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Lotononis angolensis forms nitrogen fixing, lupinoid nodules with phylogenetically unique, fast-growing, pink-pigmented bacteria, which do not nodulate *L. bainesii* or *L. listii*

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Abstract

Root-nodule bacteria that nodulate the legume genus *Lotononis* are being investigated to develop new forage species for agriculture. Bacteria isolated from nodules of *Lotononis angolensis* were fast-growing, highly mucoid and pink-pigmented, and on the basis of 16S rRNA phylogeny <94% related to other genera in the *Alphaproteobacteria*. Root-nodule bacteria isolated from other *Lotononis* species (*L. bainesii*, *L. solitudinis* and *L. listii*) resembled the more common dry, slow-growing, pink-pigmented rhizobia previously described for *L. bainesii*. These isolates could be attributed to the *Methylobacterium* genus, although not to the type species *Methylobacterium nodulans*. Further differences were uncovered with nodulation studies revealing that nodule isolates from *L. angolensis* were effective at nitrogen fixation on their host plant, but could nodulate neither *L. bainesii* nor *L. listii*. Reciprocal tests showed isolates from *L. bainesii*, *L. listii* and *L. solitudinis* were incapable of nodulating *L. angolensis* effectively. Nodule morphology for *L. bainesii*, *L. angolensis* and *L. listii* was characteristically lupinoid, with little structural divergence between the species, and with nodules eventually enclosing the entire root.

Keywords: *Lotononis*; Root-nodule bacteria; Nitrogen fixation; *Methylobacterium*; Phylogeny; Nodulation

1. Introduction

The genus *Lotononis* contains approximately 150 species of herbs and small shrubs in the tribe *Crotalarieae* of the sub-family *Fabaceae* (Van Wyk, 1991). Their distribution is mainly in southern Africa with a few species extending elsewhere in Africa, the Mediterranean and central Asia (Van Wyk, 1991). Three *Lotononis* species of current agronomic interest, *L. bainesii, L. angolensis* and *L. listii* (previously *Listia heterophylla*) are taxonomically positioned in the section *Listia,* that overall contains eight species. The remaining species are rare and in some instances considered endangered; these include *L. macrocarpa, L. marlothii*, *L. minima*, *L. solitudinis* and *L. subulata* (Van Wyk, 1991). Norris (1958, 1959) first reported rhizobia from *L. bainesii* as 'red in colour' and speculated that other species from the genus *Lotononis* may also possess pigmented rhizobia. The red or pink colouration is due to intracellular carotenoid that, together with the high resistance of these bacteria to ultraviolet irradiation, is speculated to be of ecological advantage (Godfrey, 1972; Law, 1979). Nodulation studies in the 1960s recorded effective cross-nodulation between strains isolated from *L. bainesii* and *L. listii*, but not with *L. macrocarpa* (R. Date pers. comm.). Field observations from this era suggest that these strains may not have been effective in nitrogen fixation with *L. angolensis*, as this species was recorded as "unproductive" when inoculated with the

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L. bainesii inoculant (K. Sandman, 1950, unpublished; 't Mannetje, 1967). Pink-pigmented isolates from *L. bainesii* nodules have been characterised as methylobacteria (Jaftha et al., 2002). The capacity for methylotrophic strains to nodulate was first described by Samba et al. (1999) for three *Crotalaria* species, and the name *Methylobacterium nodulans* was subsequently proposed (Sy et al., 2001). However, the classification of the microsymbionts for *Lotononis* is still in its infancy.

Within southern Australia, the *Lotononis* species have recently been identified as perennial pasture legumes with the potential to reduce ground water recharge to assist in controlling dryland salinity (Yates et al., 2006). Whereas *L. bainesii* has previously been exploited in sub-tropical areas of Australia (cv. Miles; Bryan, 1961) and Uruguay (cv. INIA Glencoe; Real et al., 2005), it has not been evaluated in Mediterranean-type climates. Although it is an unusual step to evaluate sub-tropical legume species for their adaptation to a dry Mediterranean-type climate, the increasing frequency of summer rainfall events and the availability of water stored in the root-zone may support some hardy species through a summer dry period (Howieson et al., 2007). Diatloff (1977) showed that *Lotononis* rhizobia became well-established after introduction and were stable in acidic heath sands of Queensland. This complements our studies, which indicate that *L. bainesii* isolates have the ability to persist in acid and infertile sandy soils of Western Australia (Yates et al., 2006). With renewed interest in the agronomic potential of this legume genus, it is imperative that we fully understand the various root-nodulating organisms associated with it. Hence, this study aimed to identify and characterise rhizobia isolated from the nodules of *L. angolensis, L. bainesii*, *L. listii* and *L. solitudinis*. We also report on the nodule ultrastructure of these species.

2. Material and methods

2.1. Bacterial micro-symbionts and legume hosts

The strains of rhizobia investigated are listed in Table 1, together with their host plant and collection details. All strains originally isolated from *Lotononis* nodules were authenticated on their original hosts. Cultures were grown at 28 °C on modified $\frac{1}{2}$ Lupin Agar (LA) (Howieson et al., 1988). The medium contained: (in g/L) mannitol (5.0); D-glucose (5.0); yeast extract (1.25); agar (18); and (in mM) $MgSO_4 \cdot 7H_2O$ (3.2); NaCl (1.7), $CaCl_2 \cdot 2H_2O$ (1.4); and (in μM) K_2HPO_4 (100); KH_2PO_4 (100); $FeSO_4$ (18); $Na_2B_4O_7$ (6); $MnSO_4 \cdot 4H_2O$ (12); $ZnSO_4 \cdot 7H_2O$ (0.76); $CuSO_4 \cdot 5H_2O$ (0.32) and $Na_2MoO_4 \cdot 2H_2O$ (0.54). The pH was adjusted to 6.8 with 0.1 M NaOH. WSM3686 and WSM3674 were grown for 3 days prior to inoculation while the remaining strains were grown for 7 days. Colony growth rates and morphology of strains isolated from *Lotononis* spp. were compared with the type strain *M. nodulans* (ORS2060).

The legume species and origin of seed used in glasshouse experiments are shown in Table 2. In addition to the three species of *Lotononis* (*L. angolensis, L. bainesii* and *L. listii*), *Macroptilium atropurpureum* was included because of its known promiscuous nature with a range of rhizobia (Thies et al., 1991; Perret et al., 2000; Yates et al., 2004). *L. solitudinis* could not be used in cross-inoculation and nodulation experiments because of the rarity of the species and consequent lack of seed.

2.2. General glasshouse procedures

Four days before the commencement of experiments, seeds were surface sterilised by immersion in 70% (v/v) ethanol for 60 s and then 4% (w/v) $NaHClO_4$ for 30 s,

Table 1
Origin of root-nodule bacteria used in this study

Strain	Host plant	Geographic origin	Source (year isolated)
ORS2060[a]	*Crotalaria podocarpa*	Senegal	Samba (1999)
USDA6[b]	*Glycine max*	Japan	Aso (1929)
WSM2598[c]	*Lotononis bainesii*	South Africa	Yates, Real and Law (2002)
WSM2693[c]	*Lotononis listii*	South Africa	Yates, Real and Law (2002)
WSM2799[c]	*Lotononis listii*	South Africa	Yates, Real and Law (2002)
WSM3032[c]	*Lotononis solitudinis*	South Africa	Yates, Van Wyk and Law (2005)
WSM3674[c]	*Lotononis angolensis*	Zambia	Verboom (1963)[d]
WSM3686[c]	*Lotononis angolensis*	Zambia	Verboom (1963)[d]
CB376[e]	*Lotononis bainesii*	Kenya	Botha (1954)
XCT17[f]	*Lotononis bainesii*	South Africa	Law (1982)

[a]Isolate obtained from Dr. Catherine Boivin-Masson (LSTM), France. Isolate described by Samba et al. (1999).
[b]USDA ARS National *Rhizobium* Germplasm Collection, Beltsville, USA.
[c]Western Australian Soil Microbiology (WSM) culture collection, Centre for *Rhizobium* Studies, Murdoch University, Perth, Australia.
[d]Re-isolated from mixed cultures following passage through the host.
[e]CSIRO culture collection, Brisbane, Australia (now resident at the University of Western Sydney).
[f]Agricultural Research Council (ARC)—Plant Protection Research Institute culture collection, Pretoria, South Africa.