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Isolation, taxonomic analysis, and phenotypic characterization of bacterial endophytes present in alfalfa (*Medicago sativa*) seeds

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ABSTRACT

A growing body of evidence has reinforced the central role of microbiomes in the life of sound multicellular eukaryotes, thus more properly described as true holobionts. Though soil was considered a main source of plant microbiomes, seeds have been shown to be endophytically colonized by microorganisms thus representing natural carriers of a selected microbial inoculum to the young seedlings. In this work we have investigated the type of culturable endophytic bacteria that are carried within surface-sterilized alfalfa seeds. MALDI-TOF analysis revealed the presence of bacteria that belonged to 40 separate genera, distributed within four taxa (*Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*). Nonsymbiotic members of the *Rhizobiaceae* family were also found. The evaluation of nine different *in-vitro* biochemical activities demonstrated isolates with complex combinations of traits that, upon a Principal-Component-Analysis, could be classified into four phenotypic groups. That isolates from nearly half of the genera identified had been able to colonize alfalfa plants grown under axenic conditions was remarkable. Further analyses should be addressed to investigating the colonization mechanisms of the alfalfa seeds, the evolutionary significance of the alfalfa-seed endophytes, and also how after germination the seed microbiome competes with spermospheric and rhizospheric soil bacteria to colonize newly emerging seedlings.

1. Introduction

Plant microbiomes consist in a vast diversity of microorganisms that inhabit roots, stems, leaves, flowers, fruits, and seeds (Berg et al., 2014; Philippot et al., 2013; Turner, 2013). These complex communities can be present in both external and internal tissues of plant organs, influencing plant fitness with beneficial effects such as increased biomass, metabolite production, and disease resistance (Hardoim et al., 2012.; Vorholt, 2012). Although most of these associations are still uncharacterized, some are thought to be the current expression of long-term evolutionary interactions with the host plants (Johnston-Monje and Raizada, 2011). Endophytes are among those microbes believed to participate in more intimate interactions, as they can access the plant interior without causing disease (Hardoim et al., 2015). Seed endophytes are found associated with the surface-sterilized seeds of different plants (Truyens et al., 2015), indicating that both soil and seeds can be a source of microorganisms for young plants (Johnston-Monje et al., 2014, 2016). During seed dormancy, endophytes can remain viable inside the seeds, protected from the external environment until the seeds germinate and segregate exudates; at which point the endophytes have the opportunity to initiate a colonizaton of both the spermosphere and the emerging young seedling, thus in some instances undertaking positive biotic interactions (Nelson, 2004). Current evidence suggests that different seed-borne endophytes are able to stimulate plant growth by means of auxin, 1-aminocyclopropane-1-carboxylate deaminase, or acetoin production; nitrogen fixation; or phosphorus solubilization among other mechanisms (Xu et al., 2014). In addition, a number of diverse seed endophytes have proved to inhibit different phytopathogenic fungi by producing antifungal compounds, toxins, or hydrolytic enzymes (Cottyn et al., 2001; Gagne-Bourgue et al., 2013).

At the present time, information on endophyte-access mechanisms and community assembly in seeds still remains scarce (Klaedtke et al., 2016), although in some species the seed coat has been indicated as the location where seed endophytes reside (Robinson et al., 2016). The external environment has traditionally been considered to be the

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natural source of seed endophytes, with novel data demonstrating the efficient colonization of seeds by inoculated bacteria via the flowers of the previous generation (Mitter et al., 2017). Moreover, evidence has also been garnered that plant-associated bacteria should be considered as a source of the resulting seed endophytes (Compant et al., 2011; Hardoim et al., 2012) through a mechanism compatible with the notion of a microbiome transmission across plant generations. Thus, the propagation of plant endophytes in seeds should be considered as a delicate adaptive mechanism to preserve over time the partnership characterized by positive plant-microbe interactions (Johnston-Monje and Raizada, 2011). Although seed endophytes most likely are inherited and provide the plant with an initial inoculum, conceivably beneficial to the plant fitness; those bacteria still represent the less explored portion of the plant microbiome (Truyens et al., 2015). Since endophyte isolation from surface-sterilized seeds has frequently resulted in low bacterial numbers per seed—although with a great diversity of genera (Mundt and Hinkle, 1976)—the presence microbes in seeds has been historically relegated to only transient associations of little relevance to the plant. More recently, however, the ecologic relevance of seed endophytes has been recognized and emphasized since the conservation of those bacteria among the seeds of species within the same plant family throughout evolutionary development has been demonstrated (Johnston-Monje and Raizada, 2011). So far, more than 131 bacterial genera from 4 different phyla have been identified in 25 different plant species, those being generally of agricultural significance (for a review *cf*. Truyens et al., 2015).

Leguminous plants—a major group consisting of more than 700 plant genera and having a wide geographic distribution—have been the focus of intensive studies owing to their nitrogen-fixing capability through interactions with rhizobia and their economic relevance (Ohyama, 2017). Seed endophytes of only a few species of the *Leguminosae* have been explored to date (Kremer, 1987; Rosenblueth and Martínez-Romero, 2006; Sobolev et al., 2013). In Argentina, alfalfa (*Medicago sativa*) cultivation occupies nearly 6 million ha, where that crop is mainly used as animal forage. The microbiome in alfalfa plants has been recently explored in leaves, stems, and root nodules from plants grown in different soils in order to investigate correlations between the soil bacterial community and the resulting profile of species in the corresponding endophytes (Pini et al., 2012). No studies have explored the alfalfa seed microbiome, except the early work by Handelsman and Brill (1985) that reported the isolation of *Pantoea agglomerans* (formerly *Erwinia herbicola*) from the roots of plants grown from surface-sterilized seeds of *M. sativa* var. With an aim at characterizing the seed microbiome in alfalfa, in the work reported here we have performed an exhaustive culture-based search for seed endophytes in order to characterize their diversity and principal phenotypic traits.

2. Methods

2.1. Sources and surface sterilization of alfalfa seeds

Seeds of six different varieties of *M. sativa* (alfalfa) were provided by IMYZA-INTA-Castelar, Argentina. The six varieties used in this work were referred to below as: S (Super Monarca) which was harvested in year 2010, and C (GAPP810 +), B (GAPP 909 +), F (F401Y), D (F301Y), and G (F111X) all harvested in year 2013. Seeds were locally produced in Argentina, received by IMYZA-INTA-Castelar and stored at room temperature (ca. 25 °C). We received each seed batch from INTA the same year of harvest and we preserved the seeds at 4 °C until their use in year 2015.

Of the seeds, 1 g per variety (approx. 420 seeds/g) was surface-sterilized with sulfuric acid (98,0%) for 10 min and then rinsed ten times with autoclaved phosphate-buffered saline solution (PBS; 123 mM NaCl, 9.8 mM Na_2HPO_4, 2.4 mM KH_2PO_4, pH 7.4). To verify that the surface sterilization was achieved, 100 µL of the last wash were plated on Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl; pH 7.2), and the plates incubated at 28 °C for 5 days to verify the absence of growth.

2.2. Isolation of culturable bacterial endophytes from seeds

After the sterilization and washing steps, the seeds were incubated in 30 mL of sterile PBS (4 h, room temperature) to enable the entrance of the water into the seeds and the subsequent release of seed exudates. The resulting supernatant was plated on LB media (100 µL per plate) and incubated at 28 °C for five days until the appearance of all visually different colony types from aerobic bacteria. Each of those morphologically differentiable bacterial colonies was repeatedly streaked on plates of LB media to effect a purification until a complete isolation was achieved, at which point the colony was cryopreserved at −80 °C in glycerol stocks (*i. e.*, LB media plus 25% [v/v] glycerol).

2.3. Recovery of culturable bacteria from plant seedlings derived from surface-sterilized seeds and grown under axenic conditions

To determine whether the aerobic bacteria present in seeds could colonize plant seedlings, surface-sterilized seeds were germinated in water agar (15 g/L of aqueous agar) for 48 h at 28 °C in the dark; and, once germinated, the seedlings were transferred to sterilized conical tubes containing vermiculite as a mineral support and sealed with plugs of cotton to preserve the plants from external contamination during the experiment. The individual seedlings were grown in each axenic tube. A sterilization of the plant pots was carried out three times (at 121 °C for 15 min) with a period of 48 h in between. The control of sterilization was effected in autoclaved vermiculite tubes where 100 µL of PBS buffer was mixed with the sterile support and plated in LB, in order to check for the presence or absence of colony growth.

The seedlings were incubated in a plant-growth chamber (Binder™, KBW400) with a 22-°C–16-h–light and 16-°C–8-h–dark photoperiod and were regularly irrigated with sterile Fåhraeus solution (Fåhraeus, 1957) in a laminar-flow hood. After one month, the seedlings were harvested, mashed into a mush in a sterile mortar with 1 mL of sterile PBS, and an aliquant of the overlying liquid was serially diluted 10-fold 6 times and plated in LB media. One of each of the morphologically differentiable isolates was recovered and cryopreserved, as described above.

2.4. Identification of bacterial isolates

The identification of the culturable bacterial endophytes was carried out by whole-cell matrix-assisted laser-desorption-ionization–time-of-flight mass spectrometry (MALDI-TOF MS) determinations, with an Ultraflex III UV-MALDI-TOF/TOF mass spectrometer by means of MALDI Biotyper 3.1 software (Bruker Daltonics, Bremen, Germany; Maier and Kostrzewa, 2006). The samples were prepared from a single colony of each isolate cultured on LB at 28 °C for 24 h according to the manufacturer's recommendation. Each sample was overlaid with 1 µL of a saturated solution of α-cyano-4-hydroxycinnamic acid in an organic solution (50% [v/v] acetonitrile, 2.5% [v/v] trifluoroacetic acid in water). The spectra were recorded by Flex Control 3.3 software (Bruker Daltonics) in a linear positive mode at an accelerated voltage of 19 kV in the range from 2 to 20 KDa. For each spectrum, successive shots were collected to obtain spectra with maximal absolute peak intensities ranging from about 5×10^3 to 10^4 arbitrary units. An external calibration was performed with the Bruker bacterial test standard (Bruker Daltonics). Bacterial identification was performed with MALDI Biotyper Offline classification software through the use of the score values proposed by the manufacturer, with ≥ 2 indicating species identification, between 1.7 and 1.9 genus identification, and < 1.7 no identification (Sogawa et al., 2011). Those spectra corresponding to strains or species absent in the MALDI Biotyper database were incorporated into the software as a new ‘main spectrum’ for future identifications.

When this method did not provide the reliable identification of an

isolate, a partial sequence of the 16S-rDNA gene was obtained by the polymerase-chain reaction (PCR) and a subsequent Sanger sequencing with the primers fD1/rD1 (Weisburg et al., 1991), Y1/Y2 (Young et al., 1991), 27f/1385r (Rheims et al., 2017), or R1n/U2 (Weidner et al., 1996). The partial 16S-rDNA sequences obtained were used to infer the taxonomic position of each isolate at the genus level, by alignment through a comparison with bacterial-16S entries in the Ribosomal Database Project (RDP; Cole et al., 2014) at a 95 % confidence level, and were further searched in BLASTN by means of default parameters.

2.5. Phenotypic characterization of isolates

All the different isolates were tested for 8 *in-vitro* biochemical activities related to plant-growth promotion and biocontrol. All assays were carried out in duplicate. Each isolate was grown on solid LB medium at 28 °C for 3 days and then used as an inoculum for the biochemical tests. For the principal-components analysis (PCA) of the bacterial traits associated with plant-growth promotion, the XLSTAT software package was used.

2.5.1. Mineral-phosphate solubilization

The ability to solubilize mineral phosphate was assayed on plates containing National Botanical Research Institute's phosphate growth medium (NBRIP), according to Nautiyal (1999).

2.5.2. Auxin production

Quantification of auxin production was determined from 4-day-old bacterial cultures grown in LB broth supplemented with 500 μg mL^{-1} of the acid's precursors tryptophan in the dark at 30 °C, by the use of Salkowskís reagent, as reported by Patten and Glick (2002). The amount of the acid produced was expressed as μg mg^{-1} protein (*cf.* Table S2). Total protein content of each isolate was determined by Bradford assay (Bradford, 1976).

2.5.3. Siderophore production

Siderophore production was screened by an agar-plate assay, with chrome-azurol-S agar (Louden et al., 2011). Isolates were considered to be siderophore-producing when an orange halo of diameter greater than 5 mm was formed around the bacterial colony after 5 days of incubation at 28 °C.

2.5.4. Chitinase activity

To test the chitinase activity, bacterial isolates were grown on solid medium with colloidal chitin (Cattelan et al., 1999) prepared by Shimahara and Takiguchi (1988). The colloid-containing plates contained 1.5 g of the chitin, 2.7 g of K_2HPO_4, 0.7 g $MgSO_4{\cdot}7H_2O$, 0.5 g NaCl, 0.5 g KCl, 0.13 g yeast extract, 15 g agar in 1000 mL distilled water. Chitinase production was confirmed by the generation of a clear halo around a colony after 10 days of growth at 28 °C.

2.5.5. Cellulase activity

Cellulase activity was tested by growing bacterial colonies in minimal media supplemented with 2% (w/v) CMC for 5 days at 28 °C, according to Hankin and Anagnostakis (1977). After staining with Congo Red dye for 5 min and subsequent washes with 5 M NaCl plus 0.1% (v/v) acetic acid, the generation of a clear halo around a colony indicated cellulase production.

2.5.6. Protease activity

Protease activity was assayed on solid medium containing 3% milk powder, based on the method of Brown and Foster (1970). The generation of a clear halo around a colony grown after 3 days growth on the milk-agar plates at 28 °C indicated protease activity.

2.5.7. Amylase activity

Amylase activity was detected by iodine staining and clear halo detection around bacterial colonies grown at 28 °C for 72 h in starch-supplemented media containing 10 g soluble starch, 2 g yeast extract, 5 g peptone, 0.5 g $MgSO_4$, 0.15 g $CaCl_2$, 0.5 g NaCl, 15 g agar in 1000 mL distilled water.

2.5.8. Pectinase activity

Pectinase activity was assayed by growing bacterial isolates in citrus-pectine–supplemented agar (Soares et al., 1999). After growth of the bacterial isolates for 5 days at 28 °C and staining with iodine, a clear halo around a colony indicated pectinase production.

2.5.9. Fungal inhibition

Antagonistic activity was tested against the phytopathogenic fungus *Sclerotinia sclerotiorum* by a dual-culture assay on potato-dextrose-agar medium at room temperature by a modified protocol based on Whipps (1987). The procedure stated in brief: Bacterial isolates were seeded in a 2-cm-diameter circle on a potato-dextrose-agar plate (4 g potato extract, 20 g dextrose, 15 g agar in 1000 mL distilled water) and grown for 24 h at 30 °C. A plug of fungus was then placed at the center of the circle and the plates reincubated at 28 °C for 6 days. Fungal-growth inhibition was considered positive when mycelial growth was inhibited around a given bacterial colony.

3. Results

3.1. Alfalfa (Medicago sativa) seeds from different varieties as natural carriers of taxonomically diverse culturable bacterial endophytes

In view of recent results in different plant species, we explored in detail to what extent alfalfa seeds were colonized by culturable endophytic bacteria. Exudates collected from surface-sterilized alfalfa seeds were plated on nutrient agar to estimate the number of culturable endophytes. The number of bacteria recovered from experiment to experiment ranged from 10^3-10^6 colony-forming units (CFUs).g^{-1} of seeds, with those values being quite variable compared to the numbers reported in other plant species (Mundt and Hinkle, 1976; Rosenblueth and Martínez-Romero, 2006). That observation was likewise valid for the different varieties of alfalfa used in this work. Next, in order to characterize the bacterial community of alfalfa-seed endophytes, we collected individual clones in the attempt to sample as much as possible the phenotypic diversity according to the particular colony morphology. Those clones were then identified by either mass spectrometry or partial sequencing of the 16S rDNA (Materials and Methods; Fig. 1 and Table S1). Bacterial clones from the several genera that could not be identified through the use of the commercial database of the Bruker biotyping system after the initial MALDI-TOF analysis were typified by 16S-rDNA sequencing, and the corresponding MS records were then incorporated into an in-house library which registry served for the subsequent identification of new isolates.

The analysis of the endophytes recovered from the alfalfa variety Super Monarca enabled the identification of bacteria that belonged to 24 different genera. In order to investigate to what extent the observed diversity was dependent on the variety, we isolated and characterized bacteria associated with the seeds of other 5 alfalfa varieties (*cf.* the first section of Materials and Methods for the key to the code used in Fig. 3). Though the number of genera identified increased with the extended sampling, many of the genera identified were redundant among the different varieties of alfalfa, thus supporting the notion of a conserved bacterial core colonizing alfalfa seeds. Thus, isolates from the genera *Bacillus*, *Kocuria, Arthrobacter, Arsenicicccocus*, *Microbacterium*, *Micrococcus*, and *Paenibacillus* were recovered from the seeds of at least 3 different varieties. The isolates from these genera were quite prevalent. Other isolates identified as belonging to the genus *Pantoea* were less frequent, but when found were in the highest numbers as seed endophytes. In addition to all these considerations, the analysis of more than 160 isolates from all the alfalfa varieties studied revealed a

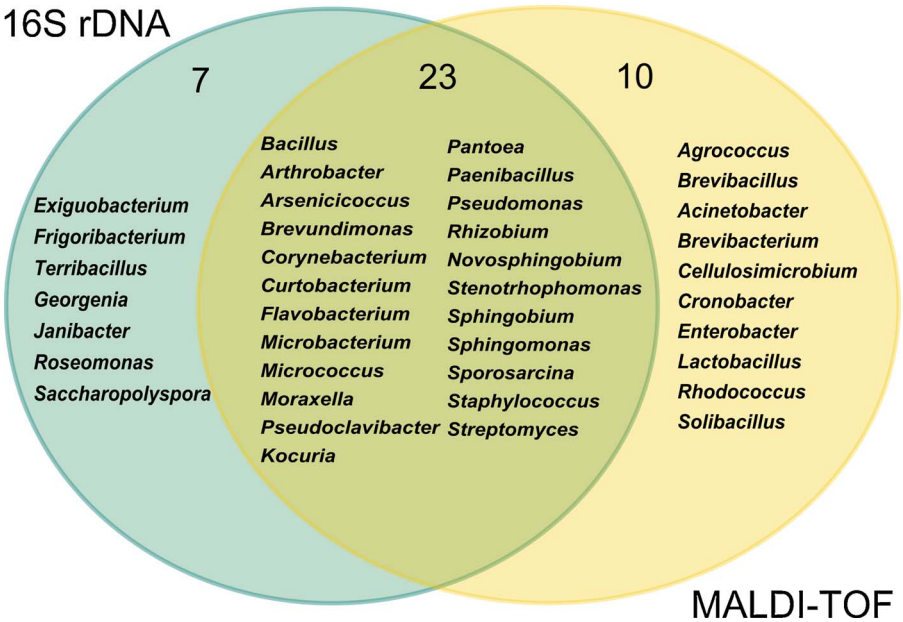
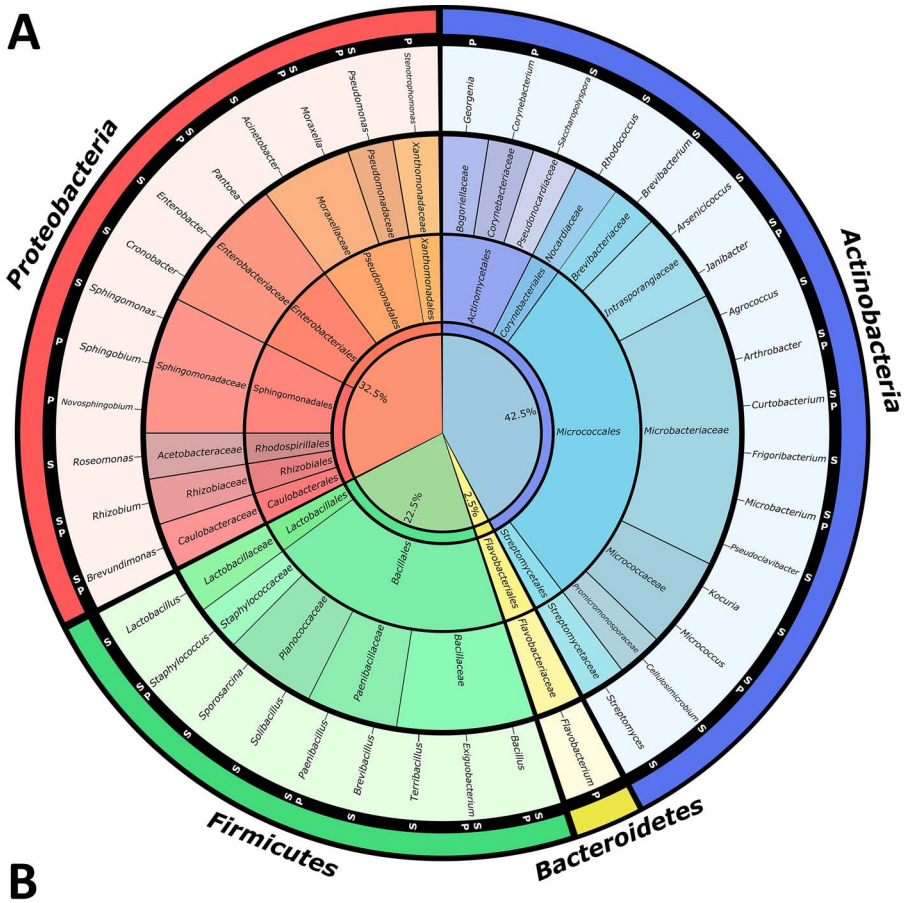


Fig. 1. Identification of bacterial seed-endophytes by UV-MALDI-TOF spectrometry and partial sequencing of 16S rDNAs. The Venn diagram represents those genera which were identified in our isolates by means of MALDI-TOF MS (red circle on the right side), partial sequencing of 16S rDNAs (blue circle on the left side), or both techniques (intersection). The 16S-rDNA sequencing was performed with those isolates that could not be typified by means of the commercial library of the Bruker-MALDI biotyping system (blue circle). Those same bacteria typified by partial sequencing of their 16S rDNA were then added to an in-house MS library in order to expand the identification capacity by the MALDI-TOF–MS approach. In order to validate the MALDI-TOF–MS results with the endophytic bacteria—many of which are not commonly found in clinical environments—we complemented the results of the Bruker biotyping system by also performing a 16S-rDNA sequencing (intersection in the Venn diagram) and found a complete coincidence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



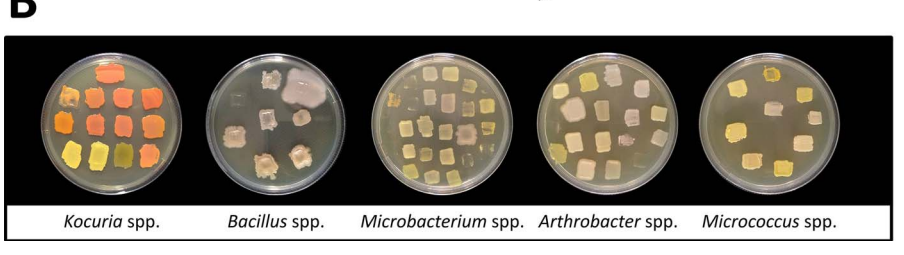


Fig. 2. Bacterial diversity in endophytic clones derived from surface-sterilized alfalfa seeds. Panel A. Percent representation of the bacterial genera present in the seed endophytes. The percentages correspond to the representation of each of the indicated phyla calculated on the basis of the number of different seed-associated genera. The phyla (here in italics) and the number of genera (in parenthesis) were as follows: *Actinobacteria* (17), *Proteobacteria* (13), *Firmicutes* (9), and *Bacteroidetes* (1). Among the *Actinobacteria*, most of the genera belonged to the order *Micrococcales* (12) with the rest belonging to the orders *Corynebacteriales* (3) and *Streptomycetales* (1). The phylum *Proteobacteria* was the most diversely represented when considered at the level of order, with genera from the orders *Enterobacteriales* (3), *Pseudomonadales* (3), *Sphingomonadales* (3), *Caulobacteriales* (1), *Rhodospirillales* (1), *Xanthomonadales* (1), and *Rhizobiales* (1). Within the *Firmicutes*, the genera were only from the order *Bacillales* (8) and *Lactobacillales* (1). *Bacteroidetes* was the least represented phylum with only one genus from the order *Flavobacteriales*. The image was created by Circos (Krzywinski et al., 2009) and edited with INSKAPE. Panel B. Morphological aspects of endophytic clones recovered from different alfalfa seeds and plants. The endophytes presented not only a remarkable taxonomic diversity (Panel A) but also a variety of growth morphologies in solid medium among the isolates from the same genus (*i. e.*, *Arthrobacter*, *Bacillus*, *Kocuria*, *Microbacterium*, or *Micrococcus*).

Phylum	Family	Bacterial genus (PGP activity)	Alfalfa variety	Seed/plant origin	Protease activity	Cellulase activity	Chitinase activity	Phosphate solubilization	Siderophore production	Amylase activity	Pectinase activity	Auxin production	Fungal inhibition	Total isolates
Actinobacteria	*Bogoriellaceae*	*Georgenia*	SM	P	0	0	0	0	0	1	1	0	0	2
	Brevibacteriaceae	*Brevibacterium*	SM	S	0	0	0	0	0	0	0	0	0	1
	Corynobacteriaceae	*Corynebacterium*	SM	P	0	0	0	0	0	0	1	0	0	1
	Intrasporangiaceae	*Arsenicicoccus*	SM, B, C	S	3	2	0	0	1	1	2	1	0	7
		Janibacter	SM, G	S, P	4	0	0	0	2	0	3	1	0	4
	Microbacteriaceae	*Agrococcus*	G	S	0	0	0	0	0	0	0	0	0	1
		Arthrobacter	SM, B, D, F, G	S, P	9	1	0	0	11	4	3	13	0	23
		Curtobacterium	SM, G	S, P	2	1	0	1	1	0	2	2	0	4
		Frigoribacterium	B	S	0	1	0	0	0	0	1	0	0	1
		Microbacterium	SM, B, C, D, G	S, P	13	7	1	1	7	13	18	16	0	34
		Pseudoclavibacter	G, SM	S	0	0	0	0	0	0	0	1	0	2
	Micrococcaceae	*Kocuria*	SM, B, C, F, G	S, P	1	2	0	1	10	4	9	13	0	14
		Micrococcus	SM, B, D, G	S, P	11	0	0	1	6	3	5	13	0	13
	Nocardiaceae	*Rhodococcus*	SM	S	0	0	0	0	1	0	0	0	0	1
	Promicromonosporaceae	*Cellulosimicrobium*	SM	S	0	1	1	0	0	1	1	1	0	1
	Pseudonocardiaceae	*Saccharopolyspora*	G	S	1	0	0	0	0	0	0	0	0	1
	Streptomycetaceae	*Streptomyces*	SM	S	0	1	0	0	1	1	1	0	0	1
Bacteroidetes	*Flavobacteriaceae*	*Flavobacterium*	SM	P	0	0	0	0	0	0	1	1	0	1
Firmicutes	*Bacillaceae*	*Bacillus*	SM, B, C, D, F, G	S, P	15	13	1	0	9	12	15	6	12	24
		Exiguobacterium	SM, G	S, P	4	0	0	1	1	3	3	1	0	4
		Terribacillus	B	S	1	0	0	0	0	0	0	0	0	1
	Lactobacillaceae	*Lactobacillus*	G	S	0	1	0	0	0	0	1	1	1	1
	Paenibacillaceae	*Brevibacillus*	F	S	0	0	0	0	0	0	0	0	0	1
		Paenibacillus	SM, B, C, G	S, P	0	6	4	0	1	2	5	0	0	9
	Planococcaceae	*Solibacillus*	D	S	0	0	0	0	0	0	0	0	0	1
		Sporosarcina	D, G	S	0	1	0	0	0	0	0	0	0	7
	Staphylococcaceae	*Staphylococcus*	SM, B, C, D	S, P	0	0	0	0	1	1	1	0	0	6
Proteobacteria	*Acetobacteraceae*	*Roseomonas*	G	S	1	0	0	0	0	0	1	0	0	1
	Caulobacteraceae	*Brevundimonas*	SM, B, D	S, P	1	1	0	0	2	0	1	0	0	5
	Enterobacteriaceae	*Cronobacter*	SM	S	0	0	0	3	2	0	0	1	0	3
		Enterobacter	SM	S	0	0	0	2	1	0	0	2	0	2
		Pantoea	SM, C, D, G	S, P	0	8	0	12	11	0	0	8	0	12
	Moraxellaceae	*Acinetobacter*	SM	S	1	0	0	0	0	1	0	1	0	4
		Moraxella	SM	S, P	0	1	0	0	1	0	0	0	0	3
	Pseudomonadaceae	*Pseudomonas*	SM, B, D	S, P	0	3	0	2	8	5	7	3	0	9
	Rhizobiaceae	*Rhizobium*	SM, C	S, P	0	0	0	1	4	1	1	6	0	9
	Sphingomonadaceae	*Novosphingobium*	SM	P	0	1	0	0	0	0	0	0	0	1
		Sphingobium	SM	P	0	0	0	0	0	0	0	0	0	1
		Sphingomonas	SM	S	0	0	0	0	0	0	0	0	0	1
	Xanthomonadaceae	*Stenotrophomonas*	SM, B, C, G	P	7	2	2	0	1	1	1	3	0	9
NI			SM, B, D, F, G	S, P	2	1	0	0	3	1	1	0	0	8
Total isolates					76	54	9	25	85	55	85	94	13	234
% genera presenting PGPA					40,0	47,5	12,5	25,0	55,0	42,5	60,0	50,0	5,0	

Fig. 3. *In-vitro* functional traits of endophytes from alfalfa, grouped by bacterial genus. The most frequent activities (columns) within a bacterial genus (rows) is highlighted in yellow. The most prevalent bacterial genus displaying a particular activity is highlighted in blue. The intersection of both categories is highlighted in green. The fourth column states the alfalfa variety (SM, B, C, D, F, G; for the key *cf*. the first section of Materials and Methods) that hosted the isolates of the designated genera. The fifth column indicates whether isolates for the specified genera were recovered from seeds (S), plants (P), or both. PGP activity, plant-growth–promoting activity.

remarkable diversity involving the presence of bacteria from 34 different genera that belonged to 4 different phyla (Fig. 2, Panel A, seed endophytes). Among those isolates, the genera that by visual inspection included the more polymorphic growth on solid media were *Microbacterium*, *Bacillus*, *Arthrobacter*, *Kocuria*, and *Micrococcus* (Fig. 2, Panel B). Our analyses revealed both a notable taxonomic diversity and a variable number of endophytes in the alfalfa seeds. That seeds from the different varieties had been preserved at 4 °C by different time periods (i.e. Super Monarca during 5 years, all the others seed during 2 years) may be a source of heterogeneity in the kind of isolates recovered from each type of seeds. Other sources of heterogeneity may arise from differences in the seed genotypes themselves, and also from differences in the plant and soil microbiomes associated to the production of each seed batch.

3.2. Colonization by seed endophytes of alfalfa plants derived from surface-sterilized seeds and grown under axenic conditions

In order to evaluate whether seed-borne endophytes were able to colonize alfalfa plants, surface-sterilized seeds were grown axenically in plastic tubes, and one month after germination the plant seedlings were analyzed to test for the presence of culturable bacteria (*cf*. Materials and Methods). The evaluation of individual plants derived from surface-sterilized seeds and grown separately under axenic conditions revealed that most had been colonized by bacteria. The endophytes associated with the aerial part of the plants accounted for *ca*. 10^5 CFUs/g of wet tissue. The taxonomic typification of the isolates recovered from the 6 different alfalfa varieties detected the presence of bacteria that belonged to fifteen of the genera that we recognized in the alfalfa-seed endophytes, together with bacteria included in 6 new genera (*Corynebacterium*, *Flavobacterium*, *Georgenia*, *Sphingobium*, *Novosphingobium*, and *Stenotrophomonas*) that had not been detected in our previous assays with seed exudates. Most likely the latter bacteria

had been poorly represented in seeds but had been efficient plant colonizers. Fig. 2, Panel A depicts which genera were represented in the colonized plants compared to the representation of those same genera as seed endophytes. Out of all the isolates recovered from both the plants and the seeds, members of the genera *Arthrobacter*, *Microbacterium*, *Micrococcus*, *Pantoea, Bacillus, Paenibacillus*, *Brevundimonas, Pseudomonas*, and *Staphyloccocus* were all found to be present in several plant varieties.

3.3. Expression of several phenotypic traits frequently related to plant-growth–promoting activities by alfalfa-seed endophytes

Seed endophytes, recovered either directly from seeds or after the colonization of alfalfa seedlings in axenic experiments, were tested for nine different *in-vitro* biochemical activities related to the promotion of plant growth and/or plant health (Table S2). The results indicated that the more prevalent plant-growth–promoting activities in the collection of alfalfa endophytes (expressed as the percentage of genera that bear the indicated activity at least in one isolate) were as follows: (Fig. 3, bottom row) pectinases (60%), siderophore (55%) and auxin production (50%), cellulases (47.5%), amylases (42.5%), proteases (40%), phosphate solubilization (25%), and chitinase activity (12.5%); with the inhibition of fungal growth (5%) being the rarest phenotypic trait. Fig. 3 demonstrates that the more prevalent activities varied depending on the specific genera under consideration; with the *Bacillus* isolates having a remarkable presence of hydrolytic enzymes and fungal-inhibitory activities and members of the *Pantoea* genus exhibiting notable phosphate-solubilization activity as well as siderophore plus indoleacetic-acid production and cellulase activity.

A PCA based on the set of activities present in each individual isolate (Fig. 4) indicated that the endophytes located at the rightmost position of the plot corresponded to seed isolates from the genus *Bacillus* (*i. e.*, the isolates *Bac*2, *Bac*3, *Bac*4, *Bac*6, *Bac*12, *Bac*22, *Bac*23, and *Bac*24 in Table S2) that combined several hydrolytic activities (proteases, amylases, pectinases, and cellulases) together with fungal-inhibitory activity (*Group A*). The upper-left region of the PCA corresponded to mostly isolates of the *Pantoea* genus together with two isolates from the *Enterobacter* and *Exiguobacterium* genera (*Pan1, Pan2, Pan3, Pan4, Pan5, Pan8, Pan10, Pan11, Pan12, Ent2*, and *Exi4*). These isolates combined a strong phosphate-solubilization phenotype with auxin production (*Group B*). A third group of isolates (*Art*4, *Koc*9, *Mcc5*, *Mcc9*, *Mic8*, *Ste1*, *Str*, *Pse3*, *Pse5*, *Pse6*, *Pse8*, and *Pse9* in Supplementary Table S2) corresponded to those characterized by a production of siderophores along with lytic enzymes or auxins (*Group C*). The last and major group of isolates in the PCA analysis corresponded to a taxonomically heterogeneous set of bacteria (33 genera) that presented various moderately expressed plant-growth–promoting activities, either individually or in combination (*Group D*). Thus, isolates from *Group D* that were able to produce indoleacetic acid (79 isolates) were taxonomically well distributed—with 19 genera presenting at least one auxin-producing isolate—and with most belonging to *Microbacterium* (16/79), *Arthrobacter* (13/79), *Kocuria* (12/79), *Micrococcus* (13/79), *Bacillus* (6/79), and *Rhizobium* (6/79). In addition to the isolates from *Group B* mentioned above that have a remarkably strong ability to solubilize phosphate (*e.g.*, *Pantoea* and *Enterobacter*), several isolates from *Group D* belonging to the genera *Cronobacter*, *Micrococcus*, *Microbacterium*, *Pseudomonas*, *Exiguobacterium, Curtobacterium*, and *Rhizobium* were also able to release soluble phosphate from $Ca_3(PO_4)_2$. With respect to siderophore production, 57 isolates from 50% of the genera included in *Group D* proved to be positive for that trait, with most of those isolates pertaining to the genera *Arthrobacter* (10/57), *Bacillus* (4/57), *Pseudomonas* (3/57), *Kocuria* (9/57), *Rhizobium* (4/57), and *Micrococcus* (4/57). In addition, different moderately expressed enzyme activities related to biocontrol and niche colonization (*i. e.*, proteases, cellulases, chitinases, pectinases, amylases) were observed in quite taxonomically diverse isolates from *Group D* (Table S2). Finally, *Group E* consisted of 42 isolates from 20 different genera that did not presented any of the studied activities.

4. Discussion

Though the biologic role of plant seeds has classically been related mainly to their capacity to preserve and protect the associated latent embryos, in the last few years several lines of evidence have presented seeds as quite efficient carriers of dormant microbes (Truyens et al., 2015). In addition to the expected presence of bacteria and other microorganisms associated with the external seed surface (Johnston-Monje et al., 2014), landmark studies with maize and rice have demonstrated that surface-sterilized seeds are nevertheless vehicles of a significant bacterial diversity whose origin, properties, and biologic roles are currently under active investigation (Hardoim et al., 2012; Johnston-Monje and Raizada, 2011). In this report, we describe the isolation and analysis of bacteria that were recovered from different varieties of surface-sterilized alfalfa seeds, which endophytes in many instances had remained alive within the seeds for more than 5 years (*i. e.*, the lapse of time from the harvesting to the processing of the seeds in this study). This observation demonstrates that the bacteria contained within the seeds can persist in a dormant state for long time periods

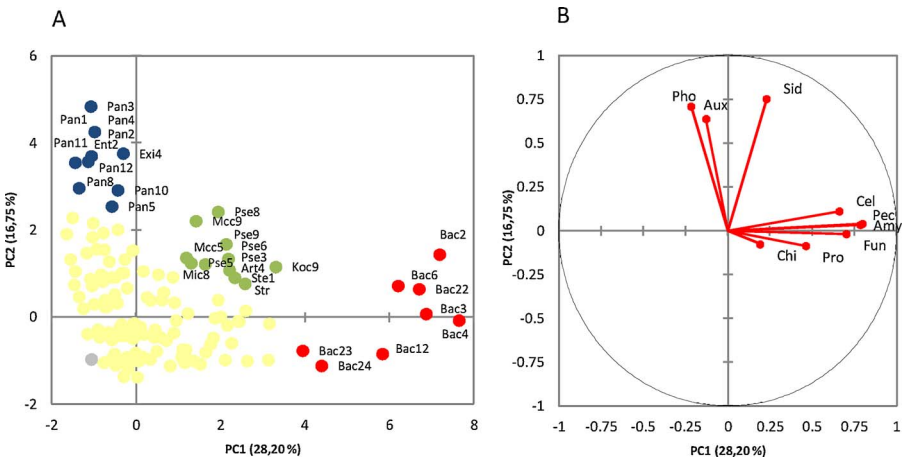


Fig. 4. Principal-components-analysis– (PCA-) based separation of isolates according to their differences in the expression of activities related to plant-growth promotion. Panel A. Vector-correlation plot between the variables examined (*i. e.*, production of auxins, siderophores, and all those traits listed in the heading of Table S2) along with the principal components of variation PC1 and PC2. The regions of specific interest were: from the center upward, a remarkable capacity to solubilize phosphates and a high auxin- and siderophore-producing activity; from the center to the right, a marked capacity for the production of extracellular enzymes (pectinases, proteases, amylases, cellulases) and for the inhibition of fungal growth. Panel B. Vector distribution in the PC1 and PC2 spaces of variation. The PCA (Pearson-n; XLSTAT software package) was performed with respect to a numerical ranking (0–3) of each phenotypic trait (variables) listed in Table S2 for each isolate. The distribution of isolates in the PC1–PC2 space (representing more than 44% of the total variation) served to separate the isolates into regions of clear dominance with respect to each of the phenotypes investigated. The color codes for the groups of isolates defined in the manuscript text are as follows: *Group A*, red; *Group B*, blue; *Group C*, green, *Group D*, yellow; *Group E*, gray. Key to the abbreviations used for the phenotypes: Pho, phosphate solubilization; Aux, auxin production; Sid, siderophore production; Cel, cellulase activity; Pec, pectinase activity; Amy, amylase activity; Fun, fungal inhibition; Pro, protease activity; Chi, chitinase activity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

when protected from environmental stress in that manner. The broad diversity of seed endophytes identified and characterized in this work demonstrated that the bacteria carried by alfalfa seeds represent a wide spectrum of taxa, which strains in many instances were able to colonize the plant at an early time after germination. The observation that the entire diversity recovered from seeds was not also found associated with plants constituted evidence for selectivity, in agreement with the well-known location-related profiles of diversity observed in other plant and animal microbiomes (Bai et al., 2015; Human Microbiome Project Consortium, 2012). Changes in the composition of the bacterial microbiome along plant ontogeny are likely the consequence of the selection for specific biochemical activities to improve the system fitness. Through the analysis of more than two hundred seed and plant endophytes of *M. sativa*, we have identified bacteria from 40 different genera. The taxonomic diversity was consistent with that reported in other plant seeds (Truyens et al., 2015); with the *Proteobacteria* being the most highly represented phylum followed by *Actinobacteria*, *Firmicutes*, and finally the less-represented *Bacteroidetes*. In agreement with a previous report by Mano et al. (2006), we observed that members of the genus *Bacillus* were more frequently found as seed endophytes than as plant colonizers (*Group A*). That *Bacillus* had not been frequently found as plant endophytes was of interest, suggesting that these bacteria might play only a limited role (if any) during and/or immediately after germination. As previously suggested by Gagne-Bourgue et al. (2013), the antifungal compounds that characterize members of the *Bacillus* genus might serve to promote plant (seedling) health and growth. The endophytes from *Bacillus*, for one, which genus is a frequent seed endophyte in several plant species (Compant et al., 2011; Gagne-Bourgue et al., 2013; Khalaf and Raizada, 2016)—together with the isolates from 19 other genera that were found here exclusively in seeds (Fig. 3, Table S1)—constitute useful germplasm to investigate the phenotypic characteristics of these endophytes underlying their preference to colonize seeds. The bacteria from the several genera with remarkable activities of siderophore production in combination with lytic-enzyme activities or auxin synthesis were sequestered as a separate group in the PCA analysis (*Group C*). In contrast to the isolates from *Group A*, bacteria from *Group B*, Group C, Group D, and Group E were found to be both seed and plant endophytes. With respect to all the groups, in addition to the types of activities under consideration, the isolates from nearly forty percent of the genera identified could be found in both the seeds and the plants. That several of the bacteria identified in alfalfa had also been found in other plant species is of interest and would be supported by the previously suggested concept of an endophytic-core microbiome (Truyens et al., 2015). Nevertheless, the data covering a much more extensive spectrum of plant species will be necessary both to more clearly define the conserved and the differing structures of plant microbiomes, and to better recognize the fundamental differences between plant and seed endophytes.

The present analysis of the alfalfa-seed microbiome indicated significant variations in both the number and the type of species found in independent analyses of the same batch of seeds, thus pointing to a complex niche whose biologic significance (*i. e.*, activities, transmission) should be further investigated. Although the exact location of the seed endophytes still remains obscure, certain authors have proposed that the seed coat generates a protective microhabitat where bacteria are preserved before their transmission to the next generation of plants (*e.g.*, Robinson et al., 2016). The vertical inheritance of microorganisms has been recently reported in diverse plant species in which seeds were demonstrated to be colonized through an internal propagation in the plant vascular tissue and in a second instance through contact with bacteria settling on the plant's flowers and fruits (Compant et al., 2011; Hardoim et al., 2012). That evidence, together with the ability of many seed endophytes to colonize seedlings, depicts seeds as natural carriers of a pioneering and likely beneficial inoculum for the young growing plants. We identified seed-associated bacteria in different alfalfa varieties and characterized their phenotypic traits *in vitro* (Fig. 2, Panel A and Table S2), which properties correlated well with known *in-vivo* plant-growth and plant-health–promoting mechanisms encountered in other studies. When seedlings emerge, the seed endophytes are in a preferential position to enter the plant and—together with other soil bacteria—to shape what will ultimately constitute the plant microbiome. In comparison with the population of external soil bacteria, the seed-associated microbiome is expected to be in an advantageous circumstance to compete for nutrients and plant space upon germination (Johnston-Monje et al., 2014; Kaga et al., 2009). Though the relevance of seed endophytes in plant colonization has yet to be quantitatively assessed, considerable evidence is available indicating that seed-borne endophytes may constitute a substantial fraction of the adult-plant microbiome (Johnston-Monje et al., 2014). We can hypothesize that many of the seed-associated endophytes are part of a long-term inherited consortium with other endophytes that uses seeds as sporadic, and protective vehicles to access and then explore new habitats. Such a dynamics would enable a vertical inheritance of a community of seed endophytes throughout generations, at the same time facilitating the adaptation and incorporation of new microbial species into the conserved core consortium. We need to emphasize that while many of the alfalfa-seed endophytes expressed one or more plant-growth–promoting activities, a significant proportion did not. Unfortunately, the natural routes of vertical inheritance of plant microbiomes have been only recently investigated. That the introduction of endophytic bacteria into the flowers of parent maize and wheat plants could drive the inclusion of those microbes into the seed microbiomes of the progeny was remarkable (Mitter et al., 2017). The existance of that mechanism enabled the possibility of manipulating the community of plant endophytes and thus producing new plant traits. New experiments with legumes should explore such scenarios, including those involving rhizobia as the endophytic candidates (since we have already found isolates from the Rhizobiaceae family as being *bona-fide* alfalfa-seed endophytes), together with conducting quantitative experiments to evaluate the relative plant-colonization efficiencies between seed endophytes and the bacteria of the microbial community that populates the rhizospheric soil.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

JLL, FA, AP performed the experiments described in the study and analyzed the data. JLL and AL wrote the manuscript. MES, MJL and WOD contributed to the manuscript writing. EJ and AL were essential for the experimental design and revision of the manuscript. All authors read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jbiotec.2017.12.020.

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