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Root nodule bacteria isolated from South African *Lotononis bainesii*, *L. listii* and *L. solitudinis* are species of *Methylobacterium* that are unable to utilize methanol

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1 **Abstract**

2 The South African legumes *Lotononis bainesii*, *L. listii* and *L. solitudinis* are  
3 specifically nodulated by highly effective, pink-pigmented bacteria that are most  
4 closely related to *Methylobacterium nodulans* on the basis of 16S rRNA gene  
5 homology. *Methylobacterium* spp. are characterized by their ability to utilize  
6 methanol and other C<sub>1</sub> compounds, but eleven *Lotononis* isolates neither grew on  
7 methanol as a sole carbon source nor were able to metabolize it. No product was  
8 obtained for PCR amplification of *mxoF*, the gene encoding the large subunit of  
9 methanol dehydrogenase. Searches for methylotrophy genes in the sequenced genome  
10 of *Methylobacterium* sp. 4-46, isolated from *L. bainesii*, indicate that the inability to  
11 utilize methanol may be due to the absence of the *mxo* operon. While methylotrophy  
12 appears to contribute to the effectiveness of the *Crotalaria/M. nodulans* symbiosis,  
13 our results indicate that the ability to utilize methanol is not a factor in the  
14 *Lotononis/Methylobacterium* symbiosis.

15

16 *Keywords: Methylobacterium, Lotononis, Methylotrophy, Root nodule bacteria.*

17

## 18 **1. Introduction**

19 Leguminous plants in the genus *Lotononis* and their associated root nodule bacteria  
20 are being studied because of their potential as well-adapted pasture legumes able to  
21 combat dryland salinity in southern Australian agricultural systems (Yates et al.,  
22 2007). The genus *Lotononis* is of mainly southern African origin, comprising some  
23 150 species of herbs and small shrubs (Van Wyk, 1991). Species in the *Listia* section  
24 are of particular interest, as they are perennial, stoloniferous and lack the poisonous  
25 metabolites found in some other species of *Lotononis* (Van Wyk & Verdoorn, 1990).  
26 The *Listia* section includes *L. angolensis*, *L. bainesii*, *L. listii*, *L. macrocarpa*, *L.*  
27 *marlothii*, *L. minima*, *L. solitudinis* and *L. subulata*. Nodulation has been described  
28 for *L. angolensis*, *L. bainesii* and *L. listii*, which characteristically form collar nodules  
29 (Norris, 1958; Yates et al., 2007).

30

31 The root nodule bacteria from *L. bainesii* were first described by Norris (1958), who  
32 reported that isolates from *L. bainesii* were red- or pink-pigmented and that the  
33 symbiosis was highly specific. These pigmented bacteria were subsequently  
34 characterized and identified as a species of *Methylobacterium* (Jaftha et al., 2002).  
35 Yates et al. (2007) further found isolates from *L. bainesii*, *L. listii* and *L. solitudinis* to  
36 be pink pigmented, highly effective, most closely related to *Methylobacterium*  
37 *nodulans* (with > 97% similarity of the 16S rRNA gene sequence) and to form a  
38 cross-inoculation group. The non-pigmented *M. nodulans* that specifically nodulates  
39 Senegalese *Crotalaria* spp. (Sy et al., 2001) is the only other *Methylobacterium*  
40 species so far reported to nodulate legumes.

41

42 Free-living methylobacteria are found in a variety of habitats, such as soil, dust, and  
43 fresh water (Green, 1992). Methylobacteria are also ubiquitous in the plant

44 phyllosphere and rhizosphere (Trotsenko et al., 2001). They promote the germination  
45 or growth of soybeans, rice and other plants, probably because of their ability to  
46 synthesise auxins, cytokinins, vitamin B<sub>12</sub> and other plant growth-promoting  
47 substances (Basile et al., 1985; Holland & Polacco; 1994, Ivanova et al., 2000;  
48 Trotsenko et al., 2001; Madhaiyan et al., 2004; Abanda-Nkpwatt et al., 2006; Ryu et  
49 al., 2006). The closeness of the association between plants and *Methylobacterium*  
50 spp. varies; epiphytes (Omer et al., 2004;), endophytes (Van Aken et al., 2004) and  
51 nitrogen-fixing symbionts (Sy et al., 2001; Jaftha et al., 2002; Yates et al., 2007),  
52 have all been described.

53

54 *Methylobacterium* spp. are characterized by their ability to utilize methanol and other  
55 C<sub>1</sub> compounds, as well as a variety of multicarbon substrates (Green, 1992; Lidstrom,  
56 2006). Utilization of carbohydrates as a sole carbon source is variable and can be  
57 used to differentiate the various species (Green, 1992). Methylo-trophy in  
58 *Methylobacterium* spp. involves over 100 genes constituting a set of metabolic  
59 functional modules (Chistoserdova et al., 2003). In the model organism  
60 *Methylobacterium extorquens* AM1, such modules involve the primary oxidation of  
61 methanol or methylamine to formaldehyde, the oxidation of formaldehyde, and the  
62 assimilation of C<sub>1</sub> products via the serine cycle (Chistoserdova et al., 2003; Lidstrom,  
63 2006). Methanol is oxidized by methanol dehydrogenase (MDH), a protein with an  
64  $\alpha_2\beta_2$  tetramer structure, a pyrroloquinoline quinone (PQQ) cofactor and a calcium  
65 ion, essential for maintaining the PQQ in its active configuration, in the active site of  
66 each  $\alpha$ -subunit (Anthony, 1996; Goodwin & Anthony, 1998). The genes encoding the  
67 MDH structural polypeptides, the specific cytochrome *c* electron acceptor, proteins  
68 essential for the insertion of the calcium ion, a regulatory protein and several proteins

69 of unknown function are transcribed in a single operon, *mxoFGIRSACKLDEHB*  
70 (Chistoserdova et al., 2003).

71

72 The *M. nodulans* isolates from *Crotalaria*, like all previously described  
73 *Methylobacterium* species, can use methanol as a sole carbon source and contain a  
74 copy of *mxoF*, the gene that codes for the large subunit of MDH (Sy et al., 2001).  
75 Isolates from *L. bainesii* are also reported as able to grow in minimal media with  
76 methanol as a substrate (Jaftha et al., 2002). In contrast, our isolates from *L. bainesii*,  
77 *L. listii* and *L. solitudinis* appeared unable to grow on methanol in minimal media.  
78 The aim of this work was thus to determine the ability of these isolates to grow on or  
79 utilize a variety of C<sub>1</sub> and other carbon substrates.

80

81 The genomes of five *Methylobacterium* spp., including the *L. bainesii* symbiont  
82 *Methylobacterium* sp 4-46, have recently been sequenced and are available on the  
83 Integrated Microbial Genomes database of the Joint Genome Institute  
84 (<http://img.jgi.doe.gov/cgi-bin/pub/main.cgi>) in either draft or finished form.  
85 *Methylobacterium* sp 4-46 is closely related to xct9 on the basis of 16S rRNA gene  
86 homology (Darryl Fleischman, personal communication). We therefore performed  
87 BLASTP searches for sequences required for methylo-trophy in these genomes, to  
88 determine the genetic basis for our isolates' inability to grow on methanol.

89

## 90 **2. Materials and Methods**

### 91 *2.1. Bacterial strains, origins and cultural conditions*

92 The bacterial strains (11 *Lotononis* isolates and four reference strains) used and their  
93 collection details are listed in Table 1. Root-nodule bacteria were isolated (Yates et

94 al., 2007) from *L. bainesii*, *L. listii* and *L. solitudinis* plants growing in eight sites  
95 across north-eastern South Africa, between latitudes 24°S and 30°S and form part of  
96 the Western Australian Soil Microbiology (WSM) collection, Murdoch University,  
97 Western Australia. Strain xct9 was isolated from a South African *L. bainesii* nodule.  
98 It is synonymous with CB376, the current commercial inoculant for *L. bainesii* in  
99 Australia (Ian Law, personal communication). These root nodule bacteria and the  
100 reference strains *Sinorhizobium medicae* WSM419 and *Bradyrhizobium japonicum*  
101 USDA6 were grown on half lupin agar (½ LA) medium (Howieson et al., 1988). All  
102 strains were stored in ½ LA plus 15% (v/v) glycerol broths at -80°C. *M. nodulans*  
103 ORS 2060 and the non-symbiont *Methylobacterium organophilum* DSM 760 (kindly  
104 supplied by Dr Catherine Boivin-Masson, INRA) were streaked onto agar plates of  
105 minimal mineral medium M72 (Belgian Co-ordinated Collection of Micro-organisms,  
106 1998) supplemented with 1% (v/v) methanol. They were then grown in broths of M72  
107 plus 1% (v/v) methanol and stored as detailed above.

108

## 109 2.2 Acidification or alkalization of ½ LA medium

110 The ability of the isolates to acidify or alkalize ½ LA medium was tested on  
111 unbuffered ½ LA agar plates, adjusted to pH 7.0 and containing 5 ml l<sup>-1</sup> of Universal  
112 range pH indicator (Vogel, 1962). The isolates and reference strains were streaked  
113 onto the ½ LA plus Universal indicator plates and colour change recorded after 7 d  
114 incubation at 28°C. Strains were scored as acidifying if the medium turned yellow  
115 (pH 6.0), alkalizing if the medium turned blue/green (pH 8) or strongly alkalizing  
116 if the medium turned blue (pH 9.0).

117

## 118 *2.3. Growth on sole carbon substrates*

### 119 *2.3.1. General growth procedures*

120 All isolates and reference strains were streaked from -80°C stocks onto fresh ½ LA  
121 plates, except for ORS2060, which was streaked onto M72 agar containing 1% (v/v)  
122 methanol. All media were adjusted to pH 7.0. All plates were incubated at 28°C for 7  
123 d. Glassware used to grow cultures (McCarty bottles and conical flasks) was  
124 soaked in a 10% (v/v) hydrochloric acid solution for at least 24 h prior to use and then  
125 rinsed twice in reverse osmosis deionized water. All broth cultures were grown at  
126 28°C with shaking (200 rpm). Lids of McCarty bottles were wrapped with parafilm  
127 prior to incubation to prevent contamination. Optical densities were read on a Hitachi  
128 U-1100 spectrophotometer.

129

### 130 *2.3.2. Growth on methanol and multicarbon substrates*

131 Isolates were tested for growth on arabinose, glucose, galactose, mannitol, succinate,  
132 glutamate and methanol as sole carbon sources. Cells were inoculated into 5 ml  
133 broths of M72 medium, supplemented with sodium pyruvate (10 mM), yeast extract  
134 (0.5 g l<sup>-1</sup>) and vitamins (thiamine HCl, 1.0 mg l<sup>-1</sup>; pantothenic acid, 1.0 mg l<sup>-1</sup> and  
135 biotin, 20 µg l<sup>-1</sup>) and grown for 40 h to an optical density at 600 nm (OD<sub>600</sub>) of  
136 between 0.6 and 0.9. The cultures were centrifuged (20 800 g for 30 s), washed twice  
137 with 0.89% (w/v) saline, resuspended in M72 medium containing vitamins (M72v)  
138 and devoid of carbon source, then added to duplicate 5 ml broths of M72v and one of  
139 the carbon substrates to a final OD<sub>600</sub> of 0.05. The concentration of all carbon  
140 substrates was 20 mM, except for methanol, where the concentration was 1% (v/v)  
141 (approximately 260 mM). Several strains were also grown in broths with 50 mM  
142 methanol. The methanol and the stock solutions of the other carbon substrates



143 (adjusted to pH 7.0 where necessary) were filter sterilized (0.2  $\mu$ m filter) and added to  
144 the autoclaved M72v medium prior to inoculation. Inoculated culture media were  
145 incubated for 10 d before a visual assessment was made. Growth on the carbon  
146 substrate was assessed as being no growth ( $OD_{600}$  was the same as for the minus  
147 carbon substrate control), poor ( $0.1 < OD_{600} < 0.2$ ), moderate ( $0.2 < OD_{600} < 0.5$ ) or  
148 abundant ( $OD_{600} > 1.0$ ). Two negative controls were used - an uninoculated control  
149 containing M72v medium and various carbon sources and a control of M72v devoid  
150 of carbon substrate but containing bacterial inoculant.

151

### 152 *2.3.3. Growth on $C_1$ sole carbon substrates*

153 Isolates were examined for growth on methanol (0.2%, v/v), methylamine (0.1%,  
154 0.2% or 0.5%, v/v), formaldehyde (0.5 mM or 1.0 mM) and formate (30 mM) as sole  
155 carbon sources in JMM medium (O'Hara et al., 1989) devoid of galactose and  
156 arabinose and with  $NH_4Cl$  (10 mM) replacing glutamate as a nitrogen source. JMM  
157 medium with succinate (20 mM) as a sole carbon source served as a positive control.  
158 Isolates were also examined for growth in medium containing succinate (20 mM)  
159 plus methanol (1.0% v/v). Stock solutions of methylamine and other carbon sources  
160 were adjusted to pH 7.0 if required. Inoculum was prepared and grown as described  
161 above, but with JMM replacing M72v medium.

162

### 163 *2.3.4. Growth of WSM2799 on formate*

164 Cells were inoculated into broths of JMM medium containing succinate (20 mM) and  
165  $NH_4Cl$  (10 mM) as carbon and nitrogen sources, and grown for 40 h to an  $OD_{600}$  of  
166 0.6. The cultures were centrifuged and washed as described, resuspended in JMM  
167 medium (containing either formate or succinate) and added to duplicate 250 ml  
168 conical flasks containing pre-warmed 50 ml JMM broths with either succinate

169 (20mM) or formate (30mM) as sole carbon source to give an initial OD<sub>600</sub> of 0.05.  
170 Duplicate samples were taken from each flask at regular intervals for OD<sub>600</sub> readings.  
171

#### 172 *2.4. Biochemical assays for utilization of methanol*

173 Cells of xct9, USDA6 and ORS 2060 were inoculated into broths of M72 medium  
174 supplemented with sodium pyruvate (10 mM), yeast extract (0.5 g l<sup>-1</sup>) and vitamins.  
175 The cultures were grown for 40 h to an OD<sub>600</sub> of between 0.6 – 0.9, centrifuged and  
176 washed as described and resuspended in M72v medium, then added to duplicate 100  
177 ml conical flasks containing 20 ml of M72v medium and either 25 mM or 100 mM  
178 methanol, to a final OD of 0.05. These and duplicate uninoculated controls were  
179 incubated for 70 h. Aliquots (1 ml) were taken from each flask at 0, 22, 40 and 70 h,  
180 growth measured as OD<sub>600</sub> and the supernatant stored at –20°C after centrifugation  
181 (20,800 g for 30 s).

182

183 Sulfuric acid (0.5M) was added to the supernatant samples (10 µl from the cultures in  
184 100 mM methanol, 40 µl from the 25 mM methanol cultures), to make a total volume  
185 of 1 ml. For ORS 2060, the 40 h and 70 h 100 mM methanol samples were also of 40  
186 µl. The samples were oxidized with potassium permanganate, excess permanganate  
187 removed with sodium arsenite (Wood & Siddiqui, 1971), and formaldehyde  
188 concentration determined by measuring OD<sub>412</sub> after reaction with Nash's reagent  
189 (Nash, 1953).

190

191 The concentration of methanol in the succinate (20 mM) plus methanol (1% v/v)  
192 media 10 d after inoculation was determined in the same way.

193

194 2.5. PCR amplification of *mxoF*

195 PCR amplification of *mxoF* was performed using the primers f1003 – 5' -GCG GCA  
196 CCA ACT GGG GCT GGT-3' and r1561 – 5' -GGG CAG CAT GAA GGG CTC  
197 CC-3'. Primers were obtained from GeneWorks Pty Ltd.

198

199 Whole cell DNA templates were prepared from isolates and reference strains. Cells  
200 were suspended in PCR-grade water to an OD<sub>600</sub> of approximately 10. An initial PCR  
201 amplification to optimize the magnesium chloride concentration of the PCR reaction  
202 mix resulted in subsequent reactions using 1.5 mM MgCl<sub>2</sub>. The total volume of the  
203 PCR reaction mix was 20 µl, consisting of 4 µl of 5X PCR polymerization buffer  
204 (Fisher Biotec), 9.8 µl of PCR-grade water, 4 µl of 7.5 mM MgCl<sub>2</sub>, 0.5 µl of each 50  
205 µM primer, 0.2 µl of *Taq* DNA polymerase (5 U µl<sup>-1</sup>) (Invitrogen) and 1 µl of DNA  
206 template. The PCR conditions for the thermocycler were an initial 4 min at 94°C; 31  
207 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; then 1 cycle of 94°C for  
208 1 min, 55°C for 1 min and 72°C for 5 min. The samples were then subjected to gel  
209 electrophoresis, or storage at -20°C.

210

211 The PCR amplification product was visualized after gel electrophoresis using a 1%  
212 (w/v) agarose in TAE gel submerged in TAE running buffer (40 mM Tris acetate, 1  
213 mM EDTA, pH 8.0) run at 80V for about 2 h. with loading dye added to each sample  
214 prior to electrophoresis. A 1 kb DNA ladder (Promega) was used as a marker. The  
215 gels were stained in 0.5 µg ml<sup>-1</sup> ethidium bromide for 40 min, destained in deionized  
216 water and visualized under UV light. Images of the gels were captured using the Gel  
217 Doc 2000 (BioRad) system.

218

## 219 2.6. Comparative genomics of *Methylobacterium* spp.

220 Homologs of genes required for methylophony were identified using BLASTP  
221 searches of sequences from the well-studied facultative methylophony  
222 *Methylobacterium extorquens* AM1 (Chistoserdova et al., 2003) against the five  
223 sequenced *Methylobacterium* spp. (*M. chloromethanicum* CM4, *M. extorquens* PA1,  
224 *M. nodulans* ORS 2060, *M. populi* BJ001 and *Methylobacterium* sp. (*Lotononis*) 4-  
225 46) and the *S. medicae* WSM419 genomes deposited in the Integrated Microbial  
226 Genomes database of the Joint Genome Institute.

## 227 3. Results

### 228 3.1. Acidification or alkalinization of 1/2 LA media

229 The *Lotononis* isolates all alkalinized or strongly alkalinized the media, consistent  
230 with organic acid utilization. USDA6 and ORS 2060 also alkalinized the media,  
231 while WSM419 caused acidification.

232

### 233 3.2. Growth on sole carbon substrates

234 Isolates from *L. bainesii*, *L. listii* and *L. solitudinis* were highly selective in their  
235 utilization of carbon sources (Table 2). None was able to utilize arabinose, galactose,  
236 glucose or mannitol as sole carbon sources. All the isolates from *Lotononis* spp. grew  
237 well on succinate and glutamate but none grew on methanol as a sole carbon source.

238

#### 239 3.2.2. Growth on C<sub>1</sub> sole carbon substrates

240 Neither the *Lotononis* isolates nor WSM419 grew on methanol, methylamine or  
241 formaldehyde at any of the concentrations supplied in the media (Table 3). ORS 2060  
242 grew on methanol, but not on methylamine or formaldehyde. Growth of the

243 *Lotononis* isolates on formate was variable. WSM2603, WSM2666, WSM2678,  
244 WSM2693, WSM3032, WSM3034 and WSM3035 did not grow, while WSM2598,  
245 WSM2660 and xct9 grew poorly and WSM2799 grew moderately well. WSM419  
246 grew poorly and ORS 2060 grew moderately well on formate. To determine whether  
247 methanol had an inhibitory effect on growth, all strains were also grown in broths  
248 containing both succinate and methanol as carbon sources (Table 3). All of the  
249 *Lotononis* isolates and WSM419, as well as ORS 2060, grew abundantly in the  
250 succinate plus methanol medium.

251

### 252 3.2.3. Growth of WSM2799 on formate

253 The growth rate of logarithmic phase cells of WSM2799 growing on formate was  
254 compared with that of succinate-grown cells by measuring OD<sub>600</sub>. The mean  
255 generation time (MGT) for WSM2799 grown on formate was 24 h, compared with  
256 5.5 h MGT when grown on succinate.

257

## 258 3.3. Biochemical assays for utilization of methanol

### 259 3.3.1. Utilization by xct9, ORS2060 and USDA6 over 70 h

260 Three strains (xct9, ORS 2060 and USDA6) were chosen for determination of  
261 methanol utilization at two different methanol concentrations (25 mM and 100 mM).  
262 The *L. bainesii* isolate xct9 was included as Jaftha et al. (2002) reported that it grew  
263 on M72 medium containing methanol as a sole carbon source. *M. nodulans* ORS  
264 2060 and *B. japonicum* USDA6 served as positive and negative controls,  
265 respectively. Over a period of 70 h and at both 25 mM and 100 mM of methanol,  
266 ORS 2060 was the only strain for which the concentration of methanol in the medium  
267 notably decreased (Fig. 1). The methanol concentrations in the cultures of xct9 and

268 USDA6 showed slight decreases equal to those in uninoculated media. Over the same  
269 period of time, and at both 25 mM and 100 mM methanol, the OD<sub>600</sub> of the ORS  
270 2060 culture increased, whereas the OD<sub>600</sub> of the xct9 and USDA6 cultures remained  
271 unchanged.

272

### 273 *3.3.2. Utilization of methanol in succinate plus methanol media*

274 The concentration of methanol in inoculated medium containing both succinate and  
275 methanol (Table 3) was determined for all strains after 10d incubation. In media  
276 inoculated with the *Lotononis* isolates or with WSM419, the methanol concentration  
277 was similar to that of the uninoculated control, but decreased by 50% in media  
278 inoculated with ORS 2060 (results not shown).

279

### 280 *3.4. PCR amplification of mxaF*

281 To evaluate the presence of methanol dehydrogenase genes in the *Lotononis* isolates,  
282 PCR amplification of *mxoF*, the gene that codes for the large subunit of methanol  
283 dehydrogenase, was performed on *Lotononis* isolates WSM2603, WSM2660,  
284 WSM2666, WSM2678, WSM2693, WSM2799, WSM3035 and xct9 and the  
285 methylotrophic strains ORS 2060 and DSM 760. The primers used have previously  
286 been shown to specifically amplify methylotrophic DNA (McDonald et al., 1995).  
287 The correct size 555-bp PCR product was obtained only for OR S2060 and DSM 760.  
288 No amplification was obtained for any of the *Lotononis* isolates, or for the reaction  
289 devoid of template DNA.

290

### 291 3.5. Comparative genomics of *Methylobacterium* spp.

292 Several different homologs of the *M. extorquens* AM1  $\alpha$ -subunit of MDH, coded for  
293 by *mxoF*, were found in each of the genomes of the five sequenced methylobacteria  
294 and of *S. medicae* WSM419. However, only the genomes of the methylophilic *M.*  
295 *chloromethanicum* CM4, *M. extorquens* PA1 and *M. populi* BJ001 had a *mxoF*  
296 homolog present in a *mxoFGIRSACKLDEHB* operon. The *M. nodulans* ORS2060  
297 *mxoF* appeared to be in a separate section of the genome, but this may be due to its  
298 genome sequence being still in a draft state. The remaining ORS2060 *mxo* genes were  
299 present in an orthologous operon. These four *mxoF* homologs had a high similarity  
300 (>88% over the length of the protein). In contrast, *mxoF* homologs found in  
301 *Methylobacterium* sp. 4-46 and *S. medicae* WSM419 were not in an orthologous  
302 operon and had a similarity of only 50% or less. No homologs of more than 50%  
303 similarity could be found in *Methylobacterium* sp. 4-46 for the MDH  $\beta$ -subunit  
304 (*mxoI*), the specific cytochrome *c* electron acceptor (*mxoG*), proteins essential for  
305  $\text{Ca}^{2+}$  insertion (*mxoACKL*) or for the protein products of *mxoSH*. Similarly, no  
306 homologs were found in WSM419 for the products of *mxoGIACKLD*.

307

## 308 4. Discussion

309 *Methylobacterium* species are described as able to grow on methanol, formaldehyde  
310 and formate (Green, 1992; Lidstrom, 2006). Sy et al. (2001) demonstrated that *M.*  
311 *nodulans*, isolated from root nodules of *Crotalaria*, was able both to fix nitrogen  
312 symbiotically and to utilize methanol as a sole carbon source. They also confirmed  
313 the presence in *M. nodulans* of *mxoF*, the gene that codes for the alpha subunit of  
314 methanol dehydrogenase. In contrast, our results from the carbon substrate utilization  
315 tests, the methanol assay and the *mxoF* PCR indicate that our isolates from *L.*

316 *bainesii*, *L. listii* and *L. solitudinis* differ from other described *Methylobacterium*  
317 species (Green, 1992; Gallego *et al.*, 2006; Kato *et al.*, 2008) in being unable to  
318 utilize or oxidize methanol. Kato *et al.* (2008) did, however, report that two of their  
319 *Methylobacterium* strains, isolated from freshwater, only grew weakly on methanol as  
320 a sole carbon source. Our findings also differ from those of Jaftha *et al.* (2002) who  
321 reported that their *L. bainesii* isolates grew in the presence of methanol as a sole  
322 carbon source. Consistent with our results, the *L. bainesii* isolate CB376 has  
323 previously been described as unable to utilize methanol (O'Brien & Murphy, 1993),  
324 but was identified as a species of *Rhizobium* at the time. The ability of the *Lotononis*  
325 isolates to grow in media containing succinate and methanol (1% v/v) indicates that  
326 their inability to grow on methanol as a sole carbon substrate is not due to any toxic  
327 effects of methanol at this concentration.

328

329 In methylobacteria, methanol dehydrogenase is essential for the oxidation of  
330 methanol to formaldehyde. The negative result for the PCR amplification of the *mxoF*  
331 gene suggests that the *Lotononis* isolates in this study lack at least one of the genes  
332 required to synthesize methanol dehydrogenase. Searches for methylotrophy genes in  
333 the sequenced genome of *Methylobacterium* sp. 4-46, isolated from *L. bainesii*,  
334 indicate that the inability to utilize methanol may be due to the absence of the *mxo*  
335 operon, which is present in the other sequenced methylotrophic *Methylobacterium*  
336 genomes, and the products of which are required for the primary oxidation of  
337 methanol (Amaratunga *et al.*, 1997; Chistoserdova *et al.*, 2003). It is likely that the  
338 *mxoF* homologs with similarity < 50% that are present in these genomes code for  
339 PQQ-dependent alcohol or glucose dehydrogenases that are not specific for methanol  
340 as a substrate.

341



342 Most species of *Methylobacterium* can only grow on a narrow range of carbohydrates  
343 (Green, 1992). The growth of the *L. bainesii*, *L. listii* and *L. solitudinis* isolates on  
344 other sole carbon sources is consistent with the substrate utilization patterns of other  
345 *Methylobacterium* species and with results obtained from other *L. bainesii* isolates  
346 (Jaftha et al., 2002) None of these isolates grew on the sugars or sugar alcohol  
347 supplied. Their ability to grow on succinate and to alkalinize ½ LA + universal  
348 indicator media also suggests that they preferentially utilize organic acids, including  
349 dicarboxylic acids, which are the usual form of carbon supplied to nitrogen-fixing  
350 bacteroids within legume nodules (Lodwig & Poole, 2003). *M. nodulans* ORS2060,  
351 in contrast, appears to be able to use methanol within the *Crotalaria podocarpa*  
352 nodule (Jourand et al., 2005).

353

354 Plants produce methanol as a by-product of pectin metabolism, with high pectin  
355 methyl esterase activity correlating with areas of rapid growth, such as seen in  
356 seedlings (Obendorf et al., 1990). Studies have suggested that the ability of  
357 *Methylobacterium* spp to utilize this methanol confers an advantage in plant  
358 colonization (Corpe & Rheem, 1989; Sy et al., 2005) and contributes to the  
359 effectiveness of the specific *C. podocarpa*/*M. nodulans* symbiosis (Jourand et al.,  
360 2005). Yates et al. (2007) have shown that the symbiosis between *L. bainesii*, *L. listii*  
361 and *L. solitudinis* and their nodulating methylobacteria is a highly effective one. Our  
362 results suggest that the inability to utilize methanol is not deleterious to effective  
363 colonization or symbiosis in the association between these methylobacteria and  
364 *Lotononis* and factors other than methylotrophy must be implicated in the specificity  
365 of the symbiosis.

366

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525



Table 1. List of strains used in this study

Isolate /strain	Host plant	Geographical origin	Collector(s)/Source	Reference
WSM2598	<i>L. bainesii</i>	Sth Africa	Yates, Real & Law (2002)	This study; Yates et al., (2007)
WSM2603	<i>L. listii</i>	Sth Africa	Yates, Real & Law (2002)	This study
WSM2660	<i>L. listii</i>	Sth Africa	Yates, Real & Law (2002)	This study
WSM2666	<i>L. listii</i>	Sth Africa	Yates, Real & Law (2002)	This study
WSM2678	<i>L. listii</i>	Sth Africa	Yates, Real & Law (2002)	This study
WSM2693	<i>L. listii</i>	Sth Africa	Yates, Real & Law (2002)	This study; Yates et al., (2007)
WSM2799	<i>L. listii</i>	Sth Africa	Yates, Real & Law (2002)	This study; Yates et al., (2007)
WSM3032	<i>L. solitudinis</i>	Sth Africa	Yates, Real & Law (2002)	This study; Yates et al., (2007)
WSM3034	<i>L. solitudinis</i>	Sth Africa	Yates, Real & Law (2002)	This study
WSM3035	<i>L. bainesii</i>	Sth Africa	Yates, Real & Law (2002)	This study
xct9 = CB376	<i>L. bainesii</i>	Sth Africa	Botha (1954); ARC <sup>a</sup>	Jaftha et al., (2002)
WSM419	<i>Medicago spp.</i>	Sardinia	Howieson (1985)	Howieson & Ewing (1986)
USDA6	<i>Glycine max</i>	Japan	USDA <sup>b</sup>	Jordan (1982)
ORS 2060	<i>Crotalaria spp.</i>	Senegal	INRA <sup>c</sup>	Sy et al., (2001)
DSM 760*		U.S.A.	INRA <sup>c</sup>	Patt et al., (1976)

<sup>a</sup>Agricultural Research Council-Plant Protection Research Institute, Pretoria, South Africa

<sup>b</sup>U.S. Department of Agriculture, Beltsville, Maryland, U.S.A

<sup>c</sup>National Institute for Agricultural Research, Montpellier Cedex 5, France

Table 2. Visual rating of growth of strains on various sole carbon sources after 10 days incubation. Growth was defined as 0 = no growth (OD < 0.1); x = poor growth (0.1 < OD < 0.2); xx = moderate growth (0.2 < OD < 0.5); xxx = abundant growth (OD > 1.0); ND = not determined.

Isolate/ Strain	Carbon Source							
	L+ Arabinose	D+ Galactose	D- Glucose	D- Mannitol	Succinate	Glutamate	MeOH <sup>a</sup> 266mM	MeOH 50mM
WSM2598	0	0	0	0	xxx	xxx	0	ND
WSM2603	0	0	0	0	xxx	xxx	0	ND
WSM2660	0	0	0	0	xxx	xxx	0	ND
WSM2666	0	0	0	0	xxx	xxx	0	ND
WSM2678	0	0	0	0	xxx	xxx	0	ND
WSM2693	0	0	0	0	xxx	xxx	0	0
WSM2799	0	0	0	0	xxx	xxx	0	0
WSM3032	0	0	0	0	xxx	xxx	0	ND
WSM3034	0	0	0	0	xxx	xxx	0	ND
WSM3035	0	0	0	0	xxx	xxx	0	ND
xct9	0	0	0	0	xxx	xxx	0	0
<b>Reference Strains</b>								
WSM419	xxx	xxx	xxx	xxx	xxx	xxx	0	ND
USDA6	xxx	xx	x	xxx	xxx	xxx	0	ND
ORS 2060	x	0	0	0	xxx	xxx	xxx	xxx

<sup>a</sup>MeOH = methanol

Table 3. Visual rating of growth of strains on various sole C<sub>1</sub> carbon sources after 10 days incubation. Growth was defined as 0 = no growth (OD < 0.1); x = poor growth (0.1 < OD < 0.2); xx = moderate growth (0.2 < OD < 0.5); xxx = abundant growth (OD > 1.0) and was determined for the following concentrations of substrates: succinate (20mM); methanol [0.2% (v/v)]; formaldehyde (0.5mM or 1.0mM); methylamine [0.1%, 0.2% or 0.5% (w/v)]; formate (30mM); succinate plus methanol [20mM plus 1.0% (v/v) respectively].

Isolate/ Strain	Carbon Source					
	Succinate	MeOH	Methylamine	Formaldehyde	Formate	Succinate + MeOH
WSM2598	xxx	0	0	0	x	xxx
WSM2603	xxx	0	0	0	0	xxx
WSM2660	xxx	0	0	0	x	xxx
WSM2666	xxx	0	0	0	0	xxx
WSM2678	xxx	0	0	0	0	xxx
WSM2693	xxx	0	0	0	0	xxx
WSM2799	xxx	0	0	0	xx	xxx
WSM3032	xxx	0	0	0	0	xxx
WSM3034	xxx	0	0	0	0	xxx
WSM3035	xxx	0	0	0	0	xxx
xct9	xxx	0	0	0	x	xxx
<b>Reference Strains</b>						
ORS 2060	xxx	xxx	0	0	xx	xxx
WSM419	xxx	0	0	0	x	xxx

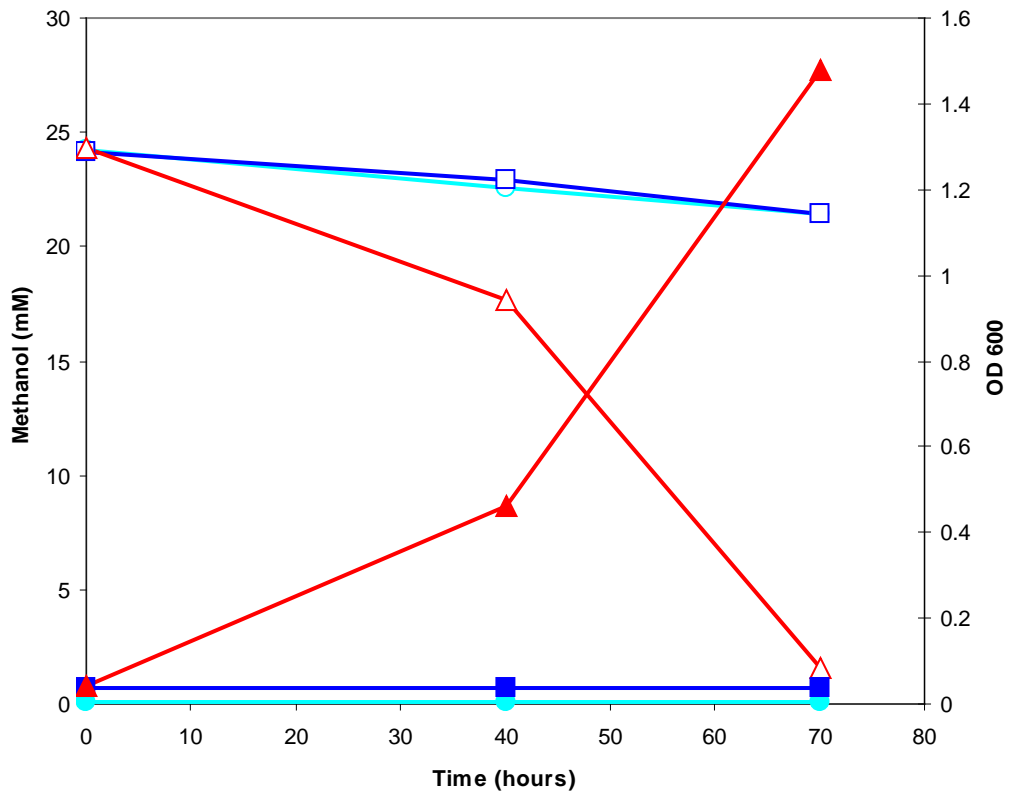


Fig. 1.

Fig. 1. Non-utilization of methanol by the *L. bainesii* isolate xct9. *Methylobacterium nodulans* strain ORS 2060 was used as a positive control. An uninoculated control was also used. Shown is growth of xct9 (filled squares), growth of ORS 2060 (filled triangles) and OD<sub>600</sub> of the uninoculated control (filled circles). MeOH concentrations in supernatants of cultures of xct9 (empty squares) and ORS 2060 (empty triangles) and of the uninoculated control (empty circles) are also shown.