Micromonospora from nitrogen fixing nodules of alfalfa (Medicago sativa L.). A new promising Plant Probiotic Bacteria.

Pilar Martínez-Hidalgo1,4, Purificación Galindo-Villardon2, Martha E. Trujillo1,4, José M. Igual3,4 & Eustoquio Martínez-Molina1,4

1Department of Microbiology and Genetics. University of Salamanca. Plaza Doctores de la Reina s/n. 37007 Salamanca, Spain, 2Department of Statistics. University of Salamanca. Plaza Doctores de la Reina s/n. 37007 Salamanca, Spain, 3Instituto de Recursos Naturales y Agrobiología de Salamanca (CSIC), Cordel de Merinas 40–52, 37008 Salamanca, Spain, 4Unidad Asociada USAL-CSIC “Interacción Planta-Microorganismo”.

Biotic interactions can improve agricultural productivity without costly and environmentally challenging inputs. Micromonospora strains have recently been reported as natural endophytes of legume nodules but their significance for plant development and productivity has not yet been established. The aim of this study was to determine the diversity and function of Micromonospora isolated from Medicago sativa root nodules. Micromonospora-like strains from field alfalfa nodules were characterized by BOX-PCR fingerprinting and 16S rRNA gene sequencing. The ecological role of the interaction of the 15 selected representative Micromonospora strains was tested in M. sativa. Nodulation, plant growth and nutrition parameters were analyzed. Alfalfa nodules naturally contain abundant and highly diverse populations of Micromonospora, both at the intra- and at interspecific level. Selected Micromonospora isolates significantly increase the nodulation of alfalfa by Ensifer meliloti 1021 and also the efficiency of the plant for nitrogen nutrition. Moreover, they promote aerial growth, the shoot-to-root ratio, and raise the level of essential nutrients. Our results indicate that Micromonospora acts as a Rhizobia Helper Bacteria (RHB) agent and has probiotic effects, promoting plant growth and increasing nutrition efficiency. Its ecological role, biotechnological potential and advantages as a plant probiotic bacterium (PPB) are also discussed.

Nodules are new organs generated mainly in roots of leguminous plants, in cooperation with alpha and beta proteobacteria developed for biological nitrogen fixation. It was initially thought that only symbiotic nitrogen-fixing bacteria could exist inside healthy N2 fixing nodules. Recent studies have shown that they are frequently populated by a broad and heterogeneous range of both gram-positive and gram-negative bacteria. Recently, the first intranodular actinobacteria have been described, but from the first description in this environment, the number of actinomycetes found has increased and in fact even new species have been described. Examples of these new findings inside nodules are Curtobacterium in Trifolium and Ornithopus; Microbacterium in Acacia, Glycyrrhiza, Medicago and Ornithopus; Micromonospora in several legumes; Streptomycies in Sphaerophis; and others. Notably, Micromonospora, which has been isolated from more than 20 different widely distributed plant species seem to have good potential as a plant-probiotic bacteria (PPB), although this remains to be studied in depth.

At our laboratory, strains of Micromonospora have been isolated from healthy plant nodules in a variety of genera of leguminous plants including M. sativa (alfalfa). Alfalfa is one of the most widely adapted agronomic crops and a cheap source of protein-rich forage with high digestibility, which is a valuable trait in economical animal husbandry. Alfalfa should be considered a key component of sustainable agricultural systems for the future because of its high yield, nutritional quality, pest resistance, and its value in soil conservation and improvement.

One of the major challenges for the twenty-first century will be sustainable crop production. Agricultural practices derived from the green revolution, defined by the use of pesticides, fertilizers and herbicides of chemical origin, together with the genetic improvement of plant germplasm, produced an increase in agricultural productivity. Decades ago, the cost and risks derived of this kind of agriculture were elucidated and as a consequence, a new agricultural revolution is now starting to develop in which probiotic microorganisms have become an alternative to chemicals. The possibilities for influencing plant growth-promoting potential applying...
microorganisms as Plant Probiotic Bacteria (PPB) agents have been largely explored. The interest of these microorganisms is clear, and today inoculants can be found on the market in several countries. Based on recent surveys, interest in the use of inoculants is also rising, suggesting that the market potential of bioinoculants will increase further in coming years.

However, it is necessary to study their ecological role and make an adequate analysis, evaluation and selection of the microbial strains used in order to obtain the desired effect and, unfortunately, the beneficial plant-microbe interaction has often been ignored in breeding strategies, even after their importance in soil ecosystems was confirmed (reviewed by Smith and Goodman). In light of the foregoing, the main goal of this study was to determine the diversity and ecological function of *Micromonospora* and analyze its plant probiotic capabilities since there is little information about it even though its biotechnological potential and also its impact in this new agricultural revolution are relevant.

Results

Bacterial isolation and morphological characterization. *Micromonospora*-like colonies were isolated from surface sterilized root nodules of naturally occurring alfalfa plants on yeast mannitol agar, along with rhizobia-like bacteria after 3-week incubation at 28°C. *Micromonospora* strains were recovered in almost all of the nodules sampled. In all, 66 strains were isolated from the sampling sites: Aldearrubia (AL) 21 strains, Babilafluente (ALFb) 11 strains, Palaciosrubios (ALFr) 19 strains, San José (ALFr) 4 strains and Tormes riverbank (ALF) 11 strains. All 66 actinobacterial strains had the morphology described for the genus *Micromonospora*; they were Gram +, filamentous, lacked aerial mycelium, and presented orange or brown colonies that darkened after around 3 weeks due to sporulation.

Genetic diversity of the *Micromonospora*-like isolates. High-resolution BOX-PCR fingerprints were obtained for the 66 actinomycetes isolated from the nitrogen-fixing root nodules of *M. sativa* (Figure 1). The amplified fragments ranged from 0.1 to 2.2 kb. Clusters based on the similarity matrix generated with Pearson’s coefficient and the UPGMA algorithm were defined at the 60% similarity level, affording 10 groups and revealing the high genetic diversity of the isolates. Figure 1 shows the diversity of the genetic profiles of the strains studied. Fifty-five strains were distributed in 10 clusters containing 2–13 strains; the remaining 11 isolates had a unique profile. No clones were found even in the strains from the same nodule. With respect to the isolation site, the strains isolated from Aldearrubia (21 strains) and the 19 strains recovered from Palaciosrubios were distributed along the entire dendrogram, they had representatives in almost every cluster; the 11 strains from Babilafluente were detected in 6 groups; the 11 strains from Tormes River bank in 8 groups and the 4 strains from San José in 4 groups. Two groups contained strains from the five different sampling sites (cluster 1 and 3). Clusters 9 and 10 (2 strains each) only contain strains from Aldearrubia, the rest of the clusters contained strains from 2 or more of the locations sampled.

According to the genetic diversity (BOX-PCR fingerprinting) and geographical origin of the isolates, we selected fifteen strains for *in planta* interaction studies.

Phylogenetic analysis and functional characterization of selected *Micromonospora* strains. Nearly complete 16S rRNA gene sequences (≥1434 nt) were obtained for the fifteen selected strains. NCBI and Eztaxon nucleotide blast searches revealed that 100% of the sequenced microorganisms were identified as belonging to the genus *Micromonospora* as suggested by their morphological characteristics.

Sequence similarities between the new isolates and currently described *Micromonospora* species ranged from 97.78 to 100%. A significant number of the isolates sequenced (approx. 87%) showed >99% sequence similarity with already described *Micromonospora* species (Table 1). The inferred phylogenetic tree based on 16S rRNA gene sequences using maximum likelihood (Figure 2) and neighbour-joining methods (Figure S1) showed that six of the isolates clustered with already described *Micromonospora* species. The tree topology generated by both maximum likelihood and neighbour-joining methods was strongly supported by bootstrap values which were similar for both methods. However, nine of the strains did not group closely with any of the currently recognized species (AL2, ALFb4, ALFr18c, ALFr19a, ALFb1, AL16, ALF4, ALFr4 and ALFr5; Figure 2). Further taxonomic work will be required to elucidate the status of these last strains.

With the exception of strain AL2 lacking pectinase activity, all of the other *Micromonospora* strains showed the ability to degrade plant cell wall components, namely cellulose, pectin and xylan (Table 2). Even though, the cellulose activity was weak in all the strains. Other components of organic matter such as proteins (cassinoise and gelatinase activities) and starch were also degraded by all the strains, being the only exception the strain ALF1, which could not degrade gelatine. Moreover, all of the tested strains showed lipase activity. They were able to degrade Tween 80. Tween 20 was strongly degraded by two strains (ALFr5 and ALFb7), weakly by nine and no hydrolytic activity was detected in four of them. Neutral and alkaline phosphatase activities were detected in all the strains but none showed acid phosphatase activity (Table 2).

Thirteen *Micromonospora* strains were able to produce IAA. AL16 and AL20 were the strains with the highest production levels (>74.8 µg/mL) whereas the strain ALF7 showed the lowest (2.9 µg/mL), the remaining had IAA production ranging from 11.3 to 47.0 µg/L (Table 2).

The ability to grow at different pH (from 4.5 to 9) was tested. All the fifteen *Micromonospora* strains grew well at a pH range of 7 to 8. None of the strains grew at pH below 5.5 nor at pH 9. We found high variability when grown at pH 6.5 (Table 2).

Effect of *Micromonospora* on plant growth and nutrient content of alfalfa. Investigating putative plant growth-promoting effects on alfalfa of the fifteen *Micromonospora* strains alone and in co-inoculation with the model strain *E. melliloti* 1021 was addressed in this part of the study. A mesocosm experiment was conducted in a greenhouse in pots containing a sandy-clay soil (Table S1) under controlled conditions of temperature, photoperiod and humidity. At harvest, shoot and root biomass, number of nodules and shoot nutrient contents were determined (Figure S2, Tables S2 and S3). The measured plant growth and nutrient content parameters were grouped together to form a data matrix of 2,560 data points (8 parameters × 32 inoculation treatments × 10 replicates).

We first used an indirect analysis (PCA) of the data [excepting number of nodules (Nod) data] to summarize the variation across all the 320 alfalfa plants tested. Figure 3a shows the distance biplot resulting from the PCA analysis. The first PCA axis explained 70.6% of the variance in the data, while the second axis accounted for 22.8%. Therefore, only the fraction of variability explained by the first axis surpasses the values predicted by the null model, indicating that the first axis describes non-random, interpretable variation in the data while the second does not. With the exception of Rdw (root dry weight) and S/R (shoot to root ratio), the remaining five response variables [Sdw (shoot dry weight), C, N, P and K] had high positive correlation (> 0.9) with the PC1 scores. Further examination of the
PCA biplot, focusing on the disposition the centroids of the 32 dummy independent variables (inoculation treatments) projected post hoc into the ordination space, reveals that the first principal component is related to E. meliloti 1021 inoculations (with and without E. meliloti 1021). Plants inoculated with E. meliloti 1021 tended to have Sdw and shoot contents of C, N, P and K higher than the E. meliloti 1021-free plants. Similarly, within the cohorts of plants inoculated and non-inoculated with E. meliloti 1021, the inoculation with specific Micromonospora strains tended to produce higher or lower values of these parameters comparing to other Micromonospora inoculation treatments and to the controls (Figure 3a). Redundancy analyses (RDA) testing for the significance of effects of the inoculation with E. meliloti 1021, the inoculation with Micromonospora and their interaction revealed statistical significance of all the three factors (Table S4).

Given the significance of the interaction effect of E. meliloti 1021 and Micromonospora inoculations (F-ratio = 2.090, P-value = 0.002; Table S4), we performed separate RDA analyses for the cohorts of plants inoculated and non-inoculated with E. meliloti 1021. In the cohort of E. meliloti 1021-free plants, redundancy analysis (RDA) revealed that the explanatory effect of the Micromonospora inoculations was highly significant according to the Monte Carlo test for significance of all canonical axis (F-ratio = 9.920, P = 0.0010). Figure 3b shows the distance biplot resulting from this RDA analysis.
The proportion of variability explained by all the constrained canonical axes was 50.8%, and 30.9% and 13.7% by, respectively, the first and second canonical axes, both being significant (Figure 3b). We undertook pair-wise RDA comparisons in order to determine which of the *Micromonospora*-inoculated treatments produced statistically significant differences when compared with the uninoculated control treatment. Table S5 summarizes the results of this set of multivariate tests. Results indicated that only three out of the 15 *Micromonospora*

| Strain | Origin       | # Accession   | Most similar *Micromonospora* type strain. (Accession number)                                      | Similarity (%) | Source       |
|--------|--------------|---------------|-------------------------------------------------------------------------------------------------|----------------|--------------|
| AL2    | Aldearrubia  | KF876220      | *M. chaiyaphumensis* MCS-1 (AB196710)                                                            | 99.72          | This work    |
| AL4    | Aldearrubia  | KF876221      | *M. viridifaciens* DSM 43909T (X92623)                                                           | 99.52          | This work    |
| AL16   | Aldearrubia  | KF876222      | *M. saelicesensis* lupac 09 (AJ783993)                                                            | 99.65          | This work    |
| AL20   | Aldearrubia  | KF876223      | *M. chokoriensis* 2-19/6 (AB241454)                                                              | 99.79          | This work    |
| ALF1   | Tormes riverbank | KF876224    | *M. humi* P0402 (GU459068)                                                                       | 99.51          | This work    |
| ALF4   | Tormes riverbank | KF876225    | *M. coxensis* 2-30-b/28 (AB241455)                                                               | 99.31          | This work    |
| ALF7   | Tormes riverbank | KF876223    | *M. saelicesensis* lupac 09 (AJ783993)                                                            | 99.86          | This work    |
| ALFb5  | Babilafuente | KF876226      | *M. aurantica* ATCC 27029 (CP002162)                                                              | 99.77          | This work    |
| ALFb7  | Babilafuente | KF876227      | *M. tulbaghiae* TVU1 (EU196562)                                                                   | 99.93          | This work    |
| ALFb1  | Babilafuente | KF876228      | *M. saelicesensis* lupac 09 (AJ783993)                                                            | 99.58          | This work    |
| ALFb4  | Babilafuente | KF876229      | *M. echinospora* ATCC 15837 (JL58332)                                                             | 97.78          | This work    |
| ALFpr18c | Palaciosrubios | KF876230   | *M. lupini* Lupac 14N (AJ783996)                                                                  | 99.31          | [37]         |
| ALFpr19a | Palaciosrubios | KF876231   | *M. saelicesensis* lupac 09 (AJ783993)                                                            | 99.51          | This work    |
| ALFr5  | San José    | KF876232      | *M. cremea* CR30 (FN658654)                                                                      | 98.62          | This work    |
| ALFr4  | San José    | KF876234      | *M. saelicesensis* lupac 09 (AJ783993)                                                            | 99.51          | This work    |

Figure 2 | Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the *Micromonospora* isolates and the closest recognized *Micromonospora* species. Bar, 0.01 substitutions per nucleotide position. Bootstrap percentages (1000 replicates) above 50% are shown at nodes.
Table 2 | Ecological, PPB related enzymatic activities and indolacetic acid production in selected strains

| Strains/Activity | AL2 | AL4 | AL16 | AL20 | ALFb1 | ALFb4 | ALFb5 | ALFb7 | ALF1 | ALF4 | ALF7 | ALFpr18c | ALFpr19a | ALFr4 | ALFr5 |
|------------------|-----|-----|------|------|-------|-------|-------|-------|------|------|------|----------|----------|-------|-------|
| Cellulase        | w + | w + | w +  | w +  | w +   | w +   | w +   | w +   | w +  | w +  | w +  | w +      | w +      | w +   | w +   |
| Xylanase         | w + | w + | w +  | w +  | w +   | w +   | w +   | w +   | w +  | w +  | w +  | w +      | w +      | w +   | w +   |
| Pectinase        | -   | -   | -    | -    | -     | -     | -     | -     | -    | -    | -    | -         | -         | -     | -     |
| Caseinase        | 1   | 1   | 1    | 1    | 1     | 1     | 1     | 1     | 1    | 1    | 1    | 1         | 1         | 1     | 1     |
| Gelatinase       | -   | -   | -    | -    | -     | -     | -     | -     | -    | -    | -    | -         | -         | -     | -     |
| Amylase          | 1   | 1   | 1    | 1    | 1     | 1     | 1     | 1     | 1    | 1    | 1    | 1         | 1         | 1     | 1     |
| Phosphatase (Acid) | -  | -   | -    | -    | -     | -     | -     | -     | -    | -    | -    | -         | -         | -     | -     |
| Phosphatase (Neutral) | 1 | 1   | 1    | 1    | 1     | 1     | 1     | 1     | 1    | 1    | 1    | 1         | 1         | 1     | 1     |
| Phosphatase (Alkaline) | 1 | 1   | 1    | 1    | 1     | 1     | 1     | 1     | 1    | 1    | 1    | 1         | 1         | 1     | 1     |
| Tween20          | w + | w + | w +  | w +  | w +   | w +   | w +   | w +   | w +  | w +  | w +  | w +      | w +      | w +   | w +   |
| Tween80          | -   | -   | -    | -    | -     | -     | -     | -     | -    | -    | -    | -         | -         | -     | -     |

Indolacetic Acid: 8.0 | nd  | 86.4 | 34.4 | 28.4 | 22.9 | 16.7 | 15.0 | nd  | 2.9  | 27.2 | 2.7 | 11.3 | 47.0 | 27.0 |

(mg/ml). In the case of phosphatases, positive activity was considered when the absorbance reading was 0.2 above that of the controls. nd, not detected.

Discussion

The number and diversity of Micromonospora strains recovered from alfalfa nodules strongly suggest that this actinobacteria is commonly associated with the symbiotic organ of legumes. Besides other microorganisms, almost all nodules selected had a population of one or more Micromonospora strains. Moreover, for each isolation experiment two sterile, non-crushed nodule was rolled over YMA agar and incubated under the same conditions as the homogenized samples in order to assess the effectiveness of the sterilization proced-
Figure 3 | Biplot representations of the results of PCA and RDA analyses. (a) PCA performed on the matrix with plant growth parameters and shoot nutrients content. RDA performed on the matrix of plant growth parameters and shoot nutrients content constrained by the matrix of bacterial inoculation treatments, either in (b) the cohort of plants singly inoculated with different strains of *Micromonospora* spp. and in (c) the cohort of plants co-inoculated with *Micromonospora* and *E. meliloti* 1021. Open circles represent the centroids of the treatments inoculated singly with each of the fifteen *Micromonospora* strains while full circles represent those of the treatments also inoculated with *E. meliloti* 1021. Star symbols represent the centroids of the control treatments without any microbial inoculation (open star) and inoculated with *E. meliloti* 1021 only (full star). Arrows represent variables measured on individual alfalfa plants: shoot dry weight (*Sdw*), root dry weight (*Rdw*), shoot to root ratio (*S:R*), number of nodules (*Nod*); and shoot contents of carbon (*C*), nitrogen (*N*), phosphorus (*P*), and potassium (*K*). The variable *Nod* is passively projected into the PCA diagram (a) but it was not included in the calculation (dotted arrow). Values on the axes indicate percentages of total variation explained by each axis and *P*-values of significance for the RDA canonical axes (b and c) obtained by Monte Carlo permutation tests (999 permutations).
dry matter and nutrient content than the non-nodulated ones. Multivariate statistics showed that plant growth and nutrition, both in nodulated and non-nodulated with alfalfa, we evaluated in a mesocosm experiment their effects on nature. To discern ecological roles of nodules13,15.

Lupinus angustifolius strains in legume root nodules. Our results are coherent monospora Micro-

noted that none of them were clones of one another, supporting the that they represented different bacterial genotypes. It should also be 66 isolates, when analysed by BOX–PCR fingerprinting, indicating that they were not related to any of the already known species66. Our results (BOX–PCR fingerprinting and 16S rRNA gene sequences) also suggest that the diversity of Micromonospora suggests that its presence is not fortuitous, but

locations sampled (Figure 1). BOX-PCR groups there are strains from more than one of the five

sequences) also suggest that the diversity of Micromonospora spp. that might have an important ecological role in nature. To discern ecological roles of Micromonospora in interaction with alfalfa, we evaluated in a mesocosm experiment their effects on plant growth and nutrition, both in nodulated and non-nodulated alfalfa plants. Multivariate statistics showed that E. meliloti 1021-nodulated plants tended, of course, to have higher values of aerial dry matter and nutrient content than the non-nodulated ones (Figure 3a). But we also found a significant effect of the inoculation with Micromonospora as well as a significant interaction between both E. meliloti 1021 and Micromonospora inoculations, indicating different behaviour of the Micromonospora strains according to the nodulation status of the plant (Table S4). In non-nodulated plants, twelve out of the fifteen Micromonospora strains produced significant multivariate differences with respect to the uninoculated control (Table S5; Figure 3b), while in nodulated plants only the treatments co-inoculated with the strains ALFb5 and ALFr18c differed significantly from the Micromonospora-free control treatment (Table S5; Figure 3c). Several actinobacteria, including strains of Micromonospora sp., had been shown to promote both shoot and root growth and nodulation in alfalfa as well as in the actinorhizal plant species Ochetophila (Discaria) trinervis and nodulation in alfalfa as well as in the actinorhizal plant species Ensifer meliloti1021. The BOX grouping provided a useful background for determining the high diversity and ubiquity of this actinobacteria inside legume root nodules. Our results are coherent with data from Lupinus angustifolius and Pisum sativum root nodules13,15.

The BOX grouping provided a useful background for determining the taxonomic relationship of the strains isolated since these groups served to select strains for 16S rRNA gene sequencing. Even though several strains have more than 99% similarity with described species, others had 16S rRNA gene sequence similarities below 99%, indicating that they were not related to any of the already known species of Micromonospora and probably represent new ones. This case has been observed previously by Trujillo and co-workers, who described two new Micromonospora species: M. lupini and M. saelicesensis, whose 16S rRNA genes were highly similar to already described species14. Our results (BOX–PCR fingerprinting and 16S rRNA gene sequences) also suggest that the diversity of Micromonospora was independent of the location where they were isolated since in several BOX-PCR groups there are strains from more than one of the five locations sampled (Figure 1).

The high diversity and ubiquity of this actinobacteria inside legume root nodules suggest that its presence is not fortuitous, but that Micromonospora might have an important ecological role in nature. To discern ecological roles of Micromonospora in interaction with alfalfa, we evaluated in a mesocosm experiment their effects on plant growth and nutrition, both in nodulated and non-nodulated alfalfa plants. Multivariate statistics showed that E. meliloti 1021-nodulated plants tended, of course, to have higher values of aerial dry matter and nutrient content than the non-nodulated ones.
Although there are few published studies on the impact of PPB on nutrient uptake systems, concomitant improvement of mineral nutrition (including N, P and K) and increase of root surface area has been described in several plant species\(^\text{35}\). With regards to N nutrition, it has been hypothesized that PPB could directly stimulate nitrite transport systems in plants\(^\text{40}\), but recent genetic studies on Arabidopsis thaliana indicate that while there are two NO\(_3^–\) transporter genes (NRT2.5 and NRT2.6) that are strongly upregulated in response to inoculation with the PPB Phyllobacterium brassicae-carum strain STM196, plant growth promotion is not linked to changes in NO\(_3^–\) uptake rate or NO\(_3^–\) distribution between roots and shoots\(^\text{41}\). However, most actinobacteria are saprophytes able to produce a wide range of extracellular hydrolytic enzymes\(^\text{2,42–44}\). All the strains we studied synthesize hydrolytic enzymes able to cleave complex nitrogen-containing polymeric substrates, such as caseinase and gelatinase (Table 2), strongly suggesting that Micromonospora can favour plant nutrition by enhancing nitrogen mineralization in soils. Nonetheless, further research is needed to fully explain the rationale for improved nitrogen nutrition in plants inoculated with Micromonospora. Moreover, all the fifteen Micromonospora showed neutral and alkaline phosphatase activities (Table 2), which can enhance the mineralization of organic phosphate in neutral or alkaline soils\(^\text{40}\) like the one used in our greenhouse experiment (pH 7.47; Table S1), thus making soil P more available to plants as suggested by higher shoot P content in some Micromonospora-inoculated treatments than in the controls (Table 3; Figure 3b, c).

In the cohort of plants nodulated by E. melliloti 1021 only two strains of Micromonospora (ALFb5 and ALFpr18c) produced statistically significant multivariate differences with respect to the Micromonospora-free control group (Table S5; Figure 3c). The success of the interaction between a PPB strain and the plant relies on a set of adaptation mechanisms by both partners, among which the phytochemical profile of the root exudates plays a fundamental role in the bacterial colonization of the root as well as in the regulation of PPB plant beneficial properties\(^\text{45}–\text{47}\). The composition of root exudates has been shown to differ in legumes depending on their nodulation status\(^\text{48–50}\), so that the biochemical environment in the rhizosphere of E. melliloti 1021-nodulated alfalfa plants might be less advantageous for Micromonospora compared with that of non-nodulated plants. Considering the soil pH, legumes are known to acidify the rhizosphere because of the release of protons following excess uptake of cations over anions during N\(_2\) fixation\(^\text{51–53}\). Only six out of the 15 Micromonospora strains tested in this study grew vigorously in vitro at pH 6.5 (Table 2) and none at lower pH values (4.5 or 5.5). Indeed, strains ALFb5 and ALFpr18c are among those able to grow at acidic pH (6.5) while the strain ALFr5, a strain that only excelled in the strains ALFb5 and ALFpr18c are among those able to grow at pH 6.5 (Table 2) and none at lower pH values (4.5 or 5.5). Indeed, rhizobial cellulases have been shown to be crucial for legume nodulation\(^\text{54}\). The ability of Micromonospora strains to produce cellulases could thus explain the increase in the number of nodules observed in co-inoculated plants compared to the control plants only inoculated with E. melliloti 1021. However, IAA production by Micromonospora may not be directly related in our study to an increase in nodulation despite of the literature.

**Conclusion.** In this study 66 Micromonospora strains were isolated, characterized using BOX-PCR and sequencing of 16S rRNA genes and selected some of them for studying their interaction with alfalfa. Our results, together with those from other authors, indicate that Micromonospora are ubiquitous in legume root nodules, presenting a very high genetic diversity. Most of them exhibit in vitro a great ability to degrade organic polymers as well as presenting a direct mechanism for plant growth promotion (IAA production). We have shown that Micromonospora could play an important ecological role in interaction with the host plant by enhancing aerial growth and nutrient contents, being an increase of N uptake by the plant a general phenomenon in the Micromonospora-alfalfa interaction. It remains to be elucidated whether these positive effects also occur in other plant species. Micromonospora engaged in tripartite interactions with E. melliloti 1021 and alfalfa increase nodulation, and some of their strains can also significantly promote the growth and nutrition of N\(_2\)-fixing plants. Contrary to most of plant growth-promoting bacteria, beneficial effects of Micromonospora do not rely on induction of plant root growth. All the above data suggests that, in general, Micromonospora can be considered as excellent PPB, although a correct selection of strain is of capital importance because of the detrimental effect that some Micromonospora may have for plant growth (i.e. strain AL4 in non-nodulated plants; Table 3). Additionally, Micromonospora is a sporulating bacterium so that it can endure in soil and harsh environments. Thus, some of their strains seem to be excellent candidates for the production of bioinoculants, which would make the use of environmentally unfriendly chemical fertilizers less intensive in a broad range of agroecosystems.

**Methods**

**Isolation and ecological characterization of Micromonospora strains.** Isolations of Micromonospora were done from surface sterilized root nodules of naturally occurring alfalfa plants from five different regions of Castilla y León (Spain).

Functional characterization of the isolated strains included: hydrolytic activities toward casein, starch, gelatin, xylan, Tween 80 and 20, cellulose and pectin; presence of acid, neutral and alkaline phosphatase activities; production of indole acetic acid (IAA); and growth under different environmental conditions. For further details on isolation and functional characterization of the isolates, see Materials and Methods in Supplementary Information.

**Genetic and phylogenetic characterization of Micromonospora strains.** BOX-PCR fingerprinting profiles from bacterial genomic DNA were obtained according to Trujillo et al.\(^\text{55}\). Similarity matrices of electrophoretic band profiles were calculated using the Pearson Correlation Coefficient followed by dendrogram construction using the UPGMA algorithm. Strain clusters were defined at the 60% level of Micromonospora (Figure 3c; Figure S2c). Furthermore, all the fifteen Micromonospora strains could be re-isolated from nodules of random plants of each co-inoculated treatment, suggesting that none of the Micromonospora strains here assayed had incompatibility with E. melliloti 1021.

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\(^\text{56}\), or by affecting the interaction between plant and rhizobia\(^\text{57–59}\). Auxins are involved in the initiation and normal development of both determinate\(^\text{60}\) and indeterminate nodules, like Medicago root nodules\(^\text{61}\). IAA production has been associated with the induction of increased nodule numbers in Medicago truncatula plants inoculated with an E. melliloti strain that overproduces IAA\(^\text{62}\) and also with nodule-like structures even in non-leguminous plants\(^\text{63–65}\). Moreover, rhizobial cellulases have been shown to be crucial for legume nodulation\(^\text{66}\). The ability of Micromonospora strains to produce cellulases could thus explain the increase in the number of nodules observed in co-inoculated plants compared to the control plants only inoculated with E. melliloti 1021. However, IAA production by Micromonospora may not be directly related in our study to an increase in nodulation despite of the literature.
**Statistical analysis.** Plant growth and nutrient content data were analysed using multivariate (PCA and RDA) and univariate (ANOVA) analyses with the CANOCO 4.5 (Microcomputer Power, Ithaca, NY) and ssps for Windows v21.0 (IBM Corp., Armonk, NY) programs. The inoculation treatments were coded as dummy variables and used as independent variable in the multivariate analyses. Significance in RDA analyses was tested using Monte Carlo permutation tests (999 unrestricted permutations) for the first canonical axis as well as for the sum of all canonical axes. In univariate comparisons, post-hoc Dunnett’s one-tailed t-tests were used to identify inoculation treatments with means significantly different from the control at \( P \leq 0.1, P > 0.05 \) or \( P > 0.01 \). For further details on the statistical analyses, see Materials and Methods in Supplementary Information for full details.

**Conclusion.** The NRT2.5 and NRT2.6 genes are involved in root development and nitrate uptake.
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Aknokledgements
This work was supported by Junta de Castilla y Leon Grant SA306A11-2 and MICINN Grant AGL2010-17380. P.M.-H. was supported by a fellowship from CSIC JAE-PRE. We thank N. Skinner for revising the English version of the manuscript.

Author contributions
Conceived and designed the experiments: P.M.-H., E.M.-M., J.M.I. Performed the experiments: P.M.-H. Analyzed the data: P.G.-V. Analyzed the BOX profiles and rrs genes: M.E.T. Wrote the paper: P.M.-H., E.M.-M., J.M.I.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Martínez-Hidalgo, P., Galindo-Villardón, P., Igual, J.M. & Martínez-Molina, E. Morosporova from nitrogen fixing nodules of alfalfa (Medicago sativa L.). A new promising Plant Probiotic Bacteria. Sci. Rep. 4, 6389; DOI:10.1038/srep06389 (2014).}

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CORRIGENDUM: *Micromonospora* from nitrogen fixing nodules of alfalfa (*Medicago sativa* L.). A new promising Plant Probiotic Bacteria

Pilar Martínez-Hidalgo¹,⁴, Purificación Galindo-Villarón², Martha E. Trujillo¹,⁴, José M. Igual³,⁴ & Eustoquio Martínez-Molina¹,⁴

¹Department of Microbiology and Genetics. University of Salamanca. Plaza Doctores de la Reina s/n. 37007 Salamanca, Spain,
²Department of Statistics. University of Salamanca. Plaza Doctores de la Reina s/n. 37007 Salamanca, Spain, 
³Instituto de Recursos Naturales y Agrobiología de Salamanca (CSIC), Cordel de Merinas 40–52, 37008 Salamanca, Spain, 
⁴Unidad Asociada USAL-CSIC “Interacción Planta-Microorganismo”.

Martha E. Trujillo was omitted from the author list in the original version of this Article. This has been corrected in the PDF and HTML versions of the Article.

The Author Contributions section now reads:

Conceived and designed the experiments: P.M.-H., E.M.-M., J.M.I. Performed the experiments: P.M.-H. Analyzed the data: P.G.-V. Analyzed the BOX profiles and *rrs* genes: M.E.T. Wrote the paper: P.M.-H., E.M.-M., J.M.I.