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2 *Solirubrobacter pauli* gen. nov., sp. nov., a mesophilic bacterium within the

3 *Rubrobacteridae* related to common soil clones

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21

21 **Abstract**

22 A novel bacterium, strain B33D1^T, isolated from agricultural soil, was
23 characterized taxonomically and phylogenetically. Strain B33D1^T was a Gram-positive,
24 aerobic rod of medium length that formed long chains on a common laboratory medium.
25 However, B33D1^T grew poorly on the surface of agar plates and was sensitive to
26 desiccation. The optimal growth temperature was 30°C (range 19-38 C). The organism
27 grew well on a variety of sugars and was capable of utilizing a few amino acids as a sole
28 carbon source. Phylogenetically, the most closely related described species to strain
29 B33D1^T was *Rubrobacter xylanophilus*, which possessed 86% 16S rRNA sequence
30 similarity. However, a number of 16S rRNA gene clones derived from soil samples
31 possessed up to 93% sequence similarity. These results placed strain B33D1^T within the
32 *Rubrobacteridae* subclass of the Actinobacteria phylum. The novel genus and species
33 *Solirubrobacter pauli* gen. nov., sp. nov., is proposed with strain B33D1^T as the type.
34

34 **Introduction**

35 Members of the phylum Actinobacteria are widespread in soils throughout the
36 world. In recent years, a number of 16S rRNA gene libraries constructed from terrestrial
37 samples revealed sequences that were phylogenetically similar to two isolates within a
38 deep branch of the Actinobacteria, *Rubrobacter xylanophilus* (Carreto *et al.*, 1996) and
39 *Rubrobacter radiotolerans* (originally *Arthrobacter radiotolerans*; Yoshinaka *et al.*,
40 1973; later reclassified in Suzuki *et al.*, 1988). Both of these organisms were notable for
41 their radiation tolerance, rare pigmentation, and thermophily. In contrast to the extreme
42 environments which were the sources of the described *Rubrobacter* species, a large
43 number of related 16S rRNA gene sequences have been recovered from moderate,
44 terrestrial environments (Furlong *et al.*, 2002; Holmes *et al.*, 2000; McCaig *et al.*, 1999;
45 Rheims *et al.*, 1996; Ueda *et al.*, 1995), suggesting a wider range of habitat for the group.
46 In this paper, we characterize an organism which is closely related to these common,
47 uncultured soil bacteria within the *Rubrobacteridae*.

48

48 **Methods and Materials**

49 **Isolation.** Strain B33D1^T was isolated from a burrow of the epigeic earthworm
50 *Lumbricus rubellus* in an agricultural soil during a previous study (Furlong *et al.*, 2002),
51 on a plate composed of 50% Difco Nutrient Broth medium (pH 7.0, ~23°C). Isolates
52 were allowed to grow for two weeks before colonies were picked. A single pink colony
53 was selected from a dilution series and maintained on the same medium. The organism
54 was stored at -70°C in medium containing 15% glycerol.

55 **Growth conditions.** Unless otherwise indicated, the optimization of growth conditions
56 for B33D1^T was carried out in undiluted Difco Nutrient Broth or NB medium. The
57 temperature range was determined using a temperature gradient incubator (Scientific
58 Industries, Inc., Bohemia, NY). The pH range of B33D1^T was determined by buffering
59 NB with 25 mM of 2-(4-morpholino)ethanesulfonic acid (MES; pH 5.5, 6.0, and 6.5), N-
60 (2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.0, 7.5, and 8.0), 3-
61 [4-(2-hydroxyethyl)-1-piperazine]propanesulfonic acid (EPPS; pH 8.5), or 2-
62 (cyclohexylamino)ethanesulfonic acid (CHES; pH 9.0, 9.5, 10.0). The pH of the medium
63 did not change during growth. Growth in various salt conditions was tested by adding 0-
64 10% NaCl to NB. Growth under anaerobic conditions was tested by sparging NB with
65 N₂ gas for 30 minutes to remove O₂ and incubating cultures in Balch tubes (Balch *et al.*,
66 1979). To test growth under microaerophilic conditions, 1% (v/v) of air was added to N₂-
67 sparged tubes of NB. The effect of increased CO₂ concentrations on B33D1^T was tested
68 by adding 1-5% (v/v) CO₂ to air in the headspace of NB medium in Balch tubes.

69 **Biochemical properties.** The ability of various compounds to serve as sole carbon
70 sources was tested in minimal medium with the carbon source added to a final

71 concentration of 0.2% (w/v). The minimal medium consisted of 1mM K₂HPO₄, 2 mM
72 NH₄NO₃, 1 mM MgSO₄, 1 % v/v trace mineral solution (Whitman *et al.*, 1986), and 1.0
73 % v/v iron solution (Whitman *et al.*, 1986) adjusted to a pH of 7.0. In some tests (for
74 example the carbon utilization of lignin-associated compounds), the high concentration of
75 iron produced a precipitate, and a 10-fold lower concentration was used. Tubes were
76 incubated at 30°C for 2 weeks before growth results were recorded.

77 The oxidase reaction of B33D1^T was tested by applying a few drops of BBL™
78 Oxidase test reagent (Becton and Dickinson and Co., Cockeysville, MD) to cells on a
79 piece of filter paper. Other tests were performed as described by Furlong *et al.* (2002).
80 **Desiccation resistance.** B33D1^T and *Deinococcus radiodurans* (ATCC 35073) were
81 grown in liquid media (NB for B33D1^T, NB + 10% dextrose for *D. radiodurans*) at 30°C
82 until growth was apparent. Wild-type *Escherichia coli* (ATCC 9637) was grown in NB.
83 Samples of the cultures, 1-3 ml, were centrifuged for 3 minutes, and the pellets were
84 resuspended in 0.4 ml of phosphate buffered saline (PBS; pH 7.2). Then 0.1 ml samples
85 were aliquoted into microfuge tubes, the tubes were centrifuged for 3 minutes, and the
86 buffer was discarded. The open microfuge tubes containing the cells pellets were placed
87 upright in a sealed mason jar containing Drierite absorbent for up to 25 days. Tubes were
88 removed at various intervals, and the cell pellets were resuspended and serially diluted in
89 PBS. *D. radiodurans* was spread onto NB + 10% dextrose plates. *E. coli* was spread
90 onto NB plates. For enumeration of B33D1^T, 1 ml of the cell suspensions were added to
91 9 mL of molten agar medium (NB + 1.5 % agar, 55 °C) and poured into a sterile Petri
92 dish. Once the plates had solidified they were sealed with parafilm and incubated at
93 30°C until colonies became visible.

94 **Fatty acid analysis.** A sample of B33D1^T was analyzed to determine the phospholipid
95 fatty acid (PLFA) content of the organism. The cell paste was extracted with the single-
96 phase chloroform-methanol-buffer system of Bligh & Dyer (1954), as modified by White
97 *et al.* (1979). The total lipid extract was fractionated into neutral lipids, glycolipids, and
98 polar lipids by silicic acid column chromatography (Guckert *et al.*, 1985). The polar
99 lipids were transesterified to the fatty acid methyl esters by a mild alkaline methanolysis
100 (Guckert *et al.*, 1985). The fatty acid methyl esters were then analyzed by capillary gas
101 chromatography with flame ionization detection on a Hewlett-Packard 5890 Series 2
102 chromatograph with a 50 m non-polar column (0.2 mm I.D., 0.11 µm film thickness).
103 The injector and detector were maintained at 270°C and 290°C, respectively. The column
104 temperature was programmed from 60°C for 2 minutes, then ramped at 10°C per minute
105 to 150°C, then ramped to 312°C at 3°C per minute. Preliminary peak identification was
106 by comparison of retention times with known standards. Detailed identification of peaks
107 was by gas chromatography/mass spectroscopy of selected samples using a Hewlett-
108 Packard 5890 series 2 gas chromatograph interfaced to a Hewlett –Packard 5971 mass-
109 selective detector using the same column and temperature program as previously
110 described. Mass spectra were determined by electron impact at 70 eV. Methyl
111 nonadecanoate (c19:0) was used as the internal standard, and the PLFA expressed as
112 equivalent peak response to the internal standard.

113 **Microscopy.** The cellular morphology of B33D1^T was observed by phase-contrast
114 microscopy, negative staining with negrosin dye, as well as electron microscopy. A
115 Nikon TE 300 inverted microscope was used to observe the cellular morphology of
116 B33D1^T. Digital photomicrographs were taken through the IP Lab Spectrum software

117 package (version 3.4.5) using an attached Princeton Instruments MicroMax high
118 resolution, cooled CCD camera. Samples for scanning electron microscopy (SEM) were
119 prepared according to the method of Hahn *et al.* (1998) except that 4% glutaraldehyde
120 was used to fix the cells. A Leo 982 field emission SEM was used to examine the cells.
121 To test for the presence of a capsule, an India ink suspension, as well as a standard
122 capsule stain was used (Benson, 1990). The Gram reaction of B33D1^T was determined
123 by a common staining technique and observed using light microscopy (Benson, 1990).
124 **Phylogeny.** The nearly complete 16S rRNA sequence of B33D1^T was obtained by
125 amplification of the genomic DNA with primers 27f and 1392r (Lane *et al.*, 1991;
126 Furlong *et al.*, 2002). Sequencing reactions were performed using a Big Dye sequencing
127 kit (Perkin-Elmer) with primers 27f, 1392r, 533f, 519r, and 907r (Lane, 1991). The
128 sequencing reaction products were run on an ABI 377 Automated Sequencer (Perkin-
129 Elmer). To construct the phylogenetic trees, the sequence was first aligned with
130 reference organisms and environmental clone sequences using the PILEUP program
131 included in the GCG software package (Genetics Computer Group). The evolutionary
132 distances between aligned sequences were determined using a Jukes-Cantor algorithm in
133 the DNADIST program of the PHYLIP software package (Felsenstein 1989). The tree
134 topology was determined using the FITCH program and the tree robustness was testing
135 using 100 replicate trees as generated by SEQBOOT within the PHYLIP set of programs.
136 The nearest phylogenetic neighbors of B33D1^T were determined by FASTA searches
137 (Pearson & Lipman, 1988) of GenBank.
138 **Mol % G+C.** The mol % G+C percentage of B33D1^T was determined by the method of
139 Mesbah *et al.* (1989). The value reported was the average of five replicates.

140 **Results and Discussion**

141 **Cellular and colonial properties.** Strain B33D1^T was a rod, approximately 1.4 μm x 0.7
142 μm, although much longer cells were occasionally observed in fresh cultures (Figure 1).
143 Older cultures generally had uniformly shorter rods. In liquid culture, cells were often
144 observed with an indentation near the center of the cell, presumably due to preparation
145 for cellular division. Also in liquid culture, cells often grew in long chains that wrapped
146 around each other producing large aggregates. No capsule was observed. Cells of strain
147 B33D1^T stained Gram-positive. No motility was observed, and no spores were apparent
148 by phase contrast microscopy.

149 Colonies of strain B33D1^T grown on Difco Nutrient Broth agar plates were round,
150 convex, with entire edges, and usually pink in color. Plates incubated at higher
151 temperatures (28°C) often produced less pigment initially, although the deep pink color
152 appeared over time. Strain B33D1^T had a low growth efficiency upon streaking on agar
153 plates, and only a few or no colonies resulted upon spread plating on solid agar surfaces .
154 However, B33D1^T did grow in a liquid culture or embedded in soft, nutrient agar. The
155 low viability of cells on the surface of an agar plate was at least partially explained by
156 desiccation sensitivity. Strain B33D1^T cells rapidly lost viability upon desiccation when
157 compared to *Escherichia coli* (which has normal sensitivity to desiccation) and
158 *Deinococcus radiodurans* (which is desiccation resistant; Figure 2). Strain B33D1^T was
159 also sensitive to high temperature. Following the incubation of a cell suspension
160 containing 10⁶ CFUs in PBS at 70°C for 30 min, no CFUs were detected by pour plating.
161 Although phylogenetically related *Rubrobacter* species were resistant to high levels of
162 radiation (Ferreira *et al.*, 1999), the apparent desiccation and heat sensitivity of B33D1^T

163 may indicate poor radiation resistance, as the phenotypes are often correlated (e.g. Billi &
164 Potts, 2002; Mattimore & Battista, 1996; Sanders & Maxcy, 1979).

165 B33D1^T appeared similar in color to other *Rubrobacter* organisms. Similarly to
166 those organisms, the pigments were not easily extracted using traditional methods.
167 However a small amount of pigment was recovered by extensive incubation of a cell
168 pellet in methanol. A visible light spectrum of these compounds in 100% methanol
169 produced maxima at 466, 493, and 526 nm. These maxima were similar to those reported
170 for pigments extracted from *Rubrobacter radiotolerans* (Saito *et al.*, 1994).

171 **Nutritional characteristics.** A variety of compounds were tested as sole carbon and
172 energy sources for strain B33D1^T. Strain B33D1^T generally grew to a low cellular
173 density even in a rich medium, where the maximum absorbance was near 0.1 at 600 nm.
174 Growth was observed on a variety of sugars including fructose, galactose, glucose,
175 lactose, mannose, sorbitol, sucrose, and xylose. Growth was not observed on cellobiose
176 and mannitol. Strain B33D1^T utilized the organic acid pyruvate, appeared to grow
177 weakly on acetate, but did not grow on citrate, malate, or succinate. Casamino acids
178 provided good growth. When tested individually, only the amino acids alanine, arginine,
179 and lysine supported growth. Glycerol was the only alcohol tested that could support the
180 growth of B33D1^T. Other alcohols that did not support growth were methanol, ethanol,
181 1-propanol, 2-propanol, butanol, isobutyl alcohol, and iso-amyl alcohol. Because
182 B33D1^T was isolated from soil, a variety of products of lignin degradation were tested as
183 possible carbon sources. Of the compounds tested, only chlorogenic acid supported
184 growth. Anthranilic acid, benzoic acid, catechol, protocatechuic acid, *p*-coumaric acid,

185 gentisic acid, ferulic acid, *p*-hydroxybenzoic acid, syringic acid, and vanillic acid did not
186 produce observable growth after two weeks.

187 B33D1^T utilized ammonium as a sole nitrogen source and could grow on
188 Casamino acids as a sole carbon and nitrogen source. It did not reduce nitrate or produce
189 urease.

190 **Other growth conditions.** A variety of pH and temperature conditions were tested.

191 Strain B33D1^T grew optimally at 28-30°C, but could grow between 19 and 38°C.

192 B33D1^T could grow at a pH between 6 and 7.5 with an optimum around 6 to 6.5.

193 B33D1^T did not grow in the absence of oxygen but grew when 1% (v/v) air was added to
194 the headspace. Because the concentration of CO₂ is often higher in soil than in the

195 atmosphere, the growth of B33D1^T under these conditions was tested. Increased CO₂

196 levels (1-5% v/v) did not significantly enhance the growth rate or yield. While

197 *Rubrobacter* strains could grow in media with increased salt concentrations (Ferreira *et*

198 *al.*, 1999; Carreto *et al.*, 1996; Suzuki *et al.*, 1988), B33D1^T did not grow when as little

199 as 1% NaCl was added to the growth medium.

200 **Biochemical and chemotaxonomic properties.** B33D1^T was catalase positive and

201 oxidase negative. Casein and Tween 80 were not hydrolyzed. B33D1^T was sensitive to

202 polymyxin, ampicillin, tetracycline, and streptomycin antibiotics. No hemolysis was

203 observed on blood agar.

204 The fatty acid profile of B33D1^T revealed no unusual compounds. The major

205 phospholipid fatty acids (PFLAs) were i16:0 (54%) and 18:1 ω 9c (36%). Trace amounts

206 of 16:0 (4%), 19:1 ω 12c (2%), 16:1 ω 7c (1%), and br17:1 (1%) were also detected. No

207 polyunsaturated PLFAs were found. Phylogenetically related *Rubrobacter* isolates

208 contain only small amounts, if any, of the PLFAs that were abundant in B33D1^T (Carreto
209 *et al.*, 1996).

210 The G+C content of the genomic DNA of B33D1^T was 71.8 % ± 0.2 (mean ± SD;
211 n = 5), which was slightly higher than the 67.6% value reported for *Rubrobacter*
212 *xylanophilus* (Carreto *et al.*, 1996) but not unexpected given the high G+C content
213 common for members of the Actinobacteria phylum.

214 **Phylogeny.** Based on 16S rRNA sequence analyses, strain B33D1^T grouped within the
215 *Rubrobacteridae* subclass of the Actinobacteria phylum, more specifically within
216 subgroup 2 as defined by Holmes *et al.* (2000; Figure 3). No other isolates have been
217 reported from this particular subgroup, although a number of environmental 16S rRNA
218 genes have been described. The most closely related sequence in GenBank by FASTA
219 analysis was an uncultivated clone (YNPFFP1; accession number AF391984) from a
220 thermal soil (93% identity over 1364 bases), while the closest characterized organism
221 was *Rubrobacter xylanophilus* (86% over 1372 bases). The other cloned sequences in
222 subgroup 2 originated from terrestrial or sediment sources similarly to B33D1^T. Given
223 the apparent ubiquitous distribution of this group in soil samples in various parts of the
224 world, there is little reason to assume an association of this organism with earthworms,
225 even though it was originally isolated from earthworm burrow soil. The nearly complete
226 16S rRNA sequence of B33D1^T was deposited in GenBank with the accession number
227 AY039806.

228 Because of its low relatedness to previously described organisms, B33D1^T
229 appears to represent a novel genus and species. This conclusion is supported by
230 phenotypic differences with representatives of the most closely related taxa (Table 1).

231 The lower temperature optimum and sensitivity to NaCl appear to be the most important
232 distinguishing features. Lastly, the poor plating efficiency and sensitivity to desiccation
233 may explain why this taxon is so poorly represented in culture collections. Presumably,
234 more representatives might be easily isolated from soil by pour plating.

235 **Description of *Solirubrobacter* gen. nov.**

236 *Solirubrobacter* (So.li.ru.bro.bac'ter. L.n. *solum* soil; L.adj. *ruber* red; M.L.n. *bacter* the
237 masculine equivalent of the GR. neut. n. *bakterion* a rod; M.L. masc. n. *Solirubrobacter* a
238 soil red rod)

239 Cells are Gram-positive, rods of medium length. Non-motile. Spores are not
240 formed. Aerobic and mesophilic. Catalase positive and oxidase negative. Grow well on
241 common sugars and a few amino acids as a sole carbon source. Sensitive to desiccation.
242 Phylogenetically within the *Rubrobacteridae* subclass of the Actinobacteria. The type
243 species is *Solirubrobacter pauli*.

244 **Description of *Solirubrobacter pauli* gen. nov., sp. nov.**

245 (pau'li. M.L.gen.n. *pauli* of *Paulus*; named for the prominent soil microbiologist Eldor A.
246 Paul). Cells are rods, 1.4 μm x 0.7 μm , and grow in long chains. Colonies are round,
247 convex, and pink in color. The G + C content of the type strain B33D1^T is 71.8 mol %.
248 The major fatty acids are i16:0 (54 %) and 18:1 ω 9c (36 %). The type strain grows at pH
249 values of 6.0 - 7.5 and temperatures between 19 – 38 °C, with optima at pH 6.5 and 28 –
250 30 °C. Fructose, galactose, glucose, lactose, mannose, sorbitol, sucrose, xylose,
251 pyruvate, acetate, casamino acids, alanine, arginine, lysine, glycerol, and chlorogenic
252 acid support growth. The organism does not grow on cellobiose, mannitol, citrate,
253 malate, succinate, methanol, ethanol, 1-propanol, 2-propanol, butanol, isobutyl alcohol,

254 iso-amyl alcohol, anthranilic acid, benzoic acid, catechol, protocatechuic acid, *p*-
255 coumaric acid, gentisic acid, ferulic acid, *p*-hydroxybenzoic acid, syringic acid, and
256 vanillic acid. The type strain is B33D1^T (ATCC BAA-492; DSMZ 14954).

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262

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Table 1. Comparison of selected properties of B33D1^T to *Rubrobacter* type strains.^a

	Strain B33D1 ^T	<i>Rubrobacter radiotolerans</i> strain JCM 2153 ^T	<i>Rubrobacter xylanophilus</i> strain PRD-1 ^T
cell size	1.4 x 0.7 µm	1-4 x 0.8-1.0 µm	1-3 x 0.9-1.0 µm
temperature optimum	28 - 30°C	46 - 48°C	60°C
pH optimum	6.0 - 6.5	7.0 - 7.4	7.5 - 8.0
mol % G+C	71.8	67.9	67.7
major fatty acid	i16:0	12-methyl-16:0	14-methyl-18:0
growth in NaCl	<1%	6%	6%
sorbitol utilization	yes	no	no
oxidase production	no	yes	yes

366 ^aData for *R. radiotolerans* and *R. xylanophilus* from Carrento *et al.* (1996), Suzuki *et al.*
367 (1988) and Yoshinaka *et al.* (1973).

368

368 **Figure legends**

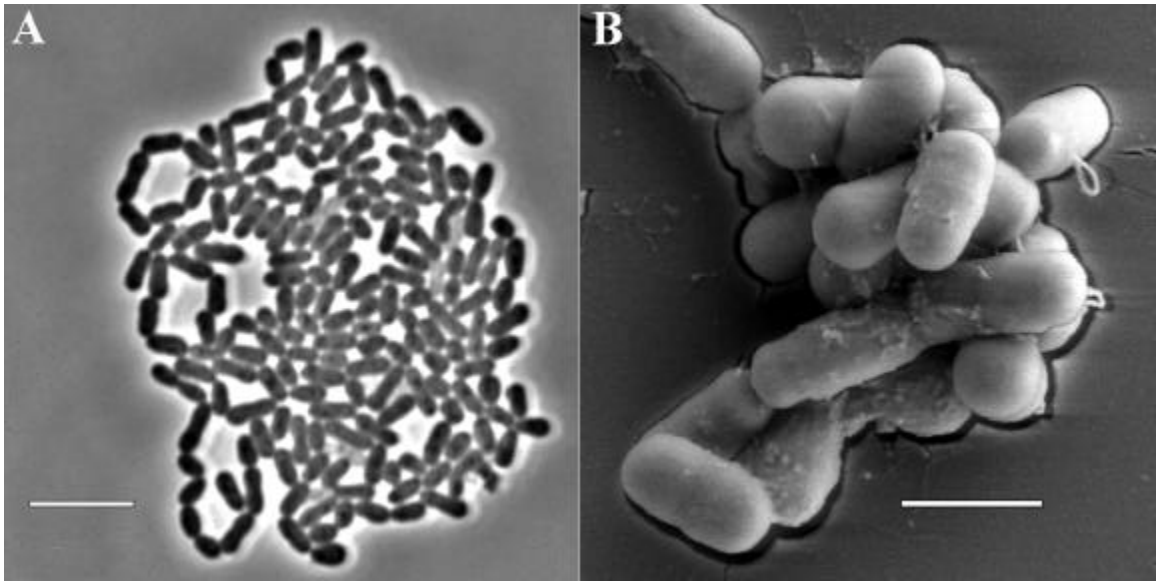
369 Figure 1. Photomicrographs of cells of B33D1^T grown in NB broth. (A) Phase contrast
370 image of an aggregate of cells. Scale bar = 5 µm. (B) SEM of an aggregate. Scale bar = 1
371 µm.

372

373 Figure 2. Effects of desiccation on B33D1^T (o, ●), *E. coli* (Å) and *Deinococcus*
374 *radiodurans* (□). Multiple lines for B33D1^T represent separate experiments.

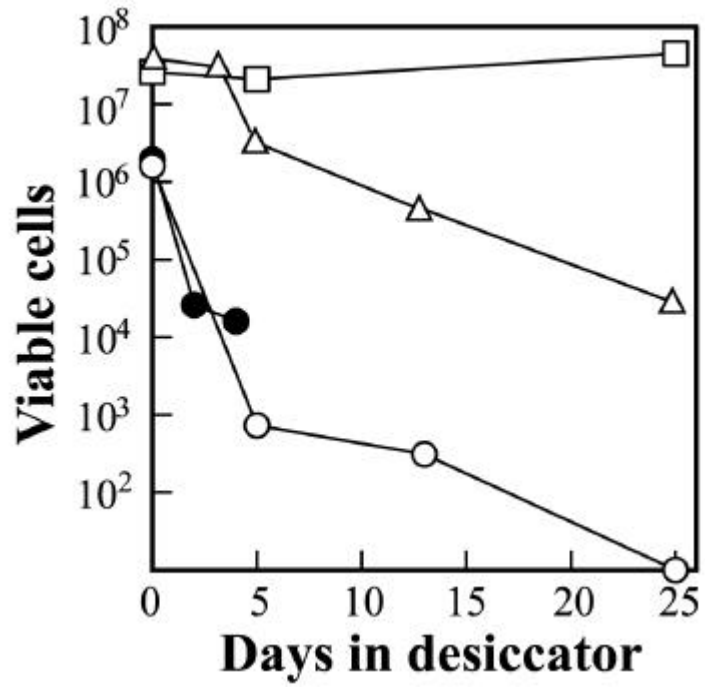
375

376 Figure 3. Phylogenetic tree of the 16S rRNA genes of B33D1^T and the closest clonal
377 and cultured relatives. Subgroup designations follow the nomenclature of Holmes *et al.*
378 (2000). Isolates are written in italics and strain B33D1^T is in bold. Closed (●) and open
379 (o) circles indicate bootstrap support of 50 and 95, respectively. GenBank accession
380 numbers are in parenthesis following each name. Only clonal sequences with nearly
381 complete 16S rDNA sequence information were used in this tree. Clones beginning with
382 TM are from a peat bog (Rheims *et al.*, 1996). Clones #649-1G9, #0319-7H2, #0649-
383 1N15, and #0319-6M6 were obtained from an Australian arid soil (Holmes *et al.*, 2000).
384 Sequence YNPFFP1 and YNPFFP59 were from a thermal soil (unpublished). Sequences
385 beginning with MC were clones from a subtropical Australian soil (Liesack and
386 Stackebrandt, 1992). Clones 480-2 and 288-2 were from soil (unpublished). BVA77 and
387 Q3-6C1 were from a landfill (Röling *et al.*, 2001) and rhizosphere soil (unpublished),
388 respectively. The tree was based on 1306 bases of aligned sequence. The scale bar
389 represents Jukes-Cantor evolutionary distance.



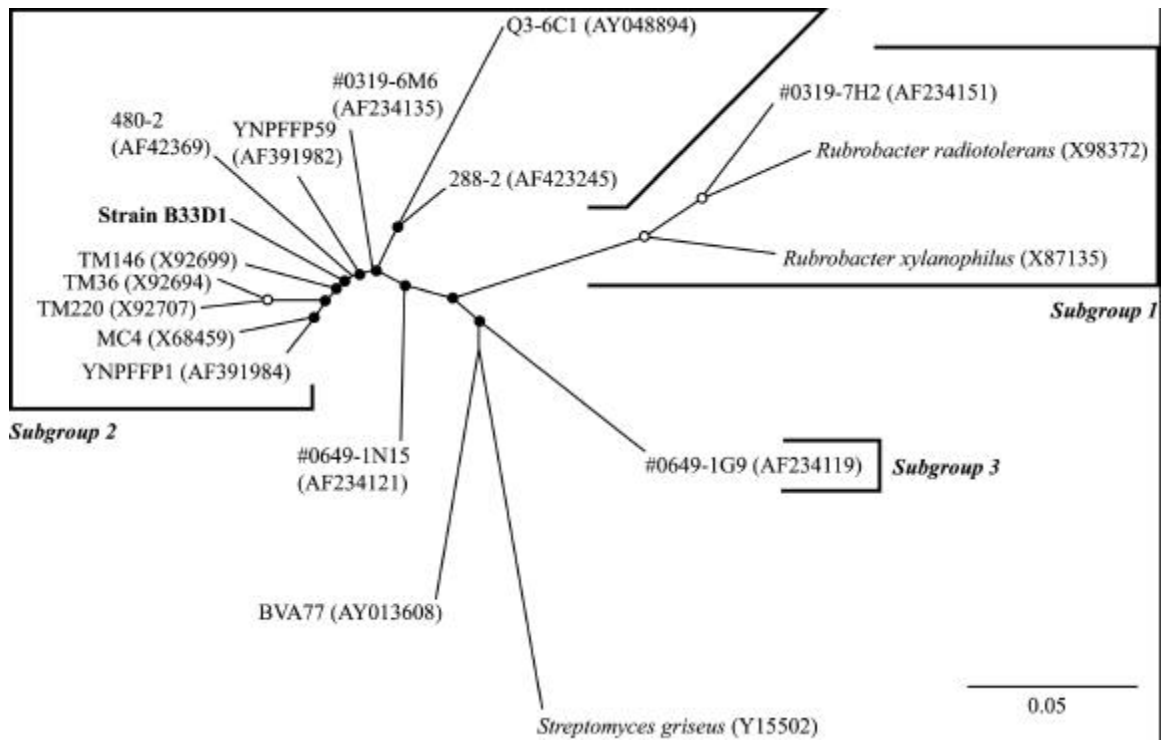
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