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Novel *Burkholderia* bacteria isolated from *Lebeckia ambigua* – A perennial suffrutescent legume of the fynbos

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ABSTRACT

We investigated symbiotic and physiological properties, and taxonomic position, of 23 bacterial strains isolated from *Lebeckia ambigua* root nodules collected from the South African fynbos region. The capacity for nodulation and nitrogen fixation on three provenances of *L. ambigua* was investigated for these strains together with several physiological characters, including growth rate on peat and in betonite clay, survival on polyethylene beads and pH tolerance. Additionally, the 16S rRNA gene phylogeny was determined. The root nodule bacteria isolated clustered in five different groups belonging to the genus *Burkholderia*, most closely related to *B. caledonica*, *B. graminis* and *B. tuberum*. Moreover there was a very strong influence of collection site on the taxonomy of the *Burkholderia* strains. The physiological characterisation revealed two promising strains, WSM4174 and WSM4184, achieved rapid growth in normal media and reached high, stable numbers in sterile peat. However, there was a worrying susceptibility to desiccation amongst these *Burkholderia*. Additionally, evidence was found for isolation of non-symbiotic strains from the nodule material collected in South Africa.

1. Introduction

Australian agriculture has relied upon N_2-fixation from rhizobia, as symbionts primarily of annual legumes, since European colonisation in the late 18thC. This N_2-fixation is currently estimated to be worth in excess of $2 billion per annum (Herridge et al., 2008). The south-west of Western Australia is a major agricultural region whose rainfall patterns appear to be changing, from a dry Mediterranean-type distribution to a generally reduced annual rainfall with a less predictable distribution (George et al., 2008). This change in climate mitigates against optimal growth from annual legumes because of a disruption to their pattern of germination and reproduction. Alternative perennial legume species are therefore being sought (Howieson et al., 2008). The target regions for our research include some 5 million ha in south-west WA receiving 250–500 mm annual rainfall, of which a portion have already experienced a decline in precipitation of 10% between 1976 and 1999, followed by 15% in the subsequent seven years (George et al., 2008), with the driest winter for 100 years recorded in 2010 (http://www.bom.gov.au/climate/current/season/wa/archive/201008.summary.shtml).

Lucerne (*Medicago sativa* L.) will not persist on many of our target soils, which are acid and infertile, and there is a paucity of well-adapted commercially available perennial, herbaceous forage legumes in regions which receive less than 500 mm annual rainfall (Howieson et al., 2008). As part of the search for legumes better adapted to these soils, we are attempting to domesticate species from southern Africa, including those from the acid, sandy soils of the fynbos biome in the Western Cape region of South Africa. We have collected seed and nodules of several genera of deep-rooted, suffrutescent, perennial legumes (e.g. *Rhynchosia*, *Lebeckia* and *Lessertia*), which although known to botanists (Boatwright et al., 2009; Van Wyk, 1991), are mostly new to agriculture. Our approach has focussed on assessing the nodule bacteria of these potential new agricultural legumes (Ardley et al., 2012; Garau et al., 2009; Gerding et al., 2012; Yates et al., 2007) because poorly adapted inoculants have historically restricted the success of new forage legumes introduced to WA soils (Parker, 1962). Several of the perennial legumes from southern Africa are nodulated by the

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relatively novel species of *Microvirga* (Ardley et al., 2012) or by Betaproteobacterial *Burkholderia* spp. (Garau et al., 2009; Phalane, 2008).

The Betaproteobacteria are a class of bacteria first reported to contain root nodule bacteria by Moulin et al. (2001). These are mainly from the genus *Burkholderia*, which are the dominant nodule occupants of species of the large sub-tropical genus *Mimosa* found in the Cerrado and the Caatinga (Bontemps et al., 2010; dos Reis et al., 2010). Rhizobial *Burkholderia* have also been found in nodules of the papilionoid legumes *Cyclopia* and the herbaceous, perennial legume *Rhynchosia ferulifolia*, both endemic to the western Cape of South Africa (Elliott et al., 2007a; Garau et al., 2009). In this manuscript we describe additional Betaproteobacteria nodule bacteria from the wild perennial fynbos legume *Lebeckia ambigua*, which we reveal are novel species of *Burkholderia*. Because rhizobial *Burkholderia* are increasingly being isolated from fynbos legumes, we raise the possibility of *Burkholderia* being nodule bacteria particularly well adapted to infertile sandy, acid soils.

2. Materials and methods

2.1. Acquisition of root-nodules, bacteria and host legumes

Four separate germplasm acquisition expeditions were made to the Western Cape of South Africa, between the latitudes of Cape Town and Springbok, from 2002 to 2007. Nodules and seed (where available) of herbaceous perennial legumes were collected and stored as previously described (Yates et al., 2004). Nodules from *L. ambigua* were collected from five sites (Table 1, Fig. S1) and returned to Australia for isolation. Bacteria were isolated from surface sterilised root nodules according to the procedure described by Yates et al. (2007). Pure cultures were deposited in the WSM Genebank (Centre for *Rhizobium* Studies, Murdoch University, Perth, Western Australia) after authentication. Seed of four representative provenances of *L. ambigua* were multiplied in the field plots at Murdoch University to provide seed for nodulation and effectiveness experiments described in this manuscript. Seed of the related legume *Lebeckia sepiaria* was provided by J.S. Boatwright, Kirstenbosch Botanical Gardens, Cape Town. The legumes used in the glasshouse experiments are listed in Table S1.

2.2. Authentication of nodule isolates (Experiment 1)

Two studies of nodulation of *L. ambigua* were performed using the axenic sand-culture system described by Howieson et al. (1995). The experiments were conducted in pasteurised soil in sterilised polythene pots, covered with sterilised alkathene beads, held in a naturally lit phytotron maintained at 22 °C during the day. This system of evaluation excludes air- and water-borne nodule bacteria from contaminating experiments.

In the first experiment, 23 strains of nodule bacteria listed in Table 1 were assessed for their capacity to nodulate three provenances of *L. ambigua* (CRSLAM-37, 39, 41, see Table S2). Three seedlings, representing each provenance, were placed in a 1 kg pot then inoculated with a single test strain as described by Yates et al. (2007). All treatments, plus uninoculated and nitrogen-supplied controls, were replicated four times in this split-plot design, and

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

Strain	Collection site	Latitude and longitude	Legume host	Exp.	Accession number[b]	Reference
***Burkholderia* sp.**						
WSM3556	5	33° 29′21″; 18° 19′36″	*L. ambigua*	1	HQ698908	This work
WSM3558	5	33° 29′21″; 18° 19′36″	*L. ambigua*	1	N.A.	This work
WSM3560	5	33° 29′21″; 18° 19′36″	*L. ambigua*	1	N.A.	This work
WSM4204	5	33° 29′21″; 18° 19′36″	*L. ambigua*	1	HQ698906	This work
WSM4205	5	33° 29′21″; 18° 19′36″	*L. ambigua*	1, 2	HE862280	This work
WSM4206	5	33° 29′21″; 18° 19′36″	*L. ambigua*	1	HQ698907	This work
WSM4289	5	33° 29′21″; 18° 19′36″	*L. ambigua*	1	N.A.	This work
WSM4290	5	33° 29′21″; 18° 19′36″	*L. ambigua*	1	N.A.	This work
WSM4291	5	33° 29′21″; 18° 19′36″	*L. ambigua*	1	N.A.	This work
WSM4292	5	33° 29′21″; 18° 19′36″	*L. ambigua*	1	N.A.	This work
WSM4293	5	33° 29′21″; 18° 19′36″	*L. ambigua*	1	N.A.	This work
WSM3617	10	32° 01′56″; 18° 47′38″	*L. ambigua*	1	HQ698903	This work
WSM3618	10	32° 01′56″; 18° 47′38″	*L. ambigua*	1, 2	HE862276	This work
WSM4182	11	31° 47′59″; 18° 37′16″	*L. ambigua*	1	HQ698910	This work
WSM4184	11	31° 47′59″; 18° 37′16″	*L. ambigua*	1, 2, 4	HE965764	This work
WSM4185	11	31° 47′59″; 18° 37′16″	*L. ambigua*	1, 4	HE965765	This work
WSM5005	11	31° 47′59″; 18° 37′16″	*L. ambigua*	1, 2, 4	HF549035	This work
WSM4174	14	31° 26′47″; 19° 8′41″	*L. ambigua*	1, 2	HQ698904	This work
WSM4175	14	31° 26′47″; 19° 8′41″	*L. ambigua*	1	HE962574	This work
WSM4176	14	31° 26′47″; 19° 8′41″	*L. ambigua*	1	HQ698909	This work
WSM4177	14	31° 26′47″; 19° 8′41″	*L. ambigua*	1, 2	HE862275	This work
WSM4178	14	31° 26′47″; 19° 8′41″	*L. ambigua*	1	HE862279	This work
WSM4180	14	31° 26′47″; 19° 8′41″	*L. ambigua*	1	HE862274	This work
WSM3937	2	33° 17′33″; 18° 26′26″	*Rhynchosia ferulifolia*	2, 4	EU219865	Garau et al., 2009
B. phymatum STM815	French Guiana		*Mimosa* sp.[a]	2	AJ302312	Moulin et al., 2001
Brad. sp. WSM471	Albany W. Australia		*Ornithopus pinnatus*	4	Gi06491	Howieson et al., 1994
M. loti WSM1293	Greece		*Lotus orn.*	4	Gi08882	Howieson et al., 2011
M. sp. WSM1497	Greece		*Bis. pelecinus*	4	AF178964	Nandasena et al., 2001
Methylobacterium sp. WSM2598	South Africa		*Listia bainesii*	4	DQ838527	Yates et al., 2007
R. leg. bv. *trifolii* WSM1325	Serifos, Greece		*Trifolium* sp.	4	Gc01039	Yates et al., 2005
R. leg. bv. *viciae* WSM1455	Mykonos, Greece		*Pisum sativum*	4	Gi06482	Howieson et al., 2000a
R. tropici CIAT899	Colombia		*Ph. vulgaris*	4	Gi05744	Graham et al., 1994
R. sullae WSM1592	Italy, Sardinia		*Sulla coronarium*	4	Gi08899	Yates et al., 1996

[a] Originally reported as isolated from *Machaerium lunatum* but not authenticated on this host, effectively nodulates *Mimosa* (Elliott et al., 2007b; Mishra et al., 2012).
[b] Either 16S rRNA gene accession number or Gold Card number, N.A. = Not Available, Exp. = Experiment, *L* = *Lebeckia*, *Bis.* = *Bisurella*, *Ph.* = *Phaseolus*, *B.* = *Burkholderia*, *Brad.* = *Bradyrhizobium*, *M.* = *Mesorhizobium*, *R.* = *Rhizobium*, *leg.* = *leguminosarum*, *orn.* = *ornithopodioides*.