Diversity of Free-Living Nitrogen Fixing *Streptomyces* in soils of the Badlands of South Dakota

Bibha Dahal[1], Gitanjali NandaKafle[1], Lora Perkins[2] and Volker S. Brözel[1,3*]

Department of Biology and Microbiology[1] and Natural Resource Management[2], South Dakota State University, Brookings, SD, 57006, USA, and Department of Microbiology, University of Pretoria, Pretoria, South Africa[3]

*** Corresponding author:**

volker.brozel@sdstate.edu

+605 688 6141

SDS 228

Box 2104A

Brookings, SD, 57007

USA

ABSTRACT

Biological Nitrogen Fixation is critical for ecosystem productivity. Select members of Bacteria and Archaea express a nitrogenase enzyme complex that reduces atmospheric nitrogen to ammonia. Several nitrogen fixing bacteria form symbiotic associations with plants, but free-living diazotrophs also contribute a substantial amount of nitrogen to ecosystems. The aim of this study was to isolate and characterize free-living diazotrophs in arid lands of South Dakota Badlands. Samples were obtained from sod tables and the surrounding base in spring and fall. Diazotrophs were isolated on solid nitrogen free medium (NFM) under hypoxic conditions, and their16S rRNA and *nifH* genes sequenced. *nifH* was also amplified directly from soil DNA extracts. The 16S rRNA gene data indicated a diversity of putative free-living diazotrophs across 4 phyla (Actinomycetes, Proteobacteria, Bacteroidetes, and Firmicutes), but ~50% of these clustered with *Streptomyces.* These *Streptomyces* isolates grew in liquid NFM in an ammonia-depleted environment. Only 5 of these yielded a *nifH* gene product using the PolF/PolR primer set. Four of these aligned with *nifH* of the cyanobacteria *Scytonema* and *Nostoc*, and the other one aligned with *nifH* of *Bradyrhizobium.* Six selected *Streptomyces* isolates, three of which were *nifH* positive by PCR, all indicated $^{15}N_2$ incorporation, providing strong support of nitrogen fixation. All *nifH* amplicons from soil DNA extract resembled Cyanobacteria. This is the first known report of diazotrophic *Streptomyce*s, other than the thermophilic, autotrophic *S. thermoautotrophicus*. *nifH* genes of these *Streptomyces* were related to those from Cyanobacteria. It is possible that the cyanobacteria-like *nifH* amplicons obtained from soil DNA were associated with *Streptomyces*.

Keywords

Diazotroph; nitrogen fixing; free-living; badlands; nitrogenase; *Streptomyces*

1. Introduction

Dinitrogen is the most abundant element in earth's atmosphere, but is biochemically unavailable to most organisms. Yet nitrogen is integral to proteins and nucleic acids, so essential for all living systems. Dissociation of dinitrogen and reduction to ammonia requires a high level of energy, catalyzed in nature by the nitrogenase enzyme complex (Canfield et al., 2010). Nitrogenase occurs in select members of many bacterial and some archaeal phyla (Young, 1992). Combined or bioavailable nitrogen can be limiting for net primary productivity (Vitousek et al., 2002). Ecosystems experience nitrogen loss through ammonia volatilization and denitrification (Peterjohn and Schlesinger, 1991; Canfield et al., 2010), suggesting that nitrogen fixers play a critical role in ecosystem productivity. The best known nitrogen fixers form symbiotic associations with plants, but are limited to select plant groups such as legumes, alders, and cycads, while the majority of plants have no known associations with diazotrophic bacteria. Free-living nitrogen fixing bacteria are not limited to growth with specific plant tax, and may, therefore, play a widespread role in nitrogen supply to ecosystems (Belnap, 2002; Yeager et al., 2007; Unkovich and Baldock, 2008).

The nitrogenase complex appears conserved. There are three genetically distinct nitrogenase systems; molybdenum nitrogenase (Nif) (Wilson et al.), the alternative vanadium nitrogenase (Vnf), and the alternative iron only nitrogenase (Anf) (Hu and Ribbe, 2015), and all are oxygen sensitive. The three nitrogenases differ in their metal content but they have structural, mechanical, and phylogenetic relatedness (Dos Santos et al., 2012). All three reduce dinitrogen by the same mechanisms, with slight variations (Eady, 1996). Among the genes that encode the nitrogenase enzyme complex, *nifH* is the most sequenced and highly conserved (Gaby and

Buckley, 2012). The currently captured genetic diversity of diazotrophs is based mainly on the N-terminal part of *nifH* sequences. The Nif Nitrogenase is a promiscuous enzyme able to reduce several substrates including acetylene to ethylene (Dance, 2013). This ability is the basis for using the acetylene reduction assay to confirm nitrogenase activity. A fourth and biochemically distinct nitrogenase which is oxygen insensitive has been reported only for *Streptomyces thermoautotrophicus* (Ribbe et al., 1997; Zhao et al., 2006). The nitrogen fixing ability of this isolate has recently been questioned as it did not incorporate $^{15}N_2$ (MacKellar et al., 2016). It remains to be determined whether any additional classes of nitrogenase exist.

Drylands such as arid, semiarid, alpine, and polar regions constitute one-third of earth's terrestrial biome (Pointing and Belnap, 2012). Much of these lands contain Biological Soil Crusts (BSCs) (Bowker et al., 2002). Extreme climatic conditions such as dryness, heat, cold and snow that limit higher plant and animal life in these areas allow for the development of BSCs. BSCs are the primary contributors of organic carbon and nitrogen sources in arid lands (da Rocha et al., 2015). They play an important role in nutrient cycling and maintain the stability of soil in arid land ecosystems (West, 1990; Belnap and Gardner, 1993). In lands with arid vegetation, BSC's contain a variety of microbes, including bacteria, algae, lichens and bryophytes (Belnap, 2002; Büdel et al., 2014). In areas with low rainfall and low inputs of nitrogen from human activity, fixation by cyanobacteria can be a common source of nitrogen (Rychert and Skujiņš, 1974; Belnap, 1996). BSCs are generally dominated by cyanobacterial populations (Belnap and Gardner, 1993; Tirkey and Adhikary, 2005; Yeager et al., 2007), but more recently, diverse groups of heterotrophic bacteria affiliated to the phyla Proteobacteria,

Actinobacteria, Firmicutes, and Bacteroidetes have been isolated from BSCs (da Rocha et al., 2015), some of them with the potential of nitrogen fixation (Abed et al., 2010).

The Badlands of South Dakota are located in an arid to semiarid region of the high Great Plains in southwestern South Dakota. Badlands were formed through differential erosion of the soil, exposing ancient sedimentary layers. South Dakota Badlands feature highly eroded formations with sparse vegetation, undisturbed mixed prairie grass, and spires with an elevation up to 992 m above sea level (Graham, 2008). Sod tables are isolated and eroded relict pediments of the once higher plains, and are usually covered with grass contributing a protective layer to the underlying soft soil, providing evidence to an earlier wetter period (Stoffer, 2003). The bases surrounding sod tables have no vegetative cover and are covered in BSC's.

No information is currently available on the microbial diversity of soils and soil crusts of the badlands of South Dakota. Since badlands display distinct geological and landscape features and have no known input of combined nitrogen, it is important to understand their free-living nitrogen fixing bacterial community. The focus of this study was to characterize the diversity of free-living diazotrophs in soils and soil crusts (matrix) of sod tables of the badlands of South Dakota, Badlands National Park, using both culture dependent and culture independent approaches.

2. Materials and Methods

2.1. Samples:

The study was conducted using soil samples obtained from sod tables of South Dakota Badlands. The climate in the study area is continental featuring extreme cold winters and hot summers. The temperature during winters may fall below -18 °C to more than 38 °C during summers, and with annual precipitation of only 40 mm (Amberg et al., 2012). The harsh climatic condition and low amount of rainfall make the growth conditions challenging for most plants (Graham, 2008). Soil samples were collected from the top of sod tables and from matrix, the surrounding base. Soil samples were collected from each site to about 5cm depth. Samples were collected at the end of the spring (May, 2014) and end of the fall (November, 2014) from eight and nine sod tables respectively. The samples were transferred to sterile Whirlpack sampling bags to avoid loss of moisture and any outside contamination, and kept at 4°C until processing in the laboratory. The pH of the soil samples ranged from 8.1 to 9.8.

2.2. Isolation of free-living putative diazotrophs from soil:

Putative diazotrophs were isolated using solid nitrogen-free medium (NFM). NFM was defined by comparing 20 published nitrogen free media and generating a consensus medium; K_2HPO_4 (0.2g/l), KH_2PO_4 (0.5g/l), $MgSO_4.7H_2O$ (0.2g/l), $FeSO_4.7H_2O$ (0.1g/l), $Na_2MoO_4.2H_2O$ (0.005g/l), NaCl (0.2g/l), Glucose (10g/l), and noble agar (15.0g/l, Difco, Catalog No. 214230). Serial dilutions were spread on triplicate freshly prepared solid NFM. Plates were incubated for 5-7 d at 28°C, rendered microaerophilic using a gas-pack (Gaspack EZ Campy, BD). Colonies

were selected based on morphology and streaked for isolation on NFM three times to ensure purity.

2.3. DNA Extraction and PCR Amplification of 16S rRNA and *nifH* from pure cultures

DNA was extracted from pure cultures using the Zymo Research Kit (Quick-gDNA™ Miniprep), and stored at -20°C. Hypervariable regions V1-3 of the 16S rRNA gene were amplified by PCR using universal primers 27F and 518 R (Table 1). The PCR was performed in 30µl reactions consisting of 0.2µl (5000U/ml) Taq polymerase (NewEngland BioLabs), 3µl (10X) PCR Buffer, 0.6µl dNTP (each 10mM), 0.6µl (10mM) each Primer, and 2.4µl (25mM) $MgCl_2$. The PCR conditions were initial denaturation at 95 ºC for 4 min, 30 cycles at 95 ºC for 30 s, 50 ºC for 45 s, and 72 ºC for 1 min, and a final elongation at 72 ºC for 10 min. The DNA sequence of PCR amplicons was determined by Sanger sequencing (Beckman Coulter Genomics, Inc). Reference sequences were selected from GenBank (Benson et al., 1993) by determining phylogenetic relatedness using the Ribosomal Database Project (Wang et al., 2007), and using NCBI BLAST (Altschul et al., 1997). The DNA sequences were aligned using Muscle (Edgar, 2004). The Maximum Likelihood method based on the Tamura-Nei model was used to analyze the evolutionary history using Mega 6.0 (Kumar et al., 2008; Tamura et al., 2013), and the bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. The sequence data is available from the GenBank database under accession numbers KX906717 – KX906807. Accession numbers of reference sequences are listed in Table S1.

Table 1. Primers used in this study.

Primers	Sequence (5'-3')	Reference
27F	AGA GTT TGA TCM TGG CTC AG	(Jiang et al., 2006)
518R	GTA TTA CCG CGG CTG CTG G	(Muyzer et al., 1993)
PolF	TGC GAY CCS AAR GCB GAC TC	(Poly et al., 2001)
PolR	ATS GCC ATC ATY TCR CCG GA	(Poly et al., 2001)
IGK3	GCI WTH TAY GGI AAR GGI GGI ATH GGI	(Gaby and Buckley, 2012)
DVV	ATI GCR AAI CCI CCR CAI ACI ACR TC	(Gaby and Buckley, 2012)
Ueda19F	GCI WTY TAY GGI AAR GGI GG	(Ueda et al., 1995)
Ueda407R	AAI CCR CCR CAI ACI ACR TC	(Ueda et al., 1995)
358F	CTC CTA CGG GAG GCA GCA GT	(Coenye et al., 1999)
CYA359F	GGG GAA TYT TCC GCA ATG GG	(Nübel et al., 1997)
CYA781R(a)	GAC TAC TGG GGT ATC TAA TCC CAT T	(Nübel et al., 1997)
CYA781R(b)	GAC TAC AGG GGT ATC TAA TCC CTT T	(Nübel et al., 1997)
536F	CAG CAG CCG CGG TAA TAC	(Coenye et al., 1999)
926F	AAC TCA AAG GAA TTG ACG	(Coenye et al., 1999)
1241F	GCT ACA CAC GTG CTA CAA TG	(Coenye et al., 1999)
1492R	CGG TTA CCT TGT TAC GAC TT	(Jiang et al., 2006)

The *nifH* PCR was performed using primer sets PolF and PolR (Poly et al., 2001), IGK3 and DVV (Gaby and Buckley, 2012), and Ueda19F and Ueda407R (Ueda et al., 1995) (Table 1). PCR was performed in 30µl reactions consisting of 0.2µl (5000U/ml) Taq polymerase (NewEngland BioLabs), 3µl (10X) PCR Buffer, 0.6µl dNTP (each 10mM), 0.9µl (10mM) each Primer, and 2.4µl (25mM) $MgCl_2$. For PolF and PolR primers, touch down PCR was performed. The first 5 cycles had annealing temperature of 63 °C with a decrement of 1°C for the subsequent cycles, and 55 °C was set as the annealing temperature for the next 30 cycles, extension at 72°C for 1 min, and final extension at 72 °C for 7 min. For IGK3/DVV and Ueda 19F/Ueda407R, the annealing temperatures were 59 °C and 52 °C respectively. Amplification using PolF - PolR primers yielded a product size of ~360bp, and for IGK3/DVV and

Ueda19F/Ueda407R primer sets the product sizes were ~400bp. The sequence data is available from the GenBank database under accession numbers KY022412 – KY022416.

2.4. DNA Extraction and PCR Amplification of *nifH* gene from soil samples and DGGE analysis:

DNA extraction from soil samples was performed in triplicate using the Power Lyzer Power Soil DNA Isolation Kit (MoBio). The *nifH* pool was amplified by PCR using PolF-PolR primers as described above. Bands of ~360 bp were excised from agarose gels, extracted using the Wizard^{R} SV Gel and PCR Clean-Up System (Promega), and used as template for generating GC-tailed amplicons by PCR using PolR and PolF with a 40 base GC clamp (CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCCC TCG GAY CCS AAR GCB GAC TC) (Rettedal et al., 2010). The *nifH* community of the soil was characterized using denaturing gradient gel electrophoresis (DGGE). The resulting PCR products were resolved in 35-65% denaturing gradient with a DCode Universal Mutation Detection System (BioRad, USA) as described previously (Muyzer et al., 1993; Rettedal et al., 2010). Electrophoresis was carried out in 1X TAE (Tris-acetate-EDTA) buffer providing a constant voltage of 70V at 60 ºC for ~16 h. The gel was stained using SYBR Gold (Molecular Probes) and de-ionized (DI) water with gentle agitation for 30 min and the gel images were acquired.

2.5. Determination of *nifH* sequences in soil samples

PCR products generated using PolF and PolR primers were cloned for sequencing. Amplicons were cleaned (DNA Wizard^{R} SV Gel and PCR Clean-Up System (Promega), ligated into the pGEM^{R}-T Vector System I (Promega) following the manufacturer's instructions, transformed

into competent *E. coli* JM109 cells, and plated onto LB agar with Ampicillin (100µg/ml), XGal (100µl of 100mM), and IPTG (20µl of 50mg/ml). All putative clones were selected, grown in LB/Ampicillin broth, and plasmid DNA extracted using the Zyppy^{TM} Plasmid Miniprep Kit (Zymo Research). Sequences were determined using Sanger sequencing (Beckman Coulter Genomics Inc.). The sequences were aligned using Muscle(Edgar, 2004), and a phylogenetic tree for *nifH* sequences was constructed as described in section 2.3. In order to obtain reference sequences we used BLAST software (v2.2.30) on Linux distribution with 5809 *nifH* sequences downloaded from Fungene (http://fungene.cme.msu.edu/) (Fish et al., 2013), and included closest matches as well as representatives from the Seed set in the alignment. The sequence data is available from the GenBank database under accession numbers KY038383 – KY038417, and accession numbers of reference sequences obtained from Fungene appear in Table S2

2.6. PCR amplification of Cyanobacterial 16S rRNA genes from soil samples

The soil DNA extract was amplified using cyanobacterial 16S rRNA gene specific primers CYA359F and an equimolar mixture of CYA781R(a) and CYA781R(b) (Table 1). The PCR was performed in 30 µl reactions consisting of 0.12µl (5000U/ml) Taq polymerase (NewEngland BioLabs), 3µl (10X) PCR Buffer, 0.6µl dNTP (each 10mM), 0.8µl (10mM) each Primer, and 2.4µl of 2.4µl (25mM) $MgCl_2$. The thermo-cycling conditions were: initial denaturation of 95 °C for 4 min, followed by 30 cycles of 93 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min, with a final extension of 72 °C for 7 min.

2.8 Acetylene Reduction Assay

Selected isolates of interest (S7-008, M4-104, M7-122, and S9A-218) were inoculated onto NFM medium slants in glass tubes and were sealed with stoppers. The headspace was flushed with 2 percent oxygen, 10 percent of the total headspace was exchanged with an equal amount of acetylene, and the cultures were incubated for 4 weeks. Reduction of acetylene to ethylene was measured with a gas chromatograph (Agilent Technologies 7890A) using a flame ionization detector and an Agilent CP7348 column. Non-inoculated slants injected with acetylene served as a negative control, and *Bradyrhizobium japonicum* USDA 110 was used as positive control.

2.7. Confirming nitrogen fixation in an ammonia free atmosphere

All the isolates allocated to *Streptomyces* by the V1-3 region of 16S rRNA gene were inoculated into liquid NFM in Erlenmeyer flasks. These were placed into a desiccator containing clinoptilolite, an ammonia scavenging zeolite (Wang et al., 2006; Liao et al., 2015). The container was then sealed using petroleum jelly, and kept at room temperature (22-26 ºC) for 10 d when cultures were inspected visually to confirm growth.

2.8. Determination of 16S rRNA gene sequence of putative *Streptomyces* isolates

The 16S rRNA genes (V1-V9) of all putative *Streptomyces* isolates were amplified by PCR using universal primers 27F and 1492R (Jiang et al., 2006). The PCR was performed in 30µl reactions consisting of 0.2µl (5000U/ml) Taq polymerase (NewEngland BioLabs), 3µl (10X) PCR Buffer, 0.6µl dNTP (each 10mM), 0.7µl (10mM) each Primer, and 2.4µl (25mM) $MgCl_2$. The thermo-cycling conditions were: initial denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C

for 1 min, 55 °C for 1 min, 72 °C for 1 min, with final extension of 72 °C for 10 min. PCR amplicons were cloned as described above. Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (QIAGEN) following the manufacturer's instructions. Sequences were determined by Sanger sequencing (Beckman Genomics Coulter, Inc.) using primers 27F, 358F, 536F, 926F, and 1241F (Coenye et al., 1999) (Table 1). Sequences were assembled and a consensus sequence created using Sequencher (GeneCodes). The consensus sequences generated were aligned using Muscle, and a phylogenetic tree was constructed as described in section 2.3. The sequence data is available from the GenBank database under accession numbers KX906808 – KX906847 and accession numbers of reference sequences from NCBI appear in Table S3.

2.9. Incorporation of $^{15}N_2$ isotope in pure cultures

In order to confirm nitrogen fixation, isolates pre-cultured in liquid nitrogen-free medium (no agar added) were transferred to fresh liquid NFM (10 ml) in a 60 mL syringe. *Bradyrhizobium japonicum* USDA 110 was used as positive control and *Escherichia coli* MG1655 as negative control. After drawing in 5 ml of air through a filter (0.2µm pore size), 45ml of $^{15}N_2$ was added. The syringes were incubated at 28 ºC for 2 weeks, when cells were harvested by centrifugation and dried at 55 °C for 4 h. The isotope ratio was determined by mass spectrometry (UC Davis, Stable Isotope Facility, Davis, California).

3. Results

3.1. Diversity of free-living putative nitrogen fixing bacteria

We obtained diverse cultures of putative diazotrophs from South Dakota Badlands using NFM agar and incubating in a reduced oxygen atmosphere. The density of culturable diazotrophs was higher in sod table than in matrix soil (Fig. 1). Analysis of variance (ANOVA) supported a significant difference between sod table and matrix soil ($p = 0.0124$). The 91 bacterial isolates selected based on unique morphology were allocated to 40 Operational Taxonomic Units (OTUs) by the V1-3 region of their 16S rRNA genes. These OTU fell into twenty bacterial genera across four phyla according to the Ribosomal Database Project (RDP) tool. The majority of the bacterial isolates belonged to *Actinobacteria* (69%), followed by *α-Proteobacteria* (23%), *β-Proteobacteria* (5%), *Bacteroidetes* (2%), *and Firmicutes* (1%) (Fig. 2). Forty-six isolates clustered with *Streptomyces*.

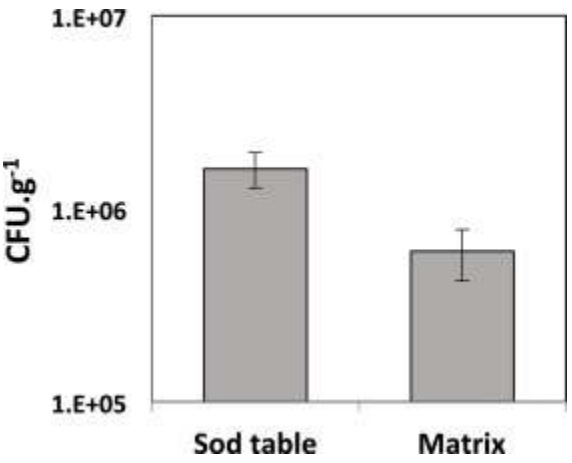

Figure 1. Culturable counts of putative diazotrophs obtained by incubating dilutions of sod table and matrix soil samples on solid nitrogen free medium in microaerophilic atmosphere at 28 °C for ~7d. Counts are the average of 17 different samples and error bars depict standard error of the mean.

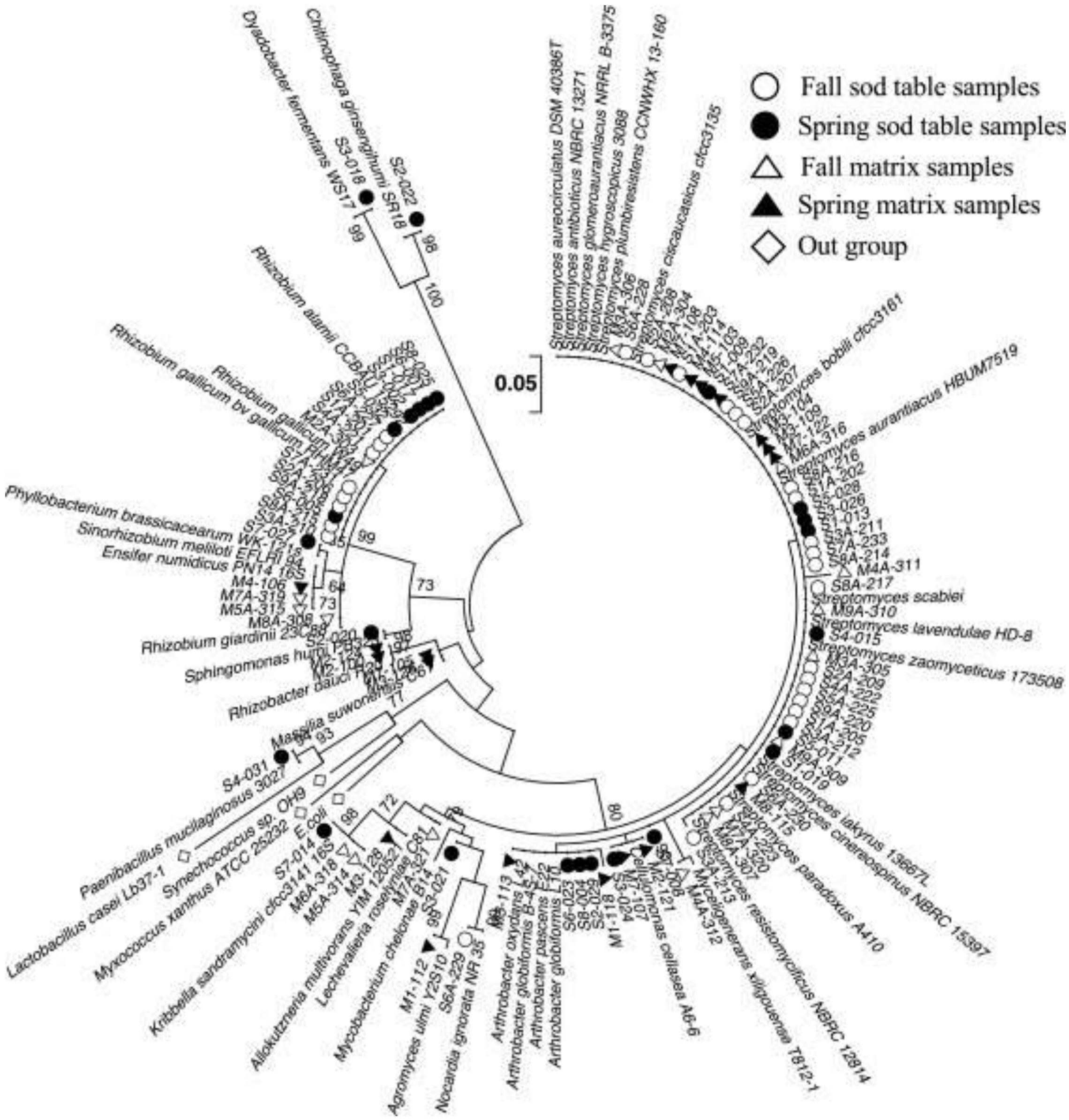

Figure 2. Maximum likelihood phylogenetic tree of 16S rRNA gene (V1-3) of putative diazotrophs isolated from sod table and matrix soils of the badlands of South Dakota based on Tamura Nei-model. Numbers represent bootstrap values of (%) 500 analyses. Bootstrap values lower than 60 are not shown.

A difference in diversity was observed between the sod table and matrix samples (Fig. 2). *Actinobacteria* and *α-Proteobacteria* were distributed in soils of both sod table and matrix, while, *β-Proteobacteria* were found only in matrix. Similarly, *Bacteroidetes* and *Firmicutes* were

found only in sod table soil. Bacterial diversity was higher in spring than in fall samples (Fig. 2). Representatives of all four phyla were isolated from spring samples, including two Bacteroidetes (*Dyadobacter* and *Chitinophaga*), four ß-Proteobacteria (*Rhizobacter* and *Massilia*), and one Firmicutes (*Paenibacillus*) isolates. Fall samples yielded only *Actinobacteria* and *α-Proteobacteria*.

3.2. *nifH* gene amplification and putative nitrogen fixation:

The presence of *nifH* in the bacterial isolates was determined using the primers PolF and PolR (Poly et al., 2001), Ueda19F/Ueda407R (Ueda et al., 1995), and IGK3/DVV (Gaby and Buckley, 2012). No samples yielded positive results with the IGK3/DVV or Ueda19F/Ueda407R primers, other than *Bradyrhizobium japonicum* USDA 110 used as the positive control. Of 46 isolates allocated to *Streptomyces* by 16S rRNA gene sequence, five yielded *nifH* amplicons. Of these, three aligned and clustered with the *nifH* of the cyanobacterium *Scytonema,* which has been allocated to Cluster I of the *nifH* gene database. A fourth clustered with another cyanobacterium, *Nostoc*, and the fifth amplicon aligned with the *nifH* of *B. japonicum,* also allocated to Cluster I, in the maximum likelihood phylogenetic tree of *nifH* (Fig. 3). In order to determine whether there were any cyanobacteria growing in syntrophic association with the *Streptomyces* cultures, cultures were streaked onto AA8 medium and incubated under light for 21d, but no growth was observed. A PCR using cyanobacterial 16S rRNA gene specific primers against genomic DNA extracts of the *Streptomyces* isolates was also negative. These results indicate an absence of cyanobacteria in the cultures.

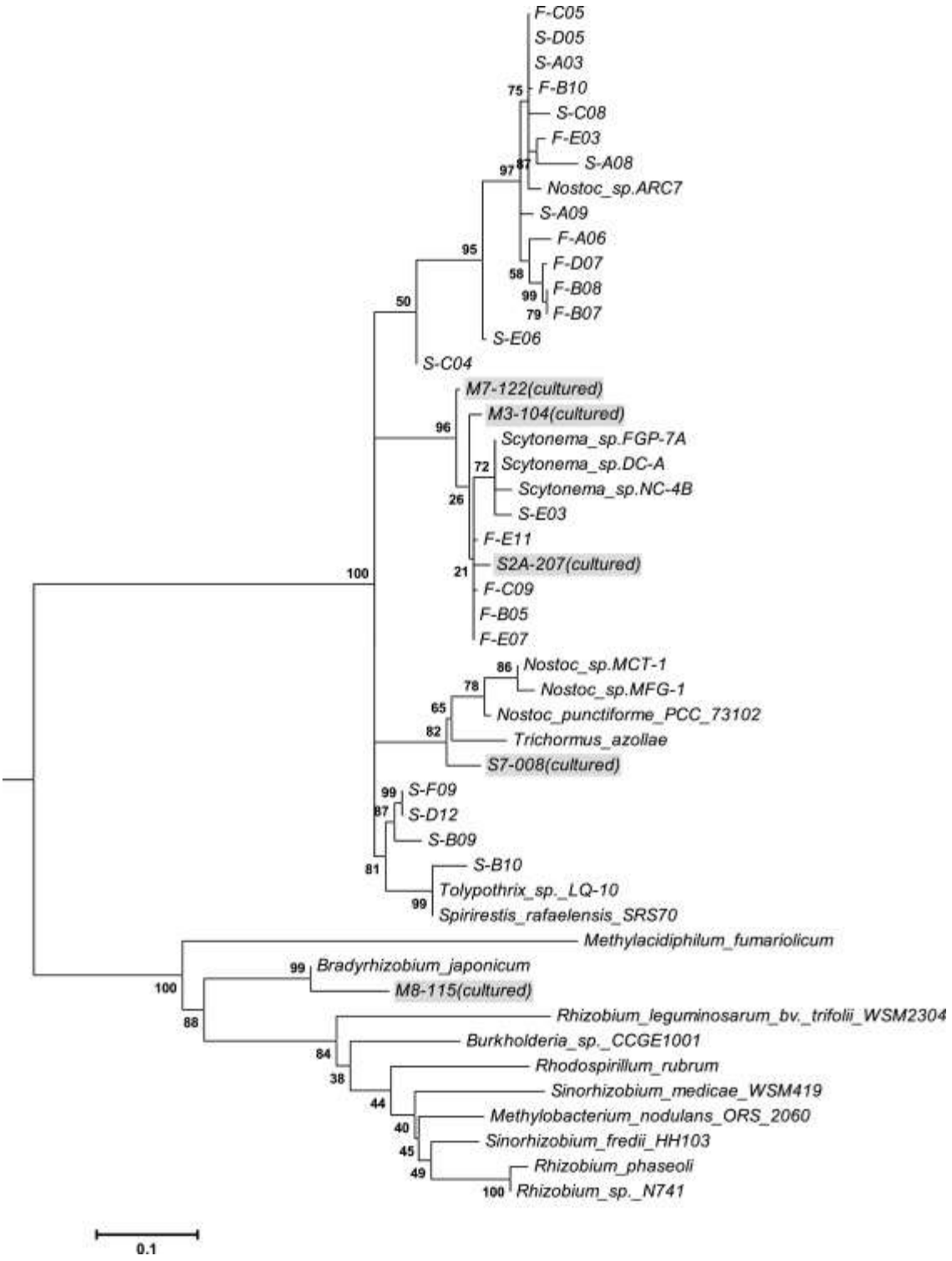

Figure 3. Maximum likelihood phylogenetic tree of *nifH* genes from cultures (highlighted in grey) and amplified from soil DNA based on Tamura-Nei model. Numbers represent bootstrap values (%) of 500 analyses.

Five putative *Streptomyces* isolates yielded *nifH* amplicons with homology to strains known to fix nitrogen. Because no mesophilic *Streptomyces* have been reported to fix nitrogen, we sought to confirm their diazotrophic nature. All 46 isolates were able to grow on NFM agar and also in NFM liquid medium. The acetylene reduction assay did not confirm nitrogen fixation in any of the *Streptomyces* isolates tested. *Rhodococcus erythropolis* is able to grow in nitrogen-free medium, possibly through scavenging ammonia from the atmosphere (Yoshida et al., 2014). In order to exclude the possibility of growth using atmospheric ammonia, we incubated liquid cultures in a sealed atmosphere container with the zeolite clinoptilolite, previously shown to bind residual ammonia (Wang et al., 2006; Liao et al., 2015). The fungus *Aureobasidium pullulans* was able to grow in NFM, but not in the ammonia depleted atmosphere with clinoptilolite. Yet, of 46 putative *Streptomyces* isolates, 45 grew in liquid NFM in the ammonia depleted atmosphere, indicating the ability to fix nitrogen. Growth appeared as distinct clumps against clear background. These clumps grew when plated on NFM.

3.3. V1-9 region 16S rRNA gene amplification of isolates allocated to *Streptomyces*

The putative *Streptomyces* clusters (Fig. 2) had poor bootstrap support based on 500 replicates. In order to better characterize these isolates their full length 16S rRNA sequence was determined. Of the forty-six isolates, two did not yield amplification using the 27F and 1492 R primers, despite multiple attempts. All of these isolates aligned to *Streptomyces* and were allocated to 16 OTUs (Fig. 4). Only five of these *Streptomyces* yielded *nifH* amplicons by PCR, highlighted with a triangle in Fig. 4. It is unclear how the other isolates were able to grow in a medium free of combined nitrogen and in an ammonia-depleted atmosphere.

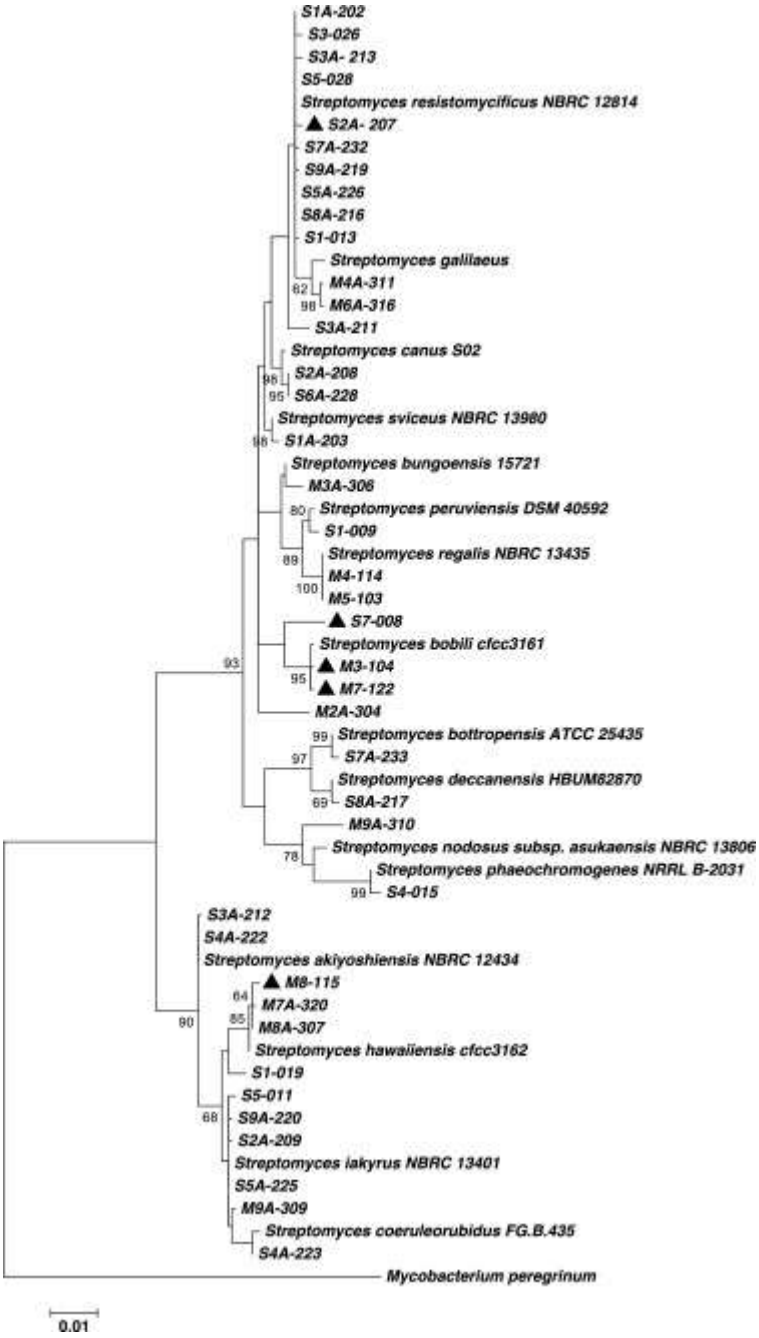

Figure 4. Maximum likelihood phylogenetic tree of 16S rRNA gene (V1-9) of diazotrophic *Streptomyces* based on Tamura-Nei model. Numbers (%) represent bootstrap values of 500 analyses. Bootstrap values lower than 60 are not shown. Triangles indicate isolates yielding *nifH* amplicons homologous to known *nifH* gene. *Mycobacterium peregrinum* was used as out group.

3.4 DGGE analysis of microbial community and sequencing of cloned DNA

DNA extraction from matrix soil was arduous, with no DNA obtained using the standard Power Soil DNA Isolation Kit (MoBio), although plating of soil on agar yielded colonies (Fig. 1). Therefore, the Power Lyzer Power Soil DNA Isolation Kit (MoBio) was used for DNA extraction. Visual inspection of DGGE results revealed a moderate number of bands for the sod table soil samples (data not shown), indicating low diversity. This caused us to opt for cloning and sequencing rather than a deeper sequencing approach. A total of 23 cloned *nifH* amplicons were sequenced and all were affiliated to Cyanobacterial dinitrogenase reductase genes (Fig. 3). These were related to *nifH* genes ascribed to five genera of cyanobacteria: *Nostoc*, *Tolypothrix*, *Scytonema*, *Trichormus* and *Spirirestis*. The presence of cyanobacteria in soil samples was confirmed using cyanobacterial 16S rRNA gene specific PCR supporting, the *nifH* findings.

3.5 Nitrogen fixation by bacterial isolates

The incorporation of stable $^{15}N_2$ isotope into the bacterial biomass in our selected isolates confirmed nitrogen fixation (Table 2). *E. coli* MG1655 did not yield any biomass in the medium and therefore could not be subjected to mass spectrometry. Three of these selected isolates had yielded *nifH* genes by PCR (PolF/R primer set), but the other three did not. None were positive for the acetylene reduction assay.

Table 2. Incorporation of $^{15}N_2$ into biomass of selected nitrogen-fixing *Streptomyces* isolates.

Isolate	**Sequence resemblance**	**% $^{15}N_2$**	***nifH* PCR (polF/R)**
S2A-207	*Streptomyces* (V1-9)	63.99	+
S7-008	*Streptomyces* (V1-9)	66.34	+
M7-122	*Streptomyces* (V1-9)	55.95	+
S3A-212	*Streptomyces* (V1-9)	78.12	-
M5-103	*Streptomyces* (V1-9)	77.43	-
S8A-217	*Streptomyces* (V1-9)	73.18	-
B. japonicum USDA 110	Positive control	52.42	+

4. Discussion

We obtained a diverse collection of nitrogen fixing bacteria from sod table and matrix soils of South Dakota Badlands belonging to Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes. As these isolates were obtained from seventeen different sod table and matrix soil sample pairs, they were not unique to a particular location, but spread across the area. Half of the putative diazotrophs were closely related to genera previously reported to fix nitrogen, including *Rhizobium, Sphingomonas* (Videira et al., 2009), *Paenibacillus* (Xie et al., 2014), *Phyllobacterium* (Rojas et al., 2001), and *Dyadobacter fermentans* (Chelius and Triplett, 2000). These nitrogen fixers are known to contain *nifH* genes. However, nitrogen fixation is not universal across any genus. None of our isolates other than the *Streptomyces* yielded *nifH* when using the PolF/PolR primer set. The sequence variability of *nifH* sequences has posed challenges to primer design. Analysis of all available *nifH* sequences pointed to IGK3/DVV as the primer set for highest probability of recovery, followed by Ueda19F/Ueda407R (Gaby and Buckley, 2012). We were unable to obtain PCR results when using these highly degenerate primers, even after evaluation with various annealing temperatures and Mg^{2+} concentrations. Only *B.*

japonicum USDA 110 as a positive control yielded amplicons. Most recent papers report use of PolF/PolR, the only primers yielding amplicons from our sod table DNA and a small numbers of *Streptomyces* isolates. There are currently no PCR primers that can cover the entire sequence variability of the *nifH* gene and capture the full diversity (Gaby and Buckley, 2012; Mirza and Rodrigues, 2012).

Over 50% of our isolates clustered with *Streptomyces* based on their partial 16S rRNA gene (V1-3). Currently, there are no known nitrogen fixing *Streptomyces*, other than the thermophilic, autotrophic *S. thermoautotrophicus* (Gadkari et al., 1992; Ribbe et al., 1997). To our knowledge this is the first report of nitrogen fixing heterotrophic *Streptomyces*. Although five of the 46 *Streptomyces* isolates yielded *nifH* gene amplicons by PCR, none displayed detectable acetylene reduction activity. This may be due to the experimental conditions, as factors including oxygen concentration, solidifying agent and incubation time all influence the level of nitrogen fixing activity (Desnoues et al., 2003; Hara et al., 2009; Mirza and Rodrigues, 2012). Even some *Rhizobium* strains known to nodulate and fix nitrogen effectively do not indicate acetylene reduction activity from pure cultures (Kuklinsky-Sobral et al., 2004). Incubation with $^{15}N_2$ of the seven selected isolates lead to ~55- 77% of ^{15}N into bacterial biomass. Our results of $^{15}N_2$ incorporation provide strong support for nitrogen fixation. It is possible that the nitrogenase of these *Streptomyces* isolates is not as promiscuous as classic Nif nitrogenase, and does not reduce acetylene.

The growth of *Streptomyces* isolates in liquid NFM in an ammonia-depleted environment and incorporation of the $^{15}N_2$ into pure cultures argue for their ability to fix nitrogen. Nitrogen fixing genes in actinobacteria have been presumed to have a narrow distribution, mostly restricted to

the genus *Frankia* (Gtari et al., 2012). More recently, molecular studies have shown the presence of *nifH* genes in other actinobacteria besides *Frankia*. (Villegas et al., 1997; Valdés et al., 2005; Gtari et al., 2007). Growth of actinomycetes in extremely oligotrophic environment is well known, especially isolates belonging to the genus *Streptomyces* (Yoshida et al., 2007). Yoshida et al. demonstrated that *Rhodococcus erythropolis* is able to grow on nitrogen free medium without fixing nitrogen by scavenging residual ammonia from the atmosphere using a high affinity uptake system (Yoshida et al., 2014). Recent work on *S. thermoautotrophicus* indicates that it does not fix nitrogen after all. It is unable to grow on noble agar, but can scavenge combined nitrogen on nitrogen free medium solidified with gellan (MacKellar et al., 2016). Although it is possible that some of our isolates possess the ability to scavenge traces of combined nitrogen, their growth on noble agar and in an ammonium-depleted environment argues for nitrogen fixation.

The homology of *nifH* from *Streptomyces* isolates to *Scytonema nifH* is unclear. Our culture independent approach indicated predominance of *nifH* homologous to cyanobacterial *nifH*. The presence of cyanobacteria in soil samples was confirmed using cyanobacterial 16S rRNA gene specific PCR, supporting the *nifH* findings. Biological soil crusts are dominated by cyanobacteria (Belnap and Gillette, 1998; Belnap, 2002; Tirkey and Adhikary, 2005; Yeager et al., 2007). It is tempting to speculate that the *nifH* genes could have transferred laterally from cyanobacteria to *Streptomyces. nifH* is a functional gene and is susceptible to horizontal transfer (Zehr et al., 2003). Horizontal transfer of *nif*HDK genes early in evolution has been reported for the cyanobacterium *Microcoleus chthonoplastes*. The *nif*HDK phylogenetic tree of *M. chthonoplastes* clusters with the *Deltaproteobacterium*, *Desulfovibrio* (Bolhuis et al., 2009). *D.*

vulgaris may be the donor for *nif* genes present in *M. chthonoplastes* because its *nif*-genes have highest similarity with *Desulfovibrio* and they are found in microbial mats habituated by Desulfovibrionaceae (Zehr et al., 1995; Sigalevich et al., 2000). The *nifH* and 16S rRNA gene trees in the genera *Rhizobium* and *Sinorhizobium*, are incompatible in many facets (Eardly et al., 1992), indicating a possibility of horizontal transfer of *nifH* genes (Haukka et al., 1998). Members of the phyla Cyanobacteria and Actinomycetes coexist together in nature, mainly in places of primary soil formation processes (Elena et al., 2006) and microbial soil crusts (da Rocha et al., 2015). The long term close associations support the notion of HGT of *nifH* between cyanobacteria and *Streptomyces*.

This study suggests that the arid Badlands of South Dakota harbor diverse free-living nitrogen fixing bacteria with the potential of novel nitrogen fixing *Streptomyces*.

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References

- Abed RMM, Al Kharusi S, Schramm A, Robinson MD. Bacterial diversity, pigments and nitrogen fixation of biological desert crusts from the Sultanate of Oman. FEMS Microbiology Ecology 2010;72(3):418-428.

- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic acids research 1997;25(17):3389-3402.
- Amberg S, Kilkus K, Gardner S, Gross JE, Wood M, Drazkowski B. Badlands National Park Climate Change Vulnerability Assessment Natural Resource Report NPS/BADL/NRR—2012/505 2012.
- Belnap J. Soil surface disturbances in cold deserts: effects on nitrogenase activity in cyanobacterial-lichen soil crusts. Biology and Fertility of Soils 1996;23(4):362-367.
- Belnap J. Nitrogen fixation in biological soil crusts from southeast Utah, USA. Biology and fertility of soils 2002;35(2):128-135.
- Belnap JGardner JS. Soil microstructure in soils of the Colorado Plateau: the role of the cyanobacterium Microcoleus vaginatus. The Great Basin Naturalist 1993:40-47.
- Belnap JGillette DA. Vulnerability of desert biological soil crusts to wind erosion: the influences of crust development, soil texture, and disturbance. Journal of Arid Environments 1998;39(2):133-142.
- Benson D, Lipman DJ, Ostell J. GenBank. Nucleic Acids Research 1993;21(13):2963-2965.
- Bolhuis H, Severin I, Confurius-Guns V, Wollenzien UIA, Stal LJ. Horizontal transfer of the nitrogen fixation gene cluster in the cyanobacterium Microcoleus chthonoplastes. ISME J 2009;4(1):121-130.
- Bowker M, Reed S, Belnap J, Phillips S. Temporal variation in community composition, pigmentation, and Fv/Fm of desert cyanobacterial soil crusts. Microbial Ecology 2002;43(1):13-25.
- Büdel B, Colesie C, Green TGA, Grube M, Lázaro Suau R, Loewen-Schneider K, Maier S, Peer T, Pintado A, Raggio J, Ruprecht U, Sancho LG, Schroeter B, Türk R, Weber B, Wedin M, Westberg M, Williams L, Zheng L. Improved appreciation of the functioning and importance of biological soil crusts in Europe: the Soil Crust International Project (SCIN). Biodiversity and Conservation 2014;23(7):1639-1658.
- Canfield DE, Glazer AN, Falkowski PG. The Evolution and Future of Earth's Nitrogen Cycle. Science 2010;330(6001):192-196.
- Chelius MKTriplett EW. Dyadobacter fermentans gen. nov., sp. nov., a novel gram-negative bacterium isolated from surface-sterilized Zea mays stems. International journal of systematic and evolutionary microbiology 2000;50(2):751-758.
- Coenye T, Falsen E, Vancanneyt M, Hoste B, Govan JRW, Kersters K, Vandamme P. Classification of Alcaligenes faecalis-like isolates from the environment and human clinical samples as Ralstonia gilardii sp. nov. International Journal of Systematic Bacteriology 1999;49:405-413.
- da Rocha UN, Cadillo-Quiroz H, Karaoz U, Rajeev L, Klitgord N, Dunn S, Truong V, Buenrostro M, Bowen BP, Garcia-Pichel F. Isolation of a significant fraction of non-phototroph diversity from a desert Biological Soil Crust. Front Microbiol 2015;6.
- Dance I. Nitrogenase: a general hydrogenator of small molecules. Chem Commun (Camb) 2013;49(93):10893-907.
- Desnoues N, Lin M, Guo X, Ma L, Carreño-Lopez R, Elmerich C. Nitrogen fixation genetics and regulation in a Pseudomonas stutzeri strain associated with rice. Microbiology 2003;149(8):2251-2262.
- Dos Santos PC, Fang Z, Mason SW, Setubal JC, Dixon R. Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. BMC genomics 2012;13:12.
- Eady RR. Structure-function relationships of alternative nitrogenases. Chemical Reviews 1996;96(7):3013-3030.

- Eardly BD, Young JP, Selander RK. Phylogenetic position of Rhizobium sp. strain Or 191, a symbiont of both Medicago sativa and Phaseolus vulgaris, based on partial sequences of the 16S rRNA and nifH genes. Applied and Environmental Microbiology 1992;58(6):1809-1815.
- Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 2004;32(5):1792-1797.
- Elena OO, Galina MZ, Natalia PC, Vladimir KO. Role of Algobacterial Associations in Soil Forming Process. The 18th World Congress of Soil Science2006.
- Fish JA, Chai BL, Wang Q, Sun YN, Brown CT, Tiedje JM, Cole JR. FunGene: the functional gene pipeline and repository. Front Microbiol 2013;4.
- Gaby JCBuckley DH. A Comprehensive Evaluation of PCR Primers to Amplify the *nifH* Gene of Nitrogenase. Plos One 2012;7(7):e42149.
- Gadkari D, Morsdorf G, Meyer O. Chemolithoautotrophic assimilation of dinitrogen by Streptomyces thermoautotrophicus UBT1: identification of an unusual N2-fixing system. J Bacteriol 1992;174(21):6840-3.
- GeneCodes. Sequencher® version 5.4 sequence analysis software. http://wwwgenecodescom/.
- Graham J. Badlands National Park Geologic Resource Evaluation Report. Natural Resource Report NPS/NRPC/GRD/NRR 2008.
- Gtari M, Ghodhbane-Gtari F, Nouioui I, Beauchemin N, Tisa LS. Phylogenetic perspectives of nitrogen-fixing actinobacteria. Archives of microbiology 2012;194(1):3-11.
- Gtari M, Brusetti L, Hassen A, Mora D, Daffonchio D, Boudabous A. Genetic diversity among Elaeagnus compatible Frankia strains and sympatric-related nitrogen-fixing actinobacteria revealed by nifH sequence analysis. Soil Biology and Biochemistry 2007;39(1):372-377.
- Hara S, Hashidoko Y, Desyatkin RV, Hatano R, Tahara S. High rate of N2 fixation by East Siberian cryophilic soil bacteria as determined by measuring acetylene reduction in nitrogen-poor medium solidified with gellan gum. Applied and environmental microbiology 2009;75(9):2811-2819.
- Haukka K, Lindström K, Young JPW. Three Phylogenetic Groups of nodA and nifHGenes in Sinorhizobium and Mesorhizobium Isolates from Leguminous Trees Growing in Africa and Latin America. Applied and Environmental Microbiology 1998;64(2):419-426.
- Hu YRibbe MW. Nitrogenase and homologs. J Biol Inorg Chem 2015;20(2):435-45.
- Jiang H, Dong H, Zhang G, Yu B, Chapman LR, Fields MW. Microbial Diversity in Water and Sediment of Lake Chaka, an Athalassohaline Lake in Northwestern China. Applied and Environmental Microbiology 2006;72(6):3832-3845.
- Kuklinsky-Sobral J, Araújo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA, Azevedo JL. Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environmental Microbiology 2004;6(12):1244-1251.
- Kumar S, Nei M, Dudley J, Tamura K. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Briefings in bioinformatics 2008;9(4):299-306.
- Liao Z-l, Chen H, Zhu B-r, Li H-z. Combination of powdered activated carbon and powdered zeolite for enhancing ammonium removal in micro-polluted raw water. Chemosphere 2015;134:127-132.
- MacKellar D, Lieber L, Norman JS, Bolger A, Tobin C, Murray JW, Oksaksin M, Chang RL, Ford TJ, Nguyen PQ, Woodward J, Permingeat HR, Joshi NS, Silver PA, Usadel B, Rutherford AW, Friesen ML, Prell J. Streptomyces thermoautotrophicus does not fix nitrogen. Sci Rep 2016;6:20086.
- Mirza BSRodrigues JL. Development of a direct isolation procedure for free-living diazotrophs under controlled hypoxic conditions. Appl Environ Microbiol 2012;78(16):5542-9.

- Muyzer G, de Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 1993;59(3):695-700.
- Nübel U, Garcia-Pichel F, Muyzer G. PCR primers to amplify 16S rRNA genes from cyanobacteria. Applied and environmental microbiology 1997;63(8):3327-3332.
- Peterjohn WTSchlesinger WH. FACTORS CONTROLLING DENITRIFICATION IN A CHIHUAHUAN DESERT ECOSYSTEM. Soil Science Society of America Journal 1991;55(6):1694-1701.
- Pointing SBBelnap J. Microbial colonization and controls in dryland systems. Nature reviews Microbiology 2012;10(8):551-62.
- Poly F, Monrozier LJ, Bally R. Improvement in the RFLP procedure for studying the diversity of nifH genes in communities of nitrogen fixers in soil. Research in Microbiology 2001;152(1):95-103.
- Rettedal EA, Clay S, Brozel VS. GC-clamp primer batches yield 16S rRNA gene amplicon pools with variable GC clamps, affecting denaturing gradient gel electrophoresis profiles. FEMS Microbiol Lett 2010;312(1):55-62.
- Ribbe M, Gadkari D, Meyer O. N2 Fixation by *Streptomyces thermoautotrophicus* Involves a Molybdenum-Dinitrogenase and a Manganese-Superoxide Oxidoreductase That Couple N2Reduction to the Oxidation of Superoxide Produced from O2by a Molybdenum-CO Dehydrogenase. Journal of Biological Chemistry 1997;272(42):26627-26633.
- Rojas A, Holguin G, Glick BR, Bashan Y. Synergism between Phyllobacterium sp.(N2-fixer) and Bacillus licheniformis (P-solubilizer), both from a semiarid mangrove rhizosphere. FEMS Microbiology Ecology 2001;35(2):181-187.
- Rychert RSkujiņš J. Nitrogen fixation by blue-green algae-lichen crusts in the Great Basin Desert. Soil Science Society of America Journal 1974;38(5):768-771.
- Sigalevich P, Meshorer E, Helman Y, Cohen Y. Transition from anaerobic to aerobic growth conditions for the sulfate-reducing bacterium Desulfovibrio oxyclinae results in flocculation. Applied and environmental microbiology 2000;66(11):5005-5012.
- Stoffer PW, 2003. Geology of Badlands National Park: a preliminary report, US Department of the Interior, US Geological Survey.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular biology and evolution 2013;30(12):2725-9.
- Tirkey JAdhikary S. Cyanobacteria in biological soil crusts of India. Current Science Bangalore 2005;89(3):515.
- Ueda T, Suga Y, Yahiro N, Matsuguchi T. Remarkable N2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of nifH gene sequences. Journal of bacteriology 1995;177(5):1414-1417.
- Unkovich MBaldock J. Measurement of asymbiotic N2 fixation in Australian agriculture. Soil Biology and Biochemistry 2008;40(12):2915-2921.
- Valdés M, Pérez N-O, Estrada-de Los Santos P, Caballero-Mellado J, Peña-Cabriales JJ, Normand P, Hirsch AM. Non-Frankia actinomycetes isolated from surface-sterilized roots of Casuarina equisetifolia fix nitrogen. Applied and environmental microbiology 2005;71(1):460-466.
- Videira SS, De Araujo JLS, Da Silva Rodrigues L, Baldani VLD, Baldani JI. Occurrence and diversity of nitrogen-fixing Sphingomonas bacteria associated with rice plants grown in Brazil. FEMS Microbiology Letters 2009;293(1):11-19.
- Villegas M, Vasquez L, Valdes M. Growth and nitrogenase activity conditions of a new diazotrophic group of filamentous bacteria isolated from Casuarina root nodules. Revista Latinoamericana de Microbiologia 1997;39(1):65-72.

- Vitousek PM, Cassman K, Cleveland C, Crews T, Field CB, Grimm NB, Howarth RW, Marino R, Martinelli L, Rastetter EB, Sprent JI. Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry 2002;57(1):1-45.
- Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and environmental microbiology 2007;73(16):5261-5267.
- Wang Y, Liu S, Xu Z, Han T, Chuan S, Zhu T. Ammonia removal from leachate solution using natural Chinese clinoptilolite. Journal of Hazardous Materials 2006;136(3):735-740.
- West NE. Structure and functions of microphytic soil crusts in wildland ecosystems of arid to semi-arid regions. Advances in ecological research 1990;20:179-223.
- Wilson KH, Wilson WJ, Radosevich JL, DeSantis TZ, Viswanathan VS, Kuczmarski TA, Andersen GL. High-Density Microarray of Small-Subunit Ribosomal DNA Probes. Applied and Environmental Microbiology 2002;68(5):2535-2541.
- Xie J-B, Du Z, Bai L, Tian C, Zhang Y, Xie J-Y, Wang T, Liu X, Chen X, Cheng Q, Chen S, Li J. Comparative Genomic Analysis of N2-Fixing and Non-N-2-Fixing *Paenibacillus* spp.: Organization, Evolution and Expression of the Nitrogen Fixation Genes. Plos Genetics 2014;10(3).
- Yeager CM, Kornosky JL, Morgan RE, Cain EC, Garcia-Pichel F, Housman DC, Belnap J, Kuske CR. Three distinct clades of cultured heterocystous cyanobacteria constitute the dominant N2-fixing members of biological soil crusts of the Colorado Plateau, USA. FEMS microbiology ecology 2007;60(1):85-97.
- Yoshida N, Inaba S, Takagi H. Utilization of atmospheric ammonia by an extremely oligotrophic bacterium, Rhodococcus erythropolis N9T-4. Journal of Bioscience and Bioengineering 2014;117(1):28-32.
- Yoshida N, Ohhata N, Yoshino Y, Katsuragi T, Tani Y, Takagi H. Screening of carbon dioxide-requiring extreme oligotrophs from soil. Bioscience Biotechnology and Biochemistry 2007;71(11):2830-2832.
- Young J. Phylogenetic classification of nitrogen-fixing organisms. Biological nitrogen fixation 1992:43-86.
- Zehr JP, Jenkins BD, Short SM, Steward GF. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environmental microbiology 2003;5(7):539-54.
- Zehr JP, Mellon M, Braun S, Litaker W, Steppe T, Paerl HW. Diversity of heterotrophic nitrogen fixation genes in a marine cyanobacterial mat. Applied and Environmental Microbiology 1995;61(7):2527-2532.
- Zhao Y, Bian SM, Zhou HN, Huang JF. Diversity of nitrogenase systems in diazotrophs. Journal of Integrative Plant Biology 2006;48(7):745-755.

Appendix A. Supplementary data

Table S1: Accession numbers of reference sequences obtained from NCBI for use in phylogenetic analysis of V1-3 region of 16SrRNA genes

Strain Name	**Accesion No.**
Agromyces ulmi Y2S10	EU221378.1
Allokutzneria multivorans YIM 120521	JQ306329.1
Arthrobacter globiformis L10	KC934755.1
Arthrobacter globiformis strain B-4S-7	JF439618.1
Arthrobacter oxydans strain L42	KC934779.1
Arthrobacter pascens E22	HF585207.1
Cellulomonas cellasea A6-6	JF496362.1
Chitinophaga ginsengihum SR18	NR_134000.1
Dyadobacter fermentans WS17	JN210905.1
Ensifer numidicus strain PN14	HM346601.1
Kribbella sandramycini strain cfcc3141	FJ883748.1
Lactobacillus casei strain Lb37-1	KM051982.1
Lechevalieria roselyniae C81	EU551683.2
Massilia suwonensis C61	HF585368.1
Myceligenerans xiligouense T812-1	KF463140.1
Mycobacterium chelonae strain B14	JX010972.1
Myxococcus xanthus ATCC25232	DQ768116.1
Nocardia ignorata strain NR_35	KM113026.1
Paenibacillus mucilaginosus 3027	JF810840.1
Phyllobacterium brassicacearum WK-121s	KF580858.1

Rhizobacter dauci strain R20	HM224398.1
Rhizobium alamii CCBAU15292	GU552885.1
Rhizobium gallicum bv *gallicum* RHM47	JQ085250.1
Rhizobium gallicum W49	JF730143.1
Rhizobium giardinii 23C88	JN624697.1
Sinorhizobium meliloti EFLRI 94	EU445256.1
Sphingomonas humi PB323	NR_112548.1
Streptomyces antibioticus NBRC 13271	AB184340.1
Streptomyces aurantiacus HBUM7519	FJ486281.1
Streptomyces aureocirculatus DSM 40386T	KF772674.1
Streptomyces bobili cfcc3161	FJ792573.1
Streptomyces cinerospinus NBRC 15397	AB184648.1
Streptomyces ciscaucasicus cfcc3135	FJ883745.1
Streptomyces glomeroaurantiacus NRRL B-3375	AB184366.1
Streptomyces hygroscopicus 3088	EF063461.1
Streptomyces iakyrus 13667L	EU741215.1
Streptomyces lavendulae HD-8	JN609386.1
Streptomyces paradoxus A410	GU479445.1
Streptomyces plumbiresistens CCNWHX 13-160	EU526954.1
Streptomyces resistomycificus NBRC 12814	AB184166.1
Streptomyces scabiei strain O3-623	EU834682.1
Streptomyces zaomyceticus 173508	EU593577.1
Synechococcus sp. OH9	AF285245.1

Table S2: Accession numbers of reference sequences obtained from Fungene for use in phylogenetic analysis of partial *nifH* genes.

Strain Name	**Accesion No.**
Bradyrhizobium_ japonicum	AH010242.2
Burkholderia_sp	CP012899
Methylacidiphilum_fumariolicum	CAHT01000076
Methylobacterium_nodulans	CP001349
Nostoc _punctiforme	CP001037
Nostoc_sp MCT-1	DQ531689
Nostoc_sp. ARC7	JX862200
Nostoc_sp. MFG-1	DQ531683
Rhizobium_leguminosarum	CP001192
Rhizobium_phaseoli	M15942
Rhizobium_sp._N741	CP013598
Rhodospirillum_rubrum	M33774
Scytomena_sp. FGP-7A	DQ531669
Scytomena_sp. NC-4B	DQ531694
Scytomena_sp.DC-A	DQ531695
Sinorhizobium_fredii	HE616895
Sinorhizobium_medicae	CP000740
Spirirestis rafaelensis SRS70	DQ531685
Tolypothrix sp. LQ-10	DQ531673
Trichormus_azollae	L34879

Table S3: Accession numbers of reference sequences obtained from NCBI for use in phylogenetic analysis of V1-9 region of 16SrRNA genes of putative *Streptomyces* isolates.

Strain Name	**Accesion No.**
Mycobacterium peregrinum	NR_114447
Streptomyces akiyoshiensis NBRC 12434	AB184095
Streptomyces bobili cfcc3161	FJ792573
Streptomyces bottropensis ATCC 25435	NR_115571
Streptomyces bungoensis 15721	JN180215
Streptomyces canus S02	HQ850405
Streptomyces coeruleorubidis FG.B.435	KF991647
Streptomyces deccanensis HBUM82870	EU841582
Streptomyces galilaeus strain WJA76	KU877598.1
Streptomyces hawaiiensis cfcc3162	FJ792574
Streptomyces iakyrus NBRC 13401	NR_041231
Streptomyces nodosus subsp *asukaensis* NBRC 13806	AB184497
Streptomyces peruviensis DSM 40592	AJ310924
Streptomyces phaeochromogenes NRRL B-2031	EU594476
Streptomyces regalis NBRC 13435	AB184400
Streptomyces resistomycificus NBRC 12814	NR_112287
Streptomyces sviceus NBRC 13980	AB184559