Microbial Ecology

Halotolerant Aerobic Heterotrophic Bacteria from the Great Salt Plains of Oklahoma

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Abstract

The Salt Plains National Wildlife Refuge (SPNWR) near Cherokee, Oklahoma, contains a barren salt flat where Permian brine rises to the surface and evaporates under dry conditions to leave a crust of white salt. Rainfall events dissolve the salt crust and create ephemeral streams and ponds. The rapidly changing salinity and high surface temperatures, salinity, and UV exposure make this an extreme environment. The Salt Plains Microbial Observatory (SPMO) examined the soil microbial community of this habitat using classic enrichment and isolation techniques and phylogenetic rDNA studies. Rich growth media have been emphasized that differ in total salt concentration and composition. Aerobic heterotrophic enrichments were performed under a variety of conditions. Heterotrophic enrichments and dilution plates have generated 105 bacterial isolates, representing 46 phylotypes. The bacterial isolates have been characterized phenotypically and subjected to rDNA sequencing and phylogenetic analyses. Fast-growing isolates obtained from enrichments with 10% salt are predominantly from the gamma subgroup of the Proteobacteria and from the low GC Gram-positive cluster. Several different areas on the salt flats have yielded a variety of isolates from the Gram-negative genera *Halomonas*, *Idiomarina*, *Salinivibrio*, and *Bacteroidetes*. Gram-positive bacteria are well represented in the culture collection including members of the *Bacillus*, *Salibacillus*, *Oceanobacillus*, and *Halobacillus*.

Introduction

The Great Salt Plains (GSP) of north-central Oklahoma are topographically and biologically diverse regions within the Salt Plains National Wildlife Refuge (SPNWR). The broad expanse of unvegetated salt plains ($\sim$65 km^2) is punctuated by small regions of sparse scrub brush. The surface of the flats is covered by a crust of evaporated mineral salts from trace to several millimeters thick deposited by continual artesian percolation of saturated Permian brine from underlying strata [39, 65]. Sodium and chloride are the main ionic constituents of the brine. The concentration of salts at the surface varies dramatically both temporally and spatially. During periods of heavy rainfall, the surface of the salt flats can be flooded with up to tens of centimeters of water, and during dry periods, a layer of crystalline salts covers much of the surface. Microbes living in GSP soils must endure variable salinities from near zero to saturated, surface temperatures often exceeding 50°C, freezing winters, alkaline pH, and unobstructed UV irradiation. It is believed that such extreme environmental stresses have pressured the selection of microbial communities that include novel taxa with remarkable survivability.

Aquatic hypersaline environments, those that have salinities greater than seawater, are more stable than hypersaline terrestrial environments. Most of these ecosystems are of marine origin and found in coastal areas, such as Solar Lake and Guerrero Negro. The microbial communities in these ecosystems are composed of microbial genera common in the ocean and include *Vibrio*, *Pseudomonas*, *Alteromonas*, and *Alcaligenes* [38]. Hypersaline aquatic ecosystems from nonmarine settings, such as soda lakes, often support very different microbial communities, due to significantly different salt compositions in these alkaline waters [40]. Solar salterns are synthetic systems for harvesting salt from brines, usually of marine origin [46, 63]. The waters are passed through a series of evaporation ponds of increasing salinity and

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the mineral salts that precipitate from these brines are harvested. The microbial populations of these synthetic systems have been the subjects of several studies, and many of the halotolerant microbes available through culture collections are from solar salterns [3, 12, 45, 46, 69]. The most concentrated brines, greater than 20% salt, are dominated by highly pigmented archaea such as *Halobacterium*, *Haloferax*, and *Haloarcula* [12, 58, 59, 69]. The more dilute brines, less than 10% salt, tend to be dominated by bacteria, such as *Vibrio*, *Acinetobacter*, and *Alteromonas* [62, 83]. In many cases, the bacteria from hypersaline aquatic systems are close relatives of marine organisms, but have increased salt tolerance.

The microbial communities of hypersaline terrestrial ecosystems may be very much different from those of hypersaline aquatic ecosystems. Hypersaline soil environments, which include salt plains, mud flats, wadis, playas, and abandoned salterns, are not always of recent marine origin, but may have similar salt compositions [15, 39, 40, 43]. Rather than mirror the microbial communities of marine ecosystems, as hypersaline aquatic systems do, hypersaline soils may be dominated by salt-tolerant relatives of typical soil bacteria [1, 28, 31, 63, 64]. *Bacillus*, *Flavobacterium*, *Alcaligenes*, *Micrococcus*, *Pseudomonas*, and *Acinetobacter* are the genera that have been most often isolated and apparently the most abundant in hypersaline soils. It is difficult to substantiate broad statements about the microbial communities of hypersaline soils, because the number of studies is very limited, and they have predominantly focused on abandoned salterns rather than unperturbed ecosystems.

The current report is a product of the Salt Plains Microbial Observatory (SPMO), which studies microbial communities of the SPNWR, a natural noncoastal thalassohaline terrestrial ecosystem. Preliminary accounts of this work have been reported previously [16, 73]. The only previous microbiological work at the GSP was directed at drug discovery, resulting in the isolation of *Bacillus* sp. str. HNGS03 that produces nitrotyramine derivatives [26]. The same group isolated an aspartate-producing anaerobe, *Vibrio aspartigenicus* (GenBank no. 36872; [79]). The current report characterizes the predominant culturable bacteria from GSP soils. Isolates from the GSP culture collection have been characterized phenotypically using biochemical, physiological, and morphological tests, and phylogenetically using 16S rDNA sequences. Given the high salinities and rapidly changing salinities, the investigation includes the determination of the halotolerance and halophilicity of the bacterial isolates.

Materials and Methods

Site Description. The GSP sampling sites are within 1.5 km of each other, south of Clay Creek, near the western edge of the barren salt flats along the north–south midline (Fig. 1). The sites are in areas of the GSP that accrue surface salt crusts from continuous recharge of saturated NaCl brine from underlying strata, and the sites are subject to flooding with freshwater during rain events. The soils of the salt flats are patchy, with areas of mud flats and raised sandy soils. Topographical features can change on time scales of days to months and the salt flats often have ephemeral streams and pools with a wide range of salinities. During a 93-day period from mid-June to late September 2001, conditions at the GSP included surface temperatures as high as 55.5°C, with median daily highs of 45.5°C. Median day-night temperature variation was 22.4°C, with a maximum variation of 30.6°C. Wind speeds ranged from a minimum of 0 to 69 km h^{-1}, with 25th and 75th percentiles of 8.6 and 21.6 km h^{-1}, respectively. Groundwater salinities ranged from a minimum of 4% to a maximum of 37%, while surface soil interstitial salinity varied from 0.3% to at least 27%. Soil pH ranged from 7.34 to 9.23 with a mean of 8.75 and a median of 9.06. The sampling sites are in regions relatively rich in nitrate and sulfate and have a low (0.5%) organic matter content (Henley, unpublished). Four sampling sites yielded bacterial isolates described in this report (Fig. 1). WP3 (N 36° 42.856′; W 98° 15.725′) and WP6 (N 36° 43.851′; W 98° 15.561′) were salt-crusted mud flats about 1.5 km apart. WP5 (N 36° 43.661′; W 98° 15.526′) and WP8 (N 36° 42.750′; W 98° 15.584′) were salt-crusted sandy sites that lie between the two mud flat locations.

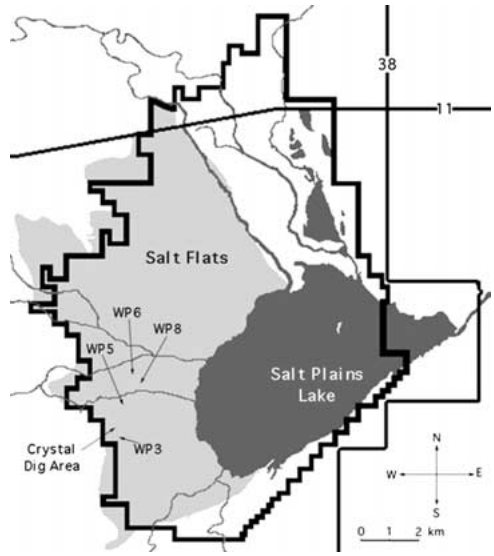

Figure 1. Map of the Salt Plains National Wildlife Refuge. The salt plains are in light gray and bodies of water are in darker gray. The arrows indicate sampling sites.

Sample Collection. Soil samples were collected from the four GSP sites during dry periods between June 2001 and August 2002. The sampling dates were 19 June,

8 July and 19 August 2002 and 22 June 2001. Sampling was limited to bulk materials in the top 10 cm of soils of the unvegetated areas of the salt plains. Bulk soil samples (50–100 g) were taken with sterile spatulas, hand spades, or PVC pipes (5 cm dia), mixed on sterile paper, and aliquoted for inoculation of cultures. Samples for establishing enrichment cultures were placed in sterile Whirl-Pak bags or directly into tubes of media, transported to an on-site laboratory at 25°C in a cooler, and processed within 1 to 2 h of collection. One-gram aliquots of soil samples were added to 30 mL of sterile media in polypropylene tubes for enrichment cultures. The tubes were capped with sterile foam plugs to provide aeration and transported at 25°C to the laboratory at Wichita State University. For spread plating and dilution plating, soil samples were taken directly to the on-site laboratory without inoculation into liquid media.

Enrichment and Isolation. Direct plating, liquid enrichment, and dilution plating were used to isolate organisms from GSP soils. The primary medium used for isolations, SP medium [64, 68], is nutrient-rich and moderately saline (10%), containing, per liter: NaCl, 98 g; KCl, 2.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.36 g; $NaHCO_3$, 0.06 g; NaBr, 0.23 g; $FeCl_3 \cdot 6H_2O$, 1.0 mg; trace minerals, 0.5 mL; Bacto tryptone, 5.0 g; yeast extract, 10.0 g; glucose, 1.0 g; final pH 7.0. A second medium (SPL) similar to SP, but not as nutrient-rich, was prepared using one-tenth the amounts of organic constituents. Enrichments in SP and SPL medium were performed at room temperature. A third medium, HM medium [64, 68] had higher salinity (25%), was used at 37°C, and contained, per liter: NaCl, 220.0 g; $MgSO_4 \cdot 7H_2O$, 10.0 g; KCl, 5.0 g; sodium citrate, 3.0 g; KNO_3, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.20 g; trace minerals, 0.5 mL; Bacto tryptone, 5.0 g; yeast extract, 1.0 g; final pH 7.3. Alkaline-tolerant isolates were obtained with a saline (10%) alkaline *Bacillus* medium (AB) [8] containing, per liter: NaCl, 100.0; K_2HPO_4, 1.0 g; Na_2CO_3, 10.0 g (filter-sterilized and added separately in a volume of 100 mL after autoclaving medium); trace minerals, 0.5 mL; Bacto tryptone, 10.0 g; glucose, 10.0 g; yeast extract, 5.0 g; final pH 9.5 to 10.0. The trace minerals contained, per liter: $Na_2MoO_4 \cdot 2H_2O$, 0.1 g; $MnCl_2 \cdot 6H_2O$, 0.2 g; $CoCl_2 \cdot 6H_2O$, 2 mg; $ZnSO_4 \cdot 7H_2O$, 0.1 g; $CuSO_4 \cdot 6H_2O$, 1 mg. For solid media, Bacto agar was added to 1.5% (w/v) prior to autoclaving.

The 30-mL enrichment tubes inoculated in the field were aseptically transferred to 250-mL Erlenmeyer flasks containing 70 mL of medium. Liquid enrichment cultures were incubated on a rotary-shaking platform (2.5-cm stroke dia) at 150 rpm. Aliquots (100 μL) were plated after 24 and 48 h. One mL of the liquid enrichment cultures was transferred to fresh liquid medium after 1 week and 100-μL aliquots were again plated after 24 and 48 h. Soil aliquots (~0.5 g) also were spread directly on to the surface of plates. Plates were kept for several weeks and representatives of new colony types were collected to capture slower growing organisms. For dilution plating, soil samples (~1 g) were added to tubes containing 10 mL of a sterile 10% (w/v) NaCl solution supplemented with 0.1% (w/v) Na metaphosphate [80], shaken for 10 min on a rocking platform, and then treated in an ultrasonic bath (Fisher; Blackstone Ultrasonics SS-2) for 2 min at 100% power. The sample was then serially diluted in sterile 10% (w/v) NaCl brine with 2 min of sonication at each step, and plated on SP medium. Plates were maintained at the desired temperature in a moist chamber to prevent rapid drying of the surface, and colonies were collected and counted after several days. Dilution plates exhibiting between 30 and 300 colonies were used for quantitation. Variance was high in dilution plate counts and individual isolate abundances could not be adequately quantified. Dilution plating experiments were only performed with soils from WP6.

Colonies arising on the plates were selected for isolation based on gross morphological and physiological features, differing in pigmentation, size, margin, or rate of growth. No effort was made to quantify colony types arising from the enrichment cultures, and attempts were made to limit the collection of apparently duplicate isolates. Selected colonies were transferred to fresh agar plates and isolated using the streak plate method. Each isolate was subjected to at least five successive streak platings to ensure clonal purity. The isolates were maintained on agar slants at room temperature in moist chambers and as 50% glycerol stocks at –70°C.

Morphological, Physiological, and Biochemical Tests. All assay media were supplemented with 10% (w/v) NaCl unless otherwise noted. All assays were incubated in duplicate at 25°C and scored at 24, 48, and 72 h unless otherwise noted, and all assays were accompanied by positive and negative controls using common laboratory strains and nonsaline media. When duplicates gave contradictory results, the assays were performed again for clarification.

All isolates were Gram-stained using the Protocol Gram-staining kit (Fisher Diagnostics) following the manufacturer's instructions. Motility was assessed by examining wet mounts of 24-h cultures at 1000× and by stab inoculation of Sulfur-Indole-Motility medium (SIM; BBL) deeps. The two assays gave the same results for all but four isolates (GSP 3, 13, 41, and 39). The addition of 3% hydrogen peroxide solution to confluent plates or smears of confluent culture on slides was used to detect catalase. Oxidase testing was performed using the BBL DrySlide system according to the manufacturer's instructions. Hydrolysis of gelatin was determined using nutrient gelatin agar (Difco) deeps, lipid hydrolysis with

Spirit Blue agar (Difco) containing homogenized olive oil and Tween 80 solution, and amylase on Starch agar (Difco) followed by flooding of plates with 25% stabilized Gram's iodine solution after a 5-d incubation. Hydrogen sulfide production was assayed using SIM medium; Kovac's reagent was added after incubation to test for the production of indole. Urease activity was determined at 5 d using urea broth with phenol red indicator. Production of acid and gas from carbohydrates was tested using 0.5% (w/v) glucose, lactose, or sucrose in culture tubes containing inverted Durham tubes, using a 10% NaCl solution supplemented with Bacto tryptone (10 gL^{-1}) and phenol red (0.018 gL^{-1}; pH 7.3). Anaerobic growth after a 7-d incubation was determined in SP medium using the GasPak system (BBL). Reduction of nitrate was determined with Nitrate broth (Difco) by detecting nitrite using napthylamine/sulfanilic acid reagents and residual nitrate with zinc dust and 6 N HCl. Salt tolerances were determined by dilution plating in duplicate on SP medium containing different amounts of NaCl, with optima representing plates where growth was first observed. Temperature tolerances were determined on duplicate SP or HM dilution plates.

PCR and DNA Sequencing. DNA extract from each isolate was prepared using a freeze–thaw technique. Cells were harvested by microcentrifugation (14,000 g for 5 min) of 2 mL of logarithmic culture and resuspended in 0.3 mL of water. The suspension was alternately frozen in liquid nitrogen (3 min) and thawed in a 90°C water bath (3 min) for six cycles, and clarified by microcentrifugation (14,000 g for 10 min). Genomic DNA in the supernatant was the target of PCR amplification of nearly complete 16S rDNA gene fragments using Bacterial primers (EUBPA: 5′-AGAGTTTGATCCTGGCTCAG-3′ and EUBPH: 5′-AAGGAGGTGATCCAGCCGCA-3′) [23]. PCR was performed in a thermal cycler (Barnstead-Thermoline or Eppendorf Mastercycler) as 100-μL reactions containing 1× PCR buffer A (Fisher) or Buffer II (ABI), 20 μM of each dNTP, 0.2 μM of each primer, 1 U of *Taq* DNA polymerase (Fisher or ABI), and 5 μL of cell extract. DNA was denatured at 95°C for 2 min, followed by 40 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final 5-min extension at 72°C. The PCR amplicons were checked by electrophoresis on 1% agarose gels.

Two or more amplicons were pooled for each set of 16S rDNA sequencing reactions in order to maximize sequencing template concentration and to allow for the detection of possible microheterogeneity in any rDNA gene copies. Amplicons were prepared for sequencing reactions using Millipore filtration units that remove excess dNTPs and the oligonucleotides used in the amplification. Flanking and internal primers (EUBPA: see above; EUB338f: 5′-ACTCCTACGGGAGGCAGCAG-3′; EUB338r: 5′-ATTACCGCGGCTGCTGG-3′; TU108: 5′-AAACTCAAAGGAATTGACGG-3′; TU108r: 5′-CCGTCAATTCCTTTGAGTTT-3′; TU10mod: 5′-GGGTTGCGCTCGTTGCGGG-3′; and TU110modr: 5′-CCCGCAACGAGCGCAACCC-3′) were used for direct sequencing of the filtered amplicons. Sequence data were obtained with the protocols and reagents that accompany the four-color PRISM BigDye kit designed for use in ABI automated DNA sequencing systems (model 373). Base calls were made using the ABI PRISM DNA Sequencing Analysis Software package (v. 3.3).

All sequence fragments generated from a given template were edited against electropherograms and then assembled into contigs using Sequencher 4.1 (Genecodes, Ann Arbor, MI). For most sequences, two to four overlapping fragments (from both the coding and noncoding strand) were used to assemble the contigs. Contextual 16S rDNA sequences for all unknown GSP sequences were identified from the published databases using BLAST [2] and by comparison with sequences deposited in the Ribosomal Database Project [18]. The GSP isolate sequences have been submitted to GenBank and appear with accession numbers AY505499–AY505536 and AY553063–AY553129.

Phylogenetic Analysis. All sequences were manually aligned using MacClade 4.05 [49]. Three sets of alignments, "B1," "B2," and "Gram-negative," were created for three separate sets of analyses, each of which was analyzed independently. Excluded from analyses were regions of each alignment that exhibited base substitutions coupled with length heterogeneity or where parallel data for some taxa were missing at the 5′ or 3′ end. Modeltest was used with PAUP 4.0 b10 [76] to identify the best fit between data and model of nucleotide substitution for use in a maximum likelihood (ML) approach to phylogenetic reconstruction. Results from analyses by Modeltest identified the General Time Reversible model (GTR) as exhibiting the best fit to the data for each alignment set. In addition, a discrete gamma model and an invariants model for the correction of site-to-site rate variation were invoked for each analysis.

In alignment "B1" comprising 1574 characters, 109 sites were excluded. Base frequencies (0.2563, 0.2284, 0.3107), the rate matrix values (0.7248, 2.8062, 1.3695, 0.4001, 4.0739), the shape parameter (alpha) for the gamma distribution (0.6431) and the percent of invariant sites (0.6704) were estimated from the "B1" data using Modeltest and PAUP. In alignment "B2" comprising 1601 total sites, 96 sites were excluded. Base frequencies (0.2233, 0.2534, 0.3301), the rate matrix values (1.1302, 2.2157, 0.9796, 0.6554, 4.1423), the shape parameter (alpha) for the gamma distribution (0.3246) and the percent of invariant sites (0.2640) were estimated from

the "B2" data using Modeltest and PAUP. In the "Gram-negative" alignment comprising 1565 total sites, 123 sites were excluded. Base frequencies (0.2454, 0.2319, 0.3108), the rate matrix values (0.7447, 1.7420, 1.1203, 0.6227, 2.8147), the shape parameter (alpha) for the gamma distribution (0.4018), and the percent of invariant sites (0.3058) were estimated from the "Gram-negative" data using Modeltest and PAUP.

All tree searches were conducted heuristically in PAUP 4.0b10 [76] using the neighbor-joining algorithm to obtain starting trees and the tree-bisection–reconnection branch-swapping algorithm to search tree space. The bootstrap was used to assess the relative support for each branch in the maximum likelihood tree for each analysis. A total of 100 bootstrap replicates were conducted heuristically using the neighbor-joining algorithm to obtain starting trees. Branch swapping for all bootstrap analyses was conducted using the nearest-neighbor-interchange algorithm in PAUP 4.0b10 [76]. The "B1" and "B2" trees were rooted using data from functional outgroups (*Bacillus haloalkaliphilus* and *Filobacillus milosensis*) identified in preliminary analyses of the data. The "Gram-negative" tree was rooted using data from *Flexibacter flexilis*. GSP isolates were grouped into 16S rDNA phylotypes if their aligned sequences (no sites excluded) exhibited sequence divergence (no sites excluded) that was less than or equal to 0.0025% (uncorrected *P*) in pairwise comparisons.

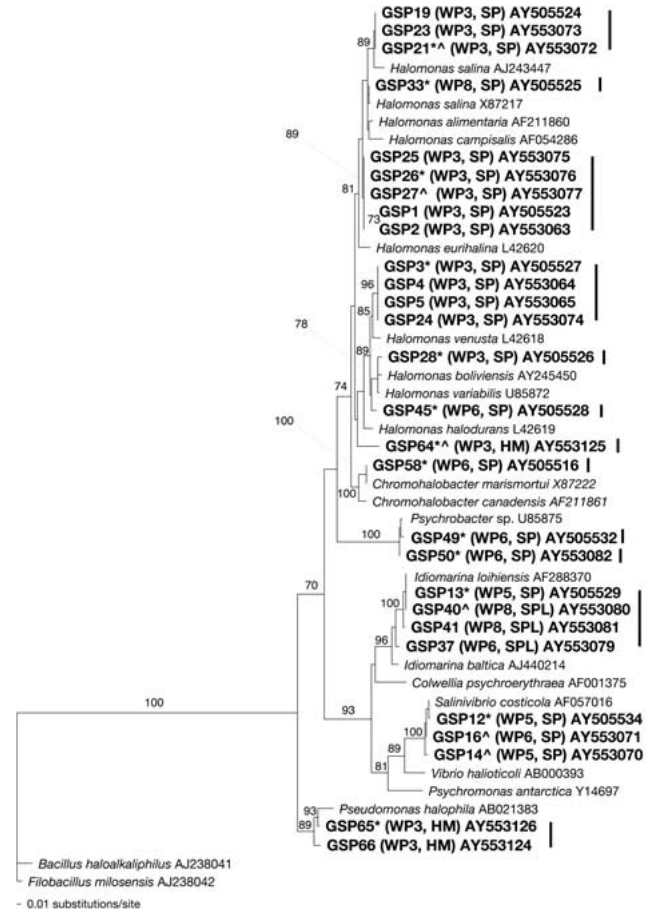

Figure 2. Optimal tree (*Gracilibacillus* node and above) from maximum likelihood analysis of 16S rDNA data for the Gram-negative alignment set of taxa. Since branch lengths are short (relative to the base of the tree), this portion of the tree was drawn separately from the OG and the basal ingroup branches. Branch lengths are drawn proportional to inferred evolutionary change (see scale at lower left). All bootstrap values ≥70 are mapped to the appropriate internode. Phylotypes are indicated by vertical bars. GSP-numbered isolates are from enrichment cultures and used for phenotypic analyses. GSP-numbered organisms with asterisks were used as the unique representative of each phylotype. MO-numbered isolates are from dilution plates. The site from which the sample was derived and the medium used for enrichment are given for each isolate. Partial sequences (~900 bases) are indicated by a (^). GenBank accession numbers are indicated for all taxa.

Results

Enrichments and Isolations. Culturing efforts yielded 105 bacterial isolates; rDNA sequences were obtained for all isolates. Approximately two-thirds of the isolates were from Gram-positive taxa. Although Gram-positive and Gram-negative organisms were found at each of the sampling sites, WP3 yielded a higher percentage of Gram-negative organisms (63%), while WP6, a similar mud flat area, yielded a higher percentage of Gram-positive organisms (84%). The phylogenetic trees shown in Figs. 2–5 have codes indicating the sampling site from which each isolate was obtained and the medium used for enrichment. The majority (71%) of the isolates reported here were obtained with SP medium (10% saline). Phylogenetic analyses described below were used to identify 46 unique phylotypes in the collection (indicated by vertical bars at right of Figs. 2–5). However, very similar, perhaps duplicate, strains were isolated that fall within each phylotype. Data from the entire collection of enrichment isolates (those with GSP designations in Figs. 2–5) and from single representatives of the unique phylotypes (GSP isolates with asterisks in Figs. 2–5) are presented separately in the column graphs (Figs. 6–9). Detailed results for each isolate can be obtained from the online SPMO database (www.okstate.edu/artsci/SPMO).

Dilution plating experiments were used to quantify the culturable bacteria in the soil community. There was considerable variance in the values obtained. Approximately 5×10^5 colony-forming units were obtained per gram of fresh soil. Some of the isolates captured from dilution plates were examined phylogenetically and appear on Figs. 2–5 with MO designations. It was not possible to accurately quantify individual isolates from dilution plates given the variable results. The culturable bacteria observed on SP and HM media plates were mainly Gram-positive organisms related to *B. lichenifor-*

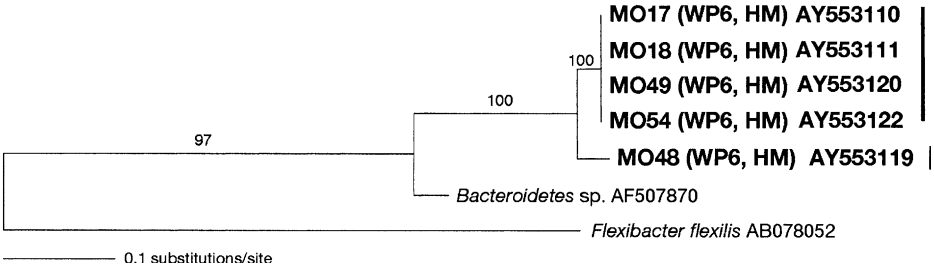

Figure 3. Basal portion of the optimal tree from maximum likelihood analysis of 16S rDNA data for the Gram-negative alignment set of taxa. Since branch lengths are long (relative to the *Gracilibacillus* node and above), this portion of the tree was drawn separately. Branch lengths are drawn proportional to inferred evolutionary change (see scale at lower left). All bootstrap values ≥70 are mapped to the appropriate internode. Phylotypes are indicated by vertical bars. GSP-numbered isolates are from enrichment cultures and used for phenotypic analyses. GSP-numbered organisms with asterisks were used as the unique representative of each phylotype. MO-numbered isolates are from dilution plates. The site from which the sample was derived and the medium used for enrichment are given for each isolate. GenBank accession numbers are indicated for all taxa.

mis, *B. subtilis*, and *B. megaterium* and Gram-negative bacteria related to *Bacteroidetes*. Dilution plating was only performed with samples from the WP6 site, so it is not surprising that *Bacillus* was most often observed. The *Bacteroidetes*, along with several isolates in the *Halobacillus* cluster (MO 22, 50, and 56) and others, were cultured on HM medium. It is interesting to note that not all of the apparently abundant bacteria were represented in the culture collection obtained by enrichment. Unfortunately, several of the dilution plate isolates were lost during subsequent transfers.

Phylogenetic Analysis of Isolates. The optimal ML trees are presented as two Gram-negative clades (Figs. 2 and 3) and two clades of Gram-positive bacteria (Figs. 4 and 5) that fit within *Bacillus* species [7]. The B1 clade (Fig. 4) mainly comprises organisms in the genus *Bacillus*, including *B. licheniformis*. The B2 clade (Fig. 5) is made of up a wider variety of genera, including *Halobacillus*, *Virgibacillus*, *Salibacillus*, and *Marinococcus*. The main Gram-negative clade (Fig. 2) is dominated by isolates related to *Halomonas* sp., while the second clade (Fig. 3) is a group of *Bacteroidetes*.

There are 13 phylotypes in the B1 clade, with eight defined by a single isolate. The most populated phylotypes within the B1 clade (Fig. 4) consist of isolates related to *B. baekryungensis*, *B. licheniformis*, and *B. subtilis*. *Bacillus baekryungensis* is a provisional species from the Yellow Sea that is represented in GenBank (AF541965; [88]), but as yet unpublished. Although well represented in the enrichment culture collection, with highly pigmented yellow or orange colonies, this species was not present in culturable bacteria captured by dilution plating. In contrast, *B. licheniformis* isolates were often observed on dilution plates, but only appear twice in the enrichment culture collection.

The B2 clade consists of 18 phylotypes, 10 of which are defined by a single isolate (Fig. 5). One cluster of enrichment isolates was related to *Halobacillus*, including three isolates from dilution plating on HM medium (MO 22, 50, and 56). A second larger cluster of isolates, all exhibiting pink colonies, are most closely related to *Bacillus* sp. KL-152 (AY030333). Eight of the other phylotypes in the B2 clade encompass all but two (GSP 69 and 77) of the twelve isolates (GSP 67–78) enriched on AB medium.

The main Gram-negative clade (Fig. 2) includes 13 phylotypes, seven of which are represented by a single isolate. The largest clusters are dominated by bacteria related to *Halomonas* species, particularly *H. salina* (pink GSP isolates) and *H. venusta* (yellow GSP isolates). Other groups of phylotypes are related to *Salinivibrio* (orange GSP isolates) and *Idiomarina*. Three of the four *Idiomarina* isolates were obtained on low-nutrient SPL medium and the *Pseudomonas* isolates were obtained from HM enrichments. The second Gram-negative clade (Fig. 3) consists of two phylotypes related to *Bacteroidetes*. All five isolates were enriched on HM medium and could not be maintained in culture.

Phenotypic Analysis of Isolates. Phenotypic analyses were performed on 76 of the bacterial isolates from GSP soils. These isolates were given strain designations that begin with "GSP." Phylogenetic analyses were used to identify 46 unique phylotypes. Nine of these were lost from culture before phenotypic analyses were completed; therefore, data from unique representatives from 37 phylotypes (marked with asterisks in Figs. 2, 3, 4, 5) are reported in Figs. 6–9. Data are presented for each test as the number of positive isolates from all 76 GSP-series organisms from enrichment cultures ("total") and from just the 37 unique representatives of the surviving phylotypes ("unique"). The numbers associated with the bars are the percentages of positive isolates within each clade. Isolates were not randomly selected from enrichment products as sampling was focused on collecting

Figure 4. Optimal tree from maximum likelihood analysis of 16S rDNA data from the B1 alignment set of taxa. Branch lengths are drawn proportional to inferred evolutionary change (see scale at lower left). All bootstrap values ≥70 are mapped to the appropriate internode. Phylotypes are indicated by vertical bars. GSP-numbered isolates are from enrichment cultures and used for phenotypic analyses. GSP-numbered organisms with asterisks were used as the unique representative of each phylotype. MO-numbered isolates are from dilution plates. The site from which the sample was derived and the medium used for enrichment are given for each isolate. Partial sequences (~900 bases) are indicated by a caret (^). GenBank accession numbers are indicated for all taxa.

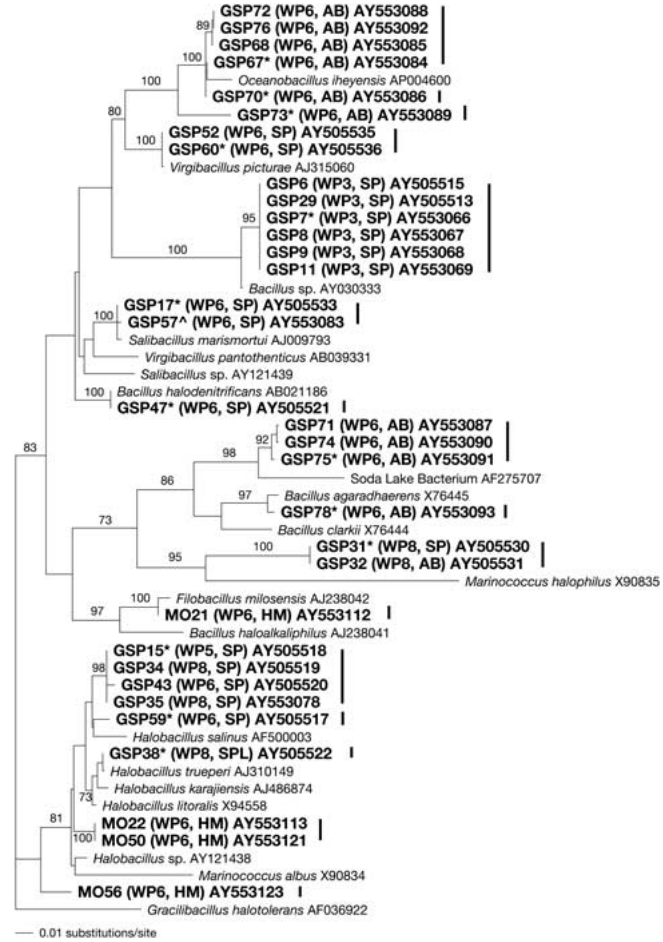

Figure 5. Optimal tree from maximum likelihood analysis of 16S rDNA data from the B2 alignment set of taxa. Branch lengths are drawn proportional to inferred evolutionary change (see scale at lower left). All bootstrap values ≥70 are mapped to the appropriate internode. Phylotypes are indicated by vertical bars. GSP-numbered isolates are from enrichment cultures and used for phenotypic analyses. GSP-numbered organisms with asterisks were used as the unique representative of each phylotype. MO-numbered isolates are from dilution plates. The site from which the sample was derived and the medium used for enrichment are given for each isolate. Partial sequences (~900 bases) are indicated by a (^). GenBank accession numbers are indicated for all taxa.

diverse bacteria. Thus, the data are not suitable for statistical analyses since the isolate collection does not necessarily reflect relative abundance within the entire community. For this reason, the data are reported separately for single representatives of each phylotype and for the total isolate collection, which apparently includes closely related or duplicate strains.

Nearly all of the bacterial isolates reacted with Gram stain in a way consistent with their phylogenetic placement (Fig. 6). Four of the GSP isolates (GSP 34, 35, 43, and 57), mainly *Halobacillus*, consistently stained Gram-negative despite repeated assessments at different salinities. This unusual finding has been reported previously in a number of bacteria, including the halophilic bacillus *Filobacillus milosensis* that has an unusual cell wall polymer type also reported for other *Halobacillus* species [72]. Five organisms from the Gram-negative clade (GSP 14, 37, 49, 50, and 58), consistently stained Gram-positive. A small percentage of B1 and Gram-negative isolates, but none of the B2 isolates, produced endospores under the conditions tested, including different salinities (Fig. 6); however, many isolates were minute enough to make assessment difficult. The ratio of bacilli to cocci was ~3:1 in the Gram-negative clade. The *Staphylococcus* (GSP22) in the B1 clade and the two *Marinococcus* (GSP

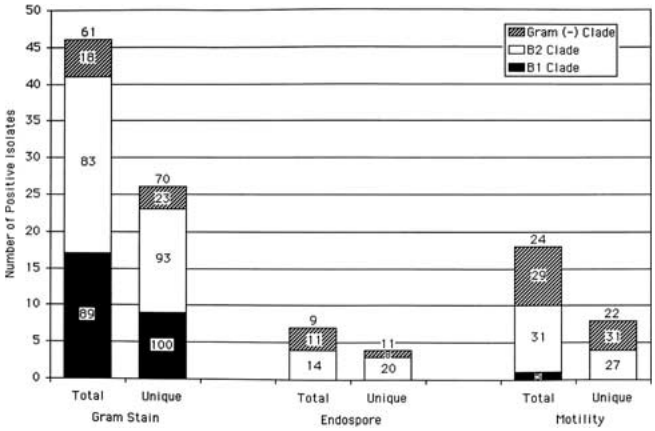

Figure 6. Prevalence of positive Gram reaction, endospore formation, and motility in GSP isolates from enrichment culture. Data are presented for each test as the prevalence of positive assays for the "total" collection of 76 GSP isolates from enrichment culture (GSP-numbered isolates in Figs. 2–5) and as the prevalence of positive assays for the "unique" representatives of each of the 37 phylotypes (GSP-numbered isolates with asterisks in Figs. 2–5). The value within each segment of the column is the percentage of positive isolates in that clade. The value at the top of each column is the percentage of positive isolates among all of the isolates in that group.

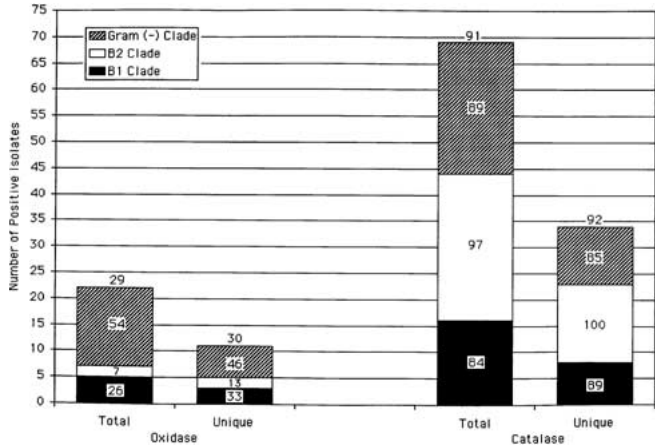

Figure 7. Prevalence of positive oxidase and catalase assays in GSP isolates from enrichment culture. Data are presented for each test as the prevalence of positive assays for the "total", collection of 76 GSP isolates from enrichment culture (GSP-numbered isolates in Figs. 2–5) and as the prevalence of positive assays for the "unique" representatives of each of the 37 phylotypes (GSP-numbered isolates with asterisks in Figs. 2–5). The value within each segment of the column is the percentage of positive isolates in that clade. The value at the top of each column is the percentage of positive isolates among all of the isolates in that group.

31 and 32) in the B2 clade were the only cocci among the 48 Gram-positive enrichment isolates. Five of the isolates (GSP 2, 30, 37, 63, and 69) formed filaments and GSP6 exhibited palisades. The AB isolates GSP 71, 74, and 75 formed long filaments of curved rods with fruiting bodies. In general, the GSP isolates were not highly pigmented, with about half producing cream or white colonies. The others were muted pink, yellow, or orange. The only brightly colored isolates were the orange *Marinococcus*, and no isolates resembled the bright reds and pinks typically associated with haloarchaea. Approximately three-quarters of the isolates were nonmotile, with the B1 cluster being relatively depleted in motile isolates (Fig. 6). Relatively more motility (42%) was observed in isolates from the sandy soils, while WP3 isolates were relatively less motile (9%).

Nearly all of the isolates were catalase-positive, while less than one-third were oxidase-positive (Fig. 7). Oxidase-positive isolates were somewhat less abundant in the B2 clade and somewhat enriched in the Gram-negative clade. Isolates from the WP3 site had a relatively lower incidence (18%) of positive oxidase reactions than the rest of the collection. All of the isolates grew to visible turbidity in SP medium (10% brine) within 24 h. Lipase was present in approximately three-quarters of the isolates (Fig. 8). All of the Gram-negative isolates exhibited lipase activity, while only about half of the Gram-positive isolates were lipase-positive. All of the isolates from WP3 were lipase-positive. Amylase and gelatinase activity was relatively rare in the isolate collection (Fig. 8). The B2 clade was particularly low in amylase- and gelatinase-positive isolates, while the B1 clade was relatively enriched in amylase-positive isolates. Those isolates from the sandy sites were enriched (42%) for gelatinase relative to isolates from the mud flats (8%) and isolates from the WP3 site were relatively depleted in amylase activity (5%). Indole production was not observed for any isolates. Protein and amino acid metabolism may be limited based on the results of gelatin liquefaction (17%) and indole production (0%), which are indicators of collagen and tryptophan metabolism, respectively. Nitrate reduction activity was distributed evenly across the clades, being present in about half of the isolates (Fig. 8). It was observed relatively infrequently (17%) in isolates from the sandy sites and relatively frequently (82%) in isolates from WP3. Urea hydrolysis was tested for 30 isolates, eight of which (27%) were positive. The balance of the tests for urease were indeterminate despite repeated assays, suggesting interference due to high salinity, although assays in 5% NaCl were also indeterminate.

Fermentation of carbohydrates was observed for about half of the isolates; however, none produced gas from carbohydrates (Fig. 9). Glucose fermentation was observed in more isolates than sucrose fermentation. Only a handful of isolates, all from the B2 clade, fermented lactose. The B2 clade was relatively enriched for fermenters overall. The Gram-negative isolates fermented sucrose relatively infrequently, preferring glucose. No

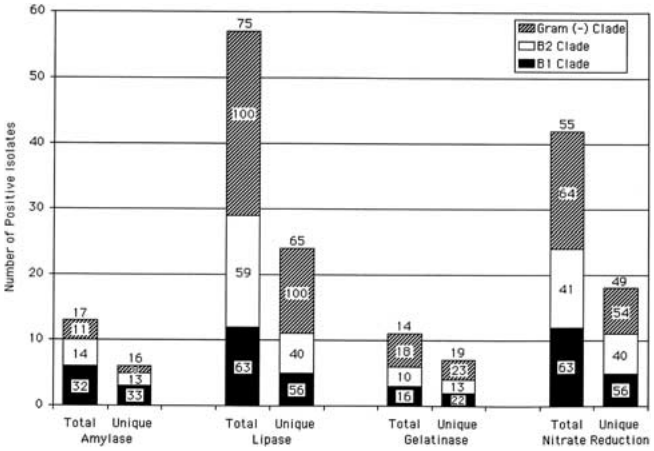

Figure 8. Prevalence of positive amylase, lipase, gelatinase, and nitrate reduction assays in GSP isolates from enrichment culture. Data are presented for each test as the prevalence of positive assays for the ''total'' collection of 76 GSP isolates from enrichment culture (GSP-numbered isolates in Figs. 2–5) and as the prevalence of positive assays for the ''unique'' representatives of each of the 37 phylotypes (GSP-numbered isolates with asterisks in Figs. 2–5). The value within each segment of the column is the percentage of positive isolates in that clade. The value at the top of each column is the percentage of positive isolates among all of the isolates in that group.

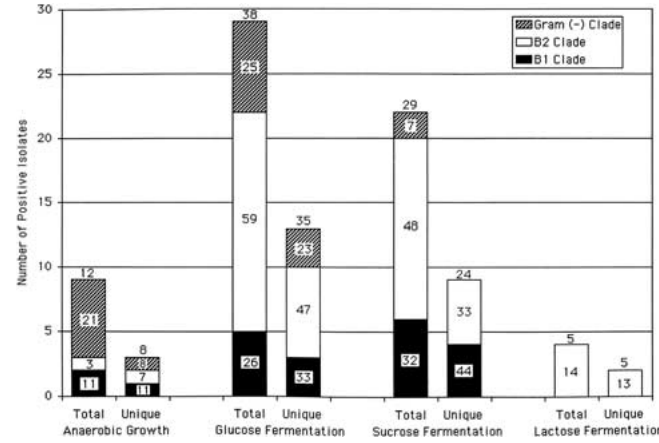

Figure 9. Prevalence of positive anaerobic growth and fermentation of glucose, sucrose, and lactose assays in GSP isolates from enrichment culture. Data are presented for each test as the prevalence of positive assays for the ''total'' collection of 76 GSP isolates from enrichment culture (GSP-numbered isolates in Figs. 2–5) and as the prevalence of positive assays for the ''unique'' representatives of each of the 37 phylotypes (GSP-numbered isolates with asterisks in Figs. 2–5). The value within each segment of the column is the percentage of positive isolates in that clade. The value at the top of each column is the percentage of positive isolates among all of the isolates in that group.

clear differences in fermentation abilities were seen between isolates from different locations. The ability to grow anaerobically was relatively rare among the isolates (Fig. 9). Tests using SIM medium showed no production of H_2S by any of isolates. SIM assays also were negative when performed at 5% NaCl (w/v). Sulfur production using Kligler Iron Agar also was negative for all isolates.

Tolerance to Environmental Stress. The unique isolates representing each phylotype were tested for their requirements and abilities for growth at a wide range of salinities and temperatures. The vast majority of the representative bacterial isolates from each phylotype were tolerant of high salinities (10% or greater), but did not require hypersaline conditions for growth (Fig. 10). Two isolates (GSP 59 and 64) required 5% salinity and two isolates (GSP 65 and 72) required 10% salinity. It is interesting to note that the halophilic GSP29 is in the same phylotype as five isolates (including GSP10) that are not halophilic and only grow in media with 15% salt or less. Six of the representative isolates had salt optima at or above 10% salinity, with all but two of the isolates (GSP 12 and 58) capable of growth at 0.1% salinity having optima below 10% salinity. Many of the isolates (84%) exhibited ranges of tolerance greater than 15% salinity, with 35% having a 20% or greater salinity range. GSP21 and GSP64 were particularly tolerant, having ranges of 25%, with GSP41 having a 30% range. GSP59 had a narrow range of tolerance (10%), but was halophilic, requiring 5% salinity, while GSP15 from the same cluster was not halophilic. All of the Gram-negative isolates grew at salinities at or above 15%, with 70% growing at or above 20% salinity. In contrast, less than 25% of the Gram-positive organisms grew at or above 20% salinity.

Most of the GSP isolates were thermotolerant, with all growing at or above 37°C and 64% growing at or above 50°C (Fig. 11). Fourteen of the isolates (35%) grew

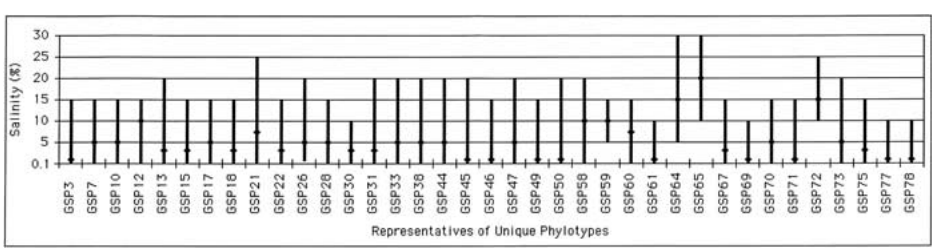

Figure 10. Salt tolerance and requirements of unique representatives of each GSP phylotype. The data presented for each isolate indicates the salinities at which visible growth occurred. The dot on each column indicates an approximate optimum salinity for growth.

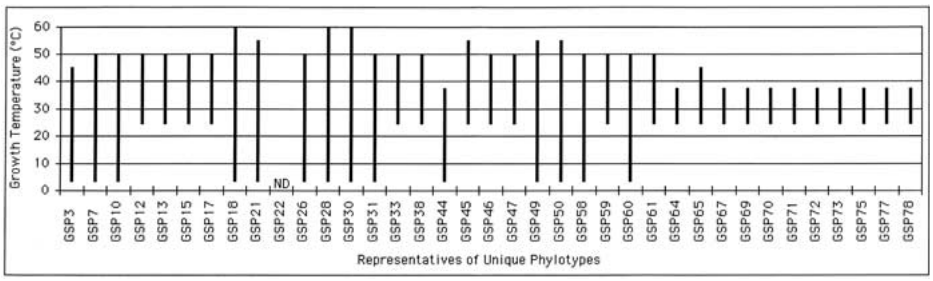

Figure 11. Temperature tolerance and requirements of unique representatives of each GSP phylotype. The data presented for each isolate indicates the temperatures at which visible growth occurred.

at 4°C. Ten of the isolates (24%) had narrow tolerance ranges (25 to 37°C), with nine of these being alkaline bacilli and the other one enriched on HM medium. It is interesting to note that the abundant organisms in the B1 clade related to *B. licheniformis* exhibited broad thermotolerance as evidenced by GSP30, but a relatively narrow range (0.1% to 10%) of halotolerance. Three representative isolates grew across the entire range from 4 to 60°C. In fact, only two (GSP 3 and 44) of the 12 isolates capable of growth at 4°C did not grow at 50°C. Fifteen of the isolates (42%) had at least a 30°C range of permissible growth temperatures. These results are not surprising given the high surface temperatures common in summer months and daily ranges of as much as 30°C.

Discussion

The initial enrichment and isolation of culturable aerobic heterotrophic bacteria from GSP soils yielded 105 isolates that represent a wide range of taxa. The culture collection is dominated by isolates that are most phylogenetically similar to bacteria that were isolated from hypersaline or desert environments. This is in sharp contrast to the only previous natural history study of saline soils by Quesada et al. at abandoned solar salterns [63, 64]. Those soils were dominated by halotolerant relatives of common soil bacteria, mainly *Flavobacterium*, *Pseudomonas*, *Alcaligenes*, *Micrococcus*, *Acinetobacter*, *Arthrobacter*, *Planococcus*, and *Bacillus*. The GSP bacterial community is rich in *Bacillus* and *Halomonas* spp., but only two *Pseudomonas* were isolated and none of the other saltern organisms were enriched, even though essentially the same media were used by Quesada et al. Their study found few overlaps between the species isolated from aquatic environments and those of the hypersaline soils. The GSP community includes many isolates closely related to bacteria found in aquatic systems. Salterns are created by recent flooding of nonsaline lands with seawater, generating evaporation pools of varying salinities, while the GSP is a very long-lived naturally hypersaline environment. Perhaps the rapid creation of salterns selected for halotolerant strains of the native nonsaline soil bacteria, while the GSP soils have developed communities rich in saline genera over longer periods of time. It is interesting to note that an early study on natural hypersaline soils near the Red Sea [31] found a culturable bacterial community that was nearly entirely (90%) bacilli, again in contrast to the saltern soil community.

The B1 clade of GSP isolates was enriched for *Bacillus* related to bacteria from dry or hypersaline environments. The cluster related to *B. licheniformis* [50] also includes *B. sonorensis* [61] from the Sonoran desert and a marine isolate (AJ000648; [48]), while the *B. subtilis* cluster includes *B. vallismortis* from Death Valley [29]. The *B. megaterium* strain related to GSP10 is from a spacecraft assembly clean-room, another potentially dry environment [82]. Three bacteria from the Yellow Sea, specifically from tidal flats that may be hypersaline, *B. baekryungensis*, *B. marisflavi* and *B. aquaemaris*, are closely related to GSP isolates [88].

In the B2 clade of GSP isolates, one cluster of phylotypes is related to the well-characterized moderate halophile *Halobacillus trueperi*, isolated from Great Salt Lake sediments [74], and *Halobacillus salinus* from a salt lake in Korea [86]. The GSP isolates from this cluster exhibited some remarkable departures from *H. trueperi*, including being oxidase-negative and hydrolyzing lipid and starch, along with the consistent Gram-negative staining of three of the GSP isolates (GSP 34, 35, and 43). The cluster including *Salibacillus* [5, 6, 85] and *Virgibacillus* [32, 33] is undergoing taxonomic revision. These species are known from the Dead Sea [6], dry wall paintings (*V. picturae*; [34]), and Salt Lake Keke (AY121439; [17]). A GSP phylotype is associated with *Bacillus* sp. KL-152 (AY030333), a halotolerant bacterium from a spacecraft assembly clean-room [82]. The characteristics of the *Salibacillus* type strain and the GSP isolates are very similar. Isolates GSP 31 and 32 are most closely related to *Marinococcus halophilus*, a moderately halophilic bacterium isolated from a solar saltern in Chile, where it plays an important role in the precipitation of carbonates [24, 30, 66].

The Gram-negative isolates in the GSP culture collection are dominated by organisms related to various members of the genus *Halomonas* (formerly *Deleya*) of the γ-Proteobacteria [4, 21, 51, 52, 84]. *Halomonas salina* is a well-characterized type strain isolated from solar salterns [11, 81], while *H. alimentaria* is from jeotgal

(Korean fermented seafood [89]), *H. variabilis* is from Great Salt Lake [25], and *H. venusta* is unpublished [20]. Several of the GSP isolates had coccoid morphologies rather than the rod shape of the type strain; however, pleomorphism is a common feature of *H. salina*, often as a function of changes in salinity or age of the culture [81]. The phenotypic characteristics of the GSP isolates matched the type strain except for the following: three isolates were nonmotile, three were oxidase-negative, all were lipase-positive, and none produced H_2S. Additionally, *Halomonas* is reported to be a strict aerobe, while four GSP isolates in this clade were capable of anaerobic growth, and one produced acid from glucose. *Chromohalobacter*, known from the Dead Sea region [52] and from jeotgal [89], is related to GSP58.

Salinivibrio costicola is a moderate halophile first isolated from salted foods [52, 53], but known from Death Valley [35]. The GSP isolates in the cluster differed from the type strain in being oxidase-negative and in one case (GSP 37) staining Gram-positive. The two GSP isolates that were closely related to *Pseudomonas halophila* [25] differed from the type strain in having higher salt tolerances, being catalase- and oxidase-negative, and gelatinase-positive. *Idiomarina* includes deep-sea (often hypersaline) and surface organisms [14, 22, 37]. One related GSP isolate consistently tested Gram-positive, three were nonmotile, and only one was gelatinase-positive, all in contrast to the reported strains. *Psychrobacter* spp. are widely known from arctic environments, the deep sea, and some temperate locations [13, 41]. The GSP isolates related to *Psychrobacter* differ significantly in phenotype, staining Gram-positive, reducing nitrate, exhibiting motility, and being oxidase-negative. It is interesting to note that the *Bacteroidetes* isolates obtained through dilution plating, most closely cluster with an isolate from Mono Lake, a meromictic soda lake [36].

All of the GSP isolates enriched on AB medium cluster with bacteria from alkaline environments. One cluster in the B2 clade is related to *Oceanobacillus iheyensis*, an extremely halotolerant alkaline deep-sea isolate whose genome has been sequenced [47, 78]. The second alkaline B2 cluster is related to an unpublished bacterial isolate from Hailaer Soda Lake [91] and alkaliphilic *B. agaradhaerens* and *B. clarkii* [57]. In the B1 clade, GSP69 clusters with the unpublished *B. alcaliinulinus* [75] and *B. krulwichiae*, a halotolerant alkaline bacterium [90]. GSP77 is related to *B. pseudofirmus*, a facultatively alkaline isolate from the deep sea [77].

Since the species distribution of the GSP isolate collection differs greatly from that of the only other extensive study of hypersaline soils, the abandoned saltern soils of Quesada et al. [63, 64], it is not surprising that there are great differences in overall phenotypic characteristics. Fewer of the GSP isolates were motile or oxidase-positive. None of the GSP isolates produced H_2S, whereas more than half of the saltern isolates did. A large percentage (75%) of the GSP isolates was lipase-positive, whereas few (4%) of the saltern isolates were. Salinity and temperature tolerances also differed greatly between the two collections. More of the GSP isolates grew at temperatures above 45°C. The GSP bacterial isolates were generally less halophilic. Whereas approximately half of the saltern isolates grew at 30% salinity, only 8% of the GSP isolates did. In addition, whereas nearly all (81%) of the GSP isolates grew at 0.1% salinity, only 23% of the saltern isolates grew at 0.5% salinity.

The earlier study of Quesada et al. concluded that the bacteria in hypersaline soils were not ''excessively specialized'' organisms. The GSP isolates clearly can live at low salt concentrations, enhancing their survival in the variable and heterogeneous environment of the GSP. However, some halophiles were isolated, indicating that local microhabitats may maintain very high salinities. In fact, the few archaeal isolates from the GSP are all halophilic, not growing below 10% salinity [16]. All of the saline soils that have been examined exhibited relatively low overall numbers of microbes, in the range of 10^5 cells per gram soil [31, 63, 64]. It is also interesting to note that the percentage of spore-forming organisms is relatively low, <20% of the GSP and saltern isolates [63, 64]. Taken together, these results suggest that many of the bacteria in hypersaline soils protect themselves from rapidly changing salinities by having a wide range of physiological salt tolerances, rather than by producing perennating structures under nonsaline conditions. Further study is needed to determine the mechanism by which the more halophilic isolates survive rain events that greatly lower salinities. Microbes that live in hypersaline ecosystems survive under difficult osmotic stresses and strong desiccation pressure that may produce much of the same cellular damage as other environmental stresses such as high UV irradiation [10, 54–56]. The same survival mechanisms, likely involving enhanced DNA repair abilities, may be working in organisms under different stresses.

Although the current study was not specifically designed to make quantitative comparisons between samples taken at different locations and times, certain distinctions can be drawn from the dataset. Dilution plating experiments were performed three times at WP6 over a period of 3 months, with each experiment yielding a similar set of dominant culturable isolates, despite intervening rain and flooding events. In addition, enrichment cultures from WP6 from two different summers yielded similar isolates, even though WP6 was subject to considerably more flooding and a changeover to sandier soils in the second year. The WP3 site yielded many Gram-negative isolates with few Gram-positive isolates, in direct contrast to the three more northerly sites. The WP3 isolates also tended to have different

metabolic capabilities, including greater lipase and gelatinase activity. It is interesting to note that the WP3 isolates had a higher incidence of nitrate reduction than isolates from WP6 did. For instance, GSP10 and GSP55 are in the same phylotype; however, GSP10 from WP3 performs nitrate reduction and GSP55 from WP6 does not. There is a gradient of nitrogen compounds at the GSP, with a northerly site such as WP6 having groundwater richer in nitrate, while a southerly site such as WP12 (N 36° 42.430′ and W 98° 15.612′) is richer in ammonia (Henley, unpublished). WP3 lies between these sites, suggesting that microbial activity may have a role in generating this gradient. It would be interesting to assay for functions associated with the nitrogen cycle in these hypersaline soils and to direct enrichment efforts toward biogeochemical functional guilds. It has been argued, based on energetics, that certain functions, particularly nitrification, are not likely to be feasible under hypersaline conditions [60]. However, insufficient effort has been directed at isolating specialized organisms from hypersaline ecosystems.

Interest in the microbes living in extreme environments has been fueled by the discovery of novel taxa in these ecosystems and by the potential for discovery of valuable enzymes, polymeric materials, and bioactive compounds [19, 27, 42]. For instance, novel antibiotics, the quinolinols, have been isolated from halophilic organisms [9, 67]. The survivability of organisms in extreme environments has implications in evolutionary biology and astrobiology. It has been suggested that life originated in a hypersaline soup of primordial evaporates [44, 70, 71]. The current study has identified culturable members of 46 phylotypes of bacteria from the GSP. The culture collection is now available to be screened for interesting activities and products. The survival mechanisms used by the isolates can be studied to better understand the ecology of variable hypersaline terrestrial environments.

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