Rhizobia with 16S rRNA and nifH Similar to Mesorhizobium huakuii but Novel recA, glnII, nodA and nodC Genes Are Symbionts of New Zealand Carmichaelinae

Heng Wee Tan1, Bevan S. Weir2, Noel Carter3, Peter B. Heenan4, Hayley J. Ridgway1, Euan K. James5, Janet I. Sprent6, J. Peter W. Young7, Mitchell Andrews1*

1 Faculty of Agriculture and Life Sciences, Lincoln University, Christchurch, New Zealand, 2 Systems Group, Landcare Research, Auckland, New Zealand, 3 Faculty of Applied Sciences, University of Sunderland, Sunderland, United Kingdom, 4 Allan Herbarium, Landcare Research, Lincoln, Christchurch, New Zealand, 5 Ecological Sciences, James Hutton Institute, Invergowrie, Dundee, United Kingdom, 6 College of Life Sciences, University of Dundee, Dundee, United Kingdom, 7 Department of Biology, University of York, York, United Kingdom

Abstract

New Zealand became geographically isolated about 80 million years ago and this separation gave rise to a unique native flora including four genera of legume, Carmichaelia, Clianthus and Montigena in the Carmichaelinae clade, tribe Galegeae, and Sophora, tribe Sophoreae, sub-family Papilionoideae. Ten bacterial strains isolated from NZ Carmichaelinae growing in natural ecosystems grouped close to the Mesorhizobium huakuii type strain in relation to their 16S rRNA and nifH gene sequences. However, the ten strains separated into four groups on the basis of their recA and glnII sequences: all groups were clearly distinct from all Mesorhizobium type strains. The ten strains separated into two groups on the basis of their nodA sequences but grouped closely together in relation to their nodC sequences; all nodA and nodC sequences were novel. Seven strains selected and the M. huakuii type strain (isolated from Astragalus sinicus) produced functional nodules on Carmichaelia spp., Clianthus puniceus and A. sinicus but did not nodulate two Sophora species. We conclude that rhizobia closely related to M. huakuii on the basis of 16S rRNA and nifH gene sequences, but with variable recA and glnII genes and novel nodA and nodC genes, are common symbionts of NZ Carmichaelinae.

Introduction

Members of the Fabaceae (the legume family) are components of most of the world’s vegetation types [1]. Many legume species have the capacity to fix atmospheric nitrogen (N2) via symbiotic bacteria (generally termed ‘rhizobia’) in root nodules and this can give them an advantage under low soil N conditions if other factors are suitable for growth [2,3,4]. New Zealand (NZ) became geographically isolated about 80 million years ago [5,6] and this separation gave rise to a unique native flora [7] that is considered to have evolved during the late Cenozoic [8,9]. There are four genera of legume, of the sub-family Papilionoideae on the main New Zealand islands, plus the strand species Canavalia rosea which occurs in the Kermadec Islands [10]. The four genera are the closely related Carmichaelia (23 endemic species), Clianthus (2 endemic species) and Montigena (1 endemic species) in the Carmichaelinae clade, tribe Galegeae, and Sophora (8 endemic species), within the tribe Sophoreae [11,12,13,14,15]. All species in all four genera are capable of nodulation, but genotypic data on the rhizobia which induce nodules on these plants are limited [4,16].

Previously, sequences were obtained for the small subunit ribosomal RNA (16S rRNA) gene of twenty bacterial strains isolated from species of the four NZ native legume genera growing in natural ecosystems [16]. Most isolates aligned with the genus Mesorhizobium either as named species or as putative novel species. Five strains from Carmichaelia, and one each from Clianthus and Montigena, were most closely related to Mesorhizobium huakuii (M. huakuii). However, this study did not assess the ability of the isolated rhizobial strains to nodulate NZ native legumes or sequence any of their N2-fixation (nif) or nodulation (nod) genes. A separate study, characterised two bacterial strains, Ca004 and Cc5, isolated from Carmichaelia australis and Carmichaelia corrigata respectively, growing in natural ecosystems, using 16S rRNA sequences [17]. Both strains aligned closely with M. huakuii and produced functional nodules on the five Carmichaelia spp. tested. In the current study, six strains isolated from Carmichaelia spp. and Montigena aligned with M. huakuii on the basis of their 16S rRNA sequences.

Here we focus on bacterial strains isolated from NZ native legumes growing in natural ecosystems in the current and previous studies which aligned closely with M. huakuii on the basis of their
16S rRNA sequences. In addition to 16S rRNA, selected ‘housekeeping’ and *nif* and *nod* genes were sequenced from the strains and their ability, and that of the *M. huakuii* type strain originally isolated from Astragalus sinicus [18,19], to nodulate species of the different NZ native legume genera and *A. sinicus* is assessed.

**Materials and Methods**

**Bacterial strains and culture media**

Ten bacterial strains isolated from species in the Carmichaelaeae growing in natural ecosystems in the current and previous studies were examined. All strains are deposited in the International Collection of Microorganisms from Plants (ICMP), Landcare Research, Auckland, NZ. Strains ICMP 18942, ICMP 18943 and ICMP 19420 from *Montigena novae-zelandiae* (Mn) and ICMP 19041, ICMP 19042 and ICMP 19043 from *C. australis* (Ca), *C. monroi* (Cm) and *C. nana* (Cn) respectively [16] and the type strain (ICMP 11069) were obtained from the ICMP collection directly. Strain ICMP 19418 (= strain Ca004) [17] was sourced from the University of York rhizobium collection.

For strains isolated in the current study, root nodules were surface sterilised in a laminar flow cabinet by immersion in 96% ethanol for 5 seconds and 5% sodium hypochlorite for 3 minutes then rinsed with sterile water. Surface sterilised nodules were squashed in sterile water then this suspension was streaked onto a yeast mannitol broth (YMB) [20] and used for preparation of subcultures for DNA extraction or inoculum.

**Sequencing of the 16S rRNA, housekeeping and symbiotic genes**

DNA was extracted from the bacterial cultures using the standard Qiagen-Gentra PUREGENE DNA Purification Kit for gram-negative bacteria. Six genes were studied: 16S rRNA, DNA recombinase *A* (*recA*), glutamine synthetase II (*glnII*), nitrogenase iron protein (*nifH*), N-acyltransferase nodulation protein *C* (*nodC*) and N-acetylglucosaminyl transferase nodulation protein C (*nodA*). Primers for PCR amplification with their sequences and sources are shown in Table 1. All primers were manufactured by Integrated DNA Technologies, Auckland, NZ. All PCR amplifications were performed using the FastStart™ Taq DNA Polymerase kit (Roche Applied Science, Auckland) optimised for annealing temperature and primer concentration, if required. The PCR products were resolved via gel electrophoresis (1% agarose gel in 1×Tris-acetate-EDTA buffer) followed by staining with ethidium bromide solution and viewing under UV light. PCR products were sequenced by the Bio-Protection Research Centre Sequencing Facility, Lincoln University, Lincoln, NZ and DNA sequence data were obtained via Sequence Scanner v 1.0 software (Applied Biosystems) and edited and assembled using DNA-MAN Version 6 (Lynnon Biosoft Corporation).

**Table 1. Oligonucleotide primers used in this study.**

| Target gene | Primer | Sequence (5'–3')* | Reference |
|-------------|--------|-------------------|-----------|
| 16S rRNA    | F27    | AGA-GTT-TGA-TCM-TGG-CTC-AG | [21] |
|             | FGPS485F | CAG-CAC-CGG-CGG-TAA | [22] |
|             | R1494  | CTA-CGG-YTA-CCT-TGT-TAC-GAC | [21] |
| recA        | 41F    | TTC-GGC-AAG-GGM-TGC-RTS-ATG | [23] |
|             | 640R   | ACA-TSA-CRC-CGA-TCT-TCA-TGC | [24] |
| glnII       | GSIII-1 | AAC-GCA-GAT-CAA-AGA-ATT-G | [25] |
|             | GSIII-2 | ATG-CCC-GAT-CGG-TTG-CAG-TC | [26] |
|             | GSIII-3 | AGR-TYT-TCG-GCA-AGG-GYT-C | [27] |
|             | GSIII-4 | GCG-AAC-GAT-CTG-GTA-GGG-GT | [28] |
| nifH        | PolF   | TGC-GAY-CCS-AAR-GCB-GAC-TC | [21] |
|             | PolR   | ATS-GCC-ATC-ATY-TCR-CCG-GA | [21] |
| nodC        | α-nodCF | AYG-THG-TYG-AYG-AGG-GTT-C | [21] |
|             | α-nodCR | CGY-GAC-AGC-CAN-TCK-CTA-TTG | [21] |
| nodA        | nodA1  | TGC-RGT-GGA-ARN-TRN-NCT-GGG-AAA | [21] |
|             | nodA3  | TCA-TAG-CTC-YGR-ACC-GTT-CCG | [21] |

*A, C, G, T = standard nucleotides; M = C or A; N = A or C or T or G; S = G or C; B = T or C or G; H = A or C or T; K = A or G or C or T; V = T or G. doi:10.1371/journal.pone.0047677.t001

**Phylogenetic analyses**

DNA sequences were aligned and Maximum Likelihood trees constructed with 500 bootstrap replications with partial deletion and an 80% coverage cut off using MEGA5 software [29]. The most closely related *Mesorhizobium* type strains and strains isolated from NZ native legumes which were closely related to *M. huakuii* on the basis of 16S rRNA and available on the Genbank sequence database (www.ncbi.nlm.nih.gov/genbank) were used for the 16S rRNA, recA, glnII, nifH, nodC and nodD trees. For the nodA tree, we also included the sequence from a strain (TM1) recently isolated from Thermopsis lupinoides and characterised as *M. huakuii* on the basis of 16S rRNA [30]. For the nodC tree, strains characterised as *M. huakuii* on the basis of their 16S rRNA sequences were used as there is no nodC sequence for the *M. huakuii* type strain in Genbank and we were unable to sequence it. MEGA5 model test was performed to select a model of nucleotide substitution and the ‘best’ model [lowest Bayesian Information Criterion (BIC) score] used for each gene. The Kimura 2-parameter (K2), gamma distribution (+G) with invariant sites (+I) model was used for 16S rRNA. The Tamura 3-parameter (T92), gamma distribution (+G) model was used for all other genes. Only bootstrap probability values ≥50% are shown. The sequences obtained in this study have been deposited in the GenBank sequence database and their accession numbers are shown in the figures.
Nodulation and N\textsubscript{2} fixation studies

Seven bacterial strains (ICMP 12690 (Mn), ICMP 18942 (Mn), ICMP 13190 (Ca), ICMP 19041 (Ca), ICMP 19042 (Cm), ICMP 19418 (Ca) and ICMP 11541 (Clp)) were selected for nodulation and N\textsubscript{2} fixation studies. Seeds of *Carmichaelia* spp. and *M. novae-zelandiae* were collected from the field site (Dry Stream, Torlesse Range, Canterbury); seeds of *Sophora* spp. and *Cliaanthus puniceus* were purchased from New Zealand Tree Seeds, Rangiora, NZ and seeds of *Astragalus sinicus* obtained from the Margot Forde Germplasm Centre, Palmerston North, NZ. All plant procedures carried out until processing of nodules, took place under sterile conditions. All seeds were, in sequence, soaked in concentrated sulphuric acid for 30–90 minutes, rinsed with sterile water then soaked in hot (\(\sim\)60\,°C) sterile water which was left at room temperature overnight. Seeds were then transferred to water agar plates. After germination, seedlings were transferred to polyeth-

![Figure 1. 16S rRNA gene phylogenetic tree of bacterial strains isolated from New Zealand native legumes and selected *Mesorhizobium* type strains.](image-url)
ylene terephthalate jars (two seedlings per jar) containing vermiculite and supplied a complete nutrient medium (pH 6.0) containing NH₄NO₃ (0.1 mM), CaCl₂ (1.0 mM), KCl (1.0 mM), MgSO₄·7H₂O (1.0 mM), NaH₂PO₄ (1.0 mM), Na₂HPO₄ (0.1 mM), FeCl₂·4H₂O (5.0 μM), H₃BO₃ (5.0 μM), MnCl₂·2H₂O (1.0 μM), Na₂MoO₄·2H₂O (0.5 μM), CuSO₄·5H₂O (0.1 μM), ZnSO₄·7H₂O (0.1 μM) and CoCl₂·6H₂O (0.02 μM). Plants were grown in a controlled environment cabinet and exposed to a 16 h photoperiod (400 μmol photons m⁻² s⁻¹) at a constant 22°C.

At 5–15 days after sowing, seedlings were inoculated with the appropriate rhizobial strain grown to log phase: uninoculated plants supplied YMB only were used as controls. There were at least 3 replicate jars per treatment. Plants were inspected at two weekly intervals for nodulation and at 30–50 days after inoculation were tested for nitrogenase activity using the acetylene reduction assay (ARA) [31]. After the ARA, rhizobial strains were isolated from three to six nodules per treatment and their 16S rRNA gene sequenced.

Results

All ten bacterial strains isolated from *Carmichaelia*, *Clanthus* or *Montigena* studied here clustered closely with the *M. huakuii* type strain and five other strains previously isolated from *Carmichaelia* or *Clanthus* spp. (ICMP 11708, ICMP 14319, ICMP 12680, ICMP 11722 and ICMP 12635) [16,32], on the basis of their 16S rRNA sequences (Fig. 1). Eight strains, ICMP 18942, ICMP 18943, ICMP 19042, ICMP 19420, ICMP 12690, ICMP 19041 and ICMP 19418 this study and ICMP 11708 were identical and showed 99.5% similarity (1200 bp) to the *M. huakuii* type strain.

The ten strains separated into four groups on the basis of their recA sequences with all four groups clearly separated from all *Mesorhizobium* type strains (Fig. 2a). The four groupings on the basis of the recA sequences held in relation to glnII sequences (Fig. 2b).

For glnII sequences, three groups (nine strains) were most closely related to, but clearly separated from, the *Mesorhizobium loti* type strain while strain ICMP 19418 (Ca) was most closely related to *M. huakuii*. Three other strains previously isolated from *Carmichaelia*...
Figure 4. nodA (a) and nodC (b) gene phylogenetic trees of bacterial strains isolated from New Zealand native legumes, selected Mesorhizobium type strains and strains characterised as Mesorhizobium huakuii on the basis of their 16S rRNA sequences. Ca = Carmichaelia australis; Cm = Carmichaelia monroi; Cn = Carmichaelia nana; Co = Carmichaelia odorata; Clp = Clianthus puniceus; Mn = Montigena novae-zelandiae. ■ indicates strains focused on in this study. Numbers on branches are bootstrap % from 500 replicates (shown only when ≥50%). doi:10.1371/journal.pone.0047677.g004

spp. (ICMP 14319, ICMP 11722 and ICMP 12635) [16] also aligned closest to M. loti.

There was little variation in the nifH gene sequences across the ten strains studied (Fig. 3). With the exception of ICMP 11541 (Clp), all strains were identical (290 bp) to the M. huakuii type strain (sequenced in the current study). The ten strains separated into two groups on the basis of their nodA sequences (Fig. 4a). The larger group (eight strains) clustered with three other strains (ICMP 11708, ICMP 11722 and ICMP 12630) previously isolated from Carmichaelia spp. or Clianthus puniceus [32], while the smaller group (two strains) grouped with strain ICMP 14319 isolated from Carmichaelia odorata [32]. The nodA sequences for the two groups showed only 66.29% similarity (530 bp) to each other but both separated clearly from all Mesorhizobium type strains and strain TMI isolated from Thermopsis lupinoides and characterised as M. huakuii on the basis of its 16S rRNA sequence [30].

All ten strains grouped closely together in relation to their nodC sequences and as for nodA sequences separated from all others on the Genbank database including those of strains characterised as M. huakuii on the basis of their 16S rRNA sequences (Fig. 4b) [33,34].

All seven Carmichaelinae strains tested and the M. huakuii type strain produced functional nodules on Carmichaelia australis, Carmichaelia kirkii, Carmichaelia petriei, Clianthus puniceus and Astragalus sinicus but did not nodulate either of the two Sophora species (Table 2). In all cases, the 16S rRNA sequence for the strain recovered from nodules after the acetylene reduction assay was identical to that of the strain used as inoculant.

Discussion

Genotypic data on the rhizobia which induce nodules on NZ native legumes are limited but it seems likely that rhizobial symbionts co-evolved with NZ native legumes in isolation from the regions of major legume evolution and as a result, may have novel characteristics. Here we focus on ten bacterial strains isolated from NZ native legumes in the current and previous studies which closely aligned with M. huakuii on the basis of their 16S rRNA sequences. It is shown that the ten strains also grouped closely to the M. huakuii type strain in relation to their nifH sequences. However, with the exception of glnII for strain ICMP 19418 (Ca), all sequences for recA, glnII, nodA and nodC were substantially different for the ten strains and the M. huakuii type strain. The ten strains separated into the same four groups on the basis of their recA and glnII sequences. For recA, the four groups clearly separated from all Mesorhizobium type strains. For glnII, three groups (nine strains) aligned closest to but clearly separated from the M. loti type strain. It is possible that Mesorhizobium evolved in NZ with variable recA and glnII genes. However, many legume species have been introduced and have naturalised in NZ since colonisation by Europeans in the 19th Century. Also, in the case of crop legumes, rhizobial inoculant, was often applied, and in some cases still is, and it has been shown that chromosomal symbiosis genes can transfer (‘lateral’ transfer) from a Mesorhizobium loti inoculant strain to indigenous M. huakuii strains in NZ soils [35]. Thus, the possibility of genetic exchange of recA and glnII genes between recently introduced rhizobia and the indigenous Mesorhizobium should be considered. Certainly, there is strong evidence that in the past, horizontal transfer of the glnII gene has occurred between major rhizobial groups [24,36], but it would require substantial further work to determine if this has happened between rhizobia in NZ over the past 150 years.

All the strains grouped closely together in relation to their nodC sequences and these sequences separated from all others in Genbank. There was no nodC sequence for the M. huakuii type strain in Genbank and we were unable to sequence it. However, the nodC sequences for the strains separated from those of other strains characterised as M. huakuii on the basis of their 16S rRNA sequences. The ten strains separated into two groups in relation to their nodA sequences, eight of the strains studied grouped with three other strains previously isolated from Carmichaelia spp. or Clianthus puniceus while two strains grouped with a strain isolated from Carmichaelia odorata [32]. The nodA sequences for both groups separated clearly from those of all the Mesorhizobium type strains.

Table 2. Host specificity of rhizobial strains used in this study.

| Strain            | Species tested | Carmichaelia | Sophora | Clianthus | Astragalus |
|-------------------|----------------|--------------|---------|-----------|-----------|
|                   |                | australis    | kirkii  | microphylla| tetrapeta |
| ICMP 18942 (Mn*)  | Nod−Fix+       | Nod+Fix+     | Nod+Fix+| Nod−       | Nod+Fix+  |
| ICMP 12690 (Mn)   | Nod+Fix+       | Nod+Fix+     | Nod+Fix+| Nod−       | Nod+Fix+  |
| ICMP 19041 (Ca)   | Nod+Fix+       | Nod+Fix+     | Nod+Fix+| Nod−       | Nod+Fix+  |
| ICMP 19042 (Cm)   | Nod+Fix+       | Nod+Fix+     | Nod+Fix+| Nod−       | Nod+Fix+  |
| ICMP 19418 (Ca)   | Nod+Fix+       | Nod+Fix+     | Nod+Fix+| Nod−       | Nod+Fix+  |
| ICMP 13190 (Ca)   | Nod+Fix+       | Nod+Fix+     | Nod+Fix+| Nod−       | Nod+Fix+  |
| ICMP 11541 (Clp)  | Nod+Fix+       | Nod+Fix+     | Nod+Fix+| Nod−       | Nod+Fix+  |
| M. huakuii        | Nod+Fix+       | Nod+Fix+     | Nod+Fix+| Nod−       | Nod+Fix+  |

*Original host: Mn = Montigena novae-zelandiae; Ca = Carmichaelia australis; Cm = Carmichaelia monroi; Clp = Clianthus puniceus. Nod− = no plants nodulated; Nod+ = all plants nodulated; Fix+ = nitrogen fixing nodules.

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and the ‘novel’ nodA sequence reported for the M. huakuii strain isolated from Thermopsis lapynoides in Kamchatka, Russia [30]. Thus, although the nifH gene of the ten strains aligns closely with that of M. huakuii, their nodC and nodD genes are novel, and it seems likely that they evolved in rhizobia indigenous to NZ.

All seven strains selected isolated from three separate genera, over three separate studies and including isolates from the North and South Islands of NZ, produced functional nodules on the three *Carmichaelia* spp. and *Clantus puniceus* tested indicating that this group of bacteria are common rhizobial symbionts of Carmichaeliaceae species in NZ. None of the strains produced functional nodules on the *Sophora* spp. tested indicating that, within NZ native legumes, they are specific to Carmichaeliaceae species. Host range in rhizobia is at least in part determined by the structure of the lipo-chitin oligosaccharides ‘nod factors’ synthesised by the products of nodulation genes such as nodA and nodC [31, 37]. Thus, it is to some extent unexpected that, although the nodA and nodC genes of the strains are novel, all seven strains tested, produced functional nodules on *Astragalus sinus*icus, and that the M. huakuii type strain isolated from *A. sinus*icus produced functional nodules on the *Carmichaelia* spp. and *Clantus puniceus*. As the strains form nodules on *Astragalus sinus*icus, they do not meet the criteria required to be considered as a different M. huakuii symbiovar [38].

Four of the strains isolated and characterised in this study (ICMP 18942 [Mn], ICMP 18943 [Mn], ICMP 19420 [Mn] and ICMP 19042 [Cm]) were obtained from plants growing in alpine scree and rock bluff habitats that have not been utilised for agricultural purposes. The 16S rRNA, and nifH genes for these strains aligned closest to *M. huakuii*, their gltA sequence aligned closest to but separate from that of *M. loti* and they had novel *nec*, nodA and nodC genes. We suggest that these strains are indigenous NZ strains that evolved with their hosts *Carmichaelia* and *Montengia* during the late Miocene, as these two legume genera diverged from a common ancestor about 5 mya [39]. Overall, we conclude that rhizobia closely related to *M. huakuii* on the basis of 16S rRNA and nifH gene sequences, but with variable *nec* and gltA genes and novel nodA and nodC genes, are common symbionts of NZ, Carmichaeliaceae.

**Author Contributions**

Conceived and designed the experiments: MA HWT PBH HJR. Performed the experiments: HWT MA PBH HJR. Analyzed the data: MA HWT PBH JPY WJ RJK JJS BSW NC. Contributed reagents/materials/analysis tools: NC BSW. Wrote the paper: MA HWT PBH HJR.
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