Ardley, J.K. , O'Hara, G.W. , Reeve, W.G. , Yates, R.J. , Dilworth, M.J. , Tiwari, R.P. and Howieson, J.G. (2009) Root nodule bacteria isolated from South African Lotononis bainesii, L. listii and L. solitudinis are species of Methylobacterium that are unable to utilize methanol. Archives of Microbiology, 191 (4). pp. 311-318.

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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Abstract

The South African legumes *Lotononis bainesii, L. listii* and *L. solitudinis* are specifically nodulated by highly effective, pink-pigmented bacteria that are most closely related to *Methylobacterium nodulans* on the basis of 16S rRNA gene homology. *Methylobacterium* spp. are characterized by their ability to utilize methanol and other C_1 compounds, but eleven *Lotononis* isolates neither grew on methanol as a sole carbon source nor were able to metabolize it. No product was obtained for PCR amplification of *mxaF*, the gene encoding the large subunit of methanol dehydrogenase. Searches for methylotrophy genes in the sequenced genome of *Methylobacterium* sp. 4-46, isolated from *L. bainesii*, indicate that the inability to utilize methanol may be due to the absence of the *mxa* operon. While methylotrophy appears to contribute to the effectiveness of the *Crotalaria/M. nodulans* symbiosis, our results indicate that the ability to utilize methanol is not a factor in the *Lotononis*/*Methylobacterium* symbiosis.

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

1. Introduction

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

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

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

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

Methylobacterium spp. are characterized by their ability to utilize methanol and other C_1 compounds, as well as a variety of multicarbon substrates (Green, 1992; Lidstrom, 2006). Utilization of carbohydrates as a sole carbon source is variable and can be used to differentiate the various species (Green, 1992). Methylotrophy in *Methylobacterium* spp. involves over 100 genes constituting a set of metabolic functional modules (Chistoserdova et al., 2003). In the model organism *Methylobacterium extorquens* AM1, such modules involve the primary oxidation of methanol or methylamine to formaldehyde, the oxidation of formaldehyde, and the assimilation of C_1 products via the serine cycle (Chistoserdova et al., 2003; Lidstrom, 2006). Methanol is oxidized by methanol dehydrogenase (MDH), a protein with an $\alpha_2\beta_2$ tetramer structure, a pyrroloquinoline quinone (PQQ) cofactor and a calcium ion, essential for maintaining the PQQ in its active configuration, in the active site of each α-subunit (Anthony, 1996; Goodwin & Anthony, 1998). The genes encoding the MDH structural polypeptides, the specific cytochrome *c* electron acceptor, proteins essential for the insertion of the calcium ion, a regulatory protein and several proteins

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

The *M. nodulans* isolates from *Crotalaria*, like all previously described *Methylobacterium* species, can use methanol as a sole carbon source and contain a copy of *mxaF*, the gene that codes for the large subunit of MDH (Sy et al., 2001). Isolates from *L. bainesii* are also reported as able to grow in minimal media with methanol as a substrate (Jaftha et al., 2002). In contrast, our isolates from *L. bainesii*, *L. listii* and *L. solitudinis* appeared unable to grow on methanol in minimal media. The aim of this work was thus to determine the ability of these isolates to grow on or utilize a variety of C_1 and other carbon substrates.

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

2. Materials and Methods

2.1. Bacterial strains, origins and cultural conditions

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

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

2.2 Acidification or alkalinization of ½ LA medium

The ability of the isolates to acidify or alkalinize ½ LA medium was tested on unbuffered ½ LA agar plates, adjusted to pH 7.0 and containing 5 ml l^{-1} of Universal range pH indicator (Vogel, 1962). The isolates and reference strains were streaked onto the ½ LA plus Universal indicator plates and colour change recorded after 7 d incubation at 28ºC. Strains were scored as acidifying if the medium turned yellow (pH 6.0), alkalinizing if the medium turned blue/green (pH 8) or strongly alkalinizing if the medium turned blue (pH 9.0).

2.3. Growth on sole carbon substrates

2.3.1. General growth procedures

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

2.3.2. Growth on methanol and multicarbon substrates

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

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

2.3.3. Growth on C_1 sole carbon substrates

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

2.3.4. Growth of WSM2799 on formate

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

(20mM) or formate (30mM) as sole carbon source to give an initial OD_{600} of 0.05. Duplicate samples were taken from each flask at regular intervals for OD_{600} readings.

2.4. Biochemical assays for utilization of methanol

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

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

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

2.5. PCR amplification of mxaF

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

Whole cell DNA templates were prepared from isolates and reference strains. Cells were suspended in PCR-grade water to an OD_{600} of approximately 10. An initial PCR amplification to optimize the magnesium chloride concentration of the PCR reaction mix resulted in subsequent reactions using 1.5 mM $MgCl_2$. The total volume of the PCR reaction mix was 20 μl, consisting of 4 μl of 5X PCR polymerization buffer (Fisher Biotec), 9.8 μl of PCR-grade water, 4 μl of 7.5 mM $MgCl_2$, 0.5 μl of each 50 μM primer, 0.2 μl of *Taq* DNA polymerase (5 U μl^{-1}) (Invitrogen) and 1 μl of DNA template. The PCR conditions for the thermocycler were an initial 4 min at 94ºC; 31 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 1 min; then 1 cycle of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 5 min. The samples were then subjected to gel electrophoresis, or storage at -20ºC.

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

2.6. Comparative genomics of Methylobacterium spp.

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

3. Results

3.1. Acidification or alkalinization of ½ LA media

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

3.2. Growth on sole carbon substrates

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

3.2.2. Growth on C_1 sole carbon substrates

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

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

3.2.3. Growth of WSM2799 on formate

The growth rate of logarithmic phase cells of WSM2799 growing on formate was compared with that of succinate-grown cells by measuring OD_{600}. The mean generation time (MGT) for WSM2799 grown on formate was 24 h, compared with 5.5 h MGT when grown on succinate.

3.3. Biochemical assays for utilization of methanol

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

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

USDA6 showed slight decreases equal to those in uninoculated media. Over the same period of time, and at both 25 mM and 100 mM methanol, the OD_{600} of the ORS 2060 culture increased, whereas the OD_{600} of the xct9 and USDA6 cultures remained unchanged.

3.3.2. Utilization of methanol in succinate plus methanol media

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

3.4. PCR amplification of mxaF

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

3.5. Comparative genomics of Methylobacterium spp.

Several different homologs of the *M. extorquens* AM1 α-subunit of MDH, coded for by *mxaF*, were found in each of the genomes of the five sequenced methylobacteria and of *S. medicae* WSM419. However, only the genomes of the methylotrophic *M. chloromethanicum* CM4, *M. extorquens* PA1 and *M. populi* BJ001 had a *mxaF* homolog present in a *mxaFGIRSACKLDEHB* operon. The *M. nodulans* ORS2060 *mxaF* appeared to be in a separate section of the genome, but this may be due to its genome sequence being still in a draft state. The remaining ORS2060 *mxa* genes were present in an orthologous operon. These four *mxaF* homologs had a high similarity (>88% over the length of the protein). In contrast, *mxaF* homologs found in *Methylobacterium* sp. 4-46 and *S. medicae* WSM419 were not in an orthologous operon and had a similarity of only 50% or less. No homologs of more than 50% similarity could be found in *Methylobacterium* sp. 4-46 for the MDH β-subunit (*mxaI*), the specific cytochrome *c* electron acceptor (*mxaG*), proteins essential for Ca^{2+} insertion (*mxaACKL*) or for the protein products of *mxaSH*. Similarly, no homologs were found in WSM419 for the products of *mxaGIACKLD.*

4. Discussion

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

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

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

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

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

Acknowledgements

J.A. is the recipient of a Murdoch University Research Scholarship.

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Table 1. List of strains used in this study

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

[a]Agricultural Research Council-Plant Protection Research Institute, Pretoria, South Africa

[b]U.S. Department of Agriculture, Beltsville, Maryland, U.S.A

[c]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[a] 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
Reference Strains								
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

[a]MeOH = methanol

Table 3. Visual rating of growth of strains on various sole C_1 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
Reference Strains						
ORS 2060	xxx	xxx	0	0	xx	xxx
WSM419	xxx	0	0	0	x	xxx

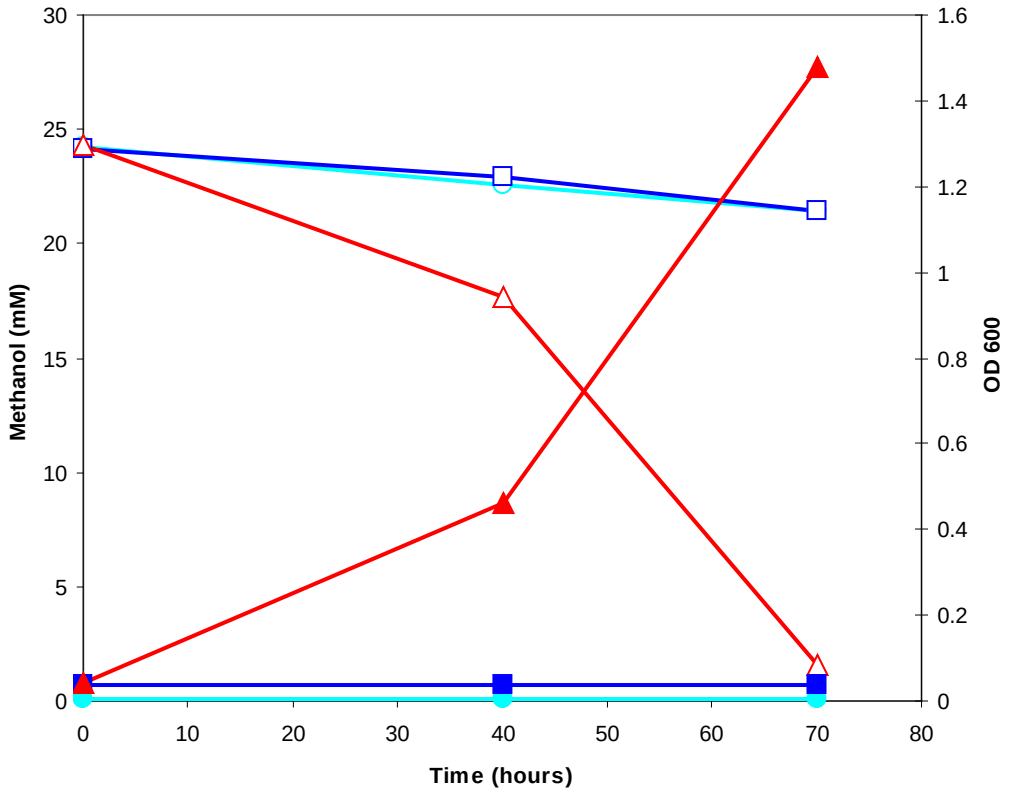
Methanol (mM)
OD 600
Time (hours)
0
5
10
15
20
25
30
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
0
10
20
30
40
50
60
70
80

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_{600} 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.