Microbial Populations and Activities in the Rhizoplane of Rock-Weathering Desert Plants. I. Root Colonization and Weathering of Igneous Rocks

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Abstract: **Dense layers of bacteria and fungi in the rhizoplane of three species of cactus *(Pachycereus pringlei, Stenocereus thurberi, Opuntia cholla)* and a wild fig tree *(Ficus palmeri)* growing in rocks devoid of soil were revealed by bright-field and fluorescence microscopy and field emission scanning electron microscopy. These desert plants are responsible for rock weathering in an ancient lava flow at La Purisima-San Isidro and in sedimentary rock in the Sierra de La Paz, both in Baja California Sur, Mexico. The dominant bacterial groups colonizing the rhizoplane were fluorescent pseudomonads and bacilli. Seven of these bacterial species were identified by the 16S rRNA molecular method. Unidentified fungal and actimomycete species were also present. Some of the root-colonizing microorganisms fixed *in vitro* N_2, produced volatile and non-volatile organic acids that subsequently reduced the pH of the rock medium in which the bacteria grew, and significantly dissolved insoluble phosphates, extrusive igneous rock, marble, and limestone. The bacteria were able to release significant amounts of useful minerals, such as P, K, Mg, Mn, Fe, Cu, and Zn from the rocks and were thermo-tolerant, halo-tolerant, and drought-tolerant. The microbial community survived in the rhizoplane of cacti during the annual 10-month dry season. This study indicates that rhizoplane bacteria on cacti roots in rock may be involved in chemical weathering in hot, subtropical deserts.**

Key words: ***Bacillus*, cactus, cardon, cholla, desert, *Ficus*, fig tree, fluorescent pseudomonads, lava degradation, nitrogen fixation, *Opuntia*, *Pachycereus*, phosphate solubilization, rock weathering, soil formation, *Stenocereus*.**

Introduction

Weathering (breakdown) of stone is caused by physical (wind, water, frost), chemical (air pollution, soil moisture, acid rain), and biological processes (Hirsch et al., 1995 a, b; Goudie and Parker, 1999). Microorganisms on rock surfaces, in cracks, and in pore spaces of sandstone and granite, sometimes form biofilms (a.k.a. bio-deteriorative patina; rock varnish) (Krumbein and Jens, 1981; De la Torre et al., 1993), which contribute to the breakdown of rocks. Microbial rock weathering is common in all climate zones, usually acts very slowly (Sun and Friedmann, 1999), and has been observed in hot (Adams et al., 1992) and cold deserts (Friedmann and Kibler, 1980) and in the Mediterranean region (Verges, 1985), Europe (Ascaso et al., 1990), North America (Friedmann and Kibler, 1980), Asia (Dahanayake and Subasinghe, 1990), and Antarctica (Friedmann, 1982). Most studies have focused on deterioration of exposed stones in buildings (Palmer et al., 1991), churches (Ascaso et al., 1990), monuments (Flores et al., 1997), and exposed rocks and cliffs (Danin, 1993). Most of these studies have described effects (Johnston and Vestal, 1993) and the microorganisms involved (Ferris and Lowson, 1997). Little is known about weathering mechanisms, except that some microorganisms produce acid in culture (Hirsch et al., 1995 b), or that organic acids were detected in weathered stones, making this mechanism likely (Palmer et al., 1991). Acids produced by microorganisms as by-products of their metabolism can dissolve rocks (Hirsch et al., 1995 a). This mechanism may increase availability of inorganic elements. These microbes include fungi growing in sandstone (Palmer et al., 1991; Hirsch et al., 1995 b), bacteria that dissolve otherwise insoluble Fe^{3+} oxides and oxyhydroxides in rocks (Adams et al., 1992), the ectomycorrhizal fungus *Laccaria laccata*, and phosphate-dissolving bacteria *Agrobacterium radiobacter* and *Achromobacter* sp. in the rhizospheres of pine and beech trees (Leyval et al., 1990). Phosphate-dissolving bacteria are abundant in cropland (Illmer and Schinner, 1995; Illmer et al., 1995; Chabot et al., 1996) and in mangrove tree roots (Vazquez et al., 2000). Precise data on weathering rates in most environments are not available (Danin and Caneva, 1990; Danin, 1993). Microorganisms involved in rock weathering include lichens (Barker and Banfield, 1998), fungi (Hirsch et al., 1995 b), cyanobacteria (Ferris and Lowson, 1997), many species of bacteria (Adams et al., 1992), and microalgae (Hirsch et al., 1995 b).

Biological weathering of rock by roots and microorganisms plays an indispensable role in maintaining a supply of inorganic nutrients for plants (Hinsinger et al., 1992; Hinsinger and Gilkes, 1993, 1995; Chang and Li, 1998). Effects of interactions between plants and rhizosphere bacteria in rock weathering and soil formation have received little attention, and are mostly speculative (Leyval et al., 1990; Berthelin et al., 1991). We previously described several species of desert plants, mainly cacti, growing without soil and noticeably weathering exposed

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cliffs, rocks, and ancient lava flows in hot desert areas of the Baja California Peninsula, Mexico (Bashan et al., 2002).

Building on that earlier work, this study explored some of the microbial species residing in the rhizoplanes of these plants, i.e., the external surface of roots together with closely adhering soil particles and debris (the rhizoplane forms a particular subdivision of the rhizosphere), and determined their activities, as it is hypothesized that microorganisms which participate in rock weathering, accelerate soil formation, and assist plant growth by supplying nitrogen, soluble phosphorus, and other essential minerals.

Materials and Methods

Sampling area, sampling techniques, size, and design

The geographical sources of plants were detailed previously (Bashan et al., 2002). Briefly, plants were taken from volcanic areas in the central Baja California Sur mountain range (Sierra de La Gigante) southwest of the town of Loreto (25° to 26°N in Mexico). Small specimens (5 – 20 cm tall) of three cacti, giant cardon (*Pachycereus pringlei* [S. Wats] Britt. and Ross), pitaya dulce (*Stenocereus thurberi* [Engelm.] Buxb. subsp. Thurberi), and choya (*Opuntia cholla* F. A. C. Weber), and the wild fig (*Ficus palmeri* S. Wats) growing within rocks and lacking any direct connection to visible soil were sampled. Usually, rock surrounding the plant roots was carefully broken open with a hammer and chisel to expose the cavity in which the plant was growing, and the entire plant, including root system, was removed manually. The shoots were discarded and the roots were handled as described later. Two or three young specimens of each species were removed from rocks during each of two sampling periods, one in the "dry" season (March 1999 with no precipitation since October 1998) and one in the "wet" season (September 1999 with 14.4 mm annual rainfall). Five root samples were taken from each plant for study with an electron microscope.

Temperature of rocks

Temperature of crevices in rocks of similar extrusive igneous rock at 5 sites spaced about 2 km apart was measured three times using a long probe portable digital thermometer (Cole Palmer, type E, model 8110-00, Chicago, IL). There is no shade at these sites, so artificial shade was provided to avoid overheating the thermometer from direct, strong sunlight (over 1800 µmol m^{-2} s^{-1}).

Field emission scanning electron microscopy (FESEM) and fluorescent vital staining

Root samples (0.5 – 1.5 cm long) were fixed with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) immediately after field sampling. Samples were kept on ice for 6 h during travel to the laboratory; then stored overnight at 4 ± 1 °C. The following day, roots were rinsed in the same buffer, and dehydrated in a series of ethanol concentrations increasing from 30 to 100% for 20 min at each step, and a final step in isoamyl acetate. After dehydration, samples were dried with CO_2 in a critical point dryer (Samdri-PVT-3B, Tousimis, Rockville, MD).

The dried samples were attached to stubs with double-sided adhesive tape and coated with 30 nm 60 : 40%, gold : palladium alloy foil in a sputter coater (Edwards S150B), and examined at 7 kV by FESEM (AmRay 3300FE, Advanced Metals Research Corp., Bedford, MA).

Vital staining of microorganisms with fluorescein diacetate on and in roots less than 1 mm in diameter was performed according to Söderström (1977), and observed with an episcopic fluorescent microscope (Labophot-2 A/2, excitation filter EX470 – 490, barrier filter BA520, Nikon, Japan).

Bacterial counts from roots

To isolate and count culturable rhizoplane weathering bacteria and fungi, roots removed from rock cavities were cut into pieces (0.5 to 1.0 cm long) in a laminar flow hood. Samples of roots (5 g per sample) from each species were placed in an Erlenmeyer flask with 50 ml distilled water and 20 glass beads and shaken (rotary incubator-shaker series 25; New Brunswick, Edison, NJ) at 200 rpm for 15 min at 30 ± 1 °C. Serial dilutions of microbial suspension were subsequently prepared in 0.06 M phosphate buffer saline (PBS) at pH 7.0 and plated (100 µl $plate^{-1}$) on Henderson's medium composed of (in g l^{-1}): K_2HPO_4 (0.4); $(NH_4)_2SO_4$ (0.5); $FeCl_3 \cdot 6H_2O$ (0.0166); peptone (1.0); yeast extract (1.0); glucose (10), pulverized rock (2.5), noble agar (32); at pH 7.0 (Henderson and Duff, 1963). Each plate contained (90 µm diameter size) particles of one of the following rock powders at 0.25% w/v: limestone (Ward's Natural Science Establishment, USA), apatite (Ward's Natural Science Establishment, USA), lava (La Purisima area, Mexico), granite (Ward's Natural Science Establishment, USA), and quartz (Ward's Natural Science Establishment, USA). Plates were incubated for 24 – 48 h at 30 ± 1 °C and culturable bacteria and fungi were counted. Fluorescent pseudomonads from the root surface were counted in King's B medium (King et al., 1954). As *in situ* microbial colonization of root microsites varied greatly, it was impractical to count microbes directly from the photomicrographs. Therefore, the exact number of non-culturable bacteria remains unknown. Several culturable bacterial strains were identified commercially (Accugenix, Newark, DE) by the standard microbiological molecular identification method for sequencing of 16S rRNA.

Isolation and characterization of bacterial isolates

To isolate N_2-fixing bacteria, roots were washed thoroughly with tap water and 2% Tween-20 detergent (Sigma) for 10 min. After washing, 0.5 cm root samples were cut, five pieces were put into a flask containing Rennie's semi-solid (3 g l^{-1} agar) N-free medium (Rennie, 1981), and incubated stationary for 6 days at 30 ± 1 °C. The bacterial pellicle that formed in the growth medium was extracted, serially diluted in PBS and plated on the same medium, solidified with 2% agar, and incubated for 48 h at 30 ± 1 °C. Different bacterial colony morphotypes were purified in a conventional manner on the same solid medium. Since the medium lacked a nitrogen source, all bacteria were assumed to be N_2-fixing. To measure the nitrogen-fixing activity of bacteria, 10% acetylene was injected into flasks showing bacterial growth. Acetylene Reduction Assay was done by gas chromatography, as described previously (Holguin et al., 1992). Results were therefore, expressed as nM ethylene $culture^{-1}$ h^{-1}.

To isolate culturable rhizoplane rock-weathering bacteria, the same technique for counting rock-weathering bacteria was used, and strains were purified in the same medium.

Lava rock was assayed for weathering by isolated bacteria as follows: volcanic rocks were submerged in 1 N HCl solution overnight at 28–33 °C to remove organic matter, rinsed several times with de-ionized water, and dried at 160 °C for 2 h. Rocks were pulverized in a mill (Sprecher and Schun, Industrial Control, Germany) and sieved to 120 µm. Four rhizoplane bacterial isolates (*Bacillus chitinolyticus*, *B. subtilis* var. 2, *Citrobacter* sp., and *B. pumilus* var. 2), three control bacteria (Plant growth-promoting bacteria [PGPB] *Pseudomonas putida* [Osburn et al., 1983; Meyer and Lindrman, 1986], the PGPB *Azospirillum brasilense* Cd, ATCC 29710 and the phosphate-solubilizing bacterium *Bacillus megaterium* [DSM 3228]) were used. Bacterial isolates were grown in nutrient broth at 30 ± 1 °C for 18 h at 120 rpm, and harvested by centrifugation at 1000 g for 20 min. Inocula were washed three times in sterile distilled water, and pellets were suspended in saline solution (0.85% NaCl) to a final concentration of 10^9 cfu ml^{-1}. Flasks containing (g l^{-1}) manitol (5), glucose (10), sucrose (5), and pulverized rock (1.5) in 135 ml de-ionized water were each inoculated with 15 ml of a bacterial suspension. Flasks were incubated for 28 days at 30 ± 1 °C in a rotary shaker (incubator shaker series 25; New Brunswick, Edison, NJ) at 150 rpm. Samples were taken every week for phosphate solubilization analysis, bacterial count on nutrient agar, and pH. After incubation, suspended pulverized rock powder was removed by centrifugation at 1000 g for 10 min, and pellet minerals (P_2O_5, K_2O, Ca^{2+}, Mg^{2+}, Na, Mn^{2+}, Fe_2O_3, Cu^{2+}, Zn^{2+}) were analyzed by EPA Method #3015-microwave nitric acid digestion (Kingston, 1994) employing an atomic absorption spectrometer (GBC Scientific Equipment, Australia). The concentrations of phosphate in the pellets were determined according to Jackson (1958). Dry pulverized rock and non-inoculated wet pulverized rock incubated in the same amount of medium and under the same conditions served as controls, and their minerals were analyzed at the same time as those from culture media. Additionally, weathering of rock particles was quantified by measuring diameters and surface areas of powder particles with an image analyzer (Image ProPlus 4.5, Media Cybernetics, Japan) before and after inoculation with bacteria.

Weathering was visually assayed with bacteria grown on Henderson's medium (Henderson and Duff, 1963) or Pikoskaya's medium composed of: (g l^{-1}): glucose (10), $(NH_4)_2SO_4$ (0.5), KCl (0.26), $MgSO_4 \cdot 7H_2O$ (0.1), yeast extract (0.5), $Ca_3(PO_4)_2$ (3.8), NaCl (0.2), $FeSO_4$ (0.05), agar (20), $MnSO_4 \cdot 4H_2O$ (0.2 mg l^{-1}) at pH 7.0 (Pikoskaya, 1948) at 30 ± 1 °C supplemented with 2.5 g l^{-1} crushed stone (120 µm) or insoluble phosphate source on solid medium for the following stones: marble [particle size 90 µm, Ward's Natural Science Establishment, USA, main ingredient calcite $CaCO_3$, dolomite $CaMg(CO_3)_2$, or a combination of both minerals], limestone (90 µm Ward's Natural Science Establishment, USA, main ingredient calcite $CaCO_3$), and insoluble phosphates [with solubility $AlPO_4$ < $FePO_4 \cdot 2H_2O$ < $Ca_{10}(OH)_2(PO_4)_6$, Spectrum, USA]. Growth and halo production on solid opaque medium were recorded every three days and measured in mm. Sometimes, no defined halo was observed, but the medium lost opacity.

The strains' ability (listed in Table 2) to dissolve phosphates was quantified according to Vazquez et al. (2000), using the phosphate source listed above as substrates in Henderson's and Pikoskaya's media. Strain selection varied with plant source. Strains were selected initially for detailed analysis on various phosphate sources according to preliminary screening for phosphate-dissolving bacteria, using calcium phosphate as substrate (Vazquez et al., 2000).

The four isolated bacteria (listed earlier) were tested for organic acid production by the test described above for evaluating dissolved minerals. Flasks were incubated for 14 days at 30 ± 1 °C. Volatile and non-volatile organic acids were analyzed by gas chromatography (Varian 6000 GC, Varian Instrument Group, Sunnyvale, CA) as described earlier (Vazquez et al., 2000).

NaCl tolerance was measured on commercial nutrient agar or Tryptic Soy Agar medium (Merck) in Petri dishes, supplemented with increasing concentrations of NaCl: 0.085, 0.171, 0.256, 0.342, 0.427, 0.513 M. Plates were incubated for 24–48 h at 30 ± 1 °C, and bacterial growth was observed.

For temperature tolerance, bacteria were grown similarly in Petri dishes containing nutrient agar or Tryptic Soy Agar medium at a temperature of 45 to 55 °C for 24–48 h. During incubation the agar medium remained moist.

Sample size and statistical analysis

At least 10 samples were taken at each field site. Details of sampling are provided in appropriate sections of the Materials and Methods. Each analysis included 8–10 replicates. Over 500 scanning electron microscopic images were taken and at least 10 different specimens were taken for particle analysis by image analyzer. Analytical data based on mean from three samples were assayed. Percentage data were arcsin converted before analysis. One-way ANOVA or Student's *t*-test at $p \leq 0.05$ were used for statistical analysis with Statistica software (StatSoft, Inc., Tulsa, OK). Mean values are accompanied by standard error.

Results

Colonization of cactus roots growing in soil-free rocks

In general, as expected, all cactus roots were colonized by microbes (Figs. 1–4). However, roots were not evenly colonized: young roots were heavily colonized (data shown below), while older roots lacked detectable microbe populations (data not shown). Roots were colonized in the 10-month dry season and in the 2-month "wet" season. Microbial colonization of plant roots, growing in the absence of measurable amounts of soil (aeroponic-like growth), was dense, and the types of microbe present were dependent on the season.

Specifically, during the dry season, the main colonizers were fungi (Fig. 1A). Some bacteria, having dense fibrous material (pili) (Fig. 1B), were also present, usually close to the fungal hyphae (Fig. 1C). During the "wet" season, most colonizers of the three cactus species detected on Henderson's medium were bacteria, and to a lesser extent fungi (Table 1). At least 3 bacterial morphotypes, including *Azospirillum*-like cells, were

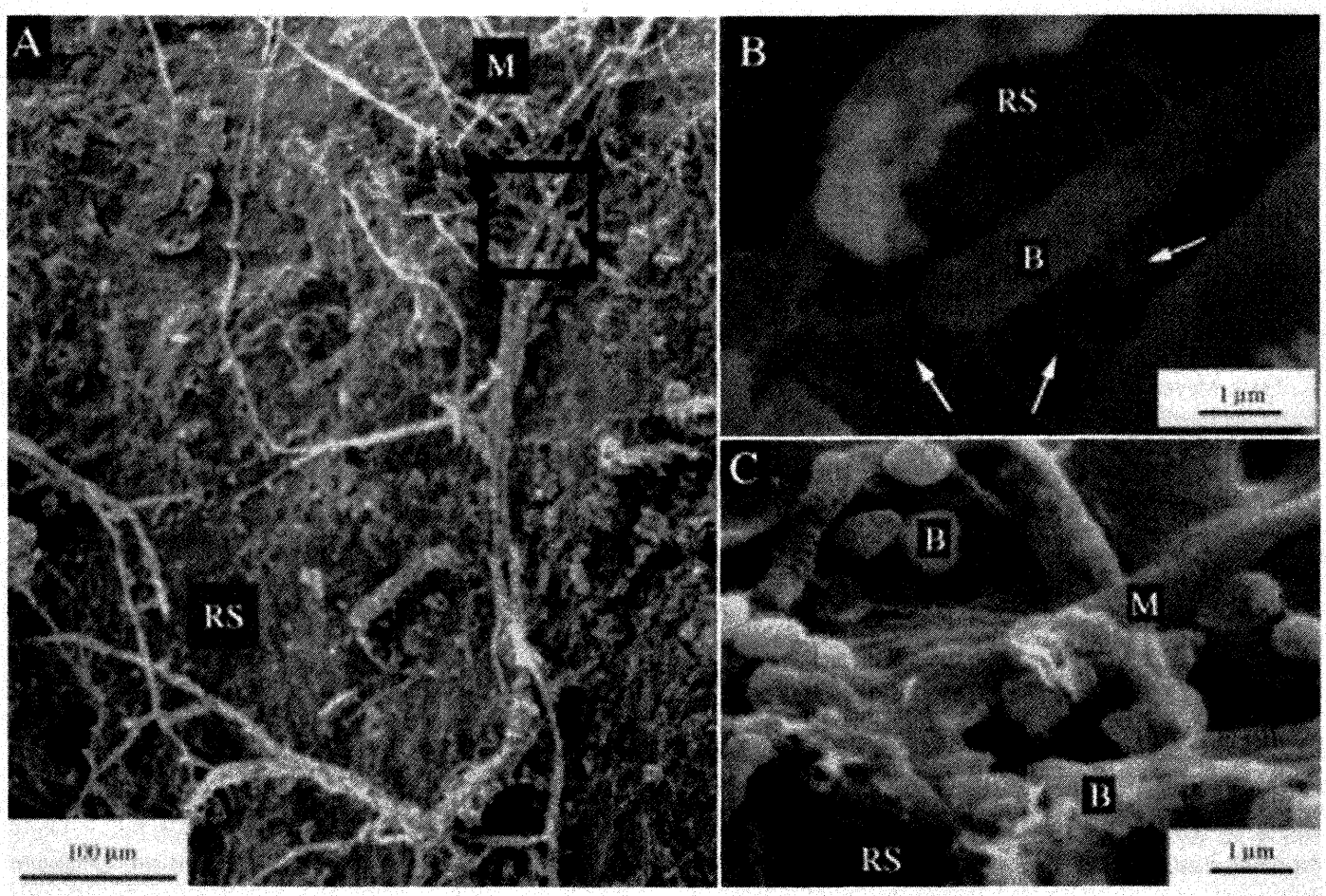

Fig. 1 Colonization of small cardon plant roots *(P. pringlei)* growing in an extrusive igneous rock cavity during the "dry season." (**A**) Coexistence of fungal mycelia and bacteria on a root surface. (**B**) Rod-shaped bacteria with abundant fibrous material (presumably pili) anchoring it to the root surface. (**C**) Higher magnification of the inset in **A**. Abbreviations: B = bacteria; M = mycelium; RS = root surface.

Table 1 Root colonization by microorganisms of two wild desert plants during the short "wet season" of September 1999, expressed as average bacterial count on Henderson's medium supplemented with different rocks, ratio of fluorescent pseudomonads to total bacteria, and number of fungal propagules

Plant species	Average bacterial count (cfu/g dw) [c] on Henderson's medium supplemented with five rock sources	Ratio of fluorescent pseudomonads to all bacteria [d]	Number fungal propagules/g fresh wt [e]
Ficus palmeri [a]	$1.62 \pm 0.042 \times 10^7$	21:163	$2.65 \pm 0.045 \times 10^6$
Pachycereus pringlei [b]	$8.0 \pm 0.193 \times 10^6$	11:1	$7.25 \pm 0.150 \times 10^5$
Pachycereus pringlei [a]	$1.19 \pm 0.018 \times 10^7$	not detected	$3.08 \pm 0.243 \times 10^6$

[a] From Sierra de La Paz.
[b] From an ancient lava flow in the La Purísima-San Isidro area.
[c] Limestone, marble, granite, apatite, quartz, and ancient lava rock mixed, separately, with Henderson's medium.
[d] Ratio between number of fluorescent pseudomonad colonies developed on King's medium and all other bacterial colonies developed on media with the five rock sources.
[e] All plates contained actinomycetes, but quantity could not be defined.

detected (Figs. **2A,B**). Fungal hyphae and bacteria shared root surfaces (Fig. **2C**), where bacteria dissolved the mucigel layer around both (Fig. **2D**). This phenomenon was observed for several bacterial morphotypes (Fig. **2F**), in which most were connected to the root surface by fibrous material or remnants of mucigel (Fig. **2E**), presumably serving as an adhesive for bacterial cells.

Several bacterial morphotypes colonized the root surfaces of young pitaya dulce plants during the "wet" season (Fig. **3**). In contrast to cardon, the roots of pitaya dulce were colonized by a greater variety of both rod-shaped (Fig. **3**) and coccal bacterial morphotypes (Fig. **3**). Fluorescent vital staining revealed numerous micro-colonies of live microorganisms (Fig. **4**). In many cases, fungal hyphae originating in roots continued growing outside, and were attached to the walls of interior stone cavities (Fig. **4**). The role of fungal activity in colonization of cavities in rocks has not been elucidated further.

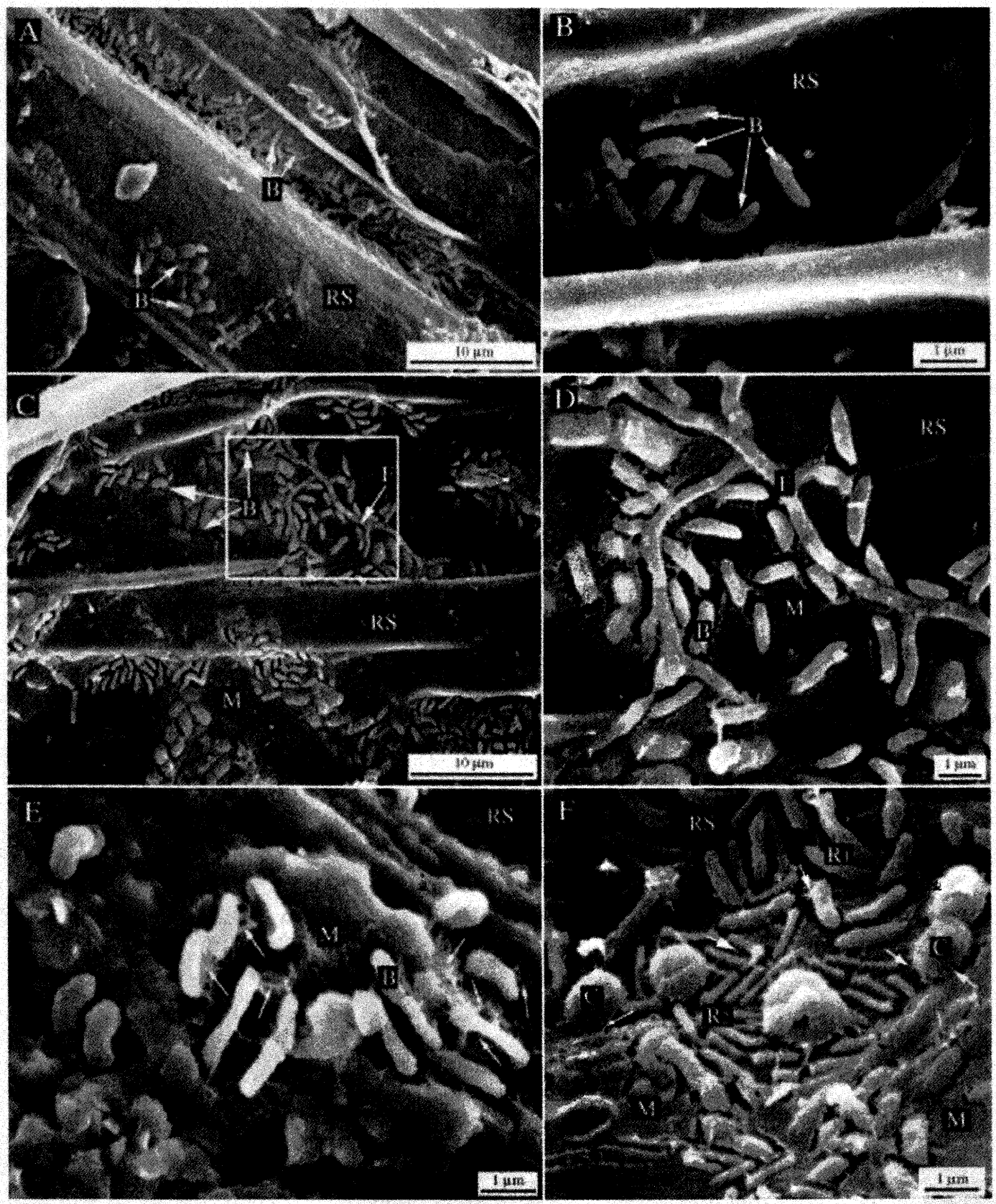

Fig. 2 Root surface colonization by microbes of a young cardon cactus *(P. pringlei)* growing in extrusive igneous rock during the "wet season." **(A)** Bacterial root surface colonization. **(B)** Higher magnification of a small section of **A**, showing *Azospirillum*-like morphotypes. **(C)** Fungal hyphae and bacteria sharing a root surface. **(D)** Higher magnification of the inset in **C**, showing partial dissolution of the mucigel layer around the microorganisms. **(E)** Small microbial colony connected to the root surface by fibrous material (arrows). **(F)** Mixed bacterial populations embedded in partially dissolved mucigel layer. Note at least two rod-shaped morphotypes, *Azospirillum*-like morphotype and a coccal morphotype. Most cells are connected to the root surface by fibrous material (arrows). Abbreviations: B = bacteria; C = coccal bacterial morphotype; F = fungal hyphae; M = mucilage substance; RS = root surface; R_1 = rod-shaped bacterial morphotype; R_2 = second rod-shaped bacterial morphotype.

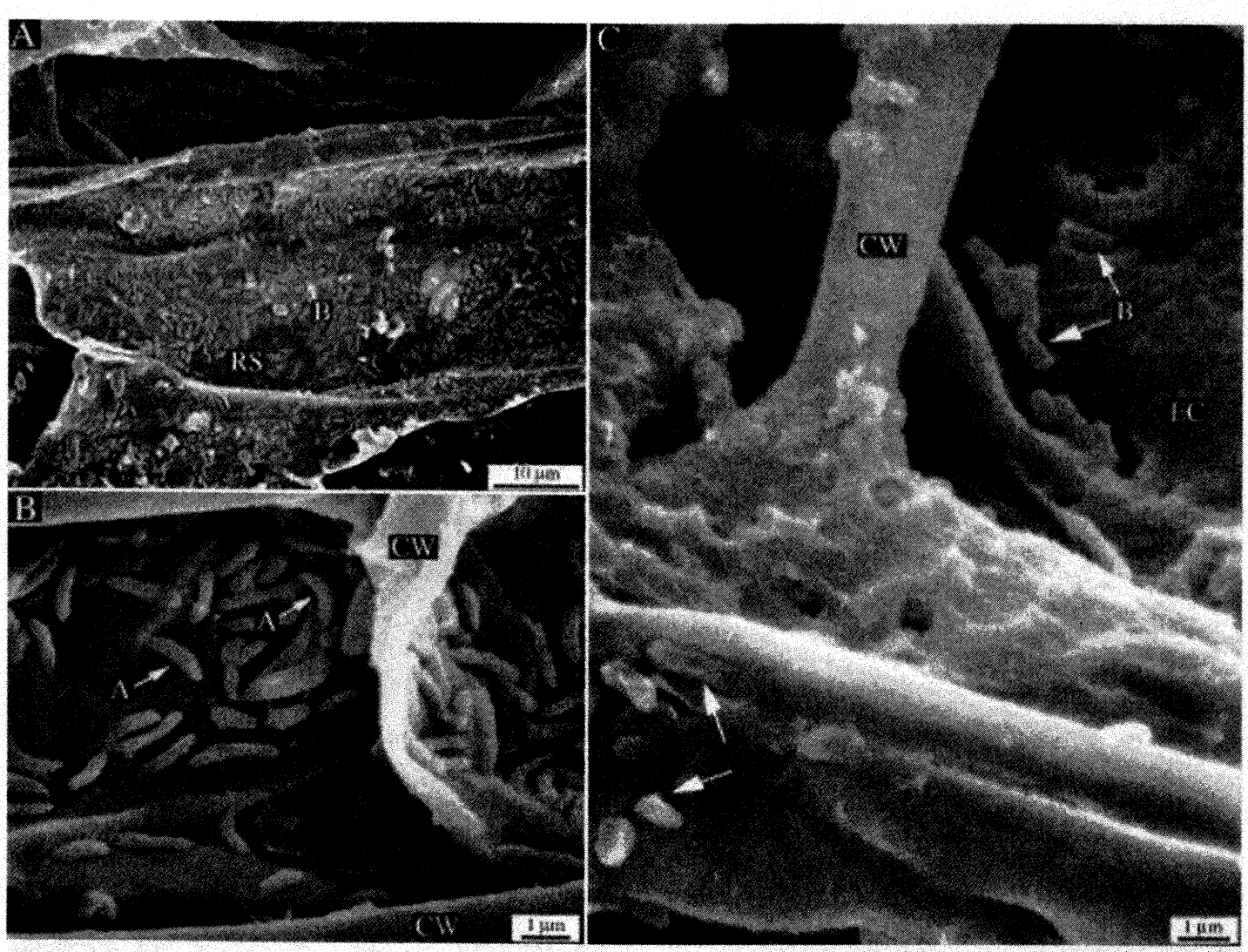

Fig. 3 Root surface colonization by microbes of a young pitaya dulce cactus *(Stenocereus thurberi)* growing in extrusive igneous rock during the "wet season". (**A**) General view showing various bacterial morphotypes. (**B**) Higher magnification of a small piece of root colonized by different bacterial rod-shaped morphotypes. (**C**) Higher magnification of a small piece of root colonized by different bacterial coccal morphotypes. Abbreviations: A = *Azospirillum*-like morphotype; B = rod-shaped bacteria; CW = cell wall; EC = epidermis cell; RS = root surface.

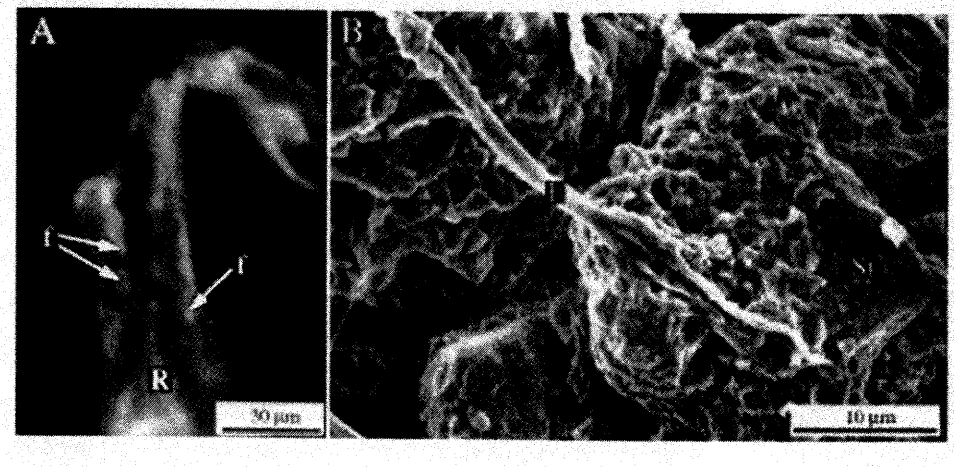

Fig. 4 (**A**) Fluorescent staining of *P. pringlei* roots. Bright spots indicate viable bacterial colonies. Original florescence is yellow-green. (**B**) Fungal hyphae originating from the surface of a seedling attached to a rock cavity where a cardon *(P. pringlei)* is growing. Abbreviations: F = fungal hyphae; f = fluorescent colony; R = root; St = stone particle.

Isolation and identification of bacteria

The recovery media for culturable bacteria varied in supplements of different rock powders. This influenced the amount of culturable bacteria recovered from each plant sample (Fig. **5**). Similar culture media used for recovery of culturable bacteria from different plants yielded different culturable populations for each culture medium (Fig. **5**). The root rhizoplane of all three cacti species were colonized (data not shown).

A large part of the bacterial population colonizing roots was fluorescent pseudomonads (Table **1**). Seven species of rhizoplane bacteria that weathered rocks in initial screening were molecularly identified as *Bacillus chitinolyticus*, *B. subtilis* var. 2, *B. fusiformis*, *B. pumillus*, *B. pumillus* var. 2, *Actinomadura oligospora*, and *Citrobacter* sp. Other strains were not identified, but are available from our culture collection.

Mineral weathering

Four identified rhizoplane bacteria, together with two PGPB species known to weather rock (as controls) were evaluated for weathering igneous rock. The clearest indication that bacteria could weather pulverized rocks was a reduction in particle diameter of powder grains after incubation (Figs. **6D,E**). *B. chitinolyticus* was the best weathering rhizoplane strain (Fig. **6A**). Particles larger than 7 µm disintegrated into smaller particles, and the number of small particles significantly increased. Similar results were obtained for *Citrobacter* sp. and *B. subtilis* var. 2 (data not shown). Incubation conditions (control flasks without bacteria), serving as a control, had much smaller effects on the reduction of particle size (Fig. **6C**). The control PGPB, *P. putida*, was capable of weathering large and small particles; and the number of smaller particles (0.1 – 2.0 µm) was far greater than the number of the larger particles that disappeared (Fig. **6B**). Incubation of rock powders in medium did not change the number of particles in the medium (from 1679560 to 1657616 particles ml^{-1} or – 1.3% change). Incubation with the control bacterium *P. putida* increased the number of particles by 15% (from 1692038 to 1946264 particles ml^{-1}), and incubation with the rhizoplane bacterium *B. chitinolyticus* increased the number of particles in the medium by 45% (from 1144464 to 1669433 particles ml^{-1}).

Significant reduction in the quantity of the nine elements tested was observed for all bacteria tested, albeit to a different extent, depending on the species. The greatest reductions among various bacterial species were observed for K_2O (up to 79.8% reduction), Ca^{2+} (up to 45.8%), Na (up to 89.2%), Fe_2O_3 (up to 30.5%), Cu^{2+} (up to 49.1%), and P_2O_5 (up to 31.2%) (Fig. **7**).

The four strains survived in pulverized lava rock culture for 28 days, and all followed close linear mortality curves with time (Fig. **8A**). During this period, the pH of all cultures decreased by 1.5 to 2.0 units, depending on the strain (Fig. **8B**). All isolated strains released orthophosphate from the pulverized lava rocks over time, but *B. chitinolyticus* released most, as did the control bacterium *P. putida* (Fig. **8C**).

Seventeen strains (seven identified) were evaluated for their ability to dissolve *in vitro* three sources of insoluble phosphate (Ca, Fe, and Al phosphates) on two solid media. None was clearly able to dissolve $AlPO_4$. Three strains had limited ability to dissolve $FePO_4 \cdot 2H_2O$, but most strains growing on Pikoskaya's medium dissolved $Ca_{10}(OH)_2(PO_4)_6$ (Table **2**). Nine rhizoplane strains dissolved pulverized marble and limestone *in vitro* to a great extent (Table **3**).

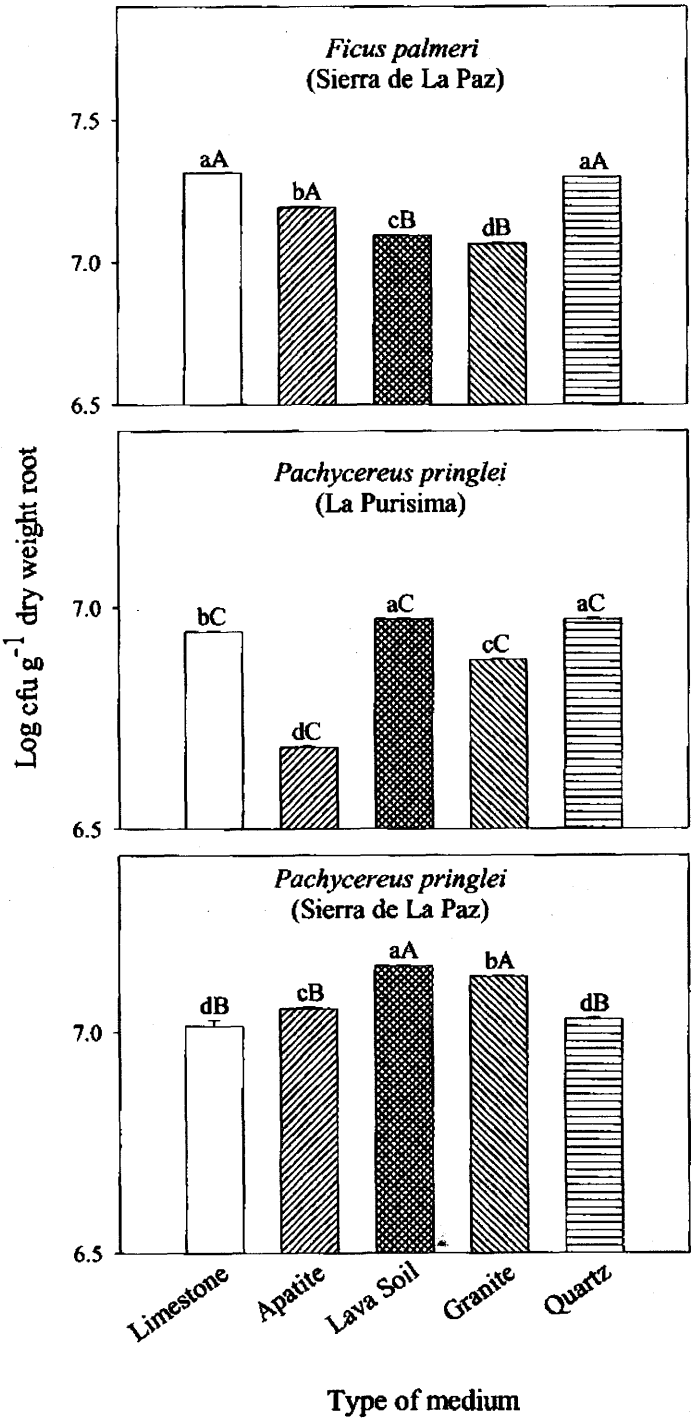

Fig. 5 Amount of bacteria colonizing roots of two wild desert plants from two desert locations during the short "wet season" (September 1999), evaluated on five different pulverized rocks added to the same culture media. Columns denoted by a different lower case letter in each sub-figure differ significantly at $p \leq 0.05$ by one-way ANOVA. Columns for each type of pulverized rock (in the three sub-figures) denoted by different capital letters differ significantly at $p \leq 0.05$ by one-way ANOVA. Bars represent standard error (SE). The absence of SE indicates a negligible value.

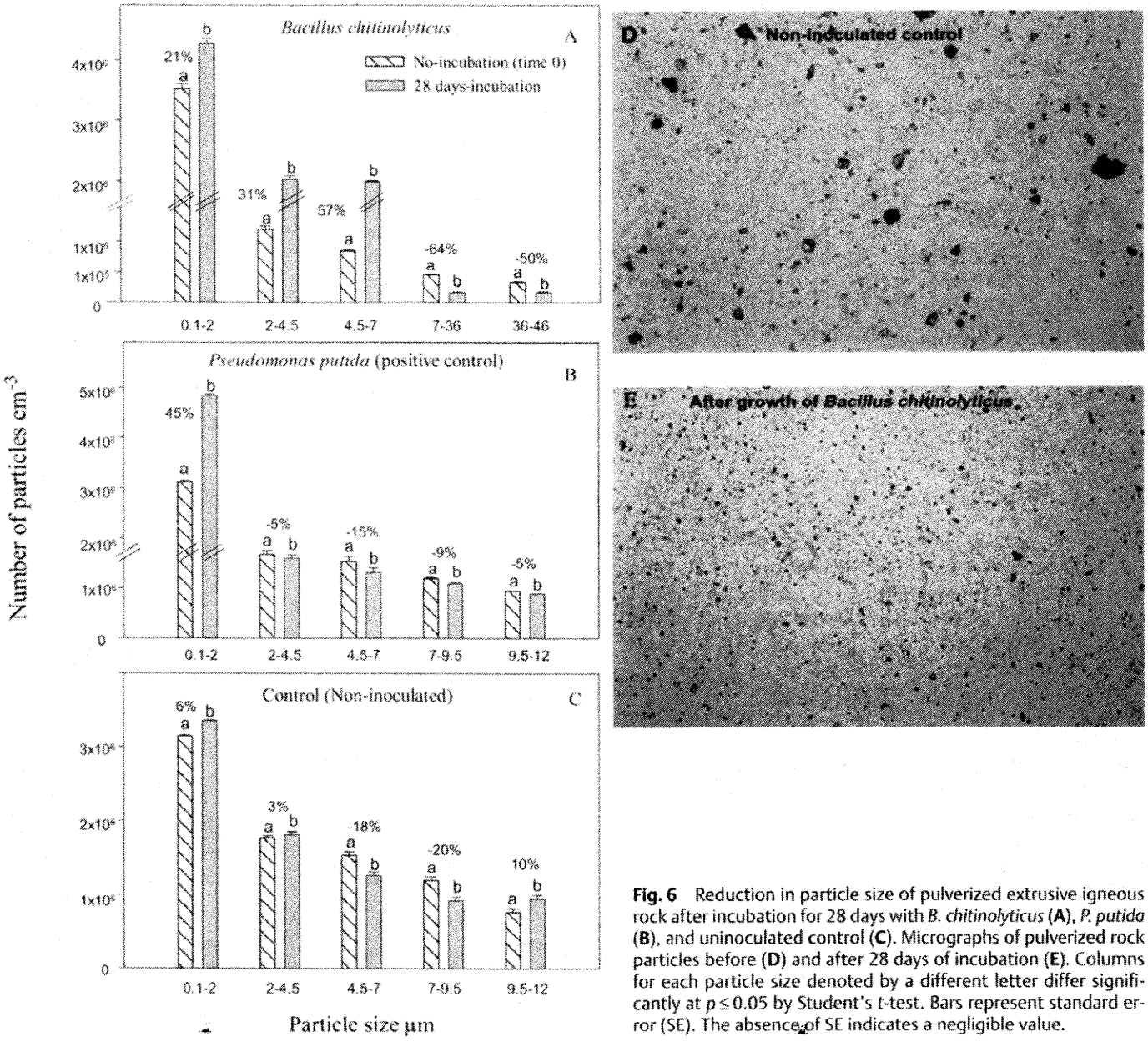

Fig. 6 Reduction in particle size of pulverized extrusive igneous rock after incubation for 28 days with *B. chitinolyticus* (**A**), *P. putida* (**B**), and uninoculated control (**C**). Micrographs of pulverized rock particles before (**D**) and after 28 days of incubation (**E**). Columns for each particle size denoted by a different letter differ significantly at $p \leq 0.05$ by Student's *t*-test. Bars represent standard error (SE). The absence of SE indicates a negligible value.

Physiological characterization of rhizoplane isolates (temperature and NaCl tolerance, N_2-fixation, and organic acid production)

While the air temperature on 17 February 2000 at 14:00 h (coldest month) was 31 °C, the internal rock cavity temperature was 40 °C. On 7 September 1999 (hottest month), the air temperature was over 42 °C and the internal rock cavity temperature was over 60 °C because there is no shade on the lava flows. Identified bacterial species growing in solid medium were tested for their ability to withstand high temperatures. None grew at 55 °C, but all grew well at 45 and 50 °C. When grown in nutrient agar and tryptic soy agar, all bacteria showed tolerance of at least 0.513 M NaCl, one of the main weathered minerals from the rock.

The 19 rhizoplane strains isolated from the cacti *P. pringlei* and *O. cholla* and wild fig tree, *F. palmeri* growing at two locations have N_2-fixing ability (acetylene reduction) *in vitro* (Table **4**).

In liquid culture supplemented with pulverized igneous rocks, the four rhizoplane strains (listed earlier) and the two positive control PGPBs, produced 12 volatile and non-volatile organic acids in large quantities. The most common acids were gluconic, succinic, and isovaleric acids (Table **5**). Additionally, each of the bacterial species produced unidentified organic acids (5 – 19) in large quantities (Table **5**, last column).

Discussion

Desert plants in the arid Baja California Peninsula of Mexico, mainly cacti, are well adapted to water scarcity and harsh climatic conditions. They are active mainly during the short,

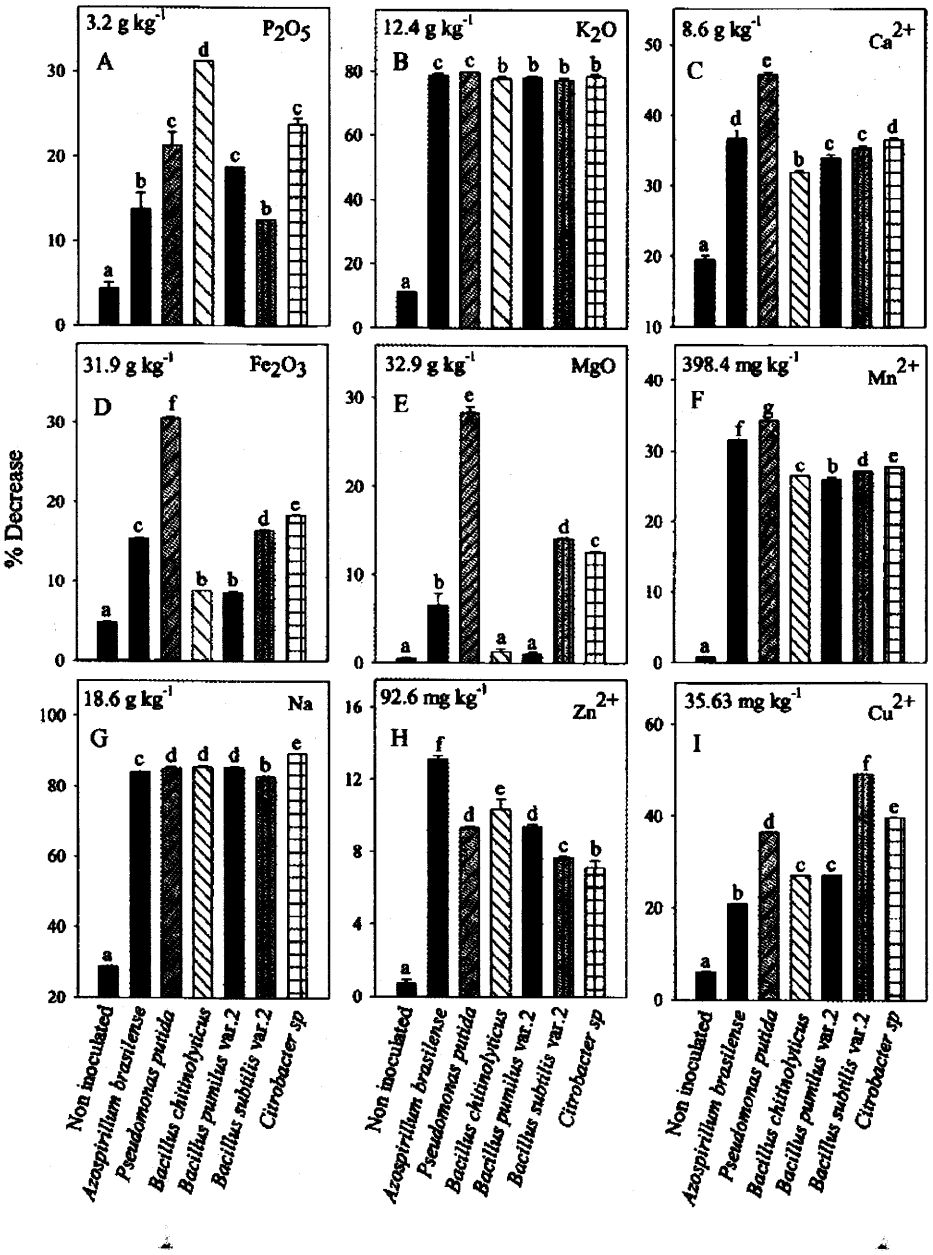

Fig. 7 Analysis of ancient lava elements before incubation and after 28 days of incubation with four bacterial species isolated from the rhizoplane of cacti and two control bacteria growing in these rocks. Number at each subfigure represents the quantity of the mineral in the rock. Results are presented as percentage decrease of the element. Columns denoted by a different letter in each subfigure differ significantly at $p \leq 0.05$ by one-way ANOVA. Bars represent standard error (SE). The absence of SE indicates a negligible value.

summer wet season, as are plants in other tropical and subtropical deserts. On alluvial plains, cacti seedlings prefer to grow under the canopy of nurse plants, such as mature mesquite, where the soil is richer in organic matter and nutrients and holds more water (Franco and Nobel, 1989; Carrillo-Garcia et al., 1999, 2000b; Bashan et al., 2000). In desert highlands, cacti seedlings grow in bare rocks and cliffs in the absence of nurse trees (Bashan et al., 2002). Our working hypothesis was that root-colonizing microorganisms on these plants participate, together with plant roots, in rock weathering and perhaps supply the plants with released inorganic nutrients and nitrogen from N_2-fixation, and that those microorganisms could withstand diverse conditions of temperature, drought, and salinity.

Microorganisms persisting in the roots of cacti in these arid, ancient lava flows are presumably drought-resistant, thermotolerant, and halo-tolerant, surviving during the 10-month dry season at elevated temperatures in rocks containing high concentrations of NaCl. The survival mechanisms are unknown since few bacterial spores were detected. Yet, large populations of live bacteria were detected in the rhizoplane by direct counting and vital staining with fluorescent dye, thus, implying an, as yet, unresolved survival mechanism of sporeless bacteria in arid zones.

Cell and colony bacterial morphotypes among the species were many and varied. Because similar culture media used for recovery of culturable bacteria from different plants yielded different bacterial populations, we concluded that different plants support different levels of colonization, and that root colonization by culturable bacteria is not homogeneous. In general, colonization of root surfaces resembled that of the plant growth-promoting bacterium *Azospirillum* sp. on other plant species: abundant fibrous material anchored microbes to root surfaces and haloes appeared in the mucigel layer covering the roots (Levanony et al., 1989; Bashan et al., 1991; Puente et al., 1999). This colonization of "aeroponically" grown

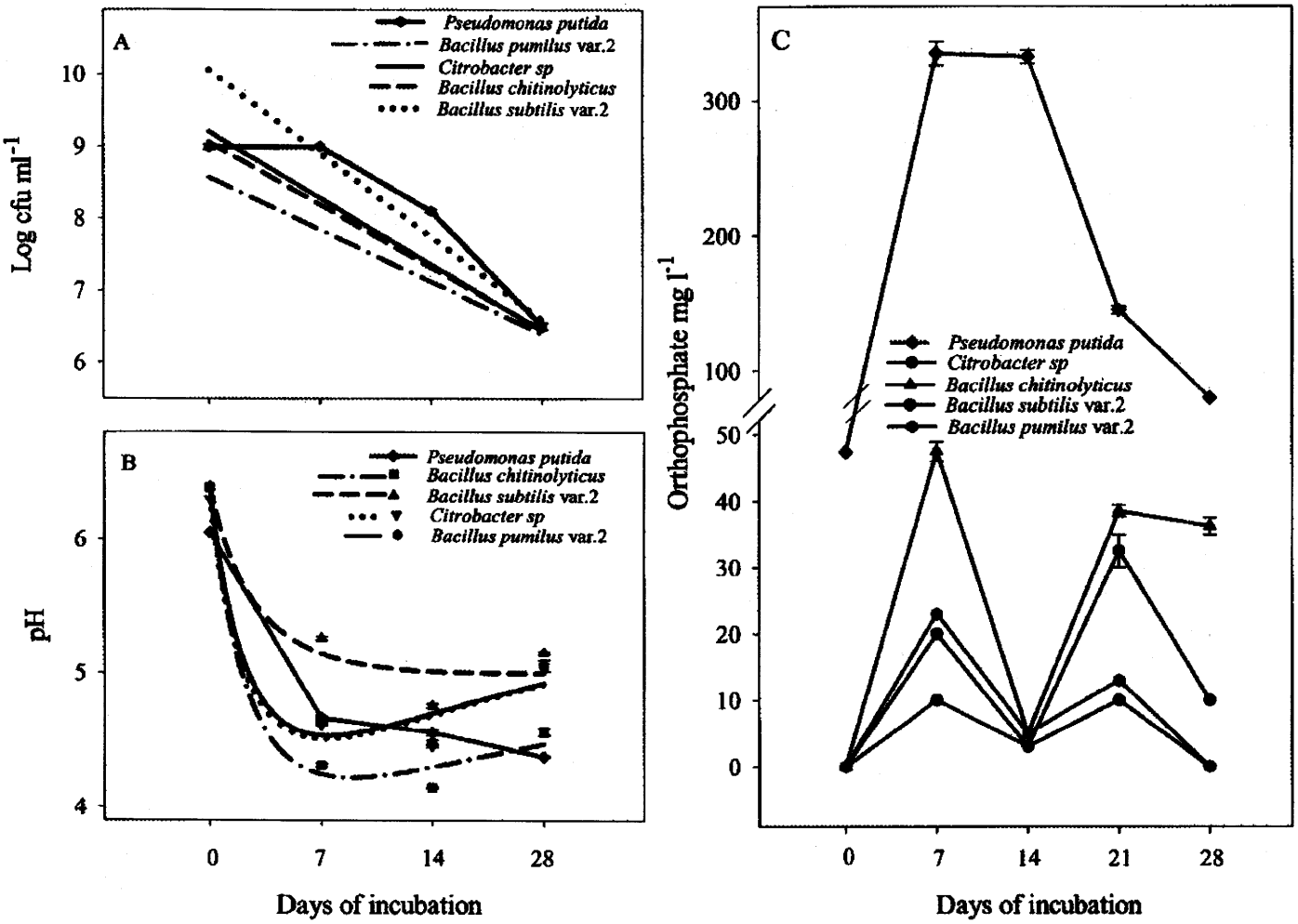

Fig. 8 (A) Linear regressions of bacterial population of four rhizoplane bacteria growing in suspension with pulverized ancient extrusive igneous rock as the sole source of minerals, with three added carbon sources. *Pseudomonas putida* served as control. Formulae of regressions are: *Bacillus pumilus* var. 2, $Y = 8.31 - 0.07\,X$, $r = -0.92$; *Citrobacter* sp., $Y = 0.93 - 0.1\,X$, $r = -0.98$; *B. chitinolyticus*, $Y = 8.71 - 0.08\,X$, $r = -0.88$; *B. subtilis*, $Y = 9.81 - 0.12\,X$, $r = -0.98$. (B) Polynomial regression of change in pH in rhizoplane bacterial culture medium. Formulae of regressions are: *B. chitinolyticus*, $Y = 6.25 - 0.28\,X + 0.01\,X^2$, $r = -0.96$; *B. subtilis* var. 2, $Y = 6.38 - 0.19\,X + 0.01\,X^2$, $r = -0.99$; *Citrobacter* sp., $Y = 6.2 - 0.24\,X + 0.01\,X^2$, $r = -0.97$; *Bacillus pumilus* var. 2, $Y = 6.28 - 0.25\,X + 0.01\,X^2$, $r = -0.97$. Bars represent SE. All regressions are statistically significant at $p \leq 0.05$. (C) Solubilization of phosphorus from rocks (release of orthophosphate with time by rhizoplane bacteria). *Pseudomonas putida* served as control. Bars represent standard error (SE). The absence of SE indicates a negligible value.

roots was detected year-round, suggesting a constant supply of nutrients from the roots to resident bacteria. The rhizoplane microorganism population was comparable to rhizosphere populations of crop plants (Sorensen, 1997). These populations far exceeded the number of bacteria normally residing in degraded rocks without plants (Adams et al., 1992; Barker and Banfield, 1998; Danin 1993; Hirsch et al., 1995 a).

Only seven strains of potential rock-weathering bacterial species were identified using molecular criteria. Clearly, many more morphotypes with similar physiological traits persist in the rhizoplane, as demonstrated in our study (Tables 1–4, unidentified strains). This limitation notwithstanding, our study identified bacterial species capable of rock weathering in ancient lava (igneous rock), marble (metamorphic rock), and limestone (sedimentary rock). Another limitation of *in vitro* studies in nutrient-rich culture media is that they show only the potential of strains for certain physiological traits. Under the nutrient-poorer conditions in the rhizosphere, these traits may not be expressed, although the cacti normally support large populations of rhizosphere bacteria (Bashan et al., 1999).

The bacteria survived in the harsh environment and proved to be thermo-tolerant, halotolerant, and possibly drought-tolerant. Moreover, these same bacteria dissolved rock and insoluble phosphates, and some fixed atmospheric nitrogen. Taken in combination, these bacteria have potential to promote growth of arid land plants under a wide variety of conditions (Puente et al., 2004).

N_2-fixing bacteria are uncommon in desert rocks lacking plants; nitrogen for endolithic microorganisms in deserts is fixed by lightning or auroras, and is then conveyed to rock cavities by occasional precipitation (Friedmann and Kibler, 1980). Nitrogen is scarce in most soils of Baja California and absent in igneous rocks (Bashan et al. 2000, 2002). Diazotrophic PGPB of the genus *Azospirillum* promote establishment and growth of cacti in eroded soils and probably contribute nitrogen to the

Table 2 Phosphate-dissolving bacteria isolated from the rhizoplane of plants growing in rocks and cultivated on media with three sources of insoluble phosphate

	Bacterial strain	Henderson's medium (mm halo)	Pikoskaya's medium (mm halo)		
		Ca	Ca	Fe	Al
Reference bacteria	*Pseudomonas putida* (positive control)	10–15 (3)	16–17 (3)	none	none
	Bacillus megaterium (positive control)	5 (16)	5 (16)	none	none
Plant species					
Giant cardon cactus (*P. pringlei*)	*Bacillus pumilus* var. 2 [a]	3 (45)	18–20 (45)	none	none
	Bacillus subtilis var. 2 [a]	16 (45)	none	none	none
	Actinomadura oligospora [b]	none	10–12 (45)	none	none
	Citrobacter sp. [a]	14–17 (45)	18–20 (45)	none	NC
	Unidentified strains				
	ICaHZ2 [NI b]	none	14 (45)	none	none
	ILCZ2 [NI b]	3 (45)	16–18 (45)	none	none
	XCaPZ102 [NI a]	none	19–20 (45)	NC	none
	VIFeCZ19902 [NI a]	none	10 (45)	5 (45)	none
	XVIIIPKCZ29902 [NI b]	10 (16)	10 (16)	none	none
	VIIFeCZ19902 [NI a]	10 (16)	NC	2 (45)	none
Wild fig tree (*F. palmeri*)	HLPLAINTDLIB1 [NI b]	none	17–18 (16)	none	none
	HLAPURINTDLIC4 [NI b]	none	15 (16)	none	none
Cholla cactus (*Opuntia Cholla*)	*Bacillus pumilus* CH008A [a]	16–17 (45)	none	5 (45)	none
	Bacillus fusiformis CH006B [a]	none	10 (45)	none	none
	Unidentified strains				
	CHLAPURCEDLIC3 [NI a]	none	15–17 (45)	none	none
	CHENDFNCE007B [NI a]	none	15 (45)	none	none
	CHENDFNCE007A [NI a]	none	10 (45)	none	none

[a] Plants from the ancient lava flow at La Purisima-San Isidro.
[b] Plants from Sierra de La Paz.
Numbers in parenthesis indicate when the first visual solubilization was detected (days).
NC = solubilization occurred, but without a defined halo.
Al = $AlPO_4$, Fe = $FePO_4 \cdot 2H_2O$, Ca = $Ca_{10}(OH)_2(PO_4)_6$, the sole phosphorus source in the medium.
NI = not identified, available from the Environmental Microbiology Group, Center for Biological Research of the Northwest, La Paz, B.C.S., Mexico.

plants (Puente and Bashan, 1993; Bashan et al., 1999; Carrillo-Garcia et al., 2000a). Growing above ground on electrical cables (Puente and Bashan, 1994), diazotrophic, endophytic *Pseudomonas stutzeri* was found in the desert epiphyte *Tillandsia recurvata* prevalent on the coastal plains of Baja California. This suggests a possible role for root-associated diazotrophs in nitrogen nutrition of desert plants. The high levels of N_2 fixation associated with the weathering of rock in this study, albeit assessed *in vitro*, are probably the result of activity of microbes in the roots of the cacti. It is likely that part of the fixed nitrogen is transferred to the plants, since they showed no sign of nitrogen deficiency, and the rock in which they live does not contain detectable nitrogen (Bashan et al., 2002). It may be possible that nutrients released or N fixed by bacteria are not necessarily available to the plants and could be leached from soil by occasional rain or taken up by the residing microorganisms.

The accelerated breakdown of rock by plants or associated biological processes, in contrast to chemical and physical breakdown, can be attributed in part to the solubilizing activity of microorganisms that colonize plant roots (Gyaneshwar et al., 1998) and the organic acids exuded by roots (Lynch and Whipps, 1990). Cardon cacti, inoculated with *A. brasilense*, excreted more protons and possibly more organic acids, which lowered the rhizosphere pH and made phosphorus more available for the plants (Carrillo et al., 2002). Other work observed that rhizosphere microflora (endo- or ecto-mycorrhizae and rhizobacteria) of maize, rice, and pine trees promote transformation of minerals (Berthelin et al., 1991). This study shows that the bacteria isolated from the rhizoplane of cacti were capable of growing on relatively insoluble phosphate powders (Fig. 8C, Table 3). Most rhizoplane bacteria dissolved calcium phosphate, which is easier to dissolve than Al and Fe phosphates (Illmer and Schinner, 1995; Illmer et al., 1995). All three phosphorus species are found in ancient lava rock (Bashan et al., 2002). This data supports a previous study (Bashan et al., 2002) that showed rock dissolution and mineral composition changes in lava rock cavities occupied by pioneer desert plants. In a different extreme climate, several Antarctic lichens weathered andesitic basalt and altered minerals as they grew, similar to the lava rock in this study (Ascaso et al., 1990).

In summary, this study showed a massive population of rhizoplane microorganisms on desert cactus roots growing in rocks in the absence of soil. These microbes, together with the roots they colonize, may perform significant weathering of volcanic and sedimentary rocks in a hot desert.

Table 3 Solubilization of marble and limestone by cactus rhizoplane bacteria

Bacterial strain	Solubilization of marble (mm halo)	Solubilization of limestone (mm halo)
Reference bacteria		
Pseudomonas putida	27–28	25–40
Bacillus megaterium	21–24	none
Azospirillum brasilense	none	none
Rhizoplane bacteria		
Citrobacter sp. [a]	25–27	27
Bacillus subtilis var. 2 [a]	18	2
Bacillus chitinolyticus [a]	16–17	17–18
Bacillus pumilus var. 2 [a]	16	15–16
Actinomadura oligospora [a]	15–18	19–21
Bacillus fusiformis HZ2 [c]	24–25	25–28
Bacillus fusiformis CH006B [b]	10	10
Bacillus pumilus CH008A [b]	18–20	19–21
CHENDFNINT005A [NI b]	18–20	17–18

None = no visual solubilization was detected.
Numbers in parenthesis indicate the first time visual solubilization was detected (days).
[a] From cardon roots.
[b] From cholla roots.
[c] From wild fig tree.
NI = not identified, available from the Environmental Microbiology Group, Center for Biological Research of the Northwest, La Paz, B.C.S., Mexico.

Table 4 N_2 fixation (acetylene reduction) of 21 rhizoplane strains isolated from three species of desert plants growing at two locations (igneous and sedimentary rocks)

Plant species	Bacterial isolate	nM ethylene culture^{-1} h^{-1}
Giant cardon cactus *(P. pringlei)*	*Bacillus pumilus* var. 2 [a]	143.88 ± 2.90
	Actinomadura oligospora [b]	141.64 ± 0.01
	Citrobacter sp. [a]	82.00 ± 0.90
	Bacillus chitinolyticus	111.37 ± 0.67
	Bacillus subtilis var. 2	176.86 ± 3.97
	Unidentified isolates	
	ILCZ2 [b]	73.14 ± 5.06
	IXFeCZ19911 [a]	95.05 ± 1.05
	gKCaZ2 [b]	83.86 ± 0.01
	IGCaZ1 [a]	104.36 ± 0.01
	IMCaZ1 [a]	70.81 ± 0.02
	IACZ1 [a]	96.91 ± 0.50
	IIICaHZ2 [b]	108.09 ± 0.11
	XIIIPKCZ29902 [b]	96.91 ± 0.01
	XVIIGCZ29911 [a]	85.73 ± 0.01
Wild fig tree *(F. palmeri)*	ILHZ2 [b]	130.45 ± 0.01
	IICaHZ2 [b]	76.41 ± 2.27
	CQHZ2 [b]	82.00 ± 0.02
	IIQHZ2 [b]	86.99 ± 0.24
Cholla cactus *(Opuntia Cholla)*	*Bacillus fusiformis* CHOO6 B [a]	104.36 ± 0.90
	Bacillus pumilus CHOO8A [a]	180.13 ± 2.15
	CHENDFNCE007A [a]	141.86 ± 2.15

Unidentified isolates are available from the Environmental Microbiology Group, Center for Biological Research of the Northwest, La Paz, B.C.S., Mexico. All values ± SE.
[a] Plants from ancient lava rocks at La Purisima.
[b] Plants from sedimentary rocks at Sierra de La Paz.

Table 5 Production of organic acids by rhizoplane bacterial strains *in vitro*

Bacterial species	Gluconic	Propionic	Isovaleric	Heptanoic	Caproic	Isocaproic	Formic	Valeric	Succinic	Oxalacetic	Oxalic	Malonic	Unidentified organic acids (No.) and range of concentrations [a]
	– µg ml^{-1} –												µV
Bacillus subtilis var. 2	133000						4500			40	80		19 (1707–4.32 × 10^7)
Bacillus pumilus var. 2	13700	410	60						90290				6 (1596–7.41 × 10^6)
Citrobacter sp.	15680		90	90	30	360			82670				13 (1807–2.95 × 10^7)
Bacillus chitinolyticus	4670		210					320		50	330	1480	22 (1547–8.41 × 10^7)
Positive controls													
Pseudomonas putida	14480												5 (1451–2.67 × 10^5)
Azospirillum brasilense	8620	2360	100	250					8150				16 (1626–2.51 × 10^7)

[a] In parenthesis: range of concentrations of unidentified organic acids.

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