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ORIGINAL PAPER

Endophytic bacteria of *Mammillaria fraileana*, an endemic rock-colonizing cactus of the southern Sonoran Desert

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Abstract The small cactus *Mammillaria fraileana* is a pioneer rock-colonizing plant harboring endophytic bacteria with the potential for nitrogen fixation and rock weathering (phosphate solubilization and rock degradation). In seeds, only a combination of culture-independent methods, such as fluorescence in situ hybridization, scanning electron microscopy, and fluorescence vital staining, detected significant amounts of non-culturable, but living, endophytic bacteria distributed underneath the membrane covering the embryo, in the undifferentiated tissue of the embryo, and in the vascular tissue. Large populations of culturable endophytic bacteria were detected in stems and roots of wild plants colonizing rocks in the southern Sonoran Desert, but not in seeds. Among 14 endophytic bacterial isolates found in roots, four isolates were identified by full sequencing of their 16S rRNA gene. In vitro tests indicated that *Azotobacter vinelandii* M2Per is a potent nitrogen fixer. Solubilization of inorganic phosphate was exhibited by *Pseudomonas putida* M5TSA, *Enterobacter sakazakii* M2PFe, and *Bacillus megaterium* M1PCa, while *A. vinelandii* M2Per, *P. putida* M5TSA, and *B. megaterium* M1PCa weathered rock by reducing the size of rock particles, probably by changing the pH of the liquid media. Cultivated seedlings of *M. fraileana*, derived from disinfected seeds and inoculated with endophytic bacteria, showed re-colonization 105 days after inoculation. Their densities decreased from the root toward the stem and apical zones. Functional traits *in planta* of culturable and non-culturable endophytic bacteria in seeds remain unknown.

Keywords Cactus · Plant colonization of rocks · Endophytic bacteria · *Mammillaria* · Phosphate solubilization · Rock weathering

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Introduction

Colonization, establishment, and continuing permanence of desert plants are part of biogenic processes that lead to accelerated weathering of rocks in arid zones of Central and North America (Puente et al. 2004a; Lopez et al. 2009). Some desert plants become established on bare rocks without the presence of soil in arid regions of Mexico (Bashan et al. 2002, 2006; Valverde et al. 2004; Lopez et al. 2009). Interactions with microorganisms assist desert plants to grow on rocks. These include association of many mycorrhizal fungi and plant growth-promoting bacteria (Puente et al. 2004b; Bashan et al. 2007). Among cacti–bacterial associates, rhizoplane bacterial populations are capable of dissolving essential inorganic nutrients from minerals, fixing atmospheric nitrogen, and promoting growth of giant cardon cacti for long periods of time (Puente et al. 2004a, b). Bacteria alone enhance metal leaching and mineral dissolution from rocks by secreting short-chain organic acids and element-specific ligands (siderophores) that are able to change pH and enhance chelation, which results in increased mobilization of many

trace elements (Fein et al. 1999; Kalinowski et al. 2004; Puente et al. 2004a).

Endophytic bacteria colonize intercellular spaces in various internal plant tissues without causing them harm (Petrini 1991; Kado 1992; Lodewyckx et al. 2002; Danhorn and Fuqua 2007). Colonization by endophytic bacteria has been described in almost all plant species examined so far (Germaine et al. 2004), mainly crop plants (Hallmann and Berg 2006). Some endophytic bacteria enhance plant growth (Sturz et al. 1997; Hardoim et al. 2008) and increase plant resistance to pathogens (Benhamou et al. 1996; 2000; Kloepper and Ryu 2006). Consequently, their commercial potential as inoculants has received substantial attention (Germaine et al. 2004), as a source of secondary metabolites for medicinal applications (Strobel 2007), and improved phytoremediation by degrading xenobiotics such as organochlorine herbicides (Germaine et al. 2006). This vast knowledge notwithstanding, endophytic bacteria of wild plants, especially desert plants, are scarcely studied. Recently, culturable endophytic bacteria that colonize roots of the giant cardon cactus were shown to directly participate in rock weathering and supply plants with the released inorganic nutrients and nitrogen by nitrogen fixation (Puente et al. 2009a, b).

Mammillaria fraileana is a small, cylindrical cactus that grows in large and small clusters. Individual plants average 10–15 cm with a 3 cm diameter, have a narrow cylindrical stem, pink flowers, and red fruit that contain numerous small black seeds (Anderson 2001). This endemic cactus is commonplace in rocky habitats along the east coast of the southern portion of the Baja California Peninsula (Wiggins 1980; Bashan et al. 2002). Many individuals grow in rock fissures or directly on the rock surface without the presence of soil (Lopez et al. 2009).

Our general working hypothesis and the rationale for this line of research was that *M. fraileana*, as a pioneer rock-colonizing cactus, harbors endophytic bacteria that can fix nitrogen and weather rocks. These functional group bacteria assist in establishing these pioneer plants by promoting plant growth and soil formation and are essential to the plants in habitats where plants normally do not grow. The first part of this hypothesis, described in this study, was assessed by using cultivation-independent and cultivation-dependent approaches. Cultivation-independent methods were used to detect natural endophytic populations in different plant organs, and cultivation-dependent methods were used to isolate potential bacterial isolates having capabilities of fixing nitrogen, degrading rock, and solubilizing phosphate in vitro. Endophytic colonization with selected bacterial isolates inoculated in plants was demonstrated with immunological, molecular, and microscopic methods.

Materials and methods

Sampling area and samplings

The sampling site (24°11′15″N, 110°17′50″W) is located on the first ridge of hills bordering the coastal plain, about 2 km north of the city of La Paz, Baja California Sur, Mexico. The climate is subtropical, hot and dry; the substrate is gray, flow-laminated rocks ranging from rhyodacite to rhyolite (Lopez et al. 2009). Ten bare rocks colonized only by *M. fraileana* were sampled. Representative fragments of each rock were collected, as described later. The entire plants were manually extracted by breaking the rocks with chisel and hammer. The cacti were transported to the laboratory within 1 h. Eight plants were used to isolate culturable bacteria from roots and shoots. For the analyses of seeds, many fruits were needed because the seeds were very small (~600 μm). Harvesting of mature fruits was done several times between 2004 and 2008 during early spring and fall, about 1–2 months after flowering. The fruits were smashed, and the seeds were extracted by hand. The seeds were rinsed thoroughly with tap water to separate them from funicular pulp. Finally, they were air-dried at 26 ± 2°C and stored at 4°C up to 6 months with no significant changes in the percentage of germination (~80%). Higher rates (~100%) could be achieved under light conditions (Lopez et al. 2009). The batches used in each experiment underwent similar period of cold storage.

Isolation of culturable bacteria from shoots and roots of wild-grown plants

Shoots and roots from plants growing in rocks were thoroughly washed with tap water to remove organic debris and soil particles. The following steps were performed under aseptic conditions: The surface of 2-cm fragments of shoots was sterilized by pretreatment with 2% Tween-20 (Sigma, St. Louis, MO) for 10 min by agitation on a rocker platform shaker (Thermo Scientific, Melrose Park, IL) at 100 cycles per minute; 10 rinses with sterile tap water; surface sterilization with 1.5% (v/v) 6% NaOCl (commercial bleach) by mechanical agitation for 5 min; and 10 rinses with sterile, distilled water. Elimination of surface bacteria was verified by placing pieces of treated shoots directly on trypticase soy agar (TSA) (Difco-BBL, Sparks, MD) and incubated at 30°C ± 1°C for 24 h. Then, the shoots were removed from the medium and the plates were re-incubated under the same conditions. The printing surface left by the shoot was periodically checked for the growth of bacterial colonies for 10 days. These shoots were then macerated with a pestle and mortar in 50 mL sterile 0.002 M phosphate buffer solution at pH 7.2 (PBS). Serial dilutions in PBS of the macerate were plated on TSA

medium and incubated at 30°C ± 1°C for 72 h. Culturable bacteria were counted. Bacteria from shoots of cacti growing in the nearby soil was isolated using the same procedure and by using two plants collected from the field.

The surface of roots was disinfected by the same procedure used for shoots, with the following modifications: 20 min of agitation in 2% Tween-20 and 10 min in 1.5% NaClO solution. Then, the cortex was removed with a scalpel, and the roots without cortex were rinsed with sterile distilled water. About 30 pieces of sterilized roots (~1 cm each) were macerated together with pestle and mortar in 50 mL PBS. Serial dilutions of macerate were plated on three different media: on the nonspecific TSA medium for general heterotrophic bacterial counts, on Rennie's medium (Rennie 1981) for potential nitrogen fixers, and on modified Pikoskaya's medium (Pikoskaya 1948) for phosphate solubilizers. The latter contained (in g L^{-1}) the following: glucose (10), KCl (0.26), yeast extract (0.5), NaCl (0.2), $MgSO_4 \cdot 7H_2O$ (0.1), $(NH_4)_2SO_4$ (0.5), $FeSO_4$ (0.05), agar (20.0), $MnSO_4$ (0.2), and supplemented, separately, with three insoluble phosphorus sources at increasing difficulty of solubilization: 0.38% $Ca_{10}(OH)_2(PO_4)_6$, 0.01% $FePO_4 \cdot 2H_2O$ or 0.01% $AlPO_4$ (Sigma) at pH 7.2–7.3. These relatively insoluble phosphorus compounds replaced the more common, relatively easy to solubilize tri-calcium phosphate (used in the original medium) to ensure that the isolates are truly phosphate solubilizers. Plates were incubated 48 h at 30°C ± 1°C, and culturable bacteria on solid TSA were counted. To verify the effectiveness of the surface sterilization procedure on roots without cortex, we performed the same procedure used for shoots.

Bacterial isolates recovered from Rennie's and Pikoskaya's media were isolated and purified by conventional and routine microbiological procedures. These isolates represent the diversity of morphotypes with abilities for fixing atmospheric nitrogen or solubilizing phosphates. From those, we selected the most promising isolates exhibiting the strongest capacities of the above features for further tests.

Identification of culturable bacteria from roots

Bacterial isolates were selected based on their capacities to fix atmospheric nitrogen or solubilize phosphate in vitro. Four morphotypes were identified by a commercial firm (Genotech, Daejeon, South Korea) by molecular sequencing of the entire 16s rRNA gene. The sequences were deposited in the GenBank (accession numbers GQ504713, GQ504715, GQ504714, and GQ504712, respectively).

Detection and enumeration of endophytic bacteria from seeds

The disinfection procedure was selected from preliminary experiments intended to eliminate bacterial growth originating from the outer seed coat and, at the same time, maintaining the viability (germination) of seeds. We assumed that viability of the seeds may also contribute to the integrity of their bacterial endophytes. Seeds were surface-sterilized through a 5-step procedure: pretreatment with 2% Tween-20 for 30 min with mechanical agitation (described earlier); 10 rinses with sterile tap water; surface sterilization with 1.5% (v/v) 6% NaOCl solution with mechanical agitation for 5 min; 10 rinses with sterile, distilled water; and immersion for 2–3 s in 100% ethanol. The seeds were air-dried under a laminar flow hood. Groups of 100 seeds were incubated, separately, in trypticase soy broth (TSB) (Difco-BBL, Sparks, MD), nutrient broth (Sigma), or placed directly on TSA or nutrient agar at 36°C (average air temperature during the summer in their natural habitat) for up to 30 days to verify the success of surface sterilization.

The 5-step procedure surface sterilization described in the previous paragraph was used to isolate endophytic bacteria from seedlings reared in a growth chamber (surface-sterilized seeds). We germinated 45 seeds on 2% nutrient agar, and these were incubated in growth chamber (Conviron CMP 3244, Manitoba, Canada) for 20 days under the following conditions: 25°C ± 1°C, 80% relative humidity, 8:16 day–night photoperiod, light intensity at 10 ± 2 μmol m^{-2} s^{-1}. Additionally, 1,200 seeds from 16 fruits (seeds with fresh funicule pulp) and ~1,000 seeds, previously extracted from wild plants and stored, were tested for the cultivation of endophytic bacteria. In no case were culturable endophytic bacteria populations obtained; hence, we explored culturable-independent methods to demonstrate vertical transmission of endophytes from seeds.

Detection of bacteria by fluorescence in situ hybridization (FISH)

Surface-sterilized seeds were incubated in a rotary shaker in trypticase soy broth up to 30 days at 36°C at 110 rpm. Aliquots of this medium were plated periodically on TSA. Since no bacterial colonies developed in this medium, the seeds were considered surface-sterilized. After 8 days of incubation, seeds collected from the liquid media were smashed with a spatula and glass slide and the outer seed coat was removed. Regarding incubation times and quantities of reagents, the internal seed tissue was analyzed by fluorescence in situ hybridization with modifications of the method of Daims et al. (2005) as follows: Tissue was fixed

in 4% paraformaldehyde (Sigma) for 2 h; the sample was attached to precoated slides with gelatin and then dehydrated with ethanol concentrations at 50, 80, and 90%, with 3 min in each concentration. Then, 10 μL of 10 μg mL^{-1} lysozyme (58,100 units mg^{-1}; Sigma) was added to the sample and incubated in the dark at room temperature, ~26°C, for 15–20 min. The dehydration step was repeated, as described earlier. The oligonucleotide probe, EUB338, routinely used to detect members of the domain *Bacteria* (Integrated DNA Technologies, Coralville, IA), (5′-GCT GCC TCC CGT AGG AGT-3′) (Amann et al. 1990), was labeled with flurochrome CY3 (excitation/emission wavelength = 552 nm/565 nm). The hybridization mixture was prepared with 2 μL of the probe (3 ng μL^{-1}) and 10 μL of hybridization buffer at 15% stringency (preparation for 1 mL:180 μL 5 M NaCl, 20 μL 1 M Tris–HCl, 649 μL ddH_2O, 150 μL formamide, and 1 μL 10% SDS). The hybridization mix was added to the tissue, and the sample was incubated in the dark for 2 h at 46°C in a hybridization oven (#5430, VWR-Boekel, Feasterville, PA). The tissue was slightly rinsed with washing buffer at 15% stringency (preparation for 50 mL: 3.18 μL 5 M NaCl, 1 μL 1 M Tris–HCl, and ddH_2O to a final volume of 50 mL; pH was adjusted to 7.0–7.2 with Tris-base buffer) and then incubated in the same buffer for 15 min at 48°C. Samples were cooled by dipping the slides for 2–3 s in ice-cold ddH_2O. Air-dried slides were temporarily mounted with anti-fadent (Citifluor AF-1, Electron Microscopy Sciences, Hatfield, PA). Study of endophytic bacteria was performed under an episcopic fluorescent microscope (Olympus BX41, Tokyo, Japan) equipped with a CY3 filter cube (U-MWIG2, Olympus). Images were saved with a digital camera (Evolution VF) and imaging software (Image Pro Plus 5.0, Media Cybernetics, Silver Spring, MD). FISH for seeds was assayed three times, using 10 seeds per assay.

Vital staining by fluorescein diacetate (FDA)

Metabolically active bacteria were studied according to the method described by Söderström (1977), with the following variations: 0.3 g seeds that did not contain surface bacteria were aseptically macerated in a pestle and mortar with 7 mL sterile PBS. The slurry was stored for 1 h at 4°C. From the supernatant, aliquots of 20 μL were collected and diluted 1:20 in sterile PBS. The diluted sample was stained with 200 μL FDA solution (dilution 1:100 20 mg of FDA in 10 mL acetone). The sample was incubated in the dark for 4 min at room temperature (~26°C). The staining solution was washed with 200 μL PBS twice by centrifugation at 14,700*g* of the sample, and then the pellet was re-suspended in PBS. Four replicates of the slurry were studied. Samples treated only with PBS were used as the negative control. Aliquots (15 μL) were mounted on slides, and metabolically active bacteria were counted under the episcopic fluorescent microscope equipped with a FITC filter (Olympus).

Active cells per fresh g of seeds were calculated from 72 individual field counts. FDA staining for active bacteria in seeds was assayed 8 times.

Detection of bacteria by scanning electron microscopy (SEM)

The same samples analyzed by FISH were used for SEM analysis. The tissue of seeds mounted on glass slides were dehydrated for 5 min in increasing concentrations of ethanol (50, 80, and 90%). The slides were air-dried, placed in a desiccator, and examined at 20 kV without additional preparation under scanning electron microscope (S-3000 N, Hitachi High-Technologies, Tokyo, Japan).

Additional evidence for colonization by inoculated endophytes was provided by field emission scanning electron microscopy (FESEM). Entire plants were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 24 h and shaken at 100 rpm, followed by vacuum for 15 min in the fixative. This extended period was necessary to allow slow penetration of the fixative into the tissues because the samples were whole plants. Plants were washed three times in the same buffer and then step-dehydrated in a series of increasing ethanol concentration (50–100%) for 2 h at each step, then 25 min in 1:1 100% ethanol/100% xylol, and finally in 100% xylol. Infiltration was done with four changes in xylol/paraffin and then 100% paraffin at 55–60°C for 2 h. Plants were embedded in paraffin, and 4- to 5-μm-thin sections were cut and laid on glass slides. Slides were prepared for FESEM by extracting the paraffin from the thin sections by immersing in 100% xylol and then washing with decreasing concentrations of ethanol (100–50% for 3 min at each step). Samples were dried with CO_2 in a critical point dryer (CPD020, Balzers Union, Balzers, Liechtenstein). Samples were coated with 30 nm 60:40% gold/palladium alloy foil in a sputter coater (Edwards S150B, Crawley, West Sussex, UK) and examined at 10 kV by FESEM (AmRay 3300 Fe, Advanced Metals Research, Bedford, MA).

Testing endophytic bacterial isolates for in vitro nitrogen fixation, phosphate solubilization, and rock weathering

Nitrogen fixation

Three-centimeter root segments from 6 plants were surface-sterilized, as described earlier. Nitrogen fixation was separately measured in bacteria from the interior of roots without cortex and also in the bacterial populations from

roots with cortex. Each tissue was homogenized in liquid absolute Rennie's N-free medium (lacking the original yeast extract) and incubated for 5 days at 30°C ± 1°C without agitation. Serial dilutions of the cultures were plated on solid Rennie's N-free medium at 30°C ± 1°C, and colony forming units (CFU) were recorded after 48 h. The isolated morphotypes were considered presumptive nitrogen fixers. These isolates and those from previous isolations were tested for nitrogen fixation using acetylene reduction assay. From the 14 bacterial isolates from roots, we tested only ten isolates that were able to grow on Rennie's medium. Eight isolates were obtained from the internal cylinder of the roots and two from roots with cortex. Flasks containing Rennie's semi-solid (3 g^{-1} agar) medium were inoculated with each bacterial isolate at a concentration of 10^9 CFU mL^{-1}. Flasks were incubated at 30°C ± 1°C, and after 2 days, 10% of the gas volume in each flask was substituted by acetylene. From each culture, serial dilutions were plated on solid Rennie's N-free medium at 30°C ± 1°C, and CFU were recorded before acetylene injection and after 48 h of culture. Nitrogen fixation was tested using the acetylene reduction assay by gas chromatography (HP Al/As 19091P-S15, FID detector) following the procedure of Holguin et al. (1992). The diazotroph *Azospirillum brasilense* Cd ATCC 29710 served as the positive control. Results were expressed in nmoles ethylene CFU h^{-1}. This experiment was repeated 4 times.

Rock weathering

Capabilities of bacterial endophytes for rock weathering were assayed by measuring solubilization of phosphate and degradation of pulverized rock. Eight isolates were selected for their ability to grow on Renie's medium and/or Pikoskaya's medium with different sources of phosphorous salts (aluminum, iron, or calcium). Phosphate solubilization capacity by bacterial endophytes from roots was tested in Pikoskaya's medium supplemented with 0.38% $Ca_{10}(OH)_2(PO_4)_6$. Each isolate was prepared at a concentration of 10^9 CFU mL^{-1}. Flasks containing liquid Pikoskaya's medium, supplemented with 0.38% $Ca_{10}(OH)_2(PO_4)_6$, were inoculated with 1 mL suspension containing bacteria and then incubated for 30 days at 36°C ± 1°C and shaken at 110 rpm (incubator shaker Innova 4340, New Brunswick Scientific, Edison, NJ). Each bacterial isolate had three replicates, and each replicate was analyzed in triplicate. The positive control was *Pseudomonas putida* (Genbank accession number EF123229; Puente et al. 2009a, b). Non-inoculated flasks incubated under the same conditions and non-inoculated flasks without incubation served as negative controls. The ability of the bacterial isolates to dissolve phosphates was quantified according to Vazquez et al. (2000). To show only solubilization by bacteria, the values of the negative controls (without bacteria) were subtracted from the samples inoculated with bacteria. The results were expressed as mg P_2O_5 removed CFU^{-1}. This parameter was used to measure the efficiency of a single cell of each isolate to dissolve phosphate. Measurements at the culture level seemed to overestimate the ability of some bacterial isolates.

The indirect capacity of *M. fraileana* to degrade rocks was measured for bacterial isolates that showed an initial ability to dissolve phosphates or fix nitrogen in vitro. The substrates for this assay (ten rocks collected from the field) were rhyodacite and rhyolite, having a mineral composition rich in volcanic glass (~90–96%), low in plagioclase (~1–8%) and pyroxene (~1–5%), and markedly rich in Si, O, Al, Na, and K (Lopez et al. 2009). Samples of each rock (250 g) were pulverized and sieved to 74 μm of final size particles (200 screen mesh). Equal amounts of ten pulverized rocks were mixed together to make a composite sample. Portions (1.5 g) of pulverized rock were sterilized by autoclave (20 min at 121°C, 15 psi) and then aseptically poured into flasks containing 140 mL one-third strength, modified liquid Rennie's medium pH 7.0. The diluted medium contained (in g L^{-1}) the following: 0.267 K_2HPO_4, 0.067 KH_2PO_4, 0.033 NaCl, 0.009 Na_2Fe EDTA, 0.008 $NaMoO_4 \cdot 2H_2O$, 1.67 manitol, 1.667 sucrose, 0.17 sodium lactate, 0.067 $MgSO4 \cdot 7H_2O$, 0.020 $CaCl_2$, and 1.67 μg L^{-1} biotine, 3.33 μg L^{-1} *p*-aminobenzoic acid (PABA), and the source of nitrogen was 0.2% NH_4Cl at final concentration. This diluted medium provided a minimal amount of carbon and nitrogen sources, such that lithotrophic metabolism was favored. Inoculum consisted of four root endophytes: *Pseudomonas putida* M5TSA, *Enterobacter sakazakii* M2PFe, *Bacillus megaterium* M1PCa, *Azotobacter vinelandii* M2Per, separately and a mix of the four endophytes. The positive control was *Bacillus pumilus* ES4 (GenBank accession number FJ032017) isolated from the giant cardon cactus *Pachycereus pringlei* and known to weather rock (Puente et al. 2009b).The treatments that involved the endophytic nitrogen fixer *Azotobacter vinelandii* M2Per lacked NH_4Cl. The negative control consisted of phosphate buffer solution (0.002 M, pH 7.2) added to the liquid medium. Each inoculum was grown in half-strength TSB at 36°C and shaken at 110 rpm for 12–48 h. Each inoculum was harvested, and bacterial suspensions were prepared in PBS to a final concentration of 10^9 CFU mL^{-1}. Flasks were inoculated with equal amounts of bacterial suspension (about 10^{10} cells) at log growth phase and incubated for 32 days at 36°C and shaken at 110 rpm. The consortium of endophytes contained 2.5 mL of each isolate. Aliquots of 3 mL were collected weekly to determine pH and bacterial count on TSA (three isolates and consortium) and on Rennie's

medium (for *Azotobacter vinelandii* M2Per). The ability of the endophytes to degrade pulverized rock was measured by the reduction in particle diameter of the pulverized rock and the increase in the number of smaller particles after incubation with bacteria. Samples of 1 mL from the flasks were diluted 1:100 in pre-filtered 3.5% NaCl solution and counted in a counter (Multisizer 3 counter, Beckman Coulter, Brea, CA). The measured particles ranged from 3 to 26 μm in diameter. Each isolate had four flasks as replicates, and each replicate was analyzed in triplicate.

Localization of inoculated bacteria in plants

Three isolates (*Pseudomonas putida* M5TSA, *Enterobacter sakazakii* M2Per, and *Bacillus megaterium* M1PCa) were used for inoculation and study of re-colonization. The isolates were selected for their ability to grow in Pikoskaya's medium and/or dissolve inorganic phosphorus in vitro.

Inoculating *M. fraileana* grown on pulverized rock with endophytic isolates

All materials in this section were sterilized using either autoclave or 70% ethanol. The test lasted 196 days. For this slow-growing plant, the extended time was needed to demonstrate that the bacteria–plant association can be detected in adult plants even when small (less than 1 cm high). Substrate for plant growth was prepared by adding 1 g pulverized rock (sieved to 74 μm) to multi-sized perlite (Supreme Perlite, Portland, OR): 3 g perlite (0.85–1.4 μm), 4 g perlite (0.53–0.85 μm), and 1 g perlite (>0.53 μm). Seeds for germination were surface-sterilized by the same procedure used for the detection of endophytes in seeds. Disinfected seeds were placed on 1% nutrient agar and incubated in a growth chamber (Model 8 15, Precision Scientific, Chicago) at 30°C ± 0.1°C, 80% relative humidity, 8:16 day–night photoperiod, light intensity 10 ± 1 μmol m^{-2} s^{-1} for 76 days. Then, seedlings with no bacterial colonies growing in the agar where the plant germinated were aseptically transferred to small black plastic pots (3.5 cm × 3.5 cm × 6 cm high) containing 9 g substrate mixture and rock described earlier. The seedlings were individually placed into small sterile units made with clear plastic and domed lids to create a micro-environment with adequate space for gas diffusion. These units were intended to minimize contamination. The plants in these units were grown in greenhouse under the following conditions: day temperature (25°C ± 5°C), night temperature (17°C ± 5°C, 80% relative humidity, 11:13 day–night photoperiod, light intensity of 340–370 μmol $m^{-2}s^{-1}$. After 15 days under these conditions, each pot of 20 seedlings was inoculated with a 1 mL suspension of bacteria (in PBS, 0.002 M, pH 7.2) at a concentration of 10^9 CFU mL^{-1}. Previously, this concentration had growth-promoting effects in other species of cacti (Puente et al. 2004a). Pots were irrigated every 15 days with 7 mL sterile deionized water. At 105 days after the plants were inoculated or not inoculated (controls), they were aseptically harvested for histological studies described later. The substrate was removed from the roots by gently soaking the plants in sterile deionized water. Then, the whole plants were surface-sterilized in a four-step procedure: 35 min of moderate agitation in 1:1 v/v of 2% chloramine and 2% Tween-20; five rinses with sterile deionized water; immersion in 70% ethanol for 30 s; and quick rinsing with 10 mL deionized water.

Production and purification of polyclonal antibodies against endophytes from roots

Antibodies for the three isolates inoculated on plants were elicited in white rabbits, as described by Levanony et al. (1987), with the following modifications. Whole cells of the three isolates were used to elicit polyclonal antibodies in F1 New Zealand × California white rabbits. Pure isolates were grown in TSB medium at 36°C and shaken at 130 rpm for 24 h. Inocula were harvested by centrifugation at 2,240 *g* for 20 min and washed 3 times with sterile PBS. Pellets were suspended in PBS. Bacteria were attenuated with cold 2.5% glutaraldehyde (Sigma), washed with PBS, and brought to a final concentration of 10^9 CFU mL^{-1}. Final bleeding via cardiac puncture was done at week 11 after immunization. Antisera were stored at −80°C until IgG purification. Antisera had an initial titer from 1:20 to 1:300, depending on antigens (bacterial strain). Specificity tests were performed by immunoagglutination. Antisera had an initial titer from 1:20 to 1:300, depending on antigens (bacterial isolates). The IgG was purified from the antisera on a spin affinity column (Montage Prosep-G, Millipore, Billerica, MA) according to the manufacturer's instructions. The purified antibodies had the following final concentrations: *Pseudomonas putida M5TSA* (850 μg mL^{-1}), *Bacillus megaterium* M1PCa (461 μg mL^{-1}), *Enterobacter sakazakii* M2PFe (900 μg mL^{-1}), and rabbits immunized with sterile PBS (461 μg mL^{-1}).

Immunostaining of tissue sections from plants inoculated with endophytes

Plants were sliced in half and fixed by vacuum infiltration for 30 min in 2.5% paraformaldehyde/0.1 M phosphate buffer at pH 7.2 and then incubated overnight at 4°C in fixative. The tissue was washed 3 times in 0.1 M phosphate buffer and then dehydrated by sequential 2-h incubations in 70, 95, 100% acetone at 4°C. Infiltration and embedding was done by using the Technovit 7100 kit (Electron

Microscopy Sciences, Hatfield, PA) according to the manufacturer's instructions. Thin sections (4 μm) were cut with a steel knife (Dorn & Hart Microedge, Villa Park, IL) on a rotary microtome (Spencer 3293, Buffalo, NY) layered onto slides (Superfrost-plus, Advanced Biotechnologies Services, Columbia, MD). The procedure for immunostaining was based on Manning and Ciuffetti (2005) with modifications as follows: Sections were blocked for 30 min at room temperature with blocking solution (0.1% Tween-20, 1.5% glycine, 2% bovine serum albumin (BSA, Biolabs, Ipswich, MA) and 3% goat serum (G9023, Sigma) in PBS: 150 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 3.3 mM KH_2PO_4 at pH 7.5. After blocking, slides were rinsed with PBS buffer supplemented with 0.25% Tween-20 and 0.8% BSA (wash buffer) followed by PBS. Sections were incubated with 50 μL of the raised antibodies diluted in PBS (1:50 for *Pseudomonas putida* M5TSA, *Bacillus megaterium* M1PCa, and *Enterobacter sakazakii* M2PFe) for 90 min at 37°C in a 100% humidity chamber. Then, the sections were washed twice, 10 min each, in high-salt PBS (350 mM NaCl, 3 mM KCl, 8 mM Na_2HPO4, 3.3 mM KH_2PO4, 0.25% Tween-20, and 0.1% bovine serum albumin at pH 7.5), and rinsed with PBS-wash buffer followed by standard PBS. Sections were then incubated with 1:500 goat anti-rabbit IgG TAMRA labeled with orange fluorescent dye (excitation/emission: 555 nm/580 nm) (AnaSpec, San Jose, CA) in PBS for 1 h at 37°C in a humidity chamber, then washed twice for 10 min each with PBS, rinsed with running deionized water for 15 min, then allowed to dry. This was then covered with Cytoseal 60 (Electron Microscopy Sciences). Tissue sections with unlabeled bacteria and fluorescent immunostaining bacteria and without primary antibodies served as the negative controls. Cross-reactions among endophytes were also verified by staining tissue sections with antibodies elicited for another endophyte used in this study. Slides were examined under a fluorescent microscope (DMRB, Leica Microsystems, Wetzlar, Germany), equipped with a TRITC 41002 filter (excitation/emission wavelength: 535/50 nm, 620/50 nm, Chroma Technology, Rockingham, VT). Complementary images were obtained with a confocal laser scanning microscope (CLSM; LSM510 Meta, Zeiss, Jena, Germany) with Axiovert 200 motorized microscope and processed with imaging software (LSM vers. 3.2, Zeiss).

Sample size and statistical analyses

The number of repetitions is indicated in each assay. For counting CFU, a single replicate in each repetition contained 6–9 separate Petri dishes. Histological analyses used five plants per bacterial isolate. Over 80 images with FE-SEM, 63 with the epifluorescence microscope, and 12 complementary images with CLSM were analyzed. For rock weathering, pH data are presented after subtracting the average value of the negative control from the inoculated cultures to ensure that the effect was due only to the inoculation with bacteria. Parametric comparison of rock degradation data and $\log_{10}$ transformation of phosphate solubilization data were performed by one-way ANOVA, followed by ad hoc Tukey HSD or Student's *t*-test analysis ($P < 0.05$) with JMP v5.1.2 statistical software (SAS Institute 1989).

Results

Culturable endophytic bacteria from adult wild plants

Population density of natural endophytic bacteria in shoots of wild plants growing on rocks was 182 ± 38 CFU g^{-1} shoot fresh weight (2,970 CFU g^{-1} shoot dry weight), while in shoots of wild plants growing in nearby rocky soil, it was 282 ± 40 CFU g^{-1} shoot fresh weight (4,610 CFU g^{-1} shoot dry weight). Recovery of endophytic bacteria from the crude macerate of roots without the cortex yielded $54.4 \pm 0.0 \times 10^3$ CFU g^{-1} root fresh weight (0.237×10^6 CFU g^{-1} root dry weight).

Detection and enumeration of non-culturable endophytic bacteria from seeds

Using standard microbiological methods for the cultivation of microorganisms, several attempts with different media and variations or disinfection procedures failed to isolate endophytic bacteria from seeds, seedlings, and fruits. Only cultivation-independent methods allowed the detection of non-culturable endophytic bacteria in seeds. During the imbibition stage, fluorescent in situ hybridization (FISH) was performed on seeds with a probe for the domain *Bacteria* and this showed natural endophytic bacteria in the internal tissue of the embryo and in the incipient vascular tissue (Fig. 1d, e). When the same seeds found by FISH were re-examined using FESEM, we found individual bacteria cells and micro-colonies emerging from underneath the membrane surrounding the embryo. In our observations, the endophytic community consists primarily of bacteria with similar rod-shaped morphology (Fig. 1b, c). Fungi were not detected as endophytes in these seeds.

The viability of endophytic bacteria from seeds was demonstrated by FDA staining, revealing populations of endophytic bacteria ($2.44 \pm 0.64 \times 10^6$ cells g^{-1} seed FW; 6.10×10^6 cells g^{-1} seed DW). Observations of fresh slides of homogenized seeds showed microbes, mainly motile rod forms.

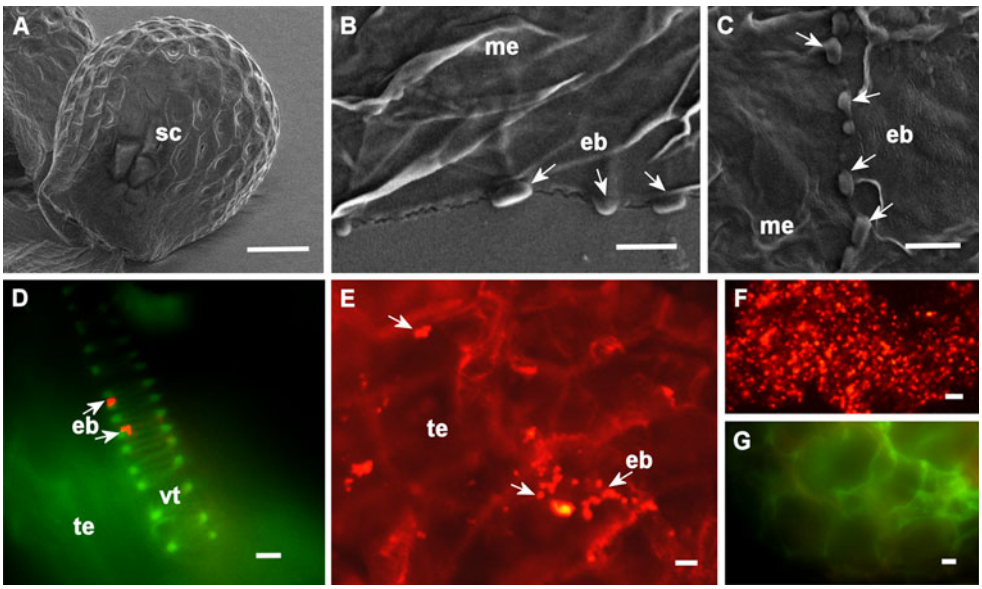

Fig. 1 Detection of non-culturable endophytic bacteria in seeds of *Mammillaria fraileana* at day 8 of imbibition. **a** View of the seed showing the seed coat by scanning electron microscope. **b, c** After eliminating the seed coat, the endophytic bacteria are shown along the edge of the outer membrane covering the embryo. **d** FISH for the detection of endophytic bacteria in the vascular tissue of seed. **e** Occurrence of endophytic bacteria in the tissue of the embryo detected by FISH. **f** Positive control for FISH using pure cell culture of *Enterobacter sakazakii* M2PFe. **g** Negative control for FISH. Embryo without fluorescent probe for the detection of bacteria. Samples from **b** and **c** are from the same seed as **d** and **e**. *Arrows* indicate bacterial cells. *EB* endophytic bacteria, *ME* outer membrane of embryo, *SC* seed coat, *TE* tissue of embryo, *VT* vascular tissue. *Bars*: **a** 200 μm; **b**, **c**, **d**, **e**, **f** 5 μm, **g** 10 μm

Nitrogen fixation

The initial number of nitrogen-fixing bacteria did not include culturable nitrogen-fixing bacteria. Therefore, after additional incubation for 48 h in Rennie's medium, the population of nitrogen-fixing bacteria varied from 0.5×10^9 CFU g^{-1} root fresh weight without cortex (2.18×10^9 CFU g^{-1}root dry weight) to $56.3 \pm 13.4 \times 10^9$ CFU g^{-1} root fresh weight with cortex (245×10^9 CFU g^{-1}root dry weight). The difference between these two values corresponds to the population in the cortex (55.8×10^9 CFU g^{-1} root fresh weight and 243×10^9 CFU g^{-1} root dry weight). Although these quantities of bacteria are definitely not the true nitrogen-fixing populations of the roots, they indicate that diazotrophic endophytes exist in these plants. Among the 10 isolates tested by acetylene reduction assay, only *Azotobacter vinelandii* M2Per, isolated from the cortex of roots, was able to reduce acetylene in vitro at $36 \pm 1 \times 10^{-6}$ nmoles ethylene CFU h^{-1} (Table 1). This rate of nitrogen fixation is ~17 times greater than the rate exhibited by the positive control (*Azospirillum brasilense* Cd ATCC 29710).

Solubilization of inorganic phosphorus

Ten tested isolates (from M1TSA to M2PCa; Table 1) were able to grow in solid media using $Ca_{10}(OH)_2(PO_4)_6$, $FePO_4{\cdot}2H_2O$, or $AlPO_4$ as the sole source of phosphorus. However, no halo was observed in solid Pikoskaya's medium with $FePO_4{\cdot}2H_2O$ or $AlPO_4$. Five isolates showed a solubilization halo in solid Pikoskaya's medium with $Ca_{10}(OH)_2(PO_4)_6$. When testing these isolates in liquid Pikoskaya's medium with $Ca_{10}(OH)_2(PO_4)_6$, these isolates dissolved phosphorus to different degrees ($F = 49.46$, $P < 0.0001$), but *Enterobacter sakazakii* M2PFe was superior in the amount (mg) of P_2O_5-removed CFU^{-1} ($1.88 \pm 0.55 \times 10^{-6}$ mg of P_2O_5 removed CFU^{-1}), whereas the positive control *Pseudomonas putida* SENDO2 was significantly lower ($14.22 \pm 7.02 \times 10^{-9}$ mg P_2O_5-removed CFU^{-1}; Table 1).

Isolation and identification of culturable endophytic bacteria from roots

The analysis of endophytic bacteria focused on root isolates because roots showed higher levels of endophytic populations than shoots. We obtained 14 isolates that represent putative morphotypes of nitrogen fixers or phosphate solubilizers. Twelve morphotypes belong to isolates from the vascular cylinder, and the other two were isolated from the cortex of roots. All the isolates were considered endophytes because they were isolated from surface-sterilized, healthy roots. Cell and colony morphology were similar; cells were mostly rod shaped and Gram negative (Table 1).

Table 1 Characterization of endophytic bacteria isolated from roots of *Mammillaria fraileana*

Bacterial isolate	Gram staining	Solubilization of $Ca_{10}(OH)_2(PO_4)_6$ in Pikoskaya's medium		Nitrogen fixation
		Solid medium (halo diameter in mm)	Liquid medium (mg P_2O_5 removed CFU^{-1})	nmoles ethylene $CFU\ h^{-1}$
Endophytic bacteria				
M1TSA[NI,1]	Gram −	NC	ND	ND
M2TSA[NI,1]	Gram −	5	ND	ND
M3TSA[NI,1]	Gram −	5	ND	ND
M4TSA[NI,1]	Gram −	NC	ND	ND
Pseudomonas putida M5TSA[1]	Gram −	1	$1.55 \pm 0.19 \times 10^{-9}$ cd	NC
M2RN2[NI,1]	Gram −	NC	$1.26 \pm 0.33 \times 10^{-9}$ d	NC
M2PAL[NI,1]	Gram −	NC	$25.43 \pm 6.6 \times 10^{-9}$ b	NC
Enterobacter sakazakii M2PFe[1]	Gram −	NC	$1.88 \pm 0.55 \times 10^{-6}$ a	NC
Bacillus megaterium M1PCa[1]	Gram +	15	$0.54 \pm 0.13 \times 10^{-9}$ d	NC
M2PCa[NI,1]	Gram −	15	$2.83 \pm 1.04 \times 10^{-9}$ cd	NC
Azotobacter vinelandii M2Per[2]	Gram −	ND	$0.77 \pm 0.07 \times 10^{-9}$ d	$36.0 \pm 1.0 \times 10^{-6}$
M1Per[NI,2]	Gram −	ND	ND	NC
M1Hom[NI,1]	Gram −	ND	ND	NC
M3Hom[NI,1]	Gram −	ND	$30.24 \pm 0.76 \times 10^{-9}$ b	NC
Reference bacteria				
Pseudomonas putida SENDO2	Gram −	ND	$14.22 \pm 7.02 \times 10^{-9}$ bc	ND
Azospirillum brasilense Cd ATCC 29710	Gram −	ND	ND	$2.09 \pm 2.5 \times 10^{-6}$

Available from the collection of the Environmental Microbiology Group, CIBNOR, La Paz, B.C.S., Mexico

Values ± SE

Original values for P_2O_5 denoted by different letters differ significantly at $P < 0.05$ by the Tukey–Kramer HSD test of $\log_{10}$-transformed data

NC bacterial growth on this medium, but without halo or acetylene reduction; *ND* not determined but culturable; *NI* not identified

[1] Isolated from the internal cylinder of the roots

[2] Isolated from roots with cortex

Four endophytes in roots were identified by comparing their 16S rRNA sequences to the GenBank database. The bacterial isolates from the cylinder of the roots were identified as *Bacillus megaterium* (100% identity at 1466/1466 pb), *Enterobacter sakazakii* (99.99% identity at 1454/1455 pb), and *Pseudomonas putida* (100% identity at 1462/1462 pb); the isolate from the cortex of roots was *Azotobacter vinelandii* M2Per (99.9% identity at 1461/1463 pb). The sequences were deposited in the GenBank (accession numbers GQ504713, GQ504715, GQ504714, and GQ504712, respectively).

Rock-weathering capacity of endophytic bacteria in vitro

To measure weathering of pulverized rhyodacite (diameter < 74 μm), the endophytes *Bacillus megaterium* M1PCa, *Pseudomonas putida* M5TSA, *Azotobacter vinelandii* M2Per, and *Enterobacter sakazakii* M2PFe were used (separately and as a consortium). All the rock slurries inoculated with bacteria significantly increased the number of smaller particles (Fig. 2). The positive control *Bacillus pumilus* ES4 (Fig. 2a), together with three endophytic bacteria, *Bacillus megaterium* M1PCa (Fig. 2b), *Pseudomonas putida* M5TSA (Fig. 2e), and *Azotobacter vinelandii* M2Per (Fig. 2f), increased significantly the number of small particles from 9 to 403%. The best rock-weathering endophyte was *Azotobacter vinelandii* M2Per, which dramatically increased the number of particles by 89% (3–5 μm:$n_{time\ 0} = 7.36 \times 10^6$ particles mL^{-1} to $n_{time\ 32d} = 15.32 \times 10^6$ particles mL^{-1}), 161% (5–8 μm:$n_{time\ 0} = 4.54 \times 10^6$ mL^{-1} to $n_{time\ 32d} = 12.92 \times 10^6$), (8–12 μm:$n_{time\ 0} = 0.51 \times 10^6$ particles mL^{-1} to $n_{time\ 32d} = 2.06 \times 10^6$ particles mL^{-1}), and 143% (12–26 μm:$n_{time\ 0} = 0.16 \times 10^6$ particles mL^{-1} to $n_{time\ 32d} = 0.48 \times 10^6$ particles mL^{-1}) (Fig. 2f). The consortium of endophytic bacteria and *Enterobacter sakazakii* M2PFe increased the number of particles to a similar level, lower than the rest of the treatments and the positive control *Bacillus pumilus* ES4 (Fig. 2c, d).

Hypothetically, acidification of the medium produces rock weathering. Differences in pH may explain

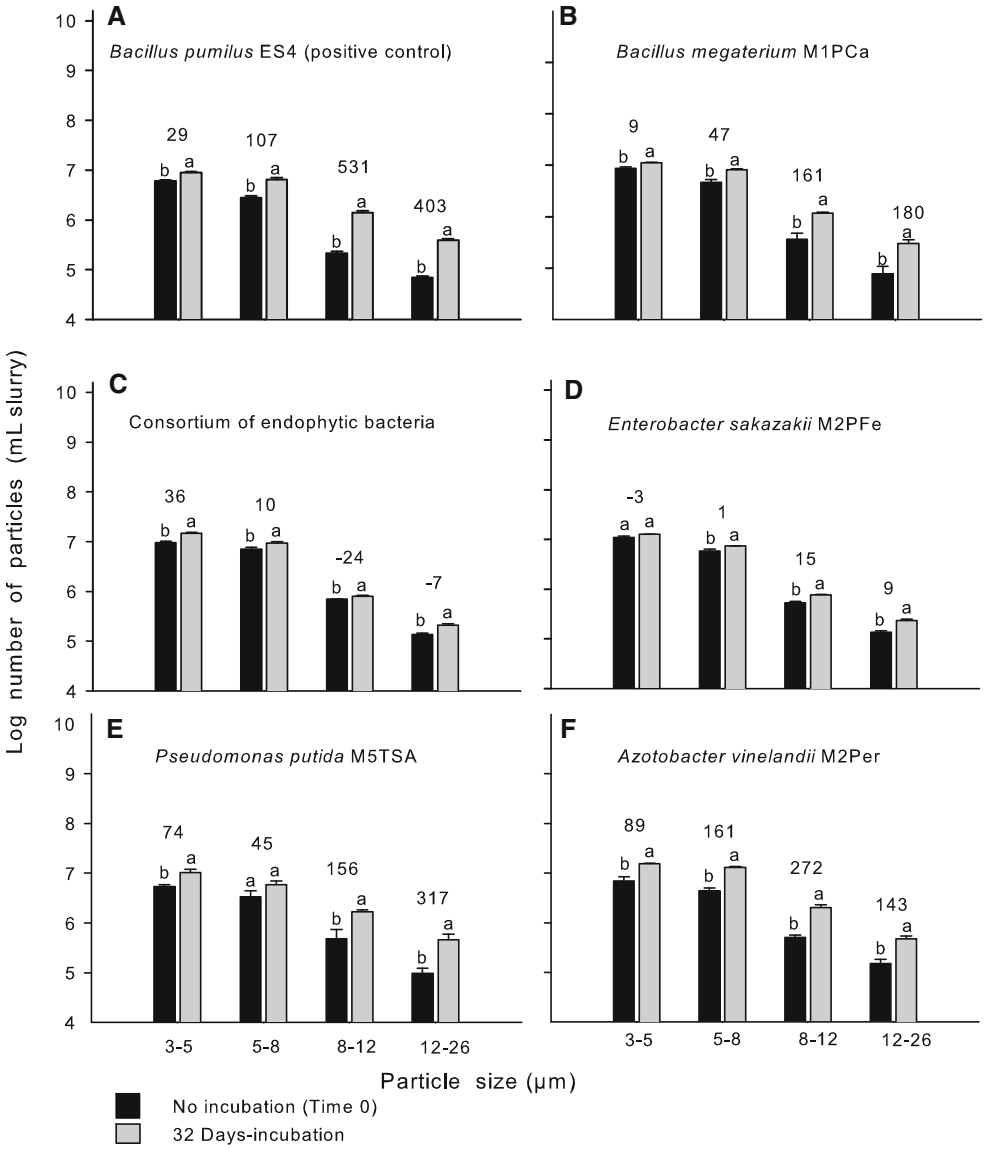

Fig. 2 Reduction in particle size of pulverized rhyodacite after incubation for 32 days with **a** *Bacillus pumilus* ES4 (positive control), **b** *Bacillus megaterium*, M1PCa, **c** Consortium of endophytic bacteria, **d** *Enterobacter sakazakii* M2PFe, **e** *Pseudomonas putida* M5TSA, and **f** *Azotobacter vinelandii* M2Per. Four replicates per inoculum and each replicate analyzed in triplicate. Values above pairs of column indicate the difference, in percentage, in the change of the number of particles. *Negative values* indicated that the percentage was higher in the non-inoculated treatment. Pairs of columns for each particle size denoted by different letters differ significantly at $P < 0.05$ by Student's *t*-test. Bars represent standard error (*SE*). The absence of a *bar* indicates a negligible SE

differences observed in the rock degradation test. Changes in pH were detected after the first week of incubation. With the exception of *Azotobacter vinelandii* M2Per, all isolates reduced the pH of the culture media by 1.68–1.86 pH units. The consortium of endophytic bacteria decreased pH by ~1.77 units, but this decrease was not significantly different from the positive control (*Bacillus pumilus* ES4) or the other two endophytes (*B. megaterium* and *P. putida*) inoculated separately. Compared with the negative control, the change in pH with *Enterobacter sakazakii* M2PFe was not significant. Interestingly, *Azotobacter vinelandii* M2Per increased the pH by 1.45 after incubation (Fig. 3a).

The six endophytes (including the positive control) survived at least 2 weeks with a minimum supply of nutrients. Some populations of endophytes increased in the second or third week of incubation (Fig. 3). Contrary to rock-weathering data presented previously, the highest populations of bacteria after 32-d incubation in the rock slurry were *Enterobacter sakazakii* M2PFe (54×10^6 CFU mL^{-1}; Fig. 3c), the consortium of endophytic bacteria (1.55×10^6 CFU mL^{-1}), and *Azotobacter vinelandii* M2Per (0.54×10^6 CFU mL^{-1}; Fig. 3b). The negative control did not show any microbial growth and remained sterile.

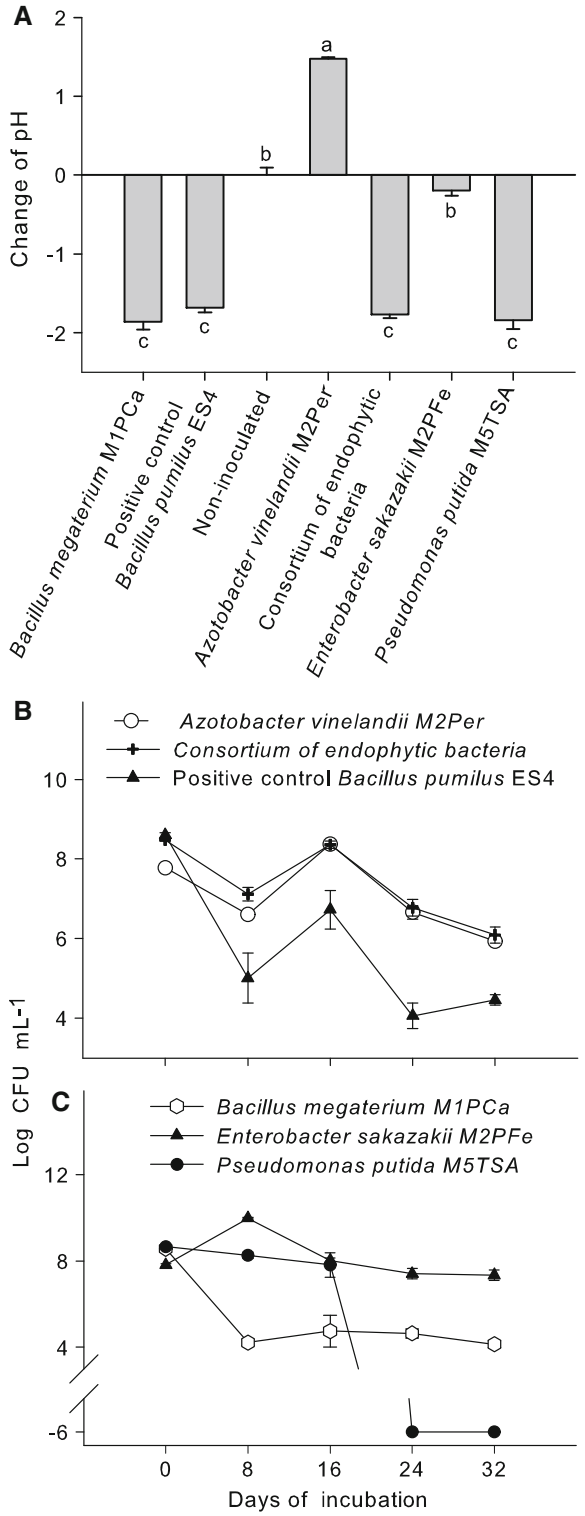

Fig. 3 **a** Change in pH in endophytic bacterial culture medium. **b**, **c** Bacterial population of endophytic bacteria cultured in suspension with pulverized rhyodacite as source of minerals supplemented with minimum source of carbon (manitol + glucose = 1%) and N (NH_4Cl = 0.2%). *Bacillus pumilus* ES4 served as positive control. Negative values of log CFU mL^{-1} indicate bacterial population = 0. Non-inoculated slurry indicates no change in the pH. *Bars* represent standard error (*SE*). The absence of a *bar* indicates a negligible SE

Colonization of cactus seedlings by inoculated endophytic bacteria

The ability of three endophytes, isolated from roots, to re-colonize roots for long periods was tested 105 d after inoculation of seedlings by pouring bacterial suspension on the substrate. At that time, surface-sterilized plants were analyzed by histological methods for specific detection and location of the original inoculum by immunolocalization under confocal laser and epifluorescence microscopes. The three isolates successfully penetrated and became established as endophytes (Fig. 4). Re-colonization by the three isolates occurred predominantly at the base of the main root (Fig. 4a, b, c), decreasing toward above-ground tissues (Fig. 4d) and toward the root tip (data not shown). Although re-colonization could not be quantified by the methods used in the colonization zone, dermal and cortical tissues had visibly higher populations of endophytic bacteria located in close proximity to the cell walls (Fig. 4a, b, c). In non-inoculated plants that served as negative controls, the immunolocalization method we used did not reveal the three inocula.

Discussion

Endophytic bacteria are ubiquitous among plants and mostly studied in agricultural crops (Lodewyckx et al. 2002). We demonstrated the presence of endophytic bacteria in the naturally grown wild cactus *Mammillaria fraileana* by isolating culturable bacteria from its roots and shoots and by detecting non-culturable endophytic bacteria in seeds. Attempts to culture bacteria from fruit, laboratory-reared seedlings, and seeds failed to recover culturable endophytic bacteria, which suggests that the population of generative organs, such as fruits, is either below limits of detection (10^1 CFU g $tissue^{-1}$) of the techniques or these bacteria are in a physiological state that do not allow culturing (Adams and Kloepper 1996; Hallmann et al. 1997). In view of the strong evidence of endogenous bacteria in seeds of herbaceous plants to hardwood trees (Bacilio-Jimenez et al. 2001; Coombs and Franco 2003; Ferreira et al. 2008) and vertical transmission of endophytic bacteria (Rosenblueth et al. 2004; Puente et al. 2009b), we explored several culture-independent methods to verify the presence of true endophytic bacteria in seeds. This fact may have an ecological role, since seeds are the main vehicle for dispersing this cactus (Bravo-Hollis and Sanchez-Mejorada 1991). Populations of endophytic bacteria from surface-sterilized seeds were detected by fluorescence in situ hybridization (FISH), scanning electron microscopy (SEM), and fluorescent vital staining (FDA). During imbibition by seeds, FISH showed microcolonies in

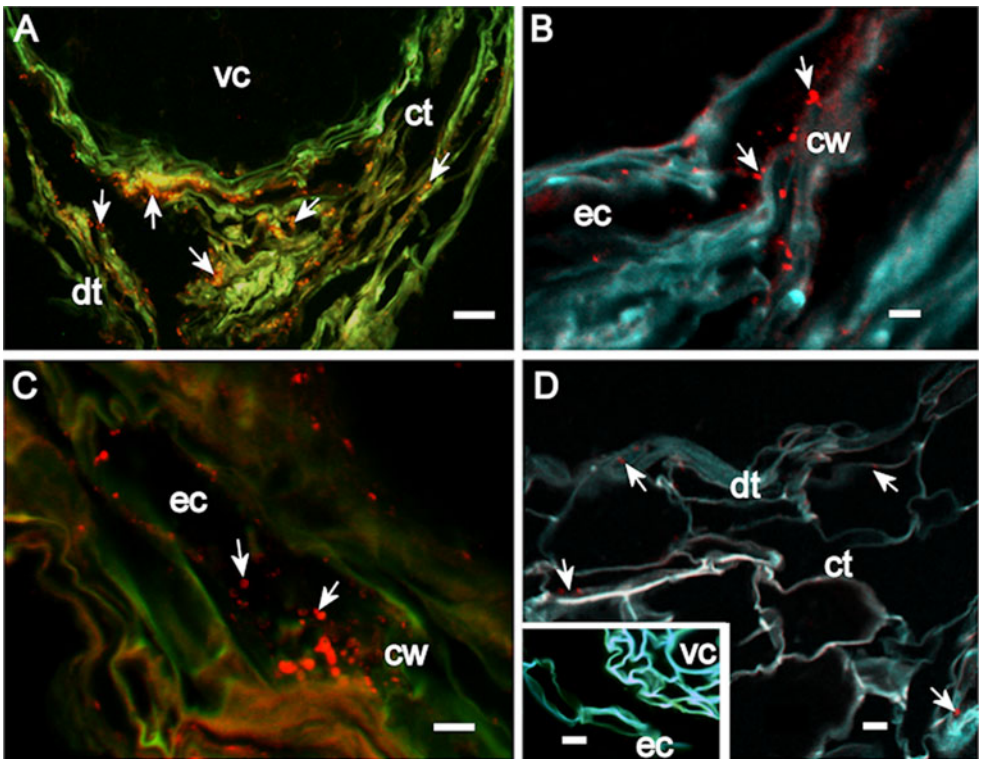

Fig. 4 **a, b** Immunolocalization of re-introduced endophytic bacteria in *Mammillaria fraileana* at day 105 after inoculation. Immunofluorescent labeling was done against *Bacillus megaterium* M1PCa, **a** epifluorescent micrograph of cross section at the base of the main root showing massive colonization of bacterial cells in dermal and cortical tissues. **b, c, d** Confocal laser scanning microscopy image showing colonization of bacterial cells close to cell walls of epidermal tissue. **c, d** Immunofluorescent labeling against *Pseudomonas putida* M5TSA, **C** Epifluorescent micrograph of root cross section indicating colonization of bacteria cells in epidermal cell. **d** Longitudinal section in the lower zone of shoot showing considerably less colonization of bacteria cells. The *insert* illustrates absence of cross-reaction among re-introduced endophytic bacteria and the elicited specific antibodies. Tissue sections with *Pseudomonas putida* M5TSA stained with fluorescent antibodies against *Enterobacter sakazakii* M2PFe. No fluorescence of bacteria is observed. **a, b, c, d** *Arrows* indicate the presence of endophytic bacteria. *DT* dermal tissue, *CT* cortical tissue, *VC* vascular cylinder, *EC* epidermal cell, *CW* cell wall. *Bars*: **a** 25 μm; **b**, **c** 5 μm, **d** and its insert = 10 μm

the undifferentiated tissue of the embryo and also in the vascular tissue. SEM showed bacteria populations distributed underneath the membrane covering the embryo; therefore, we concluded that these bacteria are genuine endophytes. Histological studies may help to determine their specific location without removing the seed coat. A similar type of colonization of the embryo during imbibition was shown for inoculated wheat seeds with actinobacteria tagged with green fluorescent protein (Coombs and Franco 2003). In our results, colonization by bacteria of the vascular system of the embryo during imbibition resembles observations in adult corn, wheat, and broccoli by Lamb et al. (1996) and Kobayashi and Palumbo (2000), who also suggested that the vascular system serves as a transport channel for some endophytic bacteria.

The levels of active endophytic bacteria found in this study by FDA are similar to the level found in seeds of the giant cardon cactus *Pachycereus pringlei* of the Baja California Peninsula (Puente et al. 2009b). The main difference is that *M. fraileana* did not harbor culturable endophytic bacteria in its seeds. This result suggests that the growth requirements of its endophytes, more than their bacterial density, are the limiting factors for isolating endophytic bacteria from seeds of *M. fraileana*. It is likely that there is a functional role for these populations of endophytes, since studies of eucalyptus trees and cardon cactus have reported culturable endophytic bacteria that are vertically transmitted to seedlings (Ferreira et al. 2008; Puente et al. 2009a) and are essential for normal growth of cacti (Puente et al. 2009a). In maturing rice seeds, endophytic bacteria exhibit attributes, such as resistance to high osmotic pressure and amylase activity, suggesting their participation in the formation and germination of seeds (Mano et al. 2006).

The population of endophytic bacteria in shoots of *M. fraileana* was far lower than average levels of endophytes in crop plants (10×10^3 CFU g fresh weight of stems) (Hallmann et al. 2006). Density of bacteria in shoots of *M. fraileana* growing on bare rocks in the field was lower than the population recovered from shoots of plants

growing in nearby rocky soil. Assuming the same pattern for roots, it is likely that low bacterial density occurs in the early stages of colonization of rocks and, as the soil develops, the bacterial community becomes more diverse. The higher density of recovered endophytic bacteria in roots, compared with that in shoots, supports the theory that endophytic bacteria originate in the rhizosphere (Hallmann et al. 1997; Misko and Germida 2002).

According to Adams et al. (1992), lithotrophic bacteria participate in the weathering of rocks in deserts. In several cases, production of numerous organic acids and lower pH are the main mechanisms responsible for decomposing rocks (Kalinowski et al. 2000; Liermann et al. 2000; Puente et al. 2006). Solubilization of minerals release essential plant elements, including phosphorus and others (Carrillo et al. 2002; Calvaruso et al. 2006; Puente et al. 2004a). In this study, accelerated degradation of rhyodacite occurred by reducing the size of particles simultaneously by reducing the pH; reducing particle size increases the reactive surfaces for mineral dissolution (Barker and Banfield 1998). Contrary to expectations, reducing the size of rock particles took place in acid and alkaline media. Increases in pH are attributed to the production of ammonia and have been related to the release of cell components when organisms die (Liermann et al. 2000). The endophytic bacteria were able to dissolve a hard form of the mineral calcium phosphate [$Ca_{10}(OH)_2(PO_4)_6$] and increase their populations. The populations grew, but without measurably releasing phosphate on even the harder-to-dissolve minerals $FePO_4{\cdot}2H_2O$ or $AlPO_4$. Since the rhyodacite substrate is low in phosphorus, it is more likely that this capacity may affect silicate minerals that release Na, K, Al, Fe, Ca, and Mg that are abundant (Lopez et al. 2009). Mechanisms for mineral solubilization by the endophytic bacteria remain unclear but may result from secreting short-chain organic acids with element-specific ligands that are able to change the pH and enhance chelation (Carrillo et al. 2002; Vessey 2003). As a consequence, dissolution of minerals outside of the plant results in the increased mobilization of elements to the plants (Puente et al. 2004a).

Nitrogen fixation is common in the rhizosphere of plants growing in soils that contain little available nitrogen (Dalton et al. 2004). The relevance of nitrogen fixation for rock-colonizing plants is considerable, since this phenomenon is uncommon in rocks without vegetation (Puente et al. 2009b), where nitrogen for endolithic microorganisms is fixed by lightning and auroras (Friedmann and Kibler 1980). Studies with desert plants of the Baja California Peninsula reveal diazotrophic endophytic bacteria *Pseudomonas stutzeri* isolated from the epiphytic plant *Tillandsia recurvata* (Puente and Bashan 1994) and various diazotrophs isolated from giant cardon cactus *Pachycereus pringlei* (Puente et al. 2004a, 2009a). This suggests a role for diazotrophs in supplying nitrogen to desert plants. Although the general nitrogen-fixing populations was significant only when roots were macerated and preincubated, only *Azotobacter vinelandii* M2Per had a significant capacity for nitrogen fixation in vitro, far better than the positive control (the diazotroph *Azospirillum brasilense* Cd). This suggests that the natural endophytic, diazotrophic bacteria may also contribute nitrogen to *M. fraileana* cacti that colonize rocks. Cardon cacti inoculated with diazotrophic endophytic or rhizoplane populations showed no sign of nitrogen deficiency even after 1 year of cultivation on rocks containing no detectable nitrogen (Puente et al. 2004b, 2009b). Further studies should focus on whether the isolates from *M. fraileana* fix nitrogen *in planta,* as shown in rice and sugarcane (Sevilla et al. 2001; Elbeltagy et al. 2001) and established associative symbiosis (James 1999). As nitrogen fixation or direct determinations of nitrogen are limited by the naturally slow growth of cacti (Nobel and Loik 1999; Puente et al. 2004b), especially in *Mammillaria* (Rojas-Arechiga and Vazquez-Yanes 2000; Lopez et al. unpublished data), alternative determinations for nitrogenase activity, such as expression of *nifH* gene or $^{15}N_2$, might be useful.

According to the definition of endophytic bacteria (Schulz and Boyle 2006), we isolated true endophytic bacteria from plant tissue whose surface has been disinfected. The capacity to re-colonize internal tissues of disinfected seedlings is an additional criterion to recognize true endophytes (Rosenblueth et al. 2004). On day 105 after inoculation, we detected internal re-colonization of endophytic bacteria in *M. fraileana,* which were healthy for the entire period. Colonization of re-introduced bacteria was remarkable in roots and decreased in aerial tissues, this pattern is similar to that found in crops (Quadt-Hallmann and Kloepper 1996; Lamb et al. 1996). Even though colonization was not quantified in this study, the frequent presence of an inoculum that formed microcolonies in peridermal and cortical tissues suggests that re-colonization was not incidental, but rather a consequence of, as yet, undefined recognition between the plant and the endophytic populations and also that the root is the main site of entry into tissues (Hallmann et al. 1997; Kobayashi and Palumbo 2000; Germaine et al. 2004).

In summary, this study revealed large quantities of non-culturable bacterial endophytes within seeds and plants of *M. fraileana.* Culturable endophytes from these sources were capable of fixing atmospheric nitrogen and solubilizing phosphate and colonizing plant tissue.

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