2016

Diversity of Free-living Nitrogen Fixing Bacteria in the Badlands of South Dakota

Bibha Dahal
South Dakota State University

DIVERSITY OF FREE-LIVING NITROGEN FIXING BACTERIA IN THE BADLANDS OF SOUTH DAKOTA

BY

BIBHA DAHAL

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Biological Sciences

Specialization in Microbiology

South Dakota State University

2016

DIVERSITY OF FREE-LIVING NITROGEN FIXING BACTERIA IN THE BADLANDS OF SOUTH DAKOTA

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biological Sciences and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Volker S. Brözel

Thesis Advisor Date

Volker S. Brözel,

Head, Department of Biology and Microbiology Date

Dean, Graduate School Date

ACKNOWLEDGEMENTS

"Always aim for the moon, even if you miss, you'll land among the stars".- W. Clement Stone

I would like to express my profuse gratitude and heartfelt appreciation to my advisor Dr. Volker Brözel for providing me a rewarding place to foster my career as a scientist. I am thankful for his implicit encouragement, guidance, and support throughout my research. This research would not be successful without his guidance and inspiration.

I would like to thank all my POS Committee for supporting me in various stages of my research and encouraging me to achieve my goal swiftly. My sincere thanks goes to my committee members Dr. Lora Perkins and Dr. Senthil Subramanian for their assistance, support, and direction when I needed. I would like to thank all the faculties and staffs of Department of Biology and Microbiology who have directly or indirectly supported me to achieve this goal.

I am thankful to my lab mate Gitanjali Nanda Kafle and my friend Tyrel Deutscher who have assisted me in my research in various ways. The conversations we had will remain in my memories.

I would like to acknowledge the funding source South Dakota Agricultural Experiment Station for its financial support.

Last but not the least, I would like to thank my husband Nirmal Paudel for his encouragement and moral support throughout my study period. My acknowledgement goes to my sister Subha Dahal and my parents who have been very supportive and encouraging.

TABLE OF CONTENTS

ABBREVIATIONS

α: Alpha

β: Beta

°C: Degrees Celsius

µ: Micro

ADP: Adenosine Di-Phosphate

ARA: Acetylene Reduction Assay

ATP: Adenosine Tri- Phosphate

BLAST: Basic Local Alignment Search Tool

BNF: Biological Nitrogen Fixation

bp: base pair

BSC: Biological Soil Crust

δ: Delta

Da: Dalton

DGGE: Denaturing Gradient Gel Electrophoresis

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleic triphosphate

EPR: Electron paramagnetic resonance

EXAFS: Extended X-ray Absorption Fine Structure

HGT: Horizontal Gene Transfer

ITS: Internal Transcribed Spacer

kDa: Kilo Dalton

LGT: Lateral Gene Transfer

M: Molar

Min: minute

mM: Milimolar

M_r: Molecular Weight

MS: Mass Spectrometry

NCBI: National Center for Biotechnology

NFM: Nitrogen Free Medium

OTU: Operational Taxonomic Unit

PBST: Phosphate Buffered Saline Tween 20

PCR: Polymerase Chain Reaction

RDP: Ribosomal Database Project

RFLP: Restriction Fragment Length Polymorphism

RNA: Ribonucleic acid

rRNA: Ribosomal ribonucleic acid

SDSU: South Dakota State University

Sec: Second

SSU: Small subunit

TGGE: Temperature Gradient Gel Electrophoresis

t-RFLP: Terminal Restriction Fragment Length Polymorphism

V: Volt

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

DIVERSITY OF FREE-LIVING NITROGEN FIXING BACTERIA IN THE BADLANDS OF SOUTH DAKOTA

BIBHA DAHAL

2016

Biological Nitrogen Fixation is carried out by a highly diverse group of members of the domains Bacteria and Archaea which express a nitrogenase enzyme complex and are termed diazotrophs. The best known diazotrophs form symbiotic associations with plants, but free-living nitrogen fixing bacteria also contribute a substantial amount of nitrogen to ecosystems, including arid lands. The aim of this study was to isolate free-living diazotrophs from the soil crusts of sod tables of South Dakota Badlands. Samples were obtained from sod tables and the surrounding base (matrix) in spring and fall of 2014. A culture dependent approach was used to isolate diazotrophs on solid nitrogen free medium (NMF) under hypoxic conditions. The 16srRNA and *nifH* genes were amplified and sequenced. This was complemented using a culture independent approach, amplifying *nifH* 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.* The *Streptomyces* isolates grew in liquid NFM in an ammonia-depleted environment in a sealed container. Only 5 of these yielded a *nifH* gene product using the PolF/PolR primer set. Of these four aligned with *nifH* of the cyanobacterium *Scytonema* (NCBI BLAST) and the other one aligned with *nifH* of

Bradyrhizobium. All six selected *Streptomyces* isolates, three of which were *nifH* positive, indicated $^{15}N_2$ incorporation providing strong support of nitrogen fixation. The culture independent method yielded a low diversity. All the *nifH* amplicons from soil DNA extract resembled cyanobacteria by NCBI BLAST. This is the first known report of a *Streptomyce*s that is diazotrophic, other than the thermophilic, autotrophic *S. thermoautotrophicus*. The *nifH* genes of these *Streptomyces* were related to those from cyanobacteria. Whether the cyanobacteria-like *nifH* amplicons obtained from soil DNA were associated with *Streptomyces,* is intriguing to speculate.

Chapter 1

1. Literature Review

1.1. Introduction

Nitrogen is the most common factor limiting growth and productivity of plants in aquatic as well as terrestrial ecosystems (Wagner 2011). Although dinitrogen is the most abundant element in the atmosphere, it is biochemically unavailable for plants and most microbes as they utilize only reduced or oxidized forms of nitrogen. The two atoms in dinitrogen are triple-bonded, requiring a high level of energy to dissociate and reduce to ammonia (Figg et al. 2012). Several biological systems are able to convert dinitrogen into other useful forms of reactive nitrogen, primarily, nitrite, and nitrate, and to incorporate ammonia into organic compounds, mainly, amino acids (Galloway 1998). There are two natural processes for the fixation of atmospheric nitrogen into the biosphere: Lightning and biological nitrogen fixation. Lightning makes about 1% ammonia of the net nitrogen fixed per year (Igarashi and Seefeldt 2003). Biological nitrogen fixation (BNF) is carried out by a group of microorganisms with sporadic distribution among the bacterial and archaeal domains (Vitousek et al. 1997) and, fixes about 50% of the total nitrogen per year (Igarashi and Seefeldt 2003). The remaining 50% is fixed industrially by the Haber Bosch process (Finan 2002). In this process atmospheric nitrogen is reduced to ammonia through reaction with hydrogen under high pressure and temperature using iron as a catalyst (Jennings 2013; Vojvodic et al. 2014). The Haber Bosch process brought a revolution in agricultural production as it fulfilled the immediate demands for nitrogen as

a fertilizer early in the twentieth century. However, the industrial nitrogen fixation contributes harmful consequences leading to nitrogen oxides emission, eutrophication of water, acidification of soil (Dixon and Kahn 2004). BNF is both economically and ecologically effective for sustainable agricultural production (Bohlool et al. 1992).

1.1.1. Biological Nitrogen Fixation

Biological nitrogen fixation is the primary source by which plants in natural ecosystems obtain their nitrogen for growth (Zehr et al. 2003). BNF is carried out by a highly diverse group of Prokaryotes (Bacteria and Archaea) termed diazotrophs (Postgate, 1987). These include free-living soil bacteria belonging to genera such as *Burkholderia*, *Azotobacter*, *Azospirillum*, *Bacillus,* and *Clostridium,* bacteria that form symbioses with legumes like *Rhizobium,* with actinorhizal plants such as *Frankia,* and *Cyanobacteria* associated with cycads (Postgate 1982). Nitrogen fixation in Archaea is restricted to methanogens (Dixon and Kahn 2004). Diazotrophs express the nitrogenase enzyme complex, which carries out nitrogen fixation. The bacterial nitrogenase enzyme complex is the only known natural system for reduction of N_2 to NH_3 (Seefeldt et al. 2009). BNF is one of the most costly metabolic processes as 16 molecules of ATP are utilized to fix one molecule of nitrogen into two molecules of ammonia (Simpson and Burris 1984).

1.1.2. Nitrogen cycle

Nitrogen fixation is an essential step in the global nitrogen cycle as it restores and compensates the overall nitrogen lost as a result of denitrification (Dixon and Kahn

2004). The element nitrogen is present in all living organisms in the form of proteins and nucleic acids (nitrogen biomolecules). Nitrogen exists either in the reduced or oxidized forms in the global nitrogen cycle (Fig. 1.1). The conversion of ammonia into oxides of nitrogen is called nitrification, which is carried out by nitrifying bacteria like *Nitrosomonas* and *Nitrobacter* which is a reversible reaction. The reductive conversion of nitrogen oxides back to dinitrogen is called denitrification and is carried out by denitrifying bacteria like *Thiobacillus denitrificans,* and *Micrococcus denitrificans.*

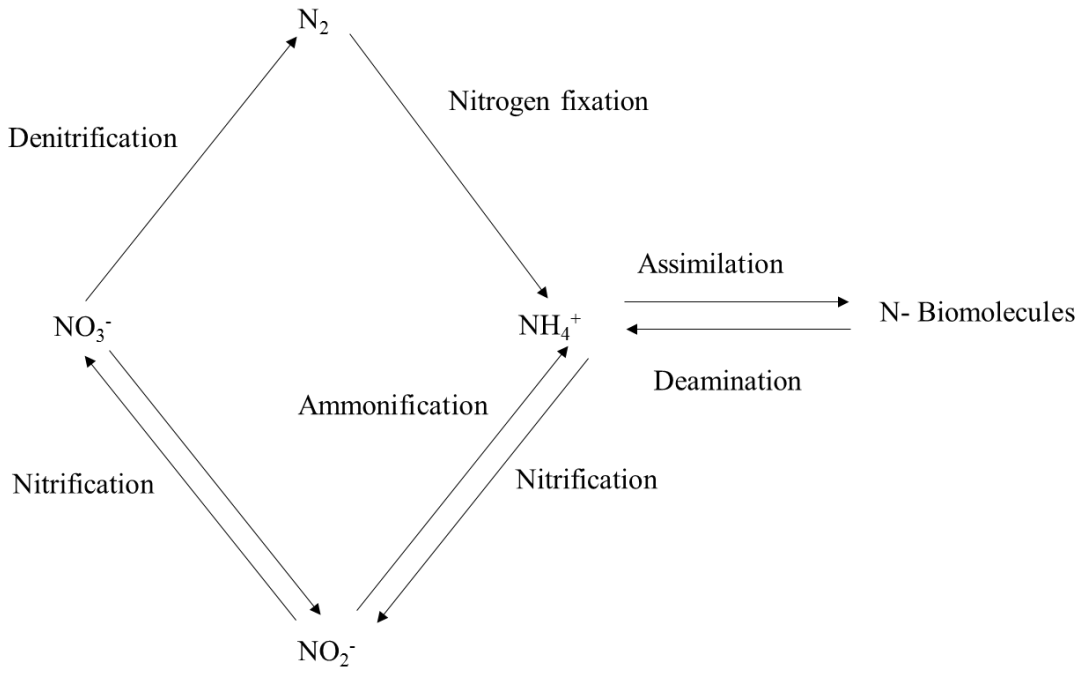

Figure 1.1 Steps involved in global nitrogen cycle (Igarashi and Seefeldt 2003).

1.2. The Nitrogenase enzyme

Nitrogenase is the enzyme responsible for biological nitrogen fixation, an important step in the global nitrogen cycle (Zhao et al. 2006; Hu and Ribbe 2015). The nitrogenase enzyme consists of two multisubunit metalloproteins: Component I, dinitrogenase which has the active site for nitrogen reduction (consists of two heterodimers with molecular weight ~240KDa), and Component II, dinitrogenase reductase (homodimer with molecular weight 60-70kDa) which couples ATP hydrolysis to interprotein electron transfer (Zehr et al. 2003; Zhao et al. 2006; Hu and Ribbe 2015). To date, four distinct nitrogenases have been reported (Eady 1996; Hofmann-Findeklee et al. 2000). There are three genetically distinct oxygen sensitive nitrogenase system, and the fourth is oxygen insensitive nitrogenase reported only for *Streptomyces thermoautotrophicus* (Zhao et al. 2006). Whether any additional classes of nitrogenase exist remains to be determined.

The three oxygen sensitive nitrogenases are: the conventional molybdenum nitrogenase (Nif) (Wilson et al.), the alternative vanadium nitrogenase (Vnf), and the alternative iron only nitrogenase (Anf) (Hu and Ribbe 2015). The three nitrogenases differ in their metal content, however, they have structural, mechanical, and phylogenetic relatedness (Dos Santos et al. 2012).

1.2.1. The Molybdenum containing nitrogenase

The molybdenum nitrogenase (Nif) is the most widely distributed and broadly studied of all nitrogenases (Rees and Howard 2000). The genes that encode the structural

components of molybdenum nitrogenase are *nifH*, *nifD,* and *nifK* (Dos Santos et al. 2012). Apart from these structural genes, there are a core of *nif* genes (*nifB, nifE, nifN, nifX, nifU, nifS, nifV, nifW, nifZ*) (Fig. 1.2), conserved across the diazotrophs and involved in the catalysis and synthesis of nitrogenase (Zheng et al. 1998; Dixon and Kahn 2004; Rubio and Ludden 2008).

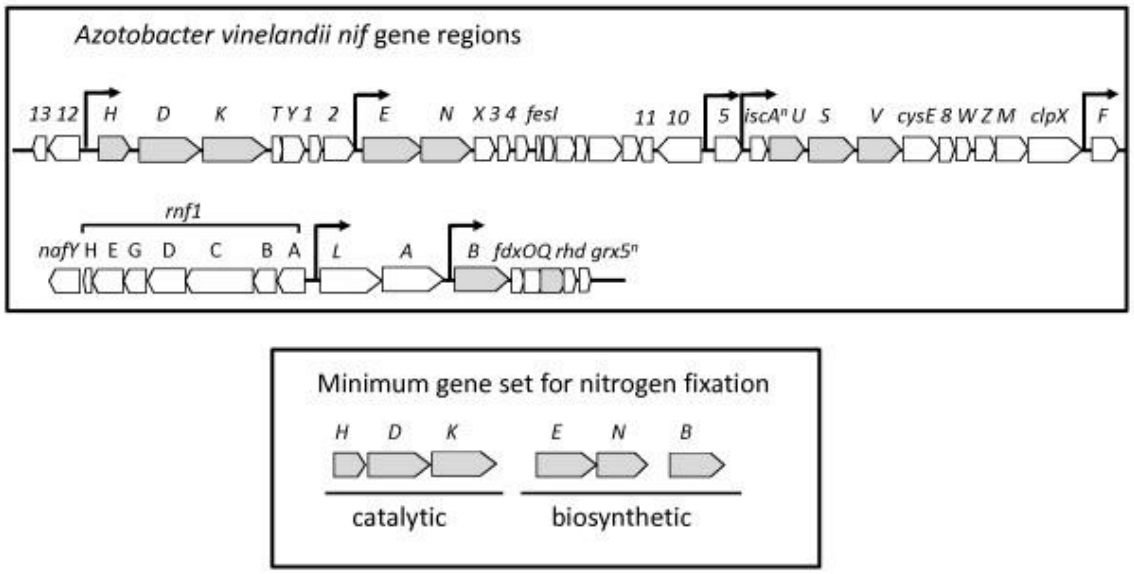

Figure 1.2. *Azotobacter vinelandii nif* gene showing minimum genes required for nitrogen fixation from (Dos Santos et al. 2012).

Mo-nitrogenase consists of two separate oxygen labile metalloproteins: MoFe protein encoded by *nifD* and *nifK,* which is an $\alpha_2\beta_2$ heterotetramer (~240kDa); and Fe protein encoded by *nifH,* which is a homodimer (~60kDa) (Zhao et al. 2006; Hu and Ribbe 2015). The Fe protein has a conserved N-terminal GXGXXG consensus nucleotide binding protein, which provides the binding site for MgATP to each of its subunit (Eady 1996). As shown in Fig. 1.3, each subunit of the Fe protein is linked by a single [4Fe-4S]

cluster (Hausinger and Howard 1983) through 2 Cys residues covalently linked to the Fe-atoms from each subunit (Eady 1996). The Fe protein is involved in the transfer of electrons to the MoFe protein during a process of MgATP hydrolysis (Howard and Rees 1994). Moreover, the Fe protein is also involved in the biosysnthesis of FeMo cofactor and is probably associated with the regulation of alternative nitrogenases (Burgess and Lowe 1996). The Fe protein encoded by *nifH* shares ~91% (Hu et al. 2012) and 60% (Eady 1996) sequence identities with the Fe protein encoded by *vnfH* and *anfH* respectively. Also the study of the Fe proteins of *A. vinelandii* and *Clostridium pasteurianum* shows that they share 69% similarity in their amino acid sequence, which indicates that the Fe protein is highly conserved (Schlessman et al. 1998).

The MoFe protein consists of two metalloclusters: the P-cluster [8Fe-7S] and the M-cluster or FeMo cofactor [Mo-7Fe-9S-homocitrate-X] (Seefeldt et al. 2009). The P-cluster is situated in the interface of the α/β-subunit and linked by 6 cysteine residues (Eady 1996) and its structure is described as two [4Fe-4S] clusters linked covalently with a shared Sulphur atom (Kim and Rees 1992; Mayer et al. 1999). The X-ray structure of P-cluster is thought as the mediator during electron transfer from the Fe protein to the FeMo cofactor (Dos Santos et al. 2012).

The overall structure of the M-cluster or FeMoco is described as having two sub-clusters: [4Fe-3S] linked to [3Fe-Mo-3S] by the X-atom at one corner and bridging through three sulfur atoms. The X-atom was identified recently in a high resolution X-ray (1.16 A) (Einsle et al. 2002). The X-atom still remains unidentified, however, depending upon its diffraction properties, heavy metals have been precluded. Its density is steady

with C, N or O, with X being one of these. (Seefeldt et al. 2009). The FeMoco site is responsible for the binding and reduction of substrate (Liang and Burris 1988; Scott et al. 1992), but how the substrate binds and reduces during catalysis is still unknown (Christiansen et al. 2001).

The overall stoichiometry of the reaction catalyzed by Mo-nitrogenase is represented in the following equation:

$$N_2 + 8H^+ + 16MgATP + 8e^- \rightarrow 2NH_3 + H_2 + 16MgADP + 16Pi.$$

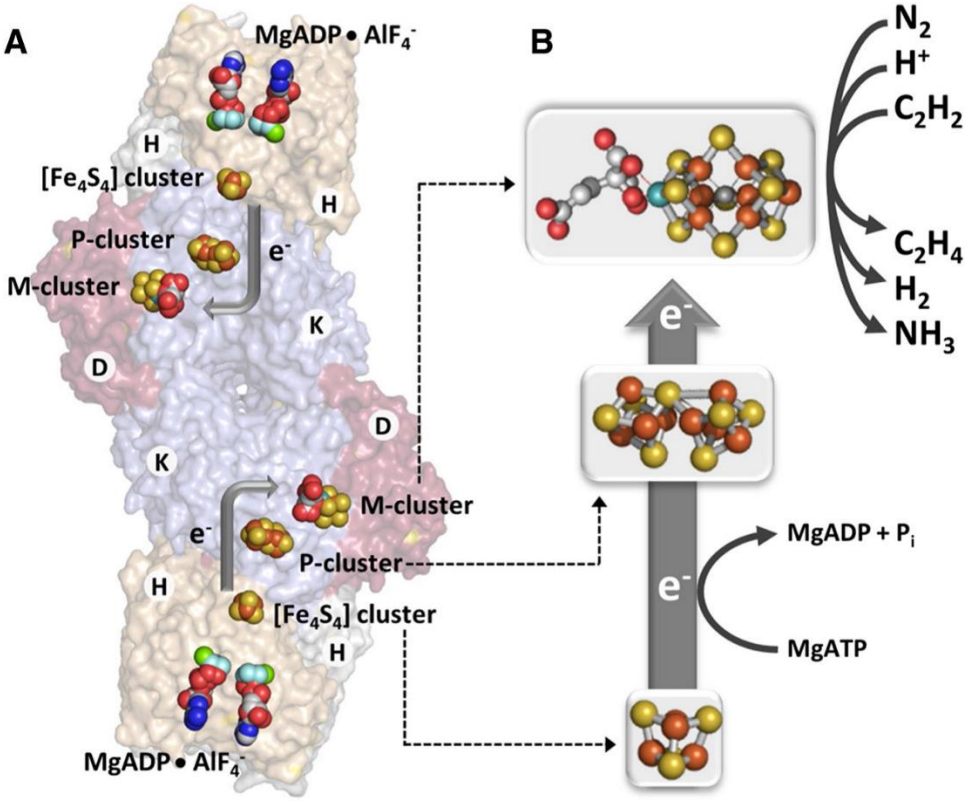

Figure 1.3. The Molybdenum Nitrogenase. Figure A shows the MoFe protein complex along with P-cluster and M-cluster. Figure B shows the transfer of electron during catalysis (Hu and Ribbe 2015).

1.2.2. The Vanadium nitrogenase

The existence of the vanadium nitrogenase system was confirmed when it was isolated from mutants of *Azotobacter vinelandii* and *A. chroococcum* that had their structural genes of Mo-nitrogenase (*nifHDK*) deleted (Hales et al. 1986; Eady et al. 1987). Since the vanadium nitrogenase is only expressed in the absence of molybdenum, it is considered the alternative nitrogenase or the "back-up" system (Rehder 2000). The cyanobacteria, *Anabaena variabilis*, also possesses Vnf (Thiel 1993). It has been suggested that V-nitrogenase works more efficiently at lower temperatures than Mo-nitrogenase (Miller and Eady 1988). Similar to the Mo-nitrogenase, the V-nitrogenase also contains two metalloprotein components, an Fe- protein (molecular weight ~64kDa), and an iron-heterometal VFe-protein (Robson et al. 1989). The Fe-protein is encoded by *vnfH* and is corresponding to the Fe-protein of Mo-nitrogenase in structure and function (Zhao et al. 2006; Hu and Ribbe 2015), sharing 91% sequence homology to the Fe protein encoded by the *nifH* (Hu et al. 2012). It also has the conserved Gly-X-Gly-X-X-Gly consensus nucleotide-binding motif, and conserved Cys ligand for subunit-linking, like the Fe-protein of Mo-nitrogenase. A ferredoxin [4Fe-4S] links the two subunits of the Fe protein of V-nitrogenase (Rehder 2000), and contains two binding sites for $Mg2^{+}$ ATP (Thorneley et al. 1989).

The structure of VFe protein is still unknown, however studies done so far suggest that it is similar to Mo-nitrogenase (Crans et al. 2004). The VFe protein is hexameric structure (α2β2δ2), encoded by *vnfD, vnfK* and *vnfG*. The δ-subunit is absent in Mo-nitrogenase (Zhao et al. 2006). The molecular weight of the VFe protein purified from *A.*

chrochococcum is ~250kDa, being uniform with $\alpha_2\beta_2\delta_2$ hexameric structure (Hu et al. 2012). The VFe protein derived from *A. vinelandii* is octameric ($\alpha_2\beta_2\delta_4$) with molecular weight ~270 kDa (Lee et al. 2009). The α- and β- subunits of the VFe protein show ~33% and ~32% sequence homology with α- and β- subunits of the MoFe protein, respectively (Hu et al. 2012).

The V-nitrogenase has a similar ligand cluster as the Mo-nitrogenase. The magnetic circular dichroism spectrum of the oxidized VFe protein shows the existence of oxidized P-clusters (Morningstar et al. 1987). The Extended X-ray Absorption Fine Structure (EXAFS) spectrum study of VFe revealed that its Fe-S and Fe-Fe interactions were similar to its MoFe cofactor (Harvey et al. 1990; Chen et al. 1993).

The utilization of ATP in vanadium nitrogenase varies for different species. The overall reaction of nitrogen fixation catalyzed by vanadium nitrogenase *for A. chrococcum* is given below:

$N_2 + 12e^- + 14H^+ + 40MgATP \rightarrow 2NH_3 + 3H_2 + 40MgADP + 40Pi$ (Eady 2003)

In comparison to Mo-nitrogenase, V-nitrogenase produces more H_2 and utilizes more ATP in reaction with N_2. There is a catalytic difference between CO and CO_2 reduction properties of V-nitrogenase and Mo-nitrogenase. V-nitrogenase can reduce CO to hydrocarbons of varying lengths: methane (CH_4), ethylene (C_2H_4), ethane (C_2H_6), propene (C_3H_6), propylene (C_3H_8), butylene (C_4H_8), and butane (C_4H_{10}), however, C_3H_6 is only produced when H_2O is replaced by D_2O (Lee et al. 2010; Hu et al. 2011; Lee et al. 2011). Mo-nitrogenase, on the other hand, cannot produce CH_4, and also the amounts of

hydrocarbons produced are very low when compared to those produced by V-nitrogenase (only 0.1% in H_2O based reactions and 2% in D_2O based reactions) (Yang et al. 2011). Also the ratio of C_2H_4/C_2H_6 formation is lower in Mo-nitrogenase than V-nitrogenase (Hu et al. 2012).

1.2.3. The Iron-only nitrogenase

The second alternative nitrogenase, the iron- only nitrogenase was first isolated by Chisnell et al. from *nifHDK* deleted strain of *A. vinelandii* (Chisnell et al. 1988). The iron-only nitrogenase is expressed only in the absence of molybdenum and vanadium nitrogenases (Crans et al. 2004), and is encoded by *anf* genes (Joerger et al. 1989) . When compared to the conventional Mo and the V nitrogenases, it has the lowest activity (Hinnemann and Norskov 2004), and is unstable and very sensitive to oxygen, making it the most difficult to study of the three nitrogenases (Chisnell et al. 1988; Crans et al. 2004). So far, the Fe-only nitrogenase has been isolated from *A. vinelandii* (Chisnell et al. 1988; Pau et al. 1993; Eady 1996) *Rhodospirillum rubrum* (Lehman and Roberts 1991; Davis et al. 1996), and *Rhodobacter capsulatus* (Schneider et al. 1991). The Fe-only nitrogenase also has the two component systems: the Fe protein, and the Fe-Fe protein. The Fe-protein, encoded by *anfH*, is a homodimer (molecular weight ~32.5 kDa), linked by a Fe_4-S_4 cluster (Zhao et al. 2006; Hu and Ribbe 2015). It is responsible for the transfer of electrons to Fe-Fe protein in Mg^{2+}ATP reaction (Schneider and Müller 2004). The *anfH* gene shares ~60% homology with the *nifH* and the *vnfH* of Mo- and V-nitrogenases respectively (Eady 1996).

The Fe-Fe protein of Fe-only nitrogenase, like the VFe protein of V-nitrogenase, is a hexamer containing three subunits: α (Mr~58kDa), β (Mr ~50kDa), and δ (Mr~ 15kDa)- subunits, encoded by *anfD, anfK*, and *anfG* (Eady 1996; Zhao et al. 2006). The intact isolation of Fe-Fe protein still remains a challenge, even though progress has been made towards the segregation of the FeFe protein from *R. capsulatus* (Eady 1996; Hu and Ribbe 2015). The EXAFS and Mossbauer spectroscopy of *R. capsulatus* indicate that FeFe cofactor has eight iron atoms giving a stoichiometry of Fe_8S_9.

1.2.4. Oxygen insensitive Streptomyces thermoautotrophicus nitrogenase

In 1992, Gadkari et al. reported that a chemolithotrophic *S. thermoautotrophicus* was able to utilize nitrogen when grown in aerobic condition with CO or H_2 and CO_2 (Gadkari et al. 1992b). The two components of this nitrogenase differ considerably from the three other nitrogenases. The dinitrogenase consists of a MoFeS protein, which is a heterotrimer and the dinitrogenase reductase is not an Fe protein but a manganese-superoxide oxidoreductase (Zhao et al. 2006). The noteworthy properties of this nitrogenase includes its inability to reduce acetylene to ethylene; its low MgATP requirement, and its insensitivity to O_2, however, they require O_2 and $O^{\cdot-}_2$ for the complete reduction of N_2 to NH_3 (Zhao et al. 2006).

The overall equation of nitrogen fixation catalyzed by *S. thermoautotrophicus* follows:

$N_2 + 4–12MgATP + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2 + 4–12MgADP + 4–12Pi$ (Ribbe et al. 1997)

The uncertainty around the ATP requirement indicates that the biochemistry of this nitrogenase is not yet well characterized. The ATP requirement appears lower than for Nif or Vnf, indicating that this nitrogenase is more efficient in its action.

1.3. Diversity of Nitrogen fixing bacteria

Nitrogen fixation occurs in members of Bacteria and Archaea with widely differing growth requirements, including heterotrophs like *Frankia* (aerobe), *Klebsiella* (facultative anaerobe), *Clostridium* (anaerobe); phototrophs like *Anabaena* and *Rhodobacter*, and chemolithotrophs like *Leptospirillum ferroxidans*. So far, ninety genera of diazotrophs are known to have the enzyme nitrogenase and are capable of converting atmospheric nitrogen to ammonia (Unkovich and Baldock 2008). Diazotrophs are found in a variety of environments: free-living in soil or in symbiotic association with legumes or guts of termites, in associative symbiosis with grass or association between actinorhizal plants and *Frankia*, and cyanobacterial association with eukaryotes (Dixon and Kahn 2004). With the increase in use of molecular and genetic tools, the organisms known to carry out biological nitrogen fixation have increased (Vitousek et al. 2013). Usually, the amount of nitrogen fixed by symbiotic association is greater than by free-living and associative nitrogen fixers (Bergersen et al. 1991). In symbiotic nitrogen fixation, energy (carbon) source is provided by the host plant and the host plant also protects the nitrogenase enzyme from oxidative deactivation (Bergersen et al. 1991). Anaerobes and facultative anaerobes convert atmospheric nitrogen into ammonia only in the absence of oxygen. Microaerophilic bacteria like *Azospirillum* can fix nitrogen in low oxygen concentrations. In aerobic bacteria such as *Azotobacter*, nitrogen fixation occurs

by lowering the concentration of oxygen intracellularly. In cyanobacteria nitrogen fixation occurs inside the heterocyst. The rate of nitrogen fixation decreases with the increase in atmospheric oxygen level (Unkovich and Baldock 2008). The division between many free-living, and symbiotic or associative diazotrophs is not explicit. Many symbiotic and associative nitrogen fixers are able to fix nitrogen in the free-living state, subject to specific growth conditions (Evans and Burris 1992).

1.3.1. Life styles of nitrogen fixers

1.3.1.1. Symbiotic association

The symbiotic association for biological nitrogen fixation occurs between heterocystous cyanobacteria and cycads, *Frankia* and root nodules of woody non-legumes, and rhizobial bacteria (like *Rhizobium*) and leguminous plants (like alfalfa, soybean). The symbiotic interaction between rhizobial bacteria and legumes to meet nitrogen requirements for growth during scarcity of mineral nitrogen (Ribeiro et al. 2015) is one of the most rigorously studied, extensively reviewed interactions (Atkins 1984; Pauly et al. 2006; Chang et al. 2009). Briefly, flavonoids secreted by roots of many legumes signal rhizobia, which in turn produce Nod factors. These Nod factors help in the recognition of bacteria by the plants. As a result of the interaction of legumes with the rhizobia, there is formation of nodules, which induce bacterial infection. The nodule provides a favorable environment for nitrogen fixation by rhizobia by limiting oxygen (Oldroyd 2013). The rhizobia inside the nodules exchange the fixed nitrogen for carbon from the legumes (Nelson and Sadowsky 2015).

1.3.1.2. Associative nitrogen fixing bacteria

Associative nitrogen fixation is performed by a number of bacteria belonging to the genera *Azospirillum, Herbaspirillum, Klebsiella, Enterobacter,* and *Paenibacillus* (Haahtela et al. 1986; Van Dommelen and Vanderleyden 2007). The associative nitrogen fixing bacteria lie between the free-living and the symbiotic nitrogen fixers with regards to the amount of the nitrogen fixed (Dart 1986). The associative nitrogen fixers have mainly been reported on roots of corn, wheat, and sugarcane. They produce carbon substrates as their exudates which help the associative bacteria to get carbon source for nitrogen fixation (Lederberg et al. 2000).

1.3.1.3. Free-living nitrogen fixers

Even though, symbiotic bacteria serve as a major group for nitrogen fixation, free-living nitrogen fixing bacteria also contribute a substantial amount of nitrogen to various ecosystems (Kahindi et al. 1997; Unkovich and Baldock 2008). Free living nitrogen fixers include various heterotrophs including members of Proteobacteria, Cyanobacteria, Firmicutes, and Archaea (Zhan and Sun 2011). They use the energy from the oxidation of organic molecules. Free-living diazotrophs have been considered to contribute to a low amount of total fixed nitrogen in comparison to the symbiotic nitrogen fixation (Stacey et al. 1992; Cleveland et al. 1999); the free-living nitrogen fixers fix about one-tenth of the total atmospheric nitrogen fixed by the symbiotic association (Unkovich and Baldock 2008). Since most nitrogenases are sensitive to oxygen, most free-living diazotrophs show anaerobic or microaerophilic behavior when they fix nitrogen (Wagner 2011).

To optimize conversion of limited energy source to ATP free-living bacteria require O_2 or another electron acceptor. Yet, nitrogenase requires protection from oxygen. This apparent conundrum suggests that free-living diazotrophs have evolved approaches to protect their nitrogenase from surrounding oxygen.

S. thermoautotrophicus is however, the only oxygen insensitive nitrogenase. In the presence of CO or H_2 and CO_2, as a source of carbon and energy, it can utilize atmospheric N_2. This remains the only known autotrophic N_2 fixer, and also the only nitrogen fixing *Streptomyces* so far.

1.3.2. Origins and evolution of nitrogenases

The distribution of the highly conserved nitrogen enzyme across the Archaea and Bacteria domains is sporadic (Young 1992). This distribution has driven to two contradicting hypotheses (Gtari et al. 2012): The first hypothesis speculates that the last universal common ancestor of Bacteria and Archaea had nitrogen fixation as an ancient function, which was transmitted vertically (Klucas et al.). However, there was a widespread loss of *nif* gene in the offspring (Fani et al. 2000; Berman-Frank et al. 2003). The second hypothesis suggests that nitrogen fixation was an anaerobic capability which showed up after oxygenic photosynthesis and was lost in most progeny over horizontal gene transfer as referred to by Gtari et al (Gtari et al. 2012).

The common pattern of transfer of genetic material from parents to offspring is the vertical gene transmission which occurs through reproduction and replication.

Horizontal gene transfer (HGT), also called lateral gene transfer (LGT) is the transfer of genetic material between strains, disjoining the vertical transfer. HGT was first observed by Fredrick Griffith in 1928, when a virulence gene was transferred from *Streptococcus pneumonia* to a non-virulent strain. This observation gave rise to the idea of horizontal gene transfer between bacteria through a transformation mechanism (Griffith 1928). HGT can be identified by a phylogenetic contradiction, irregular distribution, inconsistency in composition, or the blend of all of these (Zhaxybayeva and Doolittle 2011). Two other mechanisms of HGT are conjugation (transfer of genetic material by cell to cell contact) and transduction (transfer of genetic material via bacteriophages). HGT is an evolutionary process and has been a challenge to the study of evolutionary progeny and species (Ravenhall et al. 2015) and the difficulty in creating a phylogenetic congruency from distinct orthologous genes of diverse organisms (Koonin et al. 2001). The best example of HGT is the transfer of antibiotic resistance genes across distant phylogenetic groups (Koonin et al. 2001; Gyles and Boerlin 2014), particularly, between gram positive and gram negative bacteria (Courvalin 1994).

Nitrogenase is one functional gene susceptible to horizontal gene transfer, and is present in some plasmids of rhizobia (Zehr et al. 2003). Despite the presence of plasmids in some rhizobia, the genomic analyses of nitrogenase genes show that they are not plasmid originated (Beeson et al. 2002). Another study suggests that there was horizontal transfer of *nifHDK* gene early in evolution, in the cyanobacterium *Microcoleus chthonoplastes*. Furthermore, the phylogenetic analysis of *nif*HDK of *M. chthonoplastes* shows that they cluster with *Deltaproteobacteria*, which are closely related to *Desulfovibrio* (Bolhuis et al. 2009). Members of family *Desulfovibrionaceae* like *D.*

vulgaris may be the donor for *nif* genes present in *M. chthonoplastes* because the *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 transfer of this gene might have taken place through phages as transfer of DNA fragments via phages are familiar in *Desulfovibrionaceae* (Rapp and Wall 1987).

1.4. N_2 fixation in arid lands

Drylands constitute one-third of earth's terrestrial biome (Pointing and Belnap 2012). Extreme climatic conditions such as dryness, heat, cold and periodic extreme snow that prohibited higher plant and animal life in these areas must have provided a conducive environment for the development of biological soil crusts (BSC). BSC is colonized with diverse microbial populations depending on the soil properties over the times of erosion and stabilization. In lands of arid vegetation, these biological soil crusts are enriched with variety of microbial communities composed of bacteria, algae, lichens and bryophytes (Büdel et al. 2014). BSCs could be a major source of nitrogen in arid and semiarid lands (Belnap 2002). The nitrogen fixation in arid soils occurs generally by BSCs and free-living heterotrophic bacteria (Macgregor and Johnson 1971). Microbial communities in the BSCs perform carbon and nitrogen fixation. In areas with low rainfall and low inputs of nitrogen from human activity, nitrogen fixed by cyanobacteria can be a common source of nitrogen. Change in land use along with alterations in regional climate could possibly alter the dryland ecosystems into new ecosystems of unknown composition and function (Steven et al. 2015). Meager work has been done regarding N_2 fixing microbial communities in dry lands.

1.5. Methods for Evaluating of Nitrogen Fixation

1.5.1. Culture Dependent Methods

Nearly 99% of the total soil bacteria are estimated to be non-culturable using the standard techniques (Amann et al. 1995). Known species for which the cultivation conditions are unsuitable or are in the non-culturable phase, and unknown species which have not been cultured before due to the lack of a suitable culture approach are present (Amann et al. 1995). However, culture dependent method is still a linchpin in laboratories to study the metabolic properties and physiology of an organism (Cardenas and Tiedje 2008).

Several culture based approaches are now gaining access to the non-culturable microorganisms. A diffusion-chamber based method have led to the cultivation of several microorganisms that were previously thought non-culturable such as the groups of *Verrucomicrobia, Acidobacteria, Spirochaetes*, and *Deltaproteobacteria* (Bollmann et al. 2007). Another approach is single cell encapsulation in gel micro-droplets along with flow cytrometry, which has led to the culture of *Methanococcus thermolithotrophicus* (Zengler et al. 2002).

For diazotrophs, the nitrogen deficient conditions offer a selective advantage over other organisms that do not grow without nitrogen supply. Nitrogenase, responsible for the biological nitrogen fixation, is irreversibly inactivated at high concentrations of oxygen (Robson and Postgate 1980). Therefore, medium with a steady low oxygen concentration is suitable for the growth of diazotrophs (Mirza and

Rodrigues 2012). Diazotrophs are grown in liquid, semisolid or solid medium. Some media used for the growth of diazotrophs are Burk's nitrogen free medium, nitrogen free malate medium (Dobereiner et al. 1976), Norris glucose nitrogen free medium, Burke's Modified Nitrogen Free medium, modified Rennie medium (Atlas 2010), and many more.

1.5.2. Culture Independent Methods

The study of microbial communities by culture-dependent approach limits the view of microbial diversity as most microbes are not revealed with the culturing approach (Pace 1997). Owing to the biases of culture-dependent methods towards the microbial diversity, molecular techniques became popular since the 1980s (Su et al. 2012). The advances in molecular techniques contribute to different ways of microbial community analysis without culturing requirement. Amplification of target genes by polymerase chain reaction (PCR) has been invaluable for the culture-independent analysis of microbial communities in various environments. During the past two decades, direct amplification and analysis of small subunit ribosomal RNA (SS rRNA) gene have been used to study the microbial diversity of environmental samples (Su et al. 2012). This method comprises the PCR based techniques such as amplification of gene pools followed by sequencing, quantitative PCR (Matsuki et al. 2004), denaturing/temperature gradient gel electrophoresis (DGGE or TGGE) (Muyzer 1999), restriction fragment length polymorphisms (RFLPs) (Lee et al. 1998), and terminal- RFLPs (Schütte et al. 2008).

1.5.2.1. The 16S rRNA gene

The molecular phylogenetic tree based on ribosomal RNA gene sequences developed by Carl Woese provided a framework to study microbial diversity (Woese 1987). SS rRNA is present in all living cells and perform similar function in all cells. The 16S rRNA gene is considered as the "gold standard" for determining the diversity of microbial community from various environments: arid soils (Dunbar et al. 1999), water (Vaz-Moreira et al. 2011), lakes and rivers (Zwart et al. 2002), deep sea and underexplored biosphere (Sogin et al. 2006), air (Wilson et al. 2002), mammalian gut (Muegge et al. 2011). The 16S rRNA gene is about 1.5kb in length and has highly conserved regions, intermediate conserved regions and highly variable regions (Gray et al. 1984). The 16S rRNA gene consists of nine hypervariable regions, spanning V1 to V9, which are flanked by conserved regions (Fig. 1.4), allowing the use of universal primers to amplify target sequence (McCabe et al. 1999; Becker et al. 2004). The 16S rRNA gene can be used to identify non-cultivable microorganisms using the sequences from cultured and characterized strains (Vos et al. 2012) However, the 16S rRNA gene sequencing is not devoid of potential limitations. The resemblance of a sequence to a near identity species does not mean that the isolate can be characterized as the same species (Gevers et al. 2005). Similarly, because of the variable sequence across multiple copies of 16S rRNA gene, it may result in some degree of uncertainty in identification (Marchandin et al. 2003).

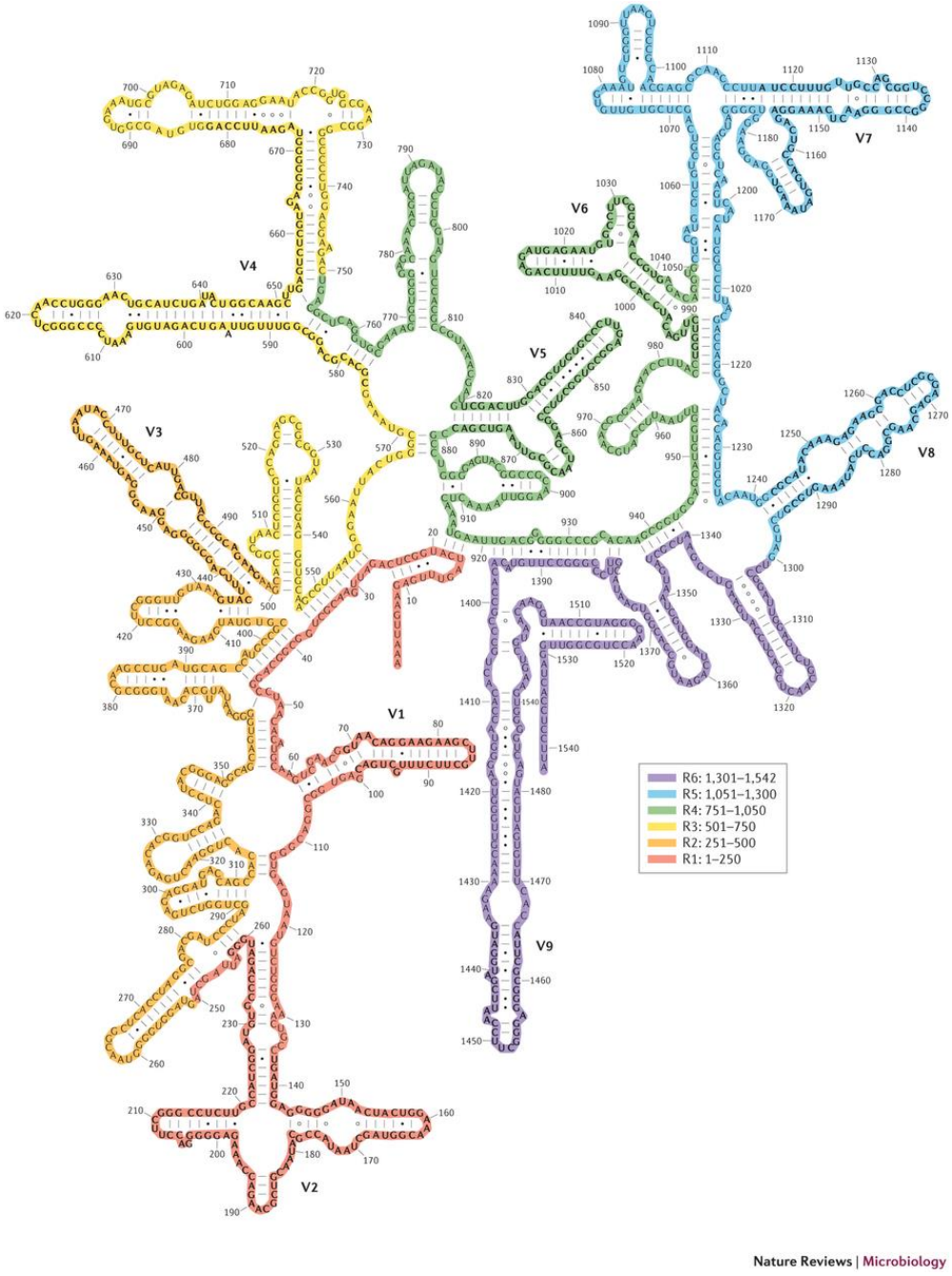

Figure 1.4. Secondary structure for 16SrRNA of *Escherichia coli* showing the variable regions V1-V9 from (Yarza et al. 2014).

1.5.2.2. The *nifH* gene to study the diversity of diazotrophs

Small subunit of ribosomal RNA gene sequencing and analysis is inadequate to elucidate the diversity of diazotrophic microbial communities, as nitrogen fixing ability is sporadically distributed across Bacteria and Archaea (Young 1992; Mehta et al. 2003). The history of gene evolution suggests *nifH* as one of the ancient extant and functioning genes. Earlier work indicated that the *nifH* tree is largely compatible with the 16S rRNA phylogenetic tree (Young 1992). More recently, it has been found that the genetic diversity of *nifH* and 16S rRNA genes differ, the strains that have >97% similarity values in 16S rRNA genes can have up to 23% dissimilarity in *nifH* (Gaby and Buckley 2014). There are five main phylogenetic clusters of *nifH* homologs (Chien and Zinder 1994). Cluster I encompasses a diverse array of *nifH* genes and includes the aerobes and facultative anaerobes belonging to the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Cyanobacteria*. Cluster II contains the alternative nitrogenase *anfH*. Cluster III contains *nifH* from anaerobes such as *Clostridium*, archaea (methanogens), and *Deltaproteobacteria* (Gaby and Buckley 2014). Clusters IV and V are considered as paralogs of *nifH,* and do not fix nitrogen (Raymond et al. 2004; Staples et al. 2007).

The diversity of diazotrophic communities has been widely studied using the culture independent methods. Among the various genes that encode the nitrogenase enzyme, *nifH* is the most sequenced and has become the marker gene for the study of diversity, phylogeny, and plethora of diazotrophs by most researchers (Gaby and Buckley 2014). All other *nif* genes are less conserved than *nifH.* As a result, many primers that target and amplify the *nifH* region of diazotrophs have been used. The

nifH gene is highly variable at the 5' end and across the last 400 bases (Fig. 1.5). Bases ~100- 450 are somewhat conserved but do not contain any regions sufficiently conserved for design of universal primers. The diversity of *nifH* genes is, therefore, studied using degenerate primers to cover the sequence variability of the *nifH* gene. Degenerate primers were first developed by Zehr and McReynolds (Zehr and McReynolds 1989). The degeneracy of primers allow the gene amplification of a wide variety of organisms. However, high degeneracy can cause amplification of unrelated sequences reducing the specificity of the PCR (Linhart and Shamir 2002). The study of phylogeny of *nifH* sequences, which encodes the Fe protein of nitrogenase, allows the identification of unknown diazotrophs (Zehr and Capone 1996). The *nifH* gene diversity has been studied in a wide variety of environments, such as hindgut of insects (Ohkuma et al. 1999), rice roots (Ueda et al. 1995), soils (Poly et al. 2001; Izquierdo and Nüsslein 2006), endophytes (Reiter et al. 2003), and oceans (Zehr et al. 1998; Langlois et al. 2008). The conserved nature of part of the *nifH* gene has made it a popular molecular technique to study the diversity of biological nitrogen fixation across diazotrophs (Zehr et al. 2003).

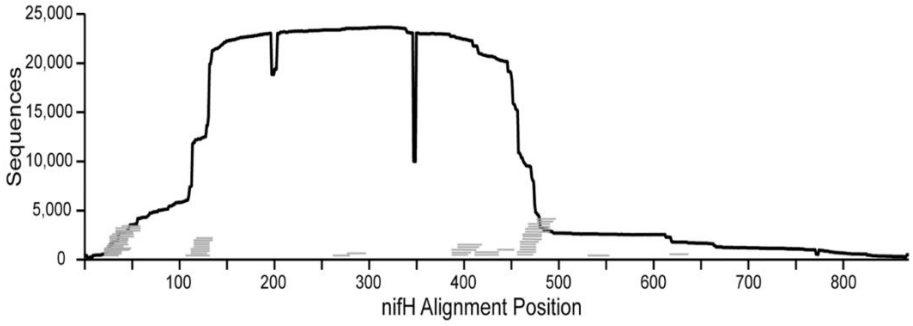

Figure 1.5. Coverage of the *nifH* gene by sequences and primers in the *nifH* database (Gaby and Buckley 2014).

1.5.2.3. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a molecular fingerprinting tool that separates double stranded DNA fragments generated by PCR. Conventional agarose gel electrophoresis resolves DNA of identical length into a single band, irrespective of sequence and base composition. Different sequences of identical length will dissociate in a denaturant gradient in relation to their base composition. A GC clamp (GC-rich sequence) is attached to the 5’ end of one of the primers that remains double stranded while the remainder dissociates when resolved through a denaturing gradient gel (Ferris et al. 1996) (Fig. 1.6 A). The GC clamp (usually 30-50 bp length) precludes the complete dissociation of the double stranded DNA fragments into single strands. The separation is based on the decreased electrophoretic mobility of dissociated, clamped DNA fragments through a polyacrylamide gel of an increasing gradient of denaturant (Muyzer and Smalla 1998). Since, the PCR products have the same size but vary in their sequence, the migration of the DNA fragments in the gel differ yielding a banding pattern (Fig. 1.6. B).

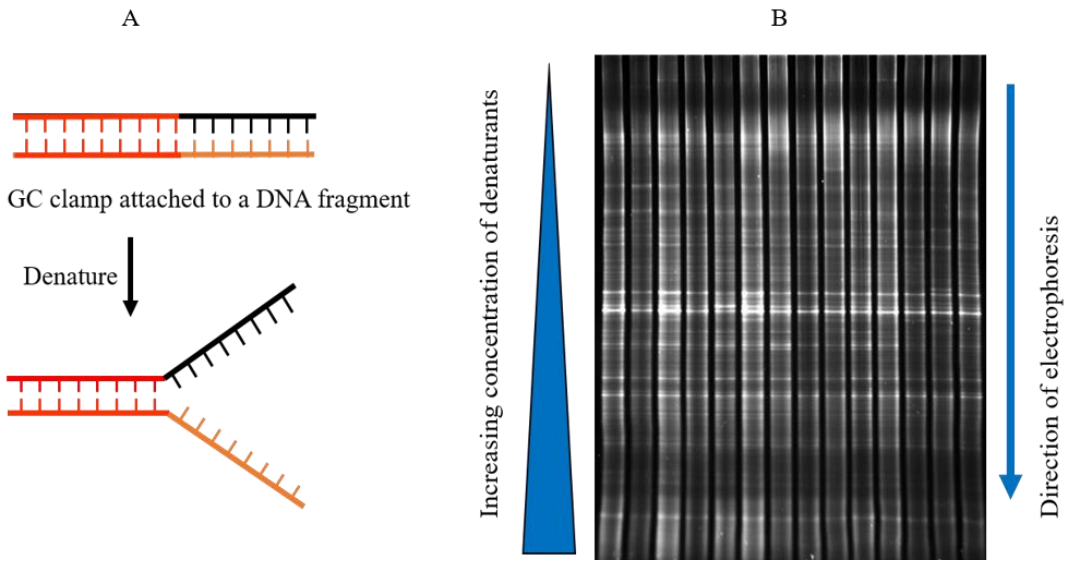

Figure 6. Principle of DGGE. GC sequence (red) attached to a DNA fragment, denaturate when run on a polyacrylamide gel of increasing denaturant, unzipping outside of the GC clamp (A). GC-clamped DNA of identical length but variable sequence forms a banding pattern when resolved through a denaturing gradient gel (B).

1.5.3. The Acetylene Reduction Assay

The acetylene reduction assay (ARA) has been widely used as a method for quantifying nitrogen fixation when Dilworth (Dilworth 1966) and Schollhorn and Burris (Schöllhorn and Burris 1967) suggested the reduction of acetylene to ethylene by nitrogenase enzyme. It is a sensitive assay, and suitable for analysis of lab and field samples to measure biological nitrogen fixation (Stewart et al. 1967; Mague and Burris 1972). The principle of ARA is based on the reduction of triple bonded hydrocarbon, acetylene (C_2H_2) to double bonded hydrocarbon ethylene (C_2H_4) by the

nitrogenase enzyme, and ethylene is detected by gas chromatography. The studies by Hardy et al. and Klucas et al., showed that the reduction of acetylene to ethylene and the reduction of dinitrogen to ammonia by nitrogenase were parallel when nitrogenase was purified from free-living bacteria as well as from legume nodule bacteroids (Hardy et al. 1968; Klucas et al. 1968). Such studies validated the use of ARA for measuring nitrogenase activity. However, ARA does not give the direct measure of nitrogen fixation, rather it is an indirect method of measurement of nitrogen fixation (Turner and Gibson 1980). Theoretically, for each molecule of dinitrogen reduced to ammonia, three molecules of acetylene are reduced to ethylene (Buresh et al. 1980). However, there are many controversies with the 3:1 ratio as this range may vary from 3 to 6.9 in soils, and for anaerobic soils the value may reach up to 25 (Hardy et al. 1973). Although, acetylene and nitrogen are iso-electronic, and have similar molecular dimensions, the difference may be because acetylene is 65 times more soluble than nitrogen (Bergersen 1970). It has been suggested by Shah et al. that the 3:1 ratio of acetylene reduction assay depends upon the ratio of the two components of nitrogenase as well as the concentration of acetylene. If the component I: component II ratio is increased, the ARA: N_2 fixation ratio decreases to 3.4 and then increases. Also, acetylene shows substrate inhibition during nitrogenase activity (Shah et al. 1975).

1.5.4. Detecting nitrogen incorporation using stable isotope $^{15}N_2$

Nitrogen has fifteen highly unstable and radioactive isotopes and two stable nitrogen isotopes: $^{14}N_2$ and $^{15}N_2$. $^{15}N_2$ is the heavier isotope and rare in the atmosphere

(0.3663% of global nitrogen), whereas $^{14}N_2$ is abundant (99.6637% of global nitrogen) (He et al. 2009). The heavy isotope $^{15}N_2$ was first used in 1941 by Burris and Miller to determine the N_2 fixation process in bacteria (Wood and McNeill 1993). However, the requirement for mass spectrometry and the need for nitrogen gas enriched with $^{15}N_2$ limited its use for about three decades (Herridge et al. 2008). The measurement of nitrogen fixation by $^{15}N_2$ is a direct method as it quantifies the total incorporation of nitrogen into the biomass over a predetermined incubation period (Bergersen 1980; Glibert and Bronk 1994). The lower detection limit of $^{15}N_2$ versus acetylene reduction assay has made it a suitable method for oligotrophic marine environment. The feasibility of the $^{15}N_2$ tracer method is due to the high precision isotopic ratio mass spectrometry (Montoya et al. 1996). The use of $^{15}N_2$ stable isotope for measurements of $^{15}N_2$ fixation are very common nowadays in the study of marine ecosystems with increasing use of mass spectrometry (Ryabenko 2013). The N_2 fixation rate yield with $^{15}N_2$ tracer addition method is generally lower when compared to the AR assay (Mulholland 2007). Also, $^{15}N_2$ incorporation requires longer incubation periods when compared to ARA to attain significant $^{15}N_2$ atom % in soils or plant tissues (Buresh et al. 1980).

Mass spectrometry (MS) is a powerful analytical technique for precise determination of mass. Many permutations of MS have been developed, for example, for determination of unknown compounds, or quantitative analysis of known compounds by measuring their mass to charge ratio (m/z). MS determines the mass of the individual molecules by transforming them into ions in vacuum, and measures the

response of their path to magnetic or electric fields (Fenn et al. 1989). A mass spectrometer consists of the following parts: a sample inlet, through which the sample to be analyzed is kept; an ion source, which generates ions from the sample/compound; mass analyzer (one or more) that selects or separates the ions; a detector that detects the ions coming from the analyzer; and lastly, a data processor that generates the mass spectrum (Hoffmann 1996).

The mass spectrometry works by measuring the composition of isotopes of nitrogen produced by combustion, carried in a helium gas stream to an open stream interface introducing a portion of gas stream to the ion source for measurement (Ryabenko 2013).

1.6. Aim of Study

The aim of this study was to characterize the diversity of free-living putatively diazotrophic bacteria in South Dakota Badlands by:

1. Isolating free living diazotrophs into culture using solid nitrogen free medium
    - Determining the diversity of putative diazotrophs using 16S rRNA gene sequencing.
    - Determining the diversity of putative diazotrophs using *nifH* sequencing.
2. Full length 16S rRNA gene sequencing of isolates allocated to *Streptomyces*.
3. Determining the diversity of putative diazotrophs using culture independent methods.

4. Evaluating whether the putative diazotrophs fix nitrogen using $^{15}N_2$ stable isotope labelling.
5. To isolate and characterize fungi able to grow in nitrogen free medium.

Chapter 2

2. Diversity of Free-Living Nitrogen Fixing Bacteria in the Badlands of South Dakota

2.1. Introduction

Nitrogen is the most common limiting factor for growth and productivity of plants in aquatic as well as terrestrial ecosystems (Wagner 2011). Although dinitrogen is the most abundant element in the atmosphere, it is biochemically unavailable for plants and most microbes as they utilize only reduced (ammonium) or oxidized (nitrate) forms. The two atoms in dinitrogen are triple-bonded, requiring a high level of energy to dissociate and reduce to ammonia (Figg et al. 2012). The bacterial nitrogenase enzyme complex is the only known natural system for reduction of N_2 to NH_3. Biological Nitrogen Fixation is sporadically distributed across the Archaea and Bacteria domains (Young 1992). Biological nitrogen fixation (BNF) is the primary source by which plants in natural ecosystems obtain their nitrogen for growth (Zehr et al. 2003). The best known diazotrophs form symbiotic associations with plants, but free-living nitrogen fixing bacteria also contribute a substantial amount of nitrogen to ecosystems (Unkovich and Baldock 2008), including arid lands (Belnap 2002; Yeager et al. 2007). Among the genes that encode the nitrogenase enzyme complex, *nifH* is the most sequenced and highly conserved gene(Gaby and Buckley 2012). The captured genetic diversity of diazotrophs is based mainly on the N-terminal part of *nifH* sequences. However, *nifH* is less conserved than the 16srRNA gene, which

necessitates the use of degenerate primers to cover the sequence variability of *nifH* gene.

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 extreme 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 (da Rocha et al. 2015). BSCs 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 of arid vegetation, biological soil crusts contain a variety of microbial community composed of bacteria, algae, lichens and bryophytes (Belnap 2002; Büdel et al. 2014).

The Badlands of South Dakota, are located in an arid to semiarid region of the high Great Plains in south western South Dakota. Badlands were formed through differential erosion of the soil, exposing the ancient sedimentary layers. South Dakota Badlands feature highly eroded badland formation regions with sparse vegetation, undisturbed mixed prairie grass, and spires with an elevation up to 992 m above sea level (Graham 2008). In areas with low rainfall and low inputs of nitrogen from human activity, nitrogen fixed by cyanobacteria can be a common source of nitrogen (Rychert and Skujiņš 1974; Belnap 1996). Change in land use along with alterations in regional climate could possibly alter the dryland ecosystems into new ecosystems of

unknown composition and function (Steven et al. 2015). The BSCs are generally dominated by cyanobacterial populations (Belnap and Gardner 1993; Tirkey and Adhikary 2005; Yeager et al. 2007). However, more recently, diverse group 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).

Till now, there is no information available to know the microbial diversity of badlands soil of South Dakota. Since badlands are a land of distinct geological and landscape features, it is important to understand the dryland nitrogen dynamics and the bacterial diversity, particularly the free living nitrogen fixing bacterial population. The focus of this study was with the soils of sod table of SD badlands. Sod tables are the isolated and eroded remains 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).

In this study, we have evaluated the diversity of free-living nitrogen fixing bacteria from the soils of the sod table and the matrix of south unit of South Dakota, Badlands National Park, using both culture dependent and culture independent approaches.

2.2. Materials and Methods

2.2.1. Study Area:

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 (0 °F) to more than 38 °C (100 °F) during summers. The average annual precipitation is 40 mm, with May and June getting the highest (~70%) precipitation in total (Amberg et al. 2012). The harsh climatic condition and low amount of rainfall make the growth conditions challenging for most plants (Graham 2008).

2.2.2. Sampling

Soil samples were collected from the top of sod tables and 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. Sampling locations, determined by GPS tracker, are listed in Appendix 1. The samples were kept in clear sterile Ziploc 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.

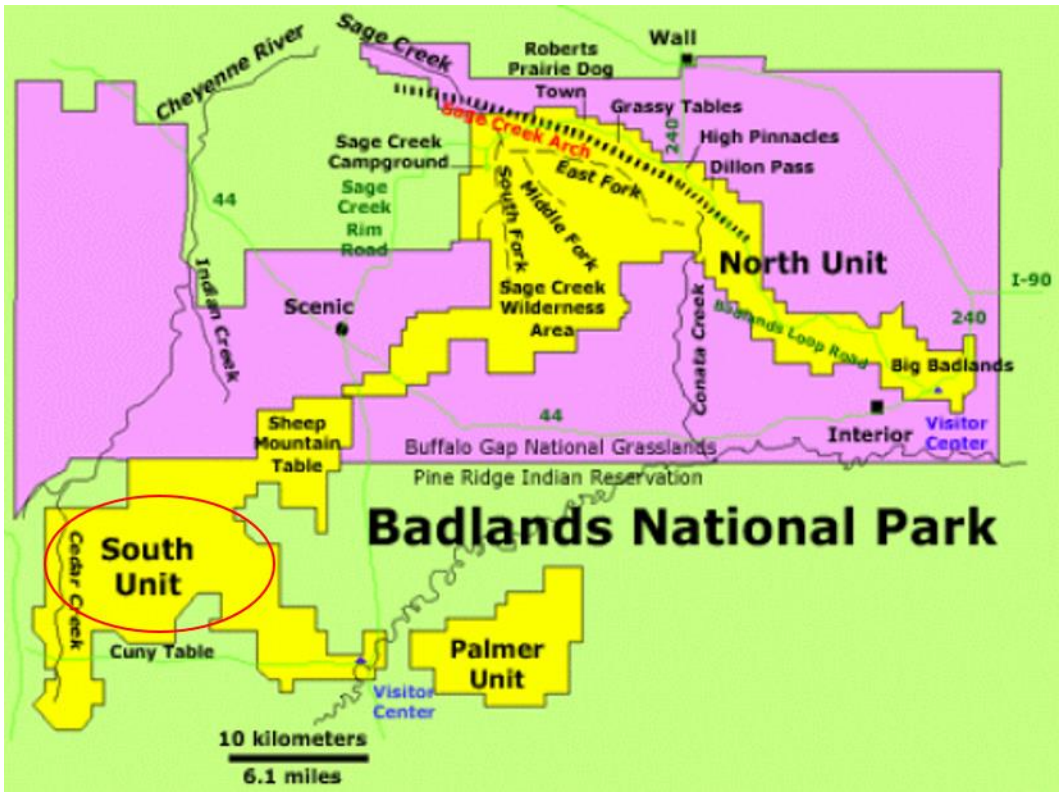

Figure 2.1. Map showing different units of Badlands National Park. The red circled area shows the sample collection site (Stoffer 2003).

Figure 2.2. A sod table from Badlands of South Dakota

2.2.3. 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 medium composition 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), noble agar (15.0g/l, Difco, Catlog No. 214230), NaCl (0.2g/l), and Glucose (10g/l). 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.

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

DNA was extracted from pure culture using the Zymo Research Kit (Quick-gDNA^{TM} Miniprep), and stored at -20°C. Hypervariable regions V1-3 of the 16S rRNA gene were amplified by PCR using universal primers 27F (AGA GTT TGA TCM TGG CTC AG) (Weisburg et al. 1991) and 518 R (GTA TTA CCG CGG CTG CTG G) (Muyzer et al. 1993). 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 (Bekman Coulter, Inc). The sequences were aligned using Omega Clustal (McWilliam et al. 2013), and a phylogenetic tree with maximum likelihood (500 bootstraps) was constructed using MEGA 6.0. (Tamura et al. 2013). 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 *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 2.1). 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 the annealing temperature of 63 °C with a decrement of 1°C for the subsequent increasing 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 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 gave a product size of ~360bp, and for IGK3/DVV and Ueda19F/Ueda407R primer sets the product sizes were ~400bp. The DNA sequences were aligned using Muscle (Edgar 2004a; Edgar 2004b), and a phylogenetic tree was constructed with Maximum Likelihood (500 bootstraps) using MEGA 6.0.

Table 2.1. Primers used for *nif*H gene amplification.

Primers	5’-3’
PolF	TGC GAY CCS AAR GCB GAC TC
PolR	ATS GCC ATC ATY TCR CCG GA
IGK3	GCI WTH TAY GGI AAR GGI GGI ATH GGI
DVV	ATI GCR AAI CCI CCR CAI ACI ACR TC
Ueda19F	GCI WTY TAY GGI AAR GGI GG
Ueda407R	AAI CCR CCR CAI ACI ACR TC

2.2.5. 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 analyzed using DGGE. The resulting PCR products were resolved in 35-65% denaturing gradient with a DCode Universal Mutation Detection System (BioRad, US) 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.

2.2.6. 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 the 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 Genomic Coulter Inc.). The sequences were aligned using Muscle, and a phylogenetic tree for *nifH* sequences was constructed as described in section 2.2.4.

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

The soil DNA extract was amplified using cyanobacterial 16S rRNA gene specific primers. Forward primer used was CYA359F (GGG GAA TYT TCC GCA ATG GG) and the reverse primer used were the equimolar mixture of CYA781R(a) (GAC TAC TGG GGT ATC TAA TCC CAT T) and CYA781R(b) (GAC TAC AGG GGT ATC TAA TCC CTT T) (Nübel et al. 1997). 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.

2.2.8. Acetylene Reduction Assay

Some selected isolates that we were interested (S7-008, M4-104, M7-122, and S9A-218) were inoculated onto NFM medium slants in glass tubes and were sealed with stoppers. For each tube, 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.

2.2.9. Incorporation of 15N2 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 CC syringe. After drawing in 5 ml of air through a filter (0.2µm), 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).

2.2.10. 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, and cultures were inspected visually to confirm growth.

2.2.11. 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 (CGG TTA CCT TGT TAC GAC TT) (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 2.2). 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 2.2.4.

Table 2.2. Primers used for 16S rRNA gene (V1-9) sequences determination.

Primers	Sequences (5'- 3')
27F	AGA GTT TGA TCM TGG CTC AG
358F	CTC CTA CGG GAG GCA GCA GT
536F	CAG CAG CCG CGG TAA TAC
926F	AAC TCA AAG GAA TTG ACG
1241F	GCT ACA CAC GTG CTA CAA TG

2.3. Results

2.3.1. Diversity of free-living putative nitrogen fixing bacteria

The density of culturable diazotrophs was higher in sod table than in matrix soil (Fig. 2.3). Analysis of variance (ANOVA) supported a significant difference between sod table and matrix soil ($p = 0.0124$). The 91 bacterial isolates were allocated to 40 Operational Taxonomic Units (OTUs) when using the V1-3 region of their 16S rRNA genes. These OTUs fell into twenty bacterial genera across four phyla according to the Ribosomal Database Project (RDP) tool. The majority of the bacterial isolates resembled *Actinobacteria* (69%), followed by *α-Proteobacteria* (23%), *β-Proteobacteria* (5%), *Bacteroidetes* (2%), *and Firmicutes* (1%) (Figs. 10 &11). Forty-six isolates clustered with *Streptomyces*. Maximum Likelihood method based on the Tamura-Nei model was used to analyze the evolutionary history (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. However, the putative *Streptomyces,* clusters based on V1-3 had poor bootstrap support (45) based on 500 replicates, taken to represent the evolutionary history of taxa analyzed. In order to confirm relatedness, full 16S rRNA sequencing was conducted (section 2.3.3).

A difference in diversity was observed in the sod table and matrix samples (Fig. 2.4). *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. 2.4). Representatives of all five phyla were

isolated from spring samples, whereas fall samples yielded only *Actinobacteria* and *α-Proteobacteria*.

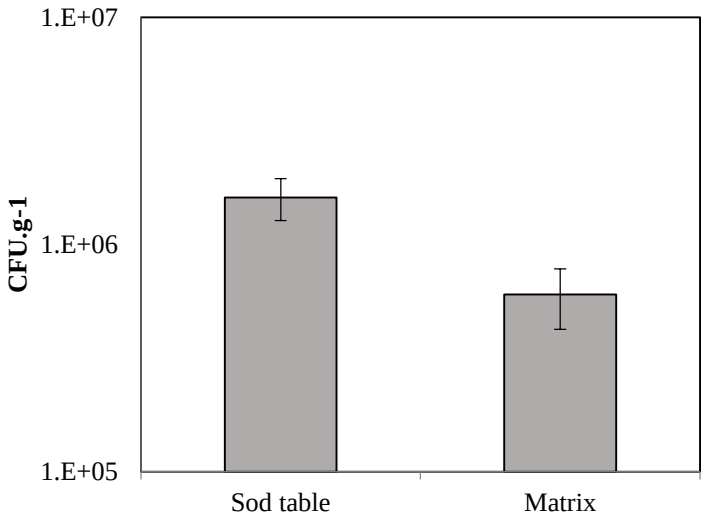

Figure 2.3. Culturable counts of putative diazotrophs obtained by incubating dilutions of sod table and matrix soil samples on solid nitrogen free medium in microaerophilic atmosphere at 28 °C for ~7d. Counts are the average of 17 different samples and error bars depict standard error of the mean.

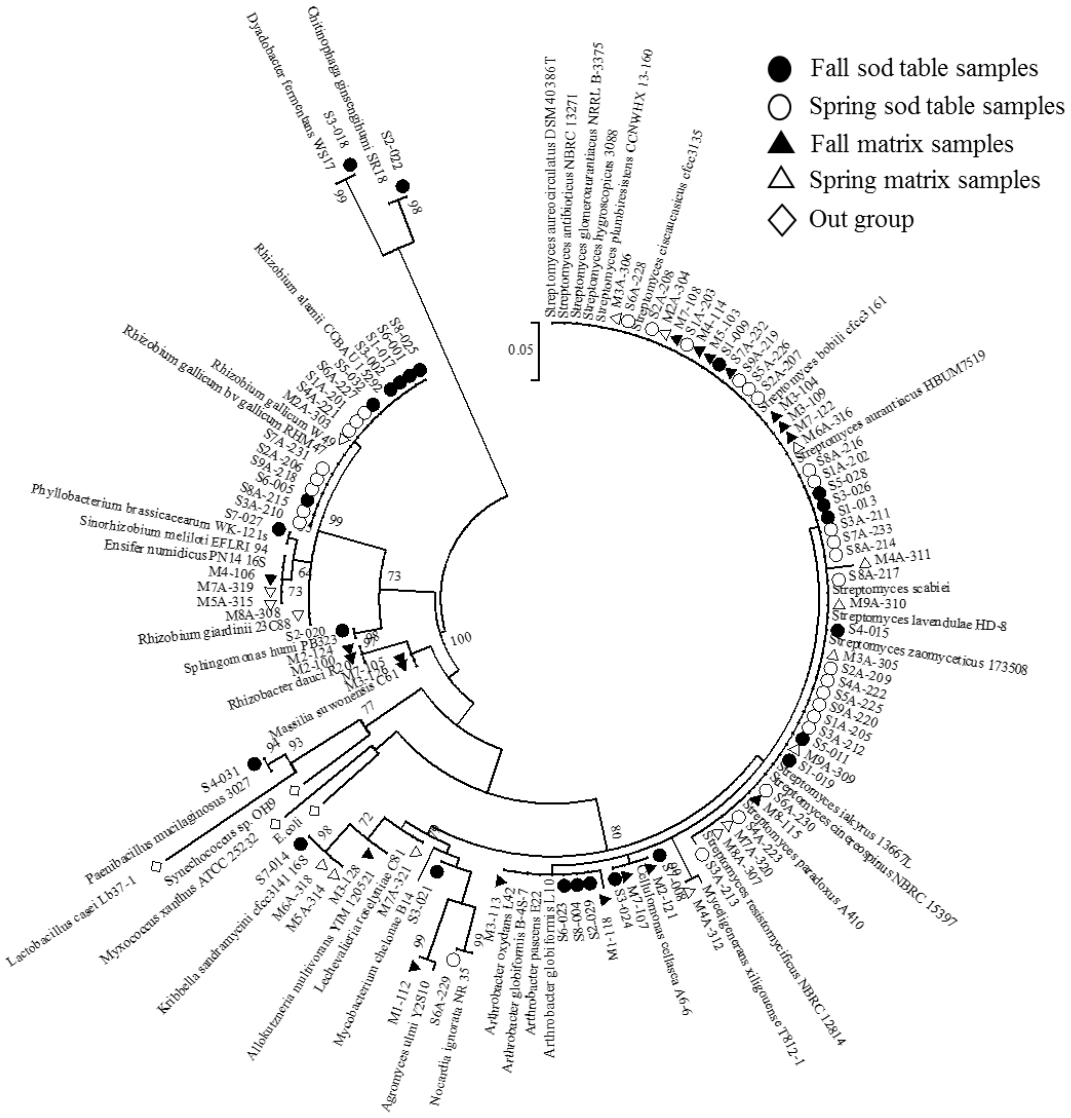

Figure 2.4. Maximum likelihood phylogenetic tree of 16S rRNA gene (V1-3) of putative diazotrophs isolated from sod table and matrix soils of the badlands of South Dakota based on Tamura Nei-model. Numbers represent bootstrap values of (%) 500 analyses. Bootstrap values lower than 60 are not shown.

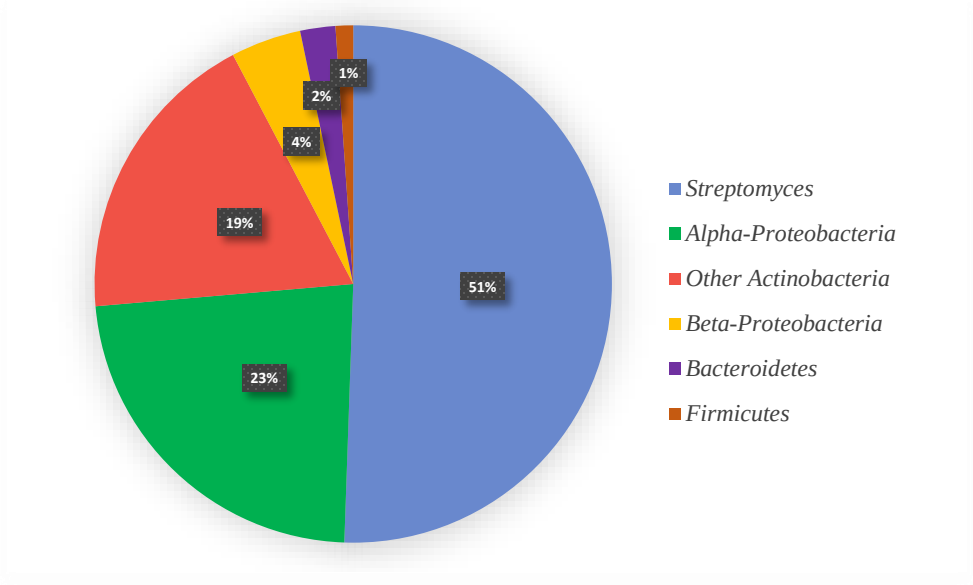

Figure 2.5. Community composition of putative diazotrophs from sod tables and matrix soil samples.

2.3.2. The *nifH* gene amplification and growth in liquid NFM with clinoptilolite

The presence of *nifH* in the bacterial isolates was tested using the primers PolF and PolR (Poly et al. 2001), and IGK3/DVV (Gaby and Buckley 2012). Of the 91 isolates, 21 showed amplification of *nifH* with the PolF/PolR primers, along with some non-specific bands. No samples yielded positive results with the IGK3/DVV primers, other than *Bradyrhizobium japonicum* USDA 110 used as the positive control. Of 17 isolates allocated to *Rhizobium*, 13 yielded *nifH* amplicons but no close homologs of *nifH* were found using BLAST (Altschul et al. 1990). Of 46 isolates allocated to *Streptomyces* by 16S rRNA gene sequence, five yielded *nifH* amplicons. Of these, four aligned and clustered with the *nifH* of the cyanobacterium *Scytonema* which has been allocated to Cluster I of *nifH* gene database. One amplicon aligned

with the *nifH* of *B. japonicum*, also allocated to Cluster I, in the maximum likelihood phylogenetic tree of *nifH* (Fig. 2.6).

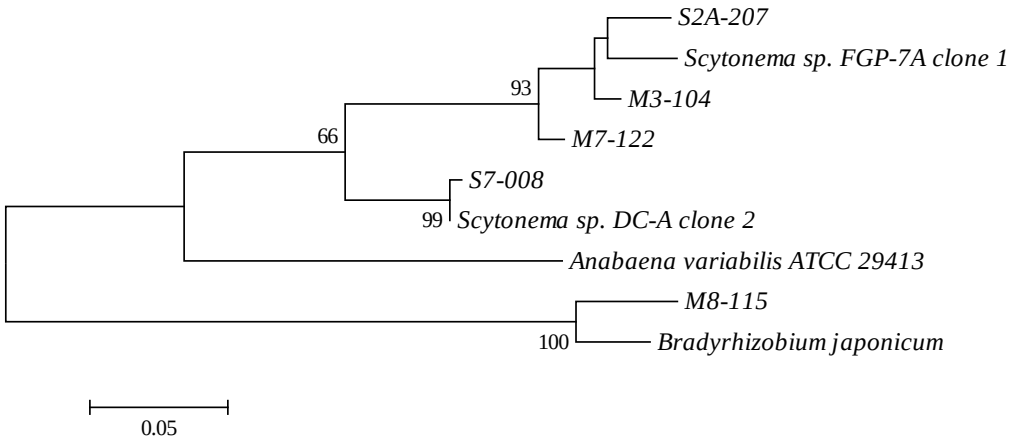

Figure 2.6. Maximum likelihood phylogenetic tree of *nifH* gene from cultures based on Tamura-Nei model. Numbers represent bootstrap values of 500 analyses represented as percentage.

Five *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. Some bacteria are able to grow in nitrogen-free medium, possibly through scavenging ammonia from atmosphere (Yoshida et al. 2014). In order to exclude 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 *Aureobascidium pullulans* was able to grow in NFM, but not in the ammonia depleted atmosphere with clinoptilolite. Yet, all but one of the

Streptomyces isolates 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.

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

Of forty-six isolates that were allocated to *Streptomyces* by 16S rRNA gene (V1-3) sequencing, two did not yield amplification using the 27F and 1492 R primers, despite multiple attempts. Of these, 40 yielded good quality sequences, and all of these aligned to *Streptomyces* and were allocated to 16 OTUs (Fig. 2.7). The putative *Streptomyces* that yielded *nifH* amplicons homologous to previously described isolates are highlighted with a triangle (Fig. 2.7). It is unclear how the other 35 isolates were able to grow in a medium free of combined nitrogen and an ammonia-depleted atmosphere.

Figure 2.7. Maximum likelihood phylogenetic tree of 16S rRNA gene (V1-9) of putative 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 kept as an out group.

2.3.4. 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 analysis revealed a moderate diversity for the sod table soil samples (data not shown). A total of thirty-five clones affiliated to the Cyanobacterial dinitrogenase reductase genes were revealed (Fig. 2.8). The clones were related to three genera of cyanobacteria: *Nostoc*, *Tolypothrix*, and *Scytonema*.

2.3.5. 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 2.3). Three of these selected isolates had *nifH* gene homologous to *Scytonema*, and all of them grew in an ammonia depleted environment in a sealed container.

Table 2.3. Nitrogen fixation by the isolates indicated by incorporation of $^{15}N_2$ into biomass.

Isolates	**Sequence Resemblance**	**^{15}N (at-%)**
S2A-207	*Streptomyces* (V1-9)	63.99243
S7-008	*Streptomyces* (V1-9)	66.34482
M7-122	*Streptomyces* (V1-9)	55.94804
S3A-212	*Streptomyces* (V1-9)	78.11928
M5-103	*Streptomyces* (V1-9)	77.42546
S8A-217	*Streptomyces* (V1-9)	73.18217
S1A-201	*Rhizobium* (V1-3)	65.41973
B. japonicum USDA 110	Positive control	52.42142

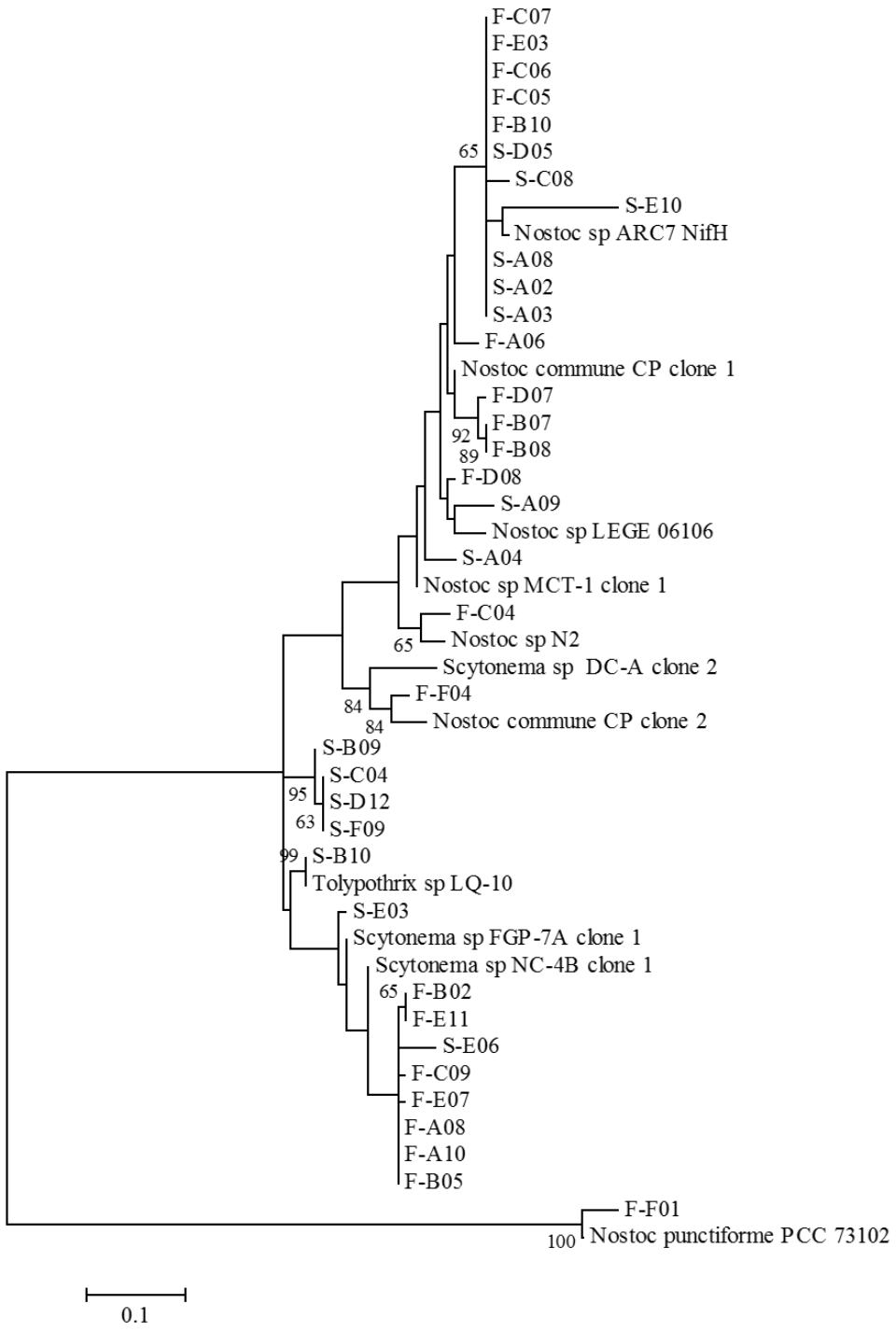

Figure 2.8. Maximum likelihood phylogenetic tree of cloned *nifH* sequences from soil DNA based on Tamura-Nei model. Numbers represent bootstrap values of 500 analyses represented as percentage. Bootstrap values lower than 50 are not shown.

2.4. Discussion

We obtained a diverse collection of putative nitrogen fixing bacteria from sod table and matrix soils of the 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. 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 *Streptomyces* yielded *nifH* when using 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 Mg2+ concentrations. Only *B. japonicum* USDA 110 as a positive control yielded amplicons. We report that most recent progress report use of PolF/PolR, the only primers yielding amplicons sod table DNA and a small numbers of *Streptomyces* isolates. There are currently no PCR primers that can cover the 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. 1992a; Ribbe et al. 1997). To our knowledge this is the first report of putative 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 provides strong support for nitrogen fixation.

The growth of these *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 in extremely oligotrophic environment is well known in actinomycetes, especially isolates belonging to the genus *Streptomyces* (Yoshida et al. 2007).

Recently, 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). While it is possible that some of our isolates possess the ability to scavenge traces of combined nitrogen, their growth in an ammonium-depleted environment argues for nitrogen fixation.

The homology of *nifH* from *Streptomyces* isolates to *Scytonema nifH* is unclear. No cyanobacteria could be cultured on BG11 agar from *Streptomyces* cultures, indicating that the *nifH* gene produced were from *Streptomyces.* Our culture independent approach indicated predominance of *nifH* belonging to cyanobacteria. 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 harbors diverse free-living nitrogen fixing bacteria with the potential of novel nitrogen fixing *Streptomyces*.

Chapter 3

3. Fungal Isolates on Nitrogen Free Medium

3.1. Introduction

The ability to fix nitrogen is sporadically distributed across the domains Archaea and Bacteria (Young 1992). Biological nitrogen fixation is carried out by only one system known, the nitrogenase enzyme complex, which is believed to be conserved through evolution (Howard and Rees 1996). There are three variants of oxygen sensitive nitrogenases, the molybdenum nitrogenase, the vanadium nitrogenase, and the iron-only nitrogenase (Zhao et al. 2006). More recently, a fourth variant, oxygen insensitive nitrogenase has been reported in *Streptomyces thermoautotrophicus,* which is a chemolithotroph, and whose nitrogenase is distinct in structure and function from the other three nitrogenases (Gadkari et al. 1992a; Ribbe et al. 1997). The discovery of this distinct nitrogenase raises the prospect of further variants of nitrogenase different from what we know so far.

Eukaryotes can only utilize nitrogen in combined forms or in symbiotic associations with the nitrogen fixing prokaryotes (Kneip et al. 2007). Fungi have been observed in several nitrogen deficient environments (Paustian and Schnürer 1987). The ability to grow in nitrogen depleted environment (oligonitrophy) has been observed in some wood decomposing fungi (Dighton et al. 2005). The potential for atmospheric nitrogen fixation by eukaryotes, particularly the fungi, has been revisited periodically over one hundred years (Lipman 1911; Duggar and Davis 1916; Metcalfe

and Chayen 1954; Brown and Metcalfe 1957; Ginterova and Maxianova 1975; Rangaswami et al. 1975; Jayasinghearachchi and Seneviratne 2004). None of these studies provided experimental support for nitrogen fixation affiliated to fungi and the methods used were not sufficiently accurate to be conclusive. This lack of definite evidence leaves the status of fungal nitrogen fixation unresolved.

The fungus *Pleurotus* spp. is able to fix nitrogen, but how it does so, is unclear (Ginterova and Maxianova 1975; Rangaswami et al. 1975). The nitrogenase activity of *Pleurotus* spp. can be detected due to an association with a diazotrophic bacteria at maximum mycelial colonization by a bradyyrhizobial strain (Jayasinghearachchi and Seneviratne 2004). Similarly, Ruiz-Herrera et al. isolated a fungus *Ustilago maydis* that grew in nitrogen free medium and showed nitrogenase activity. Later, they proved that the bacterium *Bacillus pumilus* was present intracellularly and was responsible for nitrogen fixation (Ruiz-Herrera et al. 2015).

The aim of this study was able to isolate and characterize fungi able to grow in the absence of combined nitrogen. Our study illustrates the possibility of biological nitrogen fixation in fungi.

3.2. Materials and Methods

3.2.1. Isolation of microbial communities from soil rhizosphere, root, and stem

Plant samples (*Bromus inermis*) along with the rhizospheric soil were collected from a native Prairie near Volga, SD (N 44° 22' 33.226"W 96° 57' 54.147"). The samples were brought to the laboratory, plants were kept at room temperature at 4˚C before processing.

For the isolation of fungi, plant roots were soaked in water and soil removed gently. The roots were cut off and placed into 50ml pre-sterilized conicals and phosphate buffered saline Tween 20 (PBST) was added and sonicated for 10 minutes for the isolation of rhizosphere communities (White et al. 2015).The roots were then transferred to new conicals (50ml), and rhizosphere communities harvested at 7,000 g for 10 min. The pellets were then suspended in sterile tap water.

Roots were surface sterilized by immersion in 10% H_2O_2 for 5 min, washed 3 times in sterile distilled water. The roots were then transferred to pre-sterilized blender, covered with sterile distilled water and ground for 1 min. The green part of the plant was treated as the roots. Serial dilutions were made for all three sample types viz. rhizosphere soil, roots, and green part, and pour plates and spread plates prepared using nitrogen free medium (NFM) and spread plated on to R2A agar. NFM composed of 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), noble agar (15.0g/l, Difco, Catalog No. 214230), NaCl (0.2g/l), Sucrose (10g/l), oxalic acid (2g/l), and citrate (2g/l). Spread plates of NFM

were incubated for 5-7 d at 28°C aerobically and microaerophically using a gas-pack (Gaspack EZ Campy, BD). NFM pour plates and R2A spread plates were incubated aerobically for 5-7 d at 28°C. Fungal-like colonies were selected based on morphology and streaked for isolation and purity on NFM and R2A plates.

Two fungal isolates obtained on NFM (glucose) in a previous study of soil crusts of SD Badlands (Chapter 2) were included in this study.

3.2.2. DNA Extraction and PCR Amplification of internal transcribed spacer (ITS) region, 16S rRNA gene and *nifH* gene

DNA was extracted from pure culture using SDS extraction buffer as described by (Arnold and Lutzoni 2007). PCR amplification of the internal transcribed spacer region (ITS) using primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). 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), 0.8µl (10mM) each Primer, and 2.4µl (25mM) $MgCl_2$. The PCR conditions were initial denaturation of 95 ºC for 10 min, 30 cycles of 95 ºC for 15s, at 52 ºC for 30s, and at 72 ºC for 2s, and a final elongation at 72 ºC for 10 min.

The 16s rRNA gene amplification was done spanning V1-9 region using universal primers 27F (AGA GTT TGA TCM TGG CTC AG) (Weisburg et al. 1991) and 1492R (CGG TTA CCT TGT TAC GAC TT) (Jiang et al. 2006). 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 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. Similarly, 16S rRNA gene amplification of the V1-3 region was done using universal primers 27F and 518R. PCR was performed as described in chapter 2, section 2.4. Bands of ~500bp were excised using Wizard^R SV Gel Purification and Clean-Up System (Promega) and re-amplified using the same primers. The *nifH* PCR was performed using primers PolF and PolR as described in chapter 2.

The DNA sequence of PCR amplicons was determined by Sanger sequencing (GenScript). The sequences were aligned using Muscle (Edgar 2004a; Edgar 2004b), and a phylogenetic tree with maximum likelihood (500 bootstraps) was constructed using MEGA 6.0. (Tamura et al. 2013). Reference sequences were selected from GenBank (Benson et al. 1993) using NCBI BLAST (Altschul et al. 1997).

3.2.3. Culture of fungi-like isolates on various media

The fungi-like isolates were cultured on Malt Extract Agar (MEA) and MEA with 30µg/ml cycloheximide (10mg/ml), and incubated at 28 ºC for 2-3 d. They were also cultured on NMF plates, NFM plus 30µg/ml chloramphenicol (10mg/ml stock solution) plates, in liquid NFM and in liquid NFM in a desiccator containing clinoptilolite, an ammonia scavenging zeolite (Wang et al. 2006; Liao et al. 2015). The container with clinoptilolite was then sealed using petroleum jelly, and kept at

room temperature (22-26 ºC) for ~10 d. NFM plates were incubated at 28 ºC for ~7 d both aerobically and microaerophilically and cultures were inspected visually to confirm growth (Table 3).

3.2.4. Isolation of bacterial endophytes

Each fresh fungi-like isolate was scrapped off from NFM medium and placed into a sterile centrifuge tube. Filtered autoclaved sterile water was added to each centrifuge tube and the fungi-like isolates were crushed with a sterile pestle. This suspension was plated onto MEA and MEA plus cycloheximide (10mg/ml) plates and incubated at 28 ºC for 5 d aerobically. Cultures were inspected visually for growth.

3.2.5. Microscopy

Fresh cultures of fungi were stained using SYTO 9 (Invitrogen), and fluorescence was observed using an Olympus BX53 microscope, with GFP filter for fluorescence, with excitation 460/480 and emission 495/540.

3.2.6. 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 (20 ml) in a 60 CC syringe. After drawing in 5 ml of air through a filter (0.2µm) to supply 2 % oxygen, and 30ml of $^{15}N_2$ (Sigma-Aldrich, Catalog No. 364584) 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).

3.3. Results

Eight fungal isolates grown on nitrogen free medium were allocated to five Operational Taxonomic Units (OTUs) using ITS5 and ITS4 primers as a universal DNA barcode marker for fungi. All the OTUs belonged to the phylum *Ascomycota*, and all the fungal isolates had a query cover of 100- 96%, and had similarity >96%.

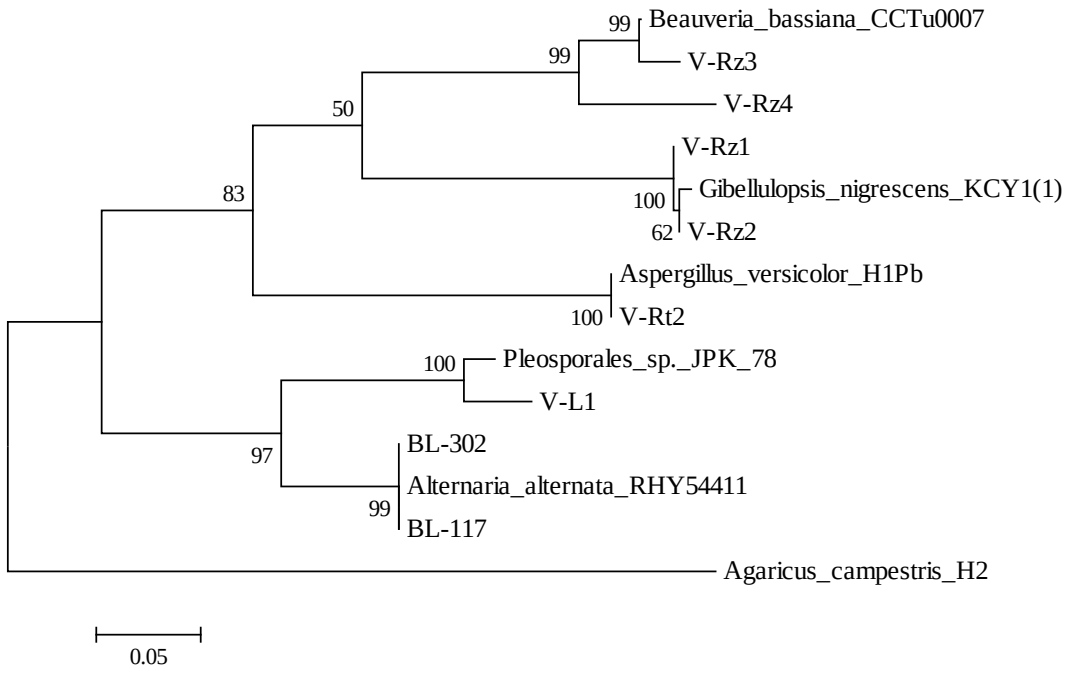

Fig. 3.1. Maximum likelihood phylogenetic tree of the ITS region of fungi based on Tamura-Nei model. Numbers represent bootstrap values of 500 analyses represented as percentage. *A. campestris* represents the out group.

Table 3.1. Source and characteristics of putative nitrogen fixing fungal isolates.

Isolates	Source	PCR amplification			Growth in various environments			
		16S rRNA (V1-9)	16S rRNA (V1-3)	*nifH* (PolF/ PolR)	NFM plates	NFM + Cm[a]	MEA + Chx[b]	Liq. NFM+ Clinoptiloli te
BL-302	Badlands matrix soil	0	-*	-	+	+	+	+
BL-117	Badlands matrix soil	0	-*	-	+	+	+	+
Rt2	*Bromus inermis* (smooth brome) (root)	0	-*	-	+	+	(+)	+
L1	*B. inermis* (leaf)	0	0	-	+	+	-	+
Rz1	*B. inermis* (rhizosphere)	0	-*	-	+	+	-	(+)
Rz2	*B. inermis* (rhizosphere)	0	+*	-	+	+	-	+
Rz3	*B. inermis* (rhizosphere)	0	0	-	+	+	+	+
Rz4	*B. inermis* (rhizosphere)	0	0	-	+	+	+	+
A. pullulans	Fungi used as a control	0	-*	-	+	+	-	_

[a]Cm= Chloramphenicol, [b]Chx= Cycloheximide, + = positive, (+) = slightly inhibited, - = negative/ no growth, 0= no amplification, -*=sequence of 500bp band not homologous to 16S, +*= sequence of 500bp band homologous to 16S.

The primers 27F and 518R used for 16S rRNA (V1-3) gene gave rise to the PCR products corresponding to 500 bp of *Streptomyces* used as a positive control (Fig. 3.2). The sequence of the 16S rRNA gene (V1-3) from Rz2 implicated the presence of a bacterial endophyte. The sequencing result showed 100% query cover and 98% identity, E value=0 with *Bacillus koreensis, B. beringensis, and B. korlensis* (Fig. 3.3). None of the other fungal isolates yielded 16S rRNA gene products, indicating absence of intracellular bacteria. Fluorescence microscopy of isolate, Rz2 stained with SYTO 9, indicated cytoplasmic bodies resembling intracellular bacteria (Fig. 3.4).

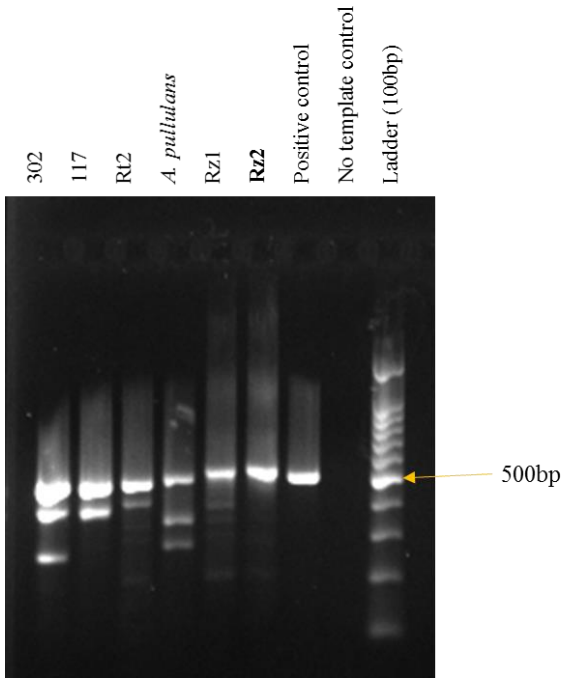

Figure 3.2. Electrophoretic pattern of the PCR products obtained using primers for the V1-3 region of 16S rRNA gene. The 500bp band and the bacterial positive control correspond to the band obtained using genomic DNA from fungi.

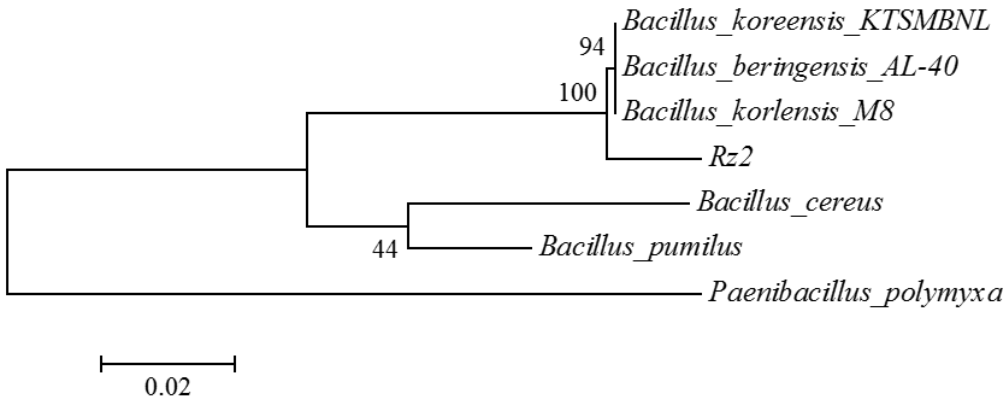

Figure 3.3. Maximum likelihood phylogenetic analysis of V1-3 region of the16S rRNA gene using MEGA 6.0. Bootstrap value of 500 replicates was used.

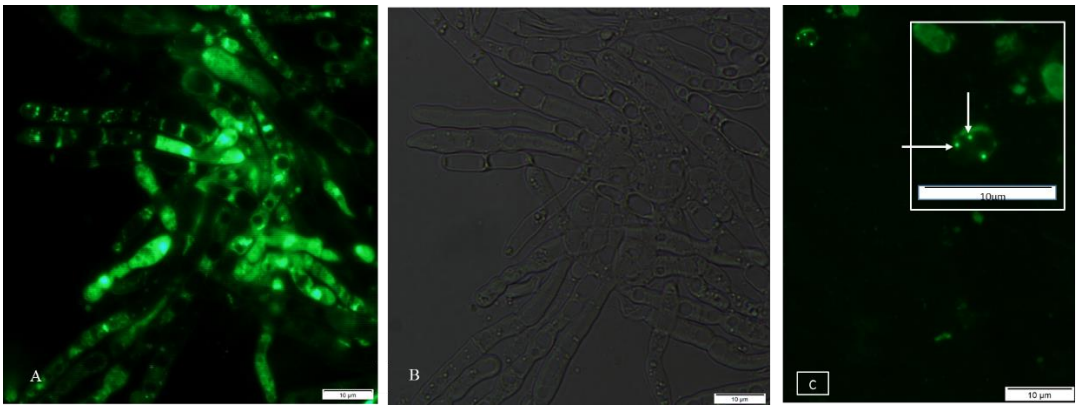

Figure 3.4. Fluorescent microscopy of fungal isolate (Rz2) stained with SYTO9 at 1,000X. (A) Fungal mycelium. (B) Bright field. (C) Inset showing presence of fluorescent cytoplasmic bodies inside the fungi.

Table 3.2. Nitrogen fixation by fungal isolates indicated by incorporation of $^{15}N_2$ into biomass.

Isolates	**Sequence resemblance**	**$^{15}N_2$ (at-%)**
B. japonicum USDA 110	Positive control	52.42142
BL-302	*B. bassiana* (ITS)	74.28161
V-Rt2	*A. versicolor* (ITS)	73.81134
V-Rz4	*A. alternata* (ITS)	75.59134

3.4. Discussion

Biological nitrogen fixation is one of the most expensive metabolic processes requiring a huge amount of energy to break the triple bond of dinitrogen, and has been reported only in prokaryotes. No eukaryotes have been reported to fix nitrogen independently or without any bacterial associations. Nitrogen fixing ability in arbuscular mycorrhizal fungi (*Glomus* spp., *Giagaspora gilmorei*, and *Endogone*

dusii) was ascribed to endophytic *Azospirillum* spp. detected in the spores of these fungi (Tilak et al. 1989). Bertaux et al. reported ectomycorrhizal fungi containing endophytic *Paenibacillus* spp. (Bertaux et al. 2003) having genes homologous to *nif* genes (Choo et al. 2003). A study of nitrogen fixation in the fungus *Pleurotus* spp. suggested that the fungi alone cannot fix nitrogen but can do so in the presence of a diazotrophic bacterium (Jayasinghearachchi and Seneviratne 2004). Ruiz-Herrera et al. isolated a pathogenic fungus *Ustilago maydis* that grew on nitrogen free medium and possessed nitrogenase enzyme. They provided first evidence for the intracellular nitrogen fixing *Bacillus pumilus* through 16S rRNA gene amplification (V1-9) and microscopy (Ruiz-Herrera et al. 2015).

Some microorganisms are able to grow in nitrogen deficient medium by scavenging ammonia or traces of combined nitrogen sources (Yoshida et al. 2007; Yoshida et al. 2014). Oligotrophic growth has been observed in some soil fungi on silica gel media without carbon and nitrogen sources (Parkinson et al. 1989; Wainwright 2012). It was intriguing to isolate fungi that grew on nitrogen free medium and even in liquid NFM in an ammonia depleted environment in a sealed container.

Despite rigorous DNA extraction by an SDS buffer extraction method, we were unable to obtain 16S rRNA gene amplification (V1-9 region), through multiple attempts and varying annealing temperatures. However, we obtained V1-3 region 16S rRNA gene amplification from Rz2 only, homologous to three *Bacillus* spp. *B. koreensis, B. korlensis*, and *B. beringensis.* In order to isolate bacteria present inside the fungi, we vigorously crushed the fresh fungal mycelia and plated on to MEA and

NFM. However, this did not yield any bacterial isolates. Further, we cultured the fungal isolates on NFM plus chloramphenicol, assuming that chloramphenicol inhibits bacterial cells, however, all the fungal isolates grew on NFM plus Cm plates. Resistance to cycloheximide was observed in some isolates (BL-302, BL-117, V-Rz3, and V-Rz4), but some fungi are resistant to cycloheximide (Ali-Shtayeh et al. 1998). Our results indicate that fungal isolate Rz2 contains an endophytic *Bacillus.* None of the other fungi growing in the absence of combined nitrogen contained detectable bacteria. All of our fungal isolates gave no *nifH* amplification but grew in nitrogen free and ammonia depleted environment which is not likely due to scavenging.

Incubation with $^{15}N_2$ of the three selected isolates, lead to ~75% of ^{15}N in biomass. As fungi generally produce ethylene, (Ilag and Curtis 1968; Dasilva et al. 1974; Chagué et al. 2002; Chagué 2010) we did not perform acetylene reduction assay. Our results of $^{15}N_2$ incorporation provides strong support for nitrogen fixation by these three fungal isolates, none of which contained detectable bacterial endophytes. This indicates a nitrogen fixing mechanism unrelated to the nitrogenase enzyme family.

Our preliminary findings indicated putative nitrogen fixing fungi that do not rely on endophytic diazotrophic bacteria. Our other finding indicated the endosymbiotic association with *Bacillus* spp. in one of the fungal isolates, Rz2, allocated to *Gibellulopsis nigrescens*.

Chapter 4

4. Conclusions and Questions for future study

4.1. Conclusion

The density of free-living culturable dizotrophs in the badlands of South Dakota was higher in sod table than in matrix soil. The culture dependent study suggested a diversity of free-living diazotrophs across 4 phyla (Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes), but ~50% of these clustered with *Streptomyces,* as determined by the 16S rRNA gene sequence.

Out of three different primer sets used to test the *nifH* gene amplification only the PolF and PolR set yielded *nifH* amplicons. Five of our isolates, allocated to *Streptomyces* by 16S rRNA gene sequence, indicated the presence of a *nifH* gene. Four of these aligned with *nifH* of *Scytonema* and one aligned with *nifH* of *Bradyrhizobium*. All the isolates allocated to *Streptomyces* by V1-3 16S rRNA gene sequence indicated a close similarity to *Streptomyces* by the full 16S rRNA gene sequence. These *Streptomyces* grew in liquid NFM in an ammonia-depleted environment in a sealed container. All of our selected *Streptomyces* isolates were able to incorporate $^{15}N_2$ into their biomass indication nitrogen fixation. The culture independent approach indicated predominance of *nifH* belonging to cyanobacteria, which was also confirmed by cyanobacterial 16S rRNA gene specific primers. Whether the cyanobacteria-like *nifH* amplicons obtained from soil DNA were associated with *Streptomyces* is intriguing to speculate. This is the first known report

of diazotrophic *Streptomyces*, other than the thermophilic, autotrophic *S. thermoautotrophicus*.

In our study of fungal isolates on nitrogen free medium, we isolated eight fungi, allocated to five OTUs using a universal DNA barcode marker for fungi, ITS5 and ITS4 primer set. All the OTUs belonged to the phylum Ascomycota. There was no amplification of 16S rRNA gene using primers 27F and 1492R despite multiple attempts, however one fungus indicated presence of endosymbiont related to *Bacillus* spp. when using 27F and 518R primers to amplify 16S rRNA gene (V1-3) indicating a possible endosymbiont of the fungus. All the fungal isolates grew in liquid NFM in ammonia depleted atmosphere in a sealed container. Three of our selected fungal isolates incorporated $^{15}N_2$ into their biomass leading to the assumption of putative nitrogen fixing fungi.

4.2. Questions for future study

The studies conducted in this thesis have opened a new realm for further investigation of free-living nitrogen fixing systems. Some questions for further investigation include:

1. Are there any other variants of nitrogenases other than the three oxygen sensitive (Nif, Vnf, Anf)?
2. Are there any other enzyme systems besides the Nif, Vnf, Anf family and the and the one oxygen insensitive *S. thermoautotrophicus* nitrogenase?

3. Is the discovery of primers that are universal to all the nitrogen fixing bacteria possible

    a) For *nif*H?

    b) For all nitrogen fixing systems?

4. How can we distinguish between a true nitrogen fixer and a scavenger of traces of combined nitrogen sources?

5. Can we develop a selective and differential medium for nitrogen fixers?

6. Does the lateral gene transfer of *nif* genes provide the ability of nitrogen fixation or are they non-functional?

7. What are the limitations of currently used methods to evaluate nitrogen fixation?

8. Do fungi fix nitrogen without diazotrophic association?

References

Abed, R.M.M., Al Kharusi, S., Schramm, A. and Robinson, M.D. (2010) Bacterial diversity, pigments and nitrogen fixation of biological desert crusts from the Sultanate of Oman. *FEMS Microbiology Ecology* **72**, 418-428.

Ali-Shtayeh, M., Jamous, R.M. and Abu-Ghdeib, S. (1998) Ecology of cycloheximide-resistant fungi in field soils receiving raw city wastewater or normal irrigation water. *Mycopathologia* **144**, 39-55.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**, 403-410.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research* **25**, 3389-3402.

Amann, R.I., Ludwig, W. and Schleifer, K.-H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological reviews* **59**, 143-169.

Amberg, S., Kilkus, K., Gardner, S., Gross, J.E., Wood, M. and Drazkowski, B. (2012) Badlands National Park Climate Change Vulnerability Assessment *Natural Resource Report NPS/BADL/NRR—2012/505*.

Arnold, A.E. and Lutzoni, F. (2007) Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? *Ecology* **88**, 541-549.

Atkins, C. (1984) Efficiencies and inefficiencies in the legume/Rhizobium symbiosis—A review. *Plant and soil* **82**, 273-284.

Atlas, R.M. (2010) *Handbook of microbiological media*: CRC press.

Becker, K., Harmsen, D., Mellmann, A., Meier, C., Schumann, P., Peters, G. and von Eiff, C. (2004) Development and Evaluation of a Quality-Controlled Ribosomal Sequence Database for 16S Ribosomal DNA-Based Identification of Staphylococcus Species. *Journal of Clinical Microbiology* **42**, 4988-4995.

Beeson, K.E., Erdner, D.L., Bagwell, C.E., Lovell, C.R. and Sobecky, P.A. (2002) Differentiation of plasmids in marine diazotroph assemblages determined by randomly amplified polymorphic DNA analysis. *Microbiology* **148**, 179-189.

Belnap, J. (1996) Soil surface disturbances in cold deserts: effects on nitrogenase activity in cyanobacterial-lichen soil crusts. *Biology and Fertility of Soils* **23**, 362-367.

Belnap, J. (2002) Nitrogen fixation in biological soil crusts from southeast Utah, USA. *Biology and fertility of soils* **35**, 128-135.

Belnap, J. and Gardner, J.S. (1993) Soil microstructure in soils of the Colorado Plateau: the role of the cyanobacterium Microcoleus vaginatus. *The Great Basin Naturalist*, 40-47.

Belnap, J. and Gillette, D.A. (1998) Vulnerability of desert biological soil crusts to wind erosion: the influences of crust development, soil texture, and disturbance. *Journal of Arid Environments* **39**, 133-142.

Benson, D., Lipman, D.J. and Ostell, J. (1993) GenBank. *Nucleic Acids Research* **21**, 2963-2965.

Bergersen, F. (1970) The quantitative relationship between nitrogen fixation and the acetylene-reduction assay. *Australian Journal of Biological Sciences* **23**, 1015-1026.

Bergersen, F., Dilworth, M. and Glenn, A. (1991) Physiological control of nitrogenase and uptake hydrogenase. *Biology and biochemistry of nitrogen fixation*, 76-102.

Bergersen, F.J. (1980) *Methods for evaluating biological nitrogen fixation*: John Wiley & Sons.

Berman-Frank, I., Lundgren, P. and Falkowski, P. (2003) Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. *Research in Microbiology* **154**, 157-164.

Bertaux, J., Schmid, M., Chemidlin Prevost-Boure, N., Churin, J.L., Hartmann, A., Garbaye, J. and Frey-Klett, P. (2003) In Situ Identification of Intracellular Bacteria Related to Paenibacillus spp. in the Mycelium of the Ectomycorrhizal Fungus Laccaria bicolor S238N. *Applied and Environmental Microbiology* **69**, 4243-4248.

Bohlool, B., Ladha, J., Garrity, D. and George, T. (1992) Biological nitrogen fixation for sustainable agriculture: A perspective. In *Biological nitrogen fixation for sustainable agriculture*. pp.1-11: Springer.

Bolhuis, H., Severin, I., Confurius-Guns, V., Wollenzien, U.I.A. and Stal, L.J. (2009) Horizontal transfer of the nitrogen fixation gene cluster in the cyanobacterium Microcoleus chthonoplastes. *ISME J* **4**, 121-130.

Bollmann, A., Lewis, K. and Epstein, S.S. (2007) Incubation of Environmental Samples in a Diffusion Chamber Increases the Diversity of Recovered Isolates. *Applied and Environmental Microbiology* **73**, 6386-6390.

Bowker, M., Reed, S., Belnap, J. and Phillips, S. (2002) Temporal variation in community composition, pigmentation, and Fv/Fm of desert cyanobacterial soil crusts. *Microbial Ecology* **43**, 13-25.

Brown, M.E. and Metcalfe, G. (1957) Nitrogen Fixation by a Species of Pullularia. *Nature* **180**, 282-282.

Büdel, B., Colesie, C., Green, T.G.A., Grube, M., Lázaro Suau, R., Loewen-Schneider, K., Maier, S., Peer, T., Pintado, A., Raggio, J., Ruprecht, U., Sancho, L.G., Schroeter, B., Türk, R., Weber, B., Wedin, M., Westberg, M., Williams, L. and Zheng, L. (2014) Improved appreciation of the functioning and importance of biological soil crusts in Europe: the Soil Crust International Project (SCIN). *Biodiversity and Conservation* **23**, 1639-1658.

Buresh, R., Casselman, M. and Patrick, W. (1980) Nitrogen fixation in flooded soil systems, a review. *Advances in Agronomy* **33**, 149-192.

Burgess, B.K. and Lowe, D.J. (1996) Mechanism of molybdenum nitrogenase. *Chemical Reviews* **96**, 2983-3012.

Cardenas, E. and Tiedje, J.M. (2008) New tools for discovering and characterizing microbial diversity. *Current Opinion in Biotechnology* **19**, 544-549.

Chagué, V. (2010) Ethylene Production by Fungi: Biological Questions and Future Developments Towards a Sustainable Polymers Industry. In *Handbook of Hydrocarbon and Lipid Microbiology* ed. Timmis, K. pp.3011-3020: Springer Berlin Heidelberg.

Chagué, V., Elad, Y., Barakat, R., Tudzynski, P. and Sharon, A. (2002) Ethylene biosynthesis in Botrytis cinerea. *FEMS microbiology ecology* **40**, 143-149.

Chang, C., Damiani, I., Puppo, A. and Frendo, P. (2009) Redox Changes during the Legume–Rhizobium Symbiosis. *Molecular Plant* **2**, 370-377.

Chelius, M.K. and Triplett, E.W. (2000) 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* **50**, 751-758.

Chen, J., Christiansen, J., Tittsworth, R., Hales, B., George, S., Coucouvanis, D. and Cramer, S. (1993) Iron EXAFS of Azotobacter vinelandii nitrogenase molybdenum-iron and vanadium-iron proteins. *Journal of the American Chemical Society* **115**, 5509-5515.

Chien, Y.T. and Zinder, S.H. (1994) Cloning, DNA sequencing, and characterization of a nifD-homologous gene from the archaeon Methanosarcina barkeri 227 which resembles nifD1 from the eubacterium Clostridium pasteurianum. *J Bacteriol* **176**, 6590-6598.

Chisnell, J.R., Premakumar, R. and Bishop, P.E. (1988) Purification of a second alternative nitrogenase from a nifHDK deletion strain of Azotobacter vinelandii. *Journal of bacteriology* **170**, 27-33.

Choo, Q.-C., Samian, M.-R. and Najimudin, N. (2003) Phylogeny and Characterization of Three nifH-Homologous Genes from Paenibacillus azotofixans. *Applied and Environmental Microbiology* **69**, 3658-3662.

Christiansen, J., Dean, D.R. and Seefeldt, L.C. (2001) Mechanistic features of the Mo-containing nitrogenase. *Annual review of plant biology* **52**, 269-295.

Cleveland, C.C., Townsend, A.R., Schimel, D.S., Fisher, H., Howarth, R.W., Hedin, L.O., Perakis, S.S., Latty, E.F., Von Fischer, J.C. and Elseroad, A. (1999) Global patterns of terrestrial biological nitrogen (N2) fixation in natural ecosystems. *Global biogeochemical cycles* **13**, 623.

Coenye, T., Falsen, E., Vancanneyt, M., Hoste, B., Govan, J.R.W., Kersters, K. and Vandamme, P. (1999) Classification of Alcaligenes faecalis-like isolates from the environment and human clinical samples as Ralstonia gilardii sp. nov. *International Journal of Systematic Bacteriology* **49**, 405-413.

Courvalin, P. (1994) Transfer of antibiotic resistance genes between gram-positive and gram-negative bacteria. *Antimicrobial Agents and Chemotherapy* **38**, 1447-1451.

Crans, D.C., Smee, J.J., Gaidamauskas, E. and Yang, L. (2004) The chemistry and biochemistry of vanadium and the biological activities exerted by vanadium compounds. *Chemical reviews* **104**, 849-902.

da Rocha, U.N., Cadillo-Quiroz, H., Karaoz, U., Rajeev, L., Klitgord, N., Dunn, S., Truong, V., Buenrostro, M., Bowen, B.P. and Garcia-Pichel, F. (2015) Isolation of a significant fraction of non-phototroph diversity from a desert Biological Soil Crust. *Frontiers in microbiology* **6**.

Dart, P.J. (1986) Nitrogen fixation associated with non-legumes in agriculture. In *Nitrogen Fixation with Non-Legumes* eds. Skinner, F.A. and Uomala, P. pp.303-334: Springer Netherlands.

Dasilva, E.J., Henriksson, E. and Henriksson, L.E. (1974) Ethylene production by fungi. *Plant Science Letters* **2**, 63-66.

Davis, R., Lehman, L., Petrovich, R., Shah, V.K., Roberts, G.P. and Ludden, P.W. (1996) Purification and characterization of the alternative nitrogenase from the photosynthetic bacterium Rhodospirillum rubrum. *Journal of bacteriology* **178**, 1445-1450.

Desnoues, N., Lin, M., Guo, X., Ma, L., Carreño-Lopez, R. and Elmerich, C. (2003) Nitrogen fixation genetics and regulation in a Pseudomonas stutzeri strain associated with rice. *Microbiology* **149**, 2251-2262.

Dighton, J., White Jr, J.F., White, J. and Oudemans, P. (2005) *The fungal community: its organization and role in the ecosystem*: CRC Press.

Dilworth, M.J. (1966) Acetylene reduction by nitrogen-fixing preparations from Clostridium pasteurianum. *Biochim Biophys Acta* **127**, 285-294.

Dixon, R. and Kahn, D. (2004) Genetic regulation of biological nitrogen fixation. *Nat Rev Micro* **2**, 621-631.

Dobereiner, J., Marriel, I. and Nery, M. (1976) Ecological distribution of Spirillum lipoferum Beijerinck. *Canadian Journal of Microbiology* **22**, 1464-1473.

Dos Santos, P.C., Fang, Z., Mason, S.W., Setubal, J.C. and Dixon, R. (2012) Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. *BMC genomics* **13**, 12.

Duggar, B.M. and Davis, A.R. (1916) Studies in the physiology of the fungi. I. Nitrogen fixation. *Annals of the Missouri Botanical Garden* **3**, 413-437.

Dunbar, J., Takala, S., Barns, S.M., Davis, J.A. and Kuske, C.R. (1999) Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Applied and Environmental Microbiology* **65**, 1662-1669.

Eady, R.R. (1996) Structure-function relationships of alternative nitrogenases. *Chemical Reviews* **96**, 3013-3030.

Eady, R.R. (2003) Current status of structure function relationships of vanadium nitrogenase. *Coordination Chemistry Reviews* **237**, 23-30.

Eady, R.R., Robson, R.L., Richardson, T.H., Miller, R.W. and Hawkins, M. (1987) The vanadium nitrogenase of Azotobacter chroococcum. Purification and properties of the VFe protein. *The Biochemical journal* **244**, 197-207.

Eardly, B.D., Young, J.P. and Selander, R.K. (1992) 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* **58**, 1809-1815.

Edgar, R. (2004a) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5**, 113.

Edgar, R.C. (2004b) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792-1797.

Einsle, O., Tezcan, F.A., Andrade, S.L., Schmid, B., Yoshida, M., Howard, J.B. and Rees, D.C. (2002) Nitrogenase MoFe-protein at 1.16 Å resolution: a central ligand in the FeMo-cofactor. *Science* **297**, 1696-1700.

Elena, O.O., Galina, M.Z., Natalia, P.C. and Vladimir, K.O. (2006) Role of Algobacterial Associations in Soil Forming Process. In *The 18th World Congress of Soil Science*.

Evans, H. and Burris, R. (1992) Highlights in biological nitrogen fixation during the last 50 years. *Biological Nitrogen Fixation New York: Chapman and Hall*, 1-42.

Fani, R., Gallo, R. and Lio, P. (2000) Molecular evolution of nitrogen fixation: The evolutionary history of the nifD, nifK, nifE, and nifN genes. *Journal of Molecular Evolution* **51**, 1-11.

Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F. and Whitehouse, C.M. (1989) Electrospray ionization for mass spectrometry of large biomolecules. *Science* **246**, 64-71.

Ferris, M., Muyzer, G. and Ward, D. (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Applied and Environmental Microbiology* **62**, 340-346.

Figg, T.M., Holland, P.L. and Cundari, T.R. (2012) Cooperativity between low-valent iron and potassium promoters in dinitrogen fixation. *Inorganic chemistry* **51**, 7546-7550.

Finan, T.M. (2002) *Nitrogen Fixation: Global Perspectives: Proceedings of the 13th International Congress on Nitrogen Fixation, Hamilton, Ontario, Canada, 2-7 July 2001*: CABI.

Gaby, J.C. and Buckley, D.H. (2012) A Comprehensive Evaluation of PCR Primers to Amplify the *nifH* Gene of Nitrogenase. *PLoS ONE* **7**, e42149.

Gaby, J.C. and Buckley, D.H. (2014) A comprehensive aligned nifH gene database: a multipurpose tool for studies of nitrogen-fixing bacteria. *Database (Oxford)* **2014**, bau001.

Gadkari, D., Morsdorf, G. and Meyer, O. (1992a) Chemolithoautotrophic assimilation of dinitrogen by Streptomyces thermoautotrophicus UBT1: identification of an unusual N2-fixing system. *J Bacteriol* **174**, 6840-6843.

Gadkari, D., Mörsdorf, G. and Meyer, O. (1992b) Chemolithoautotrophic assimilation of dinitrogen by Streptomyces thermoautotrophicus UBT1: identification of an unusual N2-fixing system. *Journal of Bacteriology* **174**, 6840-6843.

Galloway, J.N. (1998) The global nitrogen cycle: changes and consequences. *Environmental pollution* **102**, 15-24.

GeneCodes Sequencher® version 5.4 sequence analysis software. *http://wwwgenecodescom*.

Gevers, D., Cohan, F.M., Lawrence, J.G., Spratt, B.G., Coenye, T., Feil, E.J., Stackebrandt, E., Van de Peer, Y., Vandamme, P. and Thompson, F.L. (2005) Re-evaluating prokaryotic species. *Nature Reviews Microbiology* **3**, 733-739.

Ginterova, A. and Maxianova, A. (1975) The balance of nitrogen and composition of proteins in Pleurotus ostreatus grown on natural substrates. *Folia microbiologica* **20**, 246-250.

Glibert, P.M. and Bronk, D.A. (1994) Release of dissolved organic nitrogen by marine diazotrophic cyanobacteria, Trichodesmium spp. *Applied and Environmental microbiology* **60**, 3996-4000.

Graham, J. (2008) Badlands National Park Geologic Resource Evaluation Report. *Natural Resource Report NPS/NRPC/GRD/NRR*.

Gray, M.W., Sankoff, D. and Cedergren, R.J. (1984) On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. *Nucleic Acids Research* **12**, 5837-5852.

Griffith, F. (1928) The significance of pneumococcal types. *Journal of Hygiene* **27**, 113-159.

Gtari, M., Brusetti, L., Hassen, A., Mora, D., Daffonchio, D. and Boudabous, A. (2007) Genetic diversity among Elaeagnus compatible Frankia strains and sympatric-related nitrogen-fixing actinobacteria revealed by nifH sequence analysis. *Soil Biology and Biochemistry* **39**, 372-377.

Gtari, M., Ghodhbane-Gtari, F., Nouioui, I., Beauchemin, N. and Tisa, L.S. (2012) Phylogenetic perspectives of nitrogen-fixing actinobacteria. *Archives of microbiology* **194**, 3-11.

Gyles, C. and Boerlin, P. (2014) Horizontally Transferred Genetic Elements and Their Role in Pathogenesis of Bacterial Disease. *Veterinary Pathology Online* **51**, 328-340.

Haahtela, K., Laakso, T. and Korhonen, T.K. (1986) Associative nitrogen fixation by Klebsiella spp.: adhesion sites and inoculation effects on grass roots. *Applied and environmental microbiology* **52**, 1074-1079.

Hales, B.J., Case, E.E., Morningstar, J.E., Dzeda, M.F. and Mauterer, L.A. (1986) Isolation of a new vanadium-containing nitrogenase from Azotobacter vinelandii. *Biochemistry* **25**, 7251-7255.

Hara, S., Hashidoko, Y., Desyatkin, R.V., Hatano, R. and Tahara, S. (2009) 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* **75**, 2811-2819.

Hardy, R., Burns, R. and Holsten, R.D. (1973) Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biology and Biochemistry* **5**, 47-81.

Hardy, R.W., Holsten, R., Jackson, E. and Burns, R. (1968) The acetylene-ethylene assay for N2 fixation: laboratory and field evaluation. *Plant physiology* **43**, 1185-1207.

Harvey, I., Arber, J.M., Eady, R.R., Smith, B.E., Garner, C.D. and Hasnain, S.S. (1990) Iron K-edge X-ray-absorption spectroscopy of the iron-vanadium cofactor of the vanadium nitrogenase from Azotobacter chroococcum. *Biochem J* **266**, 929-931.

Haukka, K., Lindström, K. and Young, J.P.W. (1998) 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* **64**, 419-426.

Hausinger, R. and Howard, J. (1983) Thiol reactivity of the nitrogenase Fe-protein from Azotobacter vinelandii. *Journal of Biological Chemistry* **258**, 13486-13492.

He, X., Xu, M., Qiu, G.Y. and Zhou, J. (2009) Use of 15N stable isotope to quantify nitrogen transfer between mycorrhizal plants. *Journal of Plant Ecology* **2**, 107-118.

Herridge, D.F., Peoples, M.B. and Boddey, R.M. (2008) Global inputs of biological nitrogen fixation in agricultural systems. *Plant and Soil* **311**, 1-18.

Hinnemann, B. and Norskov, J.K. (2004) Structure of the FeFe-cofactor of the iron-only nitrogenase and possible mechanism for dinitrogen reduction. *Physical Chemistry Chemical Physics* **6**, 843-853.

Hoffmann, E. (1996) *Mass spectrometry*: Wiley Online Library.

Hofmann-Findeklee, C., Gadkari, D. and Meyer, O. (2000) Superoxide-dependent nitrogen fixation. In *Nitrogen fixation: From molecules to crop productivity*. pp.23-30: Springer.

Howard, J.B. and Rees, D.C. (1994) Nitrogenase: a nucleotide-dependent molecular switch. *Annu Rev Biochem* **63**, 235-264.

Howard, J.B. and Rees, D.C. (1996) Structural basis of biological nitrogen fixation. *Chemical Reviews* **96**, 2965-2982.

Hu, Y., Lee, C.C. and Ribbe, M.W. (2011) Extending the carbon chain: hydrocarbon formation catalyzed by vanadium/molybdenum nitrogenases. *Science* **333**, 753-755.

Hu, Y., Lee, C.C. and Ribbe, M.W. (2012) Vanadium nitrogenase: a two-hit wonder? *Dalton Trans* **41**, 1118-1127.

Hu, Y. and Ribbe, M.W. (2015) Nitrogenase and homologs. *J Biol Inorg Chem* **20**, 435-445.

Igarashi, R.Y. and Seefeldt, L.C. (2003) Nitrogen fixation: the mechanism of the Mo-dependent nitrogenase. *Critical Reviews in Biochemistry and Molecular Biology* **38**, 351-384.

Ilag, L. and Curtis, R.W. (1968) Production of ethylene by fungi. *Science* **159**, 1357-1358.

Izquierdo, J.A. and Nüsslein, K. (2006) Distribution of extensive nifH gene diversity across physical soil microenvironments. *Microbial ecology* **51**, 441-452.

Jayasinghearachchi, H. and Seneviratne, G. (2004) Can mushrooms fix atmospheric nitrogen? *Journal of biosciences* **29**, 293-296.

Jennings, J. (2013) *Catalytic ammonia synthesis: fundamentals and practice*: Springer Science & Business Media.

Jiang, H., Dong, H., Zhang, G., Yu, B., Chapman, L.R. and Fields, M.W. (2006) Microbial Diversity in Water and Sediment of Lake Chaka, an Athalassohaline Lake in Northwestern China. *Applied and Environmental Microbiology* **72**, 3832-3845.

Joerger, R., Jacobson, M., Premakumar, R., Wolfinger, E. and Bishop, P. (1989) Nucleotide sequence and mutational analysis of the structural genes (anfHDGK) for the second alternative nitrogenase from Azotobacter vinelandii. *Journal of bacteriology* **171**, 1075-1086.

Kahindi, J.H.P., Woomer, P., George, T., de Souza Moreira, F.M., Karanja, N.K. and Giller, K.E. (1997) Agricultural intensification, soil biodiversity and ecosystem function in the tropics: the role of nitrogen-fixing bacteria. *Applied Soil Ecology* **6**, 55-76.

Kim, J. and Rees, D. (1992) Structural models for the metal centers in the nitrogenase molybdenum-iron protein. *Science* **257**, 1677-1682.

Klucas, R.V., Koch, B., Russell, S.A. and Evans, H.J. (1968) Purification and Some Properties of the Nitrogenase From Soybean (Glycine max Merr.) Nodules. *Plant Physiol* **43**, 1906-1912.

Kneip, C., Lockhart, P., Voß, C. and Maier, U.-G. (2007) Nitrogen fixation in eukaryotes–new models for symbiosis. *BMC Evol Biol* **7**, 55.

Koonin, E.V., Makarova, K.S. and Aravind, L. (2001) Horizontal gene transfer in prokaryotes: quantification and classification 1. *Annual Reviews in Microbiology* **55**, 709-742.

Kuklinsky-Sobral, J., Araújo, W.L., Mendes, R., Geraldi, I.O., Pizzirani-Kleiner, A.A. and Azevedo, J.L. (2004) Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environmental Microbiology* **6**, 1244-1251.

Kumar, S., Nei, M., Dudley, J. and Tamura, K. (2008) MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in bioinformatics* **9**, 299-306.

Langlois, R.J., Hümmer, D. and LaRoche, J. (2008) Abundances and distributions of the dominant nifH phylotypes in the Northern Atlantic Ocean. *Applied and environmental microbiology* **74**, 1922-1931.

Lederberg, J., Alexander, M., Bloom, B.R., Hopwood, D.A., Hull, R., Iglewski, B.H., Laskin, A.I., Oliver, S.G., Schaechter, M. and Summers, W.C. (2000) *Encyclopedia of Microbiology, Four-Volume Set*: Academic Press.

Lee, C.C., Hu, Y. and Ribbe, M.W. (2009) Unique features of the nitrogenase VFe protein from Azotobacter vinelandii. *Proceedings of the National Academy of Sciences* **106**, 9209-9214.

Lee, C.C., Hu, Y. and Ribbe, M.W. (2010) Vanadium nitrogenase reduces CO. *Science* **329**, 642.

Lee, C.C., Hu, Y. and Ribbe, M.W. (2011) Tracing the hydrogen source of hydrocarbons formed by vanadium nitrogenase. *Angew Chem Int Ed Engl* **50**, 5545-5547.

Lee, I.-M., Gundersen-Rindal, D.E., Davis, R.E. and BARTOSZYK, I.M. (1998) Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *International journal of systematic bacteriology* **48**, 1153-1169.

Lehman, L.J. and Roberts, G.P. (1991) Identification of an alternative nitrogenase system in Rhodospirillum rubrum. *J Bacteriol* **173**, 5705-5711.

Liang, J. and Burris, R.H. (1988) Interactions among nitrogen, nitrous oxide, and acetylene as substrates and inhibitors of nitrogenase from Azotobacter vinelandii. *Biochemistry* **27**, 6726-6732.

Liao, Z.-l., Chen, H., Zhu, B.-r. and Li, H.-z. (2015) Combination of powdered activated carbon and powdered zeolite for enhancing ammonium removal in micro-polluted raw water. *Chemosphere* **134**, 127-132.

Linhart, C. and Shamir, R. (2002) The degenerate primer design problem. *Bioinformatics* **18**, S172-S181.

Lipman, C.B. (1911) Nitrogen fixation by yeasts and other fungi. *Journal of Biological Chemistry* **10**, 169-182.

Macgregor, A.N. and Johnson, D.E. (1971) Capacity of Desert Algal Crusts to Fix Atmospheric Nitrogen. *Soil Science Society of America Journal* **35**, 843-844.

Mague, T. and Burris, R. (1972) Reduction of acetylene and nitrogen by field-grown soybeans. *New Phytologist* **71**, 275-286.

Marchandin, H., Teyssier, C., Siméon de Buochberg, M., Jean-Pierre, H., Carriere, C. and Jumas-Bilak, E. (2003) Intra-chromosomal heterogeneity between the four 16S rRNA gene copies in the genus Veillonella: implications for phylogeny and taxonomy. *Microbiology* **149**, 1493-1501.

Matsuki, T., Watanabe, K., Fujimoto, J., Kado, Y., Takada, T., Matsumoto, K. and Tanaka, R. (2004) Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. *Applied and environmental microbiology* **70**, 167-173.

Mayer, S.M., Lawson, D.M., Gormal, C.A., Roe, S.M. and Smith, B.E. (1999) New insights into structure-function relationships in nitrogenase: a 1.6 Å resolution X-ray crystallographic study of Klebsiella pneumoniae MoFe-protein. *Journal of molecular biology* **292**, 871-891.

McCabe, K.M., Zhang, Y.H., Huang, B.L., Wagar, E.A. and McCabe, E.R. (1999) Bacterial species identification after DNA amplification with a universal primer pair. *Mol Genet Metab* **66**, 205-211.

McWilliam, H., Li, W., Uludag, M., Squizzato, S., Park, Y.M., Buso, N., Cowley, A.P. and Lopez, R. (2013) Analysis Tool Web Services from the EMBL-EBI. *Nucleic acids research* **41**, W597-600.

Mehta, M.P., Butterfield, D.A. and Baross, J.A. (2003) Phylogenetic Diversity of Nitrogenase (nifH) Genes in Deep-Sea and Hydrothermal Vent Environments of the Juan de Fuca Ridge. *Applied and Environmental Microbiology* **69**, 960-970.

Metcalfe, G. and Chayen, S. (1954) Nitrogen fixation by soil yeasts.

Miller, R. and Eady, R. (1988) Molybdenum and vanadium nitrogenases of Azotobacter chroococcum. Low temperature favours N2 reduction by vanadium nitrogenase. *Biochem J* **256**, 429-432.

Mirza, B.S. and Rodrigues, J.L. (2012) Development of a direct isolation procedure for free-living diazotrophs under controlled hypoxic conditions. *Appl Environ Microbiol* **78**, 5542-5549.

Montoya, J.P., Voss, M., Kahler, P. and Capone, D.G. (1996) A Simple, High-Precision, High-Sensitivity Tracer Assay for N(inf2) Fixation. *Applied and Environmental Microbiology* **62**, 986-993.

Morningstar, J.E., Johnson, M.K., Case, E.E. and Hales, B.J. (1987) Characterization of the metal clusters in the nitrogenase molybdenum-iron and vanadium-iron proteins of Azotobacter vinelandii using magnetic circular dichroism spectroscopy. *Biochemistry* **26**, 1795-1800.

Muegge, B.D., Kuczynski, J., Knights, D., Clemente, J.C., González, A., Fontana, L., Henrissat, B., Knight, R. and Gordon, J.I. (2011) Diet Drives Convergence in Gut Microbiome Functions Across Mammalian Phylogeny and Within Humans. *Science* **332**, 970-974.

Mulholland, M.R. (2007) The fate of nitrogen fixed by diazotrophs in the ocean. *Biogeosciences* **4**, 37-51.

Muyzer, G. (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. *Current opinion in microbiology* **2**, 317-322.

Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993) 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* **59**, 695-700.

Muyzer, G. and Smalla, K. (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek* **73**, 127-141.

Nelson, M.S. and Sadowsky, M.J. (2015) Secretion systems and signal exchange between nitrogen-fixing rhizobia and legumes. *Frontiers in Plant Science* **6**, 491.

Nübel, U., Garcia-Pichel, F. and Muyzer, G. (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. *Applied and environmental microbiology* **63**, 3327-3332.

Ohkuma, M., Noda, S. and Kudo, T. (1999) Phylogenetic Diversity of Nitrogen Fixation Genes in the Symbiotic Microbial Community in the Gut of Diverse Termites. *Applied and Environmental Microbiology* **65**, 4926-4934.

Oldroyd, G.E.D. (2013) Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Reviews Microbiology* **11**, 252-263.

Pace, N.R. (1997) A molecular view of microbial diversity and the biosphere. *Science* **276**, 734-740.

Parkinson, S.M., Wainwright, M. and Killham, K. (1989) Observations on oligotrophic growth of fungi on silica gel. *Mycological Research* **93**, 529-534.

Pau, R., Eldridge, M.E., Lowe, D., Mitchenall, L.A. and Eady, R. (1993) Molybdenum-independent nitrogenases of Azotobacter vinelandii: a functional species of alternative nitrogenase-3 isolated from a molybdenum-tolerant strain contains an iron-molybdenum cofactor. *Biochem J* **293**, 101-107.

Pauly, N., Pucciariello, C., Mandon, K., Innocenti, G., Jamet, A., Baudouin, E., Hérouart, D., Frendo, P. and Puppo, A. (2006) Reactive oxygen and nitrogen species and glutathione: key players in the legume–Rhizobium symbiosis. *Journal of Experimental Botany* **57**, 1769-1776.

Paustian, K. and Schnürer, J. (1987) Fungal growth response to carbon and nitrogen limitation: a theoretical model. *Soil Biology and Biochemistry* **19**, 613-620.

Pointing, S.B. and Belnap, J. (2012) Microbial colonization and controls in dryland systems. *Nature reviews Microbiology* **10**, 551-562.

Poly, F., Monrozier, L.J. and Bally, R. (2001) Improvement in the RFLP procedure for studying the diversity of nifH genes in communities of nitrogen fixers in soil. *Research in Microbiology* **152**, 95-103.

Postgate, J.R. (1982) *The fundamentals of nitrogen fixation*: CUP Archive.

Rangaswami, G., Kandaswami, T. and Ramasamy, K. (1975) Pleurotus sajor-caju (Fr.) Singer, a protein rich nitrogen fixing mushroom fungus. *Current science*.

Rapp, B.J. and Wall, J.D. (1987) Genetic transfer in Desulfovibrio desulfuricans. *Proceedings of the National Academy of Sciences* **84**, 9128-9130.

Ravenhall, M., Škunca, N., Lassalle, F. and Dessimoz, C. (2015) Inferring Horizontal Gene Transfer. *PLoS Comput Biol* **11**, e1004095.

Raymond, J., Siefert, J.L., Staples, C.R. and Blankenship, R.E. (2004) The natural history of nitrogen fixation. *Mol Biol Evol* **21**, 541-554.

Rees, D.C. and Howard, J.B. (2000) Nitrogenase: standing at the crossroads. *Current Opinion in Chemical Biology* **4**, 559-566.

Rehder, D. (2000) Vanadium nitrogenase. *Journal of inorganic biochemistry* **80**, 133-136.

Reiter, B., Bürgmann, H., Burg, K. and Sessitsch, A. (2003) Endophytic nifH gene diversity in African sweet potato. *Canadian journal of microbiology* **49**, 549-555.

Rettedal, E.A., Clay, S. and Brozel, V.S. (2010) GC-clamp primer batches yield 16S rRNA gene amplicon pools with variable GC clamps, affecting denaturing gradient gel electrophoresis profiles. *FEMS Microbiol Lett* **312**, 55-62.

Ribbe, M., Gadkari, D. and Meyer, O. (1997) 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* **272**, 26627-26633.

Ribeiro, C.W., Alloing, G., Mandon, K. and Frendo, P. (2015) Redox regulation of differentiation in symbiotic nitrogen fixation. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1850**, 1469-1478.

Robson, R.L. and Postgate, J.R. (1980) Oxygen and hydrogen in biological nitrogen fixation. *Annual review of microbiology* **34**, 183-207.

Robson, R.L., Woodley, P.R., Pau, R.N. and Eady, R.R. (1989) Structural genes for the vanadium nitrogenase from Azotobacter chroococcum. *The EMBO journal* **8**, 1217-1224.

Rojas, A., Holguin, G., Glick, B.R. and Bashan, Y. (2001) Synergism between Phyllobacterium sp.(N2-fixer) and Bacillus licheniformis (P-solubilizer), both from a semiarid mangrove rhizosphere. *FEMS Microbiology Ecology* **35**, 181-187.

Rubio, L.M. and Ludden, P.W. (2008) Biosynthesis of the iron-molybdenum cofactor of nitrogenase. *Annual review of microbiology* **62**, 93-111.

Ruiz-Herrera, J., Leon-Ramirez, C., Vera-Nunez, A., Sanchez-Arreguin, A., Ruiz-Medrano, R., Salgado-Lugo, H., Sanchez-Segura, L. and Jose Pena-Cabriales, J. (2015) A novel intracellular nitrogen-fixing symbiosis made by Ustilago maydis and Bacillus spp. *New Phytologist* **207**, 769-777.

Ryabenko, E. (2013) *Stable Isotope Methods for the Study of the Nitrogen Cycle*.

Rychert, R. and Skujiņš, J. (1974) Nitrogen fixation by blue-green algae-lichen crusts in the Great Basin Desert. *Soil Science Society of America Journal* **38**, 768-771.

Schlessman, J.L., Woo, D., Joshua-Tor, L., Howard, J.B. and Rees, D.C. (1998) Conformational variability in structures of the nitrogenase iron proteins from Azotobacter vinelandii and Clostridium pasteurianum. *Journal of molecular biology* **280**, 669-685.

Schneider, K. and Müller, A. (2004) Iron-Only Nitrogenase: Exceptional Catalytic, Structural and Spectroscopic Features. In *Catalysts for Nitrogen Fixation*. pp.281-307: Springer.

Schneider, K., MÜLler, A., Schramm, U. and Klipp, W. (1991) Demonstration of a molybdenum- and vanadium-independent nitrogenase in a nifHDK-deletion mutant of Rhodobacter capsulatus. *European Journal of Biochemistry* **195**, 653-661.

Schöllhorn, R. and Burris, R.H. (1967) Acetylene as a competitive inhibitor of N-2 fixation. *Proceedings of the National Academy of Sciences of the United States of America* **58**, 213-216.

Schütte, U.M., Abdo, Z., Bent, S.J., Shyu, C., Williams, C.J., Pierson, J.D. and Forney, L.J. (2008) Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. *Appl Microbiol Biotechnol* **80**, 365-380.

Scott, D.J., Dean, D. and Newton, W. (1992) Nitrogenase-catalyzed ethane production and CO-sensitive hydrogen evolution from MoFe proteins having amino acid substitutions in an alpha-subunit FeMo cofactor-binding domain. *Journal of Biological Chemistry* **267**, 20002-20010.

Seefeldt, L.C., Hoffman, B.M. and Dean, D.R. (2009) Mechanism of Mo-Dependent Nitrogenase. *Annual review of biochemistry* **78**, 701.

Shah, V.K., Davis, L.C. and Brill, W.J. (1975) Nitrogenase. VI. Acetylene reduction assay: Dependence of nitrogen fixation estimates on component ratio and acetylene concentration. *Biochim Biophys Acta* **384**, 353-359.

Sigalevich, P., Meshorer, E., Helman, Y. and Cohen, Y. (2000) Transition from anaerobic to aerobic growth conditions for the sulfate-reducing bacterium Desulfovibrio oxyclinae results in flocculation. *Applied and environmental microbiology* **66**, 5005-5012.

Simpson, F.B. and Burris, R.H. (1984) A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. *Science* **224**, 1095-1097.

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M. and Herndl, G.J. (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Sciences* **103**, 12115-12120.

Stacey, G., Burris, R.H. and Evans, H.J. (1992) *Biological nitrogen fixation*: Springer Science & Business Media.

Staples, C.R., Lahiri, S., Raymond, J., Von Herbulis, L., Mukhophadhyay, B. and Blankenship, R.E. (2007) Expression and association of group IV nitrogenase NifD and NifH homologs in the non-nitrogen-fixing archaeon Methanocaldococcus jannaschii. *J Bacteriol* **189**, 7392-7398.

Steven, B., Kuske, C.R., Gallegos-Graves, L.V., Reed, S.C. and Belnap, J. (2015) Climate Change and Physical Disturbance Manipulations Result in Distinct Biological Soil Crust Communities. *Applied and Environmental Microbiology*.

Stewart, W.D., Fitzgerald, G.P. and Burris, R.H. (1967) In situ studies on N2 fixation using the acetylene reduction technique. *Proceedings of the National Academy of Sciences of the United States of America* **58**, 2071-2078.

Stoffer, P.W. (2003) *Geology of Badlands National Park: a preliminary report*: US Department of the Interior, US Geological Survey.

Su, C., Lei, L.P., Duan, Y.Q., Zhang, K.Q. and Yang, J.K. (2012) Culture-independent methods for studying environmental microorganisms: methods, application, and perspective. *Appl Microbiol Biotechnol* **93**, 993-1003.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular biology and evolution* **30**, 2725-2729.

Thiel, T. (1993) Characterization of genes for an alternative nitrogenase in the cyanobacterium Anabaena variabilis. *Journal of bacteriology* **175**, 6276-6286.

Thorneley, R., Bergstrom, N., Eady, R. and Lowe, D. (1989) Vanadium nitrogenase of Azotobacter chroococcum. MgATP-dependent electron transfer within the protein complex. *Biochem J* **257**, 789-794.

Tilak, K., Li, C. and Ho, I. (1989) Occurrence of nitrogen-fixing Azospirillum in vesicular-arbuscular mycorrhizal fungi. *Plant and Soil* **116**, 286-288.

Tirkey, J. and Adhikary, S. (2005) Cyanobacteria in biological soil crusts of India. *Current Science Bangalore* **89**, 515.

Turner, G. and Gibson, A. (1980) Measurement of nitrogen fixation by indirect means. *Methods for evaluating biological nitrogen fixation/edited by FJ Bergersen*.

Ueda, T., Suga, Y., Yahiro, N. and Matsuguchi, T. (1995) Remarkable N2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of nifH gene sequences. *Journal of bacteriology* **177**, 1414-1417.

Unkovich, M. and Baldock, J. (2008) Measurement of asymbiotic N2 fixation in Australian agriculture. *Soil Biology and Biochemistry* **40**, 2915-2921.

Valdés, M., Pérez, N.-O., Estrada-de Los Santos, P., Caballero-Mellado, J., Peña-Cabriales, J.J., Normand, P. and Hirsch, A.M. (2005) Non-Frankia actinomycetes isolated from surface-sterilized roots of Casuarina equisetifolia fix nitrogen. *Applied and environmental microbiology* **71**, 460-466.

Van Dommelen, A. and Vanderleyden, J. (2007) Associative nitrogen fixation. *Biology of the Nitrogen Cycle*, 179.

Vaz-Moreira, I., Egas, C., Nunes, O.C. and Manaia, C.M. (2011) Culture-dependent and culture-independent diversity surveys target different bacteria: a case study in a freshwater sample. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **100**, 245-257.

Videira, S.S., De Araujo, J.L.S., Da Silva Rodrigues, L., Baldani, V.L.D. and Baldani, J.I. (2009) Occurrence and diversity of nitrogen-fixing Sphingomonas bacteria associated with rice plants grown in Brazil. *FEMS Microbiology Letters* **293**, 11-19.

Villegas, M., Vasquez, L. and Valdes, M. (1997) Growth and nitrogenase activity conditions of a new diazotrophic group of filamentous bacteria isolated from Casuarina root nodules. *Revista Latinoamericana de Microbiologia* **39**, 65-72.

Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H. and Tilman, D.G. (1997) Human alteration of the global nitrogen cycle: sources and consequences. *Ecological applications* **7**, 737-750.

Vitousek, P.M., Menge, D.N.L., Reed, S.C. and Cleveland, C.C. (2013) Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* **368**, 20130119.

Vojvodic, A., Medford, A.J., Studt, F., Abild-Pedersen, F., Khan, T.S., Bligaard, T. and Nørskov, J.K. (2014) Exploring the limits: A low-pressure, low-temperature Haber–Bosch process. *Chemical Physics Letters* **598**, 108-112.

Vos, M., Quince, C., Pijl, A.S., de Hollander, M. and Kowalchuk, G.A. (2012) A Comparison of *rpoB* and 16S rRNA as Markers in Pyrosequencing Studies of Bacterial Diversity. *PLoS ONE* **7**, e30600.

Wagner, S.C. (2011) Biological nitrogen fixation. *Nat Educ Knowl* **2**, 14.

Wainwright, M. (2012) *An introduction to environmental biotechnology*: Springer Science & Business Media.

Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and environmental microbiology* **73**, 5261-5267.

Wang, Y., Liu, S., Xu, Z., Han, T., Chuan, S. and Zhu, T. (2006) Ammonia removal from leachate solution using natural Chinese clinoptilolite. *Journal of Hazardous Materials* **136**, 735-740.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**, 697-703.

West, N.E. (1990) Structure and functions of microphytic soil crusts in wildland ecosystems of arid to semi-arid regions. *Advances in ecological research* **20**, 179-223.

White, L.J., Brözel, V.S. and Subramanian, S. (2015) Isolation of Rhizosphere Bacterial Communities from Soil. *Bio-Protocol* **5**.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications* **18**, 315-322.

Wilson, K.H., Wilson, W.J., Radosevich, J.L., DeSantis, T.Z., Viswanathan, V.S., Kuczmarski, T.A. and Andersen, G.L. (2002) High-Density Microarray of Small-Subunit Ribosomal DNA Probes. *Applied and Environmental Microbiology* **68**, 2535-2541.

Woese, C.R. (1987) Bacterial evolution. *Microbiological Reviews* **51**, 221-271.

Wood, M. and McNeill, A. (1993) 15N2 measurement of nitrogen fixation by legumes and actinorhizals: theory and practice. *Plant and soil* **155**, 329-332.

Xie, J.-B., Du, Z., Bai, L., Tian, C., Zhang, Y., Xie, J.-Y., Wang, T., Liu, X., Chen, X., Cheng, Q., Chen, S. and Li, J. (2014) Comparative Genomic Analysis of N2-Fixing and Non-N-2-Fixing *Paenibacillus* spp.: Organization, Evolution and Expression of the Nitrogen Fixation Genes. *Plos Genetics* **10**.

Yang, Z.-Y., Dean, D.R. and Seefeldt, L.C. (2011) Molybdenum Nitrogenase Catalyzes the Reduction and Coupling of CO to Form Hydrocarbons. *The Journal of Biological Chemistry* **286**, 19417-19421.

Yarza, P., Yilmaz, P., Pruesse, E., Glockner, F.O., Ludwig, W., Schleifer, K.-H., Whitman, W.B., Euzeby, J., Amann, R. and Rossello-Mora, R. (2014) Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nat Rev Micro* **12**, 635-645.

Yeager, C.M., Kornosky, J.L., Morgan, R.E., Cain, E.C., Garcia-Pichel, F., Housman, D.C., Belnap, J. and Kuske, C.R. (2007) 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* **60**, 85-97.

Yoshida, N., Inaba, S. and Takagi, H. (2014) Utilization of atmospheric ammonia by an extremely oligotrophic bacterium, Rhodococcus erythropolis N9T-4. *Journal of Bioscience and Bioengineering* **117**, 28-32.

Yoshida, N., Ohhata, N., Yoshino, Y., Katsuragi, T., Tani, Y. and Takagi, H. (2007) Screening of carbon dioxide-requiring extreme oligotrophs from soil. *Bioscience Biotechnology and Biochemistry* **71**, 2830-2832.

Young, J. (1992) Phylogenetic classification of nitrogen-fixing organisms. *Biological nitrogen fixation*, 43-86.

Zehr, J.P. and Capone, D.G. (1996) Problems and Promises of Assaying the Genetic Potential for Nitrogen Fixation in the Marine Environment. *Microbial Ecology* **32**, 263-281.

Zehr, J.P., Jenkins, B.D., Short, S.M. and Steward, G.F. (2003) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environmental microbiology* **5**, 539-554.

Zehr, J.P. and McReynolds, L.A. (1989) Use of degenerate oligonucleotides for amplification of the nifH gene from the marine cyanobacterium Trichodesmium thiebautii. *Appl Environ Microbiol* **55**, 2522-2526.

Zehr, J.P., Mellon, M., Braun, S., Litaker, W., Steppe, T. and Paerl, H.W. (1995) Diversity of heterotrophic nitrogen fixation genes in a marine cyanobacterial mat. *Applied and Environmental Microbiology* **61**, 2527-2532.

Zehr, J.P., Mellon, M.T. and Zani, S. (1998) New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (nifH) genes. *Applied and environmental microbiology* **64**, 3444-3450.

Zengler, K., Toledo, G., Rappé, M., Elkins, J., Mathur, E.J., Short, J.M. and Keller, M. (2002) Cultivating the uncultured. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 15681-15686.

Zhan, J. and Sun, Q. (2011) Diversity of free-living nitrogen-fixing microorganisms in wastelands of copper mine tailings during the process of natural ecological restoration. *Journal of Environmental Sciences* **23**, 476-487.

Zhao, Y., Bian, S.M., Zhou, H.N. and Huang, J.F. (2006) Diversity of nitrogenase systems in diazotrophs. *Journal of Integrative Plant Biology* **48**, 745-755.

Zhaxybayeva, O. and Doolittle, W.F. (2011) Lateral gene transfer. *Current Biology* **21**, R242-R246.

Zheng, L., Cash, V.L., Flint, D.H. and Dean, D.R. (1998) Assembly of iron-sulfur clusters. Identification of an iscSUA-hscBA-fdx gene cluster from Azotobacter vinelandii. *J Biol Chem* **273**, 13264-13272.

Zwart, G., Crump, B.C., Kamst-van Agterveld, M.P., Hagen, F. and Han, S.-K. (2002) Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquatic Microbial Ecology* **28**.

Appendix

1. Appendix 1. GPS locations from sod table and matrix soil samples

Samples	GPS Locations
S1 & M1	N 43° 42.889' W102°32.644', Elevation-2823
S2 & M2	N 43° 42.967' W102°32.370' Elevation-2813
S3 & M3	N 43° 42.721' W102°32.388' Elevation-2823
S4 & M4	N 43° 42.669' W102°32.476' Elevation- 2835
S5 & M5	N 43° 42.736' W102°32.487' Elevation- 2831
S6 & M6	N 43° 42.412' W102°31.944' Elevation- 2820
S7 & M7	N 43° 43.413' W102°32.008' Elevation- 2814
S8 & M8	N 43° 43.409' W102°32.167' Elevation- 2817
S1A & M1A	N 43° 45.503' W102°30.564' Elevation- 2786
S2A &M2A	N 43° 45.507' W102°30.572' Elevation- 2786
S3A & M3A	N 43° 45.513' W102°30.565' Elevation- 2787
S4A & M4A	N 43° 43.411' W102°31.947' Elevation- 2776
S5A & M5A	N 43° 43.425' W102°31.953' Elevation- 2776
S6A & M6A	N 43° 43.454' W102°32.027' Elevation- 2777
S7A & M7A	N 43° 42.889' W102°32.644' Elevation- 2787
S8A & M8A	N 43° 43.425' W102°31.953' Elevation- 2803
S9A & M9A	N 43° 42.968' W102°32.371' Elevation- 2786

2. Appendix 2. Standards analyzed with our samples

Name	N (µg)	^{15}N (at-%)	δ15N (air)
Glutamic Acid (GLU)	4.5	0.11859	
Glutamic Acid (GLU)	8.0	0.11639	
Glutamic Acid (GLU)	19.0	0.11815	
Glutamic Acid (GLU)	47.1	0.11811	
Glutamic Acid (GLU)	95.3	0.11758	
Nylon5	81.0	0.11605	
Nylon5	89.1	0.11638	
Nylon5	75.9	0.11624	
Nylon5	87.4	0.11628	
Nylon5	84.0	0.11630	
Nylon5	85.3	0.11627	
Nylon5	83.3	0.11623	
Nylon5	85.5	0.11619	
Nylon5	88.7	0.11668	
Nylon5	84.2	0.11579	
Nylon5	81.4	0.11635	
Bovine Liver	104.0	0.12462	
USGS-41 (Glutamic Acid)	100.9	0.14100	
USGS-41 (Glutamic Acid)	99.1	0.14085	
REF5 (FIVER)	73.1	5.77333	
REF5 (FIVER)	72.7	5.79482	
REF10	24.9	11.13502	
REF10	23.6	11.15819	
REF20	36.9	20.96322	
REF20	37.7	20.97780	
REF39	18.8	36.21128	
REF39	19.6	38.56760	
REF51	19.7	51.50692	
REF51	19.9	52.57343	

Standards- Known values

Name	**^{15}N (at-%)**	**d15N (Air)**	**%N**
Glutamic Acid (GLT)	0.36475	-4.3	9.52
Glutamic Acid (GLU)	0.36382	-6.8	9.52
Peach Leaves	0.36701	2.0	2.88
Nylon	0.36274	-9.8	
Nylon5	0.36254	-10.3	
Bovine Liver	0.36908	7.7	
USGS-41 (Glutamic Acid)	0.38367	47.6	9.52
REF5 (FIVER)	5.19925		
REF5 (original)	5.24850		
REF10	10.13810		
REF20	19.89340		
REF39	39.42007		
REF51	51.32540		