

Characterisation of rhizobia nodulating *Galega officinalis* (goat's rue) and *Hedysarum coronarium* (sulla)

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Abstract Many legumes can colonise low nitrogen (N) soils due to their ability to fix atmospheric N₂ via symbiotic bacteria in root nodules. *Galega officinalis* and *Hedysarum coronarium* are legumes that have naturalised and become common weeds in New Zealand. Previous work outside of New Zealand indicated that they only form effective nodules with their respective symbionts, *Rhizobium galegae* and *R. sullae*. Here, analysis of 16S rRNA and housekeeping genes, and plant nodulation tests were carried out on five selected bacterial strains isolated from root nodules of both legumes sampled at one site each. Only *Rhizobium galegae* strains were isolated from *G. officinalis* and selected strains induced effective nodules when re-inoculated onto the host plant. *Agrobacterium vitis*, *R. galegae* and *R. sullae* strains were isolated from nodules of *H. coronarium*, but only *R. sullae* induced effective nodules on this plant. Results agree with previous reports that these legume species are highly specific in the rhizobia they form effective nodules with, but further work is required to confirm this.

Keywords rhizobia, weed, legume, genotypic characterisation, 16S rRNA.

INTRODUCTION

Many leguminous plants can fix atmospheric nitrogen (N) via symbiotic bacteria (collectively termed 'rhizobia') in root nodules and this can be advantageous in a low N soil if other factors are favourable for growth (Andrews et al. 2011). Some legume species are restricted in their partner symbionts while others can be 'promiscuous' and may be nodulated by a wide range of rhizobia (Shtark et al. 2011).

A wide range of legumes has naturalised in New Zealand and for most species, it is unknown if they form effective nodules in the soils, and if they do nodulate, what are the rhizobia nodulating them? It is also not known if these legumes nodulate

with rhizobia associated with New Zealand native legumes, which are mainly *Mesorhizobium* spp. (Weir et al. 2004). The ability to fix N₂ could be an important factor in the naturalisation of specific legumes in New Zealand.

Two perennial legumes, *Galega officinalis* (goat's rue) and *Hedysarum coronarium* (sulla), have naturalised and become common weeds in New Zealand (Popay et al. 2010). Goat's rue, native to Europe and western Asia, was accidentally introduced into the country at the Manawatu river bed (Webb et al. 1988) and is now listed in the Pest Plant Management Strategies in several regions of New Zealand (Popay et al. 2010). It has

been reported to cause poisoning in sheep and other livestock (Popay et al. 2010). Sulla, native to the Mediterranean, was introduced to control soil erosion (Webb et al. 1988) and is now widely established in many places in New Zealand (Popay et al. 2010). Previous work done on both legumes sampled in their natural habitats indicated that both species have a high degree of specificity in the rhizobia they form effective nodules with. Goat's rue only formed effective nodules with *Rhizobium galegae* strains (Lindström 1989), while sulla only formed effective nodules with *R. sullae* (= *R. hedysari*) (Cabrera & Ruiz-Argueso 1979).

This paper firstly assesses if goat's rue and sulla plants form nodules in New Zealand soils. Nodules were present and bacterial strains were isolated from the nodules and characterised on the basis of 16S rRNA, *atpD* and *recA* gene sequences to establish their identity. Finally, the ability of the strains to effectively nodulate their host plants was determined.

MATERIALS AND METHODS

Isolation of bacterial strains

Root nodules of 'wild' *G. officinalis* and *H. coronarium* (5 plants each) were collected from the roadside at Palmerston North (40°19'02.50"S 175°34'22.82"E) and a roadside metal dump at Wharerata (38°51'22.52"S 177°54'13.38"E), respectively, in December 2010. Four nodules from each plant were surface sterilised and crushed in sterile water as described in Weir et al. (2004). This suspension was then streaked onto yeast mannitol agar (YMA, a recipe from Vincent (1970)) plates and incubated at 25°C in the dark for 4-5 days. Single colonies were obtained by sub-culture from each plate and a total of 20 strains inoculated in yeast mannitol broth (YMB) (Vincent 1970) were chosen on the basis of their colony morphologies for 16S rRNA characterisation.

Sequencing of 16S rRNA, *atpD* and *recA* genes

DNA was extracted from the bacterial cultures grown in YMB using the standard Qiagen-Gentra PUREGENE DNA Purification Kit for gram-negative bacteria. Firstly, the amplification and sequencing of the 16S rRNA gene were carried out on 20 bacterial strains according to Weisburg et al.

(1991). Alignment of the partial 16S rRNA gene sequences using DNAMAN Version 6 (©Lynnon Biosoft Corporation) placed the strains into five distinct groups and a representative strain from each group was used in the plant nodulation studies. The amplification and sequencing of *atpD* and *recA* genes according to Gaunt et al. (2001) and Vinuesa et al. (2005), respectively, were carried out on three bacterial strains that were found to form effective nodules in the plant nodulation studies. All PCR amplifications were performed using the FastStart™ Taq DNA Polymerase kit (Roche Applied Science, Auckland) optimised for annealing temperature and primer concentration. The PCR products were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide and viewed under UV light. PCR products were sequenced by the Bio-Protection Research Centre Sequencing Facility, Lincoln University. The DNA sequence data obtained were compared to those from GenBank using the BLASTN program (<http://blast.ncbi.nlm.nih.gov/>).

Phylogenetic analyses

DNA sequences were aligned and Maximum Likelihood (ML) trees were constructed with 1000 bootstrap replications using MEGA5 software (Tamura et al. 2007). Model test was performed and the best model was selected for each gene. The models of evolution used for 16S rRNA, *atpD* and *recA* were T92+G+I (Tamura 3-parameter with Gamma distribution with Invariant sites), T92+I (Tamura 3-parameter with Invariant sites) and T92+G (Tamura 3-parameter with Gamma distribution), respectively.

Nodulation and N₂ fixation studies

Bacterial strains isolated from *G. officinalis* (strains GR2C and GR4B) and *H. coronarium* (strains HC2C, HC2E and HC3A) were used for nodulation and N₂ fixation studies on their respective hosts. Seeds of both plants were sourced from AgResearch, Hamilton. All plant procedures were carried out under sterile conditions in a containment laboratory. Seeds were surface sterilised by soaking them in 10% commercial bleach (0.25 g/litre sodium hypochlorite) (15 min),

rinsed with sterile water and germinated on 1.5% water agar. After germination, seedlings were transferred to 400 ml polyethylene terephthalate jars covered with perforated plastic wrap (two seedlings per jar) containing 150 ml of vermiculite and supplied with 45 ml of complete nutrient medium (pH 6.0) per jar containing NH_4NO_3 (0.1 mM), CaCl_2 (1.0 mM), KCl (1.0 mM), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.0 mM), NaH_2PO_4 (1.0 mM), Na_2HPO_4 (0.1 mM), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (5.0 μM), H_3BO_3 (5.0 μM), $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$ (1.0 μM), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.5 μM), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.1 μM), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 μM) and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.02 μM). Plants were grown in a Conviron® Adaptis A1000 chamber with a 16 h photoperiod (400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at a constant 22°C. At 5-15 days after sowing, seedlings were inoculated by adding 5 ml of the appropriate rhizobial strain grown in YMB (approximately 5×10^8 cfu) into the jar. Uninoculated plants that were supplied with YMB only were used as controls. There were six replicate jars per treatment.

Plants were watered with 15 ml of the nutrient medium and inspected at 2-weekly intervals for nodulation. At 30-50 days after inoculation, the plants were tested for nitrogenase activity using the acetylene reduction assay (ARA) (Cummings et al. 2009). After the ARA, two rhizobial strains were re-isolated from 2-5 nodules per treatment and their 16S rRNA gene was sequenced.

RESULTS AND DISCUSSION

All plants of both species sampled in the field were nodulated and thus, N_2 fixation may be a factor in their successful establishment in New Zealand. Search on BLASTN indicated that the partial 16S rRNA sequences for 10 bacterial strains isolated from goat's rue were highly similar (> 99%) to *R. galegae*. The alignment of these partial sequences placed the strains into two groups with a probability value of 100%. Meanwhile, alignment of the 10 bacterial strains isolated from sulla placed them into three distinct groups and the BLASTN search indicated that they were highly similar (>99%) to *Agrobacterium vitis*, *R. galegae* and *R. sulae*. Therefore, a representative strain from each group (GR2C, GR4B, HC2C, HC2E and HC3A isolates) was selected for plant nodulation studies to assess their ability to form

effective nodules on the legume species from which they were isolated.

For goat's rue, both strains GR2C and GR4B formed nodules on the plants, while only strain HC2E formed nodules on sulla plants. 16S rRNA sequencing verified the identity of nodulating strains as the inoculant strains. All control plants of both legume species had no nodules. The ability of nodulated plants to fix N_2 was then indirectly assessed by ARA to determine the presence of effective nitrogenase (the enzyme responsible for converting N_2 to ammonia). Plants inoculated with strains GR2C, GR4B and HC2E had greater ethylene (C_2H_4) production (7.94–14.59 $\mu\text{mol C}_2\text{H}_4/\text{jar/h}$) compared to control plants and sulla plants inoculated with strains HC2C and HC3A (0.16–0.31 $\mu\text{mol C}_2\text{H}_4/\text{jar/h}$), indicating that the nodulated plants were able to fix N_2 .

Full 16S rRNA and partial *atpD* and *recA* sequences were then obtained for isolates GR2C, GR4B and HC2E as they were the only strains that formed effective nodules upon re-inoculation. Results from the phylogenetic analysis based on the maximum likelihood tree inferred for the 16S rRNA sequences indicated the placement of GR2C and GR4B (identity > 95%) within the *Rhizobium galegae* clade while HC2E (similarity > 99%) was closely aligned to *R. sulae* (Figure 1a). The other trees with partial *atpD* and *recA* sequences (Figures 1b & 1c respectively) concurred with the 16S gene tree with regard to the placement of strains GR2C and GR4B with *R. galegae*, and HC2E with *R. sulae*.

These results indicate that (i) *R. galegae* strains GR2C and GR4B can induce effective nodules on *G. officinalis* upon re-inoculation and (ii) although *R. galegae* (HC2C), *R. sulae* (HC2E) and *Agrobacterium vitis* (HC3A) strains were isolated from nodules of *H. coronarium*, only *R. sulae* induced effective nodules on this plant. In relation to the finding of *Agrobacterium vitis* and *R. galegae* in nodules of *H. coronarium*, there are many reports of nodules occupied by various bacteria that are not able to induce nodules or fix N_2 (Sprenst 2009).

In summary, *Rhizobium galegae* and *R. sulae* form effective nodules on *Galega officinalis* and *Hedysarum coronarium* respectively in New

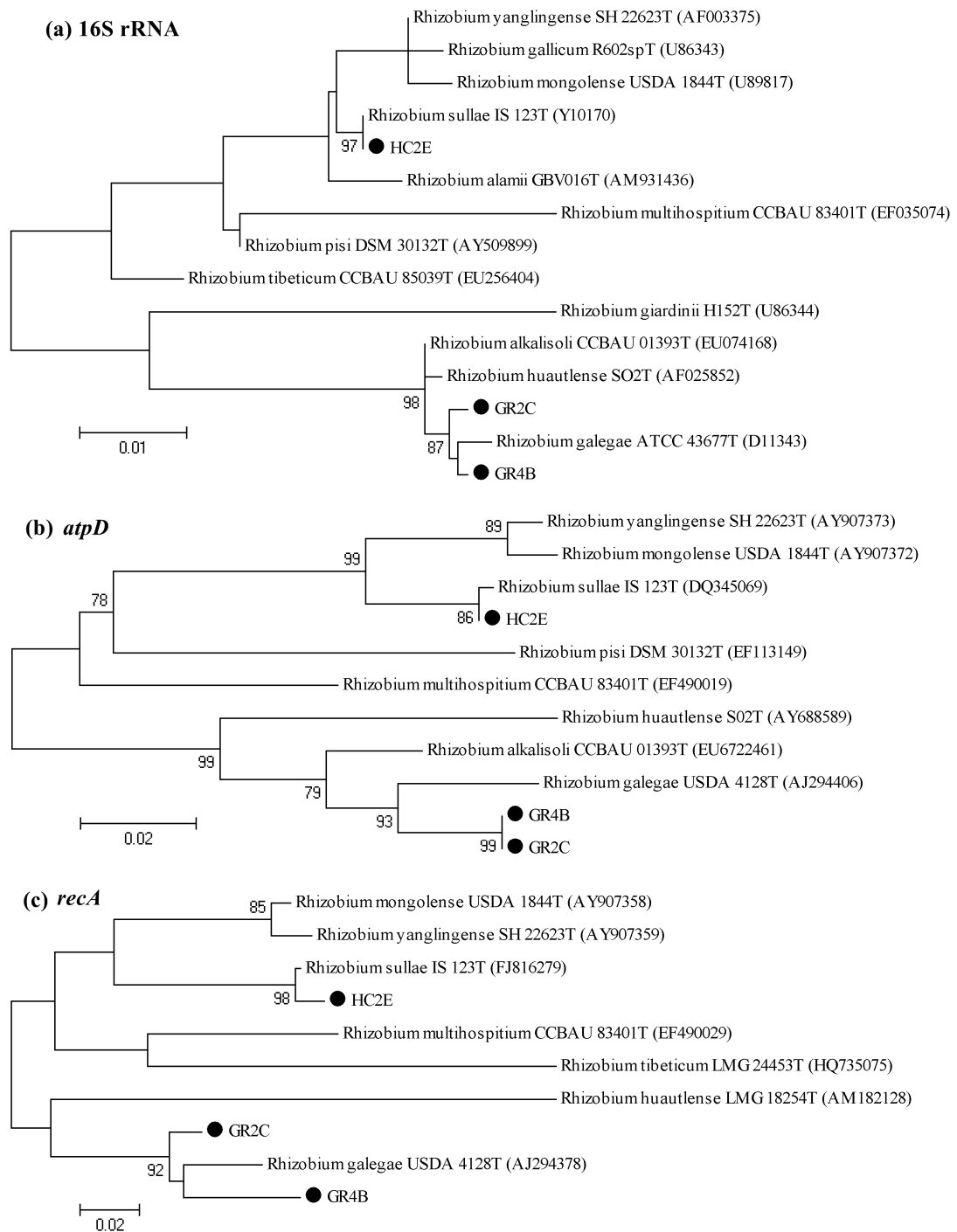


Figure 1 Maximum likelihood trees inferred for (a) 16S rRNA, (b) *atpD*, and (c) *recA* gene phylogenies, showing the relationships among the GR2C, GR4B and HC2E isolates and recognised *Rhizobium* species (type strains). Bootstrap values (%) based on 1000 replicates are shown at each node. Only bootstrap probability values >70% are shown.

Zealand and results from this study concur with previous reports on their high degree of specificity in relation to their rhizobial symbionts. *Mesorhizobium* spp. known to nodulate New Zealand native legumes (Weir et al. 2004) were not found in the nodules of *G. officinalis* and *H. coronarium*. However, further work, which includes cross-nodulation tests with native rhizobia and sampling of both legumes at various sites, is required to fully confirm the specificity of these legumes in New Zealand.

ACKNOWLEDGEMENTS

The authors thank AgResearch, Hamilton, for contributing to the funding of this project and Lincoln University for providing a Doctoral Scholarship to W.Y.Y. Liu.

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