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Rock-degrading endophytic bacteria in cacti

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ABSTRACT

A plant–bacterium association of the cardon cactus (*Pachycereus pringlei*) and endophytic bacteria promotes establishment of seedlings and growth on igneous rocks without soil. These bacteria weather several rock types and minerals, unbind significant amounts of useful minerals for plants from the rocks, fix *in vitro* N_2, produce volatile and non-volatile organic acids, and reduce rock particle size to form mineral soil. This study revealed the presence of large populations of culturable endophytic bacteria inside the seeds extracted from wild plants, from seeds extracted from the guano of bats feeding on cactus fruit, in seedlings growing from these seeds, in the pulp of fruit, and in small, mature wild plants, and are comparable in size to populations of endophytic populations in some agricultural crops. The dominant culturable endophytes were isolates of the genera *Bacillus* spp., *Klebsiella* spp., *Staphylococcus* spp., and *Pseudomonas* spp. Based on partial sequencing of the 16s rRNA gene, the isolated strains had low similarity to known strains in these genera. However, these strains have higher molecular similarity among endophytes obtained from seeds, endophytes from roots, and some bacterial strains from the rhizoplane. Seedlings developed from seeds with endophytes contain the similar species of endophytes in their shoots, possibly derived from the seeds. This study shows the involvement of endophytic bacteria in rock weathering by cacti in a hot, subtropical desert and their possible contribution to primary colonization of barren rock. This study proposes that cacti capable of acquiring diverse populations of endophytes may give them an evolutionary advantage to gain a foothold on highly uncompromising terrain.

1. Introduction

The weathering of rock to create new soil is a complex interaction of physical weathering, chemical reaction from air pollutants, soil moisture, acid rain, and biological processes (Hirsch et al., 1995a,b; Goudie and Parker, 1999). Microorganisms covering surfaces, fissures, and pore spaces of rocks sometimes form biofilms (De la Torre et al., 1993; Gorbushina et al., 2002) that contribute to the breakdown of rock. Microorganisms involved in rock weathering are lichens (Barker and Banfield, 1998), fungi (Hirsch et al., 1995b), cyanobacteria (Ferris and Lowson, 1997), many species of bacteria (Adams et al., 1992), and microalgae (Hirsch et al., 1995b). 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). Little is known about weathering mechanisms, except that some microorganisms produce acids in culture (Hirsch et al., 1995b). Organic acids have also been detected in weathering stones, making this a likely mechanism (Palmer et al., 1991).

Additionally, iron and sulfides in rocks can be oxidized by bacteria and be transformed into new minerals at great sea depths and in deserts (Bach and Edwards, 2003; Bawden et al., 2003; Edwards et al., 2003; Hossner and Doolittle, 2003). Acids produced by microorganisms, as by-products of their metabolism, can dissolve rocks and the resulting minerals benefit microbes and plants (Hinsinger and Gilkes, 1993, 1995; Illmer and Schinner, 1995; Illmer et al., 1995; Chang and Li, 1998; Vazquez et al., 2000; Yamanaka et al., 2003). However, precise data on weathering rates by biological agents in most environments is scarce.

We previously described several species of desert plants, mainly cacti, growing without soil and noticeably weathering rocky cliffs, large rocks, and ancient lava flows in hot desert areas of Baja California, Mexico (Bashan et al., 2002, 2006) and that their rhizoplane population is capable of dissolving minerals and assisting plant growth (Puente et al., 2004a,b).

This study explored endophytic bacteria residing in seeds and tissues of cacti growing in a hot desert and recorded this activity. We hypothesized that some of the seed endophytes moved to the

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rhizoplane and participated in rock weathering and transformation of minerals. As a result of this plant–bacteria association, soil formation is accelerated.

2. Materials and methods

2.1. 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 (25–26°N). Relatively young plants of the giant cardon cactus (*Pachycereus pringlei* [S. Watson] Britton and Rose), 5–20 cm tall, growing within rocks, and lacking any direct connection to a soil deposit were sampled. Usually, rock engulfing a plant was carefully broken open with a hammer and chisel to expose the cavity in which the plant was growing, and the entire plant, including its root system were extracted manually. The shoots were discarded and the roots were handled as described later. Two or three young plants were extracted from several rock sites during each of two sampling periods, one during the "dry" season (March 1999, no precipitation since October 1998) and one in the "rainy" season (September 1999; 14.4 mm rain per year). Seeds from a population of cardon were collected at a site near the junction of San Jose and El Rosario Roads (26°25′N, 112°8′W) and from a site at La Purisima–San Isidro (26°36′N, 112°6′W). Seeds were also collected from the following sites: Junction of the local roads Los Planes–El Sargento (24°3′N, 110°6′W), near the village of El Sargento (24°3′N, 110°6′W), the road to El Rosario (23°23′N, 110°20′W), near the village of El Triunfo (23°49′N, 110°9′W), near the village of San Pedro (23°29′N, 110°12′W), junction of the major highways from Todos Santos–San José del Cabo (23°25′N, 110°13′W), and the El Comitán federal preserve (17 km west of the city of La Paz). Bat feces in the El Comitán Federal Preserve were collected for extracting seeds.

2.2. Microscopic observations, light and fluorescent microscopy, and field emission scanning electron microscopy (FESEM)

Histological studies were carried out on 10-day-old cardon plantlets grown from disinfected seeds (described later). The roots and stem were fixed in FAA solution (50% ethyl alcohol:5% glacial acetic acid:10% formaldehyde, v:v:v) by applying intermittent vacuum. Afterwards, the roots were cut into 4 mm segments and dehydrated in increasing ethanol series (50%, 70%, and 95%, and two final changes at 100%). The fixed and dehydrated samples were imbedded in glycol methacrylate plastic (Feder and O'Brien, 1968), and cut (5–8 μm thick cross-sections) using a rotary microtome, 5 mm above the root emergence area. The specimens were mounted on glass slides, stained with 0.05% toluidine blue in 0.02 M sodium benzoate buffer, pH 4.4 (Strzelczyk and Li, 2000), and examined under a differential interference contrast (DIC) microscope (Olympus American, San Diego, CA). Total bacteria (living and dead, culturable and non-culturable) in the seeds were made visually distinct with the fluorescein isothiocyanate (FITC) stain method (Babiuk and Paul, 1970) and for the number of viable bacteria in the seeds by the fluorescein diacetate (FDA) stain method (Ingham and Klein, 1984). The slides were observed and counted under an episcopic fluorescent microscope (Leitz Laborlux-S, Wetzlar, Germany).

For scanning electron microscopy, root samples were initially kept for 6 h in an icebox during transport to the laboratory, and then overnight at 4 ± 1 °C. Root samples, 0.5–1.5 cm long, were then fixed with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and sliced with a sterile razor in half after fixation. 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 each, and finally with isoamylacetate. After dehydration, samples were dried with CO_2 in a critical point dryer (Samdri-PVT-3B, Tousimis Research, Rockville, MD).

An alternative technique to observe endophytic bacteria within inner root segments was also used, in which pieces of roots were flash frozen in liquid nitrogen. While still submerged in liquid nitrogen, the roots were cut and the epidermal cell wall was "peeled". Then, the roots were transferred to a vacuum evaporator for drying. The dried samples were fixed 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 then examined at 7 kV with a field emission scanning electron microscope (FESEM; AmRay 3300FE, Advanced Metals Research, Bedford, MA). Endophytes in fruit were detected in 1 mm thick pulp samples after preparation. The pulp samples were immediately frozen in liquid nitrogen, then exposed to high vacuum (50–100 mTorr vacuum) for 15–20 min, then coated with palladium, and examined at 15 kV with a scanning electronic microscope (Hitachi model S-3000N, Japan).

2.3. Bacterial isolation, identification and enumeration from seeds and plants

To isolate culturable root endophytic bacteria that are capable of rock weathering, root fragments (about 1 cm long) were extracted from rock cavities. Adhering rock particles were separated from roots by washing with sterile water. Roots were then immersed in a solution of 2% Tween-20 for 10 min with mild rotary agitation, and then thoroughly washed with sterile water. The root surfaces were then sterilized in 1.5% (v/v) 4% sodium hypochlorite solution (Aldrich) with agitation for 5 min at ambient temperature (27 ± 2 °C) and thoroughly rinsed with sterile distilled water in a laminar flow hood. Small pieces of root tissue (0.1–10 mm) that had been removed from either the side or the center of the vascular cylinder were placed on top of solid, Rennie's N-free medium (Rennie, 1981) in immuno-flasks and incubated for 10 days at 30 ± 1 °C. This is one of the most commonly used procedures to isolate nitrogen-fixing bacteria. Additionally, the root was then cut longitudinally with a sterile razor and incubated on Rennie's N-free medium, as described above. Selection criteria for isolation were: capacity to fix atmospheric nitrogen, phosphate solubilization, and dissolving of five rock types, all under *in vitro* conditions. Small pieces of root tissue were removed from the vascular area of the roots and transferred into flasks containing 25 ml Rennie's semi-solid (3 g l^{-1} agar) N-free medium (Rennie, 1981) and incubated for 6 days at 30 ± 1 °C without movement. Serial dilutions of developing microbial suspension were subsequently prepared in 0.06 M phosphate buffered saline (PBS) at pH 7.0 and plated (100 μl $plate^{-1}$) on Henderson's medium (Henderson and Duff, 1963). Each plate contained rock particles (90 μm diameter) of one of the following commercial rock powders at 0.25% w/v: limestone, apatite, granite, quartz (Ward's Natural Science Establishment, USA) and basalt (ancient lava flow at La Purisima, Mexico). Basalt was assayed as follows: rocks were submerged in 1 N HCl solution overnight at 28–33 °C to eliminate possible 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 obtain 120 μm particles. Plates were incubated for 24–48 h at 30 ± 1 °C and culturable, potential nitrogen-fixing and rock-solubilizing endophytes were isolated and counted. As *in situ* endophytic microbial colonization of root microsites varied greatly, it was impractical to count microbes directly from the FESEM photomicrographs. We used a fluorescent microscopy technique described later. We selected 17 different culturable bacterial strains according to their colony morphology and then identified