RESEARCH LETTER

The communities of tomato (*Solanum lycopersicum* L.) leaf endophytic bacteria, analyzed by 16S-ribosomal RNA gene pyrosequencing

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Received 10 October 2013; revised 5 January 2014; accepted 5 January 2014. Final version published online 3 February 2014.

DOI: 10.1111/1574-6968.12377

Editor: Paolina Garbeva

Keywords
plant endophytic bacteria; culture-independent bacterial analysis; bacterial diversity.

Abstract

Endophytic bacterial communities of tomato leaves were analyzed by 16S-rRNA gene pyrosequencing and compared to rhizosphere communities. Leaf endophytes mainly comprised five phyla, among which *Proteobacteria* was the most represented (90%), followed by *Actinobacteria* (1,5%), *Planctomycetes* (1,4%), *Verrucomicrobia* (1,1%), and *Acidobacteria* (0,5%). *Gammaproteobacteria* was the most abundant class of *Proteobacteria* (84%), while *Alphaproteobacteria* and *Betaproteobacteria* represented 12% and 4% of this phylum, respectively. Rarefaction curves for endophytic bacteria saturated at 80 OTUs, indicating a lower diversity as compared to rhizosphere samples (> 1700 OTUs). Hierarchical clustering also revealed that leaf endophytic communities strongly differed from rhizospheric ones. Some OTUs assigned to *Bacillus*, *Stenotrophomonas,* and *Acinetobacter*, as well as some unclassified *Enterobacteriaceae* were specific for the endophytic community, probably representing bacteria specialized in colonizing this niche. On the other hand, some OTUs detected in the leaf endophytic community were also present in the rhizosphere, probably representing soil bacteria that endophytically colonize leaves. As a whole, this study describes the composition of the endophytic bacterial communities of tomato leaves, identifying a variety of genera that could exert multiple effects on growth and health of tomato plants.

Introduction

Endophytic bacteria exist in a variety of plant tissues of numerous plant species without causing disease symptoms, in some cases exerting beneficial effects on their hosts (Lodewyckx *et al.*, 2002). However, quiescent endophytic bacteria can become pathogenic under certain conditions and within different host genotypes. As a consequence, it was proposed that all bacteria that colonize the interior of plants, including active and latent pathogens, can be considered as endophytes (James & Olivares, 1998). Beneficial bacterial endophytes have raised interest over the years due to their potential impact on crop production (Sturz & Nowak, 2000; Sturz *et al.*, 2000; Lodewyckx *et al.*, 2002; Rosenblueth & Martinez-Romero, 2006; Bulgarelli *et al.*, 2013). Even though endophytic bacteria capable of colonizing aerial plant organs are known (Hardoim *et al.*, 2008; Compant *et al.*, 2010; Reinhold-Hurek & Hurek, 2011), the bulk of research in this field has focused on root endophytic bacteria. Thus, the diversity of leaf endophytic bacteria and their beneficial effects on plant hosts is far from being well known. In the recent years, culture-independent methods for DNA analysis contributed to gain insight into the composition of endophytic communities in leaves of different rice (*Oryza sativa*) varieties (Ferrando *et al.*, 2012) and several plant species naturally grown in the Tallgrass Prairie Preserve in Osage County (Oklahoma; Ding *et al.*, 2013), as well as in different organs of Styrian oil pumpkin (*Cucurbita pepo* L. ssp. *pepo* var. styriaca Greb.; Fürnkranz *et al.*, 2012) and the model plant *Arabidopsis thaliana* (Bodenhausen *et al.*, 2013).

Cultivated tomato (*Solanum lycopersicum* L.) is widely grown and constitutes a major agricultural industry

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worldwide (http://faostat.fao.org). This species is well studied in terms of genetics, genomics, and breeding, thus being an excellent model for basic and applied research related to fruit quality, stress tolerance, and other physiologic traits (Gupta *et al.*, 2008; Panthee & Chen, 2010; Sahu *et al.*, 2012). Diseases are one of the main problems of the tomato industry all over the world, and the susceptibility of tomato to many pathogenic microorganisms leads to an intense use of agrochemicals (Gajanana *et al.*, 2006). Thus, biological control agents have emerged as an alternative approach for the control of tomato diseases such as bacterial wilt caused by *Ralstonia solanacearum* (Chen *et al.*, 2013) and *Fusarium* wilts caused by *Fusarium oxysporum* (Aimé *et al.*, 2008). In this way, an increased knowledge of the ecology of microbial communities associated to tomato plants will contribute to identify potential candidates for biologic control of tomato diseases and plant growth promotion. Moreover, the analysis of bacterial communities associated to tomato plants is interesting not only due to the importance of tomato as a cultivated plant, but also to the potential contribution to unravel the mechanisms that regulate the colonization of cultivated plants by beneficial microorganisms. Bacterial diversity associated to tomato leaves has been studied only in a few works (Correa *et al.*, 2007; Enya *et al.*, 2007a,b), which either focused only on epiphytic communities (Correa *et al.*, 2007) or analyzed phyllospheric communities without discriminating between epiphytic and endophytic bacteria (Enya *et al.*, 2007a,b). More recently, studies focused on the detection of human bacterial pathogens on tomato plants and provided an overview of the epiphytic microbial communities associated to different organs of this species (Telias *et al.*, 2011; Ottesen *et al.*, 2013). However, a comprehensive analysis of the bacterial diversity of tomato endophytes and the ability of soil bacteria to endophytically colonize the aerial parts of tomato plants has not been performed. The goal of this work was to analyze the communities of endophytic bacteria in leaves of tomato plants grown in soils from productive greenhouses, as well as to compare them with the community of rhizosphere bacteria, in order to identify components of the rhizosphere bacterial population with the potential to endophytically colonize leaves.

Materials and methods

Plant growth conditions

Soil was collected from the rhizosphere of tomato plants grown in greenhouses devoted to commercial production in an organic farm ('La Anunciación', GPS: WO 58°08′ 00.9″ S 34°56′43.7″) close to La Plata city (Argentina). A mixture of bulk and rhizosphere soil was obtained from sixty randomly selected plants in vegetative stage, which were spread in a total surface of around one hectare. Tomato plants (cultivar 'Platense') were cultivated in this soil for further sampling of metagenomic DNA. Seeds were placed in 3-liter pots (3 seeds per pot) that were irrigated with tap water and maintained in a greenhouse under natural light conditions during springtime (2011). Thirty days after seeding, plants were harvested and leaves were separated from the rest of the plant to obtain DNA samples from endophytic microorganisms. Soil attached to the roots of plants in vegetative stage with two fully developed leaves was gently removed and used as the source of rhizosphere DNA.

DNA extraction

Isolation of metagenomic DNA was performed on three replicate samples, each consisting of 16–18 plants. First, leaves were surface disinfected in 5% commercial bleach and 0.01% Tween 20 for 10 min and rinsed (×3) with sterile distilled water. No bacterial growth was detected after plating aliquots of the water used for the final wash on tryptic soy agar (tryptone, 17.0 g L^{-1}; soytone, 3.0 g L^{-1}; NaCl, 5.0 g L^{-1}; K_2HPO_4, 2.5 g L^{-1}; glucose, 2.5 g L^{-1}; agar, 20.0 g L^{-1}). Moreover, no amplification of 16S-rRNA gene was detected when the water used for the final wash was used as a source of DNA (data not shown). Thus, these results confirmed that the disinfection procedure was effective in eliminating both cultivable and noncultivable epiphytic bacteria, as well as potential DNA traces from the leaf surface. Subsequently, endophytic bacterial DNA was isolated from leaves as follows. Leaves were homogenized in 0.95% (w/v) NaCl with an Omnimixer 17106 (OCI Instruments), and the extract obtained was filtered (×4) through filter paper to separate bacterial cells from plant debris. The filtrate was centrifuged (10 min; 15 000 ***g***), and the pellet was used as the source for the extraction of genomic DNA from endophytic bacteria, which was performed as described by Estrella *et al.* (2009). For rhizosphere DNA isolation, 10 g of soil adhered to the roots of the above-mentioned plants was processed with the commercial kit PowerMax™ Soil (MO BIO Laboratories Inc).

Amplification and pyrosequencing

PCR optimization and pyrosequencing were performed by the AmpliconSeq Service of the Instituto de Agrobiotecnología de Rosario (Argentina). Briefly, DNA aliquots (10 and 6 ng for rhizosphere and endophytic samples, respectively) were PCR-amplified with 5-min denaturation at 95 °C, 30 cycles of −30 s at 95 °C, 45 s at 65 °C

and 60 s at 72 °C, with a final extension at 72 °C for 5 min. The bacterial 16S-rRNA gene hypervariable region V4 was amplified using RDP-TAG primers (ribosomal data project); the PCR product obtained was re-amplified with 454Adaptor-MID-TAG primers, and amplicons were purified and quantified fluorometrically with the Picogreen® kit (Invitrogen™). Emulsion PCR was performed with the GS Titanium emPCR Reagents (Lib-A) kit (Roche) after diluting template DNA to one molecule per bead.

Sequence analysis

The data set was analyzed using the QUANTITATIVE INSIGHT INTO MICROBIAL ECOLOGY (QIIME) open-source software package (Caporaso *et al.*, 2010). Several quality controls were performed during sequence processing. Sequences with a Phred score < 25 were removed, thus ensuring that the lowest quality sequences had only *c.* 0.3% probability of an incorrectly called base. Sequences < 200 bp were also excluded from the data set. Within the remaining set of sequences, the barcode and the forward primer were identified with a tolerance up to 2 and 3 incorrectly called bases, respectively. Finally, sequences with ambiguous bases and homopolymers (> 6 bases) were not considered for further analysis. After this procedure, a total of 19 403 and 16 562 sequences were obtained from rhizosphere soil and leaf samples, respectively. Sequences thus obtained were clustered based on their similarity using UClust, and each of these clusters was designated as an OTU. This process yielded a total of 3412 OTUs.

High-quality sequences thus obtained were clustered using UClust (Edgar, 2010). Representative sequences were aligned with PyNast (http://qiime.org/pynast/), and phylogenetic trees were constructed with FastTree (Price *et al.*, 2009). Bacterial taxonomy was assigned using the Ribosomal Database Project Classifier (Cole *et al.*, 2009), after filtering out chloroplastic and mitochondrial sequences derived from the contamination with plant material. Rarefaction curves based on the estimated species number (97% sequence identity threshold) were generated for operational taxonomic unit (OTU) tables that were unified to 4600 (first most indigent sample) sequences per sample for endophytic samples and 6000 sequences per sample for rhizosphere ones. Hierarchical clustering was performed on a list of 82 OTUs comprised by 68 OTUs represented at least 10 times in each sample of rhizosphere DNA and 14 OTUs represented at least once in each sample of leaf DNA. Hierarchical clustering of the percent abundance of the selected OTUs in each sample was implemented using CLUSTER 3.0 (Eisen *et al.*, 1998) and visualized in TREEVIEW (Schloss *et al.*, 2009) with the Spearman rank correlation coefficient as the similarity metric and a complete linkage clustering criterion.

Results and discussion

Leaf endophytic bacteria were represented by five phyla that comprised 99% of the community, while the remaining 1% involved six very low-abundant phyla. In this regard, *Proteobacteria* were the main component (90%) of the endophytic community (Fig. 1a). Members of this phylum have been reported to be abundant in endophytic communities of Arabidopsis and citrus leaves (Sagaram *et al.*, 2009; Bodenhausen *et al.*, 2013) and *Thlaspi goesingense* shoots (Idris *et al.*, 2004). In this way, *Proteobacteria* seem to be highly abundant in the leaf endophytic communities analyzed so far. Ottesen *et al.* (2013) reported the presence of several taxa of *Proteobacteria* as the main components of the epiphytic microbial communities of tomato leaves. Thus, our findings demonstrate that highly abundant components of epiphytic microbial communities of tomato leaves are also present in the leaf endophytic community. *Gammaproteobacteria* was the most abundant class (84%) of *Proteobacteria* in the endophytic community of tomato leaves hereby analyzed, while *Alphaproteobacteria* and *Betaproteobacteria* only represented 12% and 3% of this phylum, respectively (Fig. 1c). In addition to the highly abundant *Proteobacteria*, the endophytic community also included a small proportion of *Actinobacteria* (1.5%), *Planctomycetes* (1.4%), *Verrucomicrobia* (1.1%), and *Acidobacteria* (0.5%; Fig. 1a). These minor phyla were also reported as minor components of the endophytic communities of citrus leaves (Sagaram *et al.*, 2009) and *Thlaspi goesingense* shoots (Idris *et al.*, 2004). Therefore, the community of endophytic bacteria of tomato leaves shares several similarities, in terms of phyla composition, with endophytic communities of aerial organs of other plant species, thus suggesting that members of these phyla are adapted to the particular conditions required for the colonization of this habitat.

Rarefaction curves demonstrated that endophytic bacterial communities were less diverse than rhizosphere ones, as evidenced by differences in OTU abundance between both communities (Fig. 2). In this way, leaf samples saturated at about 80 OTUs (Fig. 2a), while rhizosphere samples continued to increase over 1700 OTUs (Fig. 2b). The low number of OTUs found within leaves showed that the diversity of bacteria that endophytically colonize this organ is low. Unknown bacteria represented 1.94% and 5.43% of the leaf endophytic and rhizosphere communities, respectively, and were excluded from the phylogenetic analysis.

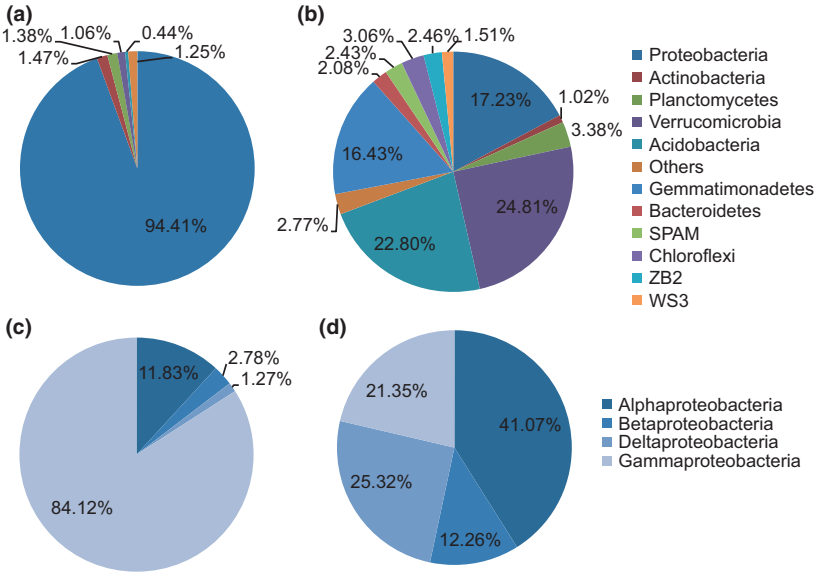

Fig. 1. Bacterial classification using RDP Classifier at 97% identity as implemented in QIIME, shown at the phylum (a and b) and class level within *Proteobacteria* (c and d) of leaf endophytic (a and c) and rhizosphere (b and d) samples. Values presented are the mean of three independent samples.

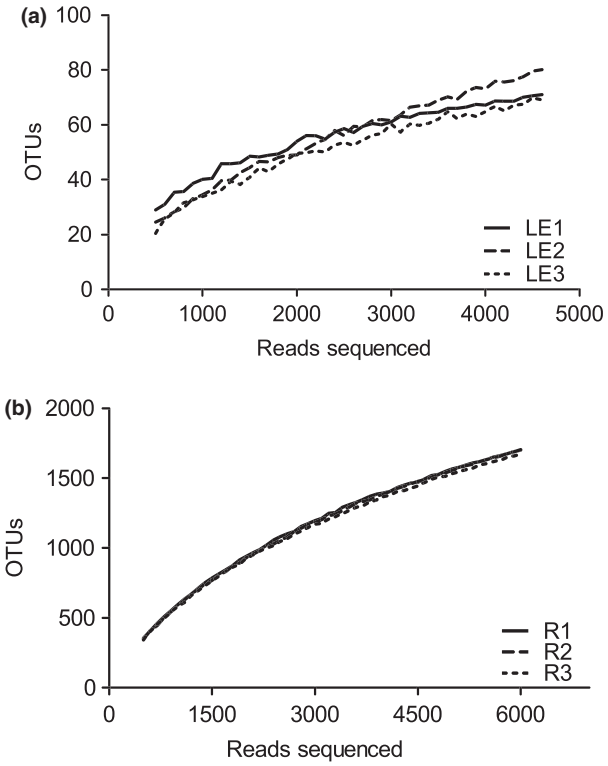

Fig. 2. Rarefaction curves for bacterial OTUs, clustering at 97% rRNA sequence similarity. Curves represent sequences for three samples of endophytic (a) or rhizosphere (b) communities from tomato plants.

The bulk of the rhizosphere community (98.5%) analyzed in the present work comprised eleven phyla, each representing at least 1% of the community, and the remaining 1.5% comprised 18 low-abundant phyla. Mayor taxonomic groups in rhizosphere soil were *Verrumicrobia* and *Acidobacteria*, which represented 24% and 23% of the community, respectively (Fig. 1b). *Proteobacteria* was the third most represented phylum in this community (17%). Although their relative abundance is variable, these phyla are usually found in soil (Shange *et al.*, 2012) and the rhizosphere of many plants (Kent & Triplett, 2002; Gottel *et al.*, 2011).

The relative abundance of major phyla strongly differed between the leaf endophytic and rhizosphere communities hereby analyzed (Fig. 1a-b). The higher abundance of *Proteobacteria* in the leaf endophytic compartment, as compared to the rhizosphere, suggests that rhizosphere members of this phylum are particularly well adapted to colonize inner plant tissues and establish as leaf endophytes. This view is supported by the fact that many *Proteobacteria* are known to establish different kinds of interactions (mutualistic, parasitic, or neutral) with plants (Bulgarelli *et al.*, 2013). However, the possibility that leaf endophytic *Proteobacteria* derived from leaf epiphytic communities cannot be discarded, but it can be concluded that a variety of *Proteobacteria* are able to endophytically colonize tomato leaves. In this regard, plants analyzed in the present work were cultured in an isolated greenhouse, with no neighboring horticultural crops and no direct exposure to the external environment. So, it seems unlikely that leaf epiphytic communities represent a significant source of endophytic bacteria in this particular study. Conclusive evidence about the origin of the endophytic bacteria hereby detected would require further work, but this issue was addressed in the hierarchical clustering analysis described in further paragraphs.

Significant differences between rhizosphere and leaf endophytic communities were also found regarding the composition of the phylum *Proteobacteria*. *Gammaproteobacteria* was the most abundant class (84%) of *Proteobacteria* in the leaf endophytic community, while *Alphaproteobacteria* and *Betaproteobacteria* only represented 12% and 3% of this phylum, respectively (Fig. 1c). On the contrary, *Alphaproteobacteria* was the predominant class (40%) of *Proteobacteria* in rhizosphere samples, followed by *Deltaproteobacteria* (25%), *Gammaproteobacteria* (20%), and *Betaproteobacteria* (15%; Fig. 1d). A high abundance of *Gammaproteobacteria* in endophytic communities has been previously reported for *Populus deltoides* (Gottel *et al.*, 2011), *Lolium perenne,* and *Trifolium repens* roots (Marilley & Aragno, 1999). Thus, the present phylogenetic analysis suggests that leaf endophytic communities of tomato plants share similarities with root endophytic communities described for other plant species, both of which are in turn different from rhizosphere communities. In this way, it is tempting to speculate that members of the root endophytic bacterial phyla are able to further colonize aerial plant organs as endophytes.

The phylum *Proteobacteria* comprises several species that promote plant growth and also act as biologic control agents of different diseases (Bulgarelli *et al.*, 2013). A study of cultivable bacteria associated to tomato leaves revealed the presence of *Proteobacteria* both in greenhouse and field-grown plants (Enya *et al.*, 2007a) and also identified *Bacillus* (phylum *Firmicutes*) and *Pantoea* (phylum *Proteobacteria*) strains with strong antifungal activity against tomato pathogens such as *Botrytis cinerea*, *Fulvia fulva,* and *Alternaria solani*. However, the phylum *Proteobacteria* also contains several species that are pathogenic on plants (Mansfield *et al.*, 2012). On this basis, the high representation of *Proteobacteria* in the community of leaf endophytic bacteria of tomato plants hereby detected could have significant implications on plant growth and health. *Gammaproteobacteria*, the most represented class of *Proteobacteria* in the endophytic community, was found to contain OTUs assigned to the genus *Acinetobacter*, *Pseudomonas,* and *Stenotrophomonas*; several other OTUs assigned to *Enterobacteriaceae* and *Xanthomonadaceae* could not be classified at the genus level.

Hierarchical clustering was performed on a subset of OTUs selected from endophytic and rhizosphere communities and segregated them in two clearly different clusters (Fig 3). A similar analysis performed on the whole set of OTUs also segregated leaf endophytic and rhizosphere samples into two different clusters (Supporting Information, Fig. S1). Interestingly, nine of the OTUs represented in all the leaf endophytic samples were absent from rhizosphere soil. One of them (2535) was assigned to the

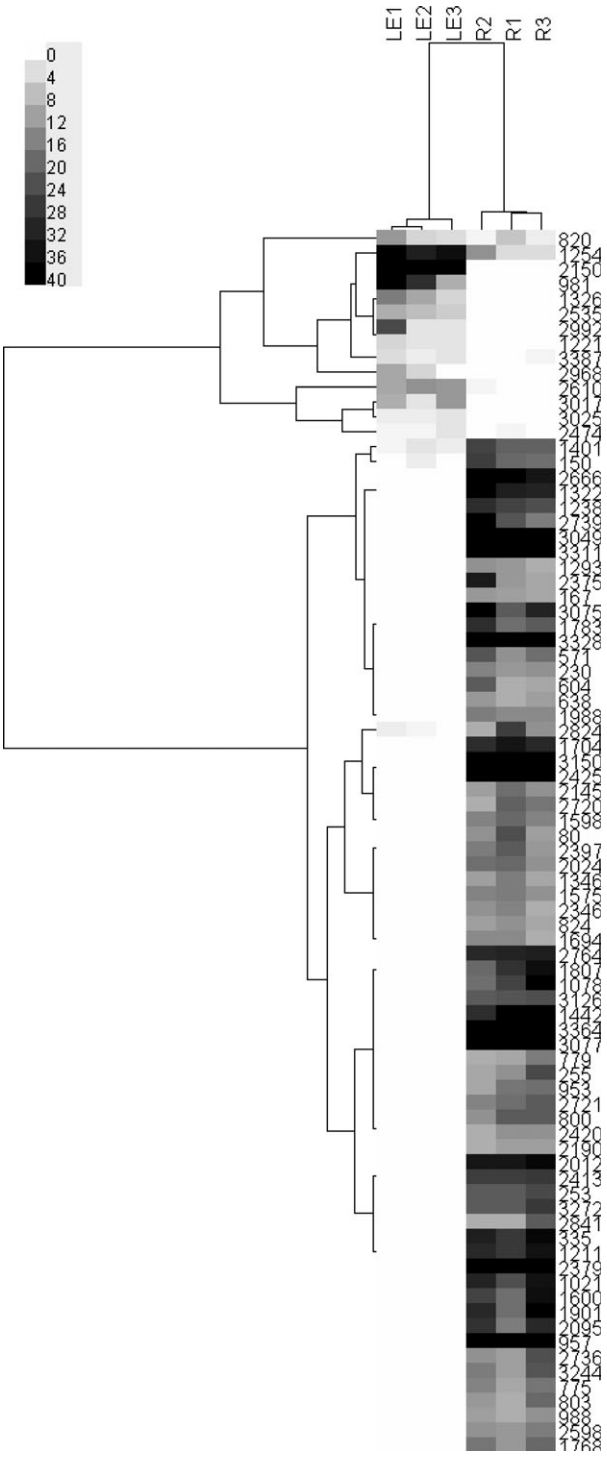

Fig. 3. Heat map and hierarchical cluster analysis of a subset of OTUs represented at least one and ten times in the three leaf endophytic (LE) and rhizosphere (R) samples, respectively. Cluster analysis completely separated OTUs according to sample provenance (leaf endophytic or rhizosphere). A similar analysis based on the complete data set is presented in Fig. S1.

genus *Bacillus* (phylum *Firmicutes*), while the remaining eight OTUs corresponded to the class *Gammaproteobacteria* (phylum *Proteobacteria*). Among these *Gammaproteobacteria*, three OTUs (2150, 3025, and 981) were assigned to the genus *Acinetobacter* (family *Moraxellaceae*), while two of them (2992 and 1326) were assigned to the genus *Stenotrophomonas* (family *Xanthomonadaceae*). Some of the remaining OTUs corresponded to unclassified members of the *Enterobacteriaceae* (1221, 450, and 2968). On one hand, it is interesting that some *Acinetobacter* species are known to promote plant growth (Peix *et al.*, 2009; Gulati *et al.*, 2010), but their use as biofertilizers is not recommended because some strains can cause severe human infections (Cerqueira & Peleg, 2011). *Stenotrophomonas* has previously been reported as a plant endophyte (Taghavi *et al.*, 2009), but some species such as *S. maltophilia* represent an increasing medical issue of multidrug resistance (Betriu *et al.*, 2001). Thus, even though the OTUs hereby analyzed could not be assigned at the species level, it was curious to find that the endophytic community of tomato leaves can harbor bacterial genera that contain human pathogens, an issue that should be kept in mind during further efforts devoted to the characterization of plant-associated microorganisms and their potential application in bioformulations. A possible explanation for the detection of OTUs unique to the endophytic community is that part of the leaf endophytic bacteria do not derive from soil and probably reach the interior of aerial plant organs by an alternative pathway to roots and the vasculature. Noteworthy, *Stenotrophomonas* and *Acinetobacter* were previously reported to be part of the epiphytic community of tomato leaves (Enya *et al.*, 2007a). Thus, it is possible that some phyllospheric bacteria of genera such as *Stenotrophomonas* and *Acinetobacter* are able to further invade inner tissues and thus become endophytes. Alternatively, the titer of some rhizosphere bacteria could be reduced to undetectable levels once they establish as endophytes.

A high proportion of OTUs were highly represented in rhizosphere but not in leaf endophytic samples. Exceptions to this trend exhibited by rhizosphere OTUs were OTUs 1401, 150, and 2824, which were also present in leaves, although in a much lesser extent. Thus, these OTUs probably represent soil bacteria able to endophytically colonize tomato leaves. OTUs 1401 and 2824 corresponded to the genera *Bradyrhizobium* (family *Bradyrhizobiaceae*) and *Microvirga* (family *Methylobacteriaceae*), both of them belonging to the order *Rhizobiales*, class *Alphaproteobacteria*, phylum *Proteobacteria*. The genus *Bradyrhizobium* comprises rhizobial species that develop symbiotic root nodules in legumes such as soybean (*Glycine max*) and peanut (*Arachis hypogaea*), while some *Microvirga* species establish a similar symbiotic process in the legumes *Listia angolensis* and *Lupinus texensis* (Andam & Parker, 2007; Ardley *et al.*, 2012). Another OTU (2474) assigned to the *Bradyrhizobiaceae* was detected in leaf endophytic samples and in low levels in the rhizosphere (Fig. 3). An additional member of the order *Rhizobiales* (OTU 820), assigned to the family *Rhizobiaceae*, was also detected both in leaf endophytic and rhizosphere samples (Fig. 3, Supporting Information, Table S1). Even though rhizobia are well known for their highly specific symbiosis with legumes, these bacteria are also present in aerial organs of nonlegumes, including tomato (Mehboob *et al.*, 2009; Bodenhausen *et al.*, 2013; Ottesen *et al.*, 2013). Moreover, *Rhizobium leguminosarum* strains were recently shown to promote growth of pepper and tomato plants (García-Fraile *et al.*, 2012). However, the strains used in the above-mentioned work were originally isolated from legume hosts. Thus, it would be interesting to evaluate the potential for plant growth promotion of rhizobia naturally adapted to the colonization of tomato plants, such as those identified in the present work.

Acknowledgements

This work was supported by grants of CONICET (PIP 0395), ANPCyT (PICT-2010-1119, PICT-2012-1716), and UNSAM (SJ10/30). M.M and F.L.P are members of CONICET. F.M.R. is a doctoral fellow of CONICET. The authors are very grateful to MsSc Mariana del Pino for allowing soil sampling in *La Anunciación* farm and Patricia Alejandra Uchiya (CIC) for valuable technical assistance. The authors declare that there are no conflicts of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Heat map and hierarchical cluster analysis of the complete OTU data set from leaf endophytic and rhizosphere communities.

Table S1. Taxonomic identity according to RDP Classifier and abundance of each OTU amplified from leaf endophytic and rhizospheric communities of tomato plants.