Rapid Evolution of Diversity in the Root Nodule Bacteria of *Biserrula pelecinus* L.

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I declare that this thesis is my own account of my research and contain as its main content work which has not been submitted for a degree at any tertiary education institution.

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Kemanthi Gayathri Nandasena

……..To my beloved parents

With much love…….

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Publications arising from this thesis

Nandasena, K. G., G. W. O'Hara, R. P. Tiwari, R. J. Yates, and J. G. Howieson. 2001. Phylogenetic relationships of three bacterial strains isolated from the pasture legume *Biserrula pelecinus* L. International Journal of Systematic and Evolutionary Microbiology **51:**1983-1986.

Nandasena, K. G., G. W. O'Hara, R. P. Tiwari, R. J. Yates, B. D. Kishinevsky, and J. G. Howieson. 2004. Symbiotic relationships and root nodule ultrastructure of the pasture legume *Biserrula pelecinus* L.-a new legume in agriculture. Soil Biology and Biochemistry **36:**1309-1317.

Abstract

Biserrula pelecinus L. has been introduced to Australia from the Mediterranean region, in the last decade due to many attractive agronomic features. This deep rooted, hard seeded, acid tolerant and insect resistant legume species provides high quality food for cattle and sheep, and grows well under the harsh edaphic and environmental conditions of Australia. In 1994, *B. pelecinus* was introduced to a site in Northam, Western Australia where there were no native rhizobia capable of nodulating this legume. The introduced plants were inoculated with a single inoculant strain of *Mesorhizobium* sp., WSM1271. This study investigated whether a diversity of rhizobia emerged over time. A second objective was to investigate the possible mechanisms involved in the diversification of rhizobia able to nodulate *B. pelecinus.*

Eighty eight isolates of rhizobia were obtained from nodules on *B. pelecinus* growing at the Northam site in August 2000, six years after introduction. These plants were self-regenerating offspring from the original seeds sown. Molecular fingerprinting PCR with RPO1 and ERIC primers revealed that seven strains (novel isolates) had banding patterns distinct from WSM1271 while 81 strains had similar banding patterns to WSM1271. A 1400 bp internal fragment of the 16S rRNA gene was amplified and sequenced for four of the novel isolates (N17, N18, N45 and N87) and WSM1271. The phylogenetic tree developed using these sequences clustered the novel isolates in *Mesorhizobium*. There were >6 nucleotide mismatches between three of the novel isolates (N17, N18, N87) and WSM1271 while there were 23 nucleotide mismatches between N45 and WSM1271.

When *B. pelecinus* cv. Casbah was inoculated with the novel isolates, five (N17, N18, N39, N46 and N87) yielded <40% of the shoot dry weight of the plants inoculated with the original inoculant (WSM1271). Novel isolates N15 and N45 were completely ineffective on *B. pelecinus* cv. Casbah.

Physiological experiments to test the ability of the novel isolates and WSM1271 to grow on 14 different carbon sources (N acetyl glucosamine, arabinose, arbutine, dulcitol, β-gentiobiose, lactose, maltose, melibiose, D-raffinose, saccharose, L-sorbose, D-tagatose, trehalose and D-turanose) as the sole source of carbon, intrinsic resistance to eight different antibiotics (ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, spectinomycin, streptomycin and tetracycline) and pH tolerance (pH 4.5, 5.0, 7.0, 9.0) revealed that the novel isolates had significantly different carbon source utilization patterns to WSM1271. However, pH tolerance and intrinsic resistance to antibiotics were similar between the novel isolates and WSM1271 except for streptomycin (100 µg/ml). Novel isolates N17, N18, N46 and N87 were susceptible for this antibiotic while the other novel isolates and WSM1271 were resistant.

Host range experiments were performed for the novel isolates N17, N18, N45, N87, WSM1271 and two other root nodule bacteria (RNB) previously isolated from *B. pelecinus* growing in the Mediterranean region (WSM1284 and WSM1497) for twenty one legumes (*Amorpha fruticosa, Astragalus adsurgens, Astragalus membranaceus, Astragalus sinicus, Biserrula pelecinus cv Casbah, Dorycnium hirsutum, Dorycnium rectum, Glycyrrhiza uralensis, Hedysarum spinosissimum, Leucaena leucocephala, Lotus corniculatus, Lotus edulis, Lotus glaber, Lotus maroccanus, Lotus ornithopodioides, Lotus parviflorus, Lotus*

pedunculatus, Lotus peregrinus, Lotus subbiflorus, Macroptilium atropurpureum, and *Ornithopus sativus*). Only isolate N17 have the same host range as WSM1271 in that they both nodulated *B. pelecinus* and *A. membranaceus,* while the other three novel isolates, WSM1284 and WSM1497 had a broader host range than WSM1271. Three isolates N18, N45 and N87 formed small white nodules on *M. atropurpureum*, in addition to nodulating the above hosts. Isolates N18 and N45 also nodulated *A. adsurgens* while N45 was the only isolate to nodulate *L. edulis*. Isolate N87 was the only isolate to nodulate *A. fruticosa*. WSM1497 nodulated *A. adsurgens, A. membranaceus, B. pelecinus* and *L. corniculatus* while WSM1284 was a promiscuous strain that nodulated 16 host species out of the 21 tested.

A 710 bp internal region of *nifH*, a 567 bp internal region of *nodA* and a 1044 bp internal region of *intS* were sequenced for N17, N18, N45, N87 and WSM1271. The sequence comparison showed that the sequences of the above three genes of the four novel isolates were identical to that of WSM1271.

Eckhardt gel electrophoresis revealed that WSM1271, three other RNB isolates from *B. pelecinus* from the Mediterranean region and isolate N18 each have a plasmid of approximately 500 kb while N17, N45 and N87 are plasmid free. Probing of the plasmid DNA from the Eckhardt gel with *nifH* and *nodA* probes indicated that these two genes were not located on the plasmid.

Furthermore, the results of this study demonstrated that 92% of the nodules on *B. pelecinus* growing in the Northam site six years after the introduction of this plant were occupied by the inoculant strain and the N_2 fixation efficiency of the progeny strains of WSM1271 remain similar to the mother culture. This study also showed that the carbon source utilization

pattern, intrinsic antibiotic resistance and pH range of the progeny strains of WSM1271 remain relatively similar, except for few variations in carbon source utilization patterns.

This thesis clearly demonstrated that phenotypicaly, genetically and phylogenetically diverse strains capable nodulating *B. pelecinus* evolved through symbiotic gene transfer from the inoculant strain to other soil bacteria within six years. The presence of *intS*, and the evidence of gene transfer between these *Mesorhizobium* strains indicates that transfer of symbiotic genes may have occurred via a symbiosis island present in WSM1271.

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