Title

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SPECIAL INVITED PAPER—RHIZOSPERE INTERACTIONS

A SURVEY OF THE MICROBIAL COMMUNITY IN THE RHIZOSPHERE OF TWO DOMINANT SHRUBS OF THE NEGEV DESERT HIGHLANDS, *ZYGOPHYLLUM DUMOSUM* (ZYGOPHYLLACEAE) AND *ATRIPLEX HALIMUS* (AMARANTHACEAE), USING CULTIVATION-DEPENDENT AND CULTIVATION-INDEPENDENT METHODS[1]

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- *Premise of the study:* Plant roots comprise more than 50% of the plant's biomass. Part of that biomass includes the root microbiome, the assemblage of bacteria and fungi living in the 1–3 mm region adjacent to the external surface of the root, the rhizosphere. We hypothesized that the microorganisms living in the rhizosphere and in bulk soils of the harsh environment of the Negev Desert of Israel had potential for use as plant-growth-promoting bacteria (PGPB) to improve plant productivity in nutrient-poor, arid soils that are likely to become more common as the climate changes.
- *Methods:* We used cultivation-dependent methods including trap experiments with legumes to find nitrogen-fixing rhizobia, specialized culture media to determine iron chelation via siderophores and phosphate-solubilizing and cellulase activities; cultivation-independent methods, namely 16S rDNA cloning and sequencing; and also community-level physiological profiling to discover soil microbes associated with the Negev desert perennials *Zygophyllum dumosum* and *Atriplex halimus* during the years 2009–2010.
- *Key results:* We identified a number of PGPB, both epiphytes and endophytes, which fix nitrogen, chelate iron, solubilize phosphate, and secrete cellulase, as well as many other bacteria and some fungi, thereby providing a profile of the microbiomes that support the growth of two desert perennials.
- *Conclusion:* We generated a snapshot of the microbial communities in the Negev Desert, giving us an insight in its natural state. This desert, like many arid environments, is vulnerable to exploitation for other purposes, including solar energy production and dry land farming.

Key words: *Atriplex halimus*; cultivation-dependent and -independent analysis; desert rhizosphere; trap experiments; *Zygophyllum dumosum*.

Microorganism diversity and complexity on the surfaces of plant leaves and especially plant roots are highly correlated with edaphic factors such as moisture, pH, climate, parent rock material, temperature, and nutrient and organic matter content (see references in Lau and Lennon, 2011; Brockett et al., 2012). However, the plants themselves also have a significant influence on the composition of their microbiomes, especially in the rhizosphere, that portion of the soil around the root

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where most microorganisms reside. Rhizosphere microbes inhabit a very different environment than those found in bulk soil, which is mostly devoid of plant material (Berg and Smalla, 2009), especially in arid environments (Aguirre-Garrido et al., 2012; Torres-Cortés et al., 2012). Plant processes, such as the release of root exudates and rhizodeposition—the latter originating from lysed or healthy intact cells (Dennis et al., 2010)—have a tremendous effect on the residents of the rhizosphere. As a result, rhizosphere soil not only holds more water than bulk soil, but also has higher nutrient levels and increased microbial biomass, both of which result from plant carbon output and microbial breakdown of organic matter and mineralization of organically bound nutrients, such as nitrogen (N) and phosphorous (P) (Herman et al., 1995; Schade and Hobbie, 2005).

The plants that live in arid, nutrient-poor environments, such as deserts, have been called "resource" (Halvorson et al., 1994) or "fertility" islands (Schlesinger et al., 1996) because they support a diversity of organisms within and below their root system in spite of the challenging conditions of desiccation and low nutrient availability. For example, some of the highly specific interactions observed between certain plants and microbes, e.g., the *Rhizobium*–legume symbiosis or the relationship between mycorrhizal fungi and roots or between endophytes and various plant tissues that are essential for plant growth, are excellent examples of coevolution modulated by host-produced signals (Berg and Smalla, 2009). Signal molecules produced by plants induce gene expression in the microbes, triggering various downstream responses that ultimately influence and alter the developmental and metabolic profiles of both partners.

The majority of studies on microbial communities have been performed in mesic environments, e.g., forests, grasslands, and agricultural fields, whereas many fewer analyses have been made of desert or other arid environment root microbiomes (Barns et al., 1999). However, with increasing demands for food as the world's human population expands, more and more desert land is being used for either food or biofuel crop production. Studies show that microbial communities in desert-farmed regions are significantly different from those in uncultivated desert soils (Köberl et al., 2011). Such comparisons have pointed out how quickly and significantly microbial communities in desert environments change.

Plants adapted to harsh environments and their association with microorganisms within these habitats makes both partners highly competitive and adaptive (Basil et al., 2004). For example, investigations of bacterial diversity in semiarid highlands in central Mexico revealed the dominance of *Actinobacteria*, *Proteobacteria*, and *Acidobacteria* in the rhizosphere of cactus plants (Aguirre-Garrido et al., 2012; Torres-Cortés et al., 2012). Deep sequencing analysis of the Atacama Desert in Chile revealed high numbers of novel *Actinobacteria* and *Chloroflexi* (the *Cytophaga-Flavobacterium-Bacteroides* [CFB] group) and low levels of acidobacteria and alpha- and beta-proteobacteria (Neilson et al., 2012). The latter groups are often the most dominant phyla in the rhizosphere of plants grown under optimal growth conditions. Actinobacteria (actinomycetes) are known for their ability to produce bioactive molecules as well as siderophores that improve plant growth and provide plants protection from root pathogens. Studies of actinomycete communities within various desert environments have revealed both their antifungal (Basil et al., 2004) and antibiotic (Hozzein et al., 2008) properties, suggesting that desert habitats are a source of novel bacteria for potentially useful metabolites and biotechnological applications.

Our investigation focuses principally on the microbial community under the canopy of *Zygophyllum dumosum* Boiss. (Zygophyllaceae) and *Atriplex halimus* L. (Amaranthaceae), two perennial plants that grow in the highlands of the Negev Desert in Israel. We employed both cultivation-dependent and -independent approaches to obtain an inventory of the microbes in the Negev Desert, which is divided into three regions: Beer Sheva-Arad in the north, the highlands in the center where our study was conducted, and Arava-Eilat in the south. Two sites in the highlands were chosen for study. The first site is removed from agricultural and residential land use and has a patchy distribution of *Z. dumosum*, the dominant shrub. The second site is adjacent to the residential community of Midreshet Ben-Gurion and is colonized by stands of *Z. dumosum* and *A. halimus*. By surveying the microbe population in these two different locales, we wanted to determine whether differences between the two sites could affect microbial distribution based on the presence of *Z. dumosum* or absence of *A. halimus* and also to investigate whether microbial community composition relative to two plants sharing the same site (same environmental conditions) would be similar.

We took a multifaceted approach toward obtaining a microbial profile of the Negev desert community using cultivation-dependent and -independent methods, as well as isolating both endophytes and epiphytes of *Z. dumosum* roots from site A and from the root tissues of both plants residing in site B. In addition, we performed a preliminary functional analysis to determine whether the bacteria obtained by the cultivation-dependent methods possessed traits that could make them useful as plant growth-promoting bacteria (PGPBs). To identify potential nitrogen-fixing, nodulating bacteria from our samples, we also conducted trap experiments. Through such trap experiments, we hoped to identify nitrogen-fixing symbionts that promoted the growth of native annual legumes and might also be used to improve the growth and productivity of crop legumes in dry environments.

MATERIALS AND METHODS

***Study sites, sampling, and handling procedures*—**Site A is a southeast-facing rocky slope (30°51′N 34°46′E; elevation 498 m a.s.l.) in the Negev Desert, west of the Sede-Boqer campus of Ben Gurion University, Israel. Although the aerial distance from campus is about 2 km, because of the topography of the area, site A is disconnected from any agricultural or residential activity. The slope is dominated by the endemic, perennial shrub *Z. dumosum* (Fig. 1). Site B (Zin) is a flat area of alluvial loess soil (30°50′N 34°47′E; elevation 477 m a.s.l.), facing the Zin valley on the south and southeast, and is very close to the residential area of Midreshet Ben-Gurion (Appendix S1, see Supplemental Data with the online version of this article). *Zygophyllum dumosum* and *A. halimus* are the dominant shrubs in this site. Although the two sites differ in topography, soil type, and their dominant perennial plants, the soils are poor in both environments, containing low levels of carbon and nitrogen, and have a neutral pH (for details, see Bachar et al., 2012).

For site A, samples under the canopy of *Z. dumosum* plants and from the open area between the shrubs (bulk soil [BS]) at the bottom, middle, and top of the hill were collected from a depth of 1–10 cm under three plants or areas and combined to form a composite sample (online Appendix S2). The samples were collected during both the wet (November–March) and dry (April–October) seasons of the years 2009–2011. Rainfall and soil temperature data are presented in online Appendix S2. Meteorological data were obtained from website http://www.bgu.ac.il/BIDR/research/phys/meteorology. The samples were placed in plastic bags on ice and brought immediately to the laboratory for analysis. Each sample was then divided into two parts. One-half of the soil samples collected from site A was frozen and kept for eDNA (environmental DNA) extraction and identification of noncultivatable bacteria. These samples were tripled-bagged,

Fig. 1. *Zygophyllum dumosum* in the Negev Desert of Israel at site A in 2010. (A) Perennial *Z. dumosum* plants dominate in a desert landscape otherwise composed of many annual plants, which proliferate after the spring rains (April). (B) Young plant (ca. 1 yr old) (April). (C) Same collection site in August. (D) Mature plant after losing its leaflets in summer (August). Note the rocky soil surface. All photographs by the authors.

and the root material was not removed. The second half was sieved (2 mm mesh) to separate the root segments from the soil.

For site B, soil samples under the canopy of *Z. dumosum* and *A. halimus* plants were collected during the wet and dry seasons of 2010–2011. Soil samples collected under the canopy of three plants of each species were combined to form a composite soil sample, and immediately transported to the laboratory for isolation and identification of cultivatable rhizosphere bacteria (online Appendix S3).

Soil extract preparation, cultivation, enumeration, and identification of viable bacteria—Five grams of sieved soil sample were placed into a 250 ml Erlenmeyer flask containing 50 ml of sterile 0.02 mol/L phosphate-buffered saline (PBS) and ten 3–4 mm glass beads. After samples were shaken vigorously by hand, they were put onto a rotary shaker for 30 min at 150 rpm. Samples were removed from shaker and allowed to stand for a few seconds, then 1 mL was transferred from the middle of the suspension to a tube containing 9 mL of PBS (for a 10^{-2} dilution). The suspension was mixed thoroughly and further diluted in series to a 10^{-8} dilution end point.

Three replicates of 0.1 mL from dilutions 10^{-3} to 10^{-7} were plated onto a rich, nonselective Luria-Bertani medium (LB; Miller, 1972) and two selective media, Rhizobium defined medium (RDM) (Vincent, 1970), a minimal medium containing sucrose, and semi-solid JMV medium, which can be diagnostic for nitrogen fixation, with the following composition g/L: 5.0 mannitol, 0.6 K_2HPO_4, 1.8 KH_2PO_4, 0.2 $MgS0_4{\cdot}7H,O$, 0.1 NaCl, 0.02, $CaCl_2{\cdot}2H_2O$, 0.05 yeast extract, 1.5 agar (Reis et al., 2004), and then incubated in the dark at 25°C. Colonies grown on each medium were counted, and the number of bacteria per gram of soil was calculated. Single colonies presumed to be nitrogen fixers based on growth on JMV medium were streaked on fresh agar plates of RDM and JMV for three consecutive transfers to verify that the isolates still grew on the N-free JMV medium.

Ribotyping of the soil bacteria used for the cultivation-based analysis was performed by Hy Laboratories (Rehovot, Israel); genomic DNA was extracted from a bacterial culture originating from a single colony, the rRNA genes were amplified using specific primers, and the PCR products were sequenced. The sequences were then compared against those of several databases using BLAST to find matches with the highest DNA sequence identity with the test sequences.

Ribosomal sequences of the closest matching species were found in the NCBI 16S ribosomal RNA database or in the NCBI nonredundant nucleotide sequence database when no matches were found in the 16S database. The sequences were then aligned with the program ClustalW, and neighbor-joining phylogenetic trees (Saitou and Nei, 1987) were generated in the program MEGA5.1 using the *p*-distance model.

***Isolation and identification of epiphytic and endophytic bacteria*—**Root segments were washed in running tap water to remove soil particles. The roots were then placed in 10 mL sterile tubes, immersed in 3 mL sterile ddH_2O (double-distilled water), and vortexed. The supernatant was used to prepare 10-fold dilutions for plating in triplicate onto LB agar. Bacterial colonies detected on these plates were designated as epiphytic bacterial isolates.

Root segments were surface-sterilized by transferring the roots to clean sterile tubes containing 95% ethanol for 30 s, then soaked in full-strength commercial bleach (5% sodium hypochlorite) for 5 min, and thoroughly rinsing 5–6 times in sterile ddH_2O. The surface-sterilized roots were blotted onto sterile filter paper and ground on ice with sterile ddH_2O (1:5, w/v). The extract was used for preparing 10-fold dilutions for plating on LB agar in triplicate. Bacterial colonies detected on these plates were designated as endophytic bacteria. To validate surface sterilization, we plated samples of the sequential water rinses onto LB plates to test for bacterial growth. All plates were kept in the dark at 25°C.

***Analysis of bacterial physiological activities*—**Cellulase activity was determined according to a protocol adapted from Teather and Wood (1982). Single colonies of the selected bacterial isolates were suspended in sterile ddH_2O and 5 μL subsamples were spotted onto LB plates containing 0.1% carboxymethyl cellulose (CMC). Plates were incubated for 72 h at 30°C in the dark. Cellulase activity was indicated by formation of a cleared zone after staining with aqueous Congo red (1 mg/mL) for 15 min and incubation in 1 mol/L NaCl for 15 min followed by rinsing with 1 mol/L HCl to make the haloes more visible.

For the phosphate solubilization assays, Pikovskaya phosphate medium (PVK; Pikovskaya, 1948) was solidified with 1.5% agar. The bacterial strains were grown in liquid tryptone-yeast extract (TY; Beringer, 1974) medium until stationary phase, then the cells were harvested by centrifugation (8000 × *g*, 10 min). After the cell pellets were washed with sterile water three times, they were diluted to an optical density of 0.2 at 600 nm (OD_{600}) in sterile water. Five microliter droplets were spotted onto the plates and allowed to dry right-side up for 20 min. The plates were then incubated upside down at 30°C for 10 d, and the size of the cleared zone around the colony was measured. The experiment was repeated three times, and the halo diameters measured.

Siderophore producers were detected using the method described by Pérez-Miranda et al. (2007). Plates of LB or TY media were inoculated with five evenly dispersed spots containing 5 μL of bacterial suspension. After incubation for 48–72 h, the plates were overlaid with molten chrome azurol S (CAS) medium, and a change of color was observed following incubation of the plates for an additional 24–48 h.

A modification of a method for detecting cellulase activity in fungi (Smith, 1977) was used to detect chitinase action by the isolates. Screw-cap tubes filled with 10 mL basal medium per L: 1.0 g $(NH_4)_2SO_4$; 0.2 g KH_2PO_4; 1.6 g K_2HPO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.01 g $FeSO_4 \cdot 7H_2O$; 0.02 g $CaCO_3 \cdot 2H_2O$ (pH 7.0 ± 0.5) and 15 g agar] were sterilized and solidified. An autoclaved top agar composed of a basal medium containing chitin azure (Sigma C3020, St. Louis, Missouri, USA) in a final concentration of 0.08% (w/v) in 10% agar was added to each tube. After the top agar solidified, each tube was inoculated with 10 μL of bacterial suspension. The tubes were incubated at 30°C. Migration of the blue dye from the top agar to the bottom agar layer indicated chitinase activity.

***Isolation of native nodule bacteria*—**Nodules were collected from the indigenous legume *Trigonella stellata* at site A and stored over silica gel. After surface-sterilization in 95% ethanol for 1 min and 5 min in 20% commercial bleach solution, followed by five washes in sterile water, the individual nodules were crushed with a sterile glass rod, and the diluted suspensions were plated onto TY or minimal salts (MS; Bumann et al., 1972) agar plates. After 3–7 d of incubation at 30°C, several colonies appeared. These were prepared for ribotyping.

***Trap experiments*—**Commonly used legume models were employed as "trap" plants to detect nitrogen-fixing bacteria (see Angus et al., 2013). Seeds were surface-sterilized in either full-strength commercial bleach for 1 h (alfalfa, lotus, sweet clover, white clover, siratro, *Mi. pudica, Me. truncatula*) or in 10% commercial bleach for 15 min (pea, cowpea) and after copious sterile water rinses were planted, depending on the seed size, in test tubes containing Jensen's agar (Jensen, 1942) with Hoagland's micronutrients minus N or in Magenta jars with Hoagland's minus N added to autoclaved 1:1 perlite to vermiculite. The plants were inoculated with the soil from under the *Z. dumosum* canopy from site A. After 17 or 29 d postinoculation (dpi), nodules were isolated from alfalfa roots (the only plant to nodulate), and sterilized as described. Colonies that appeared on plates were ribotyped using 16S universal primers. To fulfill Koch's postulates, a new set of alfalfa plants was inoculated with the colonies isolated from alfalfa nodules. The nodules were surface-sterilized, squashed, plated, and ribotyped to confirm their identity.

***ECO MicroPlate experiments for community-wide carbon source utilization*—**Soil samples from site A under the *Z. dumosum* canopy were sieved and mixed with sterile Milli-Q water (3 g of soil with 7 mL of water) and vortexed for 2 min. The mixture was allowed to settle for 40 min, and the supernatant was collected. ECO MicroPlates (Biolog, Hayward, California, USA) were inoculated with 100 μL of the supernatant per well (using an autoclaved soil and water mixture as a negative control). The plates were incubated for 5 d at either 30°C or room temperature and optical density readings were taken every 24 h using a microplate reader with a 595 nm filter (Bio-Rad model 680 Bio-Rad, Hercules, California, USA), providing an indirect measure of specific carbon source utilization by a colorimetric change due to accumulation of violet formazan. Heat maps of normalized, relative optical density in each condition were generated in R version 2.15.2 (www.r-project.org/).

***Cultivation-dependent isolation from ECO MicroPlates*—**Sieved desert soil samples from site A under the *Z. dumosum* canopy were incubated at 30°C overnight, mixed with sterile water, vortexed, and allowed to settle. A 110-μL sample of the supernatant was added to each well on the plate and incubated for 4 d at 30°C. Cultures from selected wells were collected and processed using the same method as for 16S rRNA ribotyping (i.e., eDNA isolation, 16S PCR amplification, cloning, transformation, clone verification and sequencing—see next section). Culture selection included wells that developed a significant, intermediate, or slight colorimetric change. The cultures from the wells of three identical carbon sources from the same plate were pooled and used for eDNA isolation.

***Cultivation-independent experiments*—**Negev Desert soil samples from the bottom, middle, and top of the hill (from under the plant canopy and bulk soil) from site A were sieved and stored at 4°C. Before isolation of eDNA, samples were incubated for 24 h at 30°C. The PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, California, USA) was used for the isolation of eDNA, using 0.25 g of sieved soil, according to the manufacturer's instructions with additional modifications to maximize the elimination of humic acids and other PCR inhibitors; the modifications include bead-beating, heating of samples, and repeated eDNA dilutions before PCR. The quality of eDNA was assessed by visualization on a 1% agarose gel using High DNA Mass Ladder (Invitrogen, Grand Island, NY, USA) as a reference. The 16S rRNA primers fD1 and rD1 (Weisburg et al., 1991) were used for amplification, and the PCR products were then verified for size on a 1% agarose gel, excised at minimal UV light exposure, and extracted from the gel using a PureLink Quick Gel Extraction Kit (Invitrogen). Isolated PCR fragments were cloned using the pCR 4-TOPO cloning vector and the TOPO TA cloning kit (Invitrogen, Grand Island, New York, USA). Transformation was done using One Shot TOP10 chemically competent *E. coli* (Invitrogen), and the products were plated on LB/Amp/X-gal plates and incubated overnight at 37°C. White colonies were inoculated into LB/Amp broth and incubated overnight in a shaker at 37°C. Minipreps were prepared using a PureLink Quick Plasmid Miniprep Kit (Invitrogen), and restriction fragment analysis confirmed the presence of the 16S fragment within the cloning vector. Verified clones were sequenced using Biosystems 3730 Capillary DNA Analyzer at the UCLA Genotyping and Sequencing Core Facility.

Sequenced clones were then identified using NCBI Standard Nucleotide Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990) against the NCBI 16S ribosomal RNA sequence database. Sequences of the eDNA isolates and the 16S reference sequences were aligned with ClustalW and a neighbor-joining phylogenetic tree was constructed in MEGA5.1 using the *p*-distance model and 1000 bootstrap replicates.

RESULTS

***Cultivation-dependent results*—**The study took place from September 2009 until August 2011 in two different sites. Site A is dominated by *Z. dumosum*, which is endemic to the

Saharo-Arabian phytogeographical territories. During the wet season, the plants are green and succulent, and the leaflets are actively undergoing photosynthesis (Fig. 1A). Numerous young plants, as well as ephemeral annuals, including a number of legume species, are visible in the rocky soil at this time (Fig. 1B). In the dry summer months, no other plants are visible except *Z. dumosum* plants, which after losing their leaflets use the evergreen petioles to photosynthesize (Terwilliger and Zeroni, 1994) (Fig. 1C, 1D). However, for most of the dry season, the plants are not actively growing, and photosynthesis is negligible. Then, as soil moisture increases, *Z. dumosum* leaflets rapidly grow out, and photosynthesis is re-established (Terwilliger and Zeroni, 1994).

Although the average yearly rainfall in the study area is ca. 90 mm, the total rainfall spanning the first year of the collection period was 155 mm, but often significantly less rain has been measured over the years; moreover, rain does not fall evenly (Appendix S3A). For example, in 2010, ca. 50% of the annual rainfall occurred within 1 d in January (Appendix S3B). In the following year, rainfall dropped precipitously (total < 50 mm); during the rainy season with the highest amount of rain, measuring 21.2 mm in February 2011 compared with 81.2 mm in January 2010 (Appendix S3B). The January 2010 rainfall resulted in a proliferation of annual plants in the study sites.

From the cultivation-dependent analyses, the number of colonies obtained on nonselective plates from under the plant canopy was always higher than in bulk soil (Fig. 2) as reported previously (Aguirre-Garrido et al., 2012; Bachar et al., 2012). Thirty-one bacterial species were identified from the rhizosphere soil at the *Z. dumosum* site A on the nonselective LB medium, whereas only 15 were identified in the bulk soil (Figs. 3A, 4). Significantly fewer bacteria appeared on the selective plates. The number of colonies detected on the selective medium JMV was highest when the plants resumed growth at the beginning of the rainy season (in November 2009), but colony number declined during flowering, fruit development, and seed ripening stages (January and April 2010). In the rainy (or wet) season, the bacteria detected on the selective medium RDM account for 80% of those grown on LB and the number decreased to about 30% during the dry season (Fig. 2). In the rhizosphere samples, the number of bacteria growing on LB medium remained unchanged throughout the wet season, but declined in the dry season. In samples of bulk soil, the number of bacteria on either LB or RDM had already decreased by April 2010, the end of the wet season (Fig. 2).

Of the 15 bacterial species isolated from bulk soil at site A, the majority (ca. 80%) were actinobacterial species (Figs. 3A, 4, BS-A). The rest were firmicutes, proteobacteria, and fungi. From under the *Z. dumosum* canopy soils collected at site A, 69% of the 31 total isolates were *Actinobacteria*, 20% firmicutes, 6% proteobacteria, and 3% fungi. Only one member of the *Cytophaga–Flavobacteria–Bacteroides* (CFB) group was found at site A under the plant canopy (3% of total, Figs. 3A, 4, Z-A) and none in bulk soil (BS-A). In site B under the *Z. dumosum* canopy (Fig. 4, Z-B), 41% of 22 total species were firmicutes, with the percentages of all the other three groups relatively lower than at the other sites. *Actinobacteria* accounted for 55% of the microbial species detected in the UP canopies of *Z. dumosum*

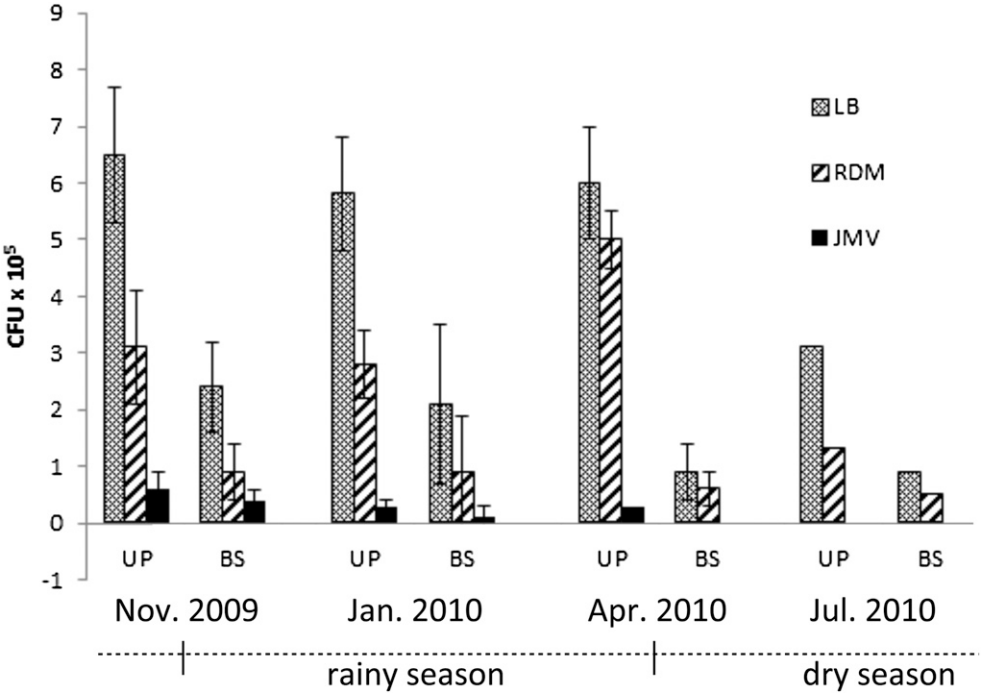

Fig. 2. Number of colony-forming units (CFU) of bacteria recovered on Luria-Bertani (LB), *Rhizobium*-defined medium (RDM), or JMV culture media. Soil samples were collected under the *Z. dumosum* canopy (UP) and from bulk soil (BS) of site A during the first year of study (2009–2010). The first rain event in 2009 occurred 30 October, and soil samples were collected 5 d later (Nov. 2009). The second sampling date was 24 January (Jan. 2010), 1 wk after a major rain (74 mm). The last rain of 2010 season occurred on 26 and 27 March, and samples were collected on 15 April (Apr. 2010). Soil samples were also collected in the middle of the dry season on 13 July (Jul. 2010). More colonies were consistently obtained on nonselective medium from under the plant canopy than in the bulk soil. The number of colonies obtained from summer samples decreased overall in the under canopy and bulk soil samples.

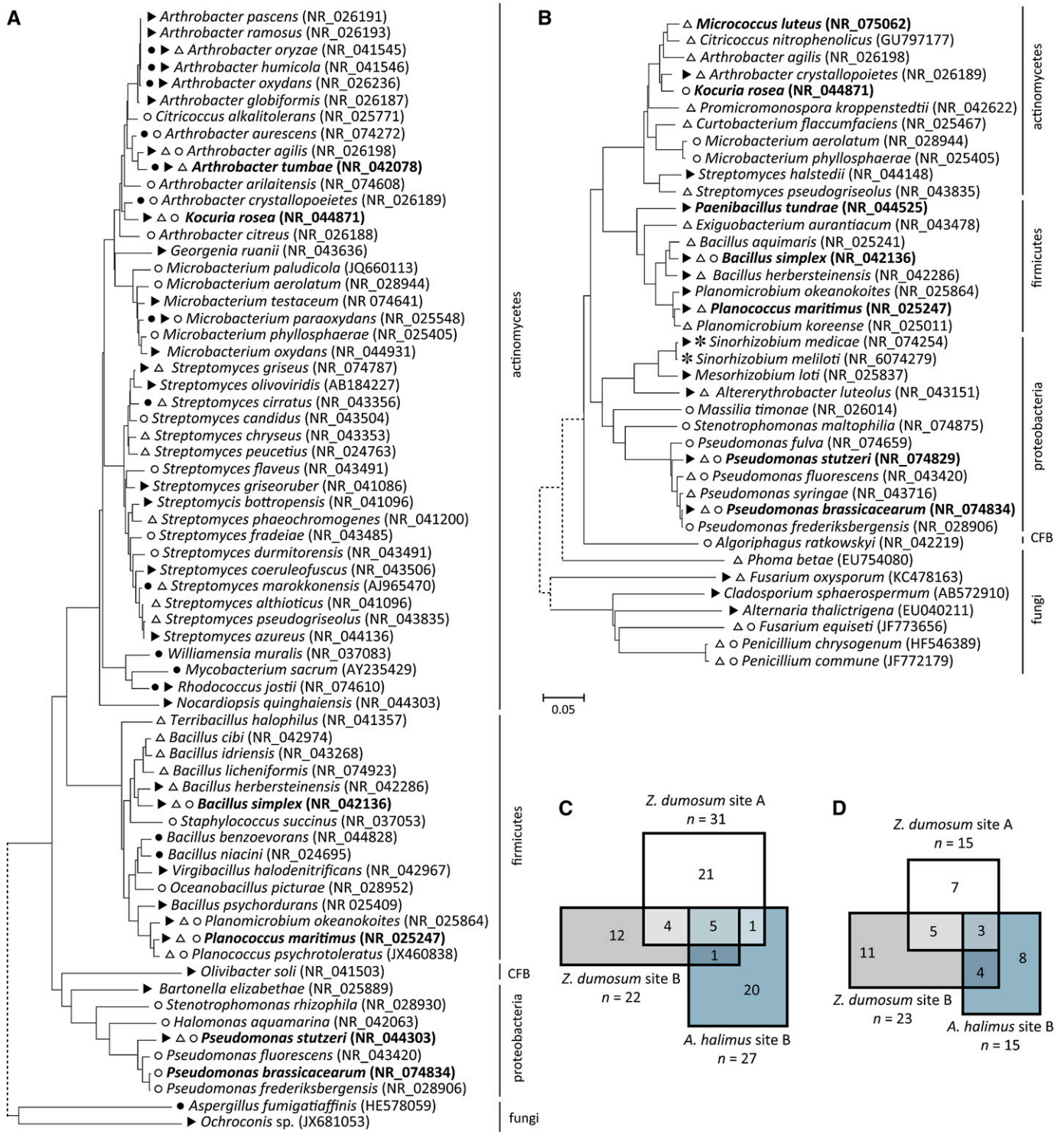

Fig. 3. Diversity of cultivatable isolates based on >97% similarity of 16S rRNA sequence. Species in bold-faced text were used for assessing physiological traits (see Table 1). (A) Phylogenetic relationship of closest sequence matches for cultivatable isolates, presumably epiphytes, found in the *Z. dumosum* rhizosphere from under the canopy of site A (▶) or site B (△), from under the *Atriplex halimus* canopy in site B (○), or from bulk soil in site A (●). (B) Putative endophytes isolated by cultivation-dependent methods from the roots of *Z. dumosum* from site A (▶), site B (△), *A. halimus* from site B (○), or legume nodule trap experiments (*). For phylogenetic trees, branches between fungi and bacteria are dashed lines and truncated to save space. (C) Overlap between cultivatable species identified under the canopy of *Z. dumosum* from sites A and B or *A. halimus* from site B. (D) Overlap of endophytic species found on the roots of *Z. dumosum* from sites A and B or *A. halimus* from site B.

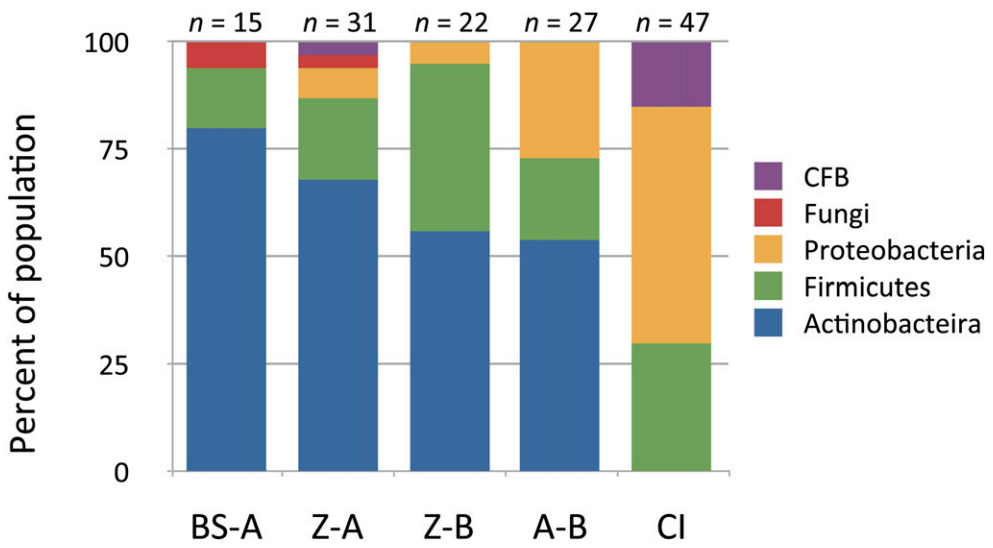

Fig. 4. Microbial community structure of cultivatable and cultivation-independent isolates in the cultivation-dependent and -independent experiments. The different colors refer to the percentage of the total number of strains identified at each location and with each method. BS-A: bulk soil, site A; $n = 15$. Z-A: under *Z. dumosum* canopy, site A; $n = 31$. Z-B: under *Z. dumosum* canopy, site B; $n = 22$; A-B: under *A. halimus* canopy, site B; $n = 27$; CI: cultivation-independent isolation method from under *Z. dumosum* canopy at site A; $n = 47$. *Cytophaga–Flavobacterium–Bacteroides* (CFB).

and *A. halimus*, growing side by side in site B, but the proportions of firmicutes and *Proteobacteria* differed in the two communities (Fig. 4, compare Z-B and A-B). The bacteria isolated from sites A and B under plant (UP) canopy soil and bulk soil (BS) from site A showed some overlap, but many fewer proteobacteria were recovered from the *Z. dumosum* UP canopy samples at site A compared to the UP *A. halimus* samples at site B (Figs. 3C, 4). Of the proteobacteria, bacteria closely related to *Pseudomonas stutzeri* were common to both sites as were relatives of the actinomycetes *Kocuria rosea* and *Microbacterium paraoxydans* and the firmicute *Bacillus simplex*.

A number of bacteria and fungi were isolated from surface-sterilized root tissue of *Z. dumosum* in sites A and B, as well as from roots of *A. halimus* in site B, suggesting that they were endophytes (Fig. 3B). Some of the same genera of actinobacteria were detected, but were related to different species based on % DNA sequence similarity than in the canopy soils. Also, a greater number of proteobacteria was detected, particularly members of the genus *Pseudomonas*. Two members of the *Rhizobiaceae*, which were closely related to *Mesorhizobium loti* and *Sinorhizobium medicae*, were also found as endophytes. Overall, the endophyte population contained a much broader representation with regards to species diversity, especially fungal diversity, than either the bulk soil or UP samples from sites A and B (Fig. 3B), with different populations in the different regions and from different plants, although the overlap was relatively higher than in the canopy soil (Fig. 3D).

***Functional studies*—**Because such a large number of diverse bacteria were recovered from under the plant canopy at site A and also as putative endophytes, we focused on only a few species for a preliminary functional analysis. Eight isolates (boldfaced in Fig. 3), representing a range of phyla and sampling sites, were examined for their ability to secrete hydrolytic enzymes, produce siderophores, and solubilize phosphate. The species analyzed included three actinomycetes, identified by 16S ribotyping to be closely related to *Arthrobacter tumbae*, a putative PGPB, *Kocuria rosea*, and *Micrococcus luteus*; three firmicutes, *Paenibacillus tundrae*, *Planococcus maritinus*, and *Bacillus simplex*; and two proteobacteria, *Pseudomonas stutzeri* and *P. brassicacearum. Burkholderia unamae* was used as a positive control for the siderophore and phosphate solubilization assays (Angus et al., 2013).

All eight isolates produced siderophores, but a species closely related to *A. tumbae* developed the greatest halo to culture diameter ratio for siderophore production. Of the eight, only the bacterium closely related to *P. stutzeri* had chitinase activity, whereas seven of eight isolates had cellulase activity, as might be expected based on the fact several were found as endophytes. Surprisingly, of the eight isolates, only one strain, a species closely related to *Paenibacillus tundrae*, solubilized inorganic phosphate when grown on PVK plates, but the amount solubilized was considerably lower than for the control *Burkholderia unamae*. The results for all tests are summarized in Table 1.

***Trap experiments and legume nodulation*—**Several commonly grown legume species including *Medicago sativa*, (alfalfa), *Lotus japonicus*, *Melilotus alba* (sweet clover), *Pisum sativum* (pea), and *Vigna unguiculata* (cowpea), *Mimosa pudica*, *Lupinus succulentus* (lupine), *Macroptilium atropurpureum* (siratro), *Medicago truncatula*, and *Trifolium repens* (white clover), were inoculated with soil from the Negev Desert, but of these, only *Medicago sativa* (alfalfa) nodulated. Five bacterial strains were isolated from the surface-sterilized nodules and used to inoculate alfalfa to fulfill Koch's postulates. *Sinorhizobium meliloti* Rm1021 was used as a positive control. Of the five unknowns, only two strains renodulated alfalfa. These bacteria were reisolated from alfalfa nodules, ribotyped, and identified as being closely related to *S. medicae* strains T10 and SWF67501 (Rome et al., 1996).

Additionally, we sought to identify native nodulating bacteria from Negev Desert legumes. Although plant cover is sparse in deserts, when rainfall is sufficient, many annual plants, particularly annual legumes, germinate, flower, and set seed before the dry season sets in. A number of legumes growing at site A were collected, identified, and prepared as voucher specimens

TABLE 1. Functional activities of a small sample of diverse bacteria from the Negev Desert Highlands assigned to certain taxonomic groups based on >98% 16S sequence identity.

Taxon	Isolated as endophyte	Cellulase activity	Chitinase activity	Siderophore release	Phosphate solubilized
Actinomycetes					
Arthrobacter tumbae	No	++	—	+++	—
Kocuria rosea	Yes	+/–	—	++	—
Micrococcus luteus	Yes	++	—	++	—
Firmicutes					
Planococcus maritimus	No	++	—	++	—
Paenibacillus tundrae	Yes	—	—	++	+
Bacillus simplex	Yes	++	—	+	+/–
Proteobacteria					
Pseudomonas stutzeri	Yes	++	+	++	—
P. brassicacearum	Yes	++	—	++	—
Burkholderia unamae	Yes	ND	ND	+++	++

Notes: +++, highest activity; ++, moderate activity; +, modest activity; +/- marginal activity; ND, not determined.

for the UCLA herbarium. These include *Trigonella stellata*, *Hippocrepis unisiliquosa* L., *Astragalus sinicus* L., *Medicago laciniata* (L.) Miller, and *Retama raetam* (Forck.) Webb. Nodules were found only on the roots of the indigenous legume identified as *T. stellata.* These were squashed after surface-sterilization, and the bacteria isolated from the nodules were identified as having 99% sequence identity to *Sinorhizobium* (*Ensifer*) *meliloti* strain RBD1 (Fig. 3B).

***Community-level physiological profiling*—**The microbial community-level physiological profile (CLPP) was examined over a period of 5 d by adding site A UP soil samples to EcoPlate wells and following the rate of utilization of 31 different carbon sources by the community over time. The metabolic patterns, presented in a heat map form (Fig. 5), show a more rapid utilization of a wide variety of carbon sources when the inoculated plates were incubated at 30°C in comparison to plates containing bacteria that were incubated at room temperature (ca. 25°C). The maximal growth was observed for the 30°C-incubated plate by day 3, whereas comparable values were recorded for the room temperature-incubated plate by day 5. These observations are consistent with the origin, composition, and dynamics of this Negev Desert microbe community, which repeatedly showed enhanced activity upon exposure to increased temperature and the ability to adapt and use a variety of substrates.

***Culturing soil bacteria using EcoPlates*—**Three replicate wells of each of the 31 carbon sources on the EcoPlate were used as a microenvironment for substrate-based selection. Soil was added to the wells, and the pooled samples were analyzed after 4 d of incubation at 30°C using 16S ribotyping. The samples chosen for analysis represented growth at either low, medium, or high optical density readings at day 4 on a variety of carbon sources. Despite the diversity of the original soil sample and the range of EcoPlate conditions, we identified six strains, all closely related to species of *Pseudomonas* (Fig. 5B).

***16S rDNA library*—**Several operational taxonomic units (OTUs), based on >97% sequence similarity, were detected (Fig. 6). The main groups of bacteria identified were members of the CFB group, the firmicutes, especially Bacillaceae, with the largest percentage being members of *Proteobacteriaceae*, especially pseudomonads (Figs. 4CI, 6). Several clones, many of which were similar to *Pseudomonas* spp., were very closely related to one another and were removed from the tree for clarity.

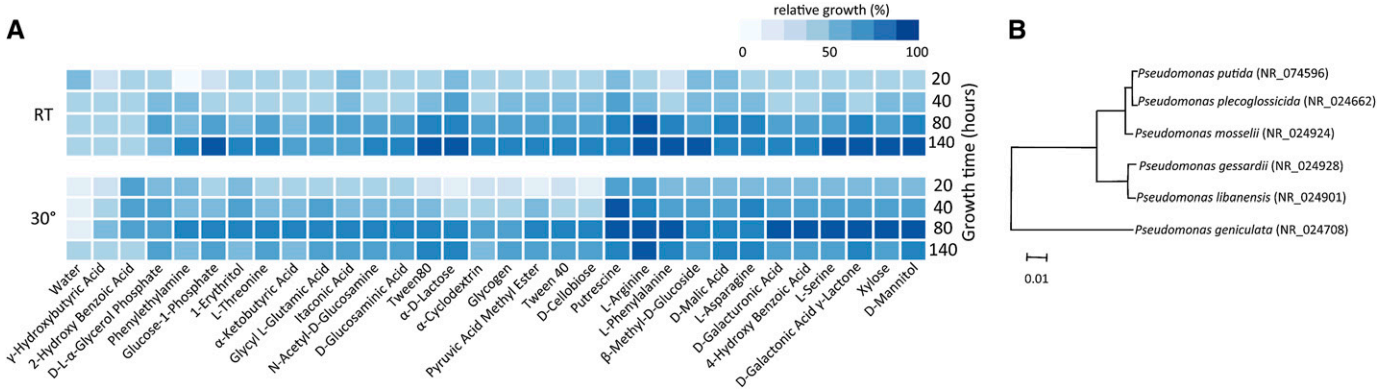

Fig. 5. Community-level physiological profile (CLPP) of the *Zygophyllum dumosum* rhizospheric soil. (A) CLPP pattern presented in a heat map form shows the rate of use of 31 different carbon sources in an EcoPlate of soil samples from under the canopy of *Z. dumosum* from site A and incubated either at 30°C or at room temperature (RT, ca. 25°C) for 5 d. Readings were taken daily. A wide variety of carbon sources was used more rapidly and maximal optical density was reached faster in plates incubated at 30°C. (B) Strains identified from pooled EcoPlate wells. Six *Pseudomonas* strains were identified using 16S ribotyping of DNA isolated after 4 days at 30°C.

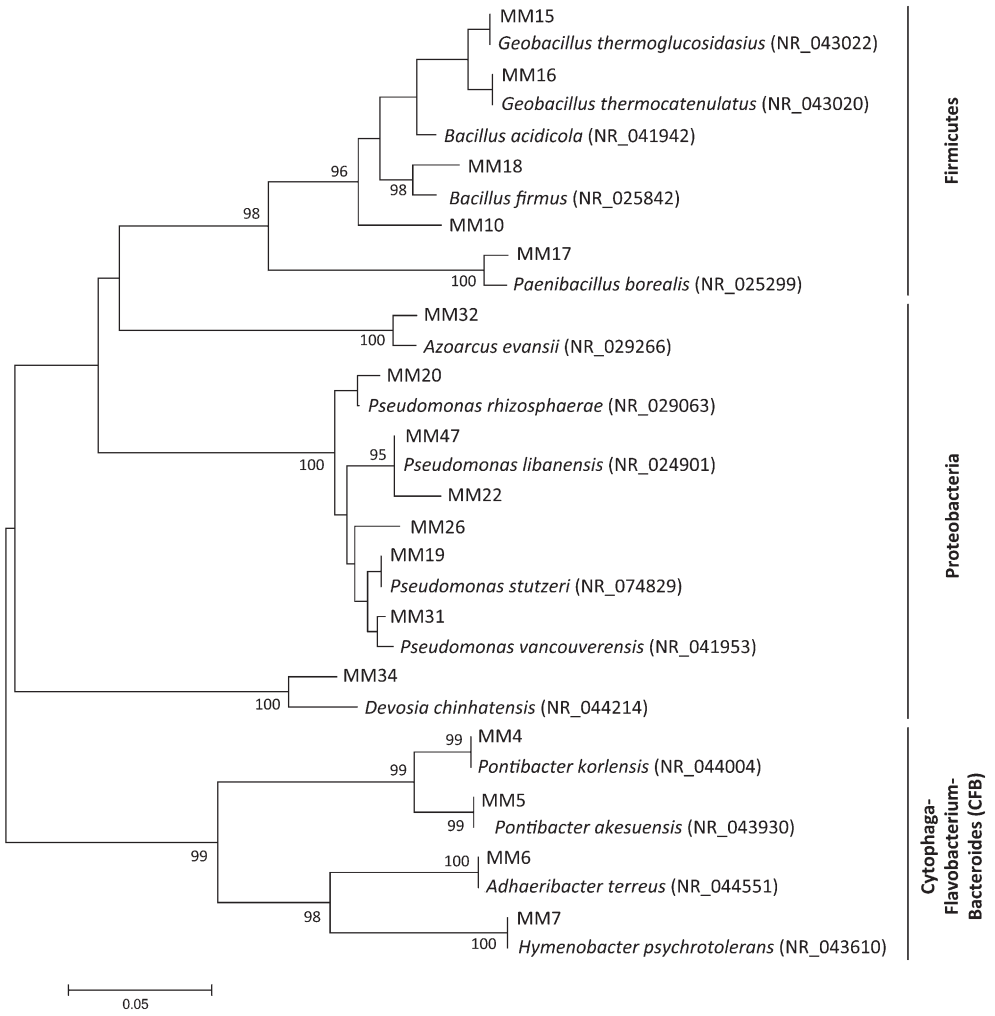

Fig. 6. Diversity of cultivation-independent isolates. Phylogenetic tree showing relationship of 16S sequences cloned from soil (MM clones) under the canopy of *Zygophyllum dumosum* from site A with closest matches to species from the NCBI 16S ribosomal RNA database. The neighbor-joining tree was generated using the *p*-distance model in MEGA5.1 with bootstrap values ≥96 displayed.

Some overlap exists between the species found in the cultivation-dependent and -independent methods; for example, for those isolates closely related to *P. fluorescens* and *P. stutzeri*.

Several of the noncultivable isolates were difficult to classify because of either low sequence similarity to known bacteria, below a value needed for meaningful comparisons, or because few closely related members have been cultured. For example, only one isolate was classified as an *Acidobacterium*, based on >98% sequence similarity to the as-yet-uncultured clone REF-081.

DISCUSSION

The Negev Desert, which occupies more than 60% of the landmass of Israel, is thought to be one of the oldest deserts in the world. In spite of low rainfall, this region is a species-rich plant community dominated by the perennial plant *Z. dumosum*. *Zygophyllum dumosum* grows on the rocky slopes in this region, whereas in the plateau areas that consist of alluvial loess soil, *Z. dumosum* and *A. halimus* grow side by side.

Several recent papers have described the microbial communities of the Negev Desert. Yu et al. (2013) investigated the microfungal community in the *Z. dumosum* and *Hammada scoparia* root zones in the northern Negev Desert using a cultivation-dependent approach and identified a number of fungal species, mainly Ascomycota, although some Zygomycota were detected. However, few fungi were detected in the bulk soil areas between the plants. In another study, the microbiomes of *Z. dumosum* and *H. scoparia* from an arid locale, and of *Noaea muchronata* and *Thymelaea hirsuta* from a semiarid site, were studied using phospholipid fatty acid (PLFA) and denaturing gradient gel electrophoresis (DGGE) analyses (Ben-David et al., 2011). Differences in the microbial communities were observed not only between the two sites, but also between the plant rhizosphere and intershrub space soil, or bulk soil, locations. A third study based on PLFA analysis corroborated the finding that bacterial abundance was significantly higher under plant canopies compared to bulk soil in the arid and semiarid sites of the Negev Desert, but found no difference regarding microbial biomass measured in a Mediterranean site (Bachar et al., 2012). The most frequently found bacteria in the last study consisted of (1) actinobacteria, most commonly detected in the

bulk soil; (2) proteobacteria, typically found under the plant canopy; and (3) acidobacteria, generally found underneath the canopy in the arid sites, but equally dispersed in the Mediterranean site (Bachar et al., 2012).

Rhizosphere microbes promote the growth of plants, particularly in harsh environments, through a number of mechanisms. We sought to identify bacterial species and characterize plant-growth-promoting behavior that may contribute to the tolerance of desert plants to a wide range of stresses. Microbes associated with *Z. dumosum* and *A. halimus* were isolated from soils sampled from two distinct locations in the Negev Desert and subjected to a multimodal approach for obtaining a broad diversity of microbes rather than maximizing the number of OTUs. To this end, we analyzed soils at different times of the year and from two distinct sites. Close relatives of the isolated microbes were identified and several physiological activities were analyzed for a small sample of these. Part of this multipronged approach included a trap experiment to identify legume-nodulating bacteria, which usually do not persist in inhospitable soils because they do not form spores.

Overall, the number of cultivatable bacteria in the BS was lower than under the canopy of the plants, regardless of the sampling season or the cultivation medium, in agreement with other reports (e.g., Aguirre-Garrido et al., 2012; Bachar et al., 2012). The total number of cultivatable bacteria grown on LB (rich medium) in the rhizosphere soil samples collected during the winter and spring (November-April) was twice that found during the summer (July) (Fig. 2). Yet, the increase in the number of bacteria grown on the selective medium RDM (clearly seen in the April sample) indicates that the community structure was changing. This change may correlate with the transition from vegetative growth to flowering and fruit/seed production, which could be related to changes in the composition of exudates released from the roots into the soil. For example, Houlden et al. (2008) observed shifts in rhizosphere microbial communities in three crop plants at different stages of their life cycles. Also noticeable is the fact that the number of bacteria in the BS decreased early (April), whereas those from UP remain high and decreased only in the dry summer. Although some seasonal effects on the microbial communities of UP and BS samples were observed, it is difficult to find any specific pattern during the 2 yr of the study because of wide fluctuations in rainfall from year to year.

Of the cultivatable isolates, minimal overlap was observed between the list of strains found in different sites under the same plant and different plants from the same site, both in the canopy region (Fig. 3C) and in endophytes (Fig. 3D). These findings suggest that both biotic (plant-related) factors as well as abiotic (edaphic) factors are involved in the shaping of the microbial communities associated with the plant root system as has been reported in other studies. We also found that a large number of microbial species, including several members of the *Rhizobiaceae*, live as putative endophytes within desert plants. In addition, most of the fungi detected by cultivation-based methods were isolated from surface-sterilized root tissues, suggesting that the endophytic life style may protect these microbes from the harsh desert conditions. However, at this time, we do not know whether any of the endophytic bacteria have plant-growth-promoting activities or whether the endophytic fungi function as mycorrhizae.

Many of the microbes isolated by the cultivation-dependent method were actinomycetes or firmicutes (Fig. 3), and of these, some were closely related to *Streptomyces phaeochromogenes*, *Planococcus psychrotoleratus*, and *Bacillus psychrodurans*, which have been reported as being cold tolerant. *Planomicrobium okeanokoites*, a CFB member, may also be cold tolerant. On the other hand, no putative cold-tolerant actinomycetes were identified through the cultivation-independent approach, but several were found to be related to members of the CFB group, e.g., *Pontibacter* spp. and *Adhaeribacter terreus*, both of which are reported as surviving in temperatures ranging from 6°–7°C to 33°–45°C (Zhang et al., 2008, 2009). Firmicutes, that are closely related to *Geobacillus thermoglucosidasius* and *G. thermocatenulatus*, known thermophiles, and capable of growing at temperatures as high as 68°C (Nazina et al., 2001), were also found. Although we have not tested whether the Negev Desert isolates exhibit the same traits, they likely do, based on the conditions that the bacteria must tolerate in the Negev Desert highlands. The air temperatures span from a low of 6°–7°C in winter months (January–February) to highs of 33°–35°C in the summer (Appendix S2). Soil temperatures may change even more, e.g., reaching almost 50°C in the summer. Thus, many of the microbes in the Negev Desert are likely to be tolerant of very severe temperature fluctuations.

In addition, many of the microbes isolated at the two sites are presumed to be salt-tolerant and/or alkaliphilic based on the fact that some isolates share sequence identity with *Nocardiopsis quinghaiensis* (Chen et al., 2008), *Oceanobacillus picturae*, *Bacillus pichinotyi* (Chowdhury et al., 2009), and *Halomonas* spp. (Mata et al., 2002), which are reported as being salt-tolerant. Although we found some isolates with high sequence identity to *Citricoccus alkalitolerans* (Li et al., 2005) and *Exiguobacterium aurantiacum* (Mohanty and Mukherji, 2008), they may not necessarily be alkaliphilic because the pH in the Negev Desert has been measured as neutral (Bachar et al., 2012).

We found five species of *Rhizobiaceae* in the Negev desert and then only by analyzing surface-sterilized root tissues for endophytes or by using trap experiments. We had expected more because at least six different legumes are found in the Negev Desert (Grünzweig and Körner, 2001). However, because the soil was compacted, we were unable to dig very deep, and thus, nodules were recovered only from *Trigonella stellata*. A diversity of nonnative legumes were reinoculated with bacteria isolated from the nodules as well as soil samples from under *Z. dumosum*, but only alfalfa was nodulated. The two species isolated from these nodules, which were closely related to *S. meliloti* and *S. medicae,* might some day serve as inoculants for providing fixed nitrogen to alfalfa varieties living in arid environments. In addition to nitrogen-fixing bacteria, several of the isolates exhibited activities typical of PGPB such as cellulose and chitin degradation and release of siderophores. However, few of the isolates solubilized rock phosphate. This may be a result of poor growth on the Pikovskaya phosphate medium (data not shown). Overall, these preliminary findings support the hypothesis that the bacterial community associated with *Z. dumosum* and *A. halimus* is likely to contribute to the plants' tolerance of the environmental conditions prevailing in the Negev Desert.

Only one isolate with close similarity to a plant pathogen, *Pseudomonas syringae* (Fig. 3B), was detected as an endophyte. Several bacteria isolated by the cultivation-dependent method were reported as "clinical isolates" based on the sequence similarity, but many were isolated as endophytes. For example, isolates showing high sequence identity to *Microbacterum paraoxydans*, *Bacillus idriensis*, and *Massilia timonae* were found in the surface-sterilized root samples (Fig. 3B). A single isolate from site A under the *Z. dumosum* canopy exhibited

similarity to *Bartonella elizabethae*, a potential canine pathogen. Overall, very few potential pathogens were detected. Nevertheless, more rigorous studies will be required to determine whether these bacteria with sequence similarity to clinical isolates are opportunistic pathogens or not.

Using a cultivation-dependent methodology, we identified a large number of actinobacteria, firmicutes, and proteobacteria, but few CFB members. We detected only one acidobacterial species, which was identified by cultivation-independent methods, perhaps because culturing this group of bacteria is still limited (Nunes da Rocha et al., 2009. However, acidobacteria are found in environments ranging in temperature, soil type, and pH (Barns et al., 1999), and thus some species will likely be present in the Negev Desert. Their lack of detection may be due either to a defect in our analysis or because the Negev Desert is inhospitable to acidobacteria for as yet unknown reasons. On the other hand, CFB members are usually not found in desert soils (Yamada and Sekiguchi, 2009). Nonetheless, the cultivation-independent analysis of the Negev Desert soils yielded more representatives from this group than did the cultivation-dependent methodology. This result may have occurred in part because CFB species are slow-growing and require longer culturing times, and thus were missed.

The isolates in the CLPP analysis are heavily dominated by *Pseudomonas* spp., which were not as prevalent as expected in the cultivation-dependent and independent studies, although more pseudomonads were found using the latter analysis. These results emphasize and reinforce the great complexity of the soil microbiome and the need to examine this heterogeneous community using different approaches and methodologies. The predominance of *Pseudomonas* among the isolates analyzed by the community-level profiling method might be due to their selection during growth in the wells of the EcoPlate, a method known to detect changes in community structure (Smalla et al., 1998). Although Classen et al. (2003) indicated that incubation temperatures do not influence CLPPs performed in EcoPlates, we found a 2-d difference between incubation at 30°C and room temperature in reaching an optimal color change. Also, it is possible that the 4-d time period used for the cultivation-dependent analysis was not long enough for a fully completed CLPP. The most parsimonious explanation for our results may be that the short period of incubation in the EcoPlate culture medium selected for faster-growing bacteria, which in our study were pseudomonads.

Several reports have addressed the obstacles, pitfalls, and variations that occur in microbial community analysis (Berg and Smalla, 2009; Pan et al., 2010; Weaver, 2012). The numerical dominance, relative abundance, and colonization patterns of a species within a microbial community may have a strong influence on selection and isolation in both cultivation-dependent and -independent methods, yielding an incomplete representation of a community. Sample collection time, conditions at the site of origin, and methodology are critical factors in experimental design and variability within those factors would yield multiple profiles of an apparently identical microbial community. Environmental DNA extraction and amplification methods may also present a biased selection toward species that are easily lysed because they are less susceptible to the effect of inhibitors such as humic acids. The overall effect of the various factors mentioned would result in a gross under-representation of a diverse rhizospheric environment, a prevalent problem in microbial ecology termed "the tragedy of the uncommon" (Bent and Forney, 2008).

In this study, we have generated a snapshot of the microbial communities in the Negev Desert during the years 2009–2010. We did this by obtaining both rhizosphere microbe sequence information and preliminary data on the phenotypes of some of the bacteria within that community. Our aim was to gain insight into the natural state of the Negev Desert. Investigations of environments such as deserts are especially critical because more and more of them are being repurposed for other uses. The exploitation of deserts for agricultural land (Köberl et al., 2011) or solar collection fields (http://blogs.kqed.org/climatewatch/tag/mojave-desert/) severely impacts both macro- and microorganismal communities. Not only is it essential to take note of the current status of these desert microbial communities, but also it is important to learn how they influence the growth and survival of their host plants and maintain biological diversity before irreversible changes are established. For one, knowledge of desert plant microbiomes will help in the management of plant rhizospheres to restore degraded desert land (de-Bashan et al., 2012). For another, we believe that "mining" for plant-growth-promoting bacteria from arid environments could provide microbes for stimulating crop growth in environments that are undergoing climate change. Arid and semiarid regions in particular are much more sensitive than other parts of the globe to climate change (Yair et al., 2008) and will need the input of dry-land-evolved microbes for soil restoration in areas of severe climate change. Strategies to find new crops and growth-promoting microbes for parts of the world where rainfall patterns are already changing are underway in Western Australia, where researchers are investigating South African legumes and their nitrogen-fixing bacteria for developing new forage crops (Howieson et al., 2013). Our study has lead to the isolation of *S. medicae* and *S. meliloti* strains that may be useful for growing alfalfa in arid environments. Further examination of the Negev Desert microbiome is likely to reveal additional candidates for promoting plant growth in challenged environments.

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