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Diversity of Free – Living Nitrogen – Fixing Bacteria in Soil of Sioux Prairie of South Dakota

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DIVERSITY OF FREE – LIVING NITROGEN – FIXING BACTERIA IN SOIL OF SIOUX PRAIRIE OF SOUTH DAKOTA

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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.

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CONTENTS

ABBREVIATIONS

α: Alpha

β: Beta

BHI: Brain-heart infusion agar

°C: Degrees Celsius

ADP: Adenosine Di-Phosphate

ARA: Acetylene Reduction Assay

ARB: from Latin *arbor*, tree

ATP: Adenosine Tri- Phosphate

BLAST: Basic Local Alignment Search Tool

BNF: Biological Nitrogen Fixation

bp: base pair

BSC: Biological Soil Crust

BSA: Bovine serum albumin

δ: Delta

Da: Dalton

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleic triphosphate

FLN: Free-living nitrogen (FLN)

HGT: Horizontal Gene Transfer

ITS: Internal Transcribed Spacer

kDa: Kilo Dalton

M: Molar

Min: minute

mM: Milimolar

M_r: Molecular Weight

µ: Micro

MS: Mass Spectrometry

NCBI: National Center for Biotechnology

NFM: Nitrogen Free Medium

OTU: Operational Taxonomic Unit

PCR: Polymerase Chain Reaction

R2A agar: Reasoner's 2A agar

RDP: Ribosomal Database Project

RFLP: Restriction Fragment Length Polymorphism

RNA: Ribonucleic acid

rRNA: Ribosomal ribonucleic acid

SDSU: South Dakota State University

Sec: Second

TSB: Tryptic soy broth

TSA: Tryptic-soy agar

V: Volt

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ABSTRACT

DIVERSITY OF FREE – LIVING NITROGEN – FIXING BACTERIA IN SOIL OF SIOUX PRAIRIE OF SOUTH DAKOTA

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There are only two natural ways by which molecular nitrogen can be available to support life, either by free-living or by symbiotic nitrogen fixing bacteria. The best studied diazotrophs form symbiotic associations with plants, primarily legumes and certain tree species, but little is known about how non-leguminous plants such as grasses obtain nitrogen in their environment. Natural prairies have few legumes, thus have less symbiotic nitrogen fixer interaction. This indicates presence of free-living nitrogen (FLN) fixation activity towards the balance of the N cycle. The objective of this study was to characterize the culturable diversity of free-living diazotrophs in native prairie. I isolated bacteria using Nitrogen Free Medium (NFM). Bacteria growing on NFM were sub-cultured with repeated steps to obtain single isolates. The phylogeny tree of 458-putative diazotrophs was determined using the 16S rRNA gene. The PolF/PolR primer set was used for *nifH* PCR to confirm the presence of nitrogenase, required for the reduction of molecular nitrogen to ammonia. Due to poor performance of multiple *nifH* primer sets various approaches to improve amplification efficiency were evaluated focusing on addition of Dimethyl Sulfoxide and Bovine Serum Albumin, and glycerol alone or combination. Of 458 putative diazotrophs, 96 yielded *nifH* by PCR. The sequences were analyzed using ARB and R. High diversity was found among FLN fixers, including

alpha-, beta-, and gamma – *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*, but the largest group were *Streptomyces.* Out of 115 *Streptomyces*, 40 yielded *nifH* amplicons aligning with those of unrelated taxa such *Mesorhizobium*, *Paenibacillus*, and *Herbaspirillium*. Whole genome sequencing of three *Streptomyces* revealed presence of *Bacillus*, indicating co-culture. Purity of the isolates obtained was confirmed using phylum-specific 16S rRNA gene primers for *Actinobacteria* and *Firmicutes* as well as alpha and beta – *Proteobacteria*. This revealed that many of the putative pure cultures had a second strain belonging to a different phylum. In conclusion, there is a considerable diversity in the free-living nitrogen fixing bacterial community in prairie soil. Additionally, our data indicate apparent bacterial bi-cultures associated with nitrogen cycling. These findings could help us understand the dynamics of nitrogen cycling in naturally sustained prairie grassland ecosystem.

Chapter 1: Literature Review

1. Diversity of Free-Living Nitrogen-Fixing Bacteria in Soil of diverse Ecosystems

1.1. Nitrogen is Important for All Forms of Life

Nitrogen is a very significant factor for all life forms and it is considered a huge part of our daily lives (Vance 2001). Nitrogen makes up a portion of amino acids and nucleic acids, both of which are required as a part of multiple structures in our bodies. In fact, 2.6 % of our body is nitrogen (Galloway and Cowling 2002). Amino acids are the primary building blocks of all proteins. Proteins include not only structural components such as tissue, muscle, and organs, but also enzymes and hormones that are extremely important for the functions of all living things. Urea is a byproduct of protein digestion (Smil 2002). Organic nitrogen, which is chemically incorporated with carbon, is significant for excretory and septic system in bodies to help in discarding food and removal of infected agents (Vance 2001). After water growth and reproduction in plants rely essentially on nitrogen, and it is the primary factor affecting plants in terrestrial and aquatic ecosystems (Vitousek, Aber et al. 1997). For instance, 20 to 40 kg N ha^{-1}per 3 to 5 month are used in crops like wheat, rice, and maize (Peoples and Craswell 1992). Plants with low levels of nitrogen show poor development. If nitrogen runs out, plants will die affecting herbivores and therefore the entire food chain.

The air consists primarily of nitrogen. Atmospheric air contains 78.1 N_2 (Cheng 2008). Eukaryotes, including plants and mammals cannot use gaseous nitrogen (N_2) because it is inert (Xiang, Wang et al. 2016). Nitrogen (N_2), also known as dinitrogen, consists of two atoms with a triple bond, needs a high level of energy to split the bonds,

and must be converted to either ammonia (NH_4^+) or nitrate (NO_3^-). Therefore, nitrogen has to go through a nitrogen cycle (Cheng 2008).

1.2. Nitrogen cycle

Nitrogen is essential to life because it is a key component of nucleic acids and proteins. Nitrogen occurs in many different forms and is cycled among these forms by a diversity of bacteria. Although nitrogen is abundant in the atmosphere as diatomic nitrogen gas N_2, it is extremely stable as previously mentioned, and conversion to other forms requires a great level of energy.

The biologically available forms NO_3^- and NH_4^+ have been limiting; however, current anthropogenic processes, such as fertilizer production, have increased the availability of nitrogen to living organisms. The cycling of nitrogen among its many forms is a complex process that involves a variety of bacteria and environmental conditions. The Nitrogen cycle can be simply defined as converting nitrogen gas by fixing it to organic nitrogen via certain microorganisms, followed by releasing it back into the environment (Vitousek, Aber et al. 1997, Cheng 2008).

In general, the nitrogen cycle has five steps: Nitrogen fixation (N_2 to NH_3/ NH_4^+), Nitrification (NH_3 to NO_3^-), Assimilation (Incorporation of NH_3 and NO_3^- into biological tissues), Ammonification (organic nitrogen compounds to NH_3), and lastly Denitrification (NO_3^- to N_2) (Fig 1.1). To understand the nitrogen cycle, we must first define each step. (Igarashi and Seefeldt 2003).

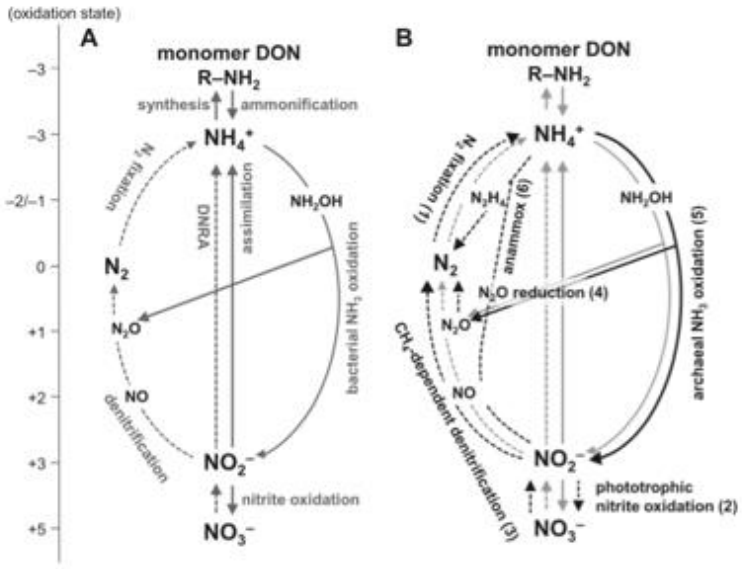

Figure 1.1 Simplified Nitrogen cycle. Retrieved from (Isobe and Ohte 2014)

Nitrogen fixation is the process by which gaseous nitrogen (N_2) is converted to ammonia (NH_3 or NH_4^+) by biological fixation through high-energy physical processes. N_2 is very stable and an extreme level of energy is required to break the bonds which link the two N atoms. N_2 can be converted directly into NO_3^- and then produce ammonia (NH_3)by reaction with hydrogen through processes that catalyze a massive amount of pressure, heat, and energy (Kanamori, Weiss et al. 1989) (Nandasena, O'Hara et al. 2007).

A greater amount of biologically available nitrogen is naturally produced by the biological conversion of N_2 to NH_3/ NH_4^+. A diverse group of bacteria and cyanobacteria have an ability to use the enzyme nitrogenase to break the bonds between the molecular nitrogen and combine it with hydrogen. Nitrogenase only can work in the absence of oxygen. The exclusion of oxygen is done by many ways. Some bacteria live underneath layers of oxygen-excluding slime on the roots of certain plants. The most substantial soil

dwelling nitrogen-fixing bacteria, Rhizobium, live in oxygen-free zones in nodules on the roots of legumes and some other woody plants. Aquatic filamentous Cyanobacteria can use oxygen-excluding or free cells called heterocyst (Berman-Frank, Lundgren et al. 2003).

Nitrification is a two-step process in which NH_3/ NH_4^+ is converted to NO_3^-. First, the soil bacteria *Nitrosomonas* and *Nitrococcus* convert NH_3 to NO_2^-, and then another soil bacterium, *Nitrobacter,* oxidizes NO_2^- to NO_3^-. These bacteria get energy through these conversions, both of which require oxygen to take place (Cheng, Lin et al. 1996). Assimilation is the process by which plants and animals incorporate NH_3 ammonia and nitrate NO_3^- formed through nitrogen fixation and nitrification. Plants take up these forms of nitrogen through their roots and incorporate them into proteins and nucleic acids. Animals are then able to utilize nitrogen from the plant tissues. Assimilation produces large quantities of organic nitrogen, including proteins, amino acids, and nucleic acids (Robertson, Goodrich et al. 2011).

Ammonification is the conversion of organic nitrogen into ammonia. The ammonia generated by this process is excreted into the environment and is then available for either nitrification or assimilation (Kanamori, Weiss et al. 1989).

Denitrification is the reduction of NO_3^- to gaseous N_2 by anaerobic bacteria. This process only occurs where there is little to no oxygen, such as deep in the soil near the water table. Hence, areas such as wetlands provide a valuable place for reducing excess nitrogen levels via denitrification processes (Seitzinger 2008) (Schipper, Gold et al. 2010).

In summary, nitrogen cycles between the air, water and soils, with many transformations occurring by specialized bacteria. Some of these transformations need aerobic conditions while others occur only under anaerobic conditions (Galloway and Cowling 2002, Cherkasov, Ibhadon et al. 2015).

1.3. Biological Nitrogen Fixation

Nitrogen fixation is a process to reduce nitrogen (N_2) in the atmosphere into ammonia (Berman-Frank, Lundgren et al. 2003). There are several methods to fix nitrogen. Some of them are natural: lightning and biological fixation. Others are industrial fixation like chemical or Haber-Bosch process to form NO_3^- or NH_3. Nitrogen fixation through lightning contributes about 1% of the ammonia fixed into the biosphere (Noxon 1976). Biological fixation is an important reaction on the earth, converting dinitrogen molecules to ammonia by a special kind of bacteria termed diaztrophs that fix about 50% of the net nitrogen per year (Vitousek, Cassman et al. 2002). The other 50% is fixed industrially by chemical processes, primarily using Haber process (Finan 2002). This process depends on iron catalysts to convert atmospheric nitrogen to ammonia. Under extreme conditions like high temperature (650-750K), high pressure around 100 bars, and hydrogen in order to weaken the dinitrogen bonds (Appl 1997). The Haber Bosch process has provided agricultural lands with much needed nitrogen fertilizers in the beginning of twentieth century (Erisman, Sutton et al. 2008). However, there are several negative impacts of industrial nitrogen fixation, including water eutrophication, soil acidification , and nitrogen oxide emission (Refsgaard, Halberg et al. 1998, Dixon and Kahn 2004). On the other hand, biological fixation is efficient for agriculture production (Bazhenova and Shilov 1995). Biological nitrogen fixation is accomplished

when dinitrogen is transformed to ammonia by an enzyme complex named nitrogenase (Fig.1.2)(Strandberg and Wilson 1967). In this process, triple bond stability makes activation and reduction very energy demanding. *Six* electrons are needed; *eight* is consumed because H_2 must be produced.

Electron donor → dinitrogenase reductase → dinitrogenase →N_2

ATP required to lower reduction potential (total 16) (Postgate 1982) :

$$8H^+ + 8e^- + N_2 \rightleftharpoons 2NH_3 + H_2$$

This process needs 16 molecules of ATP to fix one molecule of nitrogen into two molecules of ammonia (Igarashi and Seefeldt 2003):

$$N_2 + 8\,H^+ + 8\,e^- + 16\,ATP \longrightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i$$

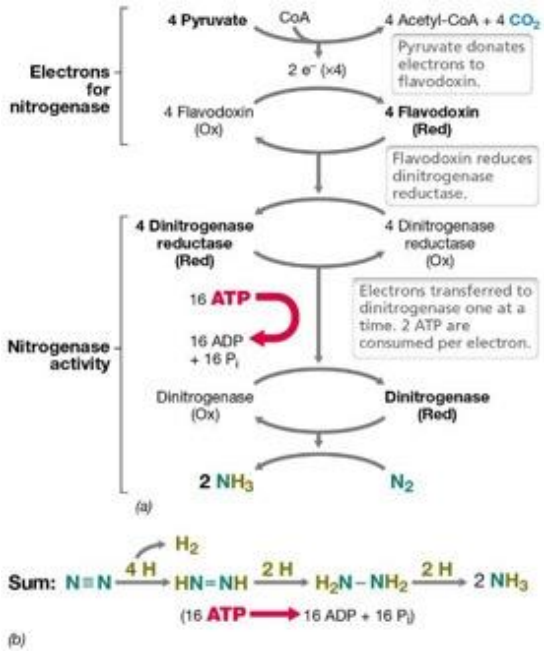

Figure 1.2 Electron flow in biological nitrogen fixation. Retrieved from (Madigan, Bender et al. 2018)

1.3.1. Biochemistry of Nitrogen Fixation

Biological nitrogen fixation was discovered by Beijerinck in 1901 (Vance 2001, Wagner 2011). Biological nitrogen fixation is the most significant natural process on the earth after photosynthesis and it can be defined as the conversion of atmospheric nitrogen (N_2) to ammonia (Robson and Postgate 1980). It is carried out by prokaryotes (Bacteria and Archaea) called diazotrophs including free living diaztrophs such as *Burkholderia, Azotobacter*, *Azospirillum*, *Bacillus*, and *Clostridium*. Many are symbiotic nitrogen fixing bacteria such as bacteria which form symbiosis with legumes like *Rhizobium*, and bacteria that form symbioses with actinorhizal plants such as *Frankia*. Some are associative nitrogen fixing bacteria like Cyanobacteria which associated with cycads (Postgate 1982). The only known Nitrogen fixers in Archaea are among the methane-producing bacterium called methanogens (Dixon and Kahn 2004).

The nitrogenase enzyme complex in nitrogen-fixing bacteria is responsible for the reduction of molecular nitrogen to ammonia (Peters, Fisher et al. 1994, Hoffman, Lukoyanov et al. 2013). It consists of two groups. Grope-1 contains three closely related, but genetically different nitrogenase enzymes which are called molybdenum (Mo) nitrogenase, vanadium (V) nitrogenase, and iron-only (Fe) nitrogenase. All three types of nitrogenase consist of two multi subunit metalloproteins, as shown in (Fig 1.3). Component I (catalytic subunit or dinitrogenase (FeMo), contains two α subunits and two β subunits, weighing approximately 240kDa), and component II (nitrogenase iron protein, or dinitrogenase reductase (Fe), contains one [Fe_4S_4] cluster and weighs approximately 60-64kDa). Both components contain iron-sulfur clusters (Studt and Tuczek 2005). This enzyme stimulates the MgATP-dependent reduction of N_2 to ammonia. Dinitrogenase links and reduces dinitrogen molecules, while dinitrogenase

reductase reduces dinitrogenase proteins. In contrast, Group-2 comprises of a single agent called *Streptomyces thermoautotrophicus* nitrogenase. It was believed to be completely insensitive to the presence of oxygen (O_2), which is an inhibitor of nitrogen fixation (Burgess and Lowe 1996). More recent reports have questioned its nitrogen-fixing activity (MacKellar, Lieber et al. 2016).

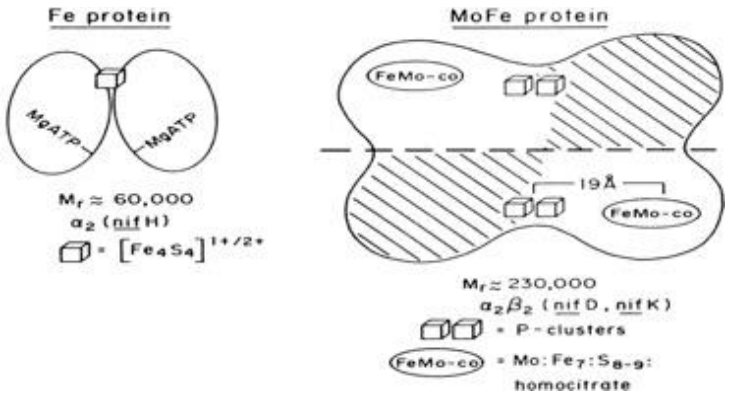

Figure 1.3 The two-component protein complexes making up nitrogenase. Retrieved from (Burgess and Lowe 1996)

1.3.1.1. Structure and the domain of molybdenum containing nitrogenase

The nitrogenase complex, as shown in the Fig 1.3, consists of two purified proteins: Protein I, the homodimeric Fe protein (nitrogenase reductase) which converts high reducing power to provide electrons in order to reduce the nitrogenase protein. The function of this protein is to transfer electrons to the MoFe protein. The transfer requires chemical energy which is result of the binding and hydrolysis of adenosine triphosphate (ATP), which is responsible of bringing the Fe protein and MoFe protein closer together to simplify electron transfer. Protein II, the heterotetrameric MoFe protein (nitrogenase) utilizes the electrons obtained to reduce N_2 to NH_3. This protein contains two α subunits, two β subunits, two iron – sulfur clusters called P- clusters which are at the interface

between the α and β subunits, and two cofactors inside the α subunits (Oelze 2000). As shown in Fig 1.4, P- clusters as the core consist of two (Fe8S7) cubes linked by sulfur atom at the center. Each P- cluster is connected to MoFe protein by cysteine remains. Each FeMo cofactor (Fe7MoS9C) holds two non-similar clusters: (Fe4S3) and (MoFe3S3), which are linked by three sulfide ions. One histidine residue and one cysteine residue can bind the FeMo cofactor to the α subunit of the protein covalently.

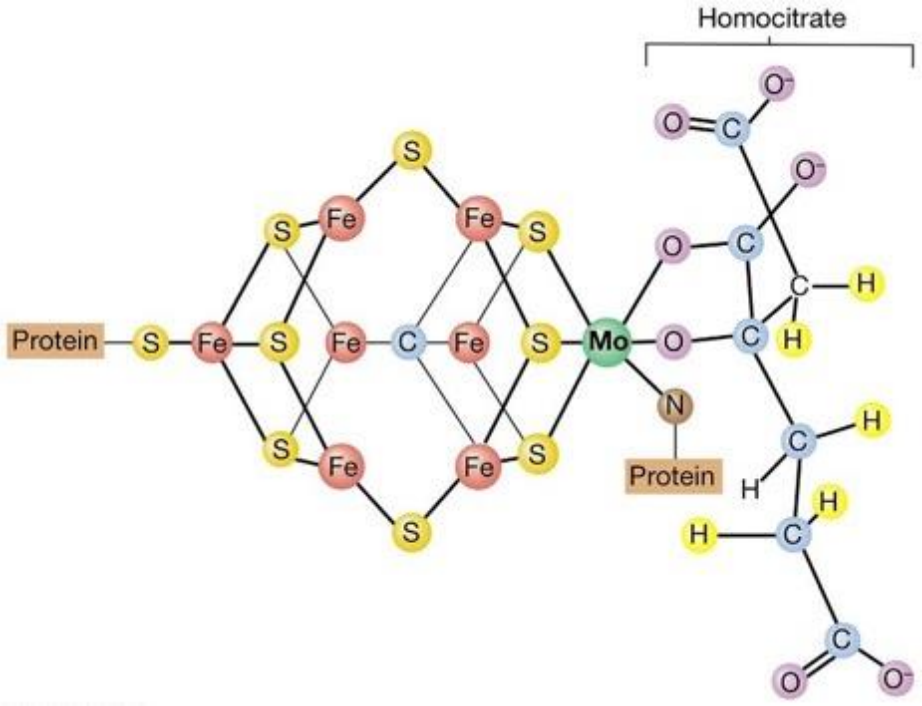

Figure 1.4 Structure of the FeMo cofactor showing the sites of binding to nitrogenase at the amino acids Cys and His. Retrieved from (Thorneley and Ashby 1989, Madigan, Bender et al. 2018)

The electrons provided from the Fe protein enter the P-cluster in the FeMo protein. Then the P-cluster transfers the electrons to the FeMo cofactors. Each FeMo cofactor then works for nitrogen fixation, with N_2 binding in the central empty space

within the cofactor. From here, Fe-N interactions lead to weakening of the highly strong triple bond that links the two nitrogen atoms, decreasing the chemical energy of activation required for reduction. Finally, Nitrogenase links each atom of nitrogen to three hydrogen atoms to produce ammonia (NH_3), which shape glutamine from glutamate. In addition, the nitrogenase reaction produces molecular hydrogen as a side product (Einsle, Tezcan et al. 2002, Lukoyanov, Pelmenschikov et al. 2007).

The net overall reaction is:

$$N_2 + 8\,H^+ + 8e^- + 16\,ATP \longrightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i$$

The molybdenum nitrogenase (Nif) is the most studied of all nitrogenases (Arber, Dobson et al. 1987). The genes encoding molybdenum nitrogenase are *nifH, nifD, nifK* (Hoffman, Lukoyanov et al. 2013). There are multiple essential *nif* genes conserved across diazotrophs that are required to catalyze the nitrogenase reactions, *nifB, nifE, nifN, nifX, nifU, nifS, nifV, nifW,* and *nifZ* (Fig. 1.5).

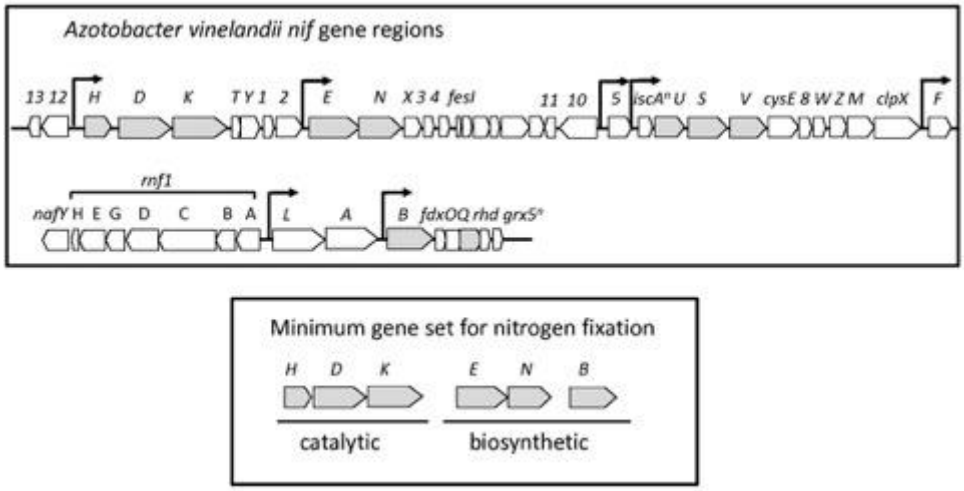

Figure 1.5 Azotobacter vinelandii nif *genes showing minimum genes required for nitrogen fixation. Retrieved from (Einsle, Tezcan et al. 2002)*

The Mo-nitrogenase from a diversity of bacterial genera displays a high level of primary amino acid sequences identity. The conservation is highest in the regions of MgATP- and metallocluster-binding sites (Schneider, Muller et al. 1995). The Mo-nitrogenase consist of two separates metalloproteins: Group-I, MoFe protein and Fe protein. The Fe-protein component encoded by *nifH*, is a homodimer (~60kDa) molecular mass. A single [4Fe-4S] cluster can link two identical subunits through 2 Cys residues covalently linked to the Fe-atoms from each unit. Each subunit has a binding site for MgATP/MgADP (Fig.1.6) (Burgess and Lowe 1996). The Fe protein is involved in the transfer of electros to the MoFe protein during the process of MgATP hydrolysis, the Fe protein is also involved in the biosynthesis of FeMo cofactor and is perhaps associated with the regulation of alternative nitrogenases (Seefeldt, Hoffman et al. 2009). Group-II, MoFe protein encoded by nifD & nifK, is an $\alpha 2\beta 2$ heterotetramer (~240kDa) molecular mass (Soboh, Boyd et al. 2010). It consists of two metalloclusters: the prothetic groups, namely, the P-cluster [8Fe-7S] and the iron-molybdenum cofactor (or FeMo-cofactor or the M center) [Mo-7Fe-9S- homocitrate-X](Seefeldt, Hoffman et al. 2009). The P-cluster is located at the interface of the α/β- subunit and linked by 6 cysteine resides (Eady 1996). In this structure, two [4Fe-4S] clusters link covalently with a shared Sulfur atom (Lobo and Zinder 1988). X-ray crystallography of P- cluster displayed that the P-cluster is a mediating factor to transfer electrons from the Fe protein to the FeMo cofactor (Hu and Ribbe 2011).

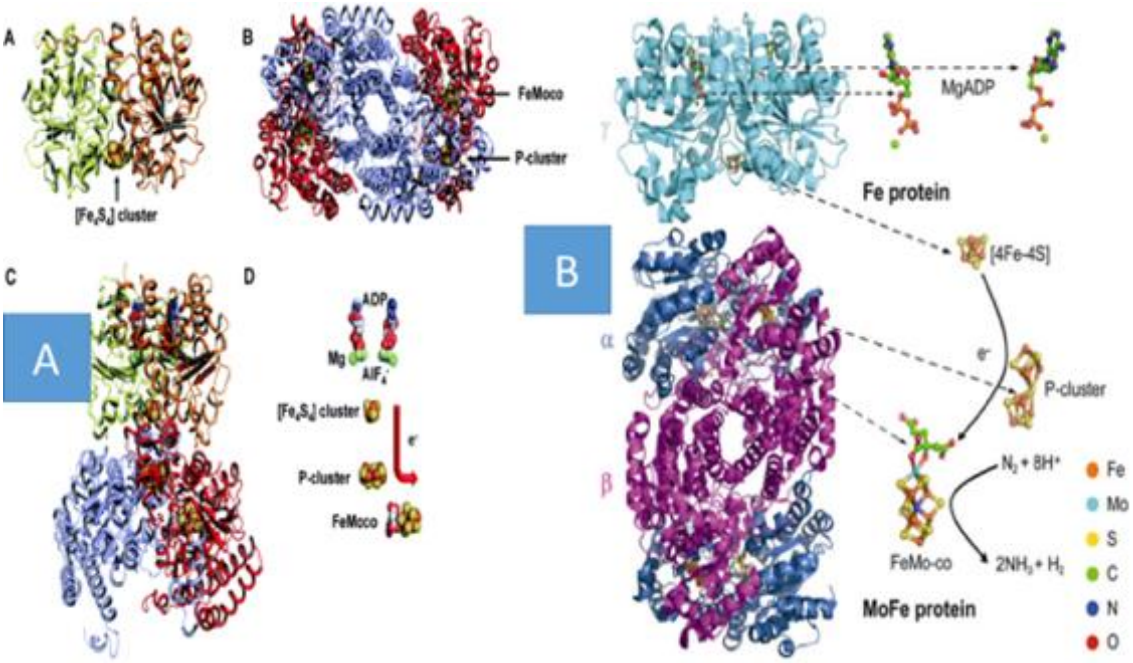

Figure 1.6 The Molybdenum Nitrogenase: Figure A shows the structure of the Fe and MoFe. Figure B shows transfer of electrons to the MoFe protein during the process of MgATP hydrolysis Retrieved from (Hu, Lee et al. 2012)

1.3.1.2. The Vanadium nitrogenase

The vanadium nitrogenase (Vnf) can be found in nitrogen fixing bacteria like *Azotobacter vinelandii* , *A. chroococcum* (Rehder 2000), and cyanobacteria like *Anabaena variabilis.* It also has Vnf (Thiel 1993, Heiniger and Harwood 2015). It is considered an alternative to molybdenum nitrogenase when molybdenum is not available (Fisher, Dilworth et al. 2006). Unlike the Mo-nitrogenase, The V-nitrogenase needs lower temperatures to work more efficiently (Miller and Eady 1988). It is similar to the molybdenum nitrogenase, consisting of two metalloprotein compounds, an Fe-protein and an iron-heterometal VFe-protein (Thiel 1993) (Robson, Woodley et al. 1989). The Fe-protein in V-nitrogenase has a close similarity to Mo-nitrogenase in structure and

function and is encoded by *vnfH* (Zhao, Bian et al. 2006, Hu and Ribbe 2011). It shares 91% homology to sequences of the Fe-protein encoded by *nifH* (Hu and Ribbe 2011). It additionally possesses the conserved Gly-X-Gly-X-X-Gly (X here refers to any amino acid) combined nucleotide-binding motif, and conserved Cys ligand for a subunit of the Fe-protein of molybdenum nitrogenase. There are two subunits of the Fe-protein of vanadium linked by a ferredoxin [4Fe-4S], and consist of two binding sites for $Mg2^{+}$ ATP (Thorneley and Ashby 1989, Rehder 2000).

The structure of the VFe protein is similar to molybdenum nitrogenase (Crans, Smee et al. 2004). Characterization of the vanadium iron (VFe) protein reveals a hexameric structure containing an alpha (2), beta (2), and delta (2), subunits or ($\alpha 2\beta 2\delta 2$), encoded by vnfD, vnfK, and vnfG. The difference between Nif and Vnf is in the subunit structure, V-nitrogenase possessing three subunits ($\alpha 2\beta 2\ \gamma 2$) while Mo-nitrogenase possesses two subunits ($\alpha 2\beta 2$). The V-nitrogenase, an alternative form of nitrogenase used in absence of molybdenum, is a binary system which is composed of iron protein and vanadium-iron (V-Fe_ protein). The V-nitrogenase is found in some members of the bacteria, for example in Azotobacter, Anabaena variabilis and Rhodopseudomonas palustris. The mode of action of V-nitrogenase during catalysis of reduction of nitrogen to ammonia is similar to its counterpart Mo-nitrogenase. The two proteins present in V-nitrogenase form a functional complex which allows the transfer of electrons required for the reduction of N2 (Hu, Lee et al. 2012).

1.3.1.3. The Iron-only nitrogenase

The Iron-only nitrogenase (Anf) is the second alternative nitrogenase. So far, Chisnell *et al* have isolated this enzyme from a *nifHDK* deleted strain of *A. vinelandii* (Chisnell, Premakumar et al. 1988), *Rhodospirillum rubruum* (Lehman and Roberts 1991), and *Rhodobacter capsulatus* (Schneider, Müller et al. 1991). This is the third nitrogen-fixing system that has been discovered till date, the other two being Mo-nitrogenase and V-nitrogenase (Crans, Smee et al. 2004). The iron-only nitrogenase works only if the molybdenum and vanadium nitrogenase are absent (Eady 1996). It is encoded by *anf* genes (Joerger, Jacobson et al. 1989). The iron-only nitrogenase is different to the molybdenum and vanadium nitrogenase. It is very sensitive to oxygen, unstable, and has the lowest activity (Chisnell, Premakumar et al. 1988, Crans, Smee et al. 2004, Kennedy, Rudnick et al. 2005). There are some organisms which have all three systems and the systems are expressed as per the availability of the metal in surroundings.

The most preferred is the Mo-nitrogenase but in unavailability of molybdenum, V-nitrogenase would be active and in absence of both molybdenum and vanadium, iron-only nitrogenase would be functioning (Smith and Eady 1993). Like V-nitrogenase, Fe-only nitrogenase also has two components of metalloprotein: component I, the Fe-Fe protein, component II, the Fe protein. The Fe-protein has obligation to transfer electrons to Fe-Fe protein in Mg_2^+ ATP reaction (Schneider and Müller 2004). The Fe-protein is a homodimer (molecular weight ~32.5kDa) joined by a Fe_4-S_4 cluster. The Fe-protein is encoded by *anfH* (Zhao, Bian et al. 2006, Hu and Ribbe 2015). Sixty % homology of the *anfH* gene is shared with the *nifH* of Mo-nitrogenase and the *vnfH* of V-nitrogenase

(Eady 1996, Noda, Ohkuma et al. 1999). Like V-nitrogenase, Fe-only nitrogenase is also a hexameric protein with three subunits ($\alpha_2\beta_2\ \delta_2$). The genes which control the synthesis of Fe-nitrogenase are *vnfD, vnfG, vnfK* (Eady 1996).

1.3.1.4. Oxygen insensitive Streptomyces thermoautotrophicus nitrogenase

Streptomyces thermoautotrophics (strain UBT1) is a thermophilic chemolithoautotroph which has been discovered as an exceptional species which contains nitrogenase that is insensitive to oxygen (Kim, Falconer et al. 1998).

Oxygen-insensitive nitrogenase was isolated from the *Streptomyces thermoautotrophicus* in 1992. This strain can grow and fix nitrogen in aerobic conditions with hydrogen and oxygen (Gadkari, Mörsdorf et al. 1992). It is a different nitrogenase enzyme from the general three forms, Mo-nitrogenase, V-nirogenase, and iron-only nitrogenase. The nitrogenase enzyme contains two components: Component I (dinitrogenase) is MoFeS which is heterotrimer, and component II is manganese-superoxide oxidoreductase instead of Fe protein (Zhao, Bian et al. 2006). This enzyme is irreversibly and rapidly damaged by oxygen; thereby, causing the aerobic nitrogen fixing microorganisms to evolve mechanisms for protecting the nitrogenase enzyme from the damage by oxygen.

This novel molybdenum-nitrogenase catalyses the reduction of nitrogen by coupling the reaction to carbon monoxide oxidation in which oxygen is reduced to superoxide. The superoxide formed is re-oxidised to form oxygen. This step transfers the electrons to the nitrogenase; thereby, reducing the nitrogen to form ammonia. Thus, the

activity of this oxygen-insensitive nitrogenase depends on the generation of an electron donor (superoxide anion) (Ribbe, Gadkari et al. 1997).

1.4. Diversity of Nitrogen fixing Bacteria

Bacteria which can fix nitrogen in the atmosphere into fixed forms of nitrogen, i.e. inorganic compounds that can be used by the plants, are known as nitrogen-fixing bacteria. These organisms play a vital role in nitrogen fixation and are termed diazotrophs (Raymond, Siefert et al. 2004). Bacteria and Archaea diazotrophs are devided into three types based on growth and oxygen requirements, heterotrophs like *Frankia* as an aerobe and *Klebsiella* as a facultative anaerobe, *Clostridium* as anaerobe, phototrophs like *Anabaena* and *Rhodobacter*, as well as chemolithotrophs like *Leptospirillum ferroxidans*. Based on the life style of diazotrophs, there are generally three categories of nitrogen-fixing (Unkovich and Baldock 2008), free-living nitrogen-fixers, symbiotic nitrogen-fixers, as well as associative nitrogen-fixers (Dixon and Kahn 2004).

1.4.1. Origins and Evolution of Nitrogenase

The origin and evolution of nitrogenase enzyme is an interesting question. In order to understand the evolutionary origin of nitrogenase, it is important that the additional selective pressures which impact the distribution and stability of diazotrophy are also considered (Young 1991). Mo-independent forms of nitrogenase (V and Fe) were responsible for N_2 fixation on early Earth because oceans were Mo-depleted and Fe-rich. Phylogenetic- and structure-based examination of multiple nitrogenase proteins suggests that such an evolutionary path is unlikely. The ancestors of nitrogenase possessed an

open cavity which had capacity to bind with the metal clusters, conferring reactivity. The availability of fixed nitrogen and some factors influencing the availability of metals in the local environment controlled the evolution of the ancestors of nitrogenase enzyme (Gtari, Ghodhbane-Gtari et al. 2012).

There are two conflicting hypotheses depended on the distribution of the conservation of nitrogenase across the Archaea and Bacteria (Young 1991): The first one considers the last global ancestor of Bacteria and Archaea had an old function in nitrogen fixation, which transmitted vertically (Klucas, Koch et al. 1968). However, the progeny widely lost *nif* gene (Berman-Frank, Lundgren et al. 2003). The second hypothesis speculates that nitrogen fixation emerged after photosynthesis in anaerobic conditions, and was lost in most the offspring over horizontal gene transfer (Gtari, Ghodhbane-Gtari et al. 2012).

1.4.2. Life Style of Nitrogen Fixers

The microorganisms that fix nitrogen are found in diverse environments including soil, water, plants, and mosses, either in free living form or in symbiotic form. These organisms convert the free nitrogen present in the atmosphere into the forms that are absorbed by plants (Vitousek, Cassman et al. 2002). This reaction occurs in these organisms as part of their metabolism. These organisms employ these nitrogenase enzyme for catalysis of nitrogen reduction into ammonia. The free-living nitrogen fixers live independently in the soil and obtain their nutrition from the soil only; however, the symbiotic nitrogen fixers live in association with the plant roots, receive energy sources from the plant roots, and in turn make nitrogen available to them. Thus, it is a mutualistic relation between the plant and symbiotic nitrogen fixers (Crews 1999). The amount of

nitrogen fixed by symbiotic and associative bacteria is higher than by free living bacteria. Symbiotic bacteria fix around 70% of atmospheric nitrogen while free-living bacteria fix 30% of dinitrogen (Pinto-Tomas, Anderson et al. 2009). In symbiotic diazotrophs, the host plant is able to provide carbon sources as an energy source and protect the nitrogenase from oxidative deactivation (Glick, Patten et al. 1999) . Anaerobic and facultative anaerobic bacteria have the capability to fix nitrogen into ammonia only in the absence of oxygen. Aerobic bacteria can fix nitrogen in the presence of low concentrations of oxygen. Nitrogen fixation rates decline with the increasing level of oxygen in the immediate atmosphere (Olivares, Bedmar et al. 2013).

1.4.2.1. Nitrogen Fixing Symbionts:

A symbiotic associative life style can occur between heterocystous cyanobacteria and cycads, *Frankia* and *Altus,* and *Rhizobium* in nodules of legumes (Soltis, Soltis et al. 1995, Zahran 1999).

Rhizobium is a nitrogen fixing bacteria which lives in symbiotic association with leguminous plants while *Frankia* makes symbiotic associations with certain non-leguminous plants (Oldroyd 2013). The first step in this association is the formation of a root nodule in which these bacteria live. The nodules are small root outgrowths which are formed by the interaction between the roots of the host plant and the bacteria (Lobo and Zinder 1988). The bacteria attach at the epidermal and root hair cells and start to multiply, and form colonies within the roots. This leads to the curling of the root hairs. The bacteria then attach the cortex from where the formation of nodule sets. Bacteria are carried to the cortex via an infection thread. After reaching the cortex, the specialized

nitrogen-fixing cells start differentiating and form the nodule. A vascular connection is established between the nodule and the host plant for commutation of nutrients. Free nitrogen in the atmosphere is converted to ammonia by the bacteria in the nodule. This ammonia formed is then changed into forms which the host plant utilizes for its growth and development (Lindow and Brandl 2003, Oldroyd 2013).

Owing to the complexity of its function, nitrogenase needs a lot of energy to transform nitrogen to different usable forms of nitrogen like ammonia (Orr, James et al. 2011). Free-living bacteria must obtain the necessary nutrients that they need to gain energy. There are other types of bacteria that have developed symbiotic associations which supply them with a source of energy and carbon for the bacterium's own synthetic reactions by providing them with sugars (Paerl, Fitzpatrick et al. 1996). In turn, the bacteria supply the plant with fixed nitrogen needed for its growth. Growth factors are discussed in the bacterial genera *Rhizobium* and *Bradyrhizobium* which have developed symbioses with the *Fabaceae* or legume family. A host plant's roots are infected with seedlings containing bacteria which then makes the plant surround the bacteria with hair on the roots. The specificity of the relationship between a plant and bacteria is regulated by identification that will hinder the wrong species of bacteria from living in a plant that it is incompatible with (Paerl, Fitzpatrick et al. 1996). Eventually, the plant where the bacteria has developed in creates a specialized structure, or nodule, during that time the bacteria grow into large forms called bacteroids. Oxygen is known to inhibit nitrogenase, which is why it is important to closely regulate the oxygen concentration inside a nodule (Dreyfus, Garcia et al. 1988).

1.4.2.2. Associative Nitrogen Fixing Bacteria

The associative nitrogen bacteria locate between free-living bacteria and symbiotic nitrogen fixers (Dart 1986). Associative nitrogen fixing bacteria refers to the associative symbiosis or interaction of rhizosphere bacteria such as *Azospirillum* with the host plant (Haahtela, Laakso et al. 1986). These bacteria make association with the roots of grasses. Apart from fixing nitrogen, these bacteria are also able to produce cytokinins and gibberellins. The associative nitrogen fixation is considered to be quite valuable for agriculture (Hansen, Nienhuiskuiper et al. 1990). This form of biological nitrogen fixation is mostly utilized in cereal crops and grasses (Lederberg, Alexander et al. 2000).

1.4.2.3. Free Living Nitrogen Fixers

Organic compounds, which are utilized and needed by plants, are incorporated when free-living nitrogen-fixing bacteria reduce the nitrogen, which is in its gaseous state, to ammonia (Orr, James et al. 2011). Aerobic bacteria (e.g. *Azotobacter*), anaerobic bacteria (e.g. *Rhodospirillum*) and cyanobacteria (e.g. *Anabaena, Nostoc*, etc) live freely in soil or in water and cause atmospheric nitrogen to become fixed. They first convert the nitrogen into ammonia and then combine it with the forms that are absorbed by the plants. The catalyst of this reaction is the enzyme complex nitrogenase. The activities of nitrogenase is highly sensitive to traces of oxygen, even in small amounts (Hu and Ribbe 2015). Free-living nitrogen fixing bacteria fix about one-tenth of the nitrogen compared to symbiotic bacteria (Unkovich and Baldock 2008). The majority of free living bacteria can fix nitrogen in anaerobic conditions (Wagner 2011).

Nearly all nitrogenase enzyme systems studied have a similar key property which is their sensitivity to oxygen. The nitrogenase enzyme is inactivated within seconds of exposure to oxygen. This is an important feature which is emphasized by the different ways in which diazotrophs have developed to avoid contact or exposure to oxygen (Sabra, Zeng et al. 1999, Ureta and Nordlund 2002). Mechanisms like high rates of respiration remove oxygen from the cell surface and reverse the conversion of the enzyme into an inactive state, helping to protect nitrogenase from oxygen exposure (Sabra, Zeng et al. 1999). In order to avoid excessive rates of oxygen transfer, effective oxygen barriers on the surface of the cell can protect nitrogenase, for example mucoid slime material (Sabra, Zeng et al. 1999). This makes the bacteria almost impenetrable to oxygen.

Some organisms have their own ways of protecting the enzyme from oxygen which are manifested in unique ways. It is only under anaerobic conditions that the *nif* genes of *Klebsiella pneumonia*, which is a facultative anaerobe, are expressed (Fouts, Tyler et al. 2008). The cyanobacteria have evolved a variety of strategies for fixing nitrogen from the atmosphere (Peoples and Craswell 1992, Mehta and Baross 2006). There are some photosynthetic filamentous cyanobacteria whose nitrogen fixation takes place in the heterocyst or specialized cells (Kumar, Mella-Herrera et al. 2010). In heterocysts, photosystem II, which is the developing step of photosynthesis involving oxygen, is absent (Lange, Belnap et al. 1998). This prevents the exposure of nitrogenase to oxygen. For cyanobacteria which are filamentous, nonheterocystous, and the unicellular cyanobacteria, as well as the phototrophic bacteria, the nitrogenase is most

commonly expressed at night when photosynthetically developed oxygen is avoided (Kumar, Mella-Herrera et al. 2010).

Even though nitrogenase fixation in most species has been restricted to anaerobic conditions, there are exceptions. *Azotobacter* has a very high respiratory rate compared to all other organisms (Oelze 2000). Conformational changes and respiratory protection enables *Azotobacter vinelandii,* which is an obligate aerobic diazotroph, to protect its nitrogenase complex (HanJaehong and 김용웅 2005). *Azotobacter* can remove all traces of oxygen through its own respiration, from its surroundings to create anoxic micro-environment (Orr, James et al. 2011).

Free-living diazotrophs provide the host organism with a nitrogen source by reducing nitrogen content (Orr, James et al. 2011). This trait of free-living diazotrophs is desired for some of the world's most important crop plants like wheat, rice, and corn. If important crop plants adopt this phenomenon, it would result in desirable effects on agricultural production where the need for fertilizer nitrogen will be greatly reduced, and therefore more environmentally sustainable (Dudeja NPS, Poonam et al. 2011).

1.4.2.4. *Streptomyces* as possible nitrogen fixers

Nitrogenases have a limitation of being susceptible to oxygen, as the presence of oxygen leads to their oxidation and proves detrimental to the process of nitrogen fixation (Zhao, Bian et al. 2006). A nitrogenase complex which is biochemically distinct and is oxygen insensitive had been reported in *Streptomyces thermoautotrophicus* (Ribbe, Gadkari et al. 1997). This bacterium, isolated from a unique environment from the soil covering a burning charcoal pile, was first identified in 1990 by a German team as a

nitrogen fixer that could operate in the presence of oxygen(Gadkari, Mörsdorf et al. 1992). Later several teams led to an indication that they could not fix the nitrogen (Kim, Falconer et al. 1998).

A recent study has identified various species of *Streptomyces* – mesophilic, autotrophs, heterotrophs, as nitrogen fixers, by $^{15}N_2$ incorporation through an alternate pathway (Dahal, Nandakafle et al. 2017). This could be an important milestone for plant biotechnologist who are in search of a good oxygen tolerant nitrogen fixing bacteria. More concrete work on its capacity and on elucidation of its mechanistic pathway can help maintain a healthy ecosystem by overcoming the use of dangerous nitrogen fertilizers (Vance 2001, Dahal, Nandakafle et al. 2017).

1.5. Nitrogen in diverse ecosystems:

Grassland is a biological community that is described by mixed herbaceous (non woody) vegetation cover, grass, and includes few trees or shrubs (Liu, Cao et al. 2016). The availability of nitrogen in grassland strongly impacts the yield of the grassland by stimulating high growth rates. Grasses tend to take up nitrogen quicker than the nitrogen is incorporated into the leaves (Qiu, Wei et al. 2013). Fixation of atmospheric dinitrogen (N_2) by specialist diazotrophs, which are found in root sheaths of cereals and grasses, may express this discrepancy. Although clues suggest that soil N_2-fixation is impacted by grass species composition and then grass-associated N_2-fixation contributes to ecosystem N availability in biofuel monocultures, N_2-fixation by grass-associated diazotrophs in natural ecosystems is practically unexplored. It has not been measured in natural grasslands, nor has fixation by free-living versus legume symbionts diazotrophs been compared at the same site. Therefore, contributions to overall ecosystem N dynamics of

N_2 fixation by grass-associated diazotrophs, as compared to legumes are still virtually unknown. A few smaller studies may explain variation in legume N_2-fixation and may or may not apply to diazotrophs in grasses (Ritchie and Raina 2016). Root mass specific fixation by grass endophytes and free-living soil bacteria may be less than that of legumes due to their exposure to increasing level of oxygen, which inhibits catalysis by nitrogenase. In general, N_2-fixation was thought to be encouraged by N-limitation, as cleared by lower net N mineralization in soils. Despite nitrogen fixation association with plants, nitrogen fixation might be limited by the supply of carbon (C) from host plants or soil organic matter, and soil elements (P, Mo) that may be limiting. Herbivory could put strong limits on abundance, leaf-area and therefore within-plant C supply to root-associated diazotrophs. Phosphate limitation can limit plant growth and C assimilation leading to reduced nitrogen fixation. N_2-fixation may alternatively be preferred at lower P where fixed N is utilized to provide phosphatases that assist extraction of P for plant uptake (Ritchie and Raina 2016).

Arid climates in desert regions cause arid soils to lose nitrogen in the form of gas. This results in deterioration of plant life where it is already scarce. Nitrogen helps sustain plant life in the desert, however the increasing temperatures hinder plant life to thrive. The dynamics of nitrogen in arid systems shows that high temperatures affect the presence of nitrogen. Nitrogen loss is due to the denitrification reaction that occur at high temperatures. Ammonia can become volatile at higher temperatures, but NO^-_3 does not. Nitrogen losses occur at higher temperature which makes the soil warmer. Warmer soil correlates to higher nitrate concentration. Nitrate would not have been converted to N_2 (Sawyer 2008). The loss of nitrogen in the desert has proven to be one of the biggest

constraints in biological activity in arid ecosystems (Cowan, Sohm et al. 2011). Higher temperatures cause nitrogen to escape from desert soils as gas. There are several different factors that contribute to nitrogen loss in the desert like constant temperature increases and shifts in precipitation patterns causes by climate change. Loss of nitrogen cause arid soils to be even more infertile, thereby being unable to support most plant life, and hindering abundance in biological activities. The combination of water with heat would greatly increase the occurrence of nitrogen loss in this type of ecosystem (Cowan, Sohm et al. 2011). Abiotic or non-biological process were found to play bigger roles in nitrogen loss compared to microbes near the surface of the soil producing more nitrogen gas that is released and dissipated in the air. The presence of ozone is formed by nitrogen oxides near the ground in deserts and contributes to air pollution which increases the greenhouse effect, which, in turn, warms the planet even more (Graedel and Crutzen 1989). Nitrogen is relatively important to plant life since a plant's chlorophyll or the compound by which plants use sunlight to produce sugars from water and carbon dioxide disappears, which results in plants being unable to sustain their own life (Raven, Evert et al. 2005).

The establishment of nitrogen as an essential element for plant growth has been monitored in different ecosystems, including forests. In deserts, scarcity of nitrogen in soils causes plant life to deteriorate. In forests, the issue stems from the overabundance of nitrogen which is potentially damaging (Rennenberg, Dannenmann et al. 2009). Nitrogen deposition uses different mediums like air, land, soil, and water to move nitrogen. The increase in atmospheric nitrogen deposition is caused by the increased emissions from burning fossil fuels, fertilizer use, and other damaging human activities (Rennenberg, Dannenmann et al. 2009). Elevated nitrogen deposition leads to chemical reactions that

make soil more acidic which restricts plant growth and increases competition for limited resources. This results in a rapid loss of local biodiversity. Forests can tolerate slightly higher levels of nitrogen deposition compared to other ecosystems before they begin to manifest the negative impacts of nitrogen deposition on biodiversity (Rennenberg, Dannenmann et al. 2009).

Nitrogen in seawater has a similar impact on biodiversity as it has on land. The nitrogen present in seawater is often a limiting nutrient for the growth of organisms. Most organisms are unable to assimilate nitrogen into their systems, but nitrogen serves as an energy source and an oxidant for marine bacteria and archaea (Paerl, Fitzpatrick et al. 1996). The ocean absorbs nitrogen gas from the atmosphere and is then transformed into various chemical compounds. The wide variety of microbes present in the ocean adapt to one or more steps in the nitrogen cycle. Every form of nitrogen in seawater is utilized by the vast number of diverse and evolved microbes either as a source of energy or as a nutrient. The oxidation of nitrogen compounds is how many types of microbes extract energy and how these microbes contribute to the reduction of the presence of limiting nitrogen in seawater (Paerl, Fitzpatrick et al. 1996).

1.6. Methods for studying of Biological Nitrogen Fixation:

Nitrogen fixation plays a critical role in production of food. This makes it important for us to study and assess the process of nitrogen fixation by using various methods. There are several methods that can be applied for assessing this process. These methods are discussed below:

1.6.1. Culture Dependent methods:

Culture dependent methods are used to study the metabolic characters and physiology of an organism. The bacteria also are cultured to increase their number so that inoculation can be performed (Cardenas and Tiedje 2008). In this approach, the process of evaluation depends primarily on the culture; thus, its name is culture-dependent. Nitrogen fixing bacteria can be isolated from the soil or from root nodules. The bacteria are then screened for identification of their activities of potential of plant-growth promotion (PGP) (Gehring and Vlek 2004). This method of evaluation introduces bias in the evaluation because the assessment of microbial community might remain incomplete because not all bacteria are culturable (Herridge and Rose 2000). Diazotrophs are grown in liquid, semisolid or solid medium like Burk's nitrogen free medium, nitrogen free malate medium (Dobereiner, Marriel et al. 1976, Doughton, Vallis et al. 1992), or Norris glucose nitrogen free medium (Atlas 2010).

The nitrogenase activity of free-living diazotrophs is affected by several factors such as supply of substrate or oxygen, mineral nutrition, pH, and the presence of N_2. The media should include essential elements for free-living nitrogen-fixing diazotrophs such as iron, molybdenum, potassium, magnesium, sulfur, phosphate, and calcium (Glick 1995). Addition of nitrogen, especially ammonia decreases the activity of nitrogenase in aerobic diazotrophs (Hartley and Schlesinger 2002). On the other hand, oxygen is very necessary for nitrogen fixation and energy supply in aerobes. Alkaline or neutral condition for growth is required for most free-living nitrogen fixing bacteria. The essential elements listed above, supplemented with a carbon source constitute a medium suitable for the selection of diazotrophs. The study by Aquilanti *et al.* determined different approaches of isolation of *Azotobacter* from soil. They concluded the isolation

of free-living bacteria with mannitol agar was the best approach to get high selectivity and reliability (Aquilanti, Favilli et al. 2004). Stella *et al.* found *Beijerinckia* medium followed by *Derxia* medium and *Ashby's* medium are suitable media for diazotrophs. They used sucrose, starch, and mannitol as carbon sources and found them a good promoter of growth for diazotrophs. Also, they described a media with the carbon sources Sucrose, L-Arabinose, and D-Mannose with some vitamins that contain nitrogen, is commonly used for growth by free-living fixing N_2 bacteria. The culturing medium used is the most important criteria to be considered (Stella and Suhaimi 2010).

Most diazotrophs required a continuously low concentration of oxygen to fix nitrogen. The preserving of continuous hypoxic conditions in the laboratories is a big challenge either in solid or liquid medium (Desnoues, Lin et al. 2003). However, the constant uptake of O_2 through high rates of microbial respiration decreases the availability of this gas in the liquid. The high O_2 concentration in the air could irreversibly damage the nitrogenase, when cells are grown on solid medium (Hatayama, Kawai et al. 2005). Based on the consideration previously described, *Döbereiner* in her study found that semisolid medium is a good selective and favored media for the growth of diazotrophs (Dobereiner, Marriel et al. 1976).

1.6.2. Culture Independent Methods

Culture conditions ideal for most bacteria have not been found, so most microbes in the soil remain uncharacterized and uncultured (Bahulikar, Torres-Jerez et al. 2014). Therefore, a culture-independent is the most suitable approach to examine the diversity of potential diazotrophs based on Polymerase Chain Reaction (PCR) amplification and

sequencing of 16SrRNA and *nifH* DNA (Zehr, Mellon et al. 1995). Many researchers have used *nifH* to study functional diversity in various environments, and evolution of diazotrophs based on 16S rRNA and *nifH* sequence (Young 1992).

These culture-independent studies can recover the presence of phylogenetically diverse potential nitrogen-fixing microorganisms (Noda, Ohkuma et al. 1999). The core of this method is amplification of target genes by PCR (community analysis) without culturing requirement, using extracted DNA as template (Thong-On, Suzuki et al. 2012). The target gene chosen must occur in all members of the group which is targeted for studying, for example all nitrogen fixing bacteria must have the targeted gene. Also, it should have conserved domains that allow amplification using universal primers as well as have sufficient variation across taxa to allow for phylogenetic analysis (Gaby and Buckley 2012). There are several steps in this approach explained below:

1.6.2.1. The 16S rRNA Gene

Phylogeny of the bacteria currently relies primarily on 16SrRNA gene as this fulfills all criteria outlined above. The molecular phylogenetic tree of bacteria relies on small subunit ribosomal RNA gene sequences (Woese 1987). All bacteria have *rRNA* as part of their ribosome, performing identical functions in all cells. The *16S rRNA* is the gold standard for determining the diversity of microbial communities from different environments (Clarridge 2004). The analysis of the16S*rRNA* gene is also useful for allocation of novel isolates to previously defined taxa. This helps in identification of the uncultured bacteria in the ecosystem (Amann, Binder et al. 1990). The gene consists of nine hyper variable regions (V1-V9) which are surrounded by conserved regions to allow

the use of universal primers to amplify the target sequences (Noller and Woese 1981). Non-cultivable bacteria and or mixtures of bacteria can be allocated to taxa by comparing the sequences obtained to databases of 16SrRNA genes (Gutell 1994). Due to the conserved nature of the 16SrRNA gene, sequences of closely related strains are similar or almost identical. In many cases, isolates can only be allocated at the genus level. For allocation to species level, and especially for differentiation of strains within species, target genes should be less conserved than the 16SrRNA gene. The partial sequencing of the V1–3 region located at the 5'- end of the 16SrRNA is sufficient and more sensitive than other variable regions for differentiation between the species of bacteria (Benga, Benten et al. 2014).

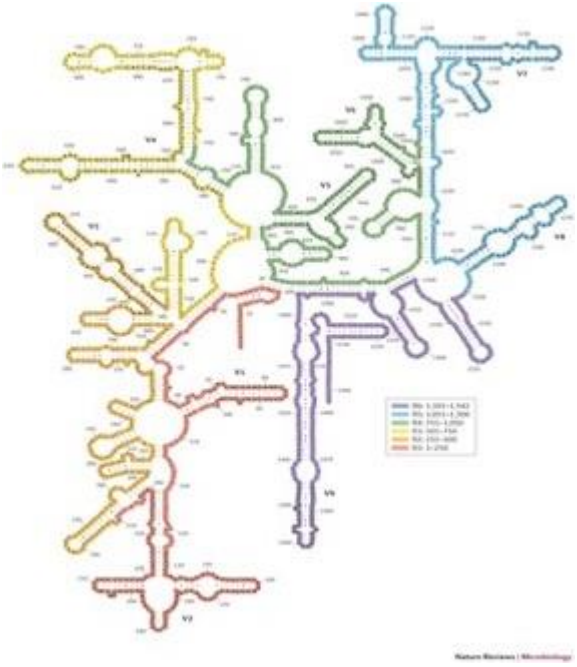

Figure 1.7 Secondary structure of 16SrRNA of Escherichia coli *presenting the nine variable regions from (Yarza, Yilmaz et al. 2014)*

1.6.2.2. Use of the *nifH* gene to Study the Diversity of Diazotrophs

The *nif* genes are a class of genes which code for enzymes involved in nitrogen fixation. These genes are found in both symbiotic as well as free-living nitrogen fixing bacteria (Harriott, Hosted et al. 1995). The expression of *nifH* gene, encoding a key structural protein of the enzyme nitrogenase is important in evaluating the potential of nitrogen fixers to fix atmospheric nitrogen. The sequence diversity and abundance of *nifH* is used for studying the abundance and diversity of the diazotrophs. *nifH* is used for classification of diazotrophs into different clusters (Clusters I-IV) which also makes it an acceptable marker for diazotroph phylogeny. Cluster I contain a diverse group of *nifH* genes and includes the aerobic and facultative anaerobic bacteria which belong to the phyla *Proteobacteria, Actinobacteria, Firmicutes, and Cyanobacteria*. Cluster II comprises of the alternative nitrogenase *anfH*. Cluster III consists of *nifH* from anaerobes like *Clostridium*, Archaea (methanogenes), and *Delta proteobacteria* (Hartmann and Barnum 2010). Clusters V and IV are paralogs of *nifH*, and they do not fix nitrogen (Raymond, Siefert et al. 2004). However, the *nifH* from *Endomicrobium Proavitum*, alignes with cluster IV, but is able catalyze nitrogen fixation. Isolation and sequencing of *nifH* from putative nitrogen fixing isolates is done to evaluate nitrogen fixation (Hoffman, Lukoyanov et al. 2013).

1.6.2.3. *nifH* gene as PCR target

Nitrogenase is likely to be an ancient enzyme, since it is distributed widely throughout bacteria and archaea (Klucas, Koch et al. 1968). There are examples of possible or probable lateral gene transfer, but most of these likely occurred early in evolution (Zehr, Braun et al. 1996). Nitrogenase genes form a closely related family that

likely arose from a common ancestor (*nifH*, *nifD*, *nifK*, *nifE*, and *nifN*, and others) (Soboh, Boyd et al. 2010). Both nitrogenase proteins are conserved, but the Fe protein, composed of two identical subunits encoded by *nifH*, is the most conserved of the *nif* genes (Bazylinski, Dean et al. 2000). There are several conserved regions in the sequence of these proteins. In the N-terminal section of NifH there is an ATP-binding site motif 'A' (P-loop) and in the central section there are two conserved cysteines which have been shown, to be the ligands of the 4Fe-4S cluster. Because it is most conserved, *nifH* has been the target of most studies. This is particularly true regarding environmental studies. Thus, there are now tens of thousands of *nifH* genes available in Genbank (Zehr 2017). Recovering these genes, their coding regions, metadata, and aligning them in a coherent manner is problematic. This is largely due to the lack of shared conventions for data storage among the major genomic repositories, and the large volume of legacy data where information is presented in an inconsistent manner (Zehr 2017). Nitrogenase genes are distributed throughout the prokaryotic kingdom, including representatives of the Archaea as well as the Eubacteria and Cyanobacteria. Although the phylogeny of *nifH* reflects the phylogeny of organisms based on ribosomal RNA genes, there are some differences (Hu and Ribbe 2015). One deeply branching cluster is anomalous and is likely to represent an independent line of evolution, and includes some sequences from gram positive organisms, such as *Clostridium* (Kanamori, Weiss et al. 1989, Chen, Toth et al. 2001). Since nitrogenase gene sequences do reflect phylogenetic affiliation, the sequence of nitrogenase genes can be used to identify the types of nitrogen-fixing microorganisms in different habitats. Nitrogenase gene sequences have been identified in complete genomes, from genome libraries and amplified from individual organisms and the environment

using the polymerase chain reaction. There are thousands of nitrogenase genes now in the public databases (Zehr 2017).

The dinitrogenase reductase gene is found in nearly every environment studied so far and environmental genetic surveys of the *nifH* gene have produced extensive datasets encompassing several tens of thousands of unique sequences, and novel sequences continue to be discovered (Hoffman, Lukoyanov et al. 2013). Such PCR-based surveys continue to serve as an important tool for studying the diazotroph diversity and, if the *nifH* transcripts are targeted (via cDNA), can also reveal transcription patterns in the environment (Hamilton, Ludwig et al. 2011, Robertson, Goodrich et al. 2011, Yan, Ping et al. 2011).

Analysis of *nifH* gene sequences is dependent on obtaining the gene from its environment, usually through PCR. Yet, the choice of primer pair can have a significant impact on the extent of diversity that is uncovered. Since the first degenerate primers were used to amplify environmental DNA, several dozens of primer pairs targeting the *nifH* gene have been designed to serve as group-specific or general *nifH* primers (Demba Diallo, Reinhold-Hurek et al. 2008). In 2012, Gaby and Buckley published an extensive review of all known primers for *nifH*, and evaluated their performance *in silico* using a comprehensive database of *nifH* sequences. Through their analysis, several primer pairs were identified for their potential to capture the largest diversity of *nifH* sequences and were tested for their ability to produce a PCR product from DNA from several diazotrophic strains and two soil samples. However, the extent to which these primers assist in capturing *nifH* diversity in environmental samples and their potential preferences of amplification has not been tested (Gaby and Buckley 2012). Furthermore,

the majority of the *nifH* sequences in public databases have sequence data between positions 100 and 500 bases (positions are relative to *Azotobacter vinelandii*), making it challenging to perform *in silico* coverage estimates of primer pairs flanking this region (Gaby and Buckley 2012, Gaby 2014).

1.6.2.4. Methods for analyzing conserved genes

Sequencing of 16S *rRNA* and *nifH* genes requires amplification using polymerase chain reaction. This method produces many copies of these genes. Successful amplification by PCR requires genomic DNA that is not too sheared, and free from inhibiting compounds (Hoffman, Lukoyanov et al. 2013).

The result of a PCR procedure is influenced by the amount of DNA in a PCR (Garibyan & Avashia, 2013). Problems like using an excessive amount of DNA in a small and tight space of a reaction vessel can lead to packed DNA which results in poor DNA amplification caused by a limited diffusion of DNA which can result in poor DNA amplification caused by a limited diffusion of Taq polymerase and other reagents, which can promote priming. To avoid amplification of nonspecific products, the concentration of target DNA should be appropriate for the number of cycles in the reaction (Canene-Adams 2013). Another challenge is the accumulation of nonspecific products in a re-amplification PCR when a high starting concentration of the template comes with a large number of cycles. To address this issue, the reduction of cycles should help (Garibyan and Avashia 2013). However, it is important to take in consideration the ratio of target DNA to burden DNA. The concentration of the target DNA should be the same across multiple analyses. Using a high concentration of the target combined with the normal, or

higher than normal, the number of cycles can cause increased accumulation of non-specific products. The accumulation of non-specific products is usually observed in a re-amplification PCR when elevated initial concentration of the PCR fragment is subjected to a large number of cycles, So the number of cycles should be reduced to avoid it. However, low concentrations of target, primer, nucleotides, and Taq are very important and recommended to ensure lower background and cleaner product (Garibyan and Avashia 2013). DNA degrading substances should be taken into consideration since these substances are abundant in the surroundings such as particles of skin or hair, or even the dust that floats in the air (Canene-Adams 2013). Nucleases can destroy DNA (Bloos and Reinhart 2013).

PCR procedures are affected by an incorrect concentration of deoxynucleotide triphosphates (dNTPs). When there are excessive dNTPs, it can hinder the PCR, preventing the formation of products (Bloos and Reinhart 2013). For primers, the recommended concentration must be used to avoid inhibiting the desired PCR product from being amplified as well as to avoid contaminating the reaction. Primer dimers need to be formed to suppress creating spurious PCR products (Garibyan and Avashia 2013).

1.6.3. Biochemical determination of nitrogen fixation

1.6.3.1. The Acetylene Reduction Assay

The acetylene reduction assay is a method used for measuring the nitrogen fixation by assessing the nitrogenase enzyme's ability to reduce the substrates possessing triple bonds. In this method, the nitrogenase enzyme reduces acetylene to ethylene gas just like the enzyme reduces nitrogen to ammonia during nitrogen fixation. This technique is particularly used for assessing the nitrogen fixation activity rate of symbiotic

nitrogen fixers. It is an inexpensive method of measuring the activity of nitrogenase enzyme (Hardy, Burns et al. 1973).

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

^{15}N is a stable isotope of nitrogen. The assimilation of $^{15}N_2$ is a method of measuring nitrogen fixation. This method employs the use of mass spectroscopy and emission spectrophotometry (Buckley, Huangyutitham et al. 2007) . Using the method of stable isotope analysis, it is possible to demonstrate how the stable isotope $^{15}N_2$ is incorporated into various amino acids which can further assist in evaluation of the rate of nitrogen fixation. Also, this help in identifying the free-living diazotrophs in the soil which are engaged in the process of nitrogen fixation. This process includes incubation of bacteria to be tested in presence of $^{15}N_2$, and then determining the ratio of ^{15}N and ^{14}N using mass spectrometer for mass analysis of nitrogen. Incorporation of stable isotope $^{15}N_2$ helps in the confirming whether a susceptible nitrogen fixing bacteria actually performs nitrogen fixation (Doughton, Vallis et al. 1992, Mulholland 2007).

1.7. The objective of this study:

The objective of this study was to capture and describe the culturable diversity of free-living diazotrophs in native prairie soil to shed some light on sources of combined nitrogen in grassland ecosystems.

Chapter 2

2. Diversity of Free-living Nitrogen Fixing bacteria in Soil of Native South Dakota Prairie

2.1. Introduction

Nitrogen is a critical factor required for all forms of life (Berman-Frank, Lundgren et al. 2003, Wagner 2011). The air consists primarily of nitrogen but eukaryotes including plants and mammals cannot use gaseous nitrogen(N_2) because it is inert (Belnap 2002). Biological fixation of gaseous nitrogen into ammonia is an important part of the global nitrogen cycle (Kim, Falconer et al. 1998). Certain bacteria and archaea known as diazotrophs are responsible for carrying out this process. Bioavailable nitrogen is the limiting nutrient in many environment and it can determine the primary productivity of plants (MacKellar, Lieber et al. 2016). Nitrogen fixers therefore play a significant role in ecosystem productivity because ecosystems can lose nitrogen through ammonia volatilization and denitrification. Diazotrophs can fix nitrogen using nitrogenase enzyme complex (Lobo and Zinder 1988). Diazotrophs fix N_2 either for their own biosynthetic needs as free living diazotrophs, such as *Burkholderia, Azobacter, Azospirillum*, *Bacillus*, and *Clostridium*, or as symbiotic partners with certain plants such as bacteria which form symbiosis with legumes like *Rhizobium,* and bacteria that form symbioses with actinorhizal plants such as *Frankia,* and associative nitrogen fixing bacteria like *Cyanobacteria* which associate with cycads (Dart 1986). The best studied diazotrophs form symbiotic associations with plants, primarily legumes and certain tree species, but less is known about the role and contribution of free living diazotrophs to natural environment such as grasslands (Gadkari, Mörsdorf et al. 1992).

All known diazotrophs have the Group-1 Mo-nitrogenase, but some organisms randomly have either or both of V-nitrogenase and Fe-nitrogenase (Einsle, Tezcan et al. 2002). For example, *Klebsiella pneumonia*, a free-living diazotrophs, has only molybdenum nitrogenase, while other species have all three enzymes like, *Azotobacter vinelandii* (Einsle, Tezcan et al. 2002). Some species have a combination of two types of nitrogenases, for example, *Azotobacter chroococcum* has the Mo- nitrogenase and V-nitrogenase, whereas *Rhodobacter capsulatus* has Mo-nitrogenase and Fe-nitrogenase (Zehr, Jenkins et al. 2003). However, Mo-containing nitrogenase is the most widely studied and is the enzyme utilized by the model *Rhizobium* with 17 *nif* genes (Schulze 2004). This complex consists of two enzymes, dinitrogenase and dinitrogenase reductase (Einsle, Tezcan et al. 2002).

The various *nif* genes vary substantially across the bacteria. The most conserved is *nifH* (Ueda, Suga et al. 1995), and this has facilitated its used as the standard marker gene for determination of diazotroph diversity (Zehr and Capone 1996, Staples, Lahiri et al. 2007). While *nifH* is the most conserved, there is still considerable variation across the bacteria. No fully conserved domains exist, so that many degenerate primer sets have been reported (Dos Santos, Fang et al. 2012, Gaby and Buckley 2012). While Gaby and Buekly have presented a suggested list based on bioinformatics analysis (Gaby and Buckley 2012), the majority of studies have reported using PolF-PolF (Dos Santos, Fang et al. 2012, Gaby and Buckley 2012). It amplified most tested strains and worked well for amplifying *nifH* from soil extracted DNA (Gaby 2014).

Nitrogenases are very oxygen sensitive, and require low cellular redox potential (Eady 1996). Symbiotic diazotrophs such as *Rhizobium* can grow aerobically, but only fix dinitrogen under reduced conditions, e.g in root nodules where they are protected oxygen scavenging leghemoglobin (Zahran 1999). In contrast, some free-living nitrogen fixers can grow and fix nitrogen in presence of oxygen (Berman-Frank, Lundgren et al. 2003), while others fix nitrogen under reduced oxygen conditions. Isolating free-living diasotrophs from Amazon forest soil under reduced oxygen conditions yielded far greater numbers and diversity (Mirza and Rodrigues 2012).

Prairie soils are part of the arid grasslands, which make up approximately 6.9% of the worlds ice-free global land, and 21% of land in the United States (Hartemink and Bockheim 2013). They are covered by a variety of grasses ranging from short grass steppe species to tall grass species. Short grasses typically resemble monoculture meadows not normally exceeding 30cm in height. Tall grass prairies are less uniform and maintains grasses growing to heights of 1-3 meters. Vegetation cover is highly dependent on moisture regime in the area. Taller grasses require more moisture (Hartemink and Bockheim 2013). Mollisoles are rich due to the accelerated decomposition of organic matter. These soils have a thick, dark, soft mollic epipedon, composed of wind-blown silts or sands that are high in calcareous material. Prairie grasses help keep soil organic matter high. Soil microbes are very productive under prairie soils (Xie, Bai et al. 2009). The extensive grass coverage, along with dense root systems and fungal mats allow an ample supply of organic matter for breaking down into various nutrients. The Nitrogen cycle is also another key reason why prairie soils are very rich and productive. Microbes are responsible for both nitrogen mineralization and immobilization through fixation.

Both processes regulate the amount of available nitrogen that is in the soil (Mulvaney, Khan et al. 2009).

No information is currently available on the diversity of nitrogen fixing bacteria in soils of temperate grasslands such as prairie. The objective of this study was to capture the culturable diversity of free-living diazotrophs in native prairie soil by characterizing their 16SrRNA and *nifH* sequences. Sioux Prairie is a preserve in the Big Sioux River valley region belonging to the Nature Conservancy. This prairie was protected from agriculture conversion, and not amended with fertilizer. Here we report isolation of diverse free-living diazotrophs from this natural prairie.

2.2. Materials and Methods

2.2.1. Sample Source

Soil samples were obtained from Sioux Prairie of South Dakota 231st Street, 44.2’8” N 96° 46’ 0” W 1720 ft Elevation. The climate is continental, featuring extreme cold winters and hot summers, between -20 ℃ and 38 ℃. The average annual precipitation is 635 mm, with May and June getting the highest (~65%) precipitation (Conservancy).

Samples were taken across three transects laid out randomly between 44.2’6” N 96° 46’ 58” W 1720 ft Elevation and 44.2’5” N 96° 46’ 58” W 1720 ft Elevation, 44.2’6” N 96° 47’ 1” W 1730 ft Elevation, and 44.2’6” N 96° 46’ 58” W 1730 ft Elevation, and 44.2’11” N 96° 46’ 59” W 1670 ft Elevation and 44.2’11” N 96° 47’ 0” W 1700 ft Elevation. Nine soil cores were taken along each of the three transects, 5m a part. Cores were 10 cm deep and 2.5 cm in diameter. Samples were placed into 50 ml sterile conical

tubes to avoid loss of moisture and any contamination, and kept at 4°C until processing in the laboratory within six h. The pH of the soil samples ranged from 8.1 to 9.8.

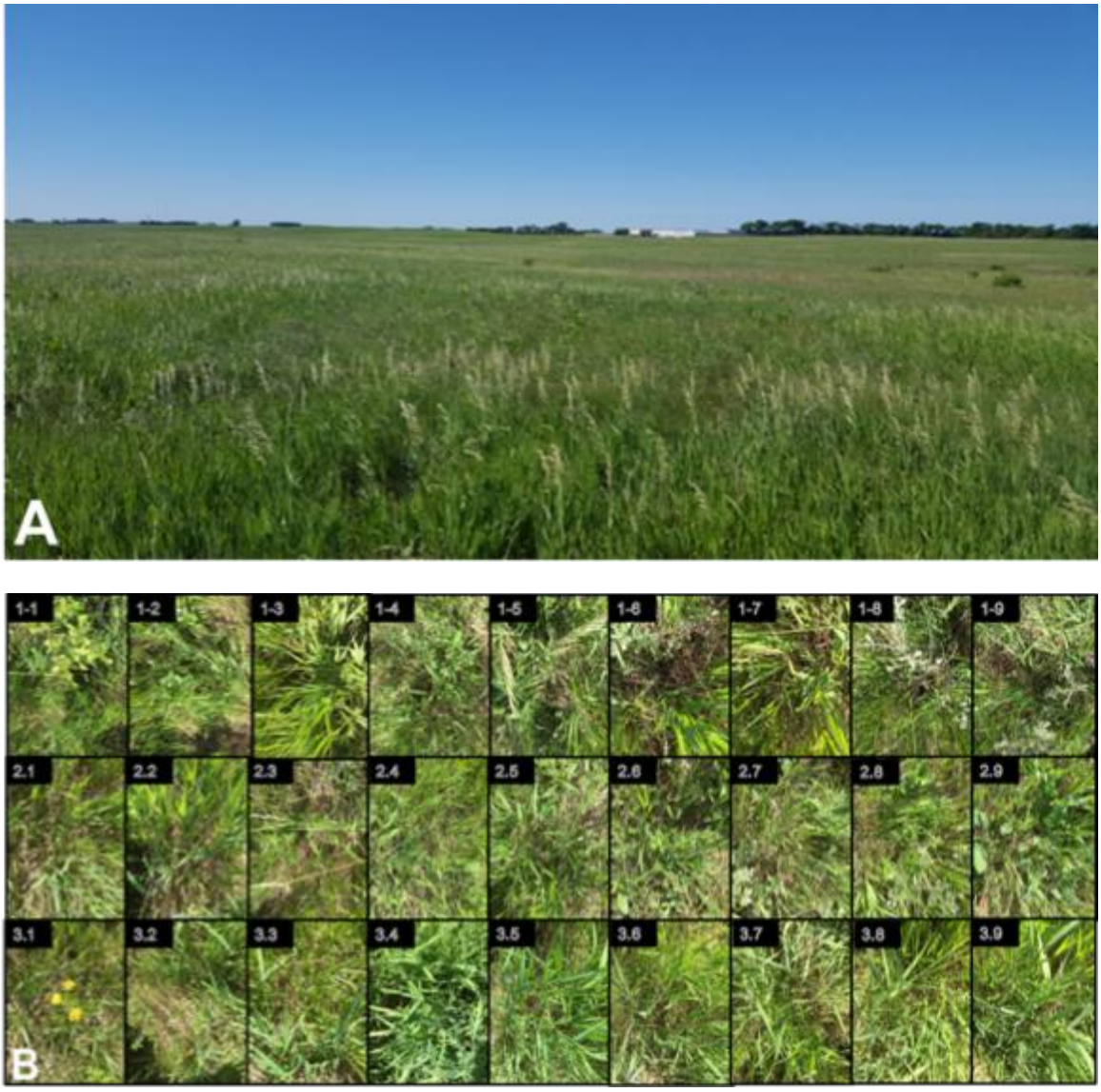

Figure 2.1 A: Images of the overall sampling at The Nature Conservancy (TNC) site at Sioux Prairie, South Dakota. B: The sets of nine specific sampling locations across each of three transects.

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

Free living putative diazotrophs were isolated using solid nitrogen free medium (NFM), solidified using Noble agar and supplemented with glucose, mannitol, arabinose, and malic acid as carbon source. NFM comprises 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, Catlog No. 214230), NaCl (0.2g/l) and was set to PH 7.2 in 500 ml $d.H_2O$. Glucose (2g/50ml), mannitol (2g/50ml), arabinose (2g/50ml), which were autoclaved separately, and malic acid (2g/50ml), was set to PH 7.2, autoclaved separately and added to NFM base after cooling to 50 °C. Soil samples were broken up and mixed on a sterile surface using a sterile spatula. Five grams was added to 45 ml of sterile deionized water in a 250 ml Erlenmeyer flask and mixed by gentle shaking for 8h at 28 °C. Sterile dilution were prepared and then 100 µl aliquote were spread onto each of six nitrogen free medium (NFM) plates. Three of each set of plates was incubated aerobically for 15d at 28 °C, and the other three were incubated under reduced oxygen using a gas-pack (Gaspack EZ Campy, BD) in a sealed container (BD GasPak ™ EZ). After incubation, bacterial colonies were counted. In order to determine whether all four carbons sources were required for growth by our isolates, each isolated subcultured onto NFM with only one of each of the four source and ability to grow recorded (Fig 2.2).

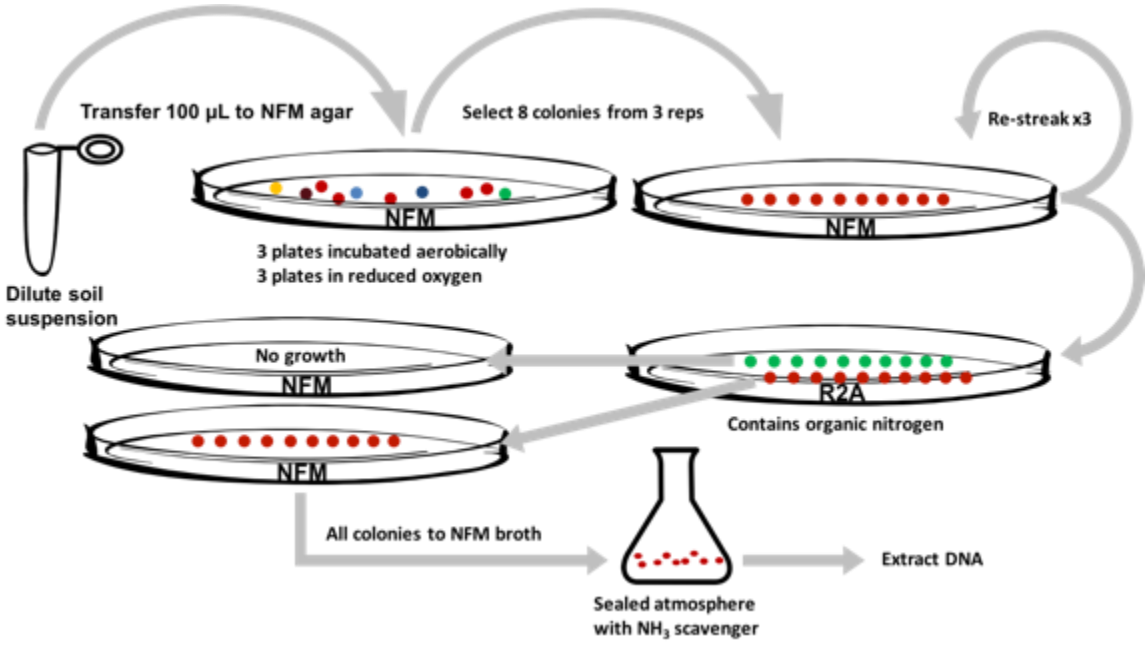

Figure 2.2 Isolation steps of free putative diazotrophs from Sioux Prairie soil.

2.2.3. Purification on R2A, Solid NFM, and Liquid NFM

Eight colonies were selected from each plate based on different morphology and then streaked on solid NFM four times to obtain single colonies and ensure purity. A total of 486 isolates were obtained. Isolates were streaked onto R2A (BD Difco ™ R2A AGAR, 218263) and incubated at 28 °C aerobically and under low oxygen conditions for one week. Some isolates formed two different colony types on R2A. To ensure their nitrogen fixing ability, both were sub-cultured again on solid NFM. All colonies able to grow on NFM agar were transferred to liquid NFM. Isolates were grown in R2A broth and supplemented with glycerol to 50%, transferred to 1.5 ml tubes, and stored at -80 °C.

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

DNA was extracted from 486 isolates pure cultures using the Microbial DNA Isolation kit (MoBio), and stored at -20°C. The DNA concentration was determined spectrophotometrically using NanoDrop. Integrity of the genomic extracts was determined electrophoretically by resolving in 0.8% agarose gel (80V, 40 min). The V1-V3 region of the 16S rRNA gene was amplified by PCR using primers 27F (AGAGTTTGATCNTGGCTCAG) (Weisburg, Barns et al. 1991) and 518R (GTATTACCGCGGCTGCTGG) (Muyzer, De Waal 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 94^{o}C for 5 min, 30 cycles at 94^{o}C for I min, 54^{o}C for 1min, and 72^{o}C for 1min, and a final elongation at 72^{o}C for 10min. Amplification was confirmed by agarose gel electrophoresis (1.2% agarose,60 V,60 min) comparing to a 100bp ladder (NewEngland BioLabs). The sequence of PCR amplicons was determined by the chain termination method (GeneScript Sequencing company). The sequences were aligned using Sina sequence Aligner and imported to ARB from where closely related species were selected as references (Ludwig, Strunk et al. 2004). The phylogenetic tree was prepared with using aBayes Branch analysis (Guindon, Dufayard et al. 2010).

2.2.5. Confirming nitrogen fixation in an ammonia free atmosphere

One hundred ninety isolates allocated to gram positive bacteria by the V1-V3 region of their16S rRNA gene were inoculated into liquid NFM in Erlenmeyer flasks, placed into a desiccator containing Clinoptilolite, an ammonia scavenging zeolite (Wang, Liu et al. 2006, Liao, Chen et al. 2015). The containers were sealed using petroleum jelly

and kept at room temperature (22-26 ºC) for 20d. Cultures were observed visually to confirm growth.

2.2.6. Amplification of *nifH* by PCR

The partial *nifH* genes were amplified by PCR primers PolF and PolR (Poly, Monrozier et al. 2001, Gaby and Buckley 2012), IGK3 and DVV (Gaby and Buckley 2012), and Ueda19F and Ueda407R (Ueda, Suga et al. 1995) (Table 2.1). PCR was performed in 30µl reactions comprising of 0.2µl (5000U/ml) Taq polymerase (NewEngland BioLabs), 3µl (10X) PCR Buffer, 0.9µl dNTP (each 10mM), 0.9µl (10mM) each Primer, and 2.5µl (25mM) $MgCl_2$. The PCR conditions gene using the primes PolF and PolR were: initial denaturation at 94ºC for 5 min, 30 cycles at 94ºC for 1 min, 55ºC for 1min, and 72ºC for 30 seconds, and a final elongation at 72ºC for 5min to yield a product size of ~360 bp. Different annealing temperatures were used till optimal amplification was attained. In order to optimize the PCR reaction, were BSA (NEW ENGLAND BioLabs Inc), 5% DMSO, and 5% Glysrol were added either alone or in combination (Strien, Sanft et al. 2013). For IGK3/DVV and Ueda 19F/Ueda407R, the annealing temperatures were 59 ℃ and 52 ℃ respectively (Gaby 2014). In addition, PCR amplification of *nifH* gene was generated using a nested protocol using degenerate primers designed to amplify *nifH*. Forward primer 19F (5 -GCIWTYTAYGGIAARGG IGG) and reverse primer nifH3 (5 -ATRTTRTTNGCNGCRTA) were used in the first amplification reaction which has reaction volume of 50 µl. The reaction conditions were as follows: 95°C for 5 min, followed by 20 cycles of 48°C for 1 min, 72°C for 1 min, and 94°C for 45 s, with a 72°C final extension step for 10 min before holding at 4°C when the reaction was terminated. For the second PCR, two different primers nifH1 and nifH2

were designed to generate forward primer nifH11 (5 -GAYCCNAARGCNGACTC) and dilution of the first PCR product template. The second PCR conditions was performed as described above except that the annealing temperature was 55°C and the reaction cycle was repeated 32 time(Table.2.1) (Yeager, Kornosky et al. 2004). In all cases *Herbaspirillum seropedicae* ATCC® 35892 and *B. japonicum* USDA 110 as a positive control.

Amplification using PolF/PolR primers gave a product size of ~360bp, and for IGK3/DVV, Ueda19F/Ueda407R, and for nifH1/ nifH2 primer sets the product sizes were ~360-400bp. The DNA sequences products were submitted to BLAST search at NCBI (Altschul, Madden et al. 1997) to obtain related sequences. Representative *nifH* sequences were obtained from the curated site FunGene. Sequences were imported into ARB (Ludwig, Strunk et al. 2004) and merged into the *nifH* database of the Zehr Lab (Zehr 2017). Sequences were aligned using the Backalign feature of ARB (Ludwig, Strunk et al. 2004). Close relatives were selected and phylogenetic analysis was done with PhyML 3.0 using aBayes branch analysis (Guindon, Dufayard et al. 2010).

Table 2.1 Primers used for *nifH* gene amplification

Primers	**5'-3'**	**Reference**
PolF	TGC GAY CCS AAR GCB GAC TC	(Gaby and Buckley 2012)
PolR	ATS GCC ATC ATY TCR CCG GA	(Gaby and Buckley 2012)
IGK3	GCI WTH TAY GGI AAR GGI GGI ATH GGI	(Gaby and Buckley 2012)
DVV	ATI GCR AAI CCI CCR CAI ACI ACR TC	(Gaby and Buckley 2012)
Ueda19F	GCI WTY TAY GGI AAR GGI GG	(Ueda, Suga et al. 1995)
Ueda407R	AAI CCR CCR CAI ACI ACR TC	(Ueda, Suga et al. 1995)
19F	GCIWTYTAYGGIAARGG IGG	(Yeager, Kornosky et al. 2004)
nifH3	ATRTTRTTNGCNGCRTA	(Yeager, Kornosky et al. 2004)
nifH11	GAYCCNAARGCNGACTC	(Yeager, Kornosky et al. 2004)

nifH22	ADWGCCATCATYTCRCC	(Yeager, Kornosky et al. 2004)

2.2.7. Validation of pure culture by 16S rRNA amplification

To determine the presence of bacteria from other phyla in my isolates, I used primer specific for the *Firmicutes* and *Actinobacteria* phyla, and α-β- and δ-*Proteobacteria*. The primer sets used are listed in Table2.2 (Pfeiffer, Pastar et al. 2014). The reaction mixture composition and PCR conditions were adopted from the protocol of amplification of V_1-V_3 region of 16S rRNA gene used for identification of diverse bacteria (Muyzer, De Waal et al. 1993). However, the annealing temperatures were first examined as mentioned in (Pfeiffer, Pastar et al. 2014) and then optimized using gradient PCR to get optimum amplification from each primer set for the positive control for each phylum Table 2.2. The annealing temperature used is listed in Table 2.3. The PCR products were visualized by electrophoresis (1.2% agarose, 60 V for 60 min) with standard 100bp ladder (NewEngland BioLabs) to compare the amplicon size. The PCR products with positive amplification were sent to Gene Script for sequencing and all allocated to genus using RDPII(Wang, Garrity et al. 2007).

Table 2.2 The species used as positive control for the specific phylum 16S rRNA

Phylum	**Species name as positive control**
Actinobacteria	*Arthrobacter aurescens* TC1 ATCC BAA-1386
Actinobacteria	*Arthrobacter aurescens* TC1 ATCC BAA-1386
Alphaprotobacteria	*Bradyrhizobium japonicum* USDA 110
Betaprotobacteria	*Herbaspirillum seropedicae* ATCC® 35892
Firmicutes	*Bacillus cereus* ATCC 14579

Firmicutes	*Bacillus subtilis* 168 ATCC 23857

Table 2.3 Primers of 16S ribosomal RNA gene of specific primers

Target group	**AL(bp)**	**Primer name**	**Primer sequences (5'->3')**	**AT(ºC)***
Actinobacteria	166	S-P-Acti-0927-a-S-17	GGRCCCGCACAAGCGGC	59
Actinobacteria	166	S-P-Acti-1154-a-S-19	GADACYGCCGGGGTYAACT	59
Alphaprotobacteria	142	S-C-aProt-0528-a-S-19	CGGTAATACGRAGGGRGYT	61
Alphaprotobacteria	142	S-C-aProt-0689-a-S-21	CBAATATCTACGAATTYCACCT	61
Betaprotobacteria	231	S-C-bProt-0972-a-S-18	CGAARAACCTTACCYACC	61
Betaprotobacteria	231	S-C-bProt-1221-a-A-17	GTATGACGTGTGWAGCC	61
Firmicutes	156	S-P-Firm-0352-a-S-18	CAGCAGTAGGGAATCTTC	57
Firmicutes	156	S-P-Firm-0525-a-A-18	ACCTACGTATTACCGCGG	57

*AT, annealing temperature; AL, amplicon length. IUPAC ambiguity codes: R (A/G), Y (C/T), W (T/A), B (C/T/G), D(A/T/G) (Pfeiffer, Pastar et al. 2014). *From gradient PCR.*

2.3. Results

Putative nitrogen fixing colonies were obtained from all 27 samples analyzed. The culturable diazotroph counts were slightly higher in reduced oxygen cultures than in the aerobic cultures, but not significant (Fig. 2.2). The highest count was from site#3 incubated in the reduced oxygen conditions. The count number at site#1 was almost same in both conditions. Analysis of variance (ANOVA) showed no significant different either between all sites or between aerobic condition and reduced oxygen conditions ($p = 0.125$).

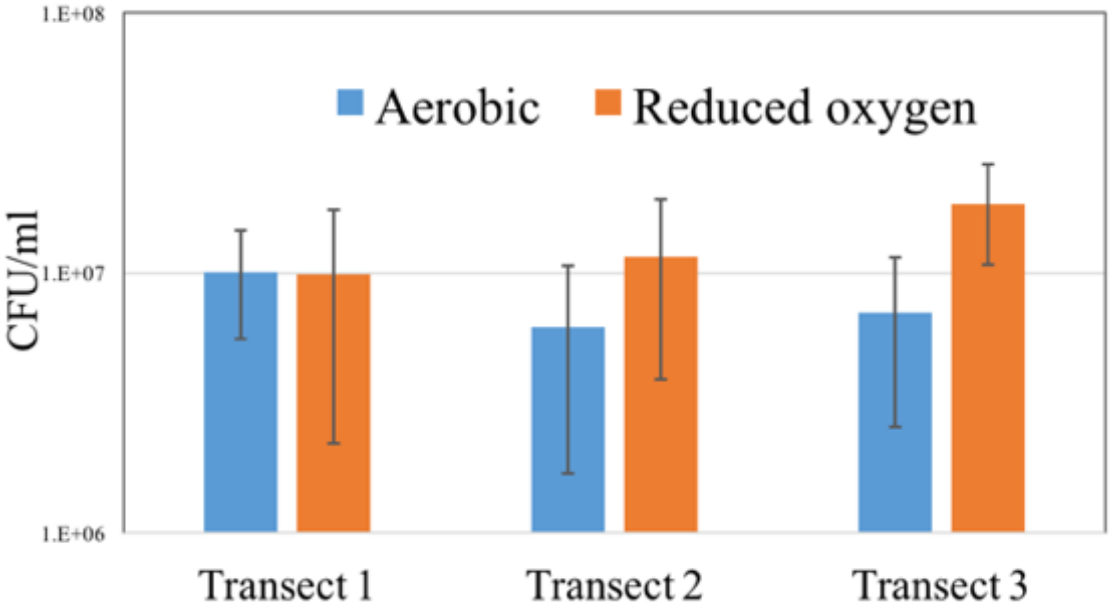

Figure 2.3 Culturable counts of free-living nitrogen fixing bacteria incubated on NFM under aerobic and reduced oxygen environment at 28°C for 14d. Error bars indicate one standard deviation of the mean.

2.3.1. Diversity of free-living putative nitrogen fixing bacteria

Eight colonies were selected at random from each of three plates incubated aerobically and three low oxygen incubated plates per sample, yielding 432 isolates. Several of these appeared as mixed cultures, yielding 486 isolates after further subculturing. Specifically, colonies streaked for purity and appearing as single culture on NFM agar by their consistent colony morphology, were streaked on R2A, a medium containing combined nitrogen. This allowed non-fixing co-cultures to appear as distinct colonies without dependence on a potential nitrogen-fixing co-culture. All distinct colonies obtained on R2A were streaked again on NFM agar to confirm their individual nitrogen-fixing ability. In several cases, colonies appeared quite different on R2A than

NFM agar, both by colony morphology and color (Fig 2.5). These traits were repeatable. This process yielded 486 putative nitrogen fixing colonies.

In select cases initial isolates appeared to grow with a fungus. Fungal growth was suppressed by culturing on both NFM and R2A agar with cycloheximide (200 μg/ml) and nystatin (40μg /ml). Absence of fungi was confirmed by amplification of the eukaryotic ITS of the internal transcribed spacer region (ITS) using primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White, Bruns et al. 1990).

Based on the V1-V3 region of their 16S rRNA genes, the 486 bacterial isolates were allocated to 45 Operational Taxonomic Units (OTUs). According to the Ribosomal Database Project (RDP) tool, these OTUs fell into 35 bacterial genera across six phyla. The largest group (36%) were *Actinobacteria* dominated by *Streptomyces*, followed by *γ-Proteobacteria* (22%)*, α-Proteobacteria* (18%), *Firmicutes* or low G+C (15%), *β-Proteobacteria* (7%), and *Bacteroidetes* (2%) (Fig 2.3). Alignment of the V1-V3 *16SrRNA* gene sequences of 486 isolates was conducted using ARB. Poorly aligning sequences were excluded. Phylogenetic analysis in PhyML using aBayles Branch analysis revealed substantial diversity across 4 phyla (Fig 2.4). The combined tree of 16S rRNA gene of all 458 free – living nitrogen fixing isolates separated the 258 gram-negative from 200 gram-positive isolates. *Streptomyces* and *Microbacterium* were dominant among the gram-positive isolates. Among the gram-negative isolates were primarily α-*Proteobacteria* and *γ-Proteobacteria* (Fig 2.4). Despite extensive steps to isolate and purify and despite displaying one repeatable colony morpho type, many cultures were later found to be mixtures of two or three OTUs (Table 2.4).

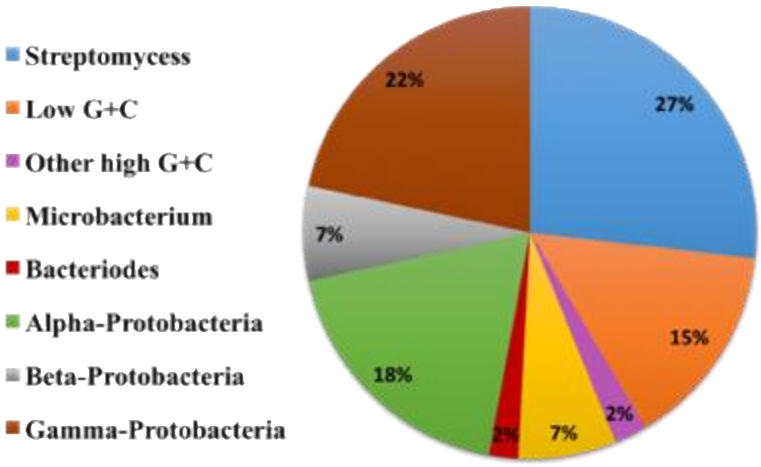

Figure 2.4 Community composition of putative diazotrophs from Sioux Prairie of South Dakota samples as allocated to phyla by their partial 16SrRNA gene sequences

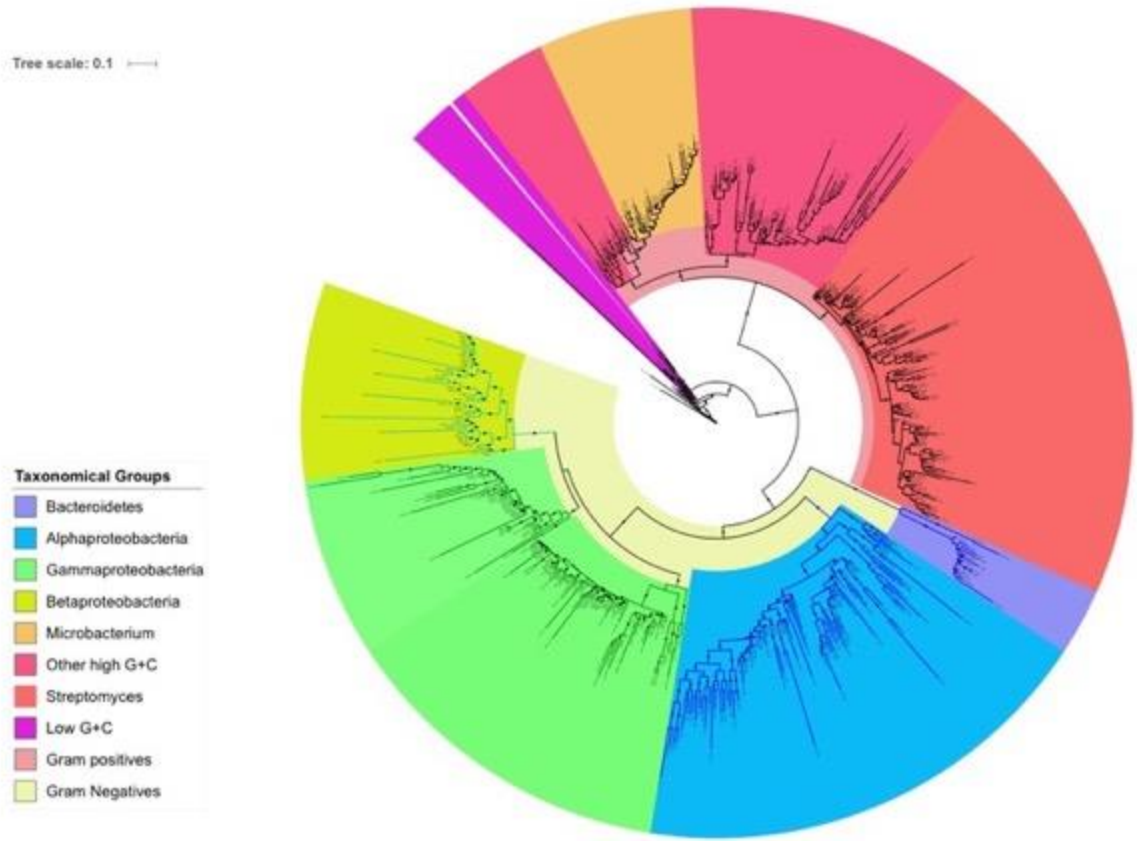
Tree scale: 0.1
Taxonomical Groups
Bacteroidetes
Alphaproteobacteria
Gammaproteobacteria
Betaproteobacteria
Microbacterium
Other high G+C
Streptomyces
Low G+C
Gram positives
Gram Negatives

Figure 2.5 Molecular phylogenetic analysis of the V1-V316SrRNA gene sequences of 458 putative diazotrophs isolates by maximum likelihood method made in PhyML using aBayes Branch analysis method. Thick dots represent bootstrap values of 80% or greater and thin dots represent values less than 80%.

2.3.2. Growth in liquid NFM in nitrogen depleted atmosphere:

A total of 163 isolates belonged to *Actinobacteria*, a phylum not well known for free-living nitrogen fixation. Importantly, 115 of these were *Streptomyces* not known much for diazotrophy. In order to exclude growth by scavenging of atmospheric combined nitrogen, cultures in liquid NFM were incubated in a sealed atmosphere container with the zeolite Clinoptilolite, previously shown to bind residual ammonia (Wang et al. 2006; Liao et al. 2015). Of these 163, 109 isolates were able to grow in liquid NFM in the ammonia depleted atmosphere, indicating the ability to fix nitrogen (Yoshida, Inaba et al. 2014). Most of the *Streptomyces* appeared as distinct clumps in clear liquid. These clumps grew when plated on NFM agar (Fig 2.5).

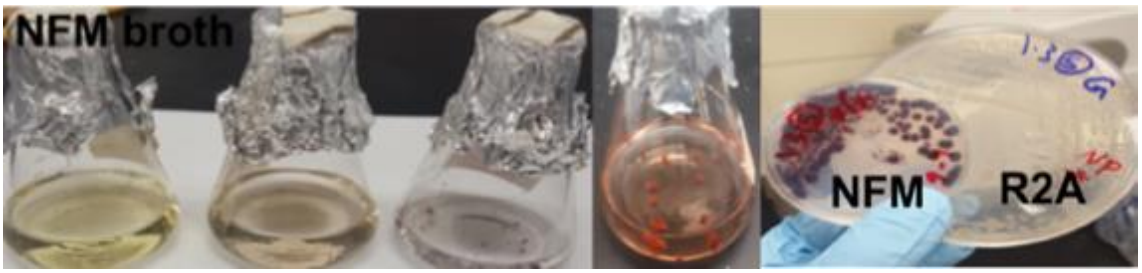

Figure 2.6 Cultures of putative diazotrophs in liquid NFM in an atmosphere depleted of ammonia using Clinoptilolite. Cultures were incubated at 28oC for 20d. Streptomyces isolates appeared morphologically different on R2A and NFM agar.

2.3.3. *nifH* gene amplification:

The presences of *nifH* genes in isolates was tested using the primers PolF and PolR (Poly, Monrozier et al. 2001, Gaby and Buckley 2012), IGK3 and DVV (Gaby and Buckley 2012), Ueda19F and Ueda407R (Ueda, Suga et al. 1995), NifH11 and NifH22, and19F and NifH3 (Yeager, Kornosky et al. 2004) (Table 2.1). The PolF-PolR primer set yielded 360 bp amplicons for 96 isolates. Comparison of the sequences using the curated

online database, Uniprot (Consortium 2014), confirmed 86 of these as *nifH*. Of the 161 *Actinobacteria,* 55 were positive for *nifH* (Table 2.3). Presence of *nifH* in *Actinobacteria* isolates was also tested using the other four primer sets, two of them part of a nested PCR. Only eighteen isolates, six *Streptomyces*, three *Microbacterium*, three *Rhodococcus*, one *Mycobacterium*, one *Stenotrophomonas,* one *Agrobacterium*, one *Nocardiodes,* one *Micromonospora,* and one *Ensifer,* yielded *nifH* with all four PCRs, and no other isolates yielded *nifH* with any primer set. While the PolF-PolR primer set was not evaluated as superior by (Gaby and Buckley 2012), it did yield by far the greatest number of positives that were then confirmed through sequencing. Most verified sequences were homologous to *nifH* genes of either *Mesorhizobium* and *Azotobacter (α-Proteobacteria), Paenibacillus (Firmicutes),* or *Herbaspirillum (β-Proteobacteria).* Only some members of the individual genera yielded *nifH,* even for genera known for N_2-fixing, such as *Rhizobium*. In *Paenibacillus,* as exception, all five isolates yielded *nifH*. This observation may have occurred either be because amplification was unsuccessful because the primers were not binding, or because no *nifH* gene was present. In many cases the *nifH* obtained was homologous to species unrelated to taxa identification by 16SrRNA, except for *Paenibacillus* Table2.4. After considering if these isolates comprised of more than one species, all isolates were subjected to a rigorous purification protocol. Based on specific phylum 16SrRNA PCR, 23/96 cultures were single strains while others comprised of two (61), three (9), or 4(3) different taxa. The genus identification by 16SrRNA obtained are detailed in Table 2.4. In case of single colony, *Phyllobacterium* yielding a *Paenibacillus nifH* gene could be a bi-culture of nitrogen- fixing *Paenibacillus,* and a non-nitrogen-fixing *Phyllobacterium*. However,

isolates had been streaked repeatedly on R2A, which supports growth of non-nitrogen fixers. This would have allowed the non-fixing *Phyllobacterium* to form colonies on its own, and facilitated separation. Furthermore, the 16SrRNA gene amplicons yielded clean sequences, also supporting single taxa. If isolates as described above (e.g. *Phyllobacterium* with *Paenibacillus nifH*) are single cultures of *Phyllobacterium* and no *Paenibacillus*, this suggests horizontal gene transfer. (Fig. 2.6) (Table 2.4).

Table 2.4 Distribution of the bacterial diversity of Phylogeny on basis of 16SrRNA gene, nifH, and phylum specific 16SrRNA for 328 that have some fixers

Allocation to genus based on 16SrRNA gene.	**Homology of *nifH* seq to genera using UniProt**	**Phylum *Firmicutes* 16SrRNA**	**Phylum *Proteobacteria* 16SrRNA**
30 *Rhizobium* (*alpha-Proteobacteria*)	11 detected by (PolF-PolR): 4 *Mesorhizobium,*3 *Paenibacillus,*3 *Herbaspirillum,* and 1 *Sinorhizobium,*	3 isolates which have *nifH* homologous to *Paenibacillus* 2 isolates which have *nifH* homologous to *Mesorhizobium* 3 isolates which have *nifH* homologous to *Herbaspirillum* 1 isolate which have *nifH* homologous to *Sinorhizobiu.* (All of them have 16SrRNA	2 isolates which have *nifH* homologous to *Paenibacillus,* have 16SrRNA *beta- Proteobacteria* homologous to *Herbaspirillum*

Allocation to genus based on 16SrRNA gene.	**Homology of *nifH* seq to genera using UniProt**	**Phylum *Firmicutes* 16SrRNA**	**Phylum *Proteobacteria* 16SrRNA**
		Firmicutes homologous to *Bacillus)*	
22 *Stenotrophomonas* (*alpha- Proteobacteria*)	6 detected by (PolF-PolR & NifH11-NifH22): 1 *Pseudomonas,*2 *Mesorhizobium,*1 *Paenibacillus,*1 *Herbaspirillum,* and 1 *Azotobacter.vinelandi,*	1 isolate which has *nifH* homologous to *Paenibacillus* 1 isolate which has *nifH* homologous to *Azotobacter.vinelandi.* (Both have 16SrRNA *Firmicutes* homologous to *Bacillus)*	1 isolate which has *nifH* homologous to *Azotobacter.vinelandi,* has 16SrRNA *beta- Proteobacteria* homologous to *Herbaspirillum*
15 *Phyllobacterium* (*alpha- Proteobacteria*)	6 detected by (PolF-PolR & NifH11-NifH22):	No evidence for co-culture	3 isolates which have *nifH* homologous to *Herbaspirillum,*

Allocation to genus based on 16SrRNA gene.	**Homology of *nifH* seq to genera using UniProt**	**Phylum *Firmicutes* 16SrRNA**	**Phylum *Proteobacteria* 16SrRNA**
	2 *Paenibacillus*,8 *Herbaspirillum*,		have 16SrRNA *beta-Proteobacteria* homologous to *Herbaspirillum*
3 *Bosea*, (*alpha-Proteobacteria*)	1 *Paenibacillus* detected by (PolF-PolF)	1 isolate which has *nifH* homologous to *Paenibacillus*, has 16SrRNA *Firmicutes* homologous to *Bacillus*	No evidence for co-culture
3 *Ensifer*, (*alpha-Proteobacteria*)	1 *Paenibacillus* detected by (PolF-PolF)	1 isolate which has *nifH* homologous to *Paenibacillus*, has 16SrRNA *Firmicutes* homologous to *Bacillus*	No evidence for co-culture

Allocation to genus based on 16SrRNA gene.	**Homology of *nifH* seq to genera using UniProt**	**Phylum *Firmicutes* 16SrRNA**	**Phylum *Proteobacteria* 16SrRNA**
5 *Shinella (alpha-Proteobacteria)*	1 *Paenibacillus* detected by (PolF-PolF)	1 isolate which has homologous to *Paenibacillus*, has 16SrRNA *Firmicutes* homologous to *Bacillus*	1 isolate which has homologous to *Paenibacillus*, has 16SrRNA *beta-Proteobacteria* homologous to *Herbaspirillum*
5 *Burkholderia* (*alpha-Proteobacteria*)	1 *Paenibacillus* detected by (PolF-PolF)	1 isolate which has homologous to *Paenibacillus, has 16SrRNA Firmicutes homologous to Bacillus*	No evidence for co-culture
2 *Agrobacterium* (*alpha Proteobacteria*)	1 *Mesorhizobium* detected by (PolF-PolF)	No evidence for co-culture	No evidence for co-culture

Allocation to genus based on 16SrRNA gene.	**Homology of *nifH* seq to genera using UniProt**	**Phylum *Firmicutes* 16SrRNA**	**Phylum *Proteobacteria* 16SrRNA**
51 *Pseudomonas* (*gamma- Proteobacteria*)	1 *Herbaspirillum* detected by (PolF-PolF)	No evidence for co-culture	1 isolate which has *nifH* homologous to *Herbaspirillum*, has 16SrRNA *beta-Proteobacteria* homologous to *Herbaspirillum*
4 *Flavobacterium* (*Bacteroidetes*)	1 *Herbaspirillum* detected by (PolF-PolF)	No evidence for co-culture	1 isolate which has nifH homologous to Herbaspirillum, has 16SrRNA beta-Proteobacteria homologous to Herbaspirillum, also has 16SrRNA *alpha-Proteobacteria* homologous to *Mesorhizobium*

Allocation to genus based on 16SrRNA gene.	**Homology of *nifH* seq to genera using UniProt**	**Phylum *Firmicutes* 16SrRNA**	**Phylum *Proteobacteria* 16SrRNA**
20 *Bacillus* (*Firmicutes*)	1 *Herbaspirillum* detected by (PolF-PolF)	1 isolate which has *nifH* homologous to *Herbaspirillum,* has 16SrRNA *Firmicutes* homologous to *Bacillus*	1 isolate which has *nifH* homologous to *Herbaspirillum,* has 16SrRNA *alpha-Proteobacteria* homologous to *Mesorhizobium*
5 *Paenibacillus* (*Firmicutes*)	5 *Paenibacillus* detected by (PolF-PolF)	5 isolates which have *nifH* homologous to *Paenibacillus* , have 16SrRNA *Firmicutes* homologous to *Bacillus*	No evidence for co-culture
115 *Streptomyces (Actinobacteria)*	33 detected by PolF-PolR 14 *Mesorhizobium*,5 *Paenibacillus,* and 14 *Herbaspirillum.*	14 isolates which have *nifH* homologous to *Mesorhizobium* 14 isolates which have *nifH*	3 isolates which have *nifH* homologous to *Herbaspirillum* , have 16SrRNA beta-Proteobacteria homologous to

Allocation to genus based on 16SrRNA gene.	**Homology of *nifH* seq to genera using UniProt**	**Phylum *Firmicutes* 16SrRNA**	**Phylum *Proteobacteria* 16SrRNA**
	7 detected by NifH11-NifH22: 4 *Clostridium*, 2 *Nostoc*, and 1 *Pelobacter*	homologous to *Herbaspirillum* (All of them have 16SrRNA *Firmicutes* homologous to *Bacillus)*	*Herbaspirillum*, also have 16SrRNA *alpha-Proteobacteria* homologous to *Mesorhizobium*
10 *Rhodococcus* (*Actinobacteria*)	1 *Paenibacillus* detected by (PolF-PolR & NifH11-NifH22)	No evidence for co-culture	No evidence for co-culture
4 *Kitasatospora*, (*Actinobacteria*)	1 *Paenibacillus* detected by (PolF-PolR & NifH11-NifH22)	No evidence for co-culture	No evidence for co-culture

Allocation to genus based on 16SrRNA gene.	**Homology of *nifH* seq to genera using UniProt**	**Phylum *Firmicutes* 16SrRNA**	**Phylum *Proteobacteria* 16SrRNA**
30 *Microbacterium* (*Actinobacteria*)	17 *Mesorhizobium* detected by (PolF-PolR & 3 of 17 detected by NifH11-NifH22)	All 17 isolates which have nifH homologous to *Mesorhizobium*, have 16SrRNA *Firmicutes* homologous to *Bacillus*	3 isolates which have *nifH* homologous to Mesorhizobium, have 16SrRNA *alpha-Proteobacteria* homologous to Mesorhizobium
2 *Nocardiodes,* (*Actinobacteria*)	1 *Paenibacillus* detected by (PolF-PolR & NifH11-NifH22)	No evidence for co-culture	No evidence for co-culture
1 *Micromonospora* (*Actinobacteria*)	1 *Paenibacillus* detected by (PolF-PolR & NifH11-NifH22)	No evidence for co-culture	No evidence for co-culture

Allocation to genus based on 16SrRNA gene.	**Homology of *nifH* seq to genera using UniProt**	**Phylum *Firmicutes* 16SrRNA**	**Phylum *Proteobacteria* 16SrRNA**
1 *Mycobacterium* (*Actinobacteria*)	1 *Paenibacillus* detected by (PolF-PolR & NifH11-NifH22)	No evidence for co-culture	1 isolate which has homologous to *Paenibacillus*, has 16SrRNA *beta-Proteobacteria* homologous to *Herbaspirillum*

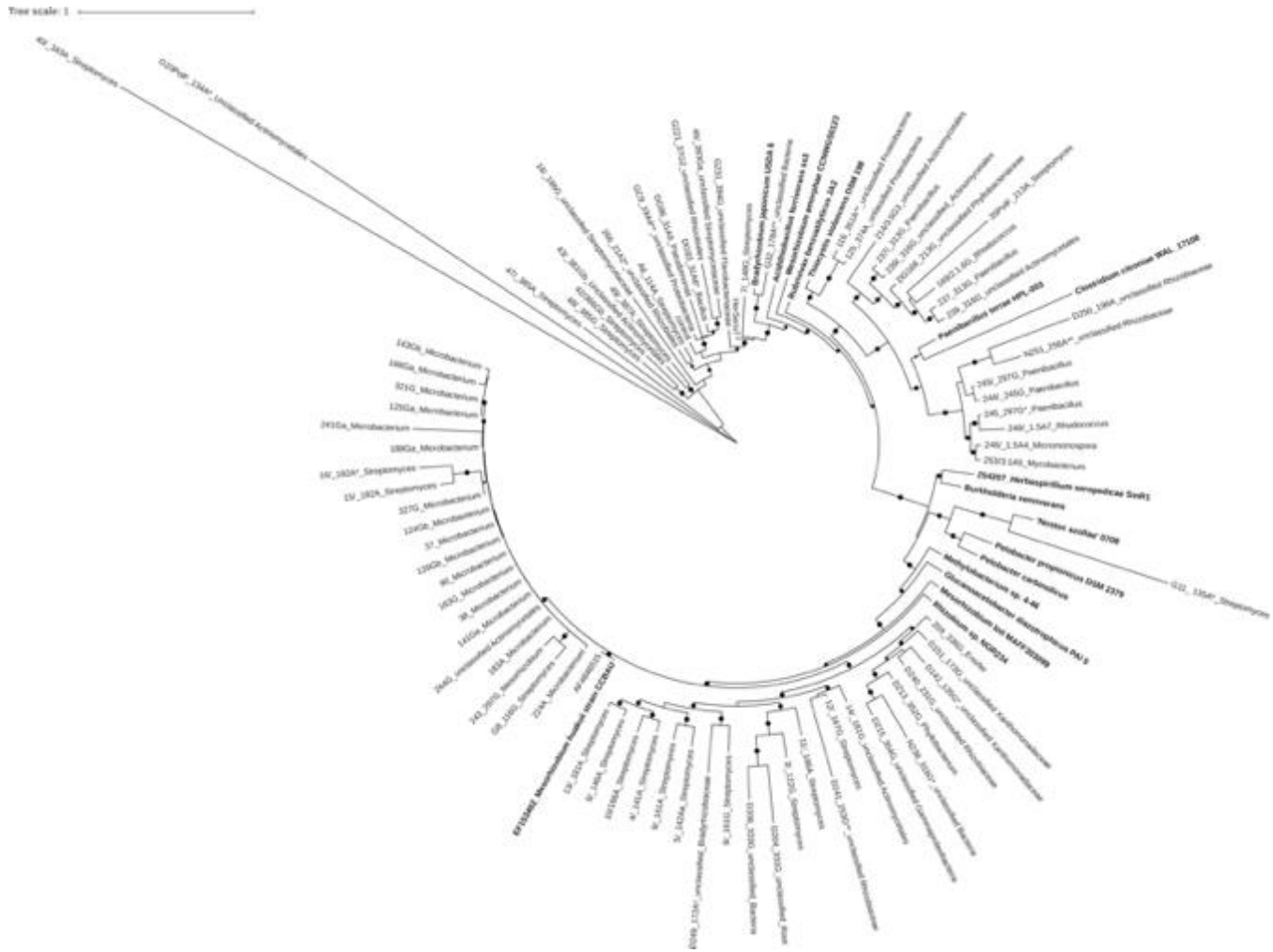

Figure 2.7 Maximum likelihood phylogenetic tree of nifH gene from cultures based on PhyML using aBayes Branch analysis method. Thick dots represent bootstrap values of 80% and thin dots represent value less than 80%.

2.4. Discussion:

The aim of this study was to obtain a broader view of free-living nitrogen fixing bacteria in a South Dakota prairie through isolation into culture. We employed a diversity-increasing approach described by Mirza and Rodriguez (2012). The generally limited input of carbon sources into soil has selected for a preponderance of slower growing bacteria. These slower growing bacteria can easily be outcompeted in soft agar by faster growing, competitive isolates, and therefore not be obtained into culture. Plating on solid medium followed by incubation under reduced oxygen atmosphere physically separates slower growing from more aggressive strains, allowing isolation of the slower growing strains, capturing a wider diversity. Many of the putative diazotrophs we obtained belong to genera that contain known nitrogen fixers, including *Rhizobium* and *Ensifer* (Videira et al., 2009), *Burkholderia* (Xie et al., 2014), *Phyllobacterium* (Rojas et al., 2001), *Paenibacillus (Shi, Wang et al. 2016)* and *Stenotrophomonas* (Chelius and Triplett, 2000). Our approach also yielded a large number of isolates from genera not known for nitrogen fixation, primarily *Streptomyces*. We did not obtain any of the classical free-living nitrogen fixers such as *Azotobacter* or *Azospirillum*. These genera may not be prevalent in prairie soil microbiome, and were also not found in Brazilian forest soil ((Mirza and Rodrigues 2012).

Nitrogen fixation is not universal across all members of any of the known diazotrophic genera, even *Rhizobium* (Mirza and Rodrigues 2012). We could detect *nifH* in members of all genera obtained. Yet only 20% of isolates yielded *nifH* by PCR. Of thirty *Rhizobium* isolates, only eleven tested PCR positive. *Paenibacillus* was the only genus with 100% *nifH* positive isolates. These results are differ from a culturing approach

from Brazilian forest soil where 79% of isolates from nitrogen free medium were *nifH* positive (Mirza and Rodrigues 2012). In contrast 39% of their isolates were acetylene reductase positive. Only 37% of our *Rhizobium* and 20% of our *Burkholderia* isolates were *nifH* positive compared to 66% of those from tropical forest (Mirza and Rodrigues 2012). Our results are in accordance with those of several other studies that reported a low rate of *nifH* recovery from isolates growing on N-free medium (Achouak, Normand et al. 1999, Berg, Roskot et al. 2002, Doty, Oakley et al. 2009). It is possible that the majority of our isolates are not nitrogen fixers. Yet our culture medium did not comprise of any nitrogen-containing compounds. The water used contained 2.57 $\mu mol.L^{-1}$ total nitrogen (Determined by the Southeast Environmental Research Center at Florida International University). The liquid NFM contained 6.42 $\mu mol.L^{-1}$ nitrogen, and the noble agar used did not contain detectable nitrogen. We excluded atmospheric ammonia in liquid culture experiments by incubating in a sealed container with Clinoptilolite, an ammonia scavenger. Bacterial growth in this environment would require nitrogen fixation, or, alternatively, the ability to scavenge residual quantities of nitrogen (Wang, Liu et al. 2006, Liao, Chen et al. 2015).

Recently, Yoshida et al. demonstrated that *Rhodococcus erythropolis* grows on nitrogen free medium without fixing nitrogen by scavenging residual atmospheric ammonia using a high affinity uptake system (Yoshida, Inaba 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 *nifH* gene varies considerably across the bacteria, and does not contain any fully conserved regions long enough for primer design. This may be why so many primer sets have been described over the past two decades, reviewed most recently by Gaby and Buckley (Gaby and Buckley 2012). There is still a need for PCR primers that capture the diversity of all isolates (Zehr 2011). Furthermore, all primers described to date are highly degenerate, decreasing specificity of amplification (Zehr and Kudela 2011, Zehr 2017). Bioinformatic analysis of 51 *nifH* primers by mapping to the complementary binding site pointed to IGK3/DVV as the primer set for highest probability of recovery, followed by Ueda19F/Ueda407R (Gaby and Buckley 2012). All these primers correspond to conserved regions encoding basic roles such as the P-loop, Switch I and Switch II. These primers did not yield many amplicons in our hands, even after changing conditions such as annealing temperature, Mg^{2+} concentrations, and reagents known to enhance PCR, such as DMSO, BSA and formamide. Only *B. japonicum* USDA 110 as a positive control and one *Streptomyces* yielded amplicons. Primers F2/R6 and nifH1/nifH2, reported widely in (Zehr and McREYNOLDS 1989, Kirshtein, Paerl et al. 1991) also performed poorly in our hands, producing smeared bands due to non-specific amplification, along with PCR product from negative controls. There are other factors which could impact the specificity of PCR primers such as primer dimerization (Brownie J,1997), hairpin formation (Singh VK, 2000), GC content (Benita Y, 2003), the location of mismatches (Bru D et al. 2008), and the thermodynamics of primer binding to the template (Polz M et al. 1998). Mismatches at the 3′ end to increase primer coverage leads to decreased primer specificity (Rose TM, 2003). We observed best results using PolF and PolR primers (Poly, Monrozier et al. 2001, Gaby and Buckley 2012), recommended

for soil bacteria (Gaby and Buckley 2012), obtaining products from 96 isolates, many of these from *Streptomyces* isolates. The PolF PolR primer set only encompassed 25% of *nifH* diversity (Poly, Monrozier et al. 2001, Gaby and Buckley 2012), with specificity to Alpha-, Beta-, and Gamma-Proteobacteria, Firmicutes, and Actinobacteria without cluster IA, Cyanobacteria, or cluster III sequences. This may explain the low positive result in part. It does not explain why primers found superior through bioinformatics approaches performed so poorly in our hands whereas PolF PolR performed the best. This may explain the low positive result in part. It does not explain why primers found superior through bioinformatics approaches performed so poorly in our hands whereas PolF PolR performed the best. All our *nifH* amplicons fell into Cluster I of the *nifH* gene database, but primers for *vnfH* and *anfH* did not yield any products. Yet it is possible that isolates negative for PolF PolR contain *nifH* aligning with clusters other than cluster I, including paralogous genes of cluster IV, not involved in nitrogen-fixation, for example, cluster IV or V genes found in bacteriochlorophyll synthesis (Rose TM, 2003). Recently *Endomicrobium proavitum* containing a cluster IV *nifH* was shown definitively to fix nitrogen (Zheng, Dietrich et al. 2016). Analysis of whole genome sequences of putative diazotrophs may yield insights into the basis for growth of these PCR-negative isolates on nitrogen fee medium.

Of 458 isolates allocated to *Actinobacteria, Proteobacteria, Bacteroidetes,* and *Firmicutes* by 16S rRNA gene sequence, 96 (20%) yielded *nifH* amplicons. Yet these amplicons aligned mostly with the *nifH* of unrelated taxa such as *Mesorhizobium, Herbaspirillum*, and *Paenibacillus* (Table 2.4). Some *Rhizobium* cutures yielded *nifH* aligning with *Paenibacillus*, as did some *Rhodococcus* isolates. *Flavobacterium* isolates

yielded *nifH* aligning to *Herbaspirillum*. Of 30 *Microbaterium*, 17 yielded nifH homologous to *Mesorhizobium*. This could be due to horizontal gene transfer as had been documented for the *nifH* of sulfate reducing delta *Proteobacteria* and for *nifH* of *Sinorhizobium* to *Ensifer* (Barcellos, Menna et al. 2007, Miyazaki, Higa et al. 2009), but could also be due to bicultures. Our isolation protocol was rigorous, entailing multiple steps to gain single cultures. All our isolates presented as colonies of identical appearance with no hints of a second taxon included. The sequencing of 16SrRNA gene amplicons yielded clean signals of high quality, indicating single cultures. Yet PCR by phylum-specific 16SrRNA gene primers indicated the presence of second cultures (Table 2.4). There are currently no fully confirmed nitrogen fixing *Streptomyces*. The thermophilic, autotrophic *S. thermoautotrophicus* initially reported as a nitrogen fixer (Gadkari, Morsdorf et al. 1992, Ribbe, Gadkari et al. 1997), was recently shown unable to fix nitrogen in a multi-laboratory study (MacKellar, Lieber et al. 2016). We have recently reported several nitrogen-fixing *Streptomyces* isolated from South Dakota Badlands (Dahal, Nandakafle et al. 2017). The 34 *nifH* from *Streptomyces* reported here, aligned to *nifH* of *Mesorhizobium*, *Paenibacillus,* and *Herbaspirillum.* In each case the co-culture of the *Streptomyces* was identified by phylum-specific 16S PCR to mirror homology of the *nifH* gene.

The pure-culture paradigm is core to reductionist microbiology, and the few cases of bacterial bi-cultures reported fall in the area of syntrophy (Sieber, McInerney et al. 2012). Extensive searches for culture-derived *nifH* sequences on NCBI revealed that this phenomenon may be more common, but is not reported. A comparison of the phylogeny of members of isolates and the *nifH* gene homology indicates that the lesser member of

the consortia is the nitrogen fixer. Soils contain diverse bacteria with different growth rates, metabolic capabilities and competitive traits. Some strains can impair the growth of others through antibiotic production or other growth-inhibiting substances (Ryan, Germaine et al. 2008). This could be due to parasitic interaction of the dominant isolate with the secondary, nitrogen-fixing one (Amann, Springer et al. 1997), and will need to be investigated further.

Chapter 3

3. Bacterial nitrogen-fixing cultures from prairie soil comprised of two-species interactions

3.1. Introduction

Biological nitrogen fixation (BNF), the reduction of atmospheric N_2 to biologically available ammonium, is the main pathway by which atmospheric N_2 enters the biosphere. Diazotrophs are the major organismal group responsible for atmospheric nitrogen (N_2) fixation in natural ecosystems. Among the nitrogenase subunits, the nitrogenase reductase subunit, encoded by *nifH*, has the most reference sequences available and has become an important marker for studying N_2 fixing bacterial communities in various environments (Zehr, Jenkins et al. 2003, Raymond, Siefert et al. 2004). Phylogenetic analysis of taxonomically identified nitrogenase genes had provided evidences of a horizontal gene transfer between bacteria and archaea (Raymond, Siefert et al. 2004).

The *nifH* gene family has been vastly studied to analyze diazotrophic microbial communities in various environments, especially in marine and soil ecosystems , resulting in novel findings on the diversity of nitrogen fixers (Izquierdo and Nüsslein 2006) . For instance, *Trichodesmium* has been found as the major N_2-fixing cyanobacteria in marine ecosystems (Zehr 2011), but a recent study proposed that there are more significant contributions by other N_2 fixers indicating substantial roles of less dominant N_2 fixers (Großkopf, Mohr et al. 2012).

N_2 fixers very diverse across multiple phyla. The more widely reported diazotrophs are members of α-β-γ and δ-*Proteobacteria, Cyanobacteria*, and *Methanobacteria*. *Clostrideacea* and *Bacillaceae* include diazotrophs of the low G+C *Firmicutes* (Shi, Wang et al. 2016), and the high G+C bacteria include divers nitrogen fixing genera, including *Arthrobacter, Agromyces, Corynebacterium, Mycobacterium, Micromonospora.* This also includes *Frankia* who fix nitrogen both in free-living and in symbiotic conditions (Sellstedt and Richau 2013). The large actinobacterial genus has previously been viewed as a nitrogen fixer except for *Streptomyces thermoautotrophicus*, a thermophilic autotrophs (Ribbe, Gadkari et al. 1997). Recently, *Streptomyces thermoautotrophicus* was reported unable to fix nitrogen (MacKellar, Lieber et al. 2016). There are scant other reports that indicate certain *Streptomyces* can fix nitrogen based on acetylene reduction activity (Ding, Sun et al. 1981). In addition, there are *Streptomyces* isolated from Taxes soil have been deposited as nitrogen fixers at ATCC by P. Jurtshuk. Recently, Dahal reported *nifH* from several *Streptomyces*, isolated from the South Dakota Badlands. The n*ifH* was homologous to *Cyanobacteria nifH*, suggesting horizontal gene transfer. The presence of *Cyanobacteria* as co-culture could not demonstrated by culturing of Cyanobacteria-specific PCR (Dahal, Nandakafle et al. 2017).

We have recently obtained 115 Streptomyces from natural prairie through isolation on free nitrogen agar (Chapter2). The aim of this study was to characterize these isolates more closely by studying their nitrogen fixing ability.

3.2. Materials and Methods

Streptomyces (115) isolated previously from prairie soil onto NFM were used in this study. All 115 grew in liquid NFM in an atmosphere depleted of ammonia using

Clinoptilolite. Diazotrophy was re-confirmed by growing them in liquid nitrogen free medium. Stock cultures were prepared by suspending liquid-grow-cultures in 50% sterile glycerol and freezing at -80^{o}C.

3.2.1. *nifH* gene amplification

nifH genes were PCR amplified using Pol F/R primers (Poly, Monrozier et al. 2001, Gaby and Buckley 2012) under the reaction condition of 15 sec denaturing at 95^0 C, 55^0 C for 1 min and 72^0 C for 30 s for 30 cycles. The PCR reaction was conducted with various modification (Fig 3.1).

Figure 3.1 Flow chart outlining the procedures followed to obtain nifH amplicons by PCR.

3.2.2. Acetylene Reduction Assay

Eighteen selected bacterial strains, which were positive for *nifH* PCR, were sub-cultured in liquid R2A medium. One colony was then inoculated into 10 ml fresh liquid NFM medium with and without 5% R2A added in 50 ml glass tubes, and sealed with neoprene stoppers. After incubation for 6 d, the gas phase of the medium was initially replaced with 2 percent fresh air and then 10 percent of the total headspace was exchanged with an equal volume of acetylene, and the cultures were incubated for 4 weeks. A 0.1mL gas sample was assayed after 10-fold dilution with O_2. Ethylene was quantified using a gas chromatograph (Agilent Technologies 7890A) using a flame ionization detector and an Agilent CP7348 column, operating at 50°C and 85°C, respectively. Non-inoculated liquid NFM was injected with acetylene and served as a negative control, and *Herbaspirillum seropedicae* ATCC® 35892 was used as positive control.

3.2.3. Incorporation of $15N_2$ isotope in pure cultures

Fourteen isolates pre-cultured in liquid nitrogen-free medium were positive for *nifH* PCR and ARA, selected and transferred to fresh liquid NFM (50 ml) in 100 ml serum bottles capped with septum caps and crimped (Fig 3.2). A sterile syringe was used to replace 10 ml air with the same amount of $^{15}N_2$ gas by injection (Cam Isotope Laboratories, Inc. [$15N_2$, 98%+]). After gentle mixing, the bottles were incubated at 28 ºC for 4 weeks. Cells were harvested by centrifugation and dried at 65 °C overnight. The mass was determined using Denver Instruments M-220D digital scale to get 0.5 - 2 mg weight, and then samples were placed into tin capsules. They were then inserted into wells of 96-well

microtiter plate and sealed with strip caps and sent to the Stable Isotope Facility at the University of California at Davis.

Figure 3.2 Cultures of Streptomyces in NFM in 100 ml glass serum vials and capped tightly with septum caps to ensure the absence of air exchange.

3.2.4. Complete Genome Sequence of *Streptomyces* sp

Strains of *Streptomyces* that showed growth on solid as well as liquid NFM and had ARA activity were selected for complete genome sequencing. The DNA was extracted using the Microbial DNA Isolation Kit (Qiagen). The concentration of DNA was quantified using a Qubit 3 Fluorometer and NanoDrop™ 2000/2000C Spectrophotometer, and the integrity was examined by resolving the samples through 0.75% agarose gel. MicrobesNG recommends a volume of 30 – 100 µl genomic DNA at a concentration of 1 – 30 ng/µl for Next Generation Sequencing. Fifty microliters of genomic DNA from seven *Streptomyces* strains qualified for complete genome sequencing with a concentration of 30-89 ng/µl. They were sent in microfuge size screw

cap tubes to MicrobesNG, Birmingham, United Kingdom for genome sequencing at 50× coverage.

3.2.5. Multilocus sequences typing of selected *Streptomyces*

To guide our *Streptomyces* species discrimination and the discovery of potentially novel species, MLST (Multilocus Sequences Typing) (Guo, Zheng et al. 2008) had constructed using concatenated tree of four housekeeping genes 16SrRNA, *atpD, recA,* and *ropB.* Sequences fragments of four housekeeping genes were obtained for 58 valid *Streptomyces* strains against eight whole genomes of *Streptomyces* strains I got. Sequences of each gene were aligned using MEGA7 software and trimmed manually at the same position before being used for further analysis. Furthermore, phylogenetic relationships among 58 *Streptomyces* strains based on four-gene concatenated sequences were constructed using the maximum likelihood method using MEGA7.

3.2.5.1. Validation of culture purity by phylum specific 16S rRNA amplification

Primers specific for 4 different phyla (table 2.3), were used to amplify of 16S rRNA genes associated with *Streptomyces* isolates (Pfeiffer, Pastar et al. 2014). The reaction mixture composition and PCR conditions were adopted from the previous protocol of amplification of V_1-V_3 region of 16S rRNA gene used for identification of diverse bacteria (Muyzer, De Waal et al. 1993). The annealing temperatures were first examined to get optimum amplification from each primer set (Table 2.3) as described in Chapter 2. The PCR products were visualized by electrophoresis (1.2% agarose, 60 V for

60 min) with standard 100bp ladder (NewEngland BioLabs) to compare the size. The PCR products with positive amplification were sent to GeneScript for sequencing.

3.2.6. Obtaining strains into single culture

The results of whole genome sequencing indicated presence of Bacillus in the Streptomyces isolates. This was confirmed by Firmicutes - specific PCR which yielded sequences aligning with Bacillus. To obtain the Bacillus into single culture we employed the following approach.

3.2.6.1. Isolation of *Bacillus* into single culture

Liquid R2A cultures of three Streptomyces isolates, were selected based on the complete genome results. Culture suspensions were incubated at 80oC (for 40 mins) in a heating block. For a second series, we incubated the culture at 90oC for 30 mins. After cooling, aliquoted, and streaked on NFM and R2A. The colonies obtained after incubation were sub-cultured on NFM. DNA was extracted and used as template to obtain 16S rRNA gene and *nifH* amplicons as described above. Firmicute and Actinobacteria specific PCR was also performed to verify absence of *Streptomyces*.

3.2.6.2. Introduction of isolated *Bacillus* with *Streptomyces*

The *Bacillus* isolated through the heat treatment were inoculated along with original *Streptomyces* isolates in Brain Heart Infusion (mixed equally with Tryptone Soya Broth) and incubated for 48 h. As a control, *Streptomyces* cultures were inoculated in separate tubes. Aliquots were streaked on NFM and R2A, and later, sub-cultured on respective medium by steaking. The results from each plate were recorded and compared.

3.2.6.3. Isolation of *Streptomyces* using filters

Streptomyces were inoculated in broth containing equal volumes of BHI and TSB. After that, 10 µl of the fresh culture was used as inoculum on to the center of NFM agar and incubated for 24 h. Sterile filters, with a pore size of 0.1, 0.2, 0.4, and 0.8 µm, were placed on top of the colony. BHI agar discs prepared in a small Petri plate, were then placed carefully on top of the filter paper and incubated at 28^{o}C for 3 days. The rich medium on the filter paper was carefully removed and placed on top of a sterile and

freshly prepared NFM; they were incubated for a week. The growth observed on each plate was carefully recorded. All the isolated cultures were subjected for 16S rRNA gene PCR using *Actinobacterial* and *Firmicute* specific primer sets, and sequenced. The isolates were also analyzed by PCR for the presence of *nifH* gene sequence. Each of the isolates were sub-cultured on NFM to evaluate their growth patterns. Gram staining was performed for the isolates obtained on top of the filter.

3.2.7. Transformation of *Bacillus* with GFP

The GFP-expressing plasmids (Table 3.2) were obtained from Bacillus Genetic Stock Center (BGSC) in *E. coli*, and were grown in LB broth for 24 h. The plasmids were extracted using the QIAprep® Spin Miniprep Kit. The concentration of plasmid was quantified using a NanoDrop™ 2000/2000C Spectrophotometer and the integrity was determined by resolving the sample in a 0.75% agarose gel. Three selected *Bacillu*s strains and a control strain were inoculated in 250ml flasks containing LB broth. The flasks were incubated at 37°C while shaking for 3 h, approximate time required for the culture to reach stationary phase (Fig 3.3). Biomass was quantified every 60 min by determining the absorbance at 600nm. In a micro-centrifuge tube, 4 µl of plasmid DNA was mixed into 50 µl of transition phase cells, assumed to be competent. The mixture was incubated on ice for 30 min. As a heat shock, each transformation tube was placed in the water bath at 42 °C for 50-60 s and then placed on ice for 2 min. LB broth (500 µl) without antibiotics was added to the culture and incubated at 37°C while shaking for 45 min. LB agar containing appropriate antibiotic, with respect to the type of plasmid used for transformation, was inoculated using 100 µl of transformed cultures (Table 3.2). The

plates were incubated overnight at 37 ºC. The growth was observed under Olympus BX53 Upright microscope.

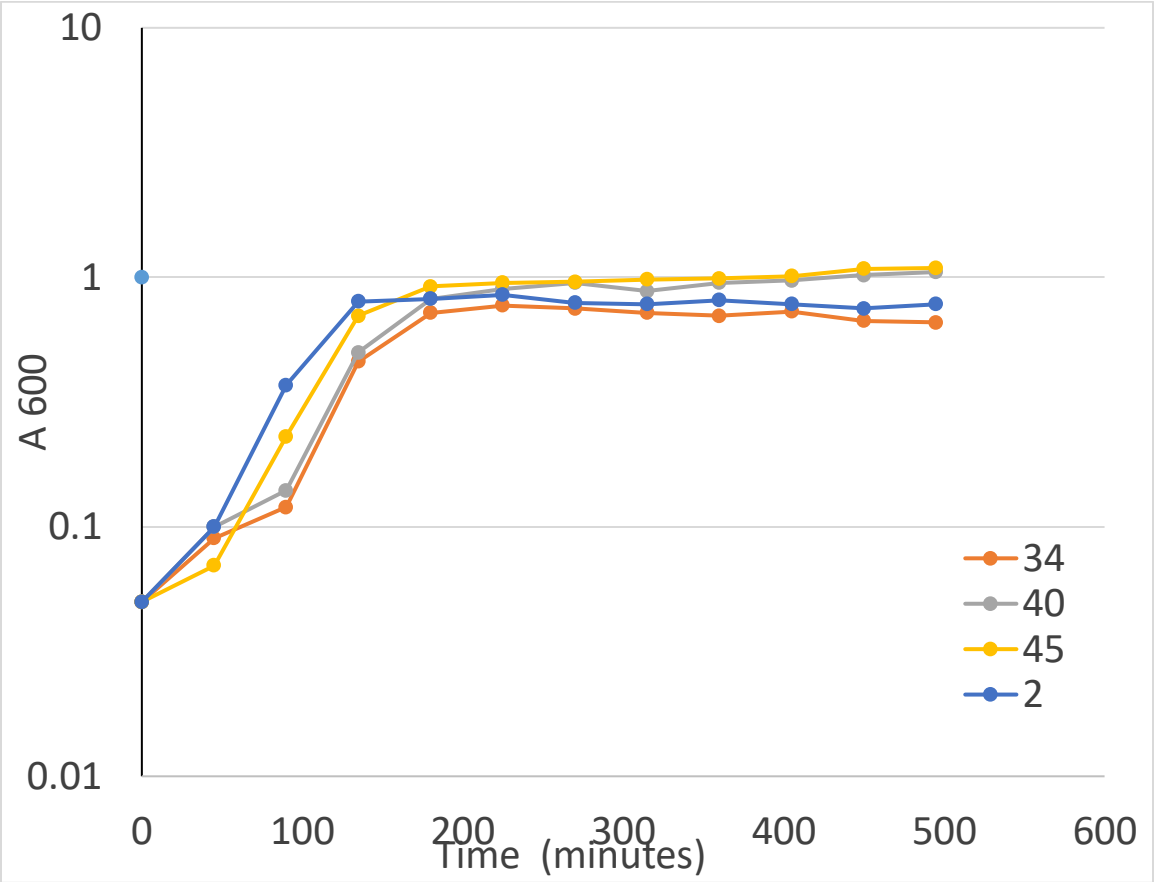

Figure 3.3 Growth curve of four Bacillus isolation in LB broth, while shaking at 37ºC.

Table 3.1 Antibiotics used for selection of Bacillus transformed with various GFP-encoding plasmids

Plasmid BGSC Accession	**Appropriate Antibiotic**
ECE152	Chloramphenicol 5μg/ml
ECE153	Spectinomycin 50μg/ml
ECE153	Chloramphenicol 5μg/ml
ECE166	Chloramphenicol 5μg/ml

3.2.8. The separation of *Streptomyces* from *Bacillus* using Chloramphenicol and Ampicillin

Based on the genome sequence data, a search was done for antibiotic resistance genes of three different *Streptomyces* co-cultures which are 40, 34, and 321. Using the mcmaster (https://card.mcmaster.ca/analyze) and ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) to narrow down some antibiotics which could be used to kill the *Bacillus*. Three *Bacillus* were inoculated in culture media with a range of concentrations of chloramphenicol to determine the required minimum inhibitory concentration. Three co-culture *Streptomyces* were inoculated in μg/ml (MIC) R2A broth containing chloramphenicol into 200 ml Erlenmeyer flask and incubated at 28^{o}C while shaking for a week. Growth was observed in each flask and recorded, and then aliquots were plated onto NFM to obtain single cultures. All the isolated cultures were subjected to 16S rRNA gene PCR using *Actinobacterial* and *Firmicute* as well as α and β-*Proteobacteria* specific primer sets, and sequenced. The isolates were amplified by PCR for the presence of *nifH* gene sequence. Furthermore, one of the *Streptomyces* cultures (40) which could grow in presence of Chloramphenicol was inoculated in R2A broth containing 5μg/ml ampicillin 5μg/ml to kill the gram positives and obtain the

Herbaspirillum in it. Aliquots from the antibiotic cultures were plated on NFM to confirm nitrogen fixation. DNA extract was of it and subjected to 16S rRNA gene PCR using *Actinobacterial* and *Firmicute* as well as α and β-*Proteobacteria* specific primer sets, and sequenced.

3.3. Results

3.3.1. *nifH* amplifications of *Streptomyces* and their pattern of growth

The *nifH* genes' presence in *Streptomyces* culture was tested using the primers PolF and PolR (Poly, Monrozier et al. 2001, Gaby and Buckley 2012) using various approaches. Of 115 isolates allocated *to Streptomyces* by 16S rRNA gene sequence, 34 yielded *nifH* amplicons. Fourteen of them aligned and clustered with the *nifH* of *Mesorhizobium*, eighteen aligned to *Herbaspirillum*, and five are homologous to *nifH* of *Paenibacillus*. They all have been allocated to Cluster I of nifH gene database (Figure 3.5). Out of 115, one hundred *Streptomyces* isolates could grow on NFM agar and in NFM liquid medium. In addition, those strains were capable of growing in nitrogen-free medium with atmosphere depleted of ammonia using atmospheric ammonia Clinoptilolite; this indicated their ability to fix nitrogen. (Wang, Liu et al. 2006, Yoshida, Inaba et al. 2014, Liao, Chen et al. 2015). The appearance of colonies of *Streptomyces* were different from one to other on NFM (Figure 3.4).

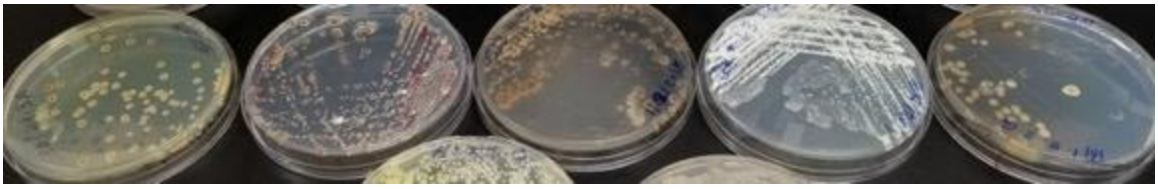

Figure 3.4 Colonies of Streptomyces *cultures on Nitrogen Free Medium.*

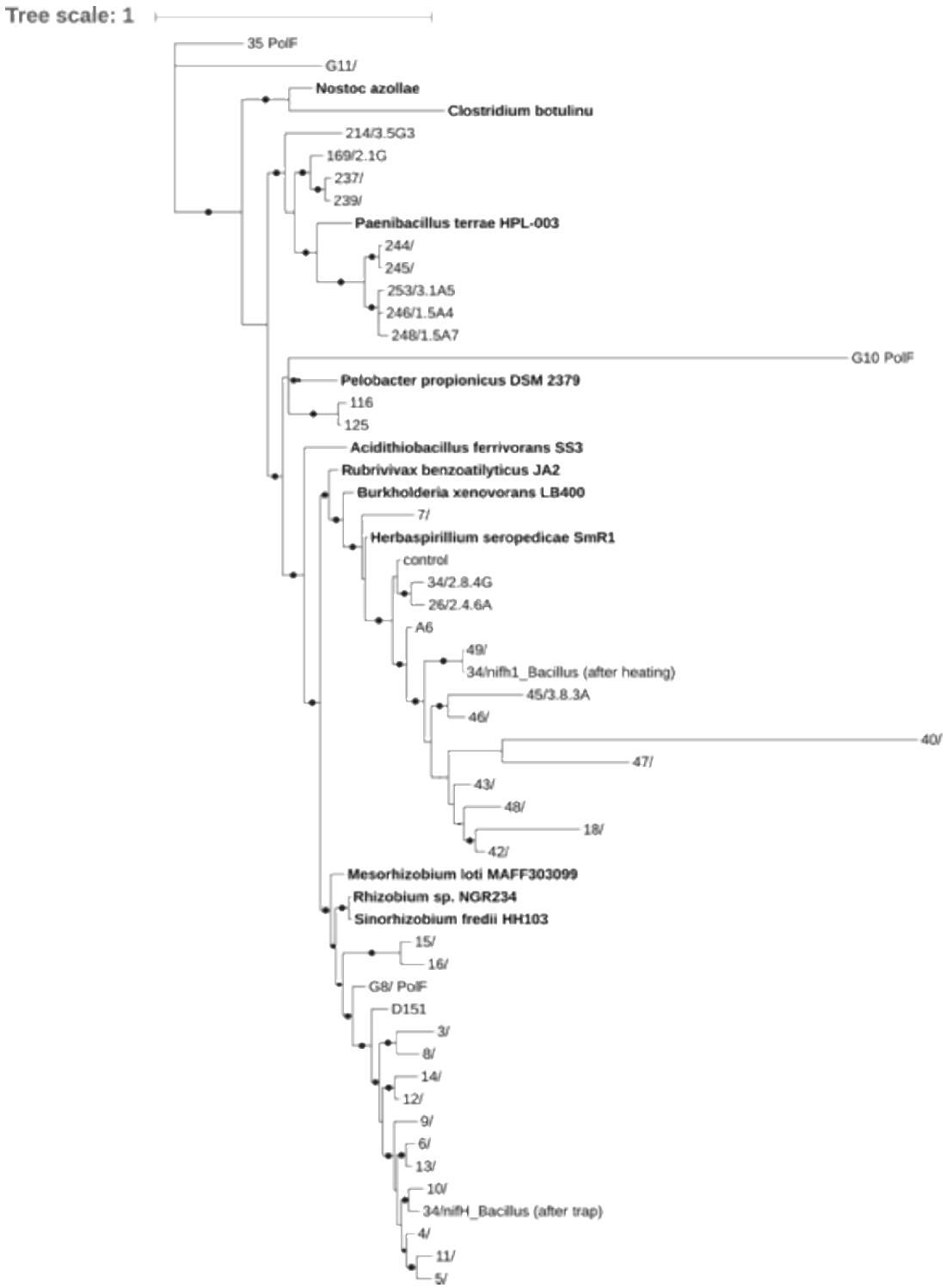

Figure 3.5 Maximum likelihood Bayesian consensus phylogenetic tree of nifH sequences from Streptomyces *cultures based on PhyML using aBayes Branch analysis method. The black dots represent more than 70% posterior probability support.*

3.3.1.1. Biochemical evidence of nitrogen fixation by acetylene reduction assay and using incorporation of $^{15}N_2$ into biomass

The acetylene reduction assay was conducted on 18 isolates in NFM medium with and without nitrogen source. Two isolates yielded ethylene peaks, indicating nitrogen fixation activity (Fig 3.5). Isolate 34 had a nifH *gene homologous to* Herbaspirillum*, and isolate 35 had a* nifH *homologous to* Paenibacillus. *Both isolates displayed a higher level of acetylene reduction than the positive control, and all of them grew very nicely in NFM an ammonia depleted environment in a sealed container. Twelve isolates showed weak acetylene reduction, and all showed good growth in an ammonia depleted environment. Their* nifH *were homologues to* nifH *in* Mesorhizobium. *The data of incorporation of stable $^{15}N_2$ isotope into the bacterial biomass in our selected isolates did not arrive yet.*

The incorporation of stable $^{15}N_2$ isotope into the bacterial biomass in our selected isolates confirmed nitrogen fixation at culture 34 as *Streptomyces* as well as at two cultures 34 and 321 as *Bacillus* (Table 3.2). These isolates had *nifH* gene homologous to *Herbaspirillum* and all of them grew in an ammonia depleted environment in a sealed container.

Table 3.2 Nitrogen fixation by the isolates indicated by incorporation of 15N2 into biomass

Isolates ID	**Sample name (Genome name)**	**^{15}N (at-%)**
34S	*Streptomyces avermitilis*	7.67818
34B	*Bacillus safensis*	5.20409
3.2.1B	*Bacillus safensis*	5.38339
Positive control	*Herbaspirillum seropedicae*	3.163706
Negative control	*E. coli*	0.369271

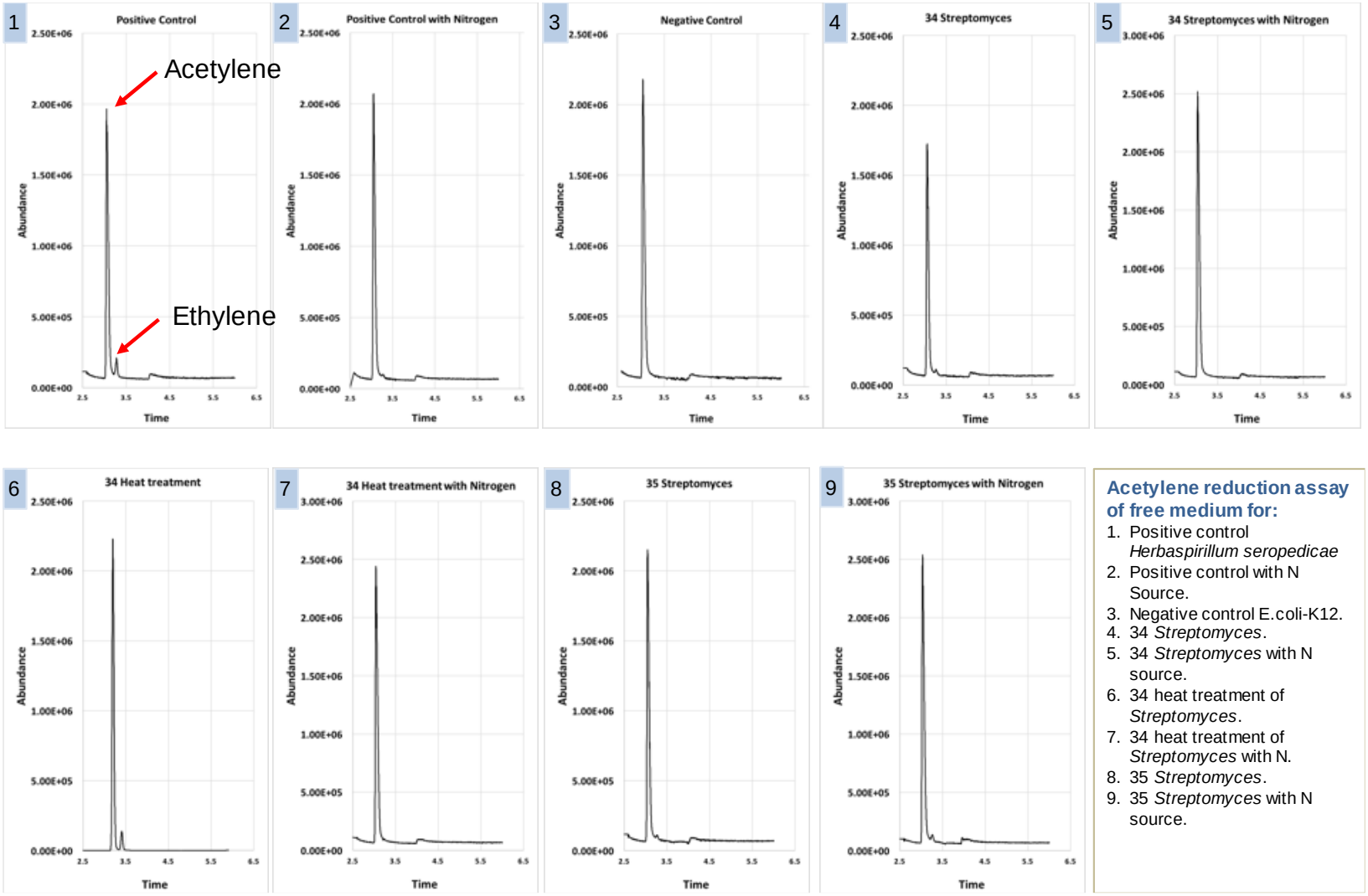

Figure 3.6 Acetylene reduction assay of isolate 34 cultured in nitrogen free medium.

3.3.2. Whole genome of *Streptomyces* sp

The seven strains selected for complete genome sequencing were sent to MicrobesNG for Illumina sequencing at 50X coverage. Interestingly, two of the seven strains contained a 16SrRNA sequence in addition to *Streptomyces*. Both align to *Bacillus,* indicating the potential of a co-culture. Local blast was used to determine if any of the genomes contained a *nifH* sequence, but no *nifH* was found. This suggested that nitrogen fixation was conducted by the co-culture which may have comprised a smaller part of the biculture escaping detection by sequencing. *Bacillus* contain many copies of the ribosomal gene, between 7 and 13, making detection more likely to phenicol resistance gene. To separate the two cultures, both heat treatment methods and TRAP experiment were carried out.

3.3.3. A Multi Locus Sequence Typing (MLST) using 16SrRNA, *atpD*, *recA*, and *ropB*

The concatenated tree of four genes was constructed for 58 valid *Streptomyces* strains against eight whole genomes of *Streptomyces* strains using MEGA 7. Interestingly, the concatenated tree has differentiated six *Streptomyces* in distinct clusters. They may seem to be unique. *Streptomyces 35* aligned to *S. aureus* which was separated into two stable branches with 100% sequence identities and shared a higher node with members of its cluster. *Streptomyces 46* aligned to *S. exfoliates* with 74% sequence identities (Fig 3.7). *Mycobacterium tuberculosis* H37Rv and *Microbacterium* were used as the outgroup.

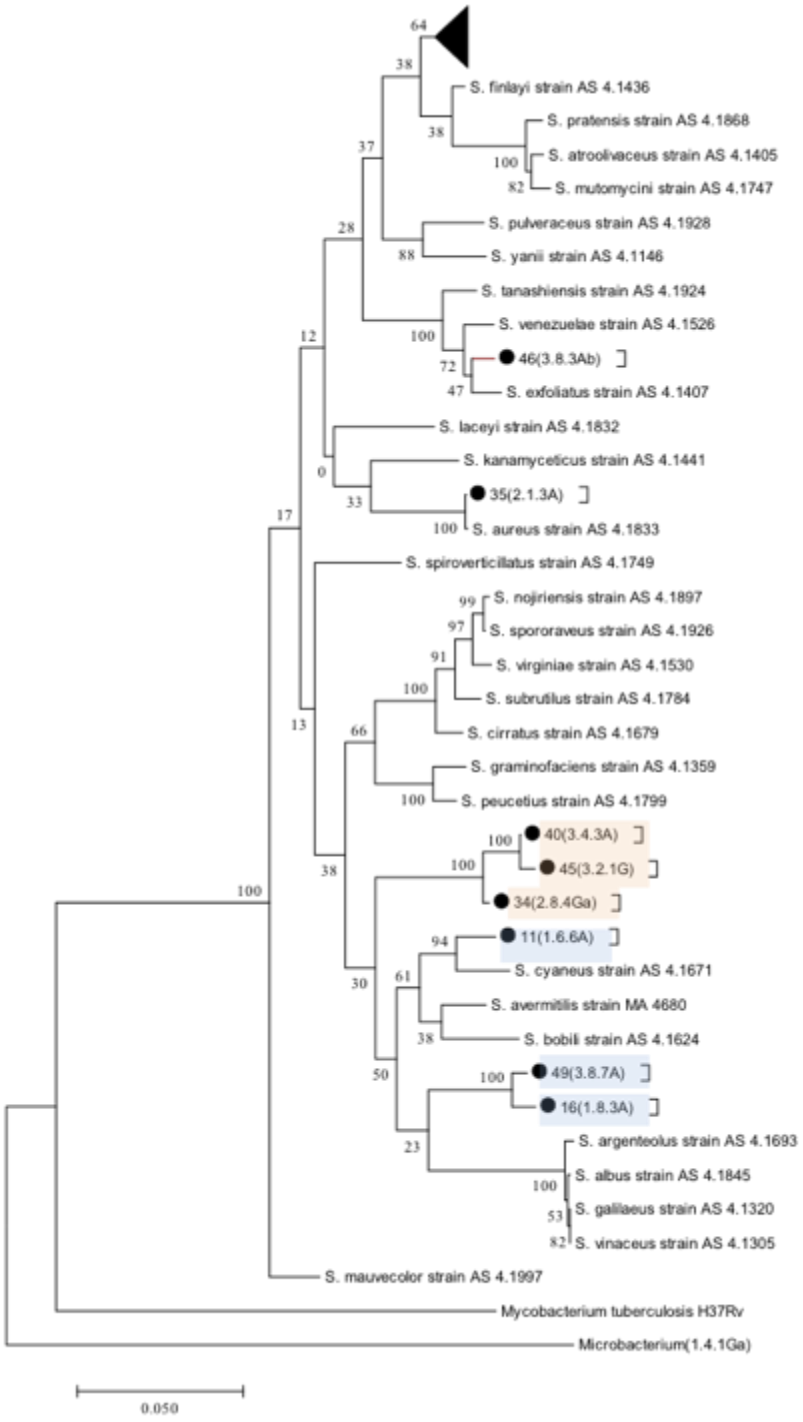

Figure 3.7 Phylogenetic relationships among 58 Streptomyces *strains based on four-gene concatenated sequences. The tree was constructed using the ML method using MEGA7. Numbers at nodes represent levels (%) of bootstrap support from 100 resampled datasets.* Mycobacterium tuberculosis *H37Rv and* Microbacterium *were used as the outgroup.*

3.3.4. Heat treatment to isolate *Bacillus* to survive

To isolate the suspected three *Bacillus* co-cultures (34, 40, and 321) were incubated for 30 min at 90°C to kill *Streptomyces*, while allowing *Bacillus* endospores to survive. Purity was only achieved after two cycles of pasteurization, possibly due to survival of some *Streptomyces* in clumps. The *Bacillus* grew on R2A and on NFM. Isolate 34 yielded the same *nifH* amplicon as the putative bi-culture gave. This supported the notion that a *Bacillus* is a nitrogen-fixing partner in the bi-culture (Table 3.3, Fig 3.6, Fig 3.7).

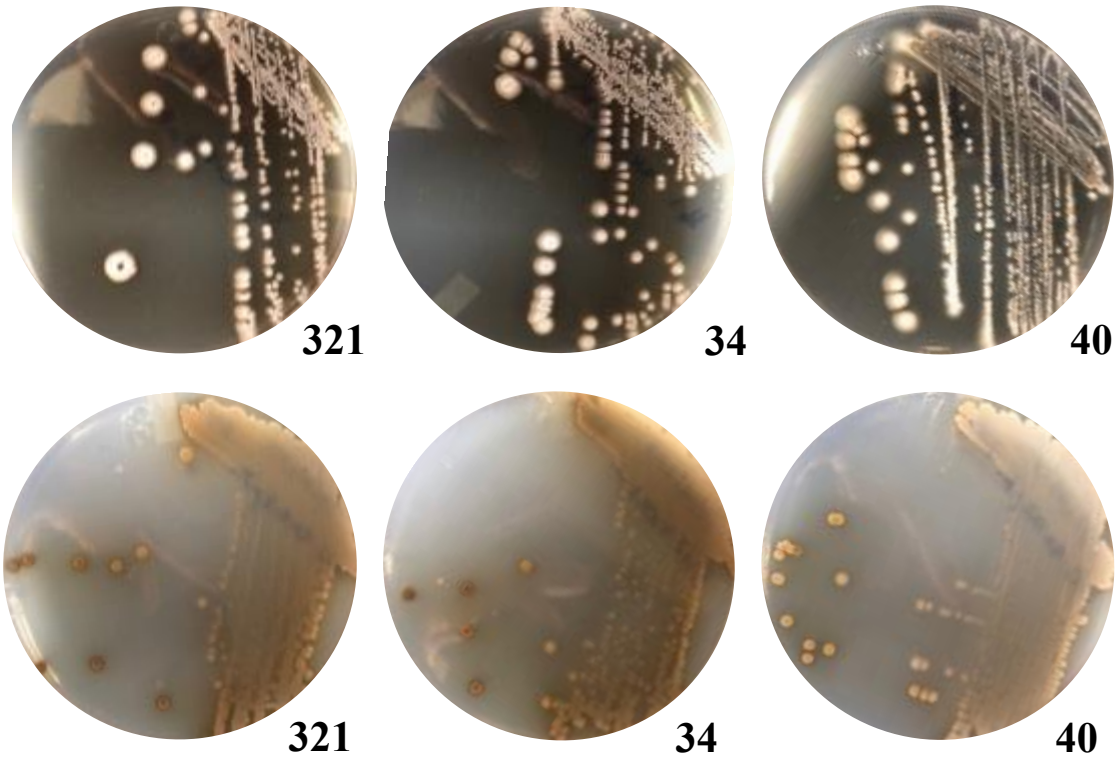

Figure 3.8 Three Streptomyces isolates (321, 34 and 40) cultured on R2A for 14d (top row), and Nitrogen Free Agar for 6d (bottom row).

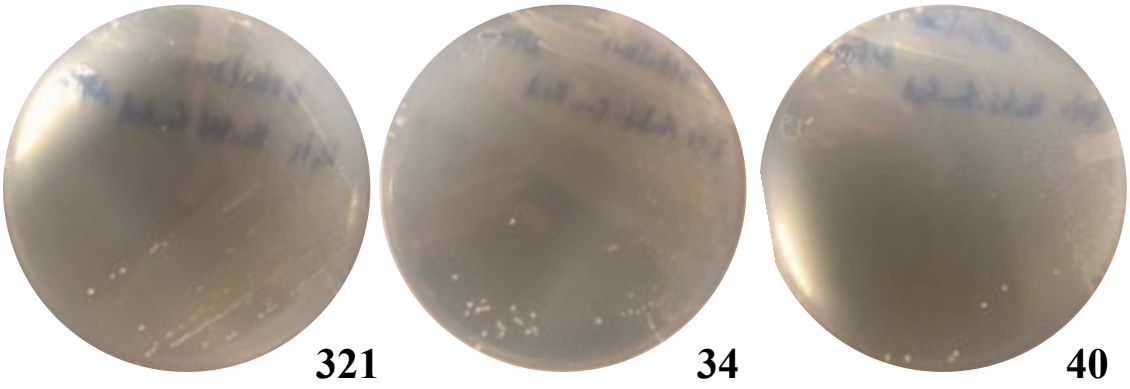

Figure 3.9 Bacillus *pure cultures obtained after 3d on NFA after treating cultures at 90°C for 30 min.*

3.3.5. Separation *Streptomyces* from *Bacillus* using a filter approach:

A membrane trap was employed to allow *Streptomyces* to grow and separate from *Bacillus*. Only culture 34 be separated into purity as outlined in Table 3.3.

Table 3.3 The result of heat treatment and trap experiment

Sample number	**34 (2.8.4Ga)**	**40 (3.4.3.A)**	**45 (3.2.1Ab)**
Original cultures			
Genome	*Streptomyces avermitilis & Bacillus safensis*	*Streptomyces avermitilis & Bacillus safensis*	*Streptomyces venezuelae /&Bacillus*
Alpha *Pro* 16srRNA	unclassified_Alphaproteobacteria	unclassified_"Proteobacteria"	unclassified_"Proteobacteria"
Beta *Pro* 16srRNA	X	unclassified_"Proteobacteria"	unclassified_"Proteobacteria"
After one pasteurization cycle			
***Firmicutes* 16srRNA**	unclassfied_Firmicues	unclassified_Bacillales	*Bacillus*

Sample number	**34 (2.8.4Ga)**	**40 (3.4.3.A)**	**45 (3.2.1Ab)**
Growth on NFM after pasteurization	*Bacillus safensis*	*Bacillus safensis*	*Bacillus*
16srRNA (V1-V3) RDPII	*Bacillus*	Unclassfied_Bacillea	unclassified_Actinomycetales
***Actino* 16srRNA**	unclassified_Actinomycetales	unclassified_Streptomycetaceae	*Streptomyces*
***Firmicutes* 16srRNA**	unclassfied_Firmicues	unclassified_Bacillales	*Bacillus*
Alpha *Pro* 16SrRNA	X	X	X
Beta *Pro* 16SrRNA	X	X	X
PolF *nifH*	nitrogenase reductase, partial *Herbaspirillum* sp	complete genome Bacillus	ABC transporter
After second pasteurization cycle			
Growth on NFM	*Bacillus safensis*	*Bacillus safensis*	*Bacillus safensis*
16SrRNA (V1-V3)	*Bacillus*	*Bacillus*	unclassified_Bacillales
***Actino* 16srRNA**	X	X	X
***Firmicutes* 16srRNA**	*Bacillus*	unclassfied_Firmicues	unclassified_Bacillales
Alpha- Prot 16SrRNA	X	X	X
Beta Prot 16SrRNA	X	X	X
PolF *nifH*	*Herbaspirillum* sp nitrogenase reductase	*Bacillus* complete genome	X
Streptomyces* mixed with *Bacillus			
***Streptomyces* mixed with *Bacillus* two-day incubation growth in broth of NFM**	*Streptomyces* growth is dominating-	*Streptomyces* is dominating but we can see *Bacillus*-	ND
***Streptomyces* mixed with *Bacillus* two-day incubation growth in broth of R2A**	*Bacillus* growth is dominating.	*Bacillus* is dominating.	
Separation *Streptomyces* from *Bacillus* using a filter approach			
First Trap Dry colony growth on NFM	✓	✓	✓
Trap *Bacilli* colony growth on NFM	slow growth	slow growth	slow growth

Sample number	**34 (2.8.4Ga)**	**40 (3.4.3.A)**	**45 (3.2.1Ab)**
Second trap of Dry colony growth on NFH	in 10 days on NFM	in 10 days on NFM	in 10 days on NFM
Second trap of Dry colony growth on TSA+Gl	it grew very fast on TSA+Gl	it grew very fast on TSA+Gl	it grew in 3 days on TSA+Gl
16srRNA (V1-V3)	*Streptomyces*	*Streptomyces*	*Streptomyces*
***Actino* 16SrRNA**	*Streptomyces*	*Streptomyces*	*Streptomyces*
***Firmi* 16SrRNA**	X	unclassfied_Firmicues	*Bacillus*
alpha +beta Prot 16rRNA	X	X	X
Second trap of *Bacilli* colony growth on NFH and TSA+Gl	Slow growth on NFM	No growth on NFM	No growth on NFM
16SrRNA(V1-V3)	*Bacillus*	*Bacillus*	*Bacillus*
***Actino* 16srRNA**	X	Unclassfied-Bacteria	X
***Firmi* 16SrRNA**	Bacillus	unclassified_Bacillaceae	Unclassified Bacillaceae
PolF *nifH*	*Herbaspirillum*	CG Bacillus	unknown

3.3.6. Transformation of *Bacillus* with GFP

Numerous attempts to transform *Bacillus* through natural competence have failed, and we need to use a different approach based on electroporation.

3.3.7. Separation of *Streptomyces* from co-culture by antibiotic treatment

The *Bacillus* obtained from heat treated co-cultures could not grow in the medium containing 5μg/ml of Chloramphenicol. Further, growth of *Streptomyces* was recorded for all three samples suspected of being a co-culture, even in presence of the antibiotic. The phylum specific - PCR results, of DNA extracts from the isolated *Stretomyces*, showed the presence 16S rRNA gene specific for *Firmicutes* for two of the samples and *Actinobacteria* for all three co-cultures. Moreover, no amplification was recorded for phylum specific PCR of Alpha-, and Beta-, *Proteobacteria*. The culture which had only *Actinobacterial* 16S was further inoculated into medium containing ampicillin, to suppress all existing gram - positive strains. However, when cultured on fresh NFM, bacterial colonies were observed, identified as an unclassified bacterium through 16S rRNA gene sequencing. The color of liquid R2A showed a unique transformation when inoculated with suspected co-culture, turning brown. In contrast, the putative pure *Streptomyces* culture was yellow in color (Fig 3.8). Colonies of *Streptomyces* culture 40 appeared different on R2A than the original culture (Fig 3.9). The initial color of the culture medium was clear in both cases.

Figure 3.10 Appearance of liquid R2A without (left) and with Chloramphenicol (right) inoculated with culture 40.

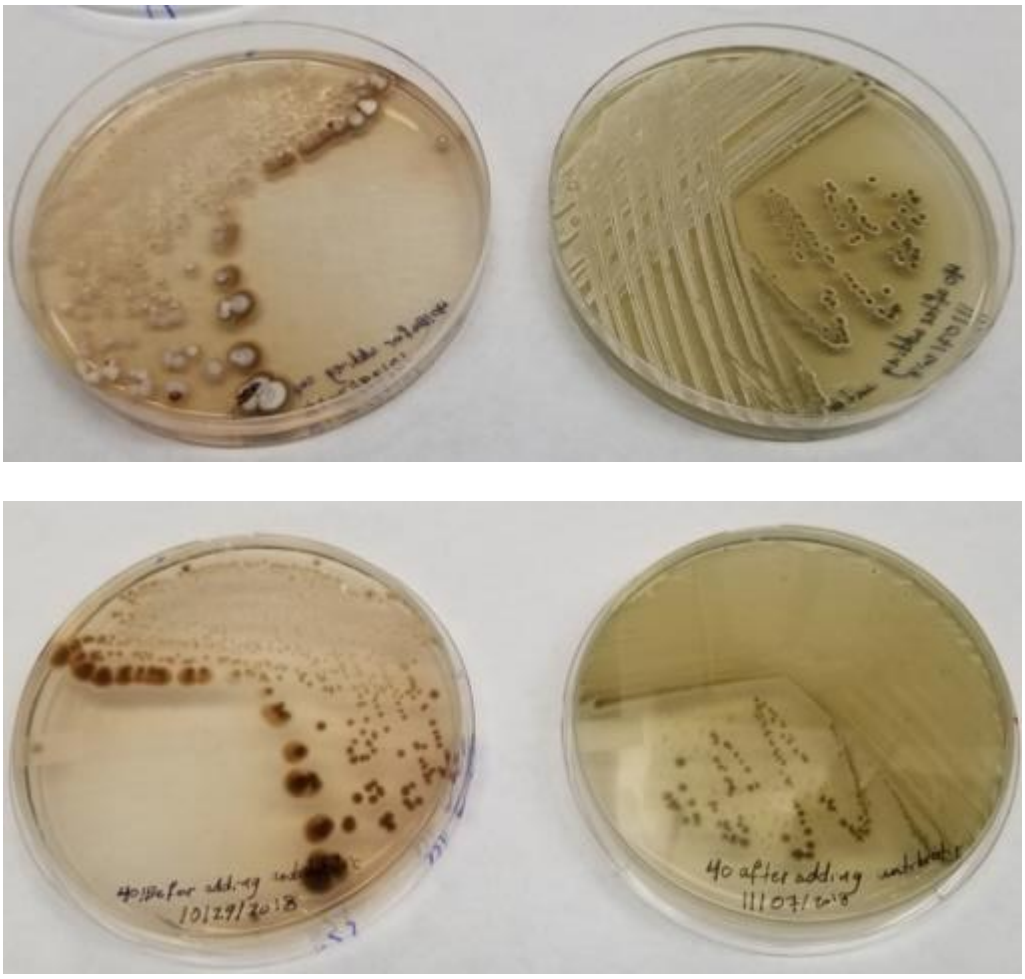

Figure 3.11 Culture 40 before (left) and after (right) chloramphenicol treatment, streaked onto R2A.

3.4. Discussion

Streptomyces are a group of aerobic heterotrophic free-living bacteria (Allen, Edberg et al. 2004, Seipke, Kaltenpoth et al. 2012), but some are symbionts associated with insects (Kaltenpoth, Goettler et al. 2006, Kaltenpoth, Yildirim et al. 2012), while others protect the fungal symbionts of their ant hosts (Haeder, Wirth et al. 2009). The

genus is not known for nitrogen fixation, but recently several isolates were reported to grow in nitrogen free medium, contain *nifH*, and incorporate nitrogen (Dahal, Nandakafle et al. 2017). The *nifH* gene encoding nitrogenase is widely accepted as marker gene for nitrogen fixation. We used the PolF -PolR primer set to explore the *nifH* gene sequences obtained from 115 *Streptomyces* cultures that appeared pure after extensive sub-culturing. Amplification of *nifH* proved challenging but use of additives such as DMSO, BSA, and glycerol to the PCR reaction enhanced amplification from the high G+C containing *Streptomyces* DNA (Strien, Sanft et al. 2013). Of 115 cultures, 34 yielded *nifH* by PCR. ARA results were positive for all 18 cultures tested, but varied between cultures. The presence of nitrogen in the medium reduced acetylene reduction, and repression of nitrogen fixation by an exogenous nitrogen source has been reported for multiple species (Dixon and Kahn 2004). The *nifH* amplicons aligned with *nifH* of *Mesorhizobium*, *Herbaspirillium* and *Paenibacillus,* suggesting a possibility of either horizontal gene transfer or co-existence with a nitrogen fixing bacterial co-culture. To investigate this further, three cultures yielding *nifH* homologous to *Bacillus*, 34, 40 and 45 were selected.

The three cultures appearing as *Streptomyces* comprised of two-species consortia. They presented as *Streptomyces* on agar and in liquid culture, even after extensive attempts at sub-culturing. Microscopic investigation of Gram-stained cultures appeared as classical *Streptomyces*. Yet all three cultures tested positive for Actinobacteria and Firmicutes - specific PCR, and amplicons aligned with *Streptomyces* and *Bacillus* respectively. The genomic sequence data also contained 16SrRNA gene sequences

aligning with *Bacillus safensis.* *Bacillus* could be isolated into single culture through intensive pasteurization (90ºC, 30 min). Sub-culturing of very old cultures onto rich agar such as Tryptone Soy agar yielded *Bacillus*, indicating outgrowth of endospores following senescence of the culture. Related most closely to *B. safensis*, these isolates grew on NFM, stained gram positive. The isolate from culture 34 displayed acetylene reduction activity, and yielded *nifH* by PCR. Intriguingly the *nifH* aligned with *Herbaspirillum,* a ß-Proteobacterium Our *Bacillus* from cultures 40 and 45, while growing on NFM, did not yield *nifH* by PCR. It has been found that a select few *Bacillus* have nitrogen fixing ability (Yousuf, Thajudeen et al. 2017). Only one isolate was reported to yield a *nifH* amplicon, but no sequence data were reported.

Streptomyces isolated into single culture appeared different on agar and in liquid culture to the biculture. It could not grow on NFM and did not yield a *nifH* gene by PCR. After following various previously published approaches to obtain *Streptomyces* into single culture did not succeed in separation of the species, an analysis of the genome sequence revealed a single antibiotic resistance gene. The *Bacillus* displayed some tolerance to chloramphenicol as well, but successive increases in antibiotic concentration cured the culture of *Bacillus*.

The three *Streptomyces* appeared to contain their *Bacillus* co-cultures, as *Bacillus* could only be obtained after death of the partner, either through heat or senescence. Mutualistic interaction among bacteria is defined in most microbiology textbooks, and many intriguing bacterial –eukaryote interactions have been studied. Yet few examples of bacterial mutualism have been studied. but few cases have been described. Cross feeding, where two or more species contributes metabolites not synthesized by their partners, is

intuitive, but there are few well characterized examples (Hillesland 2018). The *Bacillus* did not require any nutritional contributions from the *Streptomyces*, ruling out cross-feeding. Synrophy is distinct from cross feeding because the partners work together to complete energy-yielding reactions, and are, therefore obligately interdependent (McInerney, Struchtemeyer et al. 2008, Sieber, McInerney et al. 2012). As the *Bacillus*, pure cultures grew on NFM, they did not require catalytic contributions from the *Streptomyces*. Rather, the *Streptomyces* appear dependent on their *Bacillus* for growth in nitrogen deprived environments, suggesting a form of parasitism or slavery. The mechanism by which *Streptomyces* contains its nitrogen-fixing slave is unknown at present.

Bacterial fitness in the environment may depend not only on resistance to challenges by other species, but also on diverse species interactions including cross-feeding, syntrophy and parasitism. The isolation of bacterial culture that turn out to be mixed may open the door to study an array of species interactions.

Chapter 4

4. Conclusions and Questions for future study

4.1. Conclusion

A large number of diverse bacteria were obtained on the nitrogen-free minimal medium NFM. These included *alpha-, beta-, and gamma – Proteobacteria*, *Bacteroidetes, Firmicutes, and Actinobacteria*. They grew on nitrogen-free agar (NFA) and in liquid NFM in an ammonia-depleted environment in a sealed container. All isolates appeared as single cultures through extensive sub-culturing on solid and in liquid media, by displaying consistent monomorphic colonies. The PolF-PolR primerset yielded the largest number of *nifH* amplicons by PCR, whereas primer sets including IGK3/DVV, Ueda19F/Ueda407R, and for nifH1/ nifH2 yielded far fewer positives. *Streptomyces* were the largest group of FLN fixers, and 40 out of the 115-isolated yielded *nifH* amplicons. These genes were all identified as Class one nitrogenases.

The *nifH* gene profiles did not match the bacterial 16S rRNA phylogeny. The *nifH* from *Streptomyces* fell into three groups, homologous to either *Mesorhizobium* (Alfa-Proteobacteria), *Paenibacillus* (Firmicutes), or *Herbaspirillium* (Beta-Proteobacteria). Likewise, *nifH* from several proteobacterial cultures aligned with those of other taxa. Phylum-specific 16S rRNA PCR revealed that many of the isolates comprised of co-cultures. Of 96 isolates testing *nifH* positive, 23 were single, 61 were bi-cultures, 9 tri-cultures, and 3 comprised of four taxa. These results indicate that, despite rigorous procedures to obtain single cultures, most of the nitrogen fixing isolates were bacterial

co-cultures.

Genome sequencing of selected cultures identified as *Streptomyces* revealed presence of a *Bacillus safensis* in the DNA, indicating a co-culture. This was confirmed by using Firmicute-specific 16SrRNA gene primers on the cultures. *Bacillus* was obtained into single culture by intensive pasteurization (90°C, 30 min) followed by plating on NFA, allowing only endospore formers to survive. Purity of the isolates obtained was confirmed using phylum-specific 16S rRNA gene primers for *Actinobacteria* and *Firmicutes*. These PCRs confirmed absence of the partner strains. The *Bacillus* isolate grew on NFA, yielded a *nifH* amplicon and tested positive in the acetylene reduction assay. The *Streptomyces* was cured of *Bacillus* by extensive culturing in chloramphenicol. The pure culture *Streptomyces* colonies appeared different, could not grow on NFA and did not yield a *nifH* gene product. These results indicate that the *Streptomyces* cultures were bi-cultures with nitrogen-fixing *B. safensis.* Both strains can grow on their own so this is not an obligate relationship. Yet the *Streptomyces* appears to exert control over the *B. safensis*, ensnaring it in a state of slavery.

The *nifH* gene of the *B. safensis* isolate aligned with *Herbaspirrilum*, a ß-Proteobacterium. This suggests horizontal gene transfer. The isolation of bacterial culture that turn out to be mixed may open the door to study an array of species interactions.

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. Is the discovery of a universal primers of *nifH* for all the nitrogen fixing bacteria possible?
3. Is there a way to distinguish between a true nitrogen fixer and a scavenger of traces of combined nitrogen sources?
4. Could a phenomena co-culture be type or reason of horizontal gene transfer?
5. What are the efficiency's limitations of currently methods and techniques in evaluation of nitrogen fixation?
6. Could *Streptomyces* fix nitrogen alone?

Appendix

1. GPS locations from Sioux Prairie soil samples

Sampling Site	GPS Location
1.1	44.2’6” N 96° 46’ 58” W 1720ft Elevation
1.9	44.2’5” N 96° 46’ 58” W 1720ft Elevation
2.1	44.2’6” N 96° 47’ 1” W 1730ft Elevation
2.9	44.2’6” N 96° 46’ 58” W 1730ft Elevation
3.1	44.2’11” N 96° 46’ 59” W 1670ft Elevation
3.9	44.2’11” N 96° 47’ 0” W 1700ft Elevation

1. Photos of soil samples sites:

Site 1 included 9 cores:

1.1

1.2

1.3

1.4

1.5

1.6

1.7

1.8

2.1

2.2

2.3

2.4

2.5

2.6

2.7

2.8

2.9

Site 3 included 9 cores:

3.1

3.2

3.3

3.4

3.5

3.6

3.7

3.8

3.9

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