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Rhizobial genetic and genomic resources for sustainable agriculture

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ABSTRACT

Rhizobia are a diverse group of α- and β-proteobacteria that boost soil fertility by forming a nitrogen-fixing symbiosis with legumes, which is why legumes are grown in rotation with cereals in agriculture. Rhizobia that naturally populate Australian soils are largely incompatible with exotic agricultural legumes, therefore, compatible strains have been imported from all over the world for use as inoculants. An amalgamated collection of these strains, called the International Legume Inoculant Genebank (ILIG), has been established at Murdoch University, to provide a centralised strain storage facility and support rhizobial research and inoculant development (see http://ilig.murdoch.edu.au). The ILIG contains 11,558 strains representing 96 bacterial species from 778 legume species collected from >1200 locations across 100 countries. New and sometimes inefficient rhizobia evolve in the field following legume inoculation, through horizontal symbiosis gene transfer from inoculants to soil bacteria. To provide a benchmark to monitor and assess the impact of this evolution, all commercial Australian inoculant strains were genome sequenced and these data made available (PRJNA783123, see https://www.ncbi.nlm.nih.gov/bioproject/PRJNA783123/). These data, and the further sequencing of the >11,000 historical strains in the ILIG, will increase our understanding of rhizobial evolution and diversity and provide the backbone for efforts to safeguard Australia's legume inoculation program.

Keywords: horizontal gene transfer, inoculant, legume, rhizobia, sequencing, symbiosis.

The nitrogen-fixing symbiosis between diverse soil saprophytic bacteria known as rhizobia (or root nodule bacteria) and legumes is responsible for providing a substantial proportion of the biosphere's available nitrogen.[1] This symbiosis is established when rhizobia infect legume roots, differentiating into their nitrogen-fixing (or bacteroid) form, within plant-derived root nodules.[2] Bacteroids reduce dinitrogen (N_2) gas into ammonia (NH_3), secreting this source of fixed nitrogen to the host plant, in exchange for a supply of carbon from the legume. When the plant senesces, some of this fixed nitrogen makes its way into the soil, providing a source of bioavailable nitrogen for subsequent plants. For this reason, legumes are often grown in rotation with cereals in agriculture.[3–5] Symbiotic nitrogen fixation through grain legumes (e.g. chickpea, lupin, peas) and pasture legumes (e.g. clover, lucerne, serradella) is estimated to save the Australian agricultural sector A$4 billion in synthetic fertiliser costs annually, and reduce the CO_2 footprint associated with fertiliser production and application.[6]

Although bacteria in the genus *Rhizobium* are well known as legume symbionts, this capability is much more phylogenetically diverse, with 15 genera of α-proteobacteria (including *Rhizobium*) and 3 genera of β-proteobacteria engaging in legume symbioses.[7] Close to 100 species of legumes are cultivated across Australia, and, although only a small proportion of these are widely grown, their requirement for a compatible symbiotic partner can be quite specific.[8,9] To maximise the agricultural benefits of symbiotic nitrogen fixation, legumes are often inoculated with elite strains of rhizobia that specifically nodulate the target legume and are highly efficient nitrogen fixers[10–12] (Fig. 1). This is of primary importance in Australia, as our soils tend to lack rhizobia that nodulate and efficiently fix nitrogen with the exotic legumes we use in agriculture.

Since the 1950s in Australia, thousands of strains of rhizobia have been collected from legumes and soils from all over the world by various University and government agencies and stored in small, dispersed collections. To preserve and develop this genetically diverse resource critical for inoculum development, the International Legume Inoculant Genebank (ILIG) was established in 2020 at Murdoch University in Western Australia, through the financial support of the Grains Research and Development Corporation (GRDC) of Australia. The ILIG has amalgamated strain collections from all

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Fig. 1. Benefits of inoculation with *Rhizobium* sp. strain WSM4643. (*a*) Field plot comparison of uninoculated vetch (*Vicia sativa*) (left plot) and inoculated plants (right plot). (*b*) Comparison of nodulation and plant growth responses in uninoculated field pea (*Piscum sativum*) (left) with inoculated plant (right). Photos courtesy of Dr Ron Yates (Department of Primary Industries and Regional Development).

over Australia into one facility, which currently houses 11,558 strains of rhizobia representing 96 different bacterial species, isolated from 778 species of legumes that were collected from more than 1200 locations throughout 100 countries. The ILIG has an online catalogue (see http://ilig.murdoch.edu.au) to browse available information on strains within the collection and request strains of interest for research purposes. Critically, as the majority of the strains in the ILIG were collected prior to the genome sequencing era, much of their genetic potential remains unknown.

Rhizobia show a remarkable diversity in genome size and complexity. In comparison to enteric bacteria, rhizobial genomes are relatively large, ranging in size from 6 Mb to more than 10 Mb, and they can be structurally complex, consisting of a chromosome with or without multiple additional plasmids.[2] These plasmids can be very large, such as in *Sinorhizobium meliloti* 1021, which carries 1.3- and 1.7-Mb plasmids, along with its 3.7-Mb chromosome.[13–15] Genes essential to the establishment and maintenance of symbiosis (i.e. *nod* or nodulation, and *nif* and *fix* or nitrogen fixation) are typically found on plasmids (e.g. *Rhizobium* and *Sinorhizobium*) or chromosomally on symbiosis islands (e.g. *Mesorhizobium* and *Bradyrhizobium*) and are generally considered to be part of the accessory genome.[16]

For symbiotic *Mesorhizobium* spp., chromosomal symbiosis genes are encoded on mobile integrative and conjugative elements (ICEs). These symbiosis ICEs (previously referred to as symbiosis islands) were discovered when nodule isolates from the pasture legume *Lotus corniculatus* (birds foot trefoil) were found to have evolved through the acquisition of a 508-kb region from an introduced inoculant strain in New Zealand.[17] Subsequent work showed that the symbiosis ICE excises from the chromosome of a donor cell, transferring by conjugation to a recipient, and integrating into the recipient chromosome in a site-specific manner.[18] ICE transfer is a complex process that is epigenetically activated and controlled by quorum sensing.[19,20] Horizontal transfer of related symbiosis ICEs has been documented for *Mesorhizobium* in Australian soils, including inoculants used for the pasture legume *Biserrula pelecinus*[21] and grain legume *Cicer arietinum* (chickpea).[22] These studies showed that some symbiosis ICEs are tripartite in structure, whereby the ICE exists as three separate regions of DNA dispersed across the chromosome. These three regions recombine into a singular circular DNA molecule immediately prior to conjugal transfer.[23,24] Although Australian soils seem to lack native *Mesorhizobium* strains compatible with introduced legumes, they do harbour an abundance of non-symbiotic *Mesorhizobium* spp. capable of acquiring symbiosis ICEs and gaining the ability to nodulate legumes.[25] Critically, however, ICE transfer recipients are not always effective symbionts,[21,26,27] so transfer events have the potential to create strains less efficient at nitrogen fixation that can out-compete the inoculant strain for nodulation, leading to a reduced benefit of legume inoculation.

Accurately following and assessing the impact of symbiosis gene transfer in agriculture is only possible with knowledge of the inoculant genome sequence, which has not been historically available for the majority of commercially available strains of rhizobia in Australia. To close this gap, a genome sequencing pipeline was established to provide closed genomes of all commercial inoculant strains. Long-read assemblies were first created using the Oxford Nanopore Technologies MinION Mk1B, which were then polished with short-read sequences obtained from the same DNA using an Illumina NextSeq. 500 (Fig. 2). The commercial legume inoculants were sourced from the 2019 Mother Culture maintained by the Australian Inoculants Research Group (AIRG), which supplies manufacturers with a standardised and quality-controlled source of rhizobia for the inoculant industry. Sequencing from this source, rather than historical collections of these inoculant strains, ensures that the acquired genomic data for each strain directly relates to the germplasm supplied to growers.

With a breadth of strains spanning five genera, the diversity of strains used as inoculants in Australia complicates genome sequencing efforts, in that many have very different growth requirements and phenotypic characteristics.[28]

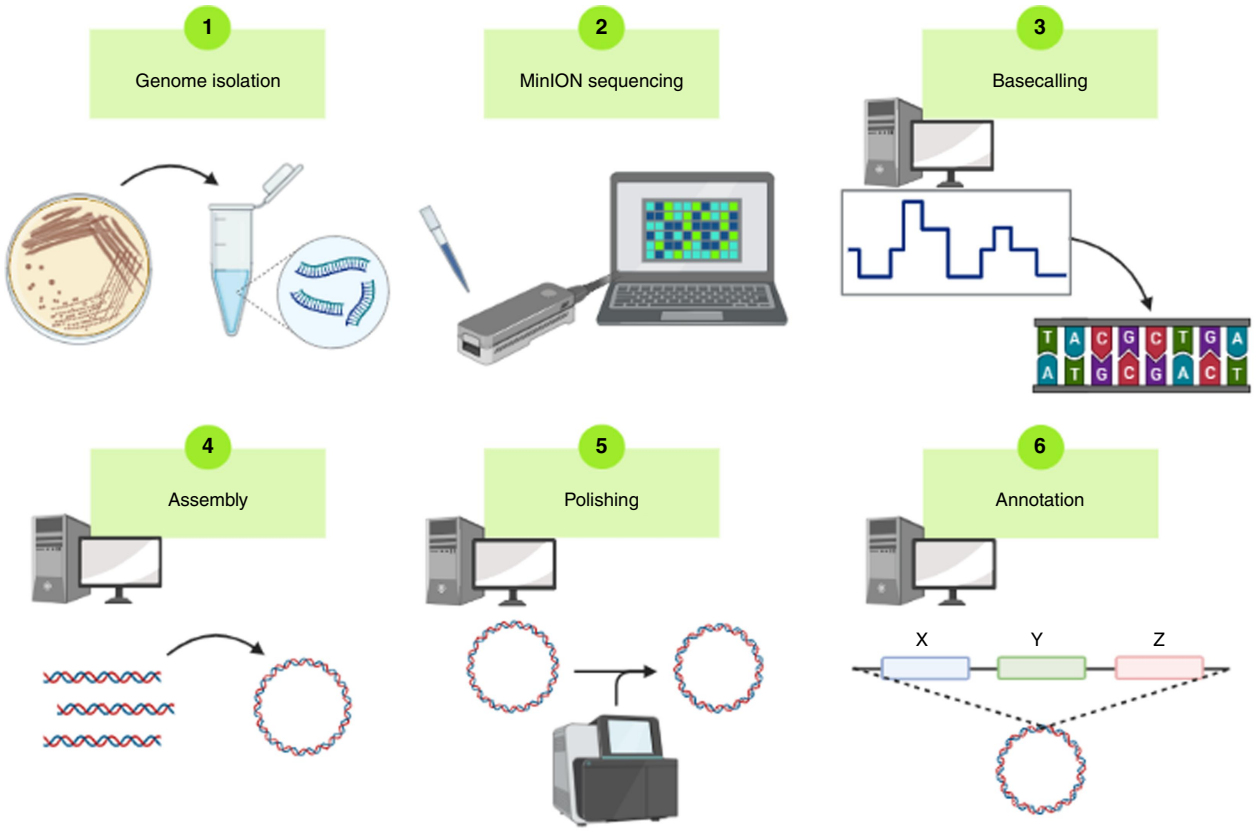

Fig. 2. Genome sequencing pipeline. The pipeline consists of six steps: (1) isolation of genomic DNA, (2) sequencing with MinION Mk1B, (3) base calling or raw data conversion to sequence data, (4) read assembly, (5) incorporation of short-read sequence for polishing and error correction, and (6) gene assignment and annotation. Created with BioRender.com.

Depending on the genus of the inoculant, different media types and extraction techniques were required for growth and isolation of genomic DNA. For many strains, thorough washing of the samples was necessary to limit carryover of cell lysate contaminants that could inhibit the sequencing reaction.

Closed genomes were completed for 38 strains, which are now available online under National Center for Biotechnology Information (NCBI) BioProject accession number PRJNA783123 (see https://www.ncbi.nlm.nih.gov/bioproject/PRJNA783123/). These genome sequences have in the first instance been used to assign more appropriate taxonomy to these strains, which is a critical first step in building a clear picture of the genetic diversity of the Australian inoculant cohort. However, many strains could not be given a designation at the species level due to a lack of closely related type strains. This reflects both the inherently novel biology of many of the inoculant organisms and the paucity of accurate genomic information currently available for many species of rhizobia. More sequencing of diverse rhizobia isolates from a range of representative legumes and biogeographical locations is therefore needed to address this deficit. Nevertheless, most inoculants showed genome architectures that were consistent with other members of their respective genera, although *Rhizobium* sp. SRDI969 was unusual in that it carried chromosomally encoded symbiosis genes, notable because *Rhizobium* spp., unlike *Bradyrhizobium* spp. or *Mesorhizobium* spp., typically carry symbiosis genes on plasmids.[29] *Rhizobium* sp. SRDI969 is new strain for faba bean (*Vicia faba*), which, alongside the new pea (*Pisum sativum*), lentil (*Lens culinaris*) and vetch (*Vicia* spp.) strain *Rhizobium* sp. WSM4643, is being released for the 2024 growing season as inoculants in Australia for these important grain legumes. This marks the first time in inoculant history that novel strains are to be released to commerce alongside their complete genome sequence. This will allow more-accurate and longer-term monitoring of inoculant efficacy and should become standard practice to also ensure the catalogue of the genetic material released as inoculants remains up to date.

This ILIG strain collection and inoculant genome sequences are invaluable resources for plant and microbe research in Australia. The ILIG collection, with its thousands of diverse root-nodule bacteria collected from around the world, will serve as the backbone for future research and development of novel inoculants for the benefit of Australian agriculture, and act as a secure repository for new isolates. However, with no genomic data on most of the historical strains in the collection, the full scope of the genetic potential of this collection is unclear. Future work will sequence the historical germplasm in the ILIG. These data will help in the development of rapid and accurate methods to monitor inoculation success, as well as identify and follow the impact of inoculant symbiosis gene transfer events on nitrogen fixation. It will also provide us with a better understanding of the dual saprophytic and symbiotic lifestyle of rhizobia and the evolutionary forces that have shaped this fascinatingly diverse group of bacteria.

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Data availability. Genome sequence data of Australian commercial inoculant strains sequenced in this project has been deposited in NCBI under BioProject accession number PRJNA783123 (see https://www.ncbi.nlm.nih.gov/bioproject/PRJNA783123/).

Conflicts of interest. The authors declare that they have no conflicts of interest.

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Biographies

MacLean Kohlmeier received his PhD from the University of Manitoba in 2020 with Prof. Ivan Oresnik. He is currently a Postdoctoral Research Fellow in Microbial Genomics in the Legume Rhizobium Sciences group at Murdoch University. His research interests include gene transfer events between soil bacteria, as well as carbon metabolism and transport, and how these features affect symbiotic establishment and efficiency.

Graham O'Hara is Director of Murdoch University's Legume Rhizobium Sciences centre in the Food Futures Institute, and co-curator of the International Legume Inoculant Genebank (ILIG). He has worked extensively in national and international projects on applied nitrogen fixation leading to the development and dissemination of inoculant rhizobia strains. His research interest is focussed on the soil ecology, mineral nutrition, physiology and stress tolerance of rhizobia, and how this affects their performance as inoculants in the field.

Joshua Ramsay carried out his PhD in 2004–2008 at the University of Otago with Prof. Clive Ronson. He was then a University of Cambridge Herchel Smith Postdoctoral Fellow from 2008 to 2011 and a Health Sciences Career Development Fellow at the University of Otago. Ramsay started his own lab at Curtin University in 2013. Dr Ramsay's investigations explore the effects of mobile genetic elements in both health related and agricultural contexts. In 2018, Dr Ramsay was awarded an ARC Future Fellowship and he is currently an Associate Professor in the Curtin Medical School and Curtin Health Innovation Research Institute.

Jason Terpolilli is Research Director of Murdoch University's Legume Rhizobium Sciences centre in the Food Futures Institute, and co-curator of ILIG. He works closely with colleagues across Australia on several projects aimed at increasing the application of effective nitrogen-fixing rhizobia for grain and pasture legumes. His main areas of research interest are in rhizobia genetics and biochemistry and in applying genomics to develop robust approaches to studying the evolution of these organisms and their effect on farming systems in the field.