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Diazotrophic Community Structure and Function in Two Successional Stages of Biological Soil Crusts from the Colorado Plateau and Chihuahuan Desert

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The objective of this study was to characterize the community structure and activity of N_2-fixing microorganisms in mature and poorly developed biological soil crusts from both the Colorado Plateau and Chihuahuan Desert. Nitrogenase activity was approximately 10 and 2.5 times higher in mature crusts than in poorly developed crusts at the Colorado Plateau site and Chihuahuan Desert site, respectively. Analysis of *nifH* sequences by clone sequencing and the terminal restriction fragment length polymorphism technique indicated that the crust diazotrophic community was 80 to 90% heterocystous cyanobacteria most closely related to *Nostoc* spp. and that the composition of N_2-fixing species did not vary significantly between the poorly developed and mature crusts at either site. In contrast, the abundance of *nifH* sequences was approximately 7.5 times greater (per microgram of total DNA) in mature crusts than in poorly developed crusts at a given site as measured by quantitative PCR. 16S rRNA gene clone sequencing and microscopic analysis of the cyanobacterial community within both crust types demonstrated a transition from a *Microcoleus vaginatus*-dominated, poorly developed crust to mature crusts harboring a greater percentage of *Nostoc* and *Scytonema* spp. We hypothesize that ecological factors, such as soil instability and water stress, may constrain the growth of N_2-fixing microorganisms at our study sites and that the transition to a mature, nitrogen-producing crust initially requires bioengineering of the surface microenvironment by *Microcoleus vaginatus*.

Biological soil crusts can comprise a significant portion of the living cover of arid and semiarid landscapes worldwide (e.g., up to 70% of landscapes of the southwest United States [4]), yet the central role that crusts play in desert ecosystem dynamics has only recently begun to gain general recognition. These complex crust communities are composed of bacteria, green algae, fungi, lichens, and mosses that together form a structured, gelatinous matrix covering and binding the uppermost layers of the soil (depth, up to 10 cm). Four morphologically distinct crust types are found in the southwest United States, depending on climate (8). Flat, cyanobacterium-only crusts occur where soils do not freeze and precipitation prevents lichens and mosses from establishing (hyperarid deserts). Lichen and moss cover increases with increasing precipitation, forming rugose crusts (arid Chihuahuan, Sonoran deserts). Frost heaving plus limited lichen-moss cover creates pinnacled crusts (Colorado Plateau), and frost heaving plus high lichen-moss cover results in rolling crusts (northern Great Basin). When heavily disturbed, all crusts lose their lichen-moss component and microtopography, thus resembling flat, poorly developed crusts found in hyperarid deserts. With time and absence of disturbance, crusts will recover to the degree allowed by climate and localized physical conditions, such as soil structure, topography, and radiation intensity. (See http://www.soilcrust.org/gallery.htm for photos of the soil crusts).

In this study, we examined both flat, poorly developed crusts and pinnacled, mature crusts from the Colorado Plateau (near Canyonlands National Park, Utah) and flat, poorly developed crusts and rugose, mature crusts from the Chihuahuan Desert (Jornada Experimental Range, N.M.). As observed by microscopy and 16S rRNA gene analysis, poorly developed crusts from these regions are typically dominated by *Microcoleus vaginatus*, whereas mature crusts contain appreciable numbers of other cyanobacteria, including *Nostoc* and *Scytonema* spp. (1, 3, 7, 37). Indeed, the UVR-absorbing pigment scytonemin, produced by *Nostoc* and *Scytonema* spp., gives the mature crusts their distinctive black coloration (14). These two crust types represent distinct stages of ecological succession, with poorly developed crusts forming relatively quickly (generally $<$10 years) following soil disturbance and mature crusts appearing much later (decades to centuries) (6).

Functionally, biological soil crusts play critical roles in soil stability and water retention and flow. They also provide habitat for soil invertebrates and germination sites for vascular plant seedlings (8, 17, 20, 45). Soil crusts also play an important role in nutrient cycling in arid and semiarid ecosystems. Carbon input via crust photosynthesis is the primary mechanism for the buildup of organic matter in many areas of sparse vegetation, especially within the plant interspaces (10, 21). Biological crusts have also been found to be a dominant source of fixed nitrogen in desert soils (19), which are typically nitrogen limited (44). Remarkably, undisturbed mature crusts are capable of fixing nitrogen at rates approximately an order of magnitude higher than those for poorly developed crusts, and mature crusts containing the lichen *Collema tenax* fix nitrogen at even higher rates (3, 5). It is estimated that mature crusts

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input approximately 9 kg of N ha^{-1} $year^{-1}$, whereas the values for poorly developed crusts range from 0 to 1.4 kg of N ha^{-1} $year^{-1}$ (3). Additionally, Evans and Belnap (18) have shown that crust disturbance in regions of the Colorado Plateau can have profound effects on nitrogen-fixing activity in the soil. Crust disturbance resulted in a 250% decrease in soil nitrogenase activity over a 30-year period in comparison to that of pristine sites, which led to a 25 to 75% loss of soil N content.

From morphological examination of biological soil crusts in combination with pure culture studies, it is thought that cyanobacteria and cyanolichens are key members of crust diazotrophic communities. *Nostoc* spp. have been implicated as the source of N_2 fixation in *C. tenax* and are thought to contribute greatly to N_2 fixation in mature crusts, along with free-living *Nostoc* and *Scytonema* spp. (3, 28, 35). In poorly developed crusts, *M. vaginatus*, or possibly an epiphytic heterotroph associated with this cyanobacterium, has been considered a source of fixed nitrogen (39).

Culture-based analysis of bacterial communities can lead to erroneous interpretations, since most bacterial species are not readily cultivated by routine methods, and, with the exception of heterocystous cyanobacteria, it is nearly impossible to identify N_2-fixing bacteria based on microscopic analysis alone. Therefore, the composition of the diazotrophic community associated with biological soil crusts has not been firmly established. Characterization of natural diazotrophic communities has been greatly enhanced by PCR amplification and sequence analysis of the gene encoding the reductase subunit of nitrogenase, *nifH*. Because phylogenetic relationships based on *nifH* sequences are largely congruent with those obtained from 16S rRNA gene data, *nifH* sequence analysis can be used to characterize the genetic diversity among natural N_2-fixing populations and to examine the nitrogen-fixing potential of microbial communities in a culture-independent manner (41, 47, 49, 50).

The goal of the present study was to use *nifH* and 16S rRNA gene sequence analysis in combination with *nifH* terminal restriction fragment length polymorphism (T-RFLP) analysis to systematically characterize the diazotrophic community associated with poorly developed and mature crusts from two climatically distinct regions of the Southwest United States, the Colorado Plateau and the Chihuahuan Desert. The purpose was to provide information concerning the underlying community structure responsible for the differences in N_2 fixation exhibited by poorly developed and mature crusts at both of these sites.

MATERIALS AND METHODS

Site and sampling characteristics. Poorly developed (*Microcoleus* spp.-dominated) and mature (mixed cyanobacteria, lichen, and moss) biological soil crusts were collected near Canyonlands National Park (38°35.08′N, 109°49.16′W) in southeastern Utah and on the Jornada Experimental Range (32°31.80′N, 106°43.41′W) in southern New Mexico during late summer/early fall 2002. The Canyonlands collection site was dominated by a pinyon-juniper woodland comprised of *Pinus edulis* (pinyon pine), *Juniperus osteosperma* (Utah juniper), and *Coleogyne ramosissima* (blackbrush). Samples were collected predominantly near blackbrush and in shrub interspaces. At the Jornada site, *Flourensia cernua* (tarbush) was the dominant plant, and samples were collected near bushes and from interspaces. The crust samples were largely devoid of plant debris and other visible organic matter. The Canyonlands soils are classified as a Rizno series loamy mixed, calcareous soil, and the New Mexico soil is a Regan series clay loam.

Previous sampling efforts of similar crusts from the Colorado Plateau show that the population structure of crusts can vary widely on even the millimeter scale (25, 46). Consequently, multiple individual samples of each crust type (poorly developed and mature) were collected from each of the Canyonlands and Jornada sites and represented an area of approximately 1 acre in each site in order to capture the heterogeneity of each site. At Canyonlands the top 5 cm of crust and soil were collected intact by pushing a 6-in-diameter ABS pipe (5 cm in height) into the ground and then sliding a flat metal sheet under it to remove the intact core. At Jornada, we were not able to insert the 5-cm pipe into the soil, so crusts were removed onto flat metal sheets and the soils to a 5-cm depth were removed by hand trowel. The crust samples were frozen immediately with dry ice and transported to the laboratory, where they were stored at −80°C. Nucleic acids were extracted from homogenized crust samples within 2 weeks of collection.

A set of 12 crust samples representing each crust type in each site (total of 48 samples) was used for all DNA-based analyses. For nitrogenase activity assay, chlorophyll *a* measurements, and microscopic analysis, either a portion of each original sample was used or additional samples were collected from the crusts as needed for each assay.

Crust nitrogenase activity. Nitrogen fixation was estimated using the acetylene reduction assay (5, 9, 27, 40). Five-centimeter-deep core samples were collected from poorly developed and mature crust samples from each site ($n = 25$ to 27 for each crust type at each site). Crusts were removed onto flat metal sheets and sealed with permagum (Virginia KMP), wetted with distilled water, sealed above with flat polycarbonate sheets containing injection ports, and then injected with enough acetylene to create a >10% acetylene atmosphere. After injection, samples were placed under fluorescent lights (~700 μmol m^{-2} s^{-1}) and incubated for 1 h at 24°C. Subsamples (4.0 ml) of the headspace were then withdrawn into vaccutainers and analyzed for acetylene and ethylene content on a Shimadzu (Kyoto, Japan) FID gas chromatograph equipped with a Hayesep-T column, using hydrogen as the carrier gas.

Chlorophyll *a* measurements. Chlorophyll *a* concentrations were estimated for eight crust samples of each crust type at each of the two sites (avoiding aboveground lichen thalli and moss tissue). Chlorophyll was extracted from the soil with ~90% acetone and measured with fluorescence and UV/visible absorption following high-performance liquid chromatography (HPLC) separation of the pigments. Samples ($n = 8$ for each crust type at each site) were collected by taking 0.5-mm cores with 10.6-mm-diameter tubes. Samples were each homogenized by gentle mixing with a mortar and pestle. One gram of each homogenized sample was transferred to a clean mortar and pestle and ground with ~1 ml of distilled H_2O, and then ~5 ml of HPLC grade acetone was added and the sample was ground again. The samples were transferred in ~90% acetone to a 7-ml tissue grinder, where they were ground until all matter was pulverized. This liquid and soil sample was transferred, using ~90% acetone, to a 20-ml glass test tube. The test tube was covered with a blanket of nitrogen, topped with parafilm, and stored at 4°C overnight. The samples were filtered on GF/F Whatman filter paper under vacuum. The liquid was filtered into a graduated test tube and evaporated to 3 ml using a gentle stream of nitrogen gas. Samples were then transferred to an autosampler vial and stored in the refrigerated autosampler chamber at 4°C until analyzed by HPLC. Quantitative and qualitative HPLC analyses were performed using a chromatography system (Waters Corporation, Milford, Mass.) as previously described (11).

Microscopic analysis of crust material. Crust samples (0.5 g) from the Canyonlands site ($n = 8$ for each crust type) were each separately ground with a mortar and pestle. Crust samples from the Jornada site ($n = 12$ for each crust type, poorly developed or mature) were pooled according to crust type and homogenized. Five subsamples from each Jornada crust mix (poorly developed or mature) were then removed and ground with a mortar and pestle. Canyonlands ($n = 8$ for each crust type) and Jornada ($n = 5$ for each crust type) samples were treated similarly hereafter for microscopic analysis.

The crust samples were hydrated with 2 ml of distilled H_2O, shaken to mix, and incubated for 30 min at room temperature. This incubation has been found helpful in separating *Microcoleus* filaments from their polysaccharide sheaths. Following the incubation, the samples were briefly shaken and 2 drops were removed from the mix and placed on a microscope slide. For each sample mix, four slides were prepared. Ten randomly selected fields were examined under magnification ×300 for each slide. Cyanobacteria that were located within the eyepiece grid of each field were enumerated and identified.

Soil crust DNA extraction. All DNA-based analyses were conducted on a set of 12 crust samples representing each crust type in each of the two sites (48 total samples). Total DNA was extracted from Canyonlands crust material (including moss or lichen material) using a modified version of the protocol described by Griffiths et al. (24). Homogenized crust material (1 g) from each of 12 cores of poorly developed crusts and 12 cores of mature crusts was added separately to 24

2-ml bead-beating tubes containing 0.1- and 0.5-mm beads (450 mg each). Next, 0.5 ml of cetyltrimethylammonium bromide (CTAB) extraction buffer (10% CTAB dissolved in 0.7 M NaCl mixed with an equal volume of 240 mM potassium phosphate [pH 8.0]) and 0.5 ml of molecular biology grade phenol-chloroform-isoamyl alcohol (25:24:1) were added to each tube. Samples were disrupted by bead beating for 2 min at room temperature and centrifuged at 4°C for 5 min at 16,000 × *g*. The aqueous-phase material was withdrawn, added to an equal volume of chloroform-isoamyl alcohol (24:1), and then mixed gently to extract residual phenol. Samples were then centrifuged at 4°C for 5 min at 16,000 × *g*. The aqueous-phase material was removed and added to a 0.1× volume of 3 M ammonium acetate (pH 5.2) and a 0.6× volume of room-temperature isopropanol. DNA was precipitated by incubating the mix at room temperature for 30 min, followed by centrifugation at room temperature for 20 min at 18,000 × *g*. The supernatant was removed. The pellet was washed with ice-cold 70% ethanol, allowed to air dry for 15 to 30 min (depending on the size of the pellet), and suspended in 75 μl of RNase-free water at room temperature for 10 to 15 min with occasional flicking of the tube.

Several variations of the Griffiths protocol were tested to extract DNA from Jornada crust material, without success. Therefore, instead of using the CTAB-organic extraction step, a similar bead-beating protocol with a sodium dodecyl sulfate-based DNA extraction was used to obtain DNA from these samples (31). One milliliter of TENS (50 mM Tris [pH 8.0], 20 mM disodium EDTA, 100 mM NaCl, 1% [wt/vol] sodium dodecyl sulfate) and crust material (0.5 g from each of 12 cores of poorly developed crusts and 12 cores of mature crusts) were added to a bead-beating tube and incubated at 70°C for 1 h. The cells were then disrupted by bead beating (as described above) and centrifuged at room temperature for 10 min at 12,000 × *g*. The supernatant was collected, and DNA was precipitated with ethanol and pelleted by centrifugation for 10 min at 13,000 × *g*. The pellet was air dried and suspended in 50 μl of distilled H_2O. All DNA stock solutions were stored at −80°C.

***nifH* and cyanobacterium-specific 16S rRNA gene PCR.** PCR amplification of *nifH* gene fragments from crust DNA was performed using a nested protocol and degenerate primers designed to amplify *anfH* (encodes the reductase subunit of alternative dinitrogenases that contain only iron in the active site), *vnfH* (encodes the reductase subunit of alternative dinitrogenases containing iron and vanadium in the active site), and *nifH* (encodes the reductase subunit of dinitrogenases with an active site containing iron and molybdenum) sequences from the vast majority of known diazotrophs. Forward primer 19F (5′-GCIWTYTAYGGIAARGG IGG) (41) and reverse primer nifH3 (5′-ATRTTRTTNGCNGCRTA) (48) were used in the first amplification reaction, which contained 40 pmol of each primer, 30 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 10 μg of bovine serum albumin, 200 μM (each) deoxynucleoside triphosphates, 2.5 U of AmpliTaq DNA polymerase, LD (Applied Biosystems, Foster City, Calif.), and 1 to 2 μl of crust DNA in a final reaction volume of 50 μl. Hot-start PCRs were carried out in an MJ Research (Waltham, Mass.) PT C-200 Peltier thermal cycler. Parameters for the reaction cycle were as follows: 95°C for 5 min, followed by 20 cycles of 48°C for 1 min, 72°C for 1 min, and 94°C for 45 s, with a 72°C final extension step for 10 min before holding at 4°C when the reaction was terminated.

For the second PCR, slight variations of primers nifH1 and nifH2 (48) were designed to generate forward primer nifH11 (5′-GAYCCNAARGCNGACTC) and reverse primer nifH22 (5′-ADWGCCATCATYTCRCC). These primers (40 pmol each) were included in a 50-μl reaction mix identical to the one described above, except that it contained 2.0 mM $MgCl_2$, and 2 μl from a 1:10 dilution of the first PCR mixture served as the template. The second PCR was performed as described above except that the annealing temperature was 55°C and the reaction cycle was repeated 32 times.

Amplification of cyanobacterium-specific 16S rRNA gene sequences was achieved using the forward primer CYA359F (5′-GGGGAATYTTCCGCAAT GGG) and reverse primers CYA781RA (5′-GACTACTGGGGTATCTAATC CCATT) and CYA781RB (5′-GACTACAGGGGTATCTAATCCCTTT) (33). Approximately 1 to 2 ng of crust DNA was added to a reaction mixture (total volume, 30 μl) containing the following: 15 μl of iQ SYBR Green Supermix (Bio-Rad, Hercules, Calif.), 22.5 pmol of CYA359F, and 11.25 pmol of each CYA781RA and CYA781RB. PCR amplification was carried out in 96-well PCR plates with a Bio-Rad iCycler as follows: 95°C for 7 min, followed by 35 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s.

Cloning, sequencing, and phylogenetic analysis. Clone libraries were generated with the TOPO TA cloning kit for sequencing and TOP10 chemically competent *Escherichia coli* (Invitrogen). Libraries were generated from two to three individual crust DNA samples from each crust type at each site, and the numbers of clones sequenced are presented in Table 1. Poor sequence quality or the presence of PCR chimeras (identified with the Chimera_Check program of the Ribosomal Database Project II [16] and by visual inspection of sequence alignments) precluded the analysis of 9 to 29% of the sequences obtained from each library. Alignment of DNA sequences was performed using Clustal X v1.81 and visually inspected with the BioEdit sequence alignment editor (26). Translations and phylogenetic analysis of sequences were performed with MEGA version 2.1 (30). The 16S rRNA gene and *nifH* DNA dendrograms were constructed using the minimum-evolution function of the software program MEGA version 2.1 with initial trees obtained by the neighbor-joining (NJ) method. Distances were calculated with the Kimura two-parameter algorithm and pairwise deletion of gaps and missing data.

TABLE 1. Clone libraries generated in this study

Site	Crust type	Library type[a]	No. of clone libraries	No. of clones/ library
Canyonlands	Poorly developed	16S cyano	3	30
Canyonlands	Poorly developed	*nifH*	3	30–35
Canyonlands	Mature	16S cyano	3	30
Canyonlands	Mature	*nifH*	3	30–35
Jornada	Poorly developed	16S cyano	2	44–46
Jornada	Poorly developed	*nifH*	2	44–46
Jornada	Mature	16S cyano	2	44–46
Jornada	Mature	*nifH*	2	44–46

[a] *nifH* and 16S rRNA gene libraries were paired for each site (i.e., DNA from the same crust samples were used to amplify both *nifH* and 16S rRNA genes).

T-RFLP analysis of *nifH* PCR products. Fluorescent *nifH* fragments were generated via nested PCR amplification (as described above) of crust DNA obtained from each of 12 cores of poorly developed crusts and 12 cores of mature crusts using 5-carboxyfluorescein-labeled nifH11 primer in the second reaction. For each crust sample, three individual PCRs were performed, which were subsequently pooled, concentrated using a speedvac, and applied to a 2.5% agarose gel for fragment separation. A 358-bp band was excised from the gel, and DNA was isolated using the Qiagen (Chatsworth, Calif.) QIAquick gel extraction kit. The purified amplicons were quantified by running subsets of samples alongside the Precision molecular mass standard (Bio-Rad) on a 2.8% agarose gel and analyzing band intensities with Science Lab 99 Image Gauge version 3.3 software (Fuji Photo Film Co., Tokyo, Japan). Fifty nanograms of purified *nifH* amplicons were digested with 2.5 U of *Mae*III (Roche Diagnostics Co., Indianapolis, Ind.) at 55°C for 4.5 h, and 1 μl of the restriction digest was heated to dryness at 95°C (2.5 min). The DNA was then suspended in 2.25 μl of loading buffer (0.25 μl of Genescan 500 TAMRA size standard [Applied Biosystems], a 5:1 mixture of deionized formamide-blue dextran, and 25 mM EDTA), denatured at 95°C for 2.5 min, and immediately placed on ice. Samples (1.5 μl) were then loaded onto a 5% denaturing polyacrylamide gel (Long Ranger Singel Packs; ABI), and fragments were separated by electrophoresis at a constant temperature (51°C) and voltage (3,000 V) for 2.5 h. Genescan version 3.1 software (ABI) was used to analyze fragment sizes and peak fluorescence intensities. Three replicate T-RFLP profiles were generated per sample for Canyonlands DNA, and duplicate T-RFLP profiles were generated per sample for Jornada DNA.

For analysis of T-RFLP data, peak areas for each sample were converted to a percentage of the total peak area (total fluorescence) for that sample. Peaks that represented at least 0.5% of the total peak area for a given sample were included in our analysis; peaks below this threshold were assigned a value of 0%. The peak area percentage values from the replicate T-RFLP profiles were then averaged to yield a quantitative representation of the occurrence of *nifH* fragment types in each crust DNA sample. Peak area fluorescence values ranged from 500 to 200,000 fluorescence units. Peaks included in the analysis were easily assigned to individual peaks in each profile by visual inspection and could be readily aligned with peak data from the other profiles.

***nifH* quantitative PCR.** Primers CY81F (5′-GYGCTGTNGAAGATATWG AAC) and CY226R (5′-GCCGTTTTCTTCCAAGAAGTT) were designed to amplify a 145-bp fragment from each of the *nifH* sequence types that we had recovered from Canyonlands biological soil crusts by PCR and cloning. Quantitative PCR amplifications were carried out in 30-μl reaction mixtures containing 15 μl of iQ SYBR Green Supermix, 15 pmol of each CY81F and CY226R, and approximately 12 ng of crust DNA as template. PCR amplification of each sample was carried out in triplicate in 96-well PCR plates with a Bio-Rad iCycler as follows: 95°C for 7 min, followed by 45 cycles of 95°C for 30 s, 55°C for 25 s, and 72°C for 30 s. The Icycler iQ Optical System Software Version 3.0a (Bio-Rad) was used to analyze data, and the PCR baseline subtraction curve fit function was used to automatically determine threshold cycle (C_t) values. Indi-

TABLE 2. Estimates of microbial biomass associated with biological soil crusts

Crust source	Microbial biomass[a]	
	Chlorophyll *a* content (μg g of soil^{-1})	DNA extracted (μg g of soil^{-1})
Canyonlands, poorly developed	10.8 ± 1.6[b]	12.3 ± 2.1
Canyonlands, mature	20.8 ± 0.4	28.6 ± 4.3
Jornada, poorly developed	5.9 ± 1.3	8.2 ± 1.3
Jornada, mature	5.8 ± 0.8	19.6 ± 0.9

[a] Chlorophyll *a* was used as an estimate of cyanobacterial biomass, and the amount of DNA extracted was used as an estimate of the total microbial biomass.
[b] Values are reported as means ± standard errors (n = 8 for chlorophyll *a* measurements; n = 12 for DNA measurements).

vidual PCR amplifications were repeated at least two times with template DNA obtained from each of 10 cores of poorly developed crusts and 10 cores of mature crusts from each site.

Nucleotide sequence accession numbers. Sequences were deposited in GenBank under accession numbers AY360961 to AY360977 for *nifH* clusters and AY360978 to AY361002 for representative cyanobacterium 16S rRNA gene sequences.

RESULTS

Microbial biomass and nitrogenase activity associated with biological crusts. Two methods were used to obtain estimates of the microbial biomass associated with the Canyonlands and Jornada crust samples. First, chlorophyll *a* concentrations were measured to estimate the free-living cyanobacterial biomass (34). Second, the bead-beating DNA extraction procedures used in this study preferentially isolate microbial DNA based on the size of the beads used (http://www.biospec.com) and do not effectively disrupt lichen thalli or moss tissues. Thus, the amount of DNA extracted from crust material was used to provide a rudimentary estimate of the associated microbial biomass.

Both the cyanobacterial and total microbial biomass measured in Canyonlands crusts were approximately two times higher in mature crusts than in poorly developed crusts, as determined by chlorophyll *a* and DNA content, respectively (Table 2). With Jornada crusts, approximately 2.5 times more DNA was extracted from the mature crust material than from poorly developed material, yet essentially no difference was observed between the amount of chlorophyll *a* extracted from the two crust types at this site. With mature and poorly developed crust samples from Canyonlands and poorly developed crust samples from Jornada, the ratio of chlorophyll *a* to DNA extracted from a given sample (wt/wt) averaged 0.7 to 0.9, whereas the ratio averaged 0.3 for the Jornada mature crust samples. Although it is difficult to provide a satisfactory explanation for the low chlorophyll *a*/DNA ratio measured in the Jornada mature crust material from the existing data, it is possible that fungus-, heterotrophic bacterium-, or lichen-associated DNA comprised a significantly higher proportion of the total extracted DNA in the Jornada mature crust samples than in the others.

Consistent with results of previous studies (21), nitrogenase activity measured by acetylene reduction was greater in mature crust material than in poorly developed crust material collected from both the Canyonlands (10-fold increase) and Jornada (2.6-fold increase) sites (Fig. 1).

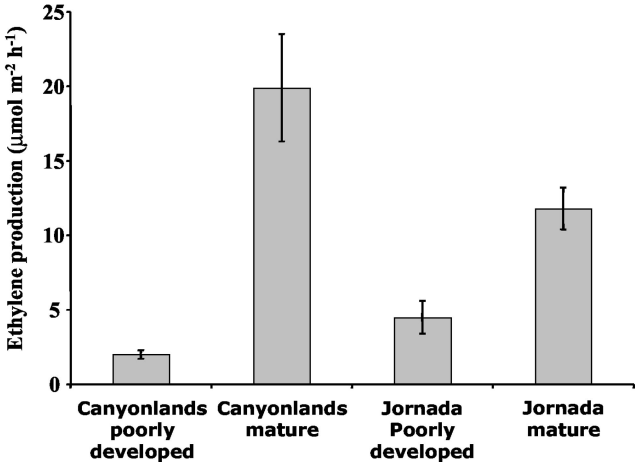

FIG. 1. Nitrogenase activity associated with poorly developed and mature crusts at the Canyonlands and Jornada sites measured with the acetylene reduction method. The bars represent the mean ± standard error for 25 to 27 crust cores. The means of the poorly developed and mature crust activities at each site were significantly different from each other as determined by Student's t test.

***nif*H sequence analysis.** To determine the putative identities of bacterial species that carry out nitrogen fixation in the Canyonlands and Jornada crust samples, *nifH* PCR products were obtained from both sites, cloned, and sequenced (Table 1). The majority (90%) of sequences from both sites were tentatively classified as *Nostoc*-type *nifH* based on BLASTn searches and phylogenetic analysis of the sequences (Fig. 2). The *Nostoc*-like *nifH* sequences were grouped into 12 distinct clusters (A to L) by distance analysis of DNA sequences. Sequences within each cluster were at least 95% similar (most were >98% similar), and all *Nostoc*-type *nifH* sequences were at least 84% similar to each other. Although the *nifH* DNA sequence clusters were obviously distinct from one another by DNA comparison, several of the consensus sequences representing each cluster encoded identical polypeptides (A and D, H and I). Additionally, some of the translated *nifH* sequences were identical to known sequences. The consensus NifH sequences of clusters B, E, and J were identical to those from *Scytonema* sp., *Nostoc* sp. N2, and *Anabaena* sp. I1, respectively. Three NifH consensus sequences obtained in the present study were identical to NifH protein sequences previously identified in bacteria isolated from Canyonlands crust material (39). The consensus sequence for cluster F was identical to that from Utah soil crust isolate M, and the consensus sequence for clusters H and I was identical to that from Utah soil crust isolate B.

Seven percent (20 of 283) of the *nifH* sequences comprised cluster O and were most closely related to *nifH* from the uncultured bacterium NIS10-1, which was recovered from dead stands of the smooth cordgrass, *Spartina alterniflora*, in a salt marsh on Sapelo Island, Ga. (32). However, 13 of the sequences in this cluster were recovered from just one of the mature crust samples from Canyonlands. Other *nifH* sequences recovered from the crusts were most closely related to *nifH* sequences from the cyanobacteria, *Microcoleus chthonoplastes* (cluster N) and *Bradyrhizobium* sp. IRBG2 (cluster P). Finally, four sequences obtained from Jornada poorly developed crust

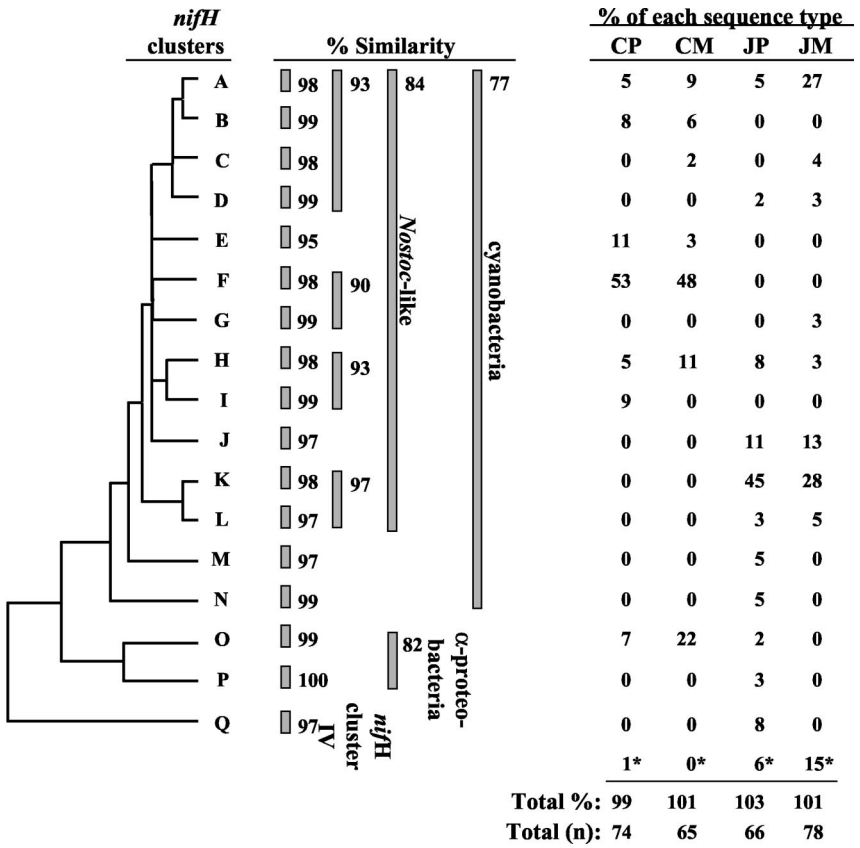

FIG. 2. Sequence analysis of *nifH* clones recovered from biological soil crusts. Sequences were grouped into clusters (A to Q) based on visual inspection of alignments, distance data, and NJ trees. Similarity (percent) was determined between all members of each group(s). Clusters were assigned to known taxa (*Nostoc*-like, cyanobacteria, α-proteobacteria, or cluster IV) based on BLASTx searches and NJ tree analysis. The percentage of each *nifH* sequence type recovered from each crust type and site is listed in the table next to the corresponding *nifH* cluster. Values with an asterisk represent sequences that could not be readily assigned to any cluster (all were *Nostoc*-like sequences, however). Abbreviations: CP, Canyonlands poorly developed; CM, Canyonlands mature; JP, Jornada poorly developed; JM, Jornada mature.

samples comprised cluster Q and grouped deeply within cluster IV of *nifH* sequences as defined by Chien et al. (15). These sequences are very divergent, and it is unknown if they encode functional NifH proteins (13, 15).

T-RFLP analysis of *nif*H sequences. Using small clone libraries, it is difficult to quantify the relative abundance of sequence types within a population, and it is expensive and time consuming to construct and analyze numerous, large clone libraries. However, with certain functional genes or limited-scope 16S rRNA gene surveys, T-RFLP analysis can provide a semiquantitative representation of sequence distribution (richness) and relative abundance (evenness) between samples in a timely and affordable fashion. By determining a computer-simulated, theoretical terminal restriction fragment (TRF) size for each *nifH* sequence type (clusters in Fig. 2) identified in our *nifH* clone libraries, individual TRFs could be paired with *nifH* sequence types. Therefore, the relative contribution of each *nifH* sequence type to the total *nifH* sequence pool for a given crust sample could be established via T-RFLP analysis. Because we retrieved and analyzed approximately 140 sequences each from Canyonlands and Jornada, the *nifH* sequence clusters shown in Fig. 2 should represent the majority of amplifiable *nifH* sequence types found at these two study sites. Preliminary analysis of *nifH* sequences obtained from Canyonlands crust material led us to use *Mae*III to generate T-RFLP profiles for optimal differentiation of the various *nifH* sequence types present in the crusts.

The results shown in Fig. 3 demonstrate that the frequencies of the theoretical *MaeIII* TRFs (determined by in silico digestion of *nifH* clones) were similar to the observed *MaeIII* TRF frequencies (determined from T-RFLP profiles) in pooled samples from poorly developed and mature crusts collected at both the Canyonlands and Jornada sites. These results gave us confidence that *nifH* T-RFLP data could be used to approximate the relative abundance of *nifH* sequence types P, Q, N, E, H, O, I, and F within crust samples from the Canyonlands and Jornada sites. Sequence types A, B, C, D, G, J, K, L, and M (all Nostoc-type *nifH*, except the cyanobacterial-like cluster M) were all expected to yield a single 266-bp TRF with the *MaeIII* digestion; therefore, they could not be discriminated from one another.

Subsequently, T-RFLP analysis was used to examine the distribution and relative abundance of *nifH* sequences amplified from 12 mature and 12 poorly developed crust cores collected from each of the Jornada and Canyonlands sites. The *Mae*III profiles generated from mature and poorly developed crusts from Canyonlands were similar, with the only major difference being the increased abundance of two TRFs, 54 and

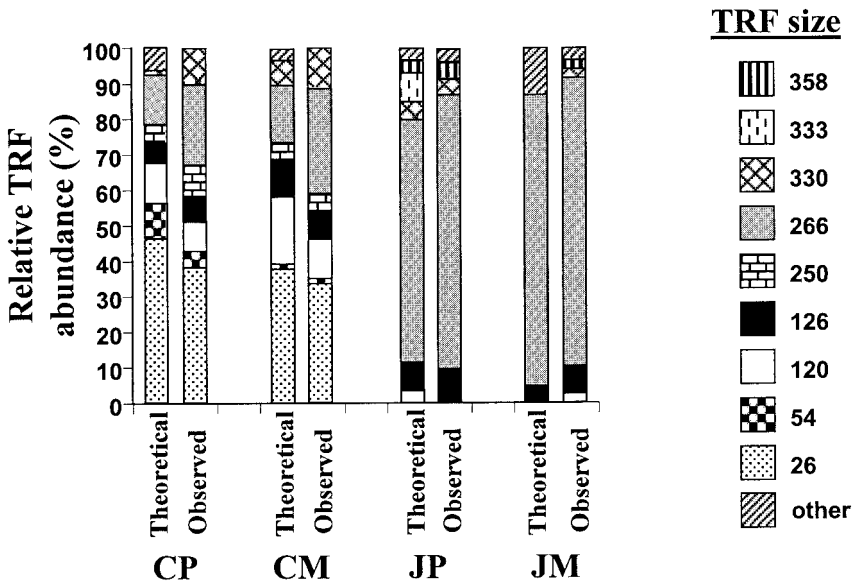

FIG. 3. Comparison of the theoretical frequencies of *nifH* TRFs versus the observed frequencies of *nifH* TRFs in Canyonlands and Jornada crusts. Bars labeled Theoretical represent the average theoretical TRF abundance (percent) determined by in silico *Mae*III digestion of *nifH* clone sequences obtained from DNA isolated from the same crust cores used for the T-RFLP analysis described below (three Canyonlands clone libraries for each crust type, two Jornada clone libraries for each crust type). Bars labeled Observed represent the average observed TRF abundance (percent) determined from T-RFLP profiles of three poorly developed or three mature Canyonlands crust cores (CP and CM, respectively) or two poorly developed or two mature Jornada crust cores (JP and JM, respectively).

250 bp in length, in most of the poorly developed crust profiles (Table 3). Likewise, the mature and poorly developed crust profiles generated from Jornada samples were similar to each other. The only noticeable difference was the presence of an additional 120-bp TRF in some mature crust profiles and a slightly greater abundance of 330- and 358-bp TRFs in the poorly developed crust profiles. The results presented in Table 3 suggest that the differences between *nifH*-containing members of the crust are much greater between geographic sites (Canyonlands and Jornada) than between crust types (poorly developed versus mature). However, comparisons between sites (Canyonlands versus Jornada) should be approached judiciously because different extraction procedures were utilized to obtain crust DNA from the two sites and because the sequence analysis did not consist of an equal sample size for each site.

A total of 10 distinct TRFs were identified in crust material from the Canyonlands and Jornada sites. Three TRFs (26, 266, and 330 bp) represented 70 to 80% of the *nifH* sequences obtained from the Canyonlands poorly developed and mature crust samples. One *nifH* TRF (the 266-bp TRF) comprised approximately 80% of the *nifH* sequences retrieved from both Jornada crust types. Inspection of standard deviations displayed in Table 3 and the raw T-RFLP data revealed that the

TABLE 3. Average abundance (%) of each *nifH* TRF observed for poorly developed (P) and mature (M) crust samples from the Canyonlands and Jornada sites

nifH TRF size (bp)	Corresponding *nifH* sequence cluster[b]	Putative phylogenetic classification[c]	Avg *nifH* TRF abundance (%)[a]			
			Canyonlands		Jornada	
			P	M	P	M
358	P	α-Proteobacteria	0	0	3.7 ± 4.9	1.5 ± 2.4
330	N	Cyanobacteria	11.8 ± 4.2	12.6 ± 3.0	4.4 ± 1.9	1.9 ± 0.4
266	A, B, C, D, G, J, K, L, M	*Nostoc*/cyanobacteria[d]	22.9 ± 11.0	28.9 ± 5.3	79.0 ± 4.9	82.8 ± 4.3
250	E	*Nostoc*	10.1 ± 6.2	2.5 ± 2.5	0.4 ± 0.9	0.3 ± 0.6
126	H	*Nostoc*	5.5 ± 1.7	6.0 ± 1.8	9.2 ± 4.1	7.8 ± 3.1
120	O	α-proteobacteria	7.6 ± 8.2	7.2 ± 5.9	0	2.4 ± 3.4
113	—[e]	Unknown	0	0	1.6 ± 0.7	1.8 ± 0.8
107	—	Unknown	0.6 ± 0.3	0.6 ± 0.2	1.2 ± 0.4	1.2 ± 0.3
54	I	*Nostoc*	4.8 ± 2.7	0.5 ± 0.4	0	0
26	F	*Nostoc*	36.0 ± 7.4	41.1 ± 7.4	0	0

[a] Values represent the average peak fluorescent area of each TRF from 12 individual crust samples including standard deviations ($n = 12$).
[b] *nifH* sequence cluster(s) (Fig. 2) expected to yield the corresponding TRF size.
[c] Phylogenetic classification of *nifH* sequence clusters was based on BLASTX searches and NJ tree analysis.
[d] Sequence clusters A, B, C, D, G, J, K, and L were all classified as *Nostoc*-like *nifH,* whereas sequence cluster M was classified as cyanobacterium-like *nifH.*
[e] —, *nifH* sequences were not recovered that yielded expected TRF sizes of 113 or 107 bp.

TABLE 4. *nifH* copies in crust material determined by quantitative PCR

Crust source	No. of *nifH* copies/ng of DNA[a]	No. of *nifH* copies/g of soil[b]
Canyonlands, poorly developed	93 ± 19	1.1×10^6
Canyonlands, mature	685 ± 152	2.0×10^7
Jornada, poorly developed	223 ± 92	1.8×10^6
Jornada, mature	1719 ± 582	3.4×10^7

[a] Values represent the means ± standard errors for 10 crust cores.
[b] Assumes a 100% extraction efficiency of DNA from crust material.

occurrence and abundance of most TRFs was reasonably consistent across all 12 samples examined for each crust type. However, the average abundance of the 120-bp TRF in both mature and poorly developed crusts from Canyonlands was skewed upward due to the overabundance of this TRF in two to three samples from each crust type. This was also the case with the 120-bp TRF obtained with the Jornada mature crust samples. Also, the abundance of the 358-bp TRF was skewed upward in both Jornada poorly developed crusts and mature crusts due to the overabundance of this TRF in one to two samples from each crust type.

Quantitative *nifH* PCR. Quantitative PCR was used to determine the relative amounts of *nifH* sequences in mature and poorly developed crusts from both the Canyonlands and Jornada sites. The efficiency of the CY81F-CY226R primer set was tested separately on each *nifH* sequence type recovered in our crust clone libraries. With the exception of the rare *nifH* sequence types represented by clusters N, O, and Q, the *nifH* sequence types exhibited similar amplification efficiencies with this primer pair (data not shown).

A standard curve mix for the *nifH* quantitative PCR was prepared by mixing purified plasmid DNA, containing *nifH* inserts representing each of the sequence types obtained from the cloning and sequencing experiments, in ratios to approximate the *nifH* sequence distribution found in the poorly developed and mature crusts from the Canyonlands and Jornada sites. The *nifH* copy number in each standard curve mix was calculated, and an appropriate dilution series was prepared to generate the standard curve samples for each quantitative PCR. The standard curve generated with these primers was linear for *nif*H concentrations covering 8 orders of magnitude, and the reaction was specific for the expected 145-bp fragment, with the exception of primer dimer formation that was evident after 35 cycles (data not shown). C_t values for all of the crust samples tested in this study were between 24 and 31 cycles, well within the standard curve. It was also demonstrated that the amplification efficiency of the standard curve mix (at concentrations yielding C_t values of 15 to 22 cycles) was not altered when the PCR mixture was spiked with crust DNA samples (data not shown). Thus, the effect of any inhibitory substances that may have been present in the crust DNA samples (e.g., humic acids) was negligible on the *nifH* quantitative PCR assay.

The quantitative PCR protocol was used to determine the total copy number of amplifiable *nifH* sequences in DNA extracted from 10 poorly developed crust samples and 10 mature crust samples from both Canyonlands and Jornada (Table 4). Notably, the *nif*H copy number per unit of DNA was approximately 7.5 times larger in the mature crusts from both sites than from the corresponding poorly developed crust material, suggesting that the number of diazotrophic species relative to other microbial species is greater in mature crusts than in poorly developed crusts for both the Jornada and Canyonlands sites. Additionally, a rough estimate of the population size of N_2-fixing bacteria in biological soil crusts from the Jornada and Canyonlands sites can be derived from this data. Assuming a DNA extraction efficiency of 100% from crust material and the presence of two *nifH* gene copies per bacterial cell, data from our *nifH* quantitative PCR experiments set the number of diazotrophs in the biological soil crusts from the Jornada and Canyonlands sites within the range of 5×10^5 to 2×10^7 cells g of soil^{-1}.

Cyanobacterial 16S rRNA gene analysis. Because the majority of *nifH* sequences recovered from crusts were putatively identified as cyanobacterium-like *nifH*, we performed sequence analysis of cyanobacterial 16S rRNA genes amplified from poorly developed and mature crusts. The sequences were grouped into eight distinct clusters (Fig. 4). Although branching order was unstable, the clusters shown in Fig. 4 remained intact, whether the sequences were analyzed by the NJ or maximum-likelihood methodology (data not shown).

The majority of sequences recovered from all crusts were related to *Microcoleus* spp., most closely to *M. vaginatus* (Vaucher) Gomont (Table 5). Other sequences that grouped within the larger *Microcoleus* cluster were most closely related to isolates of *Microcoleus sociatus*, *Microcoleus steenstruppi*, and *Phormidium murrayii*. The 16S rRNA gene sequence pools from the poorly developed crusts of Canyonlands and Jornada contained 95 and 100% *Microcoleus*-like sequences, respectively, while mature crusts from these sites contained 74 and 93% *Microcoleus*-like sequences, respectively. Within our broadly defined *Microcoleus* cluster (Fig. 4), a set of sequences formed a smaller cluster (containing JL76, DGE band 11B, etc.) that was most closely related to sequences from *M. steenstruppi* isolates but was clearly distinct from known members of this group.

Nostoc- and *Scytonema*-like sequences were not recovered from poorly developed crust material from either site, while 4% (3 of 70) and 5% (2 of 43) of the sequences recovered from the Canyonlands and Jornada mature crusts, respectively, were *Nostoc*- and *Scytonema*-like (Table 5). Fourteen percent (10 of 70) of the sequences recovered from Canyonlands mature crust belonged to the Crust A cluster (Fig. 4), which is distantly related to the *Nostoc* and *Chroococcidiopsis* groups as determined by BLASTn searches and DNA distance analysis.

Morphological examination of cyanobacterial composition in crusts. Microscopic analysis of the biological soil crusts revealed a different picture of cyanobacterial composition than did 16S rRNA gene analysis (Table 6). In particular, the calculated percentage of *Nostoc* spp. and *Scytonema* spp. among all cyanobacteria was much higher in each of the crust types examined as determined by microscopic analysis than as determined by the 16S rRNA gene method. However, the percentage of *Nostoc* spp. and *Scytonema* spp. comprising the total cyanobacterial community was always found to be greater in mature crusts than in poorly developed crusts at a given site, regardless of the method of assessment.

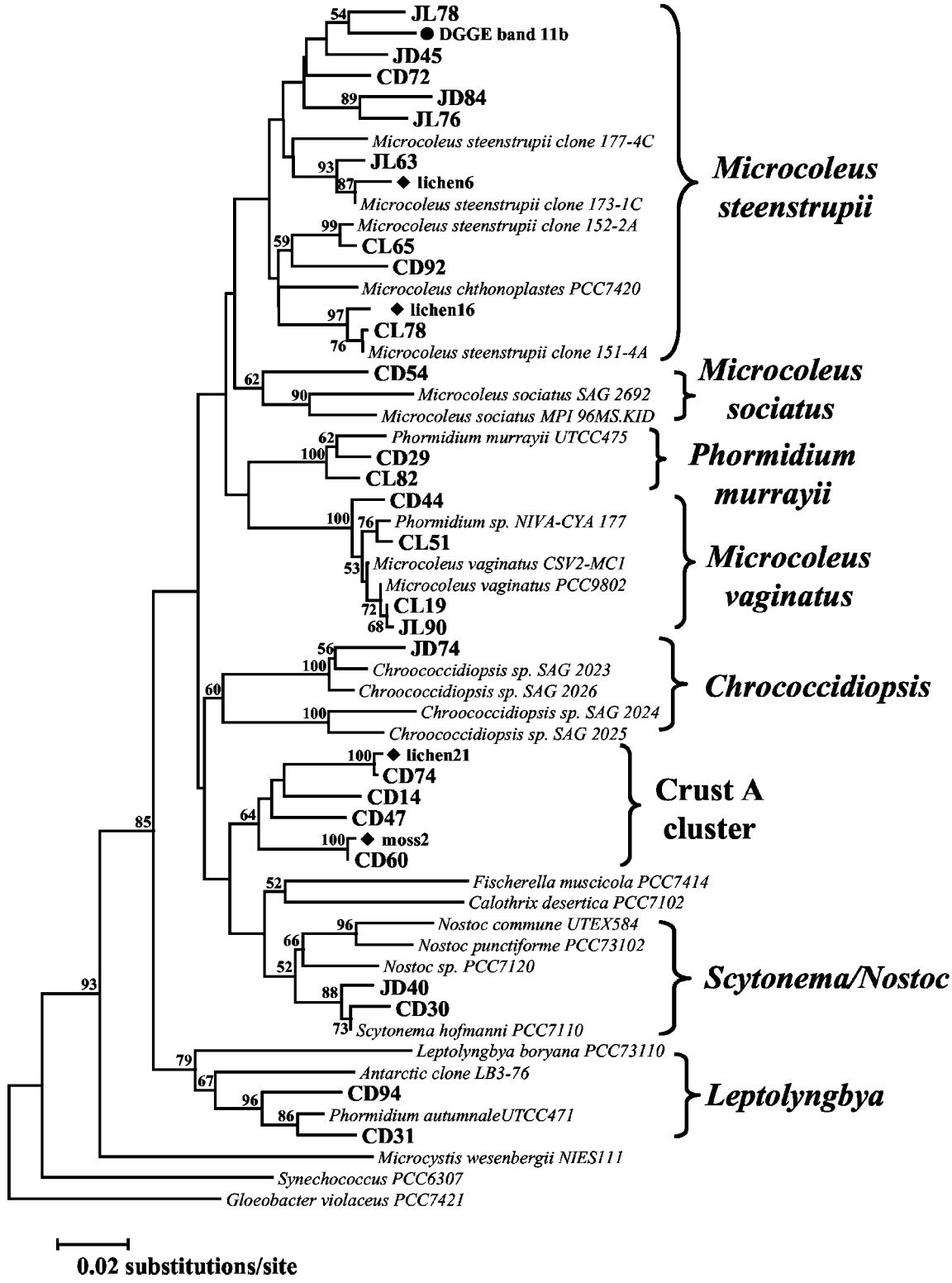

FIG. 4. Dendrogram of the cyanobacterial 16S rRNA gene sequences from biological soil crusts. The dendrogram was constructed using the minimum evolution function of the software program MEGA version 2.1, utilizing the NJ method to construct the initial tree. Distances were calculated with the Kimura two-parameter algorithm and pairwise deletion of gaps and missing data. Bootstrap values from 100 resamplings are shown as a percentage above internal nodes if larger than 50. The 16S rRNA gene sequence from *Gloeobacter violaceus* was used as the outgroup. Prominent sequence clusters were labeled and are shown on the right. Sequences recovered from soil crusts in this study are labeled in bold: CL, Canyonlands poorly developed; CD, Canyonlands mature; JL, Jornada poorly developed; JD, Jornada mature. Sequences with symbols were recovered from soil crusts from the Colorado Plateau in previous studies: ● (23) and ♦ (37).

TABLE 5. Distribution of cyanobacterium 16S rRNA gene sequence clusters in biological soil crusts

16S rRNA gene cluster[a]	% of sequences in cluster for crust source			
	Canyonlands, poorly developed	Canyonlands, mature	Jornada, poorly developed	Jornada, mature
M. vaginatus	92	53	80	44
M. steenstrupii	3	17	18	37
M. sociatus	3	3	2	9
P. murrayii	4	3	0	0
Nostoc/*Scytonema*	0	4	0	5
Crust A	0	14	0	0
Chroococcidiopsis	1	0	0	5
Leptolyngba	0	4	0	0
Other	0	1	0	0
Total (%)	103	99	100	100
Total (*n*)	73	70	51	43

[a] Sequences were assigned to clusters based on phylogenetic analysis as illustrated in Fig. 4 and inspection of sequence similarities.

TABLE 6. Cyanobacterium composition by morphotype in biological soil crusts

Cyanobacterium morphotype	% Composition for crust source[a]			
	Canyonlands, poorly developed	Canyonlands, mature	Jornada, poorly developed	Jornada, mature
Microcoleus spp.	85.4 ± 12	71.6 ± 9	85.4 ± 4	49.2 ± 19
Nostoc spp.	14.3 ± 12	24.3 ± 8	8.7 ± 3	17.2 ± 15
Scytonema spp.	0.3 ± 1	4.1 ± 4	2.4 ± 3	33.6 ± 20

[a] Percent composition is reported as the mean ± standard error (Canyonlands, $n = 8$; Jornada, $n = 5$; see Materials and Methods).

DISCUSSION

Biological soil crusts are thought to play an important role in nitrogen deposition in arid and semiarid ecosystems. However, characterization of the diazotrophic community associated with the crusts has been largely limited to culture- and microscopy-based investigations. To more systematically investigate the N_2-fixing communities in biological soil crusts, we employed a polyphasic molecular approach, utilizing T-RFLP, clone sequencing, and quantitative PCR to examine these complex assemblages. By comparing *nifH* T-RFLP and *nifH* sequencing data sets, we were able to match abundant TRF peaks with *nifH* sequence types with a reasonable degree of confidence and thus examine the composition and relative abundance of N_2-fixing microorganisms within two successional stages of soil crusts from both the Colorado Plateau and Chihuahuan Desert.

The majority of *nifH* sequences retrieved from both sites (>90% by sequence analysis, >80% by T-RFLP analysis) were most closely related to *Nostoc*-type *nifH*. Although several *Nostoc* sequence types constituted a significant proportion of the *nifH* pool recovered in this study (cluster F, Canyonlands; A, J, and K for Jornada), 12 distinct *Nostoc*-type sequence types were identified. Additionally, the *nifH* T-RFLP data (Table 3) indicated that the composition of the N_2-fixing community remained fairly uniform across a given site (approximately 1 acre) regardless of crust type (poorly developed or mature), with the exception of the rare occurrence of organisms containing *nifH* sequences represented by the 120- and 358-bp TRFs (clusters O and P). Because the 120- and 358-bp TRFs most likely represent *nifH* sequences most closely related to those from α-proteobacterium species known primarily as rhizosphere-dwelling microbes, it is possible that these sequences were obtained from organisms associated with a very small root section or other unseen piece of plant debris.

Unquestionably, *nifH* analysis is a powerful method of examining natural assemblages of N_2-fixing organisms; however, it does have limitations. For example, even though phylogenetic relationships based on *nifH* sequences are largely congruent with those obtained from 16S rRNA gene data (47, 50), the *nifH* sequences recovered in the present study cannot be assigned to a given species with absolute certainty. Indeed, a phylogenetically diverse cluster of 16S rRNA gene sequences (Crust A cluster) was identified in this study (and in a previous study [37]) that may represent species capable of N_2 fixation, given its phylogenetic relationship to the *Nostoc* and *Chroococcidiopsis* groups (Fig. 4). However, we find it unlikely that a significant proportion of the *nifH* sequences recovered in this study belong to members of the Crust A cluster, because Crust A-type sequences were found only in Canyonlands mature crusts, whereas almost all *nifH* sequences obtained from Canyonlands mature crusts were equally abundant in poorly developed crust material. The occurrence of multiple *nifH* gene copies in diazotrophic bacteria can also introduce confounding factors for phylogenetic analysis based on *nifH* sequences (49). To address these issues, efforts are currently under way to culture N_2-fixing cyanobacteria from the crusts and to link 16S rRNA gene sequences and *nifH* sequences to single organisms.

Our *nifH* PCR primers and protocol are capable of amplifying a wide variety of *nifH* sequence types, including many bacterial species unrelated to the cyanobacteria. In soils at 30-cm depth and in association with grass rhizospheres at Canyonland sites, we found the *nifH* sequences to be primarily composed of *Paenibacillus* types, *Rhodobacter* types, and a cluster of novel, deeply branching *nifH* sequences (C. M. Yeager and C. R. Kuske, unpublished data). *anfH* sequence types were also recovered from those soils. Soils from a Ponderosa pine forest were found to have a completely different composition when compared to the soil crusts reported here, where less than 0.5% of the *nifH* sequences were cyanobacterial (C. M. Yeager, D. E. Northup, and C. R. Kuske, unpublished data).

Overall, our observations provide compelling evidence that nitrogen fixation is predominantly carried out by a collection of heterocystous cyanobacteria, presumably *Nostoc* or closely related species, in both poorly developed and mature crusts at the Canyonlands and Jornada sites. In accordance with these results, we have measured rates of N_2 fixation in these crust types under both lighted and dark conditions (Belnap, unpublished data), with the highest rates occurring in the light. Upon darkening, rates are maintained for a few hours, declining to zero within 4 to 6 h (despite moisture being kept constant). These findings are consistent with other studies of N_2 fixation in cyanobacteria, where it has been hypothesized that carbon is depleted over time in the dark and thus ultimately limits dark N_2 fixation in these organisms (29, 36). Were heterotrophic fixation an important process in these soil crusts, a substantial

decline in activity would not be expected to occur under darkened conditions within 4 to 6 h.

Although the composition of the diazotrophic community did not differ appreciably between poorly developed and mature crusts at either the Canyonlands site or the Jornada site, real-time PCR data suggested that the diazotroph population size did vary between crust types. The *nifH* copy number was approximately 7.5-fold higher in DNA extracted from mature crusts than from an equivalent amount of DNA extracted from poorly developed crusts at both the Canyonlands and Jornada sites. Our results indicate that not only does the total microbial population increase during the progression from poorly developed to mature crust (as measured by total extracted DNA) but the proportion of the diazotrophs within the microbial community increases as well. A proliferation of an existing N_2-fixing community during the transition from a poorly developed crust to a mature crust could explain the observed increase in nitrogenase activity in mature crusts relative to poorly developed crusts. However, it should be noted that nitrogenase activity and/or expression could be differentially controlled in the poorly developed crusts versus the mature crusts because of differences in localized conditions, such as levels of O_2, NH_3, or pH. In other words, the nitrogenase activity per *nifH* copy number may differ between the two crust types.

In N-limited terrestrial ecosystems, N_2-fixing organisms often serve as abundant and early colonizers that then give way to other species over time (43). However, our study and previous microscopic and molecular studies demonstrate that the cyanobacterial community in poorly developed desert crusts is composed almost exclusively of species closely related to *M. vaginatus* (Vaucher) Gomont (1, 23, 37). *M. vaginatus* is not known to fix N_2 (38, 39), and we found no evidence from our *nifH* sequence analysis to support the idea that an abundant, heterotrophic, N_2-fixing organism(s) forms a consortial relationship with *M. vaginatus* (39).

In soil crusts the large and mobile *M. vaginatus* secretes copious amounts of polysaccharide-based sheath material that glues soil particles together, counteracting erosional forces and providing a stable microhabitat for other species (7, 8, 23). In addition, sheath material from *M. vaginatus* can hold up to eight times its weight in water, dramatically increasing the water-holding capacity of desert soils and effectively, the time period in which *M. vaginatus* and other microorganisms are metabolically active following a precipitation event (7, 12). This sheath material also binds essential nutrients in a manner that prevents leaching downwards into the soil profile but that is still readily available for uptake by organisms (8). Thus, the soils at our study sites are not initially dominated by N_2-fixing organisms but instead are dominated by one that can tolerate the nutrient-poor and unstable soils of these arid regions. Our results, in combination with those from other studies (11, 22), suggest that it is not until after *M. vaginatus* has engineered the soil landscape that the N_2-fixing community is able to proliferate and enrich the surrounding soil with N.

In terrestrial ecosystems where cyanobacteria proliferate, such as deserts, grasslands, tundra, and various open woodlands, the ecological controls of biological N_2-fixing activity have been well studied and are, for the most part, remarkably similar (2). Foremost, sufficient levels of moisture, followed by light and favorable temperatures, are required for optimal rates of N_2 fixation. Other factors influencing rates of biological N_2 fixation in cyanobacterium-dominated communities include season, pH, salinity, soil chemistry, grazing, snow cover, and organismal interactions. However, little is known about the factors controlling diazotrophic community structure in these ecosystems. Indeed, one of the major uncertainties associated with global climate change models is the response of N_2-fixing communities to various permutations and the resulting effect on ecosystem biogeochemistry (42). Using *nifH* T-RFLP, clone sequencing, and real time PCR, we have established a baseline from which we can effectively track the composition and abundance of N_2-fixing organisms within biological soil crusts at our study sites. We intend to use these tools and baseline data to analyze the cause-and-effect relationships between environmental changes (particularly those involving temperature and moisture) and the structure and function of N_2-fixing communities within biological soil crusts.

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