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REGULAR ARTICLE

Medicago root nodule microbiomes: insights into a complex ecosystem with potential candidates for plant growth promotion

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Abstract

Purpose Studying the legume nodule microbiome is important for understanding the development and nutrition of the plants inhabited by the various microbes within and upon them. We analyzed the microbiomes of these underground organs from both an important crop plant (*Medicago sativa*) and a related legume (*M. polymorpha*) using metagenomic and culture-based techniques to identify the main cultivatable contributors to plant growth enhancement.

Methods Using high-throughput sequencing, culturing, and *in planta* techniques, we identified and analyzed a broad population of the bacterial taxa within *Medicago* nodules and the surrounding soil.

Results Fifty-one distinct bacterial strains were isolated and characterized from nodules of both *Medicago*

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species and their growth-promoting activities were studied. Sequencing of 16S rRNA gene amplicons showed that in addition to *Ensifer*, the dominant genus, a large number of Gram-positive bacteria belonging to the Firmicutes and Actinobacteria were also present. After performing ecological and plant growth-promoting trait analyses, selecting the most promising strains, and then performing in planta assays, we found that strains of *Bacillus* and *Micromonospora* among others could play important roles in supporting the growth, health, and productivity of the host plant.
Conclusion To our knowledge, the comparison of the biodiversity of the microbiota of undomesticated vs. cultivated *Medicago* roots and nodules is novel and shows the range of potential Plant Growth-Promoting Bacteria that could be used for plants of agricultural interest. These and other nodule-isolated microbes could also serve as inoculants with rhizobia with the goal of replacing synthetic fertilizers and pesticides for sustainable agriculture.

Keywords *Medicago* · Root nodule microbiome · Plant growth promoting bacteria · Bacterial inoculants

Introduction

The ecosystems that surround or inhabit the plant body, i.e. its collective phytobiomes, determine plant health and productivity in response to specific external environments. Phytomicrobiomes house a vast array of microorganisms, both fungal and bacterial, which colonize all parts of the plant, but especially the roots (Kaplan et al. 2013; Quiza et al. 2015). Bacteria make up the most abundant and diverse component of the phytobiome (Leach et al. 2017) and can be considered to act as a second genome within the eukaryotic plant (Berendsen et al. 2012). The composition of the microbial communities of roots has been determined for a number of different plants by using high-throughput sequencing of the *rrs* amplicons of the total prokaryotic communities followed by phylogenetic analyses (Ahrenhoerster et al. 2017; Schlaeppi et al. 2014; Xiao et al. 2017). Many of these studies focus on some of the major changes that occur in response to environmental fluctuations that result in biotic and abiotic stress (Agler et al. 2016; Cobo-Díaz et al. 2015; Fernández-González et al. 2017; Xiang et al. 2014).

Legume root nodules contain a highly specialized phytomicrobiome in which atmospheric nitrogen is converted into ammonia by rhizobia within plant tissues. However, nitrogen-fixing rhizobia are not the only inhabitants of legume nodules; many different species of non-rhizobial bacteria are also present (Aserse et al. 2013; Martínez-Hidalgo and Hirsch 2017; Muresu et al. 2008). High-throughput sequencing studies of the composition of these specialized, internalized microbial communities, e.g., of *Lotus japonicus* (Zgadzaj et al. 2016), *Medicago truncatula*

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(Yaish et al. 2016), and *Vigna unguiculata* (cowpea) (Leite et al. 2017; Mukhtar et al. 2020), suggest that nodule PGPM (plant growth-promoting microorganisms) play important roles in the plant's growth and development either via expression of plant growth-promoting (PGP) properties or biocontrol activity (BCA) (Martínez-Hidalgo et al. 2014, 2015). In addition to the inherent properties of the microbes, studies on *V. unguiculata* nodules show that soil characteristics also have a significant effect on the types of non-rhizobial genotypes populating nodules (Leite et al. 2017; Mukhtar et al. 2020). The traits expressed by nodule-isolated Plant Growth-Promoting Bacteria (PGPB) (Bashan and Holguin 1998) not only enhance crop production in agricultural fields, but also over time help restore soil health.

Sequencing root nodule metagenomes provides a novel and important approach to understanding the identities of the microorganisms living within this highly specialized organ. However, if we are to formulate optimal bacterial consortia or engineer phytomicrobiomes for use in sustainable agriculture, the microbes must be cultured in order to determine their effectiveness on plants and survivability in the soil. Legumes select rhizobial bacteria from the soil that will be most useful for their growth and development in nitrogen-deficient soils (Batstone et al. 2020), but non-rhizobial microbes, also known as nodule-associated bacteria (NAB), are isolated from nodules as well (reviewed in Martínez-Hidalgo and Hirsch 2017). Towards this end, we not only determined the diversity of bacteria within nodule microbiomes, but also isolated and cultured a large number of the NAB. Although some have considered the NAB to be "free-loaders" or "cheaters", it might then be expected that over time the NAB would be selected against. However, mutualisms rarely go extinct (Frederickson 2017), and thus it is likely that field-grown legume nodules always incorporate NAB within their tissues; if not exactly identical microbes, then others with similar functions depending on the soil environment. In our studies of cowpea nodules grown in Pakistan (Mukhtar et al. 2020), not only several rhizobial genera (*Mesorhizobium*, *Ensifer*, and *Bradyrhizobium*) but also various NAB species were cultured from surface-sterilized root nodules. Examples are PGPB species such as *Paenibacillus* and *Bacillus*, the actinomycetes *Streptomyces* and *Frankia*, as well as a number of *Pseudomonas* species, including *P. putida*, *P. fluorescens*, and other genera with PGPB activity. Similar results have been observed consistently in other studies in our laboratories.

Here we expand upon our previous study by examining the population of NAB within indeterminate *Medicago* nodules and comparing them to our studies on cowpea, a legume that develops determinate nodules. In contrast to cowpea soils collected from a saline agricultural area, the *Medicago* nodules were isolated from a working farm on the campus of California Polytechnic University in Pomona (Cal Poly Pomona), CA (soil is nutrient-rich due to periodic fertilizer addition) and also from soils of the Mildred E. Mathias Botanical Garden (MEMBG) located on the University of California, Los Angeles (UCLA) campus. This soil is more acidic as well as significantly more nutrient-poor than the Pomona agricultural soil. Nodules of *M. sativa* (alfalfa) were the source of cultivatable bacteria from Cal Poly Pomona, whereas nodules of *M. polymorpha*, commonly found in California, were the source for the MEMBG microbes.

Our goal is to discover prospective "helper" bacteria by selecting nodule-isolated strains from cultivated alfalfa plants and wild *Medicago* plants, the latter not normally used in agriculture, with the highest potential for growth promotion when coinoculated with rhizobia. A comparative analysis of these characteristics will allow the establishment of valid criteria for the in vitro selection of coinoculants (De-la-Peña and Loyola-Vargas 2014; Pérez-Montaño et al. 2014). We have also endeavored, when possible, to find the genes that could be responsible for the various plant growth-promoting (PGP) properties involved in stimulating plant growth and survival. We have done this: 1) via bioassays using published protocols that suggest PGPB function, e.g. plate assays, etc. (Martínez-Hidalgo et al. 2015; Maymon et al. 2015; Schwartz et al. 2013); and more importantly, 2) by testing the effects of inoculating the various isolates with and without rhizobia on plants grown under non-optimal conditions (Mukhtar et al. 2020).

Materials and Methods

Soil collection, physiochemical analysis, and preparation of plant samples Soil was collected in 2017, 2019, and 2021 from the Mildred E. Mathias

Botanical Garden (MEMBG; 34° 3′ 51.4866" N; 118° 26′ 39.2562" W), UCLA, Los Angeles, CA and in 2017 and 2019 from the California Polytechnical University farm, Pomona, CA (34° 2′ 42.27" N, 117° 48′ 45.108" W). Approximately 1 kg of soil from different and separated areas in the alfalfa field was collected to make composite soil samples per each site, which were stored at 4° C until sent to Waypoint Analytical (formerly Soil and Plant Laboratory, Inc.), Anaheim, CA, USA for nutrient analysis. The 2019-collected alfalfa rhizosphere soil and MEMBG soil collected in 2021 were used for eDNA analysis. For these studies, eDNA was isolated from 0.25 g of Pomona or MEMBG rhizosphere soil using the Qiagen DNeasy PowerSoil Pro kit. The analysis of the bacterial groups in the soil was performed by an external service: MR DNA (Showalter, TX) using 16S rRNA gene sequencing and the Illumina platform (San Diego, CA). Triplicate PCR reactions were not used for this amplicon taxonomic profiling as this has been shown to be unnecessary (Marotz et al. 2019).

Culture-independent analyses *M. polymorpha* nodules collected early in the season were small whereas later-collected nodules were mature and effective, based on their pink–red color, and subsequently separated into branched or elongated types. The nodules were thoroughly washed with sterile distilled water to remove any attached soil, then were carefully removed from the roots, and surface-sterilized with commercial bleach at 25% for 3 min. After the sterilization process, the nodules were rinsed in sterile distilled water 7 times, and then crushed aseptically using a sterile glass rod. Approximately 0.25 g of macerate was processed using the PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA). The eDNA was stored at -20 °C and sent to the Joint Genome Institute on dry ice for paired-end sequencing using the KAPA-Illumina library creation kit (KAPA Biosystems) and the HiSeq-2500 Illumina platform (San Diego, CA). A data cleaning process was applied to all sequences prior to analysis. Low-quality bases with a Phred quality value lower than 20 were trimmed off the read ends.

To study the nodule microbiome's taxonomic composition, the 16S rRNA gene sequences were extracted from the metagenomic shotgun sequencing data using a mapping-based method modified from Shi et al. (2015). The sequences were aligned with paired reads against the rRNA database (Greengenes v13.5, non-redundant precalculated OTU references, 97_otus from PICRUSt) (DeSantis et al. 2006; Langille et al. 2013). Lastly, the alignments were performed using Bowtie2 (Langmead and Salzberg 2012) to identify mappable sequences, which were included in the further analysis if their similarities to the references were 80% or above. The abundance of each species in each sample was calculated by counting the number of base pairs covering the genome of the organism, normalized by the genome size.

Cultivation-dependent analyses Mature alfalfa nodules (*M. sativa* L.) were collected when the plants were flowering. The plant studied for both cultivation-dependent and -independent analyses was *M. polymorpha*, a common legume in Southern California. It is native to the Mediterranean region but is abundantly distributed in disturbed and agricultural areas in California (Jepson and Hickman 1993). The nodules were surface-sterilized following the same protocol explained in culture-independent methods and then macerated using a sterile glass rod to collect the nodule bacteria. For validation of the success of the sterilization process, 20 µl of sterile water from the seventh wash were plated and the plates incubated; no growth was observed. For the bacterial suspension, different dilutions were prepared and then plated on yeast mannitol agar (YMA) (Somasegaran and Hoben 2012), Tryptone Yeast Extract (TY) (Beringer 1974) and SA1 (Trujillo et al. 2005). After 3 weeks of incubation, colonies that were morphologically different from one another were selected and harvested under axenic conditions. They were then checked for purity and re-streaked onto SA1 medium to obtain pure cultures. After isolating and plate-purifying the bacteria grown from the nodules, they were subjected to a number of biochemical and physiological assays.

Physiological assays Culturable bacteria were characterized for siderophore production, phosphate solubilization, cellulase activity, pectinase activity, growth in nitrogen-free medium, and were cultured on various carbon sources. They were also checked for halotolerance and ability to grow in media of different pH values as previously described (Martínez-Hidalgo et al. 2014). CAS medium was used to assay siderophore production (Alexander and Zuberer

1991); and PVK medium (Pikovskaya 1948), with modifications (Bashan et al. 2013), for determining phosphate solubilization ability. Cellulase activity was detected using CMC plates and halotolerance was tested using SA1 medium adjusted with several NaCl concentrations: 1%, 3%, and 5% (w/v). The SA1 medium was also pH-adjusted to 4, 5, 6, 8, and 9 to test the bacterial isolates for pH tolerance.

Chitinase detection was performed in an in vitro test based on the cellulase activity detection method used by Smith (1977) and modified by Kaplan et al. (2013). The disassociation of an azure dye from a chitin conjugate was used to observe chitinase activity and a total of seven replicates were performed for each strain tested. Results were noted as positive or negative based on the color of the medium after 3 weeks of incubation at 30ºC. The isolates with the most promising PGPB phenotypes were chosen for in planta experiments.

In planta co-inoculation Alfalfa seeds (cultivar Aragón or Vernal) were sterilized with full-strength commercial bleach and germinated at 30ºC for 24 h in water agar plates. The alfalfa seedlings were then transferred to plastic pots and incubated in the UCLA Plant Growth Center under controlled environmental conditions (light hours 16/8, temperature 23ºC/18ºC, humidity 60%). The substrate used was a 1:1 tyndallized mix of Seramis® and vermiculite. The tyndallization procedure was modified from Tyndall (1877) such that the substrate was heated 3 times during 3 consecutive days in an autoclave up to 100ºC for 50 min. For each trial, the selected PGP strains were co-inoculated with *Ensifer meliloti* 1021 (Meade et al. 1982). Two positive control treatments were included—plants inoculated only with *E. meliloti* 1021 and plants co-inoculated with *E. meliloti* 1021 and a known PGPB, namely *Micromonospora* pr18 (Martínez-Hidalgo et al. 2014). A negative control (the uninoculated treatment) was also included. Eight to ten replicates were used for the controls, and ten replicates per experimental treatment were set up. The nodule-isolated strains tested as coinoculation partners were *Bacillus* 1u117, PSB43' and 1SA(ca)5, *Ensifer* USAF6 and USAF17, *Micromonospora* USAFONa4 and UTRUM1, *Ochrobactrum* 1u19 and 2u24, and one isolate each of the following: *Oceanobacillus* UTRUM2, *Streptomyces* USAFOC20 and *Variovorax* 2u118 (Table 2).

Inoculation of plants with the selected strains was performed when the first true leaf appeared. Colonies of each isolate were suspended in sterile deionized water and adjusted to a McFarland number six standard. One mililiter of the bacterial suspension was inoculated at the base of the alfalfa seedling growing in the Seramis®-vermiculite mixture. Plants were uprooted after the start of flowering, and the following parameters were measured or calculated for each plant: dry root and shoot biomass, root and shoot length, number of nodules, and the shoot:root ratio.

Statistical analysis Statistical studies were performed using ANOVA and conducted in IBM SPSS 22 and RStudio 0.99. Post-hoc LSD and Dunnett's one-tailed t-tests were used to identify inoculation treatments with means significantly different from the control at $p \leq 0.05$.

16S rRNA analysis of isolates DNA was extracted from cell cultures using the REDExtract-N-Amp™ PCR ReadyMix (Sigma Aldrich) following the manufacturer's instructions. The amplification of *rrs* for each strain were performed using primers rD1: CCGGGATCCAAGCTTAAGGAG and fD1: CCG AATTCGTCGACAACAGAGT and REDExtract-N-Amp™ PCR ReadyMix (Sigma Aldrich), following the manufacturer's recommendations. PCR products were subjected to electrophoresis in 1% agarose gels containing ethidium bromide and Tris–acetate EDTA buffer. The amplified bands were excised and purified using Invitrogen PureLink™ Quick Gel Extraction Kit according to the manufacturer's instructions. Sequencing was performed by Macrogen, Inc., using a BigDye Terminator Cycle Sequencing Kit according to the manufacturer's instructions and using the primers described previously. The isolates were identified using the Ez-Taxon server (Kim et al. 2012; Yoon et al. 2017) on the basis of partial (~750 bp) 16S rRNA sequence data. Sequence data has been submitted to the GenBank database under accession numbers from MW722906 to MW722933 and MZ015721 to MZ015742.

Phylogenetic tree construction Sequenced type strains similar to the isolates were obtained from

EZ-Taxon website and added to the sequences obtained for the isolates described herein. For rooting the tree, the *rrs* gene from *Catellatospora citrea* was added to the analysis. The *rrs* sequences were aligned using ClustalW (Thompson 1997) and the tree was constructed using the Maximum Likelihood method based on the Kimura 2-parameter model, in MEGA6 software (Tamura et al. 2011).

Results

Physicochemical properties of *Medicago*-planted soils The alfalfa soil collected from Cal Poly Pomona differed from the MEMBG soil in several properties including salinity, which was measured as the expected electrical conductivity (Ece), pH, available nitrogen (N), phosphorous, and other factors (Table 1). Moisture, SAR (the level of sodium compared to calcium and magnesium), etc. were all close to the normal range for growing crops. The 2017- and 2019-collected Pomona soil samples were comparable to each other with regard to phosphorous (19 and 15 ppm), but not for other nutrients because of fertilizer addition to the field in 2019. In contrast, the MEMBG composite soil collected in 2019 was more acidic and lower in P, Mg, K, Ca and other nutrients, showing that the Botanical Garden soil was deficient in several essential elements (Table 1). Unlike the Cal Poly Pomona field soils, the MEMBG soils are infrequently amended (J. Munch, pers. comm.). However, Cu, Mn, Zn and Fe levels were higher than in the Pomona soil.

Culture-independent methods

Rhizosphere soil and nodule microbiomes. The Cal Poly Pomona alfalfa rhizosphere collection of soil made in 2019 was used to determine the total soil microbe population based on eDNA analysis of a composite soil sample collected from several adjacent sites in a defined area. The dominant phyla detected were Actinobacteria, Proteobacteria, Firmicutes, and Acidobacteria, which together made up more than 75% of the soil microbiome sequences (Fig. 1). Less highly

Table 1 Physicochemical characteristics of *Medicago* rhizospheric soil samples

Characteristics	MEMBG (2019) composite sample	Pomona (2017) composite sample	Pomona (2019) composite sample
Salinity (ECe) (dS/m)	0.3	0.7	1.4
Sodium Adsorption Ratio (SAR)	1.35	0.94	0.67
Sodium (Na) (meq/L)	1.6	1.6	1.8
Boron (B) (ppm)	0.33	0.08	0.19
Half Sat. Moisture Content (%)	32	20	20
pH	6.6	7.9	7.3
Organic Matter (% OM)	2.2	2.2	2.7
Available N (ppm)	11	18	33
NO_3 (ppm)	5	11	29
NH_4 (ppm)	6	7	4
P (ppm) (Olsen)	5	19	15
K (ppm)	74	106	123
Ca (ppm)	885	2477	1880
Mg (ppm)	188	363	263
Cu (ppm)	2	0.9	1.1
Zn (ppm)	12	1	3
Mn (ppm)	4	2	2
Fe (ppm)	25	4	5
Total Exchangeable Cations (TEC) (meq/kg)	61	155	113

represented groups were also detected including Chloroflexi, Planctomycetes, Verrucomicrobia and others.

MEMBG rhizosphere soil was not collected until 2019 due to a *M. polymorpha* population crash in 2014. Analysis of the eDNA showed that sequences of Proteobacteria, Actinobacteria, and Acidobacteria dominated the rhizosphere population similar to the alfalfa rhizosphere, and sequences from Firmicutes, Gemmatimonadetes, Bacteroidetes, Nitrospira and Thaumarchaeota were detected in both soils (data not shown). Nevertheless, the diversity of the bacteria in the MEMBG rhizosphere soil was not as great as that of the Cal Poly Pomona soil, which may be related to the differences in the amount of fertilizer added.

M. polymorpha nodules collected in 2017 that came from a site outside the MEMBG were used for an initial analysis of their microbiomes to test the methodology; *Ensifer* spp. (alpha-proteobacteria) sequences dominated in these preliminary tests (data not shown). The population of MEMBG *M. polymorpha* plants had recovered by 2020 at which time the nodules were collected and analyzed. Figure 2 shows the profiles of the bacteria within the MEMBG-collected nodules. A large percentage (ca. 70%) of *Ensifer* spp., the typical nodule-forming and nitrogen-fixing symbiont of *M. polymorpha*, was detected in the nodules. Although DNA sequences of *Ensifer*/*Sinorhizobium* were the most abundant bacteria in the nodular microbiome, they were not the only nodule inhabitants. Other alpha-proteobacteria in the Hyphomicrobiales were also detected depending on the site and the year of soil collection. As Fig. 2 shows, DNA sequences for members of the families 1) Rhizobiaceae [*Rhizobium*, *Agrobacterium, Hoeflea* (formerly *Agrobacterium;* Peix et al. 2005); *Shinella* (Lin et al. 2009)]; 2) Phyllobacteriaceae [*Mesorhizobium, Aminobacter* (herbicide-degrading; McDonald et al. 2005), *Nitratireductor* (Jang et al. 2011)], as well as bacteria in the Caulobacteraceae, namely, *Phenylobacterium* (Tiago et al. 2005) were also identified in the MEMBG-collected nodule DNA. In addition, DNA from a potential pathogenic species, *Ochrobactrum* (Holmes et al. 1988), now *Brucella* (Brucellaceae) as well as from a possible herbicide-degrading species, *Rhizorhabdus* (Francis et al. 2014) (Sphingomonadaceae) was also detected in nodule macerate.

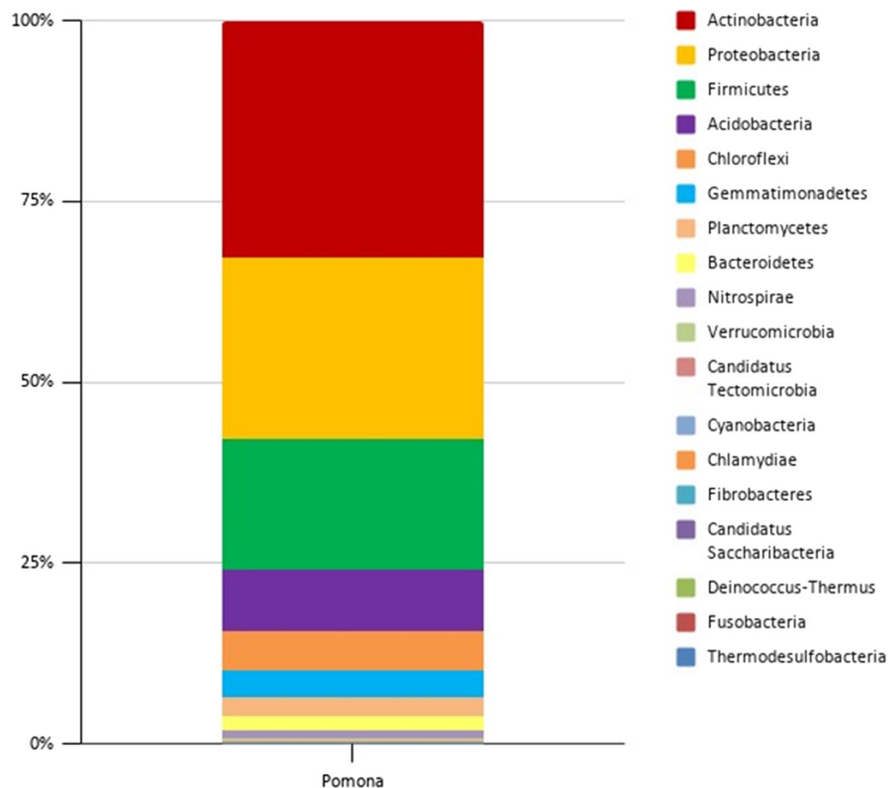

Fig. 1 *Medicago sativa* rhizosphere soil microbiome

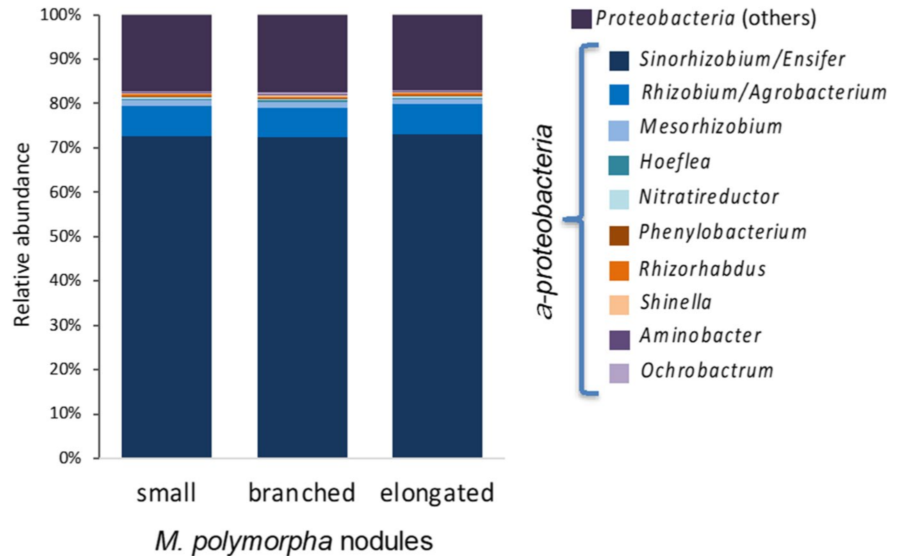

Fig. 2 *Medicago polymorpha* nodule microbiome

Culture-dependent methods

Bacterial isolation and 16S sequence analysis. Bacterial colonies were obtained from surface-sterilized root nodules of both *Medicago* species. Fifty-one strains in total were isolated from the sampling sites: 27 strains were isolated from the nodules collected in the UCLA MEMBG and 24 strains were obtained from nodules of alfalfa plants collected from the field at Cal Poly Pomona.

Partial 16S rRNA (*rrs*) gene sequences were obtained for all strains. NCBI and Ez-Taxon nucleotide blast searches revealed that the microbes within root nodules are diverse, based on the finding that several distinct species belonging to a wide range of genera were isolated. Table 2 illustrates the most related type strains based on *rrs* gene sequence analysis. In addition to *Ensifer* (responsible for the induction of the nitrogen-fixing nodules on alfalfa and related genera), numerous Gram-positive bacteria belonging to the phyla Firmicutes and Actinobacteria were isolated. *Ensifer,* with 22 isolates, was the most represented among the isolated strains based on the *rrs* gene sequences, and most were identified as *E. meliloti* although *E. medicae* or *E. arboris* were also found. Additional Gram-negative strains were identified including four strains of *Ochrobactrum* and a single representative of *Variovorax*. Sixteen isolates were identified as Firmicutes consisting of 14 different strains of *Bacillus*, one strain of *Oceanobacillus*, and one of *Paenibacillus*. Finally, 5 representatives of Actinobacteria of the genera *Micromonospora* (2 strains), *Pseudonocardia* (one strain) and *Streptomyces* (2 strains) were isolated. Sequence similarities between the new isolates and currently described bacterial type strain species ranged from 94.51 to 100% (Table 2).

Phylogenetic analysis of isolated strains. A tree using the closest type strain of currently described *Ensife*r species showed that the strains grouped in the same branch as the type strains to which they were most closely related. In fact, almost 70% of the strains had sequence similarity values that matched 100% with the *E. meliloti* and *E. arboris* type strains. However, some of the strains did not group with any currently recognized species. Further taxonomic work will be required to elucidate the status of these strains (Figure S1). Phylogenetic trees were also constructed for the remaining most-represented genera using the closest type strains currently described for *Bacillus*, *Ochrobactrum* and *Micromonospora* species (Figures S2, S3 and S4).

Ecological and PGP related activities. Functional characterization. Phenotypic analyses were conducted for all isolates (Table 3). The different nodule isolates were tested in vitro for a variety of PGPB traits such as siderophore production, phosphate solubilization, the strain's ability to grow on media containing cellulose, pectin, or xylan as the sole carbon source, as well as ability to grow in a medium without

Table 2 Geographical origin and 16S rRNA gene sequence analysis of nodule-isolated strains in this study

Strain	Origin	Host plant	Accession number	Most similar bacterial type strain	Similarity
1u117	Pomona	*M. sativa*	MZ015721	*Bacillus altitudinis* 41KF2b	96.05
PSB43’	MEMBG	*M. sativa*	MW722920	*Bacillus altitudinis* 41KF2b	100
PSB32	MEMBG	*M. sativa*	MZ015723	*Bacillus cereus* ATCC14579	100
PSB33	MEMBG	*M. sativa*	MZ015724	*Bacillus cereus* ATCC14579	100
PSCA15	MEMBG	*M. sativa*	MZ015725	*Bacillus dabaoshanensis* GSS04	99.6
15Sd13	MEMBG	*M. sativa*	MZ015726	*Bacillus licheniformis* ATCC14580	94.51
1SA(ca)5	MEMBG	*M. sativa*	MZ015728	*Bacillus safensis* FO-36b	99.21
USAFON2	MEMBG	*M. polymorpha*	MW722910	*Bacillus siamensis* KCTC13613	99.93
1SB5	MEMBG	*M. sativa*	MW722931	*Bacillus simplex* NBRC15720	99.86
USAFONa16	MEMBG	*M. polymorpha*	MW722912	*Bacillus stercoris* D7XPN1	99.68
1SD10	MEMBG	*M. sativa*	MZ015722	*Bacillus stercoris* D7XPN1	99.62
USAFOC6	MEMBG	*M. polymorpha*	MW722907	*Bacillus subtilis* subsp. *inaquosorum* KCTC13429	99.87
PSCA21	MEMBG	*M. sativa*	MZ015729	*Bacillus tequilensis* KCTC13622	99.86
1SD11	MEMBG	*M. sativa*	MW722925	*Bacillus zhangzhouensis* DW5-4	100
USAF1	MEMBG	*M. polymorpha*	MW722918	*Ensifer arboris* LMG14919	100
USAFON1	MEMBG	*M. polymorpha*	MW722917	*Ensifer arboris* LMG14919	100
USAF6	MEMBG	*M. polymorpha*	MW722911	*Ensifer medicae* WSM419	100
1u10	Pomona	*M. sativa*	MZ015730	*Ensifer meliloti* LMG6133	100
1u111	Pomona	*M. sativa*	MZ015731	*Ensifer meliloti* LMG6133	100
1u113	Pomona	*M. sativa*	MW722929	*Ensifer meliloti* LMG6133	100
1u114	Pomona	*M. sativa*	MZ015732	*Ensifer meliloti* LMG6133	100
1u115	Pomona	*M. sativa*	MZ015733	*Ensifer meliloti* LMG6133	100
1u116	Pomona	*M. sativa*	MW722924	*Ensifer meliloti* LMG6133	100
1u118	Pomona	*M. sativa*	MW722933	*Ensifer meliloti* LMG6133	97.45
2u110	Pomona	*M. sativa*	MW722932	*Ensifer meliloti* LMG6133	100
2u15	Pomona	*M. sativa*	MZ015734	*Ensifer meliloti* LMG6133	100
2u16	Pomona	*M. sativa*	MZ015735	*Ensifer meliloti* LMG6133	100
2u17	Pomona	*M. sativa*	MW722930	*Ensifer meliloti* LMG6133	100
2u18	Pomona	*M. sativa*	MZ015736	*Ensifer meliloti* LMG6133	100
2u27	Pomona	*M. sativa*	MZ015737	*Ensifer meliloti* LMG6133	100
4650D	Pomona	*M. sativa*	MW722921	*Ensifer meliloti* LMG6133	100
4650F	Pomona	*M. sativa*	MZ015738	*Ensifer meliloti* LMG6133	99.7
4677A	Pomona	*M. sativa*	MZ015739	*Ensifer meliloti* LMG6133	99.7
PSB71	MEMBG	*M. sativa*	MW722923	*Ensifer meliloti* LMG6133	99.72
USAF16	MEMBG	*M. polymorpha*	MW722906	*Ensifer meliloti* LMG6133	99.85
USAF17	MEMBG	*M. polymorpha*	MW722909	*Ensifer meliloti* LMG6133	98.1
USAFONa4	MEMBG	*M. polymorpha*	MW722914	*Micromonospora echinofusca* DSM43913	99.37
UTRUM1	MEMBG	*M. polymorpha*	MW722915	*Micromonospora inositola* DSM43819	99.79
UTRUM2	MEMBG	*M. polymorpha*	MW722908	*Oceanobacillus caeni* S-11	98.97
1u19	Pomona	*M. sativa*	MZ015740	*Ochrobactrum anthropi* ATCC49188	99.85
2u114	Pomona	*M. sativa*	MW722927	*Ochrobactrum anthropi* ATCC49188	100
2u24	Pomona	*M. sativa*	MW722926	*Ochrobactrum anthropi* ATCC49188	100
2u13	Pomona	*M. sativa*	MW722922	*Ochrobactrum cytisi* ESC1	95.66
USAFONa6	MEMBG	*M. polymorpha*	MW722919	*Paenibacillus polymyxa* ATCC842	99.73
2u210	Pomona	*M. sativa*	MZ015741	*Pseudonocardia carboxydivorans* Y8	100
USAFOC17	MEMBG	*M. polymorpha*	MW722913	*Streptomyces naganishii* NBRC12892	98.86

Table 2 (continued)

Strain	Origin	Host plant	Accession number	Most similar bacterial type strain	Similarity
USAFOC20	MEMBG	*M. polymorpha*	MW722916	*Streptomyces sparsogenes* NBRC13086	99.42
2u118	Pomona	*M. sativa*	MZ015742	*Variovorax paradoxus* IAM12373	100

nitrogen. Most isolates exhibited five or more PGPB traits, but very few produced siderophores. Those that did were several *Ensifer* strains, the two *Micromonospora* isolates (UTRUM1 and USAFONA4), and *Bacillus* isolate PSCA15.

Phosphate solubilization ability was detected in 14 of 17 isolates using the methodology of Bashan et al. (2013), which requires testing in media containing other metal-phosphate compounds, namely Al-P and Fe-P, in addition to tricalcium phosphate (TCP). Of the 17 tested, 9 isolates solubilized all three phosphate sources, 5 solubilized both TCP and Al-P, one was effective for TCP and Fe-P, and 3 isolates did not solubilize any of the metal-P compounds (Table 4).

Chitinase activity and other traits. The two *Micromonospora* isolates were tested for chitinase activity, but each exhibited a different response. USAFONA4 showed no chitinase activity in the assay whereas UTRUM1 exhibited definite chitinase activity (Table 5).

The ability to grow at different pH levels (from 4.5 to 8) was tested and all the strains except *Bacillus* PSCA15 and *Ensifer* USAF1 grew at pH 9, and 98% of the strains could be cultured at pH 5.5. There was a high variability in growth when the bacteria were cultured in media of pH 4.5. Taken together, these results show a high adaptation to different edaphic conditions. Most strains exhibited a high tolerance to salinity although the *Ensifer* isolates were less tolerant of salinity and low pH (Table 3).

Twelve strains were selected according to functional characterizations, phylogenetic analyses, and *rrs* gene sequencing (Tables 2–5). Based on the data obtained, the strains selected as coinoculation partners for the in planta tests were: *Bacillus* spp. (1u117, PSB43' and 1SA(ca)5); *Ensifer* spp. (USAF6 and USAF17); *Micromonospora* spp. (USAFONa4 and UTRUM1); *Ochrobactrum* spp. (1u19 and 2u24); *Oceanobacillus* sp. (UTRUM2); *Streptomyces* sp. (USAFOC20); and *Variovorax* sp. (2u118). The selected strains and their closest type strains are listed in Table 2.

Plant tests. Studies were conducted in a growth chamber with controlled temperature, photoperiod, and humidity. Plants were collected at the beginning of flowering. At the end of the experiment, we analyzed the parameters considered to be important for assessing the growth of alfalfa plants: shoot (SDW) and root (RDW) dry weight, shoot length (SL), root length (RL), shoot:root ratio (S:R) and the number of nodules (Nod). However, SDW and RDW gave the best estimates of the increase in growth brought about by the potential PGPB.

Univariate (ANOVA) analyses on the dependent variables were performed to compare differences within the control treatment and those co-inoculated with the selected strains. In response to the inoculation of these strains, the number of nodules on the plant root increased (Table 6; bold-faced), but these differences were statistically significant only for strains *Bacillus* PSB43', *Micromonospora* USAFONa4 and UTRUM1, and *Oceanobacillus* UTRUM2.

In addition, co-inoculation treatments resulted in a significantly higher shoot biomass increase than the control treatments. The mean shoot dry weights (SDW) of the co-inoculated plants were greater than the values found for the control plants except for *Variovorax* strain 2u118 (Table 6). In the treatments involving co-inoculation between *Ensifer* and the isolates *Bacillus* PSB43', and *Micromonospora* USAFONa4 and UTRUM1, there was a statistically significant increase in growth with respect to the control. The results of the root dry weight (RDW) measurements are similar. The RDW was always higher in the co-inoculation treatments, but this increase was significant only for strains *Ensifer* USAF17, *Micromonospora* UTRUM1, and *Ochrobactrum* 2u24.

Similarly, shoot length also showed a positive effect in all co-inoculation treatments but only reached statistical significance for *Bacillus* PSB43' and *Micromonospora* UTRUM1 strains. Co-inoculated plants also showed a higher shoot-to-root

Table 3 Ecological and PPB related activities in selected strains

Medium Strain	C source CMC	C source Pectin	C source Xylan	N free medium	Casein	Sidero-phores production	Phosphate solubilisa-tion	pH 4.5	pH 5.5	pH 6	pH 7	pH 8	pH 9	NaCl 1%	NaCl 3%	NaCl 5%
Bacillus sp 1u117	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+
Bacillus sp 15Sd13	-	-	w	-		-	+	-	+	+	+	+	+	nd	nd	nd
Bacillus sp 1SA(ca)5	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Bacillus sp 1SB5	-	-	w	-	**w**	-	+	-	+	+	+	+	+	nd	nd	nd
Bacillus sp 1SD10	-	-	w	-	nd	-	-	+	+	+	+	+	+	nd	nd	nd
Bacillus sp 1SD11	-	-	w	-	nd	-	+	+	+	+	+	+	+	nd	nd	nd
Bacillus sp PSB32	+	-	-	-	**w**	-	-	+	+	+	+	+	+	+	+	+
Bacillus sp PSB33	+	-	-	+	nd	-	+	-	+	+	+	+	+	nd	nd	nd
Bacillus sp PSB43’	+	-	+	-	**w**	-	+	-	+	+	+	+	+	+	+	+
Bacillus sp PSCA15	-	-	w	-	nd	+	-	-	+	+	+	+	-	nd	nd	nd
Bacillus sp PSCA21	+	-	-	+	nd	-	-	+	+	+	+	+	+	nd	nd	nd
Bacillus sp USAFOC6	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+
Bacillus sp USAFONa16	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
Bacillus sp USAFON2	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
Ensifer sp 1u10	+	+	-	+	nd	-	-	-	+	+	+	+	+	-	-	-
Ensifer sp 1u111	+	+	-	+	nd	-	-	-	+	+	+	+	+	-	-	-
Ensifer sp 1u113	+	-	-	+	-	-	-	-	+	+	+	+	+	-	-	-
Ensifer sp 1u114	+	-	-	+	nd	+	+	-	+	+	+	+	+	-	-	-
Ensifer sp 1u115	+	-	-	+	-	-	-	-	+	+	+	+	+	-	-	-
Ensifer sp 1u116	+	-	-	+	nd	+	-	-	+	+	+	+	+	-	-	-
Ensifer sp 1u118	+	+	-	+	nd	-	+	-	+	+	+	+	+	-	-	-
Ensifer sp 2u110	+	+	-	+	nd	-	-	-	+	+	+	+	+	+	-	-
Ensifer sp 2u15	+	-	-	+	-	+	-	-	+	+	+	+	+	+	-	-
Ensifer sp 2u16	+	-	-	+	-	-	-	-	+	-	+	+	+	+	-	-
Ensifer sp 2u17	+	-	-	+	nd	+	+	-	+	+	+	+	+	-	-	-
Ensifer sp 2u18	+	+	-	+	-	+	-	-	+	+	+	+	+	+	-	-
Ensifer sp 2u27	+	-	-	+	-	-	-	-	+	-	+	+	+	+	-	-
Ensifer sp 4650D	+	-	+	+	-	+	-	-	+	+	+	+	+	-	-	-
Ensifer sp 4650F	+	-	-	+	nd	-	-	-	+	+	+	+	+	-	-	-

Table 3 (continued)

Medium Strain	C source CMC	C source Pectin	C source Xylan	N free medium	Casein	Siderophores production	Phosphate solubilisation	pH 4.5	pH 5.5	pH 6	pH 7	pH 8	pH 9	NaCl 1%	NaCl 3%	NaCl 5%
Ensifer sp 4677A	+	+	-	+	-	-	-	-	+	+	+	+	+	-	-	-
Ensifer sp PSB71	-	-	nd	-	nd	-	+	-	-	+	+	+	+	-	-	-
Ensifer sp USAF1	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
Ensifer sp USAF16	+	-	-	+	-	-	-	-	-	-	+	+	+	+	-	-
Ensifer sp USAF17	+	+	-	+	-	-	+	-	+	+	+	+	+	+	+	-
Ensifer sp USAF6	+	+	nd	+	nd	+	-	-	+	+	+	+	+	-	-	-
Ensifer sp USAFON1	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	-
Micromonospora sp USAFONa4	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Micromonospora sp UTRUM1	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Oceanobacillus sp UTRUM2	+	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+
Ochrobactrum sp 1u19	+	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+
Ochrobactrum sp 2u114	+	-	-	-	**w**	-	-	-	+	+	+	+	+	+	+	+
Ochrobactrum sp 2u13	+	-	+	-	-	-	-	-	+	+	+	+	+	+	+	+
Ochrobactrum sp 2u24	+	+	-	+	-	-	+	+	+	+	+	+	+	+	++	+
Paenibacillus sp USAFONa6	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	-
Pseudonocardia sp 2u210	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Streptomyces sp USAFOC17	+	-	+	+	+	-	-	-	+	+	+	+	+	+	+	+
Streptomyces sp USAFOC20	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-
Variovorax sp 2u118	+	-	-	+	-	+	-	-	+	+	+	+	+	-	-	-

"W" means "weak", "nd" means "not determined"

Table 4 Phosphate solubilization activity of selected strains shown on plates containing tricalcium phosphate (TCP), aluminum phosphate (Al-P) and ferric phosphate (Fe-P)

Strain	TCP	Al-P	Fe-P
Pseudonocardia sp 2u210	+	+	+
Streptomyces sp USAFOC20	+	+	+
Bacillus sp PSB 43'	+	+	+
Bacillus sp PSB 33	-	-	-
Bacillus sp 15Sd13	+	+	-
Bacillus sp 1SA(ca)5	+	-	+
Bacillus sp 1SD11	+	+	+
Bacillus sp 1SB5	-	-	-
Bacillus sp USAFOC6	+	+	+
Paenibacillus sp USAFONa6	+	+	+
Ensifer sp USAFON1	-	-	-
Ensifer sp 1u118	+	+	-
Ensifer sp PSB 71	+	+	+
Ensifer sp USAF17	+	+	-
Ensifer sp 1u114	+	+	+
Ensifer sp 2S(ca)3	+	-	-
Ensifer sp 2u17	+	+	-
Ochrobactrum sp 2u24	+	+	+

(S:R) ratio, but no significant differences were observed between treatments for this parameter (Table 6).

Discussion

The dominant groups obtained by cultivation methods are usually *Actinobacteria*, *Firmicutes*, and *Proteobacteria* (alpha, beta, and gamma). However, to our knowledge, few experiments have been done to find the optimal non-rhizobial strains isolated from legume nodules to add to a rhizobial inoculum. We reasoned that PGPM (or PGPB if solely bacteria), which are found in unamended fields following the sowing of inoculated seeds, might be good candidates. If non-rhizobial, environmentally stable strains are present in nitrogen-fixing nodules, by looking for optimal plant yield under the conditions under which the crops are grown, it may result in a strategy that seeks out potential “helper” strains that could stimulate plant growth as well as restore soil microbial diversity, especially in poor soils.

Studying the nodule microbiome. Typically, there is a discrepancy between the diversity of the bacteria in nodule metagenomes and that acquired from cultivation-dependent experiments. In part, this is because of the lack of optimal cultivation methods to grow the diverse assortment of bacteria associated with plants. A variety of microbes are present in root nodules as shown by the fact that several distinct species belonging to a wide range of genera were isolated. Their differences from previously described type strains suggest that they are new strains belonging to species already described or in some cases (*Ensifer* strain USAF17) may be a completely new species (Table 2). Phylogenetic trees based on 16S rRNA gene sequences (Suppl. Figures) show that the strains belonging to each genus compared to the type strains of closely related species are different, and it is possible that some of the isolates represent new species.

As expected, most of the isolated strains belong to the genus *Ensifer*, the major nitrogen-fixing symbiont of *Medicago* species, but the phylogenetic tree topology suggests that strains USAF17 and 1u118 could

Table 5 Chitinase activity in selected *Micromonospora* and *Bacillus* strains

Strain	Chitinase activity	Reference
Medicago isolates		
Micromonospora sp UTRUM1	+ +	This study
Micromonospora sp USAFONa4	-	This study
Bacillus sp PSB43'	-	This study
Other isolates		
Micromonospora sp ALFpr18c	+	Martínez-Hidalgo, et al (2014)
Micromonospora sp L5	-	Niner et al. (1996)
Bacillus sp 30 N-5	-	Schwartz et al (2013)
Promicromonospora sp Lb5-2	-	Unpublished strain

“+” means chitinase activity was detected, while “+ +” means chitinase activity was higher compared to the regular positives

Table 6 Relative efficiency of growth parameters of alfalfa plants inoculated with *Ensifer meliloti* 1021 alone (control treatment) or in co-inoculation with selected strains isolated from *Medicago* root nodules

Strain	S:R	SDW RE	RDW RE	SL RE	RL RE	Nod RE
Control	*2,09*	*100*	*100,00*	*100,00*	*100,00*	*100,00*
Coinoculations						
Bacillus sp 1u117	1,27	103	110,40	110,11	85,85	129,46
Bacillus sp PSB 43'	2,02	**143,84****	141,28	**123,19*****	98,28	**163,46*****
Bacillus sp 1SA(ca)5	2,13	109	100,91	103,21	96,39	126,78
Ensifer sp USAF6	1,49	114	110,52	103,92	99,09	117,85
Ensifer sp USAF17	1,22	127	**150,26***	97,72	111,28	104,92
Micromonospora sp USAFONa4	2,25	**159,82****	148,26	106,26	89,16	**176,17*****
Micromonospora sp UTRUM1	1,95	**162,43****	**173,96***	**119,7****	**84,27****	**153,94****
Ochrobactrum sp 1u19	1,31	107	112,09	113,00	97,01	97,67
Ochrobactrum sp 2u24	1,30	131	**145,14***	108,86	105,51	99,86
Oceanobacillus sp UTRUM2	1,92	110	120,17	95,27	90,63	**144,4***
Streptomyces sp USAFOC20	1,67	107	127,18	89,48	90,14	133,16
Variovorax sp 2u118	1,34	88	89,92	106,18	88,26	120,47

Control treatment corresponds to alfalfa plants inoculated only with *E. meliloti* 1021

S:R, shoot to root ratio; SDW RE, shoot dry weight relative efficiency; RDW RE, root dry weight relative efficiency; SL RE, shoot length relative efficiency; RL RE, root length relative efficiency; Nod RE, nodulation relative efficiency

Relative efficiency: differences between treatments versus control plants expressed in percentages

Within columns, treatment in bold type were higher than their control treatment (in italics) according to a Fisher protected LSD test at $P \leq 0.1$ (*), $P \leq 0.05$ (**) and $P \leq 0.01$ (***)

be new species of the genus *Ensifer* (Figure S1). The second most isolated genus, based on the number of strains detected, was *Bacillus*. Strains PSB32 and PSB33 are closely related to the *B. cereus* group (Figure S2., which includes a large number of species with pathogenic potential (Didelot et al. 2009). Several isolates belong to the genus *Ochrobactrum* (now *Brucella*) (Figure S3) and are closely related to species previously isolated from legume root nodules of *Cytisus* and *Lupinus* (Trujillo et al. 2005; Zurdo-Pineiro et al. 2007). Two *Micromonospora* strains were isolated, UTRUM1 and USAFONa4 and they may represent new species (Figure S4). *Micromonospora* bacteria are frequently isolated from legume nodules (Trujillo et al. 2010). In fact, a wide diversity of strains belonging to this genus has been isolated from *Medicago* nodules in different countries (Australia, Spain) and different soils (Martínez-Hidalgo et al. 2020). Beneficial endophytic *Streptomyces* have been isolated from plant roots and leguminous nodules (Coombs and Franco 2003; Le et al. 2016; Mukhtar et al. 2020; Vo et al. 2021), and as shown in this study, two *Streptomyces* strains were also isolated from *Medicago* root nodules. *Streptomyces* (isolated from nodules and roots) and *Mesorhizobium ciceri* coinoculation increased the dry weight of chickpea (Vo et al. 2021) supporting the hypothesis that nodule isolates are effective inocula.

In summary, a large number of diverse Gram-positive and Gram-negative bacteria belonging to 10 different genera were obtained. The diversity and abundance of populations of bacteria inside legume root nodules suggest that non-rhizobial presence could play important ecological and agricultural roles.

The strains selected to investigate their competence as PGPB in co-inoculation with the model strain *E. meliloti* 1021 were tested on alfalfa under growth chamber conditions and several of the selected strains promoted nodule formation in alfalfa (Table 6). The strains tested have synergy with *E. meliloti*, and probably other species of *Ensifer*, and may yield a benefit for the plant hosting them, but additional studies are needed. If there indeed is a benefit for the microbes, it suggests that the mutualistic interaction that is established occurs not only between *Ensifer* and the plant, but also between other bacterial strains in combination with *Ensifer* and the plant. Nevertheless, the possibility also exists that these nodule endophytes may not play a significant role in legume growth and

development (Mayhood and Mirza 2021). More studies are needed. However, it is highly likely that soils in which legumes are grown are much more likely to house effective nodule endophytes that re-establish an effective symbiosis when coinoculated with rhizobia.

We measured several growth parameters used in agronomy to evaluate the productivity of alfalfa in response to the nodule isolates. The results showed that several of the tested strains have PGP potential, including *Bacillus* PSB43', *Micromonospora* USAFONa4 and UTRUM1, and others. We selected *Bacillus* and *Micromonospora* strains for further study because they stood out from the rest in enhancing plant growth and nodule numbers under experimental conditions (Table 6). Although no other strains in co-inoculation with *Ensifer* statistically surpassed the control treatment in enhancing plant growth, a trend towards an increase in the number of nodules following co-inoculation was observed (Table 6), suggesting that a major effect of co-inoculations on legume performance could be due to an improvement in nodulation. Plant growth-promoting bacteria can increase nodulation in legumes through different mechanisms, including the production or degradation of phytohormones involved in nodule initiation and organogenesis (Fox et al. 2011), or by affecting the interaction between plant and rhizobia (Madhaiyan et al. 2006; Merzaeva and Shirokikh 2010; Radwan et al. 2002). Our selected strains might use one or more of these mechanisms to promote legume nodulation, and we are investigating these possibilities.

One of the most common effects described for PGPB is the formation of broader root systems, which allow them to explore a greater volume of soil increasing the uptake of water and nutrients (Vacheron et al. 2013). However, in this study we have not found significant differences between co-inoculated and control plants with regards to S:R ratio (Table 6) and thus the plant growth promotion cannot be ascribed to larger root systems in co-inoculated plants. This lack of increase in the size of the root system has been described previously by Martínez-Hidalgo et al. (2014). The strains *Bacillus* PSB43' and *Micromonospora* USAFONa4 and UTRUM1 seem to have boosted plant growth by means other than increasing root system dimensions. The higher S:R ratio in co-inoculated plants indicates that the better shoot growth cannot be related to a higher uptake of nutrients produced as consequence of a larger root system, but rather to a possible increase in uptake efficiency. Studies on mineral nutrition in plants, especially mineral availability and uptake, have been neglected despite their importance not only for agriculture, but also for the environment (Camelo et al. 2011).

So far, it is difficult to determine definitively which PGP capabilities are shared among the isolates and if they relate to soil type or conditions. Previous studies have shown the plant actively selects its optimal consortium of bacteria, expending significant resources in the process. Approximately 17% of the total photosynthate of the plant is transferred to the rhizosphere via root exudation (Nguyen 2003), which indicates that the plant strongly influences the composition of its microbiome, more than the soil. Root exudates thus play a pivotal role in the selection of microorganisms in both rhizospheres and plant tissues, which allows some bacteria to be more competitive in the rhizosphere (by increasing their growth by means of the exudates) thereby directing microbial colonization and persistence (Eilers et al. 2010).

Because many soils are not optimal for plant growth, the capacity of symbiotic rhizobia to produce metal-scavenging compounds, such as siderophores, is also important and has been well studied (Fabiano and O'Brian 2010). Studies by Moreau et al. (1995) suggest that soybean bacteroids transport iron citrate. Other metallophores have been discovered that are encoded in the *Bradyrhizobium japonicum* genome, e.g., for nickel uptake, and *Micromonospora* is also reported to produce metallophores (Ortúzar et al. 2020). *Micromonospora* is also well known for being one of the most important secondary metabolite producers in the Actinobacteria group and the two strains studied here synthesize siderophores as does *Bacillus* PCSA15 and several *Ensifer* isolates (Table 3). Endophytic *Micromonospora* also synthesize phosphatases and auxins (Martínez-Hidalgo et al. 2014), demonstrating that nodule isolates act as probiotic microbes by improving not only crop yield, but also forage quality (Martínez-Hidalgo et al. 2014). *Micromonospora* alfalfa nodule isolates have also been described (Martínez-Hidalgo et al. 2015) as agents for biological control because they combine direct antifungal activity and the ability to prime the immunity of plants against plant pathogens. Moreover, the evidence for chitinase activity in *Micromonospora* sp. UTRUM1 suggests that this strain may also be a

useful biocontrol agent (Table 5). Currently, chemical fungicides, insecticides, and nematicides are the primary means of controlling plant disease-causing agents. However, due to the potential damage that these chemicals cause to the environment and human and animal health (Damalas and Eleftherohorinos 2011; Chiesa et al. 2016), new strategies are needed to replace or reduce their use. In this context, chitinolytic microorganisms are sustainable alternatives to chemical pesticides in the control of pathogens. Bacterial chitinases weaken and degrade the cell walls of many pests and pathogens, thus exhibiting antibacterial, antifungal, insecticidal, or nematicidal activity (Edreva 2005; Liu et al. 2010; Sadeghi et al. 2006; Suryanto et al. 2014; Tahtamouni et al. 2006; Veliz et al. 2017; Zhong et al. 2015). They have also been found to provide protection against pathogens in several different settings, and in some cases amending the soil with chitin is sufficient to decrease pathogen effects (Buxton et al. 1965; Cretoiu et al. 2013). Also, in this study, *Bacillus* strain PSB43' is shown to be an excellent candidate for use as an inoculant because of its ability to increase plant productivity by significantly increasing nodulation in alfalfa.

Together, these data suggest that some *Medicago* nodule isolates are excellent PGPB, particularly *Micromonospora* and *Bacillus* strains; both are Gram-positive and form spores crucial for the survival of inocula, and thus more likely to be developed as coinoculants. Many of our isolates produce metabolites in vitro that seem to be related to their PGP capabilities in planta, such as siderophore, chitinase, phosphatase and cellulase production. Trials for the detection of these compounds could be used to determine PGP potential in isolates. In addition, many other isolates could also serve as excellent candidates for use as bioinoculants in that they can substitute for, or supplement various synthetic soil amendments currently being used at very high levels. It should be noted that we have also isolated several PGP strains from wild plants (*M. polymorpha*). These results show us the importance of exploring wild plants as an additional source for obtaining microbial strains useful in agrobiotechnology. Using PGP bacteria from wild relatives could be a logical step to recover or add bacteria to damaged soils. We also observed that the source of the isolates does not hinder their PGP effect on either wild or domesticated plants. In fact, six isolates from *M. sativa* and *M. polymorpha* were found to be potential PGPR, suggesting that *M. polymorpha* is an excellent source for cultivated species such as alfalfa. Further studies concerning the microbes' viability in storage in peat or other substrates or as liquid inoculants, as well as their activity in field trials need be conducted to confirm the trend of the results reported here.

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Declarations

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