrates (adjusted to pH 7.0 where necessary) were filter sterilized (0.2 μm filter) and added to the autoclaved JMM medium (devoid of carbon source) prior to inoculation. Inocula were prepared by washing stationary cultures twice with 0.89 % (w/v) saline then resuspending cells in JMM medium devoid of carbon source. The resuspended cells were added to duplicate 5 ml broths containing one of the carbon substrates to a final OD_{600nm} of 0.05. Inoculated culture media were incubated for 14 days at 28 ºC on a gyratory shaker before a visual assessment was made. Glassware

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

API –20E (bioMérieux) test strips were used to determine utilization of various substrates and acid production from sugars. Inocula were prepared from fresh plate culture resuspended in sterile RODI water containing either vitamin solution (Egli & Auling, 2005) or yeast extract (0.005 % (w/v)) for the *L. angolensis* and *L. texensis* strains, respectively. Strips were prepared in accordance with the manufacturer's protocols and read after incubation at 28 ºC for 40 hours. Oxidase activity was detected by applying fresh plate culture to filter paper impregnated with a solution of 1% (w/v) tetramethyl-p-phenylenediamine HCl and 0.1% (w/v) ascorbic acid. Catalase activity was determined on fresh plate culture using 3% (v/v) hydrogen peroxide solution. Tests for nitrate reduction were performed on cell cultures grown for 24 hours at 28 ºC in shaking TY broths supplemented with KNO_3 (1 g l^{-1}), using a method modified from Kohlerschmidt *et al.* (2009), in which 0.8 % (w/v) 8-aminonaphthalene-2-sulphonic acid (Cleve's acid) replaced 0.5 % (w/v) *N*,*N*-dimethyl-1-naphthylamine. Determination of starch hydrolysis was performed on TY agar supplemented with 0.4 % (w/v) soluble starch. Oxidative or fermentative catabolism was determined according to the method of Hugh & Leifson (1953), with

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

Electron micrographs of the *L. angolensis* and *L. texensis* strains showed rod shaped cells, surrounded by a capsule (Supplementary Fig. S3, available in IJSEM Online). Lut5 and Lut6^T did not possess flagella. On Biolog GN2 microplates, the carbon sources oxidized by the *L. angolensis* and *L. texensis* strains spanned most of the 11 designated carbon source categories (Garland & Mills, 1991), with none of the polymer, alcohol, phosphorylated chemical or amine substrates being oxidized. The range of substrates oxidized within each category was, however, quite narrow. Only 9 of 28 carbohydrates and 7 of 24 carboxylic acids gave positive results. Oxidation of amino acids varied according to strain, with 12 of the possible 20 amino acid sources being utilized by at least one strain. Results for the full list of substrates are given in Supplementary Table S5, available in IJSEM Online. Detailed phenotypic characteristics are given in the species descriptions.

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

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

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

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

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

WSM3557^T and WSM3693^T were unable to nodulate *Crotalaria juncea, Indigofera patens, Lotus corniculatus, Lupinus angustifolius,* or *Macroptilium atropurpureum.* WSM3693^T elicited and was reisolated from non-fixing nodules on the promiscuous hosts *Acacia saligna, Phaseolus vulgaris* and *Vigna unguiculata* (Amrani *et al.*, 2010; Broughton *et al.*, 2000) and on the South African *Indigofera frutescens.* WSM3557^T was also able to form ineffective nodules on some *P. vulgaris* plants, but could not be reisolated. Lut5 and Lut6^T were unable to nodulate *L. angolensis, Listia bainesii, Listia heterophylla, Lotus corniculatus, Lupinus angustifolius* or *V. unguiculata,* but formed ineffective nodules on *A. saligna* and *P. vulgaris.* Reisolates were obtained only for Lut5 from an *A. saligna* nodule. Both WSM3557^T and WSM3693^T were able to ineffectively nodulate *Lupinus texensis*. WSM3557^T was the most effective strain for nitrogen fixation on *L. angolensis* (J. Ardley, unpublished data).

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

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

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

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

Description of *Microvirga lupini* sp. nov.

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

Cells are strictly aerobic, asporogenous, Gram-negative non-motile rods (0.4-0.5 x 1.0-2.2 µm). Grows well on YMA, ½ lupin agar, TY agar and nutrient agar. On ½ LA after three days at 28 ºC, colonies are pale orange, convex, smooth and circular, with entire margins, 0.5-1.5 mm in diameter. Grows from 10-43 ºC; optimum temperature is 39 ºC and mean generation time at this temperature is 1.8 hours. Best growth is at 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 extract is an absolute requirement for growth in minimal media. The main cellular fatty acids are 18:1 ω7c and summed feature 2 (16:1 iso I / 14:0 3 OH / unknown 10.938). Ubiquinone Q-10 is the major respiratory lipoquinone. Positive for catalase and urease and weakly positive for tryptophan deaminase and acetoin production. Oxidase, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, indole and hydrogen sulphide production are negative, as is utilization of citrate. Gelatin and starch are not hydrolysed. Nitrite is not produced from nitrate. Acid is produced from growth on L-arabinose but not from growth on α-D-glucose or D-mannitol. Partially resistant to ampicillin, chloramphenicol, gentamicin and streptomycin and sensitive to kanamycin, nalidixic acid, rifampicin, spectinomycin and tetracycline. Assimilates L-arabinose, D-cellobiose, D-fructose, α-D-glucose, D-mannitol, acetate, succinate, glutamate, ethanol and *p*-hydroxybenzoate. The G + C content of the type strain is 61.9 %.

The type strain, Lut6^T (= LMG26460^T = HAMBI 3236) and other strains were isolated from N_2-fixing nodules of *Lupinus texensis* collected in Texas, USA.

Description of *Microvirga lotononidis* sp. nov.

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

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

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

The type strain, WSM3557^T (= LMG26455^T = HAMBI 3237) and other strains were isolated from N_2-fixing nodules of *Listia angolensis* originally collected in Zambia.

Description of *Microvirga zambiensis* sp. nov.

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

Cells are strictly aerobic, asporogenous, Gram-negative rods (0.4-0.5 x 1.0-2.2 µm), motile with one or more polar flagella. Grows well on YMA, ½ lupin agar, TY agar and nutrient agar. On ½ LA after three days at 28ºC, colonies are cream coloured, convex, smooth, mucilaginous and circular, with entire margins, 0.5-1.5 mm in diameter. Grows from 15-38 ºC; optimum temperature is 35 ºC and mean generation time at this temperature is 1.7 hours. Best growth is at pH 7.0-8.5 (range 6.0-9.5) and 0.0-0.5 % (w/v) NaCl (range 0-1.5 % (w/v)). Yeast extract or the vitamin mix detailed in Egli and Auling (2005) is an absolute requirement for growth in minimal media. The main cellular fatty acids are 18:1 ω7c and 19:0 cyclo ω8c. Ubiquinone Q-10 is the major respiratory lipoquinone. Positive for catalase and urease and weakly positive for acetoin production. Oxidase, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, indole and hydrogen sulphide production are negative, as is utilization of citrate. Gelatin and starch are not hydrolysed. Nitrite is produced from nitrate. Acid is produced from growth on L-arabinose but not from growth on α-D-glucose or D-mannitol. Resistant to

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

The type strain, WSM3693^T (= LMG26454^T = HAMBI 3238) was isolated from N_2-fixing nodules of *Listia angolensis* originally collected in Zambia.

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Table 1. List of strains.

Strain	Synonym (Derived from)	Host	Geographical source (Collector)	Reference or source
Llb5 *Microvirga lupini*		*Lupinus texensis*	Texas, USA	This study
Lut5 *Microvirga lupini*		*Lupinus texensis*	Texas, USA	Andam & Parker (2007)
Lut6^T *Microvirga lupini*		*Lupinus texensis*	Texas, USA	Andam & Parker (2007)
WSM3557^T *Microvirga lotononidis*	(CB1322)	*Listia angolensis*	Chibala/Fort Jameson Zambia (Verboom)	Eagles & Date (1999)
WSM3674 *Microvirga lotononidis*	(CB1323)	*Listia angolensis*	Chibala/Fort Jameson Zambia (Verboom)	Eagles & Date (1999) Yates *et al.* (2007)
WSM3686 *Microvirga lotononidis*	(CB1297)	*Listia angolensis*	Chibala/Fort Jameson Zambia (Verboom)	Eagles & Date (1999) Yates *et al.* (2007)
WSM3693^T *Microvirga zambiensis*	(CB1298)	*Listia angolensis*	Chibala/Fort Jameson Zambia (Verboom)	Eagles & Date (1999)
TFB^T *Microvirga flocculans*	LMG 25472^T		Gunma Prefecture Japan	Takeda *et al.* (2004)
TE2^T *Chelatococcus asaccharovorans*	LMG 25503^T		Switzerland	Auling *et al.* (1993)
FaiI4^T *Microvirga subterranea*	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	+	-	-
p-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: phosphatidyldimethylethanolamine, 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.

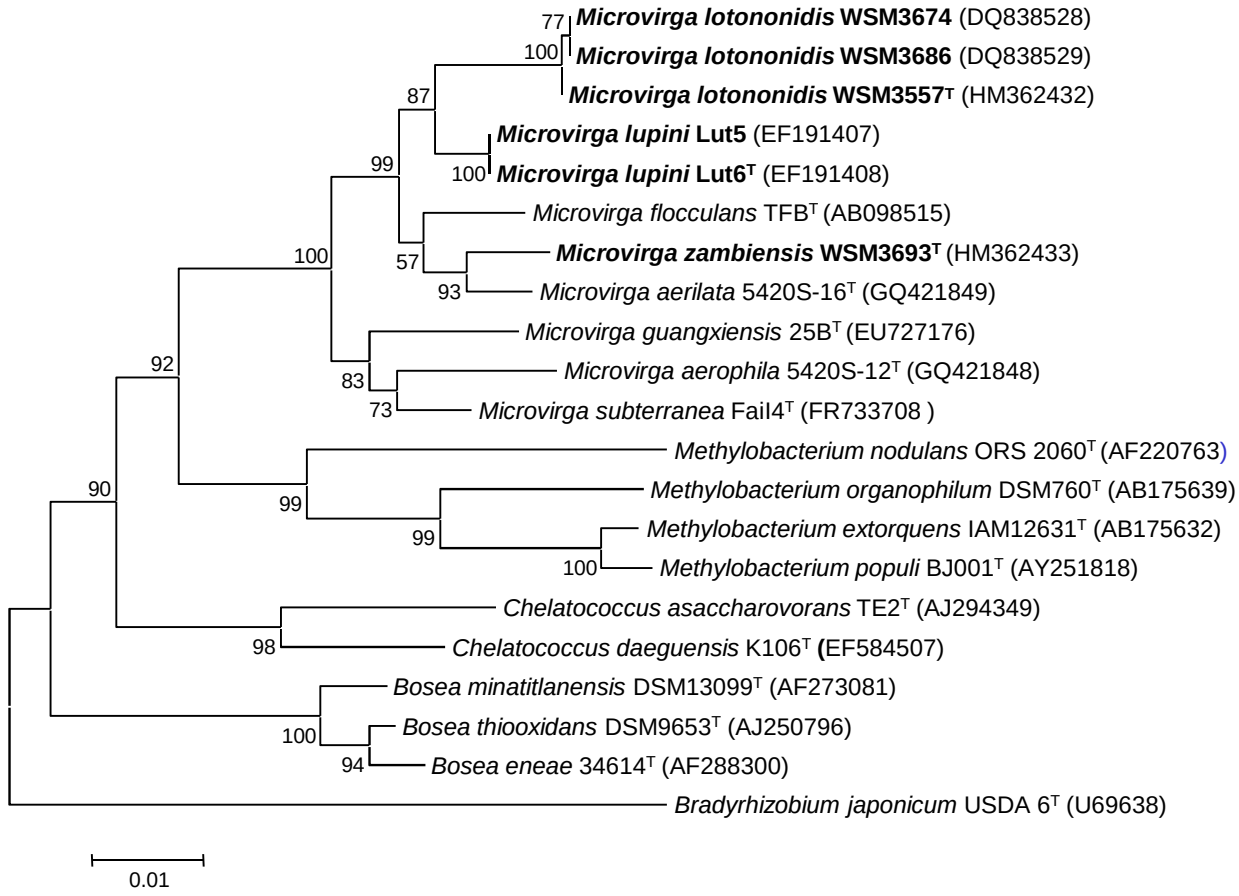
77
100
87
99
100
100
57
93
92
83
73
90
99
99
100
98
100
94
Microvirga lotononidis WSM3674 (DQ838528)
Microvirga lotononidis WSM3686 (DQ838529)
Microvirga lotononidis WSM3557T (HM362432)
Microvirga lupini Lut5 (EF191407)
Microvirga lupini Lut6T (EF191408)
Microvirga flocculans TFBT (AB098515)
Microvirga zambiensis WSM3693T (HM362433)
Microvirga aerilata 5420S-16T (GQ421849)
Microvirga guangxiensis 25BT (EU727176)
Microvirga aerophila 5420S-12T (GQ421848)
Microvirga subterranea FaiI4T (FR733708)
Methylobacterium nodulans ORS 2060T (AF220763)
Methylobacterium organophilum DSM760T (AB175639)
Methylobacterium extorquens IAM12631T (AB175632)
Methylobacterium populi BJ001T (AY251818)
Chelatococcus asaccharovorans TE2T (AJ294349)
Chelatococcus daeguensis K106T (EF584507)
Bosea minatitlanensis DSM13099T (AF273081)
Bosea thiooxidans DSM9653T (AJ250796)
Bosea eneae 34614T (AF288300)
Bradyrhizobium japonicum USDA 6T (U69638)
0.01

Fig. 1.