IDENTIFICATION OF MAJOR INCREASED T-RFs IN T-RFLP PROFILES OF HYDROGEN TREATED SOIL SAMPLES USING MOLECULAR FINGERPRTING TECHNIQUES

by

Yue (Lanie) He

A Thesis Submitted to Saint Mary's University, Halifax, Nova Scotia in Partial Fulfillment of the Requirements for the Degree of Masters of Applied Science

November, 2008, Halifax, Nova Scotia

Your file Votre référence
ISBN: 978-0-494-46942-2
Our file Notre référence
ISBN: 978-0-494-46942-2

Certification

Identification of major increased T-RFs in t-RFLP profiles of hydrogen-treated soil samples using molecular fingerprinting techniques

by

Yue He

A Thesis Submitted to Saint Mary's University, Halifax, Nova Scotia,
in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Applied Science

December 15, 2008, Halifax, Nova Scotia

Examining Committee:

Approved: Dr. Gavin Kernaghan, External Examiner
Department of Biology, Mount Saint Vincent University

Approved: Dr. Zhongmin Dong, Senior Supervisor
Department of Biology

Approved: Dr. Doug Strongman, Supervisory Committee Member
Department of Biology

Approved: Dr. Harry Murray, Supervisory Committee Member
Department of Biology

Approved: Dr. Kathy Singfield, Supervisory Committee Member
Department of Chemistry

Approved: Dr. Genlou Sun, Program Representative

Approved: Dr. Kevin Vessey, Dean of Graduate Studies

ABSTRACT

IDENTIFICATION OF MAJOR INCREASED T-RFs IN T-RFLP PROFILES OF HYDROGEN TREATED SOIL SAMPLE USING MOLECULAR FINGERPRING TECHNIQUES

By Yue (Lanie) He

In a previous study, twenty hydrogen-oxidizing bacterial strains showing positive plant root elongation effects were isolated. However, T-RFLP work showed that none of these isolates contributed to hydrogen induced TRF increases compared with air treated soil samples.

To study which bacterial groups are responsible for the major increased T-RFs in T-RFLP profiles of hydrogen-treated soil samples, a 16S rDNA library from hydrogen-treated soil samples was constructed utilizing the same DNA used to generate T-RFLP profiles. It showed that the major bacteria groups responsible for the observed hydrogen uptake in soil might belong to the phylum Actinobacteria. Comparisons between the T-RFLP profiles and the 16S rDNA library showed that the T-RFLP fingerprinting method does have the ability to reflect the diversity and structure of numerically dominant bacterial populations within complex microbial communities such as those found in soil.

Nov16th, 2008

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Zhongmin Dong, for giving me the wonderful opportunity to study with him in the past two years. He has given me valuable insights and guidance in my research as well as patience and support in everything.

I would like to thank my committee members, Dr. Kathy Singfield; Dr. Douglas Strongman and Dr. Harry Murray for their patience, reviews and willingness to engage in this academic exercise.

I would like to thank Ms. Jing Yang for her precious valuable help in my molecular work.

I would like to thank technicians in Biology Department, Saint Mary's University who devoted their kind help for my studies, experiment and life: Carmen Cranley and Heidi de Boer.

I would like to thank Amber Golding, Bryan Flinn and Jennifer Lau for revising my thesis and Amber Golding, who I have worked with during the past years for your friendship and willingness to help all the time.

I would like to thank my friends, Jianyin Huang, Tongning Yang and Zehua Zhou for their contributions in the format of my thesis. Without their help, the thesis would not

turn out as it is now.

I would like to thank Mr&Mrs Wayne&Pat Barre, my PAPA and NANA, from Ontario, for their generous support and considerate care for me ever since the minute I landed on this foreign country. They have been cherishing me as their own. If it were not for them, I do not think I would be able to pull myself through the past two years in this country. I will be forever heartily grateful for everything they have done for me.

Finally, I would like to thank my parents and my sister and brother-in-law for their unconditional love and support. They have never asked anything of me except to be happy. I would especially thank my sister, Ying He, for always being there encouraging me, especially the 120 days when I struggled with my thesis writing. This degree is as much of an accomplishment for her as it is for me.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVATIONS

A	ampere
ACC	1-aminocyclopropane-1-carboxylate
ARDRA	Amplified Ribosomal DNA Restriction Analysis
bp	base pair
BSA	bovine serum albumin
BSF8/20	bacterial universal forward primer (5'-AGAGTTTGATCCTGGCTCAG-3')
BSR534/18	bacterial universal reverse primer (5'-ATTACCGCGGCTGCTGGC-3')
CE	capillary electrophoresis
CTAB	hexadecyl-trimethylammoniun bromide
DAPG	2,4-diacetyphloroglucinol
DC	direct current
DGGE	denaturing gradient gel electrophoresis
6-FAM	phosphoramidite fluorochrome 5-carboxyfluorescein
HUP	uptake-hydrogenase
IAA	indoleacetic acid

ISR	induced systemic resistance
M	mol per litre
mA	milliampere
mg	milligram
mM	millimol per liter
MSA	mineral salt agar
PCR	polymerase chain reaction
PGPR	plant growth promotion rhizobacteria
ppm	parts per million
RDP	ribosomal database project
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TRF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
µl	microlitre
µmol	micromole
µm	micrometer
v/v	volume : volume

1. Introduction and Literature Review

1.1 Soil Microbial Community

With the ever-increasing growth of global populations, great amounts of effort and funding have been spent to ensure sufficient food supply for the global human population. However, some agricultural measures taken to increase food production worldwide have already created a series of adverse effects on the environment by turning more and more areas into arable lands by using a wide variety of inorganic fertilizers. Pesticides and herbicides have caused problems such as soil erosion, loss of water resources and development of pathogen resistance to the applied agents (Gerhardson, 2002). A big challenge for researchers and farmers has been how to enhance plant growth without endangering the sustainability of environment.

A fundamental part of environmental sustainability is the sustainability of soil. Soil health is of critical importance to soil sustainability. Soil health is defined as "the capacity of soil to function as a vital living system to enhance biological productivity, promote environmental quality and maintain plant and animal health" (Doran and Zeiss, 2000). Soil health is closely related to soil microbial activity and affected by such soil additives as fertilizers. Recently, there has been growing interest in replacing inorganic fertilizers with beneficial soil microorganisms. In order to expand the beneficial effects of soil microbial activity to its fullest potential, a better understanding of the factors influencing microbial diversity and activities is essential. A good understanding of soil bacterial diversity and bacterial function is crucial for sustainable development of the soil-plant ecosystem.

Nitrogen fixation by rhizobia and other nitrogen fixing bacteria has been known for a long time for their effects on plant growth promotion. What is not as well understood and less appreciated is the potential beneficial effect that the soil microbial community could exert on plant growth. One obvious benefit is the improved access to nutrients that some microbes can provide for above-ground plants. A better understanding of how crops interact with soil microbes could provide more environmental sustainable strategies to increase crop productivity.

1.1.1 The effect of plants and related factors on the soil microbial community

'Above-ground' plant species diversity is of ecological importance and 'under-ground' microorganism diversity has long been suggested to be strongly associated with 'above-ground' diversity (Marcel *et al.*, 1998). Grassland soil bacterial composition has been found to be significantly influenced by the level of plant diversity (Grüter *et al.*, 2006). Moreover, significantly different microbial communities and a clear discrimination of carbon sources utilized by them have been observed in soils on which wheat, ryegrass, bentgrass and clover were grown (Grayston *et al.*, 1998). It has been demonstrated that different types of plants select specific compositions of rhizosphere bacterial communities around their roots (Kaiser *et al.*, 2001; Kruske *et al.*, 1997). This might be explained by differences in the amount and type of exudates and nutrients found to be released from the different roots (Jaeger *et al.*, 1999; Rangle-Castro *et al.*, 2005). The compounds of root exudates vary with plant growth and development, which means the structure of

rhizosphere microbial communities change with the growth and development of plants. It has been clearly demonstrated by di Cello *et al.*, (1997) and Seldin *et al.* (1998), both of whom reported the variation of bacterial communities in the maize rhizosphere occurred during the process of plant growth. This phenomenon has also been observed by Gyamfi *et al.* (2002). Kennedy *et al* (2004) showed that the overall microbial community structure of an upland acidic grassland soil changed with the addition of nitrogen and lime, using terminal restriction fragment length polymorphism (T-RFLP),

Soil microbial diversity and structure has been reported to be influenced by soil type (Girvan *et al.*, 2003) and soil management practices like crop rotation, tillage, herbicide, fertilizer application and irrigation (Pankhurst *et al.*, 1995; van Veen *et al.*, 1997; Curci *et al.*, 1997; Boddington and Dodd, 2000; Sun *et al.*, 2004). Many studies have demonstrated it is through the changing organic and energy resources excreted by plant roots or provided by soils that different soil management practices induced variation of microbial diversity and composition in soil (Giri *et al.*, 2005; Maloney *et al.*, 1997; Semenov *et al.*, 1999; Garbeva *et al.*, 2004). Soil microbial populations and structure have been reported to respond to different tillage systems (Peixoto *et al.*, 2006). In one particular study, enzyme activity was found to be much higher in soil tilled by shallow ploughing as compared to soil tilled by deep ploughing (Curci *et al..*, 1997). Legume plants grown on soil were found to exert a positive effect on organic matter levels and thus induce active bacterial population changes (Girvan *et al.*, 2003).

1.1.2 The effect of soil microbial community on 'above-ground' diversity

Just as plants have an effect on soil microbial communities, soil microbes have a profound influence on plant health and productivity (Bloemberg and Lugtenberg, 2001). It has long been recognized that the soil microbial communities and their activity are major contributors to soil health due to their vibrant participation in pivotal processes taking place in soil. These include maintenance of soil structure; decomposition of residual soil organic matter; recycling of plant nutrients and energy; pest control and suppression of soil borne diseases; soil aggregation and degradation of environmental pollutants and contaminants (Parkinson and Coleman, 1981; Vn Elsas and Trevors, 1997). Soil microbial diversity may represent the ability of a soil to cope with external perturbations (Bardgett *et al.*, 2002; Johnsen *et al.*, 2001).

Most rhizosphere microbes can exert not only beneficial but also detrimental effects on the plant-microbe ecosystem via the induction of a myriad of plant-microbe and/or microbe-microbe interactions (Bowen and Rovira, 1999). It is well known that some rhizosphere microbial pathogens can have a destructive impact on crop yields. It has been reported that plant-parasitic nematode diseases caused yield losses of 11.8% in pearl millet, 12% in groundnut and 7% in sorghum in many parts of the world in the past decade (Sharma and McDonald, 1990).

In contrast to these microbial pathogens damaging the plants, rhizosphere beneficial microbes benefit plant growth by competing with plant pathogens for nutrients or ecological root niches; producing inhibitory allelochemicals such as

secondary metabolites (antimicrobial metabolites and antibiotics), extracellular enzymes (antibiotics) and triggering host plant induced systemic resistance (ISR) so that plants are armed to fight against pathogen infection.

Beneficial rhizosphere microbes can improve soil fertility in a variety of ways. First, through biological nitrogen fixation: the ability of nitrogen fixing microorganisms to reduce atmospheric nitrogen to ammonia for plant use, which has been well documented for decades; Second, through decomposition of the organic matter entering the soil; Third, through an increase of mineral nutrients (e.g. phosphorus) available to plants; for example, AM (Arbuscular Mycorrhizal) fungi infection is associated with the provision of phosphorous to plants in exchange for organic carbohydrates (Smith and Read, 1997). Because of the 'plant growth promoting' attributes of some beneficial rhizobacteria, they are termed plant growth promoting rhizobacteria (PGPR). To increase plants access to nutrients and thereby increase crop yield without endangering the environment, scientists have been dedicated to finding more biofertilizers and have been investigating the mechanisms of how these PGPR enhance plant growth.

1.2 The Mechanisms of Plant Growth Promoting Rhizobacteria (PGPR) to Promote Plant Growth

Bacteria that inhabit the rhizosphere can either exert a negative influence on plant growth as in the case of pathogenic bacteria (Lugtenberg *et al.*, 1991; Persello-Cartieaux *et al.*, 2003) or promote plant growth as in plant growth promoting bacteria (PGPB), whose growth is in turn enhanced by plant root activities (Barea *et*

al., 2004). Plant growth promoting rhizobacteria (PGPR), a group of PGPBs, are a group of root-colonizing bacteria with the capacity to enhance plant growth primarily by increasing seed emergence, plant weight, crop yield and contributing to disease control (Klopper *et al.*, 1980; 1992). According to the general mechanisms by which they increase plant growth, PGPR can be divided into three groups: biofertilizers which assist directly with nutrient uptake (Bashan and Holguin, 1998); bioprotectants which control the growth of plant pathogens and deleterious rhizoshphere microorganisms (Kloepper *et al.*, 1999; 2004); and biostimulants which stimulate or suppress certain plant hormones (Esitken *et al.*, 2006; Farag *et al.*, 2006).

1.2.1 Biofertilizers

Some PGPR are classified as biofertilizers because they have the ability to provide plants nutrients which are otherwise not available for plants. For instance, both symbiotic and free-living nitrogen-fixing bacteria are able to supply plants with nitrogenous compounds by reducing nitrogen to ammonia. Significant amount of nitrogen is fixed by legume-rhizobium symbiosis in which the nitrogen fixing strains are grouped within the genera Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium, and Allorhizobium (Bloemberg and Lugtenberg, 2001).

Another group of symbiotic nitrogen fixing bacteria comparable to rhizobium is *Frankia*, a genus of filamentous *actinomycete*, which induces nitrogen fixing root nodules on certain plants distributed in eight families going by the general name of actinorhizal plants (Tjepkema and Torrey, 1979; Baker and Seling, 1984).

Free-living nitrogen-fixing bacteria are a group of bacteria which fix nitrogen without developing a symbiotic relationship with plant roots, falling into a wide variety of genera *Azomonas, Bacillus, Beijerinckia, Clostridium, Corynebacterium, Derxia, Herbaspirillum, Klebsiellas, Pseudomonas* etc. (Kennedy *et al.*, 2004; Tilak *et al.*, 2005); Some bacteria associated with groups of *Bacillus, Pseudomonas* and *Rhizobium* are able to make phosphorus, another essential growth element for plants, available for plant roots by solubilizing phosphates (Dey *et al.*, 2004; Tilak *et al.*, 2005; Alikhani *et al.*, 2006);

Under iron-limiting conditions, some PGPR have the ability to make iron available for plants by producing siderophores to deprive iron from pathogenic fungi whose siderophores have lower affinity with ferric iron or from heterologous siderophores produced by cohabiting microorganisms (Loper and Henkels, 1999; Whipps, 2001). Here the siderophore producing PGPR can also be regarded as bioprotectants as they can potentially limit the growth of pathogenic fungi which might infect the crop. Sulfur oxidizing bacteria are able to oxidize sulfur and promote plant sulphur uptake.

1.2.2 Bioprotectants

Some PGPR are known as bioprotectants because they promote plant growth indirectly through biological control of pathogenic microorganisms, thereby reducing the need for agrochemicals. The mechanisms by which PGPR suppress pathogen growth varies among different PGPR. Some PGPR compete for nutrients and spaces with phytopathogens intensified in the nutrient rich niches along the root surface

(Duffy, 2001).

Some PGPR eliminate the growth of pathogens and cut down the rate of root infection by synthesizing allelochemicals, such as the iron-chelating siderophores described above; antibacterial and/or antifungal metabolites like DAPG (2, 4-diacetylphloroglucinol) produced by pseudomonades (Défago, 1993); oligomycin A produced by Streptomyces (Kim *et al.*, 1999); zwittermicin A and kanosamine produced by Bacillus (Milner *et al.*, 1995; 1996; Bloemberg and Lugtenberg, 2001).

Some PGPR elicit plant growth by stimulating plant defensive responses, such as the production of polyphenolic compounds, defensive enzymes and salicylic acid, an important defense compound found in higher plants (Chakraborty *et al.*, 2006; Domenech *et al.*, 2007; Saravanakumar *et al.*, 2007).

It has been recently discovered that some PGPR have the capacity to degrade a group of small signaling molecules collectively named autoinducers, an important mediator in quorum sensing (QS) upon which most plant pathogenic bacteria depend on for activating genes for their malicious (virulence) factors (Molina *et al.*, 2003; Von Bodman *et al.*, 2003; Uroz *et al.*, 2003).

Some PGPR control the growth of plant pathogens through triggering a defense system termed induced systemic resistance (ISR). ISR enduces plants infected by pathogens with a stronger defense system by fortifying epidermal or cortical cell walls (Duijff *et al.*, 1997); forming new structural barriers at the pathogen frequently attack sites due to the deposition of callose and the accumulation of phenolic compounds (Benhamou *et al.*, 1996; Kloepper *et al.*, 2004); thus inducing

accumulation of plant pathogen relevant enzymes like chitinases, peroxidases and polyphenol oxidase (Chen *et al.*, 2000).

1.2.3 Biostimulants

PGPR which elicit plant growth by synthesizing certain plant growth promoting hormones or by suppressing particular plant growth inhibiting hormones are called biostimulants. Plant growth promoting hormones include auxins (Loper, 1986; Taiz and Zeiger, 1991); cytokinins (Taiz and Zeiger, 1991; Timmusk *et al.*, 1999) and gibberellins (MacDonald *et al.*, 1986).

The most important member of the auxin family in higher plants is indole-3-acetic acid (IAA), which is responsible for tropisms (growth responses) and regulation of cell elongation in stems and coleoptiles (Taiz and Zeiger, 1991). Cytokinins are involved in such important plant processes as the regulation of plant cell division, cell enlargement; shoot and root morphogenesis, nutrient mobilization and delay of senescence (Taiz and Zeiger, 1991; Kieber, 2002). The ratio of auxin to cytokinin was found to play a decisive role in the differentiation of plant tissues and cell division; cytokinin synthesis is regulated by auxin in *Arabidopsis thaliana* (Nordstrom, 2004).

In terms of hormone stimulation, it has been shown that two different bacterial strains released volatile compounds leading to growth promotion in *Arabidopsis thaliana*; both used cytokinin pathways, one ethylene-dependent, the other ethylene-independent (Farag *et al.*, 2006).

An example of plant hormone suppression as a mechanism of PGPR is the

ability of some bacterial species to decrease levels of ethylene, an important gaseous plant hormone involved in many stages throughout the plant growth cycle including seed germination, root hair development, root nodulation, flower senescence, abscission and fruit ripening (Taiz and Zeiger, 1991; Johnson and Ecker, 1998). Inhibition of ethylene helps promote nodulation in most legumes (Hunter, 1993) and increase root elongation in nonlegume plants; increased root elongation means better access to nutrients in soils (Shah *et al.*, 1998).

1.2.4 Some of the mechanisms of H_2-oxidizing bacteria isolates to promote plant growth

The PGPR of interest in the present study are the H_2-oxidizing bacteria, of which only a few species have been successfully isolated, characterized and identified (Maimaiti *et al.*, 2007). With this new knowledge comes a new understanding of some of the ways in which PGPR increase plant growth and yield. For instance, some H_2-oxidizing bacteria are in possession of ethylene biosynthesis inhibitors such as (ACC) deaminase (1-aminocyclopropane-1-carboxylate); this enzyme cleaves ACC, a precursor of ethylene (Taiz and Zeiger, 1991) into α-ketobutyrate and ammonia, disrupting the ethylene production pathway (Hontzeas *et al.*, 2006; Zhang, 2006). ACC deaminase activity has been observed in strains of *Variovorax paradoxus* and *Flavobacterium johnsoniae* (Zhang, 2006; Maimaiti *et al.*, 2007).

Rhizobitoxine, another ethylene biosynthesis inhibitor, is first found in certain rhizobia strains (Erdman *et al.*, 1956), it blocks the action of ACC synthase, thereby disrupting the ethylene biosynthesis pathway one step earlier than ACC deaminase

(Sugawara *et al.*, 2006). This chemical's activity has been found in *Bradyrhizobium elkanii*, *Bradyrhizobium japonicum* (Hunter, 1993) and *Burkholderia sordidicola* species of H_2-oxidizing bacteria (Zhang, 2006; Maimaiti *et al.*, 2007). In root elongation experiments with spring wheat, isolates producing increases of up to 254% in root length have been found to contain rhizobitoxine activity (Maimaiti *et al.*, 2007). Clearly, these PGPR hold great promise in the challenge to minimize or extinguish inorganic chemical fertilizer use, but we must learn more about them before they can be utilized to their fullest potential.

1.3 The Effect of Legume Crop Rotation on Soil Microbial Community

Soils are always complex and variable. They can affect structure of microbial populations either directly by providing a specific habitat for selecting microbial populations or indirectly by influencing plant root activities. Plus the fact that it is highly responsive to environmental and anthropogenic factors such as pH; soil particle size; vegetation type, soil microbial community diversity and structure is complex and variable.

It was found that similar bacterial communities tend to present in the similar soil types through comparing DGGE patterns of 16 different soil samples from different geographical locations. Some other studies (Groffman *et al.*, 1996; Buyer *et al.*, 1999) also indicated that the property of soil would exert marked influence on microbial populations in rhizosphere.

Among these factors, the impact of legume crop rotation on soil microbial community structure and diversity is the motivation for the current study. Legume

plants are noteworthy for their ability to fix atmospheric N_2 because of their symbiotic relationship with rhizobacteria found in root nodules. They are able to significantly minimize the utilization of inorganic fertilizer N, an accomplishment first achieved by Martinus Beijerinck, a Dutch microbiologist in 1887. Farmers started to grow cereal crop in rotation with legume plants to increase crop yield as early as 2000 years ago (Gras, 1925; Wolf, 1977). Many experiments have demonstrated that crop rotation with legume plants can improve crop yield by at least 20% (Bullock, 1992).

Researchers generally attribute the benefits of rotation to the residual N_2 left by symbiotic N_2-fixation of previous legume plants for subsequent non-leguminous cereal crop grown on the soil (Baldock *et al.*, 1981; Pierce and Rice, 1988). However, studies on legumes and nitrogen fixation showed that residual N_2 only accounts for the 25% of the growth enhancement (Hesterman *et al.*, 1986; Bullock, 1992). This surprising finding stimulated many researchers to look for the factors responsible for the major benefits seen in crop rotation with legumes and several reasons other than residual N_2 have been subsequently proposed:

1. Rotation with legumes may exert a better control of soil born parasites and pathogenic microorganisms and increase the population of beneficial arbuscular mycorrhizal (AM), rhizobacteria or rhizospheric fungi (Schippers *et al.*, 1987; Fyson and Oaks, 1990; Bagayoko *et al.*, 2000);

2. Legumes may help the mineralization and acquisition of nutrients required for plant growth such as phosphorus (P) and Potassium (K) (Bullock, 1992);

3. Legume rotation is able to suppress nematodes and enhance the uptake of

phosphorous (P) and nitrogen (N) of the subsequent crops (Buerkert *et al.*, 2002).

The composition of the soil bacterial community structure has been significantly changed and species diversity has been greatly increased as a result of legume rotations (Lupwayi *et al.*, 1998; Alvey *et al.*, 2003). In addition, higher total microbial biomass and carbon and nitrogen are found in soils which have a rotation cropping history (Granastein *et al.*, 1987). Rotational soil has been found characterized by its higher in pH, mineral N and lower microbial C/N ratio and a generally higher fungal biomass in the rhizosphere of rotation sorghum compared to continuous grown sorghum (Marschner *et al.*, 2003). Populations of pathogen nematode were consistently smaller in rotation sorghum than continuous sorghum in sorghum/groundnut cropping systems (Bagayoko *et al.*, 2000). Crop-legume rotation has been discovered to support a larger, more active and more diverse soil microbial community than cereal-cereal rotation. This is to be expected as we could expect that the dominant bacterial community associated with previous legumes would exert some temporary influence on the rhizosphere bacterial community of the subsequent crop, at least during early growth.

There should be many factors explaining the increase in soil microbial diversity after rotation with legume plants. One hypothesis is that a major factor might be the H_2 gas released by legume root nodules in the process of biological nitrogen fixation; this is known as the "hydrogen fertilization theory" proposed by Dong and colleagues (Dong *et al.*, 2003).

1.3.1 The Effect of Hydrogen Metabolism on the Soil Microbial Community

1.3.1.1 HUP^+ (hydrogen uptake hydrogenase) ---an advantage or a disadvantage?

Hydrogen gas (H_2) is produced when protons are reduced by nitrogenase in the process of nitrogen fixation in legume root nodules, accounting for about 35% of the energy flowing through the nitrogen-fixing enzyme (Hoch *et al.*, 1957; Schuler and Conrad, 1991; Hunt and Layzell, 1993). Only 40-60% of the electron and ATP flow through nitrogenase was transferred to nitrogen (Schubert and Evans, 1976). Hydrogen evolution competes with nitrogen fixation for energy and therefore was considered as a major factor affecting energy efficiency in the symbionts (Schubert and Evans, 1976).

Some symbiotic rhizobacteria were found to possess the enzyme uptake hydrogenase (HUP) which is able to oxidize the hydrogen and therefore recover at least part of the energy used in hydrogen evolution (Dixon, 1967; 1968). These kinds of legume root nodules, whose symbiotic bacteria possess HUP, are therefore named as hydrogen uptake hydrogenase positive (HUP^+) stains of rhizobium. However, it is surprising to know that many of the most productive nitrogen-fixing symbioses do not have hydrogen uptake hydrogenase, termed as (HUP^-), and the hydrogen produced just diffuses out from the nodules into the soil (Uratsu *et al.*, 1982).

It was firmly believed that legume-rhizobacteria symbioses that contain

HUP^+ are more energy efficient as it makes the energy recyclable, while the release of H_2 from HUP^- nodules into the soil was considered a wasteful process from a metabolic point of view (Eisbrenner and Evans, 1983). Surprisingly, in contrast, most strains of commercial Rhizobium inocular lacked hydrogen uptake hydrogenase (HUP^-) (Schubert and Evans, 1976; Keyser *et al.*, 1984). What is even more interesting is that the majority (75%) of the nodulate soybean grown in the US contain a HUP^- rhizobia strain in their root nodules (Uratsu *et al.*, 1982), as well as most known alfalfa and clover symbioses (Dong and Layzell, 2002).

Why would evolution, agricultural crop breeders and a selection of optimal nitrogen fixing bacteria favor HUP^- symbiosis, the "less efficient one", over the "more efficient" HUP^+? What is the biological role for the large amount of released H_2? Researchers could not help but speculate that the "loss of hydrogen" might exert some beneficial effects on the rhizobacteria or the plant or both (Uratsu *et al.*, 1982; Eisbrenner and Evans, 1983). In our experience, however, this speculation did not start to take shape until Dong and Layzell proposed in 2002 that H_2 released by HUP- nodules might function as a soil biofertilizer and help promote crop growth (Dong and Layzell, 2002).

This was subsequently confirmed by a series of experiments conducted by comparing the growth of various crops which were previously treated with H_2 under laboratory conditions or with HUP^- soybean nodules grown in soil under field conditions. Their controls were soils exposed to the same rate of air under laboratory conditions and bulk soil under field conditions respectively. The dry weights of

seven-week old soybean, spring wheat, canola and barley were 15% to 48% greater in H_2 treated soil compared to their controls (Dong *et al.*, 2003). The tiller number of seven-week old barley and spring wheat grown in the field were increased 36% and 48% respectively (Dong *et al.*, 2003). Furthermore, the dry weight of barley grown in rotation with soybean inoculated with JH47 (a HUP^- strain of *Bradyrhizobium japonicum*) increased compared with that of JH (HUP^+) strain of *Bradyrhizobium japonicum*. All these results showed that the metabolism of H_2 evolved from root nodules had some positive effect on stimulating plant growth and suggest that this is another major reason for the rotation benefits with legume crops (Dong *et al.*, 2003) However, the mechanism by which H_2 metabolism benefits plant growth was not clearly understood.

1.3.1.2 Hydrogen consumption by soil hydrogen oxidizing bacteria

Despite the profuse evolution of H_2 into soil, no H_2 gas was detected in the soil column around pigeon peas (LaFavre and Focht, 1983) The H_2 was believed to be consumed within a few centimeters from the nodule surface after its release (LaFavre and Focht, 1983). Soil consumption of H_2 resulted in several significant changes in soil. Changes such as higher H_2 oxidation kinetics (LaFavre and Focht, 1983); an increase in microbial biomass in the rhizosphere (Popelier *et al.*, 1985) and greater O_2 uptake rates and chemoautolithotrophic CO_2 fixation were found (Dong and Layzell, 2001). All these changes provide a satisfactory explanation for the plant growth promotion effect of H_2 liberated from HUP^- legume root nodules but still do not

elucidate the root of hydrogen fertilization theory, which warrants further study.

It was suggested the H_2 released into the soil was conserved by hydrogen oxidizing bacteria and a strong positive correlation was found between the population size of hydrogen oxidizing bacteria and concentration of H_2 around Pigeon Pea nodules, i.e. the higher the hydrogen concentration is, the larger the population of hydrogen oxidizing bacteria is and the higher hydrogen uptake ability (LaFavre and Focht, 1983). This was in agreement with the enriched H_2-oxidizing bacterial population found around the HUP^- nodules in soybean and alfalfa later (Popelier *et al.*, 1985; Wong *et al.*, 1986; Cunningham *et al.*, 1986). Experiments performed by Dong and Layzell in 2001 showed the causative agent responsible for hydrogen uptake in the soil can utilize hydrogen to fix carbon chemoautolithotrophically; this agent did not start to massively uptake hydrogen until the soil was treated for about 7 days (Dong and Layzell, 2001); this indicates the possibility that the causative agent responsible for hydrogen uptake activity might need some adaptation time to "regain consciousness" or increase in numbers or for enzyme synthesis (Trevors, 1985; McLean and Dong, 2002; Irvine *et al.*, 2004; Dean, 2004). H_2 gas also caused an increase in the populations of springtails (O. Collembola) and insects in the soil (Dong and Layzell, 2002). It was hypothesized that the increase in density of springtails was an indicator of the increased soil bacterial population (Dong and Layzell, 2002). This substantiates Favre and Focht's argument back in 1983 that hydrogen evolved from HUP^- nodules is an additional organic matter and energy input into the plant-soil ecosystem (LaFavre and Focht, 1983).

It was interesting to find that the hydrogen uptake ability of this agent disappeared when the soil physical structure was destroyed, but recovered with re-provided H_2, indicating the soil bacteria responsible for hydrogen uptake in the soil might possess a colonial structure or a long filamentous shape like that of Actinobacteria, which is easily destroyed by mechanical action (MacLean and Dong, 2002).

Along with other studies, it was confirmed that the causative agent responsible for the increased hydrogen uptake rate in hydrogen treated soil is hydrogen oxidizing bacteria, which is also stimulated by hydrogen treatment (McLearn and Dong, 2002)

1.3.2 Aerobic hydrogen oxidizing bacteria

1.3.2.1 The known taxonomic composition of aerobic hydrogen oxidizing bacteria

The aerobic hydrogen-oxidizing bacteria comprise a group of bacteria that are able to use H_2 as their electron donor, with O_2 as the electron acceptor, and to fix CO_2 and so grow chemolithoautotrophically. As a group, these aerobic hydrogen oxidizing bacteria are very heterogeneous in terms of taxonomy, comprising diverse species from at least 15 genera, including Pseudomonas, Alcaligenes, Azospirillium, Derxia, Flavobacterium, Microcyclus, Paracoccus, Renobacter, Rhizobium, Xanthobacter, Arthrobacter, Bacillus, Mycobacterium, Nocardia and Hydrogenomonas (Seiler, 1978; Bowien and Schlegel, 1981). The discovery of an obligatorily chemolithautotrophic

streptomycete named *Streptomyces thernoautotrophicus* was originally reported in 1990; this species observed to be able to oxidize both H_2 and CO (Gadkari *et al.*, 1990).

1.3.2.2 The metabolism category of hydrogen oxidizing bacteria

The mixotrophic metabolic system of hydrogen oxidizing bacteria allows them the ability to grow both autographically with H_2 as the sole source of energy and reducing power and heterotrophically on organic compounds. When in presence of both H_2 and organic compounds, they choose to grow organoheterotrophically (Schink and Schlegel, 1978; Aragno and Schlegel, 1992). The enzyme that mediates the hydrogen oxidizing activity of these aerobic hydrogen oxidizing bacteria is hydrogenase, an enzyme that catalyzes the reversible oxidation of molecular hydrogen and reduction of electron acceptors like oxygen, according to the following equation: $2H_2+O_2 \rightarrow 2H_2O$. Therefore, it was reasonable to expect that H_2 oxidizing bacteria to be found in a habitat where both hydrogen and oxygen are available (Aragno and Schlegel, 1981). A gradient of decreasing H_2 concentration resulted in a similar gradient of decreasing H_2-oxidizing bacteria (LaFavre and Focht, 1983).

1.3.2.3 The cultivation of hydrogen oxidizing bacteria

Hydrogen oxidizing bacteria can grow on low nutrient media as long as they are provided with an air mixture of H_2, O_2 and CO_2 (Aragno and Schlegel, 1981). This feature has allowed researchers to cultivate aerobic hydrogen-oxidizing bacteria

by simple direct plate counting techniques and *via* liquid enrichment cultures using soil, mud or water samples as inocular under purely autotrophic conditions (Aragno and Schlegel, 1981). Researchers have been trying to isolate and investigate the physiology of aerobic hydrogen oxidizing bacteria for more than 30 years. German microbiologists have successfully isolated various species of hydrogen oxidizing bacteria belonging to 5 Gram-positive and more than 15 Gram-negative genera (Aragno and Schlegel, 1981; Lechner and Conrad, 1997). Maimaiti *et al.* (2007) managed to isolate and characterize 19 strains of these aerobic hydrogen oxidizing bacteria from soil exposed to hydrogen gas under lab conditions and from soils adjacent to HUP^- soybean nodules under field conditions; these 19 strains of hydrogen oxidizing bacteria were identified and assigned to the *Variovorax*; *Flavobacterium* and *Burkholderia* genera (Maimaiti *et al.*, 2007).

However, Zhang (2006) found that none of these isolates matched the major increased TRF peaks in T-RFLP profiles generated from either hydrogen-treated soils or soils sampled from around HUP^- nodules (Zhang, 2006), indicating that the hydrogen-oxidizing bacteria responsible for the hydrogen uptake in hydrogen treated soils and legume planted soils have not been isolated. There may be several reasons accounting for this: Firstly, the soil microbial population is so numerous and diverse that a single gram of soil may contain up to 10^9 microorganisms, representing potentially thousands of different species (Bollon *et al.*, 1993); particularly 99% of the microbial population in the rhizosphere (Dandurand and Knudsen, 1999) are not amenable to cultivation, so it is now recognized that the direct plate counting method

adopted by previous researchers can only detect a very small portion of the real population (Brock, 1987);

Secondly, it has been found that some soil bacteria showing hydrogen oxidizing ability (i.e. possessing an active hydrogenase) are not able to grow autotrophically (Conrad, 1988; Kluber *et al.*, 1994), indicating that not all hydrogen-oxidizing bacteria can be enumerated by traditional cultivation methods.

Finally, the physiology, genetics and ecology of soil-borne hydrogen-oxidizing bacteria is not well understood and information is scarce in the literature (LaFavre and Focht, 1983; Cunningham *et al.*, 1986; Dugnani *et al.*, 1986; Schuler and Conrad, 1991), making their further study somewhat difficult.

Thus there is an urgent need to turn to an alternative, culture-independent method to investigate these bacterial species. The application of certain molecular approaches, especially those based on surveys of PCR-amplified genes (collectively called molecular fingerprinting), has provided a powerful adjunct to the traditional cultivation-based methods and has allowed for rapid investigation of the soil microbial communities to be made. One approach in particular that has been frequently used to characterize various kinds of microbial communities is Terminal Restriction Fragment Length Polymorphism, also known as T-RFLP (Liu *et al.*, 1997)

1.4 Hydrogenase

1.4.1 [NiFe] Hydrogenase

Hydrogenases play an important role in microbial energy metabolism, allowing microorganisms to use H_2 as their sole energy source and fix CO_2 for

autotrophic growth. Hydrogenases have been commonly found in archaea, bacteria and eukaryotes (hydrogenosomes of protozoa and chloroplasts of green algae) (Vignais *et al.*, 2001) Although there are various types of hydrogenases found, they can generally be divided into two main groups: metal-containing hydrogenase and metal-free hydrogenase; the metal containing hydrogenase are further subdivided into three classes: [NiFe] hydrogenases; [Fe] hydrogenases and [NiFe (Se)] hydrogenases (Fauque *et al.*, 1988).

The first isolated and characterized hydrogenase belongs to the [NiFe] class, a heterodimeric protein consisting of a large nickel-iron center unit and a small subunit which contains three iron-sulfur clusters. The [NiFe] hydrogenase is able to catalyze both H_2 production and consumption with cytochrome C3 acting as the electron donor and/or acceptor (Cammack *et al.*, 1994).

One of the best characterized aerobic hydrogen oxidizing bacteria is *Alcaligenes eutrophus* (Friedrich, 1990). This bacteria species contains two different types of [NiFe] hydrogenases: a membrane-bound hydrogenase, consisting of two non-identical subunits, which is linked to a respiratory chain on the periplasmic side of the cytoplasmic membrane and is responsible for transferring electrons to the chain with the help of a b-type cytochrome (Schink and Schlegel, 1979; Schwartz and Friedrich, 2006); and a NAD-linked soluble cytoplasmic hydrogenase composed of four non-identical subunits (Schneider and Schlegel, 1976). The soluble hydrogenase catalyzes the oxidation of H_2 coupled with the reduction of NAD^+ to NADH, which was also found in a Gram-positive, facultative hydrogen oxidizing bacteria: *Nocardia*

opaca 1b, which does not possess a membrane-bound hydrogenase, but only the soluble cytoplasmic hydrogenase (Reh and Schlegel, 1981; Goodfellow, 1992).

Studies in recent literature also proposed that some bacteria possess a third class of hydrogenase called the regulatory hydrogenase; this apparently is an essential component of a complex hydrogen sensor, which is thought to comprise part of a multi-component signal transduction system (Cammack *et al.*, 2001).

The widespread [NiFe] hydrogenase is a priority for research as it plays a fundamental role in H_2 metabolism in all aerobic hydrogen oxidizing bacteria examined so far (Schwartz and Friedrich, 2006).

1.4.2 Some hydrogenases require activation or synthesis

Some hydrogen oxidizing bacteria possess active hydrogenase that are expressed constituently while others must be activated or biosynthesized; but differs among various bacteria depending on the growth conditions or other physicochemical factors. For instance, in the *Alcaligenes eutrophus* (mentioned above), the transcription of both its membrane-bound hydrogenase and NAD-linked cytoplasmic hydrogenase is regulated in response to hydrogen concentration and growth rate (Friedrich *et al.*, 1981). These hydrogenases are only transcribed when the hydrogen concentration reaches a certain threshold. This is similar with the *Hydrogenobacter thermophilus* TK-6 strain, a strain showing efficient hydrogen-oxidizing ability, yet requiring a hydrogen concentration of 75% to express all four of its hydrogenase gene clusters (Ueda *et al.*, 2007). In contrast, the expression of hydrogenase activity in free-living *Bradyrhizbobium japonicum* is strongly influenced by a combination of

quantity of nitrogen, concentrations of carbon substrates and decreased oxygen tension in the medium (Maier *et al.*, 1978).

1.5 Culture and Isolation of H_2 oxidizing bacteria

Given the importance of the soil microbial community in maintaining soil sustainability, microbiologists have been investigating the relationships between soil microbial diversity and soil sustainability since the 1960s (Harrison *et al.*, 1968). Pure cultures derived from colonies that survived under the lab conditions have been successfully and extensively studied all through the 20th century. Although the suspicion was stated as early as 1968 that those cells that were forming colonies were unrepresentative of the total bacterial community (Vagn Jensen, 1968), much of our basic knowledge of soil bacteria, as well as the discovery of many important antibiotics, have come from investigations of pure cultures (Alexander, 1977; Paul and Clark, 1996). Besides, previous studies have consistently demonstrated that direct cultivation and culture-independent methods generally sample different fractions of the bacterial community (Wilson and Blitchington, 1996; Chandler *et al.*, 1997; Suzuki *et al.*, 1997).Therefore cultured isolates are still very important in developing our understanding of bacterial physiology, genetics and ecology (Palleroni, 1997).

1.5.1 Medium for isolating H_2-oxidizing bacteria

Selecting the right medium that encourages the growth of H_2-oxidizing bacteria while at the same time discouraging the growth of other bacteria make a key step in beginning isolation. Previous studies have shown that the hydrogen uptake rate increases very slowly at first up to 7-10 days of growth, when it experiences a rapid

increase, indicating that the hydrogen-oxidizing bacteria are a group that exhibits slow growth (Dong and Layzell, 2001). So, if a nutrient-rich medium, whereon both rapid-growth copiotrophic bacteria and slow-growing hydrogen-oxidizing bacteria (slow growing bacteria) can grow is used, it can be anticipated that the copiotrophic bacteria will take over the whole plate before hydrogen oxidizing bacteria can ever form their colonies. Fortunately, as most of the aerobic hydrogen oxidizing bacteria are autotrophs or facultative autotrophs, an organic-free mineral medium can be successfully developed for isolation of this group of bacteria (Aragno and Schlegel, 1981). Furthermore, in natural habitats, soil hydrogen oxidizing bacteria would need a growing surface to form colonies. According to all the known characteristics of hydrogen oxidizing bacteria, a solid mineral medium would be a suitable medium for isolation of representatives of this group.

1.5.2 Incubation condition

Aerobic hydrogen-oxidizing bacteria are able to grow on an organic free medium as long as they are provided with a gaseous atmosphere with a combination of H_2, O_2 and CO_2 (Veldkamp, 1970; Maire *et al.*, 1978; Aragno and Schlegel, 1981; Dong and Layzell, 2001). Researchers have tried to isolate hydrogen oxidizing bacteria from legume fields by using a closed jar under an atmosphere consisting of H_2, O_2 and CO_2 in the ratio of 8:1:1(Kluber *et al.*, 1995; Conrad and Seiler, 1979; Aragno and Schlegel, 1981; Schuler and Conrad, 1990). In the present study, the open flow gas system was used.

1.5.3 Testing for H_2 uptake capacity of isolates

Four methods have been applied to detect the hydrogen uptake ability of isolates

Firstly, there is the TTC (2, 3, 5-triphenyl-2H-tetrazoliumcholoride) reduction test. Isolates in possession of a hydrogenase (i.e. showing hydrogen oxidization capacity) are believed to have the ability to irreversibly reduce water-soluble, uncolored TTC to the water-insoluble, red triphenylformazane while isolates without hydrogenase do not; this assay has been used in the past to differentiate colonies with and without hydrogenase activity (Schlegel and Meyer, 1985). However, although the TTC test is convenient and inexpensive, it is not reliable in mixed cultures of unknown bacteria as some endogenous electron donors in isolates without a hydrogenase can also reduce TTC and thereby give false positive results (Kluber *et al.*, 1995)

Secondly, there is the DNA-DNA-hybridization test. DNA probes in the DNA-DNA-hybridization technique are able to detect the presence of both active and silent hydrogenase genes. However, since the DNA probes were originally designed based on known hydrogenase genes, they only cover a small part of all possible hydrogenase genes. It would be expected that some bacteria with hydrogen oxidation activity may not emit a hybridization signal with DNA probes derived from certain hydrogenase genes (Kluber *et al.*, 1995)

Thirdly, we have the autotrophic growth test. Aerobic hydrogen oxidizing bacteria are known for their ability to grow autotrophically with H_2 as the sole energy

source (Aragno and Schlegel, 1992), so this property has been applied as an indicator of H_2 oxidation activity.

Finally, there is the H_2 oxidation test. In this assay, gas chromatography (GC) is applied to test the capacity of isolates for hydrogen oxidation. With the H_2 oxidation test, bacteria have been detected, both those with active hydrogenases and those possessing easily inducible hydrogenases (Kluber *et al.*, 1995). So, although it is time-consuming, this test is the most reliable and straightforward test for detection of hydrogen oxidation activity and has yet to be substituted with any superior technique.

1.6 The Application of the Terminal Restriction Fragment Polymorphism Length (T-RFLP) Method

Firstly developed to identify *mycobacteria* (Avaniss-Aghajani *et al.*, 1996), the T-RFLP method has now been widely used to monitor the diversity, structure and dynamics of microbial communities in a wide range of habitats, including various kinds of soil microbial populations (Dunbar, *et al.*, 2000; Graff and Conrad, 2005; Hartmann and Widmer, 2006, 2008); bioreactor sludge (Liu *et al.*, 1997); the human intestinal microbial community (Matsumoto *et al.*, 2005); infant and rat fecal communities (Wang *et al.*, 2004; Kaplan *et al.*, 2001) and aquatic communities (Moeseneder *et al.*, 1999; Danovaro *et al.*, 2006)

1.6.1 How T-RFLP profiling works

The phylogenetic marker for T-RFLP analysis comes from the small-subunit (SSU) rRNA genes, usually the 16S ribosomal RNA (rRNA) gene or 16S rDNA for a bacterial community. The main advantage of using the 16S rRNA gene as a marker is

that it contains both highly conservative regions found in all bacteria and regions that are variable enough to differentiate one species from another (Knox *et al.*, 1998). The T-RFLP profiles are created using a specific set of molecular protocols. Firstly, community genomic DNA is extracted from an environmental sample. Then the extracted DNA is used as a template for PCR amplification of 16S rRNA genes used with either universal or domain-specific primers, depending on the downstream application. Thirdly, the amplified PCR products are cut with restriction enzymes with a 4bp recognition site, or four-cutters (Liu *et al.*, 1997); One of the primers, usually the forward one, is fluorescently labeled so that the fragments with fluorescent dye can be detected by fluorescence after separation via gel electrophoresis as the fourth step (Liu *et al.*, 1997; Blackwood *et al.*, 2003).Finally, the fluorescence intensities and corresponding peak sizes (measured in base pairs) are recorded by a DNA sequencer (Blackwood *et al.*, 2003).

Microorganisms in a community are thus differentiated based on the different TRF sizes they generate, which in turn creates a fingerprinting pattern unique to that specific community. It should be noted that the use of multiple restriction enzymes for T-RFLP analysis of complex microbial communities increases the method's resolving power and provide more information about the community composition (Kaplan and Kitts, 2003). While the method has demonstrated its robust potential and high-throughput capability to analyze variations of complex microbial communities from diverse environmental samples (Liu *et al.*, 1997; Clement *et al.*, 1998; Marsh, 1999; Osborn *et al.*, 1999; Kirk *et al.*, 2004; Hartmann *et al.*, 2005),

there are limits on its capacity to assess microbial community diversity due to biases inherent in the method.

1.6.2 Bias susceptibility in T-RFLP profiling

To begin with, as a PCR based method, T-RFLP is susceptible to all biases inherent in the PCR amplification technique. It has been observed that variations in initial DNA template concentration; the number of PCR cycles and the annealing temperature can all result in different T-RFLP patterns, indicating visualized compositions of the microbial community can be easily changed due to biases inherent in these in PCR parameters (Osborn *et al.*, 1999), such as template reannealing with an increasing number of PCR cycles (Suzuki and Giovannoni, 1996) and preferential gene amplification of specific templates can also become issues (Polz and Cavanaugh, 1997).

Besides PCR biases, the composition of T-RFLP patterns can also be influenced by downstream restriction enzyme cutting. The presence of additional T-RFs resulting from inadequate digestion of PCR products has been encountered in T-RFLP profiles of both pure cultures (Bruce, 1997) and environmental samples (Osborn *et al.*, 1999; Song *et al.*, 2002), consequently leading to overestimation of diversity in the community. Unfortunately, bias due to incomplete digestion may not even be eliminated by increasing the amount of restriction endonuclease used (Osborn *et al.*, 1999)

Furthermore, formation of false or pseudo T-RFs resulting from non-digested single-stranded 16S rRNA gene amplicons has also been reported to bias T-RFLP

profiles (Qiu *et al.*, 2001; Egert and Friedrich, 2003; Matsumoto *et al.*, 2005; Graff and Conrad, 2005). These single-stranded DNAs are formed during PCR amplification and their terminal restriction sites are not accessible to restriction enzymes (Egert and Friedrich, 2003). Fortunately, they can be completely removed by application of single-strand-specific mung bean nuclease prior to T-RFLP analysis (Egert and Friedrich, 2003).

Another difficulty is that different phylogenetic groups may produce T-RFs of the same length, while closely related or even nearly identical bacterial sequences could yield very different T-RF sizes (Schmitt-wagner *et al.*, 2003; Graff and Conrad, 2005; Hartmann and Widmer, 2006), which can seriously impeded phylogenetic inference based on T-RF sizes. A single T-RF peak in a profile may represent more than one bacterial species in the microbial community. Applying more than one restriction enzyme may help resolve the problem (Osborn *et al.*, 1999)

Last but not least, discrepancies have been commonly observed between T-RF lengths read from T-RFLP profiles (observed TRF length) and T-RF length predicted from clone libraries or extensive databases of existing sequences (sequence-determined TRF length), making taxonomic assignment of particular T-RFs in complex profiles difficult (Brunk *et al.*, 1996; Liu *et al.*, 1998; Ludemann *et al.*, 2000; Hackl *et al.*, 2004; Matsumoto *et al.*, 2005; Hartmann and Widmer, 2006) Kaplan and Kitts (2003) termed this discrepancy as "TRF drift", the drift being equal to the observed TRF length minus the sequence-determined TRF length. They also reported this variation is due to differential migration of ladder and sample DNA;

subtle differences in molecular weight, either from purine content or dye label; or fluctuations in ambient temperature (Kaplan and Kitts, 2003) can result in TRF drift. Variations resulting from differential migration can be corrected by using the equations generated by them in their work (Kaplan and Kitts, 2003); Changes due to temperature fluctuations can be prevented simply by maintaining a constant experimental temperature. However, biases caused by purine content or dye label may still remain and may result in a TRF drift of approximately -7 to +1 base pair (Kaplan and Kitts, 2003).

1.6.3 T-RF drift and 'matching windows'

Due to the problems of TRF drift discussed above, a 'matching window' is required when assigning 16S rDNA clones to specific peaks in T-RLFP profiles of PCR products amplified from the same DNA from which the 16S rDNA library was generated. The range of the matching window changes according to different research requirements. Investigators have adopted different ranges of TRF drift to compensate for the discrepancies generated in their research according to their needs, but within scientific and reasonable ranges.

For instances, Kaplan and Kitts (2003) demonstrated the observed TRF length is within an average of -3±1.28bp of the sequence-determined T-RF length and they recommend using a matching window of at least ±2bp on the ABI 310 Genetic Analyzer (Kaplan and Kitts, 2003). Due to the nature of gel separation, Braker and colleagues (2001) suggest allowing the observed TRF length to be within ±2bp of the computer-simulated T-RFs of 16S rDNAs of archaeal species (Braker *et al.*, 2001). In

the above studies, sequence-determined T-RF lengths were predicted from the Ribosomol Database Project rather than from *in silico* digestion of each clone sequence.

Kaplan adopted a matching window of +1 to -4bp when he used the T-RFLP method to monitor the changes in the microbial populations of rat feces fed with NCFM (Kaplan *et al.*, 2001). Hartmann and Widmer observed that the experimental T-RF sizes of each individual clone differed from theoretical sequence determined T-RF sizes by an average of 3.5±1.9bp (Hartmann and Widmer, 2006). A matching window of -4 to +3bp was selected in a phylogenetic assignment of 16S rDNA clones from three forest soils to the predominant T-RFs in their corresponding bacterial community level T-RFLP profiles (Hackl *et al.*, 2004); these T-RF drifts are between the $T\text{-}RF_{exp}$ of each clone from experimental T-RFLP profiles and its corresponding community T-RFLP profile. However, in the present study, as the *in vitro* T-RF pattern (the experimental T-RFLP profile compiled from the $T\text{-}RF_{exp}$ of each clone) of individual samples was not tested alongside the *in silico* determination of the expected size of the T-RFs, the existence of pseudo-TRFs cannot be excluded (Egert and Friedrich, 2003).

1.6.4 The necessity of a polyphasic approach to assess microbial diversity

According to the above, assessing microbial community structure and diversity solely by the T-RLFP fingerprinting methods seems inadequate.A polyphasic approach, using both culture-based and culture-independent methods, is

likely to produce more complete information on the composition of soil microbial communities (Hill *et al.*, 2000). Furthermore, it has been shown that generating a 16S rDNA clone library combined with T-RFLP statistical analysis provides the most detailed and reliable information on the composition and diversity of microbial communities (Osborn *et al.*, 1999; Dunbar *et al.*, 2000; Venter, 2004; Janssen, 2006).

Thus, in order to obtain more accurate information on the structure and diversity of H_2-treated soil microbial communities, a combination of community level T-RFLP profiling with 16S rRNA gene clone libraries constructed in parallel from the same sample will be required, as will the matching window described above, to synthesize results generated from both of these techniques.

1.7 16S rRNA Gene Clone Library

The 16S rRNA gene clone library resembles the terminal restriction fragment length polymorphism analysis of 16S rRNA genes in that they use the same molecular marker (the 16S rRNA gene) and that they share the same purpose: namely to assess bacterial community structure and diversity. Compared with the T-RFLP fingerprinting technique, the sequencing of 16S rRNA genes, although much slower and more expensive, resulting in a clone library of an environmental sample DNA, can provide a much more refined elucidation of microbial community structure and composition, especially where highly complex microbial communities such as those found in soils is concerned, as each phylotype can be precisely identified and quantified. The superior resolution of sequence analyses of 16S rRNA gene libraries over that of T-RFLP analyses has been demonstrated (Dunbar *et al.*, 2000).

The cloning and sequencing of a 16S rDNA library is executed so as to isolate total community DNA from an environmental sample such as soil. This DNA is then used as a template for PCR amplification of the 16S rRNA genes (using universal bacterial primers); these genes are thereafter utilized directly for library construction. The clone library is then screened either according to the results of a T-RFLP community profile analysis, generated from the same DNA used to construct the library (Moyer *et al.*, 2004), or else is screened based on sequence variations (Bond *et al.*, 1995).

1.7.1 Biases inherent in the 16S rRNA gene cloning techniques and their corresponding solutions

Like the T-RFLP fingerprinting technique, the cloning of 16S rRNA genes also starts with PCR amplification of targeted DNA fragments, thus it is also subject to all biases inherent in PCR amplification. The sources of potential biases and artefacts in PCR amplification may include contamination with foreign DNA from, for example, the air or a researcher's hands, which may interfere with amplification of the desired DNA template; thus careful handling, such as wearing gloves when performing PCR, is of critical importance. Also, primers can bind to themselves, forming hairpin loops; primers may also anneal to a non-specific targeted area because of high annealing temperature; these problems can be solved by making sure the primers do not have termini that can bind to each other and by lowering annealing temperatures (Ishii and Fukui, 2001); The formation of chimeric molecules from the recombination of two different DNA molecules with high similarity is also a

potentiality, but this artefact can be minimized or eliminated by increasing the primer elongation time and decreasing the number of PCR cycles (Wang and Wang, 1997; Qiu *et al.*, 2001); Another problem that may be encountered is the generation of point mutations due to a high intrinsic misincorporation rate of Taq DNA polymerase (Gelfand, 1992) and deletion mutations caused by the stable secondary structure of the 16S rRNA gene (Cariello *et al.*, 1991), point mutation is not of great concern as the maximum misincorporation rate would only lead to a 0.3% sequence divergence (Wintzingerode *et al.*, 1997). Furthermore, these issues can be circumvented by decreasing the template concentration (Qiu *et al.*, 2001). The formation of heteroduplexes, it has been observed with a corresponding increase in the proportion of heteroduplexes correlated with increases in template species diversity, PCR cycles and template concentration (Qiu *et al.*, 2001). Heteroduplexes are non-avoidable during the process of PCR amplification of a mixture of homologous genes. If a heteroduplex molecule of 16S rDNA is cloned and transformed, two heteroduplex molecules of 16S rRNA genes will be produced and multiply with the propagation of the plasmid. Fortunately, it can be removed by excising the bands corresponding to heteroduplexes from a non-denatured polyacrylamide gel, or digested by a T7 endonuclease (Qiu *et al.*, 2001); Finally, there can be problems with the cloning system; an alteration of taxon composition in a 16S rRNA gene library was reported with the change of cloning system given that the DNA from which they were both constructed was the same (Rainey *et al.*, 1994).

1.7.2 16S rRNA gene library: the current most robust fingerprinting technique

Despite the above mentioned biases and artefacts, 16S rRNA gene clone libraries have been successfully used to characterize microbial diversity and discern different compositions in microbial communities (Bond *et al.*, 1995; Dunbar *et al.*, 2000; Hackl *et al.*, 2004; Graff and Conrad, 2005; Hartmann and Widmer, 2006). While research should continue on developing methodologies that can rapidly characterize microbial community diversity, it is still proposed that a 16S rRNA gene library will give us the most comprehensive picture of a soil microbial community with careful planning and regulation of experimental conditions.

1.8 The Objectives of the Present Study

It has been previously shown that the hydrogen released from HUP– legume nodules into soil is conserved by a group of hydrogen-oxidizing bacteria that show plant growth promotion effects. The present study identifies the hydrogen-oxidizing bacteria responsible for the major increased T-RFs in T-RFLP profiles of hydrogen-treated soil samples, with the study objectives as follows:

- To monitor variations in soil microbial community structure induced by hydrogen metabolism via comparisons between T-RFLP profiles of hydrogen-treated soil samples and air-treated soil samples under lab conditions.
- To construct and sequence a 16S rDNA library from the same DNA preparations from which the 16S rRNA genes for T-RFLP profiles of hydrogen-treated soil were PCR amplified.

- To match 16S rDNA clones with major increased T-RFs in T-RFLP profiles of hydrogen-treated soil samples through generating in silico T-RFLP profiles of the library.
- To isolate the hydrogen-oxidizing bacteria from hydrogen-treated soil and characterize them using Gram staining and 16S rRNA sequence analyses.

2. Materials and Methods

2.1 Culture and Isolation of H_2-oxidizing Bacteria

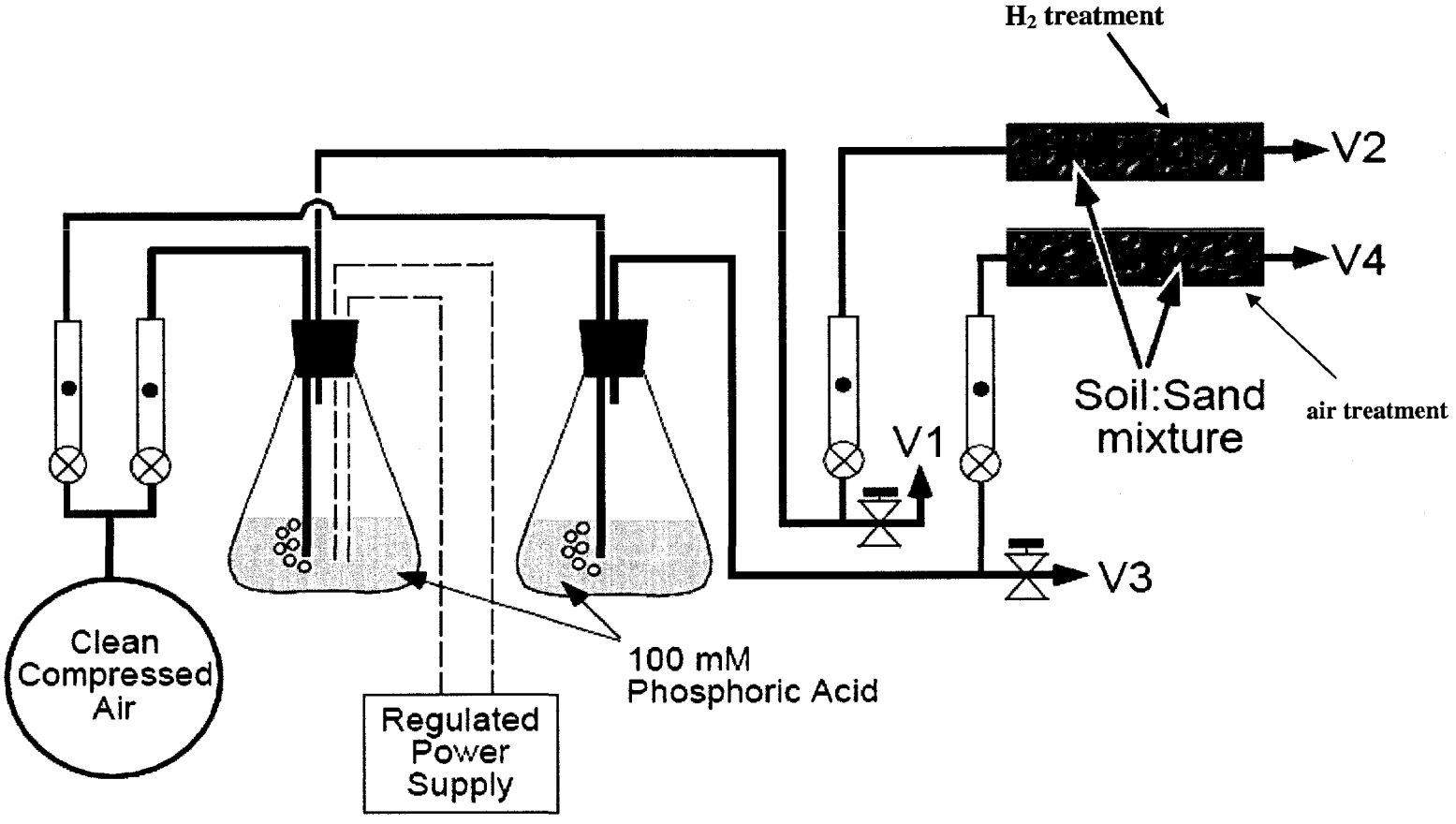
2.1.1 Samples for isolating H_2-oxidizing bacteria

Soil samples were collected from the Plumdale Facility, NSAC (Nova Scotia Agricultural College) Experimental Farm, Truro, Nova Scotia in the summer of 2006. They were put through the following procedures before being used for H_2 oxidizing bacteria isolation and DNA extraction: a total of 50 mL of soil and fine sand were mixed in the ratio of 2 to 1 (v/v ratio) and autoclaved for 20 min. One gram of pre-H_2 treated soil showing a significantly high hydrogen uptake rate was mixed together with 3 mL of autoclaved distilled water (dH_2O) in a test tube. 1 ml of supernatant from the test tube was used to inoculate the 50 mL of prepared soil and then the soil was transferred to a 60 mL syringe; the following four syringes were prepared in the same way as the first one. These syringes were labeled as LH1, LH2, LH3, LH4 and LH5 and were treated with a gas stream containing approximately 3000 ppm H_2 generated by electrolysis for 3 weeks using the H_2 treatment system as described by Dong and Layzell (2001) (Figure 1). In the air treatment system (Figure 2) with the same flow rate (3000 ppm), five syringes were prepared the same way as described

above and labeled as LA1, LA2, LA3, LA4 and LA5.

Figure 1: A simplified diagram of the hydrogen treatment system (Modified from Dong and Layzell, 2001).

The hydrogen gas is produced by hydrolysis in the first flask being equipped with a regulated power supply to provide a direct electric current. The second flask acts as a control (air treatment). Air is provided at a stable rate to both flasks. For hydrogen gas treatment, the hydrogen enriched gas stream (V1) was connected with the soil column before venting to the atmosphere at (V2). For air treatment, the air (V3) was connected with the soil column before venting to the atmosphere (V4).


H_2 treatment
V2
V4
Soil:Sand mixture
air treatment
V1
V3
Clean Compressed Air
Regulated Power Supply
100 mM Phosphoric Acid

2.1.2 Measurement of soil H_2 uptake rates

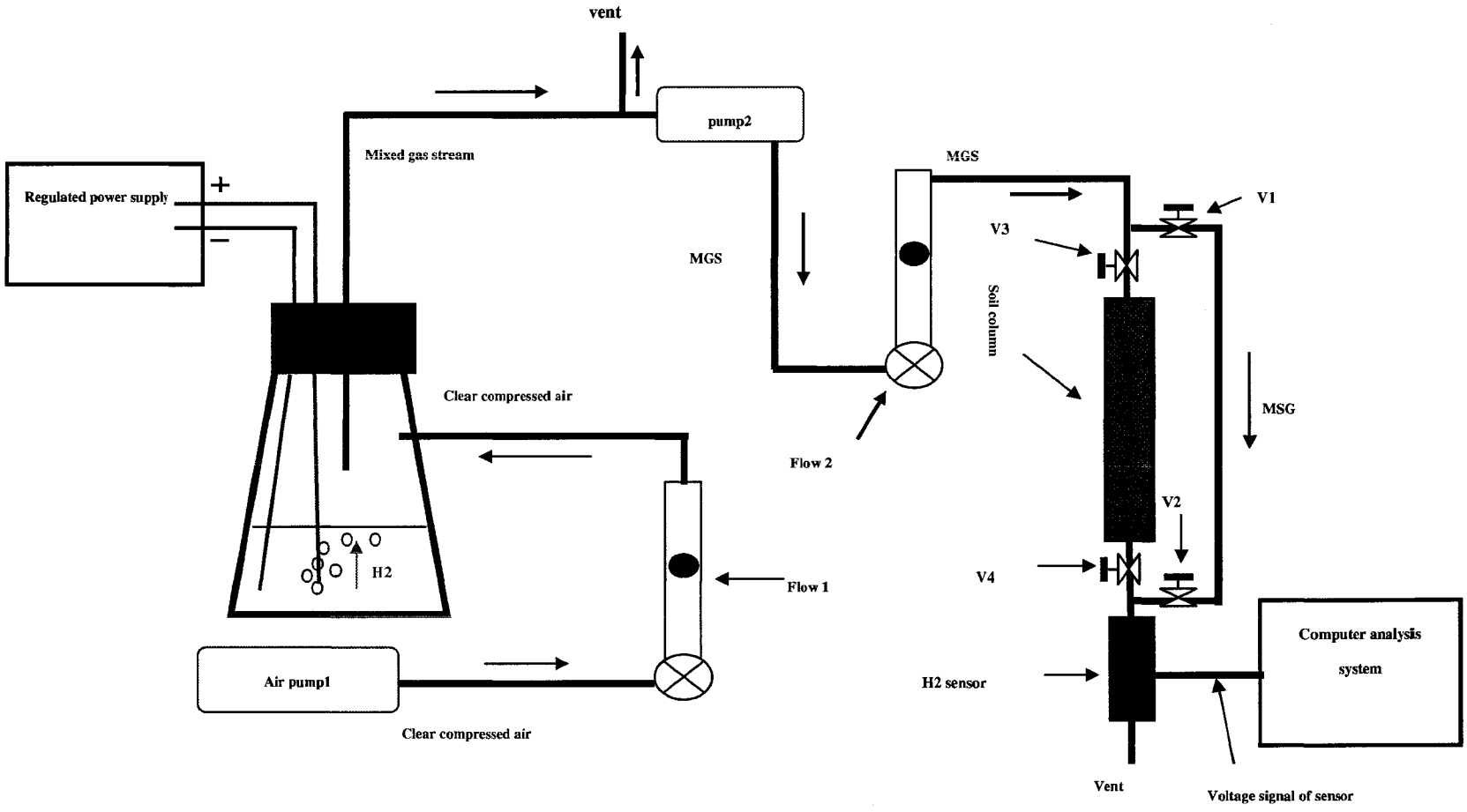
The hydrogen uptake capacity of each soil sample had to be measured before it could be used for hydrogen-oxidizing bacterial isolation. This capacity is measured as the difference between the concentrations of hydrogen before and after passing the soil sample, which were recorded by a H_2 sensor (Model S121, Quibit Systems Inc) using the Data Logger program as described by Dong and Layzell (2001).

The hydrogen sensor is a ceramic tube equipped semi-conductor device, which with a 10K resistor are the two major components of a circuit supplied with a five voltage DC. The resistance of the semiconductor changes with the concentration of hydrogen which varies with its combustion with oxygen in the passing gas stream. The voltage on the 10K resistor changes with the variation of the resistance of the semi-conductor and was recorded by the Data Logger Program as the computer analysis system (Dong and Layzell, 2001) (Figure 2). Thus the concentration of hydrogen in the passing gas stream can be calculated by comparing the voltage value monitored by the program with a standard curve of hydrogen concentration versus voltage on the 10K resistor.

The standard curve is needed for the conversion of voltage to concentration as the computer output is voltage, not H_2 concentration. After the measurement of hydrogen uptake rate of the soil samples, 0.1 g and 1 g of soil from the first syringe (the one closest to the H_2 treatment system) was removed for isolation of hydrogen-oxidizing bacteria and soil DNA extraction respectively.

Figure 2: A simplified diagram of the hydrogen uptake capacity measurement system.

The hydrogen gas is generated in the flask equipped with a regulated power supply to provide a direct electric current. Air is provided at a stable flow rate by both pumps and combined with hydrogen gas to make a mixed gas stream before passing the soil column or hydrogen sensor. Valves 1, 2, 3 and 4 are operated to make the sensor determine the concentration of hydrogen in the mixed gas stream before and after passing the soil column. MGS=mixed gas stream.


vent
pump2
Mixed gas stream
Regulated power supply
+
−
MGS
MGS
V1
V3
Soil column
MSG
Clear compressed air
Flow 2
V2
H2
V4
Flow 1
Computer analysis system
Air pump1
H2 sensor
Clear compressed air
Vent
Voltage signal of sensor

2.1.1.1 Standard curve of Voltage *versus* Hydrogen Concentration (ppm)

The amount of electrolytic hydrogen (Z: μmol/min) in the flask (Figure 2) was calculated using the following equation:

$$Z\ (\mu mol/min) = (3.00*104*C*I) / Av \quad \text{----------①}$$

C (Coulomb Constant): $6.24*10^{18}$ (A^{-1});

I (current of electrolysis): mA;

Av (Avogadro Constant): $6.02*10^{23}$ (mol^{-1}).

From Equation①, the following equation was derived to calculate the concentration of electrolytic hydrogen in the mixed gas stream (H: ppm):

$$H\ (ppm) = [1.00*103*Z*Gc*(273.15+T)]/(273.15*FR1) \quad \text{----------②}$$

Z (amount of electrolytic hydrogen per minute): μmol/min;

GC (gas constant): 22.41 L/mol at 0 °C and 1 atmosphere pressure;

T (temperature): °C; FR1 (Flow Rate One): ml/min.

A series of mixed gas streams with gradient hydrogen concentration (from 0.55 ppm to 147 ppm) were generated by regulating the current of electrolysis and Flow Rate One. Then V1 and V2 were opened and V3 and V4 were closed to allow the mixed gas stream to pass the hydrogen sensor directly. Finally, voltage across the 10K resistor in hydrogen sensor was recorded by the computer analysis system when the mixed gas stream with known hydrogen concentration passed the hydrogen sensor (Figure 2). Based on Matlab, a standard curve of voltage versus hydrogen concentration (ppm) was fitted with an exponential function: ppm (H_2) = a*e (b*v) [v: voltage, e=2.718282].

2.1.1.2 Hydrogen uptake rate of both air-treated and H_2-treated soil samples

Firstly, the concentration of electrolytic hydrogen in the mixed gas stream before passing the soil column ([H]in: v) was determined by passing the mixed gas stream by the hydrogen sensor directly (turning on V1 & V2 and turning off V3 & V4). Then, the concentration of electrolytic hydrogen in the mixed gas stream after passing the soil column ([H]out: v) was measured when V3 & V4 were turned on and V1 & V2 were closed (Figure 1)

2.1.2 Isolation of H_2 -oxidizing bacteria

2.1.2.1 Culture condition

The medium used for isolating H_2-oxidizing bacteria was mineral salt agar (MSA).: (10% (w/v, hereinafter) $NaNO_3$ 20 mL ; 12% K_2HPO_4 10 mL ; 10% $MgSO_4$ 5 mL; 10% KCl 5 mL; 14% KH_2PO_4 1 mL; 1% $Fe_2(SO_4)_3$. H_2O 1 mL; add 0.02 g yeast extract and 15 g agar in 1000 mL distilled water (dH_2O) autoclaved. The open gas flow-through system was used for bacterial incubation at room temperature. The hydrogen gas, almost 3000ppm, was generated by electrolysis in atmospheric air and delivered to the incubation bucket where plates were stored.

2.1.2.2 Bacterial isolation

0.1 g hydrogen treated soil was mixed together with 10 mL autoclaved distilled water (dH_2O) and serially diluted (10^{-2}-10^{-12}). The dilutions were plated onto MSA medium. 0.05 g autoclaved soil was spread over each inoculated plate to create a growing surface for the bacteria. These plates were incubated in the open gas flow-through system as described above for three weeks.

The colonies were checked every two days and the visible bacterial colonies were picked and transferred to new MSA plates. The visible colonies on these plates were further subcultured by transfer to new MSA plates to reduce the number of unwanted bacteria and so increase the purity of the colonies. These last plates were incubated under the same conditions as the originals. The number of transfers was dependent on the number and kind of undesirable bacteria present and the consistency of the colonies. Bacterial cultures from the new MSA plates were inoculated on the MSA slant tubes (5 mL MSA medium in each 17-mL test tube) and incubated for 5-7 days before testing for hydrogen uptake ability of the isolates.

2.1.2.3 Test for hydrogen uptake ability of isolates

Test tubes on which all isolates grew under hydrogen treatment were tested, as well as an uninoculated test tube and a test tube with isolates grown under air treatment, which were used as controls. The hydrogen-oxidizing isolates grown in slant tubes and the control tubes were individually flushed with H_2 (<3000 ppm) for 30 seconds. Immediately thereafter, the plastic caps on the tubes were replaced by gas-tight rubber caps wrapped with parafilm to avoid gas leaks. 0.5 mL gas was extracted from each test tube with a gas-tight syringe immediately after H_2 was flushed in, and was injected into a gas chromatograph (GC) system connected to a H_2 sensor to measure the initial H_2 concentration.

These tubes were then incubated at room temperature for the intervals of 30 min, 1 h, 2 h, 3 h and overnight. The changes in H_2 concentration with each bacterial isolate grown, as well as that of the control tubes, were measured at the end of each interval. The H_2 concentration of the tubes on which hydrogen-oxidizing bacteria were grown showed greater changes over time compared to the control tube,

indicating they were utilizing H_2.

2.1.3 Identification and characterization of H_2-oxidizing isolates

2.1.3.1 Gram-staining

First, circles were marked on the backs of slides and two drops of dH_2O were transferred to the circles using an inoculation loop. Bacterial isolates were picked out from plates, transferred to the slides with the loop and then mixed evenly with the water. Specimens were air dried and quickly flamed with an alcohol lamp. Slides were placed on a rack and then flooded with crystal violet for 60 seconds. Slides were then washed with dH_2O water for 5s. Next the slides were flooded with the iodine solution and again rinsed with dH_2O water for 5s. Ethanol was then added to slides as a decolorizer drop by drop until the violet-blue color was gone; this was done to ensure there were no false results from either over-or under-colorization. Slides were rinsed in dH_2O water for 5 s and then flooded with safranin (a counterstain) for 60 s to allow the bacteria incorporate the stain. Gram positive cells incorporated little or no counterstain and remained blue-violet in appearance. Gram negative bacteria, on the other hand, took on a pink color and were easily distinguishable from the Gram positives. Again, slides were rinsed with water for 5s to remove all excess dye and then air dried or blotted gently with bibulous paper before viewing under a compound microscope with the 100X objective and immersion oil.

2.1.3.2 16S rRNA gene sequence analysis

For Gram negative isolates, DNA amplification was carried out immediately gram staining with colony PCR by using the following universal primers: bacterial 16S forward primer: (BSF8/20 5'-AGAGTTTGATCCTGGCTCAG-3') and bacterial

16S reverse primer: (BSR1541/20 5'-AAGGAGGTGATCCAGCCGCA-3'). Each 20 µL colony reaction mixture contained: 15 µL PCR water (molecular biology reagent, Sigma-Aldrich Canada Ltd, Oakville, ON), 2 µL of 10X ThermolPol Reaction Buffer (New England Biolabs Ltd., Pickering, ON), 1 µL of 10mM dNTP (dATP, dTTP, dCTP, dGTP; New England Biolabs Ltd, Pickering, ON), 1.5 µL each of 20µM 6-FAM-5'-BSF8/20 and BSR1541/20 (Applied Biosystems, Foster City, CA) and 0.5 µL of 5U/ µL Taq DNA polymerase (New England Biolabs Ltd., Pickering, ON). PCR amplification reactions were carried out in a Bio-rad iCycler thermal cycler (Bio-rad Laboratories, Inc., Hercules, CA) with the following cycling conditions: three minutes of denaturation at 94 °C, 35 cycles of 45 seconds at 94 °C for denaturation, 45 seconds at 58 °C for annealing, and 45 seconds at 72 °C for primer extension, with a final cycle of primer extension at 72 °C for 10 minutes. The PCR product was purified with the Qiaquick PCR Purification Kit (QIAGEN Inc., Mississauga, CA) using the manufacturer's protocol and sent for sequencing (Marcrogen Inc., Seoul, SK). The obtained sequences were compared with similar sequences in the Genbank database using the BLAST program (NCBI).

For Gram positive isolates, whose cell walls are much thicker than that of gram negative species, genomic DNA has to be extracted first before amplification. Gram positive isolates bacterial cells were washed with 1.5 ml distilled water and transferred to a microcentrifuge tube. This was centrifuged at the 14, 000 rpm at room temperature using a (model info for the centrifuge that I used) for 2 minutes, and then the pellet was resuspended in 576 µl TE buffer (100mM pH 8.0; Tris-cl, 1mM pH 8.0 Na_2EDTA).This resuspension was mixed with 3 µl proteinase K (20 mg/ml) and 30 µl 10% sodium dodecyl sulphate (SDS). The mixture was incubated at 60 °C for 30 seconds. An equal volume of phenol was then added to the resuspension and the

mixture was vortexed at maximum speed. The upper layer was then removed and added to an equal volume of chloroform-isoamylalcohol (24:1) to remove the phenol. 0.6 volumes of isopropanol were added to the sample. The sample was incubated for 1hr at -80 °C or at -20 °C overnight. Following a centrifugation at 1,2000g for 20 minutes at 4 °C to pellet the DNA, genomic DNA was washed in 70% ethanol and then air dried. The pellet was resuspended in 100-200 µl sterilized TE buffer. The conditions for DNA amplification of gram positive isolates were the same as the colony PCR described above except that 13 µl PCR water and 2 µl genomic DNA were used in the PCR reaction mixture. This PCR product was purified with the Qiaquick PCR Purification Kit (QIAGEN Inc., Mississauga, ON) following the manufacturer's protocol and sent for sequencing at Marcrogen Inc., Seoul, SK. The obtained sequences were compared with similar sequences in the Genbank database using the BLAST program (NCBI).

2.2 The Effect of Hydrogen Metabolism on Soil Microbial Community Structure

2.2.1 DNA extraction from both air-treated and H_2-treated soil samples

H_2- and air-treated genomic soil DNA was extracted respectively (using two independent extractions for each soil sample) from 1g soil taken out from the first syringe as described above, using PowerSoil™ DNA isolation Kits (MO BIO Laboratories, Inc., Solana Beach, CA)using the manufacturer's protocol.

1g of soil sample was added to the PowerBead solutions and gently vortexed to mix. 60 µL of solution C1, containing SDS and other disruption agents required for cell lysis, was added to the PowerBead tubes and these were inverted several times or vortexed briefly. The PowerBead tubes were then secured horizontally on a flat-bed

vortex pad with tape and vortexed at maximum speed for 10 min. Following a centrifugation at 10,000 x g for 30 seconds at room temperature (18-20 °C, ambient O_2), the supernatant was transferred to a clean 2 mL collection tube. 250 μL of Solution C2, containing a reagent to precipitate non-DNA organic and inorganic material, was added to the collection tube and vortexed for 5 seconds, followed by an incubation at 4 °C for 5 min. The tubes were then centrifuged at room temperature for 1 min at 10,000g. 600 μL of supernatant was transferred to another clean 2mL collection tube. 200μL of Solution C3, similar to Solution C2, was added and the tubes were vortexed briefly, followed by incubation at 4 °C for 5 min. Following a centrifugation at 10,000g for 1 min, 750 μL of supernatant was transferred to a third collection tube. 1200 μL of Solution C4 (which makes making DNA bind to silica in the presence of a high salt concentration) was added and there tubes were vortexed for 5 seconds. The mixture of supernatant and Solution C4 was loaded onto a spin filter and centrifuged at 10,000g for 1 minute at room temperature (a total of three loads were required due to the limited volume of the spin filters). Following the discard of flow-through, 500 μL of Solution C5, an ethanol based wash solution, was added to further clean the harvested DNA which was now bound to the silica filter membrane. The tubes were centrifuged at 10,000g for 30 seconds. After the flow-through was discarded, the spin filter was centrifuged at 10,000g for 1 minute to remove residual ethanol. The spin filter was put into a clean 2mL collection tube. 50 μL of Solution C6, a sterile elution buffer, was added to the center of the white filter membrane, with an additional centrifugation at 10,000g for 30 seconds, to release the DNA from the filter membrane. DNA concentration and purity were examined using a UV/visible spectrophotometer (Ultrospec 3100 pro).

2.2.2 PCR of 16S rRNA genes

16S rDNA genes amplification was carried out using the following bacterial universal primers: BSF8/20 with a fluorescent dye, 6-FAM (phosphoramidite fluorochrome 5-carboxyfluorescein), labeled at the 5' terminus (6-FAM-5'-AGAGTTTGATCCTGGCTCAG-3') and unlabeled BSR534/18 (5'-ATTACCGCGGCTGCTGGC-3'). The expected length of products was 527 base pairs (bp).

2.2.2.1 PCR conditions

Each 50-μL reaction mixture contained: 38.5 μL PCR water (molecular biology reagent, Sigma-Aldrich Canada Ltd, Oakville, ON), 5 μL of 10X ThermolPol Reaction Buffer (New England Biolabs Ltd., Pickering, ON), 1 μL of 10mM dNTP (dATP, dTTP, dCTP, dGTP)(New England Biolabs Ltd., Pickering, ON) 1.5 μL of 20mM 6-FAM-5'-BSF8/20 and BSR534/18 (Applied Biosystems, Foster City, CA), 0.5 μL of 5U/ μL Taq DNA polymerase (New England Biolabs Ltd., Pickering, ON) and 2 μL template DNA. PCR amplification reactions were carried out in the Bio-rad iCycler thermal cycler (Bio-rad Laboratories, Inc., Hercules, CA) with the following cycling conditions: three minutes of denaturation at 94 °C, 35 cycles of 45 seconds at 94 °C for denaturing, 45 seconds at 55 °C for annealing, and 45 seconds at 72 °C for primer extension, and a final cycle of primer extension at 72 °C for 10 minutes. Multiple PCR reactions from the same DNA sample were pooled together to minimize PCR-induced random biases. PCR products were purified with the QIAquick PCR purification Kit (QIAGEN Inc., Mississauga, ON) using the manufacturer's protocol.

2.2.3 Generation of community TRF profiles of air- and H_2-treated soil samples

PCR products from three DNA amplification replicates were pooled together to reduce PCR bias (Polz and Cavanaugh, 1998). Approximately 200 ng of fluorescently labeled purified PCR amplification products were digested with one of the four restriction endonucleases (the apostrophe shows the cleavage site): BstUI (CG'CG), HaeIII (GG'CC), HinfI (G'ANTC), and MspI (C'CGG) (New England Biolabs Ltd., Pickering, ON).

Digestion occurred in a 50-μL reaction system (Osborne *et al.*, 2006; Dunbar *et al.*, 2000; Kitts, 2001; Liu *et al.*, 1997). Each 50-μL reaction mixture contained 38.25μL PCR water (molecular biology reagent, Sigma-Aldrich Canada Ltd, Oakville, ON); 5μL of 10X ThermolPol Reaction Buffer (New England Biolabs Ltd., Pickering, ON); 0.5μL of restriction endonucleases (New England Biolabs Ltd., Pickering, ON) and 6.25μL of template DNA. Each 50-μL reaction mixture was loaded into a sterilized 0.5mL PCR tube and incubated at 37 °C overnight (for approximately 10 hours) except samples digested with BstUI, which were incubated at 60 °C overnight.

Digested PCR products were purified with the QIAquick Nucleotide Removal Kit (QIAGEN Inc., Mississauga, ON) following the manufacturer's protocol. Finally, fluorescently labeled terminal restriction fragments (T-RFs) were analyzed by comparison to an internal standard using a model ABI3730 automated DNA sequencer (Applied Biosystems, Foster City, CA) at the University of Calgary's University Core DNA Services, the Faculty of Medicine, University of Calgary, Calgary, AB, Canada. Thus, four separate TRF profiles for air-treated samples and another four T-RF profiles for H_2-treated soil DNA samples were generated.

2.2.4 Analysis of T-RF profiles and data Sets

TRF profiles were analyzed using GeneMarker V1.4 software (SoftGenetics LLC, USA). Three parameters were usually used for TRF profile comparison: fragment length in nucleotides, this being the apex position of each peak on a base pair scale relative to a DNA size ladder (GeneScan 500 LIZ Size Standard, Applied Biosystems, Foster City, CA), the peak height and the area under the peak in fluorescence units (FU).

Community T-RFLP profiles was characterized by both the number of peaks and the areas of the peaks. The area of any one peak is calculated by integrating the fluorescence units under that peak, and the total area for any profile is the amount of the areas of all peaks between 50 bp and 500 bp, for the purpose of avoiding detection of primers and/or uncut large fragments. The relative abundance of each T-RF is determined by calculating the percentage between the peak area of each peak and the total peak area of all peaks in one profile.

Moreover, T-RFLP profiles were analyzed by the presence or absence of one specific T-RF. T-RFs were considered identical if their fragment sizes varied in a range of ±1-2 bp among different gels and /or lanes of the same gel (Graff and Conrad, 2005).

2.3 Identification of Bacteria Contributing to Significantly Increased TRF peaks

2.3.1 Construction of 16S rRNA gene libraries

Cloning of the target genes was completed with the CloneJET™ PCR Cloning system (Fermentas Inc. Burlington, ON, CA) in accordance with the manufacturer's instructions (see below). A total of 192 clones of 16SrRNA genes

from H2-treated soil sample were analyzed in this study.

2.3.1.1 PCR amplification of 16S rRNA gene fragments

The DNA templates used for construction of clone libraries were the same DNA preparations from which the 16S rRNA genes for TRF analysis were amplified. Three independent PCRs were performed for each soil sample in a total volume of 50 μL by using the unlabeled primer non-fluorescent labeled BSF8/20 (5'-AGAGTTTGATCCTGGCTCAG-3') and BSR534/18 (5'-ATTACCGCGGCTGCTGGC-3') and the conditions described for PCR were the same as those given above. 16S rRNA gene PCR products were pooled together and put through gel electrophoresis. The sharp clear bands at approximately 500 bp were cut and purified by gel extraction using a DNA gel extraction kit (#0513 DNA Gel Extraction Kit (Fermentas Inc. Burlington, ON, CA).

2.3.1.2 Ligation

16S rRNA gene amplicons were ligated into the pJET^{TM}/blunt cloning vector (Fermentas Inc. Burlington, ON, CA) via the following protocol: Firstly, the blunting reaction was set up within an 18-μl reaction system in a 0.5mL PCR tube. The 18-μL reaction system contained the following reagents: 10 μL 2Xreaction buffer; 1-2 μL of purified PCR product; up to 17 μL of nuclease-free water and 1 μL of DNA blunting enzyme. The tube was vortexed briefly and centrifuged at 14,000 rpm at room temperature for 3-5 s. Secondly, the mixture was incubated at 70 °C for 5 min, and then it was chilled briefly on ice. Thirdly, the following reagents were added to the blunting reaction mixture to set up a 20-μL ligation reaction system: 1 μL of pJET^{TM}1.2/blunt cloning vector (50ng/ μL) and 1 μL of T4 DNA ligase (5U/ μL).

The mixture was vortex briefly and centrifuged at 14,000 rpm at room temperature for 3-5 s. Finally, the ligation mixture was incubated at room temperature (22 °C) for 30 min.

2.3.1.3 Transformation

The ligation products were transformed into Escherichia coli DH 5α™ cells according to the following procedure: To begin with, a tube of DH 5α™ cells was thawed on ice. A 1.5-mL microcentrifuge tubes was set on wet ice, the half frozen cells were gently mixed with a pipette tip, and aliquot of 50 µL cells was transferred to the 1.5-mL tube. Unused cells were refreezed in dry ice/ethanol ice for 5 min before storage at -80 °C freezer. 5 µL DNA ligation mixture (DNA concentration=10ng/µL) was added to the cells and mixed gently. The tube was then incubated on ice for 30 min before the cells were heat shocked for 20 seconds in a 42 °C water bath without shaking. The tube was then set on ice for 2 min before 950µL of SOC medium which was pre-warmed at 37 °C for 30 min was added. The tube was then incubated in a 37 °C water bath for 1h at 225 rpm. 20 to 200µL of ligated Escherichia coli DH 5α™ cells were plated out on the pre-warmed LB-ampicilin (100 µg/ml) plates, which were then incubated overnight at 37 °C after. The remaining transformation reaction was stored at +4 °C for future use.

2.3.2 Screening for inserts of expected size (500bp): colony PCR

Colonies grown on LB-ampicillin plates were randomly selected for transfer onto fresh plates that were labeled in divisions designated as A1-A12; B1-B12…H1-H12, etc., the usual design for colony PCR plates. The PCR master mix was comprised of the following reagents : 16.2 µL of PCR water (molecular biology reagent, Sigma-Aldrich Canada Ltd, Oakville, ON); 2 µL of 10X ThermolPol

Reaction Buffer (New England Biolabs Ltd., Pickering, ON); 0.4 μL of 10mM dNTP (dATP, dTTP, dCTP, dGTP) (New England Biolabs Ltd., Pickering, ON), 0.6 μL each of 20 mM 6-FAM-5'-BSF8/20 and BSR534/18 bacterial primers(Applied Biosystems, Foster City, CA) and 0.2 μL of 5 U/μL Taq DNA polymerase (New England Biolabs Ltd., Pickering, ON).

Each colony was picked and dipped (with sterilized toothpicks) into the PCR master mix and simultaneously streaked onto a corresponding division on the appropriate new plates. PCR was carried out to amplify cloned inserts with the following conditions: three minutes Hot Start at 94 °C for initial denaturation, followed by 25 cycles of 30 seconds at 94 °C for denaturing, 45 seconds at 55 °C for annealing, 2 min at 72 °C for primer extension, and a final cycle of primer extension at 72 °C for 3 minutes.

Colony amplification results were verified by gel electrophoresis of aliquots of 5 μL PCR products mixed with 1μL of loading dye in 1.0% agarose in 0.5XTBE buffer. Colonies showing bands at 500 bp in the agarose gel were regarded as the ones possessing the expected 16S rDNA insert (i.e. positive clones) and were marked "+" on the corresponding plate division and picked for following plasmid DNA extraction.

2.3.3 Plasmid DNA extraction of positive clones

The colonies marked "+" were picked and inoculated in test tubes with 3mL of LB medium supplemented with ampicilin (50 mg/mL). They were incubated for 14 hours at 37 °C in a shaker at 200-250 rpm. The bacterial culture was harvested by centrifugation at 8000 rpm (6800 x g) in a microcentrifuge (conventional table top) for 2 min at room temperature.

All the remaining medium was removed before the pelleted cells were resuspended in 250 μl of the Resuspension Solution (GeneJET^{TM} Plasmid Miniprep

Kit, Fermentas Inc. Burlington, ON, CA). The bacteria were resuspended completely by vortexing or pipetting up and down until no cell clumps remained. The cell suspension was transferred to a microcentrifuge tube to which 250 µl of the Lysis Solution was added. The Cell suspension and lysis solution were mixed thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. The mixture could not be vortexed in case of chromosomal DNA shearing, nor could it be incubated for more than 5min in order to avoid denaturation of supercoiled plasmid DNA. The clear mixture was added to 350 µl of Neutralization Solution and mixed immediately and thoroughly by inverting the tube 4-6 times. The neutralized bacterial lysate was cloudy and viscous, following a centrifugation at 14,000 rpm at room temperature for 5 min to pellet cell debris and chromosomal DNA. The supernatant was transferred to the supplied GeneJET™ spin column by decanting and centrifuging for 1min. The flow-through was discarded and the column was placed back into the same collection tube. 500 µL of the Wash Solution (diluted with ethanol prior to first use) was added to the GeneJET™ spin column and centrifuged at 14,000 rpm at room temperature for 30-60 seconds and the flow-through was discarded. The column was placed back into the same collection tube and the wash procedure was repeated using 500 µL of the Wash Solution. The flow-through was discarded and the tube was centrifuged for an additional 60 seconds to remove residual Wash Solution, which is essential for avoiding residual ethanol in plasmid preps.

The GeneJET™ spin column was transferred into a fresh 1.5-mL microcentrifuge tube. 50 µL of the Elution Buffer was added to the center of the spin column membrane to elute the plasmid DNA. This was incubated for 2 min at room temperature to ensure the DNA was dissolved in the elution buffer and then centrifuged at 14,000 rpm at room temperature for 2 min. An additional elution step

with Elution Buffer was carried out to increase the yield of recovering residual DNA recovered from the membrane. The column was discarded and the purified plasmid DNA was stored at 4 °C before being shipped for DNA sequencing.

2.3.4 DNA sequencing

The insert DNA was sequenced by Marcrogen Inc. (Seoul, SK) with an automated ABI 3700 sequencer. The sequencing reactions were performed with the pJET^{TM}1.2 forward sequencing primer (5'-CGACTCACTATAGGGAGAGCGGC-3'); the sequences obtained were manually proofread and corrected, if necessary. Chimeric sequences (sequences without target genes) were removed from further analysis. The 16S rRNA gene sequences (most approximately 500 bp) were compared to the available sequences in NCBI (National Center for Biotechnology Information) database by using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.*, 1990) in order to ascertain their closest relatives.

The closest relatives were picked out according to the max identity. If the one which has the biggest max identity with our inquired sequence is unidentified clone, then the next biggest identity was picked out till the identified ones were found. The ones that were not identified till the max identity went under 50% were termed unknown sequences.

2.3.5 Phylogenetic assignment by matching computer simulated terminal restriction enzyme digestion with real digestion

Computer-simulated RFLPs (with the various terminal restriction enzyme combinations) were generated employing the region of the 16S rDNA contained between the PCR priming sites described by Moyer *et al* (1994). Again, the terminal restriction enzymes used in the current study were as follows: BstUI; HaeIII; HinfI;

and MspI (New England Biolabs Ltd., Pickering, ON). Restriction sites for each enzyme were given above.

The sequences that each terminal restriction enzyme interacted with were manually picked out using the 'locate' function of Microsoft Word and the fragment sizes of each clone were counted from the first nucleotide of forward primer BSF8/20 (5'-AGAGTTTGATCCTGGCTCAG-3') to the first restriction cutting sites(the closest ones from the forward primer BSF8/20) of each terminal restriction enzyme. Occasionally, it occurred that the entire 16S rDNA gene sequence did not show a single cutting site for a specific enzyme. In this case, the fragment sizes were regarded as 527 bp (the length of the entire target sequence).

In order to identify the bacteria contributing to the significantly increased TRFs in the community TRFLP profile, a graph of the Number of Clones versus Fragment Sizes for each of the four terminal restriction enzymes was generated with the corresponding community TRFLP profile of H_2-treated soil. The bacterial groups that corresponded to the predominant T-RFs were identified by associating the T-RF peaks in the T-RFLP profile of hydrogen treated soil sample to clones in the 16S rRNA gene library derived from the same sample, since the terminal-restriction fragment (T-RF) length can be calculated from a known sequence, as described above.

However, the problem is that the chances that sequence-determined T-RF exactly matches T-RF length in community T-RFLP profile are very small. For instances, in our current study, the smallest "TRF" shift observed is 0.1 bp and the biggest is 4.7 bp. In conjunction with what is discussed in Chapter 1, a matching window of -4 to +3 bp was adopted in the current study (the range equals to the observed T-RF length minus T-RFseq).

3. Results

3.1 Effects of Hydrogen Metabolism on Soil Microbial Community Structure

3.1.1 Standard Curve of Voltage *Versus* Hydrogen Concentration

Based on the original data and Equation 1 and 2, the concentrations of electrolytic hydrogen in a mixed gas stream (in ppm) were calculated. For each concentration of hydrogen, a relative voltage across the hydrogen sensor was detected by computer system (Table1). Using Matlab V7.1, the standard curve was generated by the voltages across the hydrogen sensor (v) plotted against hydrogen concentrations (ppm) (Figure 3). The standard curve was fitted as an exponential function and described by the following equation:

$$ppm[H_2] = 0.1527e(1.7158v) \quad \text{----------} \quad ③$$

$ppm[H_2]$ =concentration of hydrogen

v=voltage across the hydrogen sensor

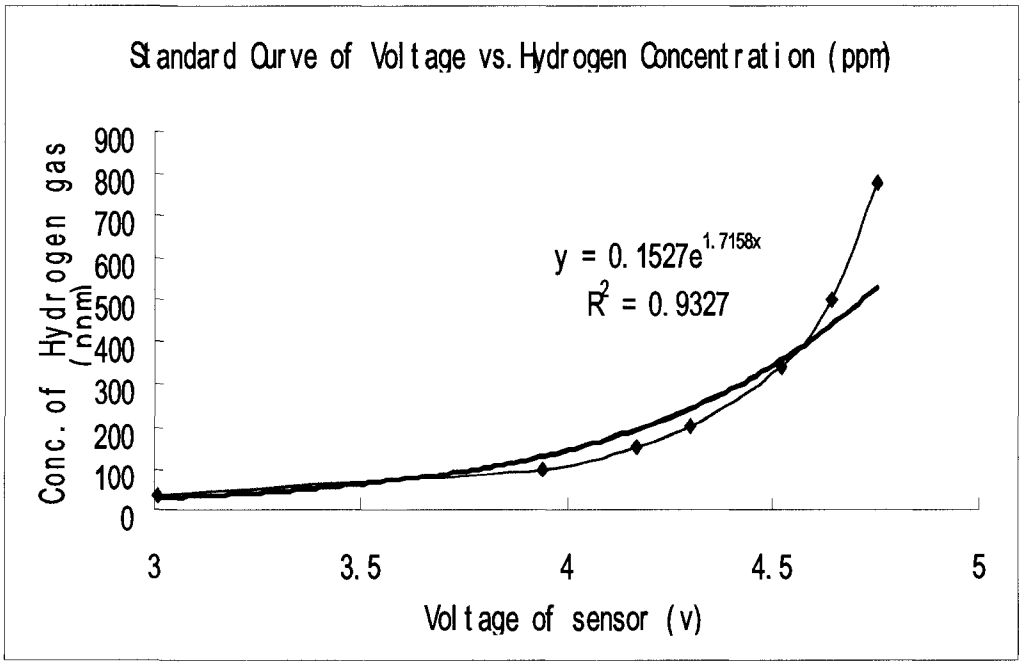
e=universal constant (2.718281828)

Table 1: Raw data used to generate the standard curve of voltage across the hydrogen sensor *versus* hydrogen concentration (in ppm)

The experiment was duplicated. The concentration of hydrogen in a mixed gas stream (ConH2) was calculated by using Equations 1 and 2. FR1= Flow Rate One; FR2=Flow Rate Two; V= voltage across hydrogen sensor.

Current (mA)	T(°C)	FR1 (ml/min)	FR2 (ml/min)	$ConH_2$(ppm)	V (v)
10.18	27	100	43	779.6	4.75
6.55	27	100	43	501.6	4.64
4.46	27	100	43	341.6	4.52
2.62	27	100	43	200.6	4.30
1.99	27	100	43	152.4	4.17
1.26	27	100	43	96.5	3.94
0.46	27	100	43	35.2	3.01

Figure 3: Standard curve of voltage across the hydrogen sensor *versus*. Hydrogen concentration (in ppm) [H_2] =0.1527e $^{(1.7158v)}$ (R^2=0.9327)



3.1.2 Hydrogen Uptake of air-treated and H_2-treated soil samples

As shown in the Table 2, the H_2-treated soil samples showed significantly higher H_2 uptake rates than those of air treated soil samples, indicating the H_2-treated soil samples can be used for the isolation of hydrogen oxidizing bacteria and for studying the effects of hydrogen metabolism on the soil bacterial community structure.

Table 2: Hydrogen uptake rates of air-treated and H_2-treated soil samples

The current was set at 1.05mA for both samples in this experiment. Flow Rate Two (FR2) was set at 41mL/min in this experiment. Voltage-in equals the voltage corresponding to the concentration of hydrogen without passing the soil column; Voltage-out equals the voltage corresponding to the concentration of hydrogen after passing the soil column. LH1~LH5 are hydrogen treated soils in 5 syringes and LA1~LA5 are air-treated soils in 5 syringes. (See Chapter 2)

Soil syringe	Room Air	Voltage-in (V) [for both samples]	Voltage-out (V) [hydrogen-treated]	Voltage-out (V) [air-treated]
LH1&LA1	0.82	3.56	1.22	3.3
LH2&LA2	0.6	3.52	1.47	3.47
LH3&LA3	0.72	3.56	1.96	3.18
LH4&LA4	0.76	3.39	2	3.14
LH5&LA5	0.61	3.45	2.19	3.42

3.2 Culture and Isolation of H_2-oxidizing Bacteria

3.2.1 Gram staining

Gram staining revealed YH3 and YH22 to be Gram positive bacteria while isolate YH26 is a Gram negative bacterial species.

3.2.2 The identification of H_2 -oxidizing bacterial strains using 16S rRNA gene sequence analysis

The DNA amplification of tested H_2-oxidizing strains (YH3, YH22 and YH26) using the primers BSF8/20 and BSR1541/20 showed clear distinct bands at approximately 1500 bp in a 0.7% agarose gel. These1500 bp bands were cut from the gel, purified and sent for sequencing. Obtained sequences of tested H_2-oxidizing bacterial strains were compared with similar sequences in the Genebank database using the BLAST program (NCBI). A BLAST search of GeneBank showed that YH3 is a *Micrococcus sp.* (EF540464); YH22 is a *Nocardioides sp.* (AB373748) and YH26 is a *Flavobacterium sp.* (AM922192). Sequence information is attached below.

3.3 TRF profiles of air-treated and H_2-treated soil samples

In this study, the changes in soil microbial community structures and diversity were monitored by T-RFLP analysis of PCR amplified 16S rRNA genes derived from four restriction enzymes (HaeIII, HinfI, MspI and BstUI).

3.3.1 Generation of TRF profiles

Both DNA extracted from both air-treated soil sample and H_2-treated soil samples showed sharp bands above 10 kbp and smeared DNA bands below 10 kbp in 0.8% agarose gels, which suggested that the size of DNA fragments in soil DNA extraction s were bigger than 10 kbp. Therefore, they qualified as templates for amplifying 16S rRNA genes.

It was found that most PCR products, versus the total DNA extracted from soil samples concentrated and formed a sharp bands at approximately 500 bp in 1.2% agarose gels, and the remainder appeared up as some smeared bands located between 500 bp and 700 bp in the same concentration gels. Therefore, most PCR products were considered to be copies of 16S rRNA genes because they shared the same bp size as the anticipated PCR products of the target 16S rRNA genes.

The sharp bands at approximately 500 bp contributed by PCR products was weaker or disappeared entirely in 2% agarose gels after PCR products were digested with the different REs (BstUI, HaeIII, HinfI, or MspI) at their optimal incubation temperatures for 8 to 10 hours. Furthermore, the digested PCR products contributed several weak bands below 500bp in 2% agarose gels. This indicated that PCR products were potentially completely digested by the REs and these digested PCR products were considered to be of sufficient quality to generate TRF profiles.

3.3.2 Comparison of TRF profiles between air-treated and H_2-treated soil samples

In order to evaluate the soil microbial community structure in both air-treated and hydrogen-treated soil samples, and to compare changes of community diversity upon hydrogen treatment, T-RFLP community profiles of their bacterial 16S rRNA gene fragments were generated and analyzed. In order to compare these two T-RFLP profiles, fragment size (T-RF length) and peak intensity (the percentage of individual peak area in total peak area) were used as the two parameters functioning as the axes. Peak intensity was calculated by comparing the individual peak area of T-RFs to the sum of all peak areas in the electropherograms. However in the current study, only the peaks whose intensity increased after hydrogen treatment were of interest and analyzed.

Figure 4-7 demonstrates the typical extent of differences we observed in community T-RFLP patterns of both air-treated and hydrogen-treated soil samples after digestion by the four restriction enzymes. Regardless of the enzyme used to create the TRF pattern, visual inspection of these four figures shows an apparent increase in the number of peaks and the total peak area in hydrogen-treated soil T-RFLP pattern. The hydrogen-treated T-RFLP profiles yielded 43, 42, 17, and 17 peaks (unique T-RFs) after digestion with HaeIII, HinfI, MspI and BstUI respectively, while only 33, 31, 14 and 16 peaks were detected in the corresponding air-treated T-RFLP profiles.

To study the hydrogen-induced variation of soil bacterial community structure, the focus was kept on the peaks whose intensity (peak area divided by the sum of all peak areas in a pattern) increased more than 1%, which was defined as a large increase for the purpose of this study (Table3-6). Peaks with fragment sizes varying within a range of ±1bp were considered as the same peak in two corresponding T-RFLP profiles due to the possible biases derived from the two different gels (Graff and Conrad, 2005). Here it is important to realize that the increase of peak height or even peak area does not necessarily equate with an increase of peak intensity, due to the increase of the total peak area. The variations of peak intensity are shown in column D of Table 3-6. "D" is defined as the difference between a percentage of a specific peak in a hydrogen-treated T-RFLP profile and that of the corresponding peak in an air treated T-RFLP profile, calculated as H2% minus A%.

The T-RFs of 206.1 bp and 217.2 bp (HaeIII); T-RFs of 311.1 bp, 320.6 bp, 326.1 bp and 497.9 bp (HinfI); T-RFs of 138.6 bp, 451.6 bp and 482.4 bp (MspI) and T-RFs of 220.9 bp and 482.4 bp (BstUI) were exclusively observed in hydrogen

treated T-RFLP profiles (Figure 4-7; Table 3-6). Table 3 shows that in HaeIII profiles, a peak at 63 bp increased by 28%, the largest difference observed, with a relative increase of 560% (relative increase is calculated by dividing D over Air% and is defined as the difference compared to the percentage of the same peak in hydrogen treated T-RFLP profiles); this indicated a tremendous increase in the number of bacteria whose first HaeIII restriction site is at a distance of 63 bp from the fluorescently labeled forward end (Figure 4 and Table 3).

In HinfI profiles, there are significantly increased peaks: a peak at 320.6 bp increased by 8.5%, with an infinity relative increase; a peak at 321.9 bp increased by 16.5%, with a relative increase of 367% and a peak at 323.9 bp increased by 12%, with a relative increase of 300%.

The most marked change was observed in MspI profiles, where T-RFs at 141 bp showed increases of 18%, with a relative increase of 1095%.

Table 6 demonstrates that in BstUI-generated T-RFLP profiles, a peak at 482.4 bp increased by 13.7%, with an infinity relative increase; a peak at 106.8 bp increased by 12.4%, with a relative increase of 620% and a peak at 224.3 bp increased by 8%, with a relative increase of 368%. These peaks were the major focus of investigation and corresponding bacterial species were identified as part of the present study.

An infinity relative increase indicates there is no such peak in air-treated soil T-RFLP profiles and the T-RFs are derived from novel bacterial groups being stimulated after hydrogen treatment.

In general, variations in T-RFLP profiles used to determine hydrogen effects on the soil microbial community were due to either differences in relative abundance, the presence and absence of individual T-RFs or both.

Figure 4-7 Electropherograms of HaeIII (Figure 1), HinfI (Figure 2), MspI (Figure 3) and BstUI (Figure 4)-derived TRF Profiles of bacterial communities from hydrogen treated and air treated soil samples. The top figure is generated from air treated soil samples and the bottom figure is generated from hydrogen treated soil. TRF sizes (rmu: relative migration units) are on the X axis; The Y axis shows the peak intensity in fluorescent units. Scales for the Y axes have been adjusted individually for ease of comparison.

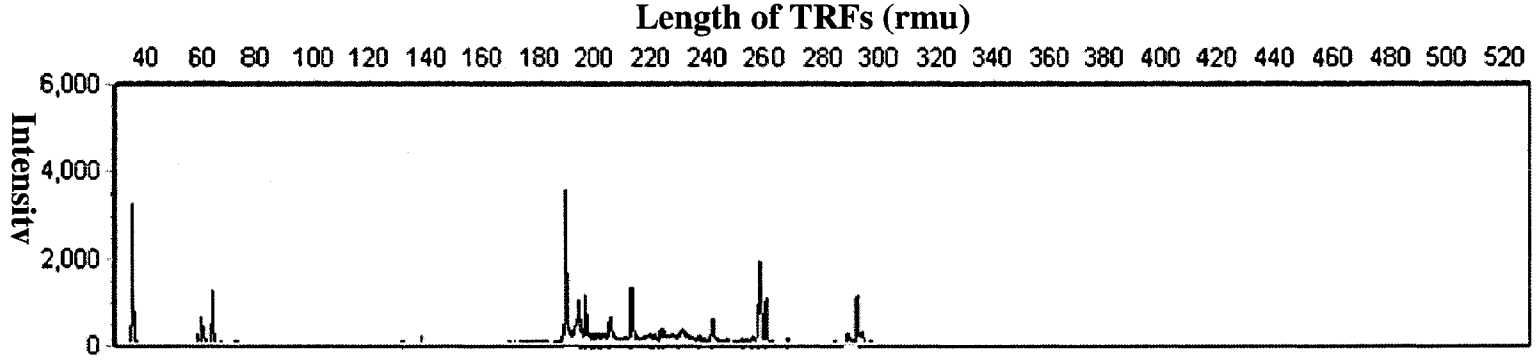
Table 3-6 Intensity variation of major increased TRF peaks in hydrogen-treated soil samples compared to air-treated soil samples.

Table 3-6: HaeIII-, HinfI-, MspI- and BstUI-generated data sets. D is the difference in intensity (percentage of total area) of TRF peaks between hydrogen- and air-treated soil samples. D/Air is relative intensity increase.

Figure 4 Electropherograms of HaeIII-derived TRF Profiles of bacterial communities from hydrogen-treated soil samples and air treated soil samples.

Sample 1: AHa.fsa

Dye: Blue - 33 peaks



Sample 2: HHa.fsa

Dye: Blue - 40 peaks

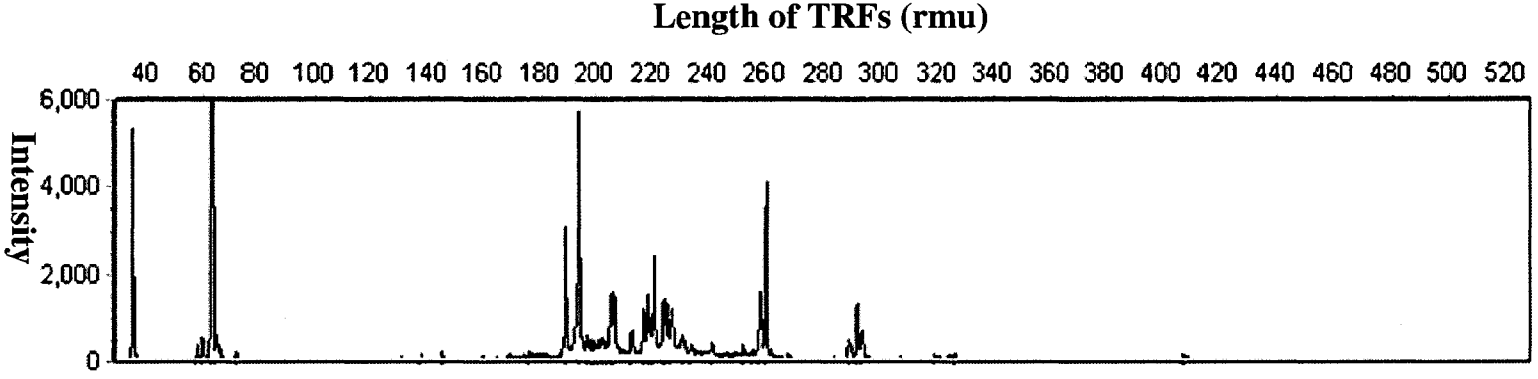


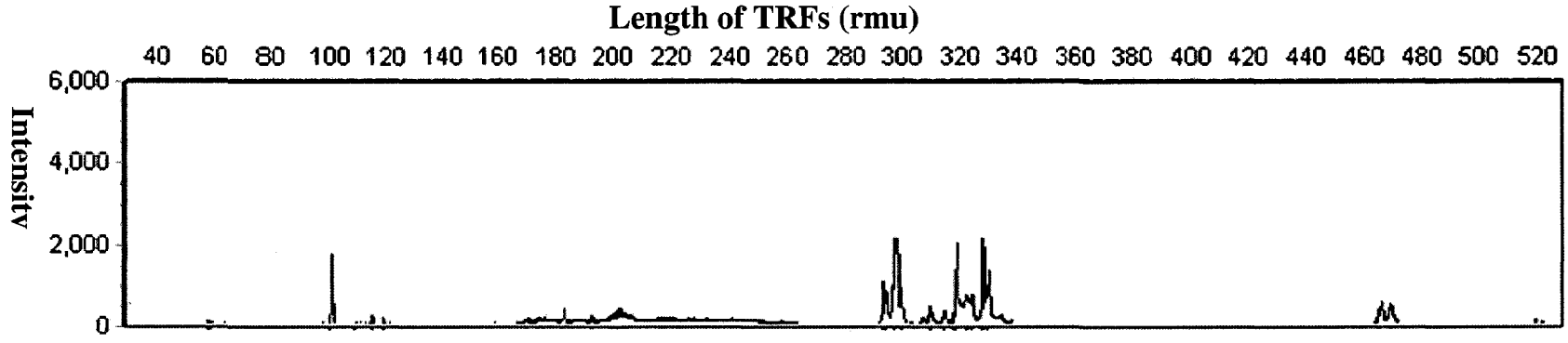
Table 3: Intensity variation of major increased TRF peaks in hydrogen-treated soil samples compared to air-treated soil samples in HaeIII-generated T-RLFP profiles

Fragment Size (±1 rmu)	Air (Percentage of the total area)	H_2 (Percentage of the total area)	D (H_2%-A %)	D/Air%
63	5	33	28	560
193.4	4	9	5	125
206.1	0	3	3	N/A
217.2	0	2.4	2.4	N/A
218.6	1.6	3	1.4	87.5
220.7	1.2	4	2.8	233
226.9	1.5	2.6	1.1	73.3
260.5	4	6	2	50

Figure 5 Electropherograms of HinfI-derived TRF Profiles of bacterial communities from hydrogen-treated soil samples and air treated soil samples.

Sample 1: AHi.fsa

Dye: Blue - 31 peaks



Sample 2: HHi.fsa

Dye: Blue - 41 peaks

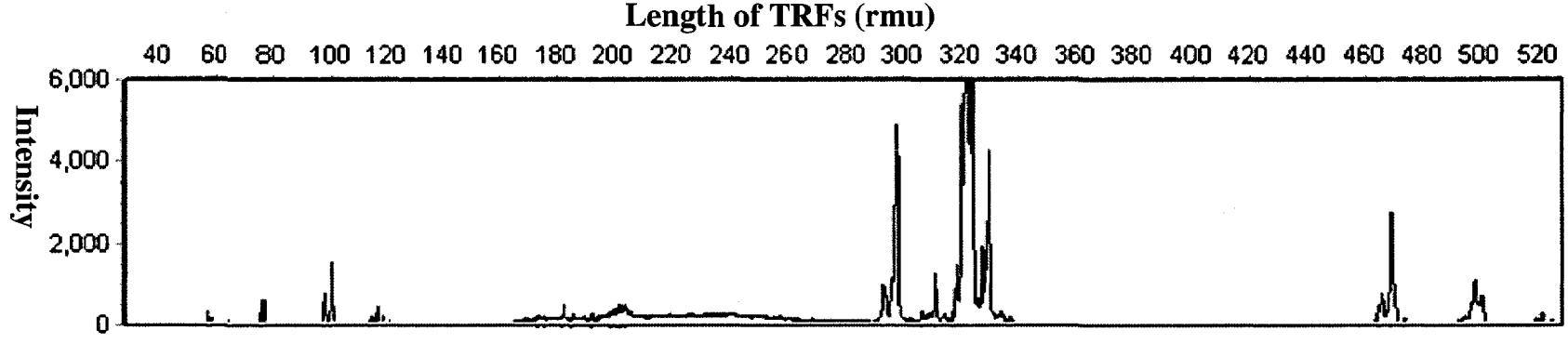
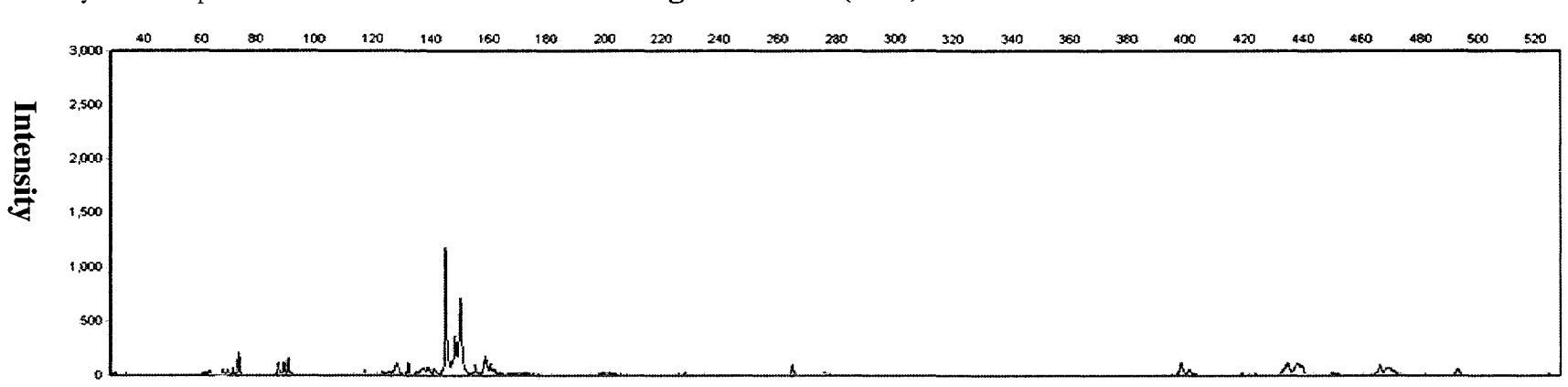


Table 4: Intensity variation of major increased TRF peaks in hydrogen-treated soil samples compared to air-treated soil samples in HinfI-generated T-RLFP profiles

Fragment Size (±1 rmu)	Air (Percentage of the total area)	H_2 (Percentage of the total area)	D (H_2%-A %)	D/Air%
311	0	1.9	1.9	N/A
320.6	0	8.5	8.5	N/A
321.9	4.5	21	16.5	367
323.9	4	16	12	300
326	0	1.2	1.2	N/A
497.9	0	2.66	2.66	N/A

Figure 6: Electropherograms of MspI-derived TRF Profiles of bacterial communities from hydrogen-treated soil samples and air-treated soil samples

Sample 1: AM.fsa
Dye: Blue-14 peaks



Sample 2: HM. fsa
Dye: Blue-17 peaks

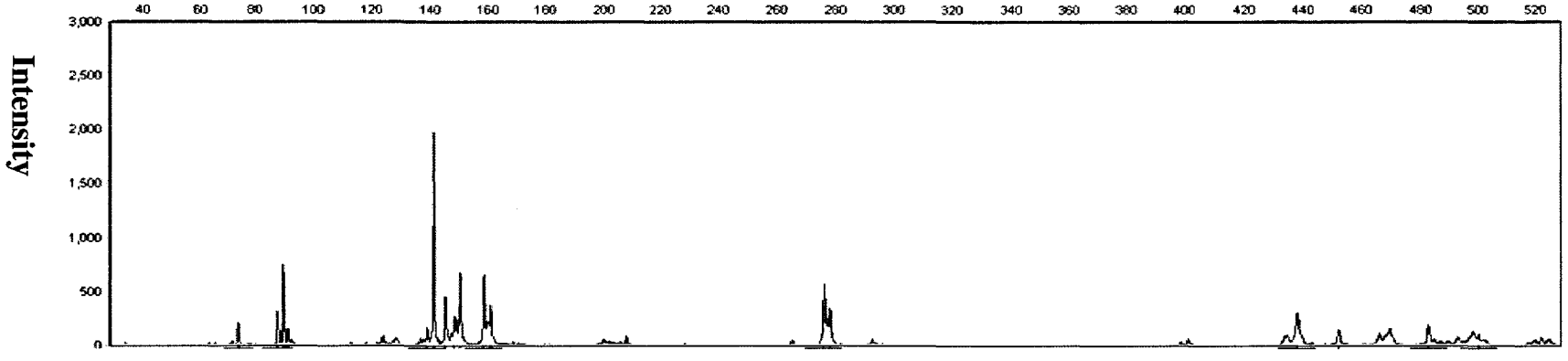
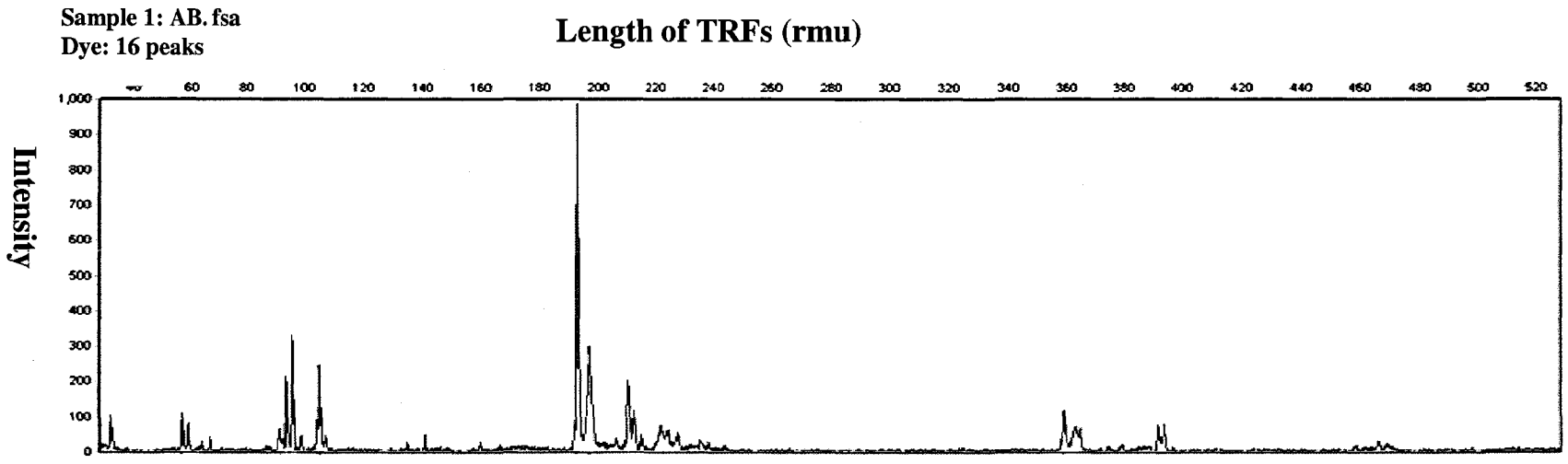


Table 5: Intensity variation of major increased TRF peaks in hydrogen-treated soil samples compared to air-treated soil samples in MspI-generated T-RLFP profiles

Fragment Size (±1 rmu)	Air (Percentage of the total area)	H_2 (Percentage of the total area)	D (H_2%-A %)	D/Air%
89.5	2.3	6.6	4.3	187
138.6	0	2	2	N/A
141	1.7	19.7	18	1059
158.1	4.7	8.2	3.5	75
160.5	2.6	4.3	1.7	65
275.8	1	6.6	5.6	560
277.6	0.5	5.4	4.9	980
451.6	0	2.5	2.5	N/A
482.4	0	4	4	N/A

Figure 7: Electropherograms of BstUI-derived TRF Profiles of bacterial communities from hydrogen-treated soil samples and air-treated soil samples

Sample 1: AB.fsa
Dye: 16 peaks



Sample 2: HB.fsa
Dye: 17 peaks

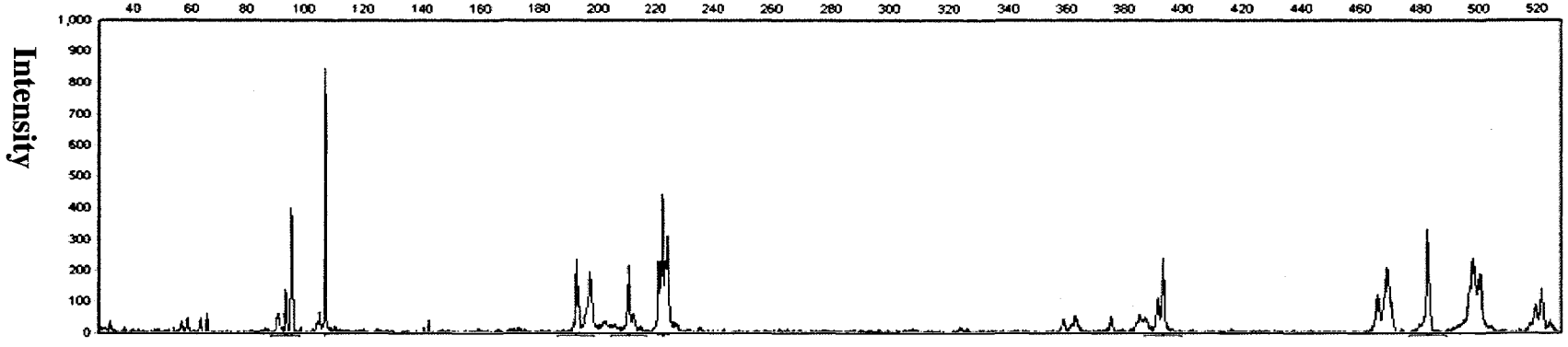


Table 6: Intensity variation of major increased TRF peaks in hydrogen-treated soil samples compared to air-treated soil sample in BstUI-generated T-RLFP profiles

Fragment Size (±1 rmu)	Air (Percentage of the total area)	H_2 (Percentage of the total area)	D (H_2%-A %)	D/Air%
106.8	2	14.4	12.4	620
220.9	0	6	6	N/A
224.3	2.2	10.3	8.1	368
393.3	3.7	8.2	4.5	122
482.4	0	13.7	13.7	N/A

3.416S rDNA library of H_2-treated soil samples

In order to determine what taxonomic group of these hydrogen-oxidizing bacteria was responsible for the major increases in hydrogen-treated soil samples over time, a clone library of bacterial 16S rRNA gene fragments was constructed. The DNA templates used for construction of this 16S rDNA library were the same DNA preparations from which the 16S rRNA genes for T-RFLP profiles hydrogen treated soil were amplified.

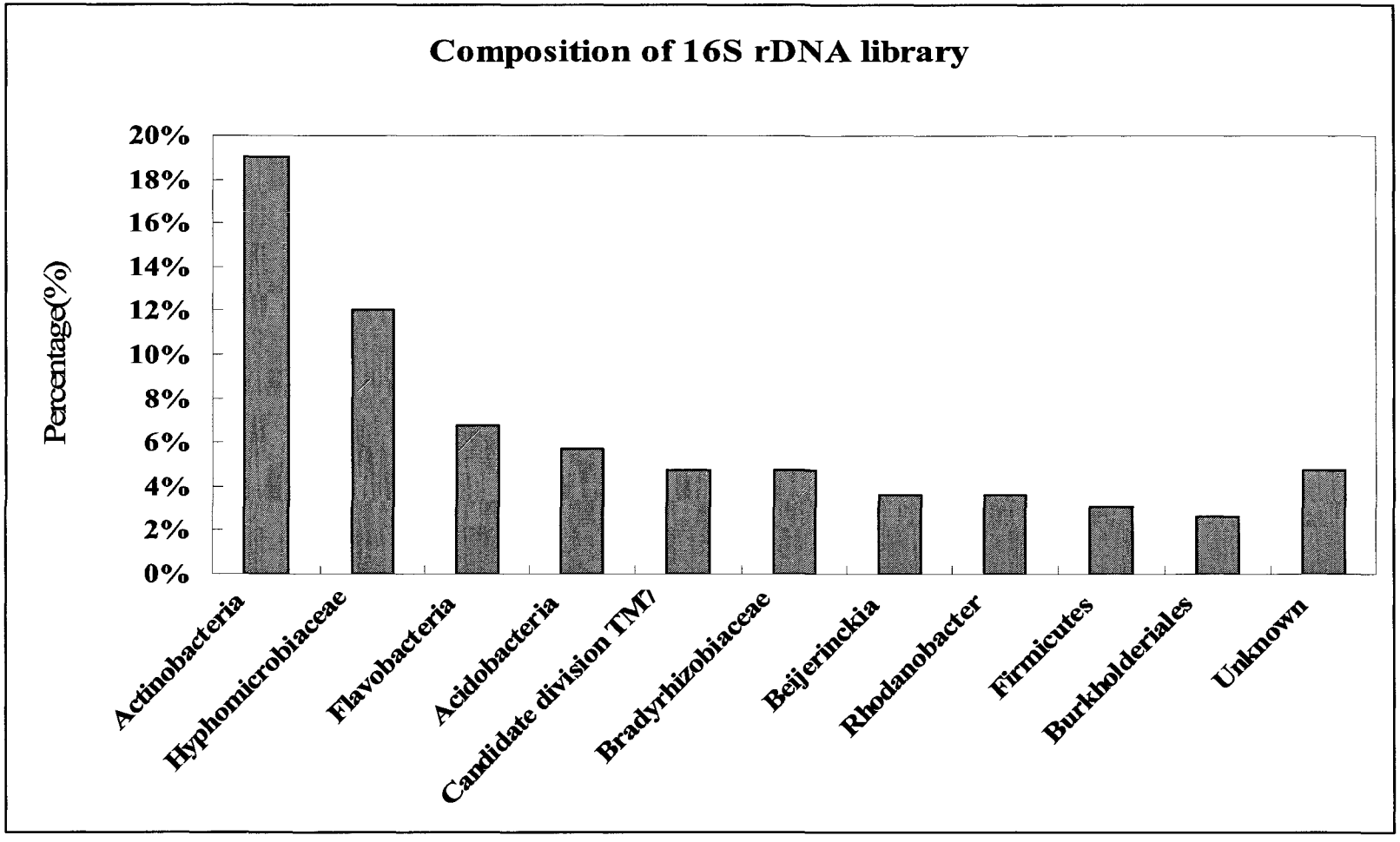
Originally, a total of 228 clones were generated. The 228 clones were randomly selected and sequenced: random selection ensures the library represents (or at least closely reflects) the relative composition of each predominant member within the entire microbial community. It also made possible the assignment of phylotypes to specific T-RFs in T-RFLP profiles.

In a total of 228 bacterial clones analyzed, 36 non-16S rRNA gene sequences were detected and excluded for further analysis. The majority of these sequences (95%) showed at least 90% similarity to the known sequences deposited in NCBI database after using BLAST, while the similarity of the remaining sequences ranges from 86% to 89%.

Among the 192 remaining clones, those affiliated with the *Actinobacteria* division were the most abundant, comprising about 19% of the total, followed by clones grouping within the Hyphomicrobiaceae family (12%). Clones representing members of the Flavobacteria division were the third most abundant, at approximately about 6.8%. The remainder were related to members of the Acidobacteria (5.7%); Bradyrhizobiaceae (4.7%); Candidate division TM7 (4.7%) Beijerinckia (3.6%); Rhodanobacter (3.6%); Firmicutes (3%) and Burkholderiales (2.6%). Among these, the Bradyrhizobiaceae; Candidate division TM7; Beijerinckia and Rhodanobacter

belong to the Proteobacteria. There were nine clones that could not be classified into any known bacterial division (Figure 8).

Figure 8: The phylogenetic distribution of the 16S rDNA library derived from H_2-treated soil. The percentage of clones is presented. The phylogenetic divisions fall within different taxonomic classifications due to variations in identification level of BLAST.


Composition of 16S rDNA library
Percentage(%)
20%
18%
16%
14%
12%
10%
8%
6%
4%
2%
0%
Actinobacteria
Hyphomicrobiaceae
Flavobacteria
Acidobacteria
Candidate division TM7
Bradyrhizobiaceae
Beijerinckia
Rhodanobacter
Firmicutes
Burkholderiales
Unknown

3.4.1 Standard curve of Number of Clones *versus* Sample Number

In order to check whether the number of clones we sequenced in the present study was sufficient to represent the H_2-treated soil microbial community, a standard curve of Number of Clones versus Sample Number was generated by plotting the number of different clones against sample number (from 1 to 192). Each standard curve of Number of Clones *versus* Sample Number corresponds to a terminal restriction enzyme.

3.4.2 Graphs of Number of Clones *versus* Sequence-determined T-RF length

In order to identify the bacteria responsible for the significantly increased T-RFs in the community T-RFLP profile of H_2-treated soil, graphs of the Number of Clones versus Sequence-determined T-RF length were generated by plotting the number of clones against computer-simulated restriction digestion-derived fragment sizes (see below). Again, each graph of Number of Clones *versus* Sequence-determined T-RF length corresponds to a terminal restriction enzyme.

3.5 Computer-simulated T-RFLP analysis of 16S rRNA Gene clones---Identification of Bacterial Groups Responsible for Significantly Increased T-RFs

To identify the major increased T-RFs after hydrogen treatment, a 16S rRNA gene clone library of 192 clones was constructed from the same DNA templates used in the T-RFLP analysis of hydrogen-treated soils, followed by T-RFLP screening of these clones.

T-RFLP analysis (or RFLP analysis) of 16 rRNA gene clones provides the

most detailed, reliable information about the composition of microbial communities (Dunbar *et al.*, 2000). The increase in intensity of TRF peaks suggests that hydrogen metabolism may have induced the development of distinct microbial populations in the soil, thus contributing to these markedly increased TRF peaks. Most of these bacteria were dormant in the soil samples until they were exposed to hydrogen treatment of a certain concentration.

In a computer simulation, 16S rRNA gene sequences from 192 hydrogen treated soil clones were cleaved with any of the four restriction enzymes used in this study. The length of these theoretical T-RFs was calculated as the distance from the fluorescently labeled end (the first base of the forward primer) to the first endonuclease cutting site. The bacterial groups that corresponded to the predominant T-RFs were identified by associating the T-RF peaks in the T-RFLP profile of hydrogen treated soil sample to clones in the 16S rRNA gene library derived from the same sample using the matching window of -4~+3 explained in Chapter 1 and 2, since the terminal-restriction fragment (T-RF) length can always be calculated from a known sequence.

In HaeIII-derived T-RFLP profiles (Figure 4; Figure 17), the T-RF of 63bp increased significantly (from 5% to 33%, a relative increase of 560% and became dominant in the T-RFLP profile from the hydrogen-treated soil (Table 3; Table 7). According to what is discussed in Chapter 1 and 2 and our data, the peak at 63bp in T-RFLP profile could correspond to the 67bp peak in HaeIII generated graph of Number of Clones *versus* Sequence-determined T-RF length, shifted by 4bp (Table 7).16S rDNA clone library indicates this peak represents the *Mycobacterium sp.* (7); the *Nocardioides sp.* (1); the *Kribella sp.* (1); the Pseudonocardiaceae (3); the Actinobacteria (1) and one unidentified clone. Most of the environmental sequences

were closely affiliated with *Mycobacterium sp.* within the Mycobacteriaceae family.

In HinfI digested T-RFLP profiles (Figure 5; Figure 18), for the T-RFs of 321.9 bp and 323.9 bp, both of them show an individual peak increase when compared to their counterparts in the air-treated T-RFLP profile, with the peak at 321.9 bp increasing by 16.5% and the peak at 323.9 bp increasing by 12%(Table 4; Table 8), both marked increased peaks. By the same token, the peak at 321.9 bp in T-RFLP profile corresponds to the 322 bp peak in HinfI generated graph of Number of Clones versus Sequence-determined T-RF length, shifted by only 0.1bp; the peak at 323.9 bp corresponds to the 324 bp peak in HinfI generated graph of Number of Clones *versus* Sequence-determined T-RF length, also shifted by only 0.1 bp (Table 8) In this case, the clones whose T-RFseq determined by HinfI is 322 bp representing the peak at 321.9 bp in T-RFLP profile were related to members of the *Mycobacterium sp.* (6); the *Streptomyces sp.* (1) and the *Catellatospora* (1). And clones whose T-RFseq determined by HinfI is 324 bp representing the peak at 323.9 bp were affiliated with members of the *Nocardioides sp.* (4); the *Mycobacterium sp.* (2); the *Actinoplanes sp.* (1); the Pseudonocardiaceae (2); the Actinobacteria (1); the Oxalobacteracea (1).

Clones contributed by the Actinobacteria were strongly associated with *Mycobacterium sp.*, *Streptomyce sp.*, *Pseudonocardia sp.*, *Catellatospora sp.*, *Nocardioides sp.*, *Hongia sp.* or *Kribella sp.* or Micromonosporaceae (family). A sizable number of clones in the library are associated with two or more phylotypes based on their sequence information according to the NCBI database using BLAST. For example, clone number 139 has 100% similarity with *Acidobacteria* (DQ450718.1) and 99% similarity with *Actinobacteria* (EF220638.1); clone number 189 is 95% identical with *Nocardia* (AB108773); *Pseudonocardia* (DQ448726) and *Mycobacterium* (DQ973806.1).

Surprisingly, investigations of MspI generated T-RFLP profiles and data sets (Figure 6; Figure 19; Table 5; Table 9) found T-RFs of 141 bp show the largest increases (18%) but show affiliation with only 9 clones (T-RFseq=143 bp, shifted by 2 bp), while a proximate peak (at 144.9bp) was assigned with 18 clones(T-RFseq=148 bp, shifted by 3.1 bp). Among the 9 clones assigned to the T-RFs of 141 bp, 8 of them were affiliated with Candidate Division TM7 and only 1 was related to the Gemmatimonadetes. The 18 clones associated with the peak at 144.9 bp in T-RFLP profile were affiliated with the *Beijerinckia sp.* (7); the *Phaeospirillum sp.* (1); the Hyphomicrobiaceae (6); the Micrococcaceae (3) and the Acidobacteria (1). T-RFs of 89.5bp, with an increase of 4.3%, correspond to peak at 93 bp in MspI generated graph of Number of Clones *versus* Sequence-determined T-RF length (Table 9). This peak was assigned with 6 clones identified as *Flavobacteria* (Table 9).

The other significantly increased and interesting peaks at 158.1 bp, 160.5 bp, 275.8 bp and 277.6 bp in MspI generated T-RFLP profile correspond to the peaks at 159 bp; 161 bp, 275 bp and 277 bp respectively (shifted in a range of -1~+1 bp). All these peaks were assigned with clones identified as the *Actinobacteria* without a single exception (Table 9): clones whose first computer-simulated MspI cutting site at a distance of 159 bp were affiliated with the *Mycobacterium sp.* (5); the *Streptomyces sp.* (1) and the *Catellatospora* (1); clones whose first computer-simulated MspI cutting site at 161bp were associated with the *Nocardioides sp.* (2); the *Psedonocardia sp.* (2); the *Hongia sp.* (1); the *Kribbella sp.* (1); the *Parvularcula sp.* (1) and one unidentified clone; both peaks at 275 bp and 277 bp in the MspI generated graph of Number of Clones *versus* Sequence-determined T-RF length were assigned with 3 clones identified as *Mycobacterium sp.* each (Table 9).

In Figure 7; 20 and table 6, the BstUI generated T-RFLP data sets, T-RFs

of 106.8 bp are the most abundant peak in hydrogen treated T-RFLP profiles, increasing from 2% in air-treated T-RFLP profiles to 14.4%. Computer-simulation T-RFLP analysis of the 16S rDNA libraries with BstUI showed T-RFs of 106.8 bp corresponds to clones whose first BstUI cutting site at a distance of 109 bp from the first base of the fluorescently labeled forward primer, representing the Proteobacteria and most of the clones were closely related to the *Candidate Division TM7* (42%)and the *Hyphomicrobiaceae* (37%). At the same time, T-RFs of 220.9 bp and 224.3 bp increased 6% and 8.1% respectively (Table 6; Table 10). As expected, clones falling into these two peaks with $T\text{-}RF_{seq}$ equaling to 221 bp and 223 bp are all related to members of the *Actinobacteria* except one clone assigned to the 224.3 bp peak is *Parvularcula sp.* The clones assigned to the T-RFs of 220.9 bp were affiliated with the *Mycobacterium sp.* (5); the *Streptomyces sp.* (1) and the *Catellatospora sp.* (1); the clones assigned to the T-RFs of 224.3 bp were related with the *Mycobacterium sp.* (2); the *Nocardioides sp.* (2); the *Pseudonocardia sp.* (2); the *Kribbella sp.* (1); the *Actinoplanes sp.* (1) and the *Parvularcula sp.* (1) (Table 10). Interestingly, the largest increased peak in this profile, involving T-RFs of 482.4 bp, was found in none of these clones. This may be due to pseudo-T-RF formation (see above)

Table 7-10: Corresponding highly abundant T-RFs in H_2-treated soil community T-RFLP profile to dominant phylogenetic groups represented by 16S rDNA clones derived from the same soil sample (HaeIII, HinfI, MspI and BstUI generated data sets). TRF lengths observed in profiles have been reported to differ from sequence-determined TRF lengths, depending on subtle differences in molecular weight, either from purine content or dye label (Kaplan and Kitts, 2003; see Chapter 1). Thus, to allow comparison of the TRF lengths of the clones with the TRFs observed in the community profiles, the true fragment lengths of the clones were determined by subjecting them to TRF analysis. (Data maked by yellow are associated with increased T-RFs)

Table 7 HaeIII-generated data sets.

16S rDNA gene library			Community T-RFLP profiles				Identification (Phylogenetic groups)
Fragment Size range (bp)	Number of Clones	Percentage (%)	Bpk (bp)	Air (%)	H_2 (%)	D (H_2%-Air%)	
39	33	17	34.3	6.2	7	0.8	Hyphomicrobiaceae; Bradyrhizobiaceae
67	14	7.3	63	5	33	28	*Mycobacterium sp.; Nocardioides sp.; Kribbella sp.;* Pseudonocardiaceae;
191	13	6.8	188.7	12.4	4.6	-7.8	Hyphomicrobiaceae; Micrococcaceae; *Beijerinckia sp.;*
195	6	3.1	193.4	4.3	9.3	5	*Bradyrhizobium sp.*; Hyphomicrobiaceae
207	7	3.6	206.1	0.3	3.3	3	Candidate division TM7
214	5	2.6	217.2	2.4	2.4	2.4	Acidobacteria
219	3	1.6	218.6	1.8	3.2	1.4	Actinobacteria
224	5	2.6	220.7	1.2	4	2.8	Actinobacteria
226	3	1.6	225.1	2.2	2.7	0.5	*Nocardioides sp.; Mesorhizobium*
229	3	1.6	226.9	1.5	2.6	1.1	Hyphomicrobiaceae; Rhodospirillaceae; Burkholderiaceae
258	11	5.7	258.4	7.6	3	-4.6	Flavobacteria; Sphingobacteriaceae; Burkholderiaceae
260	6	3.1	260.5	4	6	2	*Flavobacteria*

Table 8 HinfI-generated data sets

16S rDNA gene library			Community T-RFLP profiles				Identification (phylogenetic groups)
Fragment Size range(bp)	Number of Clones	Percentage (%)	Bpk (bp)	Air (%)	H_2 (%)	D (H_2%-Air%)	
300	21	10.9	297.8	16.5	10.5	-6	Hyphomicrobiaceae
312	13	6.8	311.1	0	1.9	1.9	Candidate division TM7; Acidobacteria
321	10	5.2	320.6	0	8.5	8.5	Hyphomicrobiaceae; Burkholderiaceae; *Beijerinckia sp.;*
322	11	5.7	321.9	4.5	21	16.5	*Mycobacterium sp.; Streptomyces sp.; Pseudonocardia sp.*
324	11	5.7	323.9	4	16	12	*Nocardioides sp.; Mycobacterium sp.; Actinoplanes sp.;*Pseudonocardiaceae
330	9	4.7	329.7	6.3	6.8	0.5	*Rhodanobacter sp.; Frateuria sp.; Dyella sp.;* Hyphomicrobiaceae
N/A	N/A	N/A	468.9	5.3	5.6	0.3	N/A
N/A	N/A	N/A	497.9	0	2.66	2.66	N/A

Table 9 MspI-generated data sets

16S rDNA gene library			Community T-RFLP profiles				Identification (phylogenetic groups)
Fragment Size range(bp)	Number of Clones	Percentage (%)	Bpk (bp)	Air (%)	H_2 (%)	D (H_2%-Air%)	
77	3	1.6	73.7	5.3	3	-2.3	Gemmatimonadetes
91	11	5.7	87.4	3.5	4.4	0.9	Flavobacteria; Sphingobacteriaceae; Burkholderiaceae
93	6	3.1	89.5	2.3	6.6	4.3	Flavobacteria
143	9	4.7	141	1.7	19.7	18	candidate division TM7
148	18	9.4	144.9	24.3	5.2	-19	*Beijerinckia sp.*; Micrococcaceae; Hyphomicrobiaceae;
150	6	3.1	148.1	12.5	4.6	-8	Acidobacteria; *Sphingomonas sp.*
152	17	8.9	150	27.5	7.7	-19.7	Hyphomicrobiaceae; *Bradyrhizobium*
159	7	3.65	158.1	4.7	8.2	3.5	*Mycobacterium sp.; Streptomyce sp.*
161	8	4.2	160.5	2.6	4.3	1.7	*Mycobacterium sp.; Pseudonocardia; Nocardioides sp.*
275	3	1.6	275.8	1	6.6	5.6	*Mycobacterium sp.*
277	3	1.6	277.6	0.5	5.4	4.9	*Mycobacterium sp.*
452	2	1	451.6	0	2.5	2.5	*Frateuria sp.*
485	1	0.5	482.4	0	3.98	3.98	*Sphingomonas sp.*; Burkholderiaceae

Table 10 BstUI-generated data sets

16S rDNA gene library			Community T-RFLP profiles				Identification (phylogenetic groups)
Fragment Size range(bp)	Number of Clones	Percentage (%)	Bpk (bp)	Air (%)	H_2 (%)	D (H_2%-Air%)	
92	3	1.6	91.1	2.9	2.7	-0.2	Actinobacteria
97	7	3.6	93.3	5.8	2.9	-2.9	Sphingomonadaceae
99	11	5.7	95.4	10	8	-2	*Bradyrhizobium sp.;* Rhodospirillaceae
109	19	9.9	106.8	2	14.4	12.4	*Hyphomicrobiaceae; Bradyrhizobiaceae; Candidate division TM7;*
195	17	8.9	192.5	25.6	5.2	-20.4	*Beijerinckia sp.;* Hyphomicrobiaceae; Micrococcaceae
199	6	3.1	197.1	22	8.5	-13.5	Hyphomicrobiaceae
211	10	5.2	210.4	10.8	5.8	-5	*Rhodanobacter sp.; Frateuria sp.;* Acidobacteriaceae
221	7	3.6	220.9	0	6	6	*Mycobacterium sp.; Streptomyces sp.; Catellatospora*
223	9	4.7	224.3	1.9	10	8.1	*Mycobacterium sp.; Nocardioides sp.; Kribbella sp; Actinoplanes sp.;* Pseudonocardiaceae;
396	6	3.1	393.3	3.7	8.2	4.5	*Flavobacteria*
N/A	N/A	N/A	482.4	0	13.7	13.7	N/A

4. Discussion

4.1 Hydrogen Induced Variation of Bacterial Community Structure Detected by TRFLP Method

The increased number of peaks and peak area observed in hydrogen treated soil T-RFLP profiles compared with those in air treated T-RLFP soil profile (Figure 4-7) should correspond to hydrogen treatment as the effect of hydrogen on soil bacterial community structure was the only parameter tested in this experiment. Although not a final proof, this close correlation provides strong evidence that hydrogen was the factor responsible for the observed changes in bacterial community structure.

4.1.1 The functions of hydrogen

Hydrogen is an energy source and reductant of critical importance for many bacteria species, archaea and anoxygenic phototrophs (Schwartz and Friedrich, 2006). Hydrogen-oxidizing bacteria are able to use hydrogen as their sole energy source for chemoautotrophic growth via a hydrogenase enzyme and in conjunction with the electron transport system (Trevors, 1985). Both biogenic and abiogenic H_2 production can support growth of H2-utilizing prokaryotes (Schwartz and Friedrich, 2006). Hydrogen evolved from HUP- nodules as a byproduct of nitrogen fixation in legume plants may benefit low-fertility soil as a form of additional energy and nutrient input in the legume-soil system (LaFavre and Focht, 1983). Hydrogen, as an electron donor, plays an important intermediary role in the decomposition of soil organic matter, yet

variations in composition of organic matter in soil can have a profound influence on bacterial diversity and species composition (Lynch, 1990; Blackwood *et al.*, 2003). This may help explain why the T-RFLP pattern differs between air-treated and hydrogen-treated soil samples.

4.1.2 Bacterial population changes observed after hydrogen treatment

According to what is discussed above, it is reasonable to suggest that hydrogen treatment of soil may induce bacterial population changes by stimulating the growth of new bacterial species and inhibiting the growth of others. This is supported by LaFavre and Focht's (1983) observation that the increase in the number of hydrogen-oxidizing bacteria is coupled with a similar increase in hydrogen concentration in close proximity to H_2 evolving nodules (LaFavre and Focht, 1983). An enhanced hydrogen oxidizing activity was also observed in soils adjacent to soybean root nodules showing H_2-evolving ability (Popelier *et al.*, 1985). Up to 12 times more hydrogen-oxidizing *Acinetobacter spp.* were found on the surface of soybean nodules inoculated with HUP- mutants of *Bradyrhizobium japonicum*, compared with those found in wild-type HUP^+ *B. japonicum* inoculated soybean nodules (Wong *et al.*, 1986). Observed population increases in insects and springtails (O.Collembola) in hydrogen treated soil are an indicator of enriched soil bacterial populations due to hydrogen treatment (Dong and Layzell, 2002). Cunningham and colleagues concluded that H_2 evolution from alfalfa root nodules can exert a significant impact on the surrounding microenvironment after

increases in abundance and diversity of hydrogen-metabolizing bacteria were observed compared to that found in three other soil environments where little H_2 is generated (Cunningham *et al.*, 1986). Combining the above with the results of our study, it is reasonable to suggest that hydrogen treatment of soil increases the number and diversity of bacteria inhabiting in the soil.

4.1.3 T-RFLP profiling---a molecular tool to assess soil microbial community variations

It is quite likely the stimulated soil bacteria responsible for the major intensity increases in T-RFs is a group of hydrogen oxidizing bacteria; the T-RFLP fingerprinting technique would seem to be suitable for monitoring effects on soil microbial communities and may even be capable of quantifying a microbial community's species diversity. That T-RFLP profiling is able to rapidly differentiate four microbial communities without the need to generate and analyze 16 S rDNA clone libraries has been demonstrated (Dunbar *et al.*, 2000). A number of investigators have confirmed that T-RFLP analysis is a promising and high-throughput technique for the assessment of variations in soil microbial communities, providing results generally consistent with T-RFLP analysis of individual clones in the 16S rRNA gene libraries (Hackl *et al.*, 2004; Graff and Conrad, 2005; Hartmann and Widmer, 2006). The relative abundance of T-RFs in T-RFLP profiles quantitatively reflected precise template ratios in an artificial community of four pure methanogen cultures when targeting archaeal SSU rRNA

(Lueders and Friedrich, 2003). When it comes to complex microbial communities like those found in soil, whether T-RFLP community profiling is able to reflect real community composition or not requires further verification (see below). The next question raised then is to discover what phylogenetic group these hydrogen-oxidizing bacteria belong to.

4.2 Actinobacteria---an Important Member of Soil Bacterial Community after Hydrogen Treatment

4.2.1 Reasons for the inability of library to reflect complete picture of microbial diversity

The bacterial diversity reflected in our 16S rRNA gene library derived from hydrogen-treated soil samples is predictably less when compared with large-scale soil community diversity, which is believed to be composed of thousands of different species (Roselló-Mora and Amann, 2001), indicating a large portion of undetected bacterial diversity. This is not surprising, for a library of less than 400 clones cannot reasonably represent a complete picture of microbial diversity in soil due to the immense wealth of microorganisms in soil (Torsvik *et al.*, 1990; Curtis *et al.*, 2004). That the number of clones in our 16S rRNA gene library is insufficient to completely represent soil bacterial diversity can also be judged from the figures showing the Number of Clones versus the number of sequences sampled (Figure 9-12). Only the second curve (figure 10) derived from digestion with the HinfI restriction enzyme, approaches a leveling off when the

sample number reaches 200. All three of the other curves are still increasing, indicating the number of clones in the present study is too low. Also, the primers used to amplify the 16S rRNA genes in the soil samples, though termed universal, were designed according to all known bacterial 16S rDNA sequences, thus only representing a subset of total species diversity within the entire soil bacterial community. Furthermore, none of the universal 16S rDNA primers have been shown to be complementary to all known bacterial sequences (Brunk *et al.*, 1996). What is more, it is unlikely the mixture of clones would quantitatively reflect the relative abundance of taxons in the natural microbial world unless we assume that amplification and cloning efficiencies were the same for all molecules (Rainey *et al.*, 1994; Suzuki and Giovannoni, 1996), given the fact that the gene copy number is variable among different species to begin with (Farrelly *et al.*, 1995). Thus, it has to be assumed that the phylotype diversity represented in a 16S rDNA library of 192 clones is far from embracing all the bacterial species in the hydrogen-treated soil community.

However, sequence analysis of 16S rRNA gene clones can and has been used to identify the most dominant populations of bacterial communities in three natural forest soils (Hackel *et al.*, 2004).In a similar way, and like previous analysis, we assume our 16S rRNA gene library did capture the predominant members of the hydrogen-treated soil microbial community. Predominant members in a microbial community have been suggested to play the most important ecological roles in the community (Bardgett,

2002; Dunbar *et al.*, 2002), although the question has been raised and critically discussed based on whether the predominant species are at the same time the functional species in the community (Bengtsson, 1998).

Figure 9-12: Rarefaction curves of clone sequences *in silico* cleaved by the endonucleases HaeIII (Figure 8), HinfI (Figure 9), MspI (Figure 10) and BstUI (Figure 11). The red diagonal line represents the 1:1 relationship where each screened clone is unique.

Figure 9. Clones *in silico* cleaved by HaeIII at GG'CC

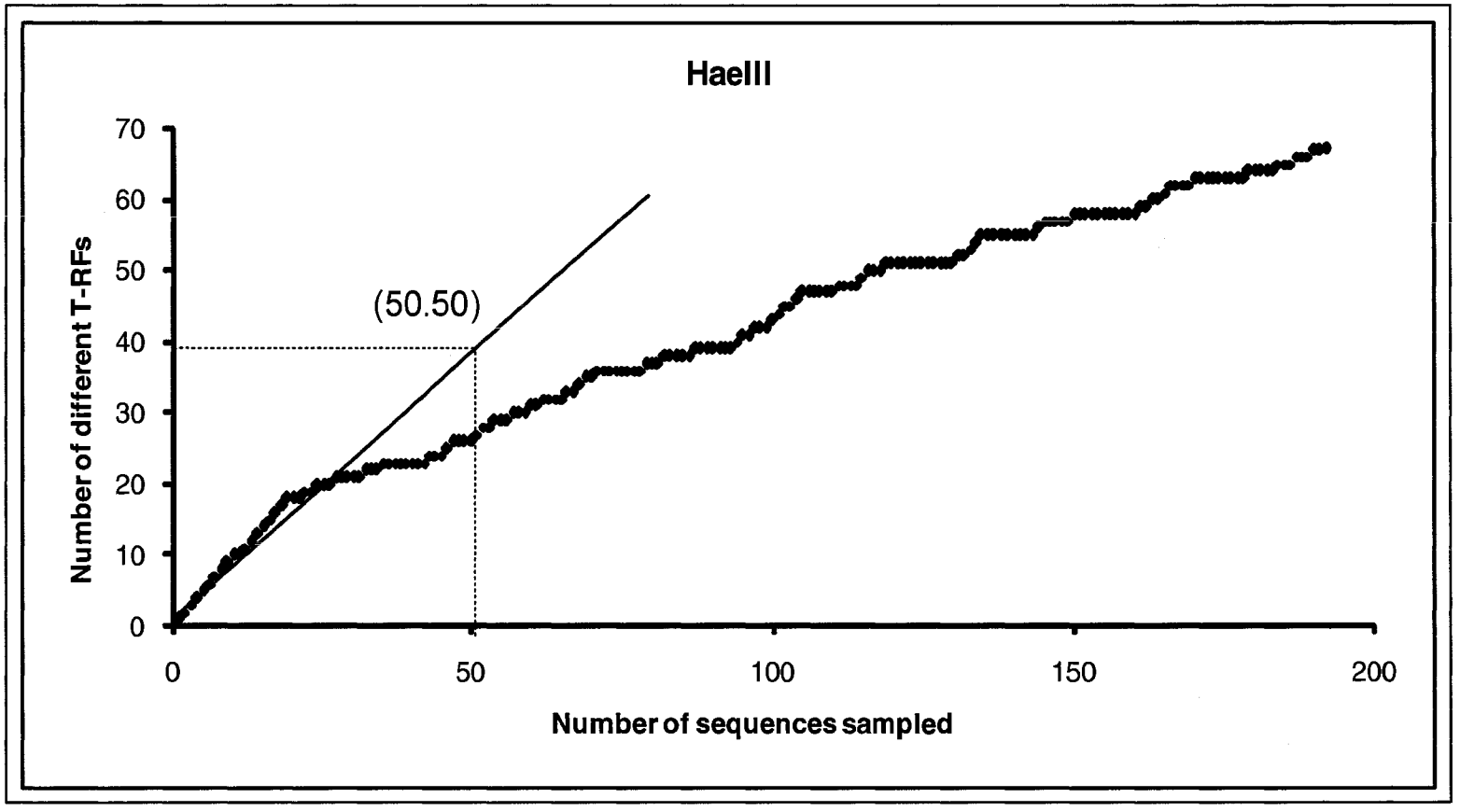


Figure 10. Clones *in silico* cleaved by HinfI at G'ANTC

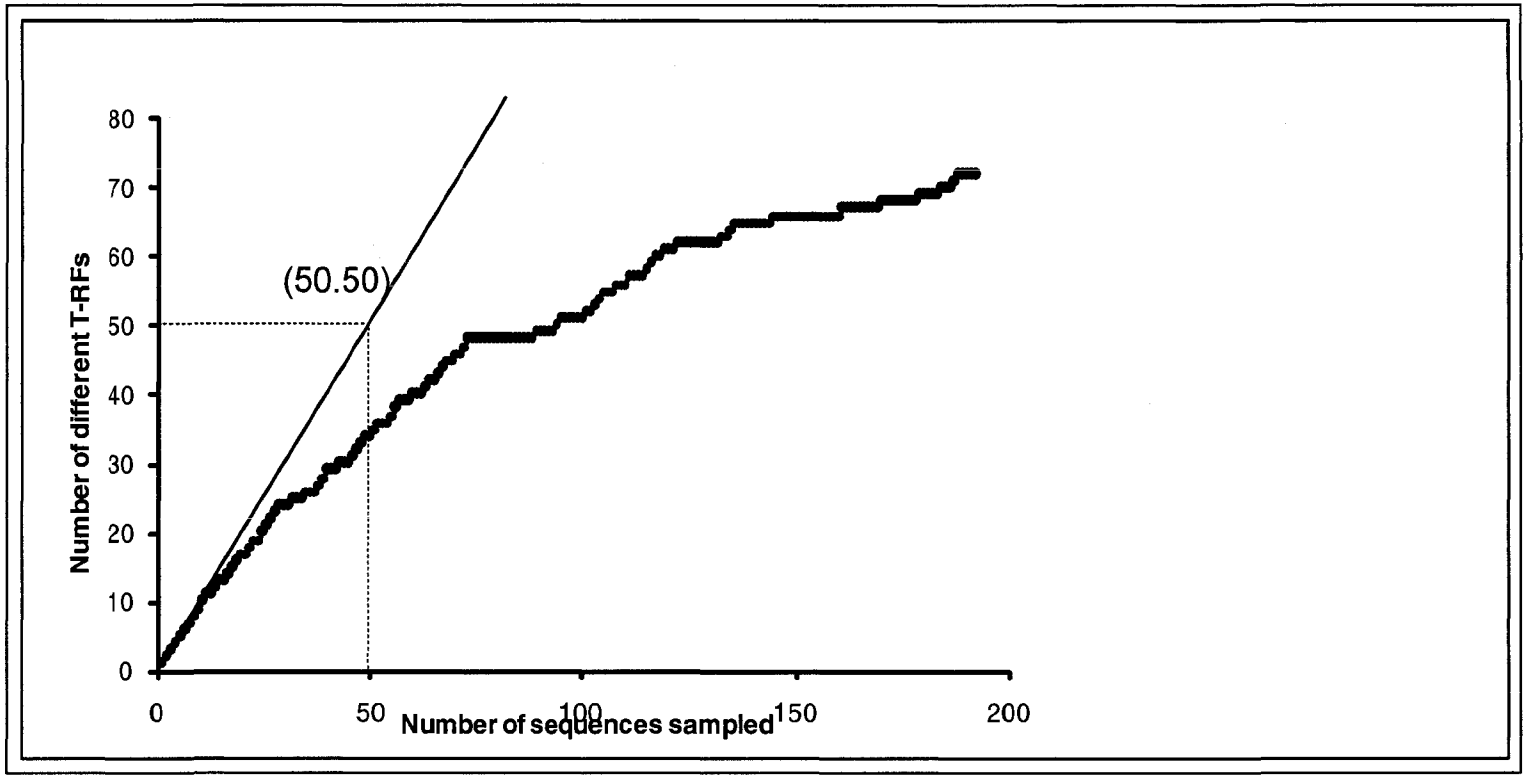


Figure 11. Clones *in silico* cleaved by MspI at C'CGG

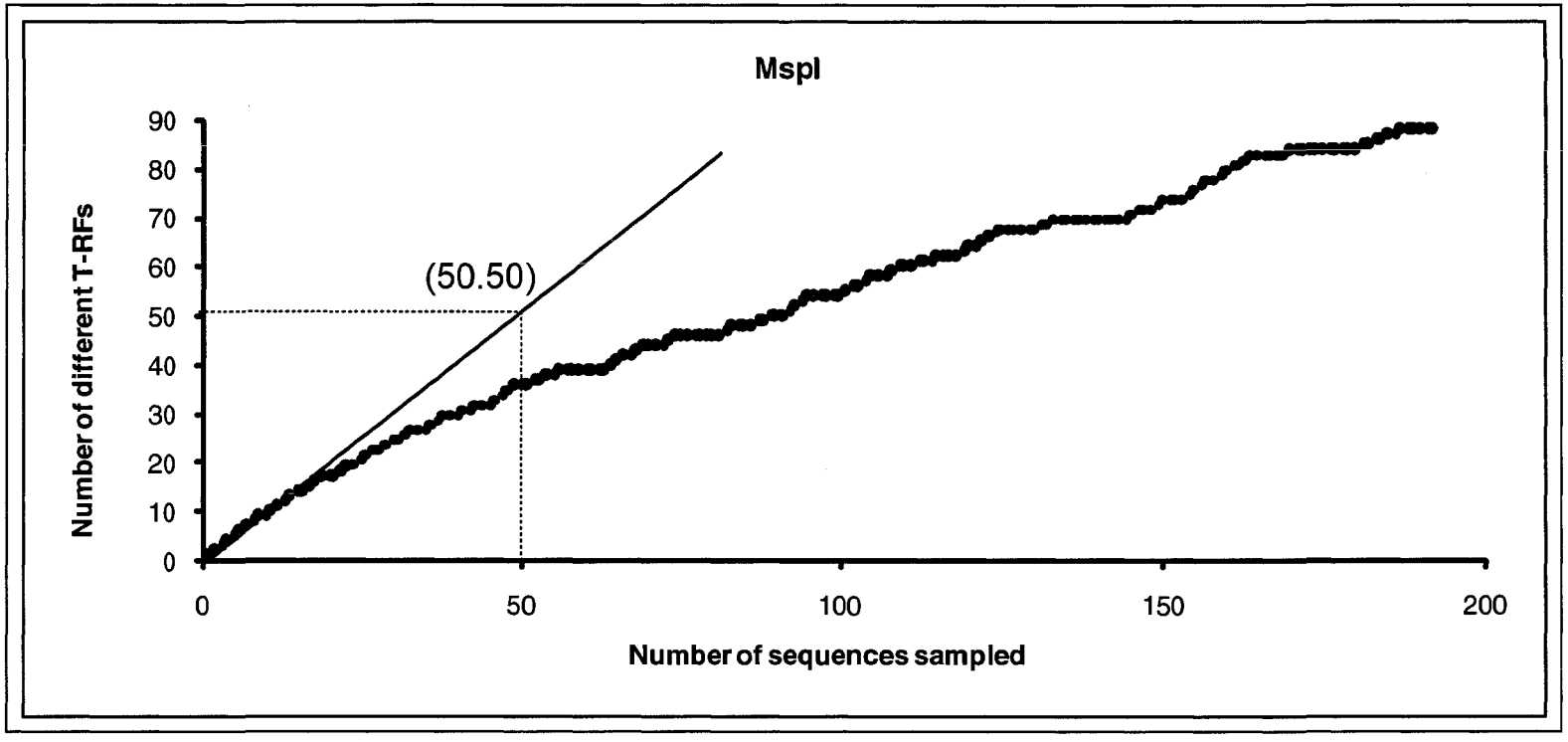
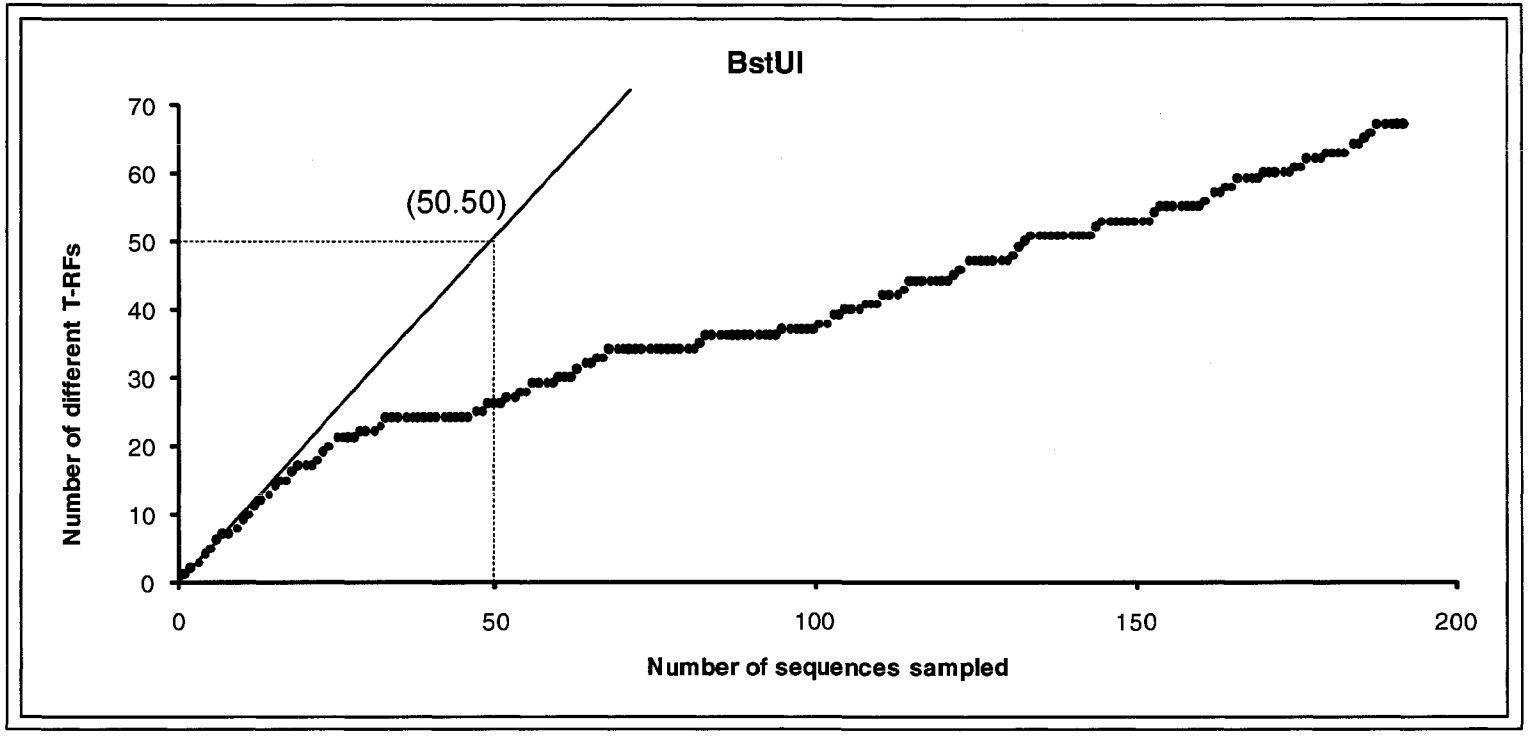


Figure 12. Clones *in silico* cleaved by BstUI at CG'CG



4.2.2 Actinobacteria---an important member of soil bacterial community after hydrogen treatment

Based on the above, the affiliation of a large number of clone sequences with Actinobacteria in the library indicates that this may be an important member of the bacterial community within hydrogen-treated soil sample and that this bacterial group is mainly hydrogen induced. Previous studies have demonstrated that significantly increased hydrogen-oxidizing activity is found in both hydrogen-treated soil and soil adjacent to HUP- nodules showing hydrogen-evolving capacity (LaFavre and Focht, 1983; Popelier *et al.*, 1985; Wong *et al.*, 1986; Cunningham *et al.*, 1986). Furthermore, when compared with T-RFs in T-RFLP profiles of air treated soil samples, the T-RFs exhibiting the most marked increases in intensity matched the T-RFs predicted from the 16S rRNA gene sequences of *Nocardia* (AB126875)and *Pseudonocardia* (AJ252828) deposited in NCBI GenBank (Zhang, 2007); both *Nocardia* and *Pseudonocardia* are members of the *Actinobacteria* group. Additionally, genes encoding hydrogenases have already been found in Nocardia, Mycobacteria, Streptomyces, Flavobacteria, Pseudomonas and Rhizobium (Seiler, 1978; Bowien and Schlegel, 1981; Reh and Schlegel, 1981; Gadkari *et al.*, 1990; Goodfellow, 1992). Finally, it was suspected that long filamentous bacteria like actinomycetes might be responsible for soil hydrogen uptake when it was found that the hydrogen uptake ability of the soil disappeared when the physical structure of soil was destroyed (McLean and Dong, 2002). Candidate Division TM7 has been reported to possess a sheathed-filament morphotype and is regarded as one of the three lineages of bacteria

which possess an authentic gram-positive cell envelope besides Actinobacteria and low G+C gram-positive divisions (Hugenholtz *et al.*, 2001).

4.2.3 Actinobacteria---the possible major group stimulated by hydrogen treatment

At the same time, the fact that *Actinobacteria* corresponded to most of the major increased T-RF peaks with all four the restriction enzymes derived from hydrogen treated soil T-RFLP profiles is unlikely to be coincidental and probably indicates that the major bacteria group stimulated by hydrogen metabolism is Actinobacteria; this is in agreement with McLean and Dong's speculation in 2002 that the bacteria responsible for the main hydrogen uptake rate in hydrogen-treated soils might have a colonial or long filamentous structure like that of *Actinomycetes* (McLean and Dong, 2002). Further to this, Ye Zhang's personal communication with Osborn implied five strains of Pseudonocardia isolated from hydrogen-treated soil showed great hydrogen uptake ability and plant growth promotion effect. Zhang (2006) also found that several T-RF peaks which showed marked intensity increases in profiles from hydrogen treated soils possibly matched TRF peaks predicted from 16s rDNA sequences of Nocardia (AB126875) and Pseudonocardia (AJ252828) published in NCBI GenBank (Zhang, 2006). Moreover, the present study also showed that *Nocardioides sp.* and *Micrococcus sp.* (members of Actinobacteria) consumed large amounts of hydrogen unparalleled by other species under same conditions described in above. Jiamila Maimaiti (2007) isolated hydrogen oxidizing bacteria

which were identified as *Variovorax paradoxus*, *Flavobacterium johnsonae* and *Burkholderia sordidicola*. Zhang (2006) found that none of these isolates has the same TRF length as that of TRF peaks showing significant intensity increases. This is not surprising according to the present cloning results, which show that among our 192 clones, only 3 clones are affiliated with Burkholderiaceae and 13 are pooled into the Flavobacteria division.

4.2.4 The first attempt to isolate hydrogen oxidizing bacteria

Jiamila Maimiati (2007) first started isolation of hydrogen oxidizing bacteria in our lab. As it mentioned in 1.3.2, aerobic hydrogen oxidizing bacteria is able to use H_2 as the electron donor, with O_2 as the electron acceptor and to fix CO_2 to grow chemolithoautotrophically. So hydrogen oxidizing bacteria could grow on a non-organic carbon media which encourage the growth of hydrogen oxidizing bacteria while inhibit the growth of other bacteria. The mineral salt agar (MSA) media with fungicide in it was first used in the isolation of hydrogen-oxidizing bacteria under the open flow-through system. The reason that fungicide was added in is some fungi grow so fast that they would take over the whole plate before hydrogen oxidizing bacteria could even form colonies. However, little did Jiamila know that the fungicide she added in might have killed the phyla *Actinobacteria* as the branching filaments or colonial structure of the *Actinobacteria* is somewhat similar to the mycelia of the unrelated ray fungi (Krassilnikov, 1938).While the phylum Actinobacteria is the possible candidate we suspected to contribute to major increased

T-RFs after hydrogen treatment. This probably explains why none of 21 isolates that Jiamila (2007) had belong to the Actinobacteria and has the same TRF length as that of TRF peaks showing significant intensity increases (Zhang *et al.*, 2007).

4.2.5 The possible reasons that Actinobacteria were not isolated in large quantity

In the present study, the MSA media was still used but without fungicide. Two hydrogen oxidizing bacteria belonging to the *Actinobacteria* (one is *Nocardioides sp.* and the other is *Micrococcus sp.*) have been successfully isolated. However, the possible reasons that members of the phyla *Actinobacteria* were not able to be cultured in large quantity are as follows

To begin with, it is very hard for some subclass of the phylum Actinobacteria to form colonies on laboratory media. As illustrated in the previous part, only about 1% of the $> 10^9$ bacterial cells in one gram of soil can be cultured in microbiological media. However, the recent adoption of nontraditional media: VL55; Xylan and DNBG, has successfully isolated many representatives of previously rarely isolated groups (Janssen *et al.*, 2002; Sait *et al.*, 2002; Joseph *et al.*, 2003). So probably speaking of majority of the bacterial cells in soil as "unculturable" is relative.

Furthermore, the incubation time may not be long enough for those slow-growing bacteria like the *Actinobacteria* to form viable counts on mineral salt media (MSA) on which some other fast growing bacteria might have already taken over. What is more, as illustrated above, some *Actinobacteria* have colonial structure

or form branching filaments. During the process of dilution and inoculation, the filaments or colonial structure might be broken by pipetting, which may make the slow-growing *Actinobacteria* grow even slower as it takes them some time to recover their broken filaments. The number of visible colonies is believed to increase with the increase of the length of incubation time, especially on low nutrient concentration media (Jensen, 1968). It was found that members of previously rarely cultured groups became predominant on appropriate media inoculated with soil after extending incubation time up to 3 months (Davis *et al.*, 2004). While in the present study, the maximum incubation time is two months; in Jiamila's (2007) isolation of hydrogen oxidizing bacteria, the normal incubation time is only 21 days.

Last but not least, the inoculum size could also exert certain effect on the viable counts of isolation. It is generally believed the number of viable colonies appearing on each plate increase with the decrease of inoculum size (Jansen, 1962; 1968; Olsen and Bakken, 1987). When the inoculum size is too big, i.e., the dilution level is too low (for instance 10^{-8}), more than one bacterial cell (10 if 10^9 bacterial cells are assumed in one single gram of soil) will be on one plate, leading to the overgrowth of other bacterial cells rather than the slow-growing Actinobacteria. On the other hand, however, when the inoculum size is too small, i.e., the dilution level is too high (like 10-12 in the present study), chances are that some plates may not even get one single soil bacterial cell per plate as one single gram of soil contain up to 10^9 bacterial cells (Bollon *et al.*, 1993)

4.3 Reasons that TRF peaks are not Represented in Clone Libraries and Vice Versa

4.3.1 Different matching windows applied in researches

Two approaches have been used previously to identify T-RFs of interest in community T-RFLP profiles: 16S rRNA gene sequence databasing (Clement *et al.*, 1998; Kaplan *et al.*, 2001) and the composition and investigation of 16S rRNA gene libraries of microbial communities (Dunbar *et al.*, 2000; Ludemann *et al.*, 2000; Hackl *et al.*, 2004; Graff and Conrad, 2005; Harmann and Widmer, 2006, 2007) In some investigations as illustrated in chapter 1, sequence-determined T-RF length were predicted from Ribosomal database project (RDP) rather than from *in silico* digestion of each clone sequence (Braker *et al.*, 2001; Kaplan *et al.*, 2001; Kaplan and Kitts, 2003). Lengths of predominant bacterial and archaeal 16S rDNA T-RFs were theoretically compared to those of aligned sequences in the Ribosomal Database Project. The matching windows these researchers utilized are for picking out sequences from RDP database corresponding to the predominant T-RFs within T-RFLP profiles. In the second situation (investigation of 16S rDNA library), there are two instances: in vitro T-RFLP analysis of each clone ($T\text{-}RF_{exp}$) and *in silico* digestion of each clone ($T\text{-}RF_{seq}$). In some researches (Hackl *et al.*, 2004; Hartmann and Widmer, 2006), T-RF drift is between $T\text{-}RF_{exp}$ of each clone from experimental T-RFLP profiles and its corresponding community T-RFLP profiles. So the matching windows were used to make up for the discrepancies between $T\text{-}RF_{exp}$ and T-RFs in

community T-RFLP profile. For the situation in the present study, the matching window of -4 to +3bp was selected to match computer simulated digested 16S rDNA clones to the predominant T-RFs in hydrogen-treated soil T-RFLP profiles. While in the present study, the predominant T-RFs in hydrogen-treated soil microbial community T-RFLP patterns were identified by association with computer-simulated (*in silico*) T-RFs of corresponding 16S rRNA gene clones, in which the matching window of -4 to +3 was applied as illustrated in Chapter 3.

4.3.2 TRF Peaks that are not vovered by clone library and vice versa

All the clones were chosen for identification of predominant T-RFs. The T-RFs determined by computer-simulated restriction digestion of most clones corresponded to T-RF peaks observed in the community T-RFLP profile of hydrogen treated soil, which demonstrated the general consistency between the two approaches (Figure 13-16). However, a few predominant T-RF peaks observed in the community T-RFLP profile, such as the first peak (of unknown base pair location due to the resolution of T-RFLP analysis beyond 50bp) in the MspI digested T-RFLP profile and the 482.4bp peak in the BstUI digested T-RFLP profile were not represented in the clone library (Figures 15, 16; Tables 9, 10). A similar occurrence happens to T-RFs of 468.9bp and 497.9bp in HinfI digested T-RLFP profiles respectively, neither has any clone affiliated with them, and 275.8 bp, 451.6 bp and 482.4 bp in MspI digested T-RFLP profiles respectively, each has only a couple of clones affiliated with them (Tables 8, 9). At the same time, the significant peak observed at 39bp in the HaeIII

generated TRFseq clone graph (Figure 13, Table 7) corresponds to a much smaller peak in the community T-RFLP profile. This is also seen with the peak at 312bp in the HinfI-generated data sets (Figure 14, Table 8).

4.3.2.1 Reasons that may explain the above situations

To begin with, as it was readily seen, it mostly happens with large TRF fragments of almost 500bp, it is reasonable to assume some phylogenetic groups do not have cutting sites of the four restriction enzymes (HaeIII, HinfI, MspI and BstUI) applied in the present study.

Furthermore, the discrepancies may be due to sampling and/or cloning biases in the library construction and/or PCR-inherent biases in both the T-RFLP profiling method and the generation of the 16S rDNA library as indicated in the previous section. For example, there may be biases in the contributions of the various bacterial groups to libraries, which may result from the following: Firstly, the DNA extraction efficiency varies among different bacteria; Secondly, an individual cell has its own unique number of copies of 16S rRNA or 16S rRNA genes. Finally, preferential amplification of some sequence types over others is a possibility (Frostegard *et al.*, 1999).

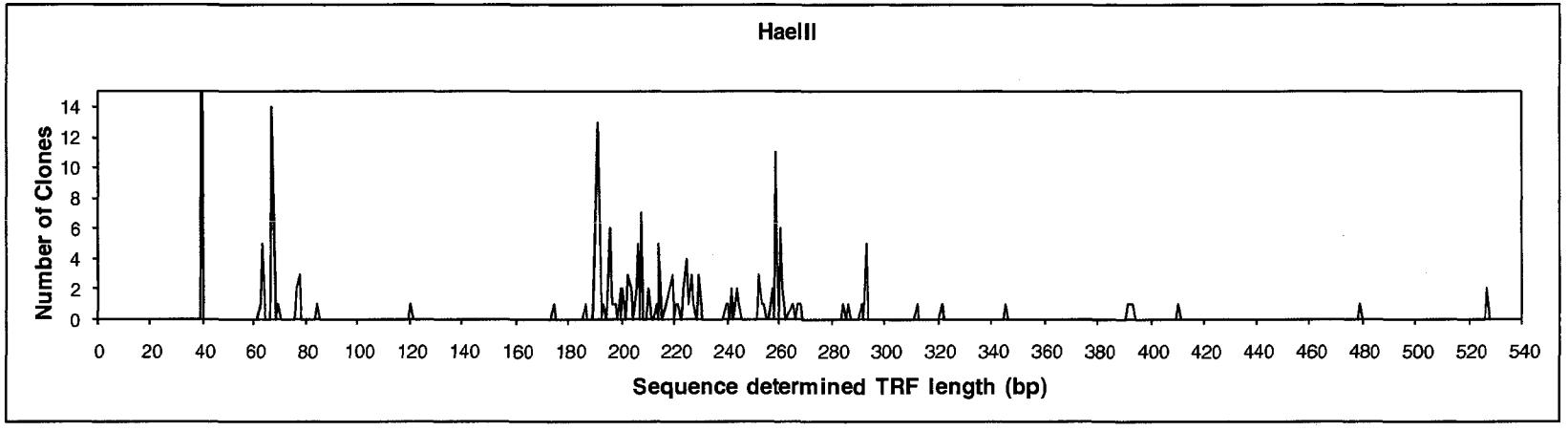
It is not uncommon for TRF peaks to not be represented in clone libraries and for clones producing TRF fragments to not be observed in the community TRF pattern (Dunbar *et al.*, 2000; Wang *et al.*, 2004; Phelps *et al.*, 1998), neither are the inconsistencies observed between frequency of clone abundance and its corresponding abundance in community TRF patterns (Lueders and Friedrich., 2000).

There also exists the potential that these specific TRF peaks not represented in the clone library are possibly pseudo T-RF peaks. Pseudo T-RFs, resulting from uncut fluorescently labeled single-stranded amplicons, can be present and are usually seen as additional peaks in T-RFLP profiles as additional peaks, leading to overestimated interpretations of diversity within community T-RFLP patterns (Egert and Friedrich, 2003). Graff and Conrad (2005) reported 29% of their clones showed formation of pseudo-T-RFs when they created a 16S rRNA gene library of 281 clones to investigate the impact of flooding on soil microbial communities, indicating the creation of pseudo-T-RF occurs at a fairly high frequency (Graff and Conrad, 2005). Egert and Friedrich have suggested all clones be checked for their in vitro digestion T-RF pattern to facilitate the detection of pseudo-T-RFs (Egert and Friedrich, 2003). However, because this study did not generate in vitro T-RF formation patterns of each individual clone beside the *in silico* determination of T-RF size, how many pseudo-T-RFs were potentially formed can not be quantified.

More clones need to be analyzed for clearer identification of each T-RF peak and a better reflection of the total community diversity in the complex microbial communities such as soil. It is believed in the ideal situation, i.e. unlimited numbers of clones, the two graphs would be identical.

Figure 13-16: Comparison of T-RFs of amplified 16S rRNA genes to 16S rDNA clone fragments from *in silico* digestion of sequences (HaeIII-, HinfI-, MspI- and BstUI-generated profiles).

Figure 13. HaeIII-generated profiles



Sample 1: HHa.fsa

Dye: Blue - 40 peaks

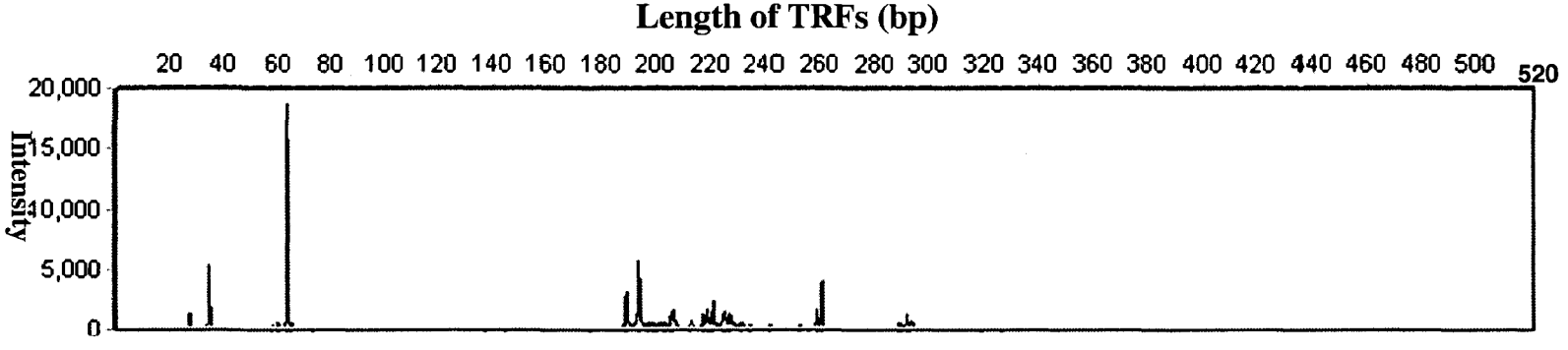
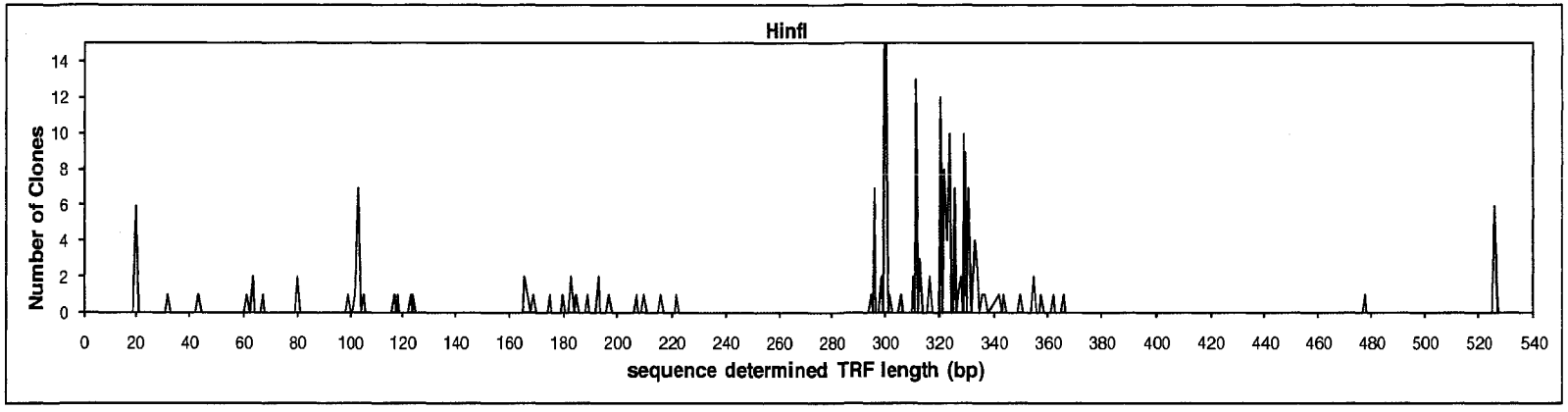


Figure 14. HinfI-generated profiles



Sample 1: HHi.fsa
Dye: Blue - 41 peaks

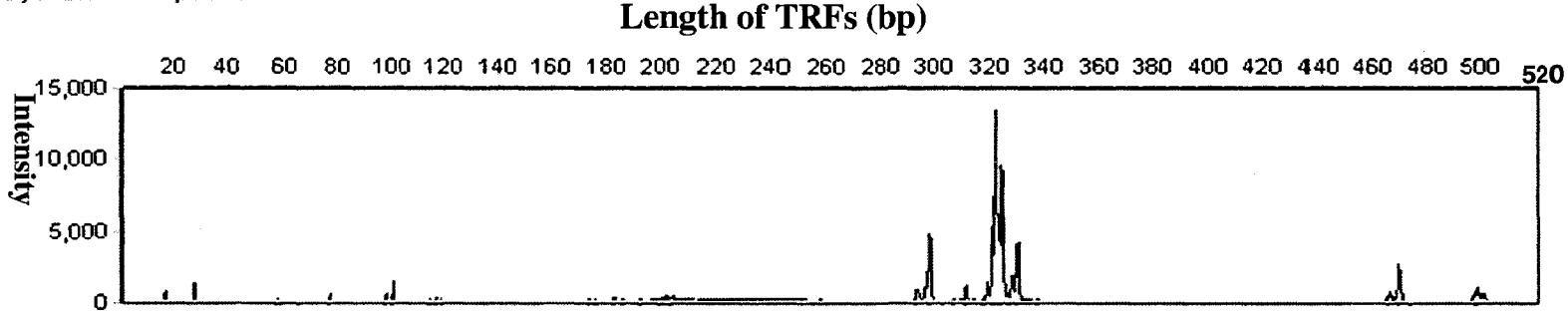
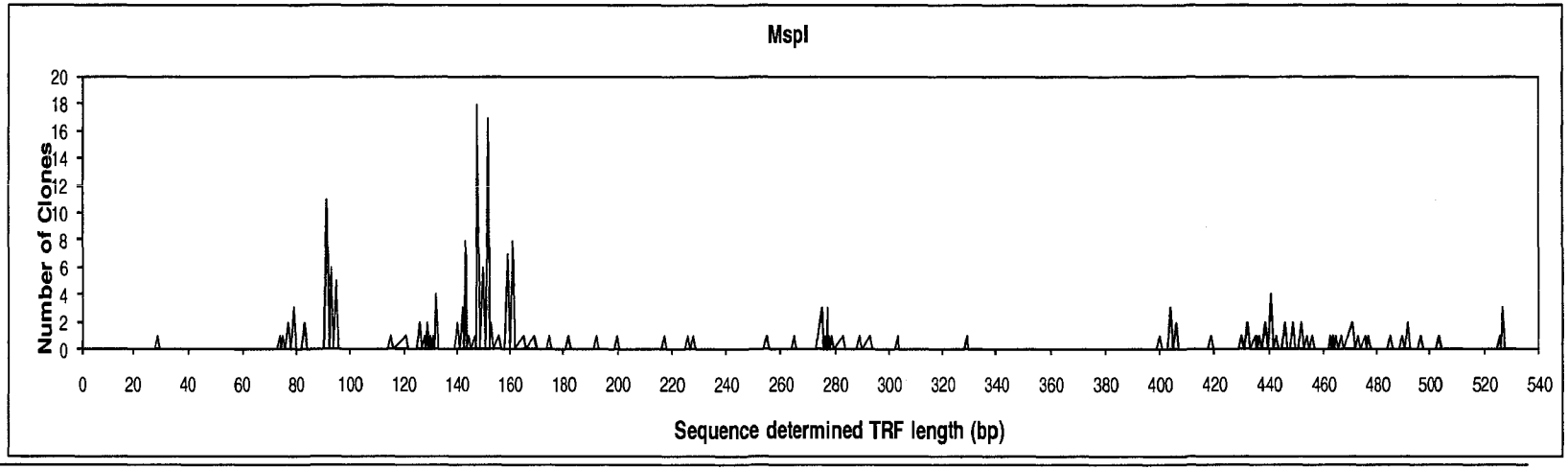


Figure 15. MspI-generated profiles



Sample 1: HM.fsa

Dye: Blue - 17 peaks

Length of TRFs (bp)

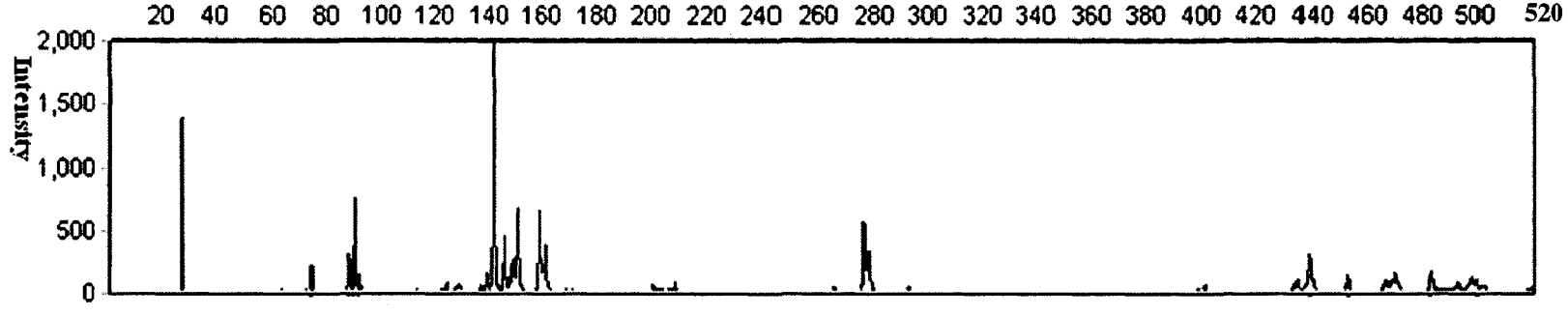
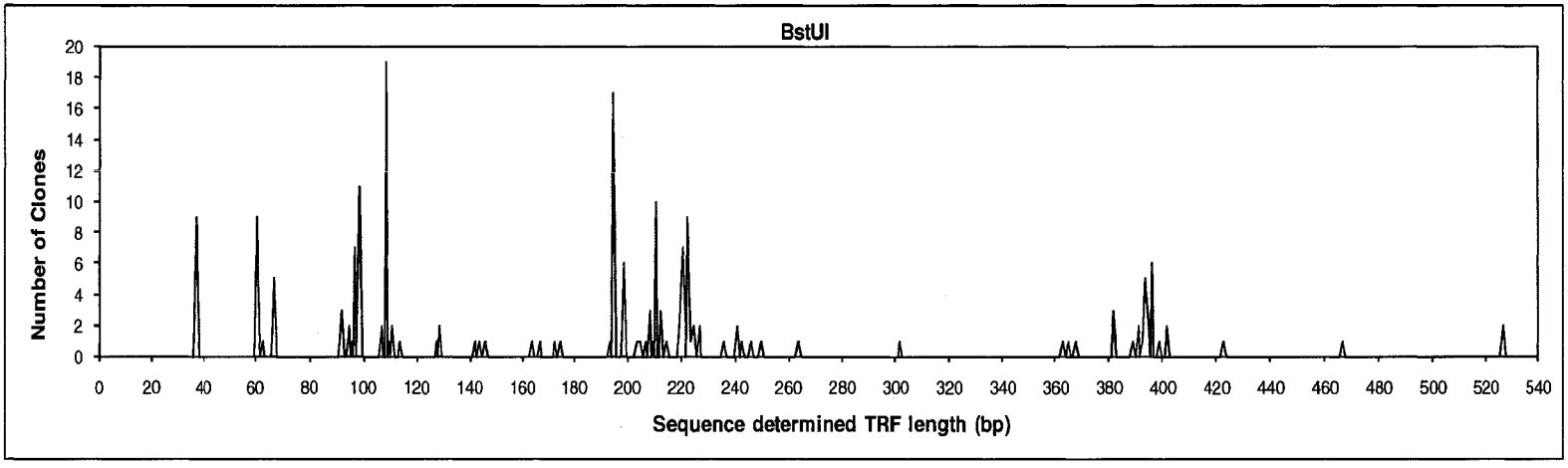
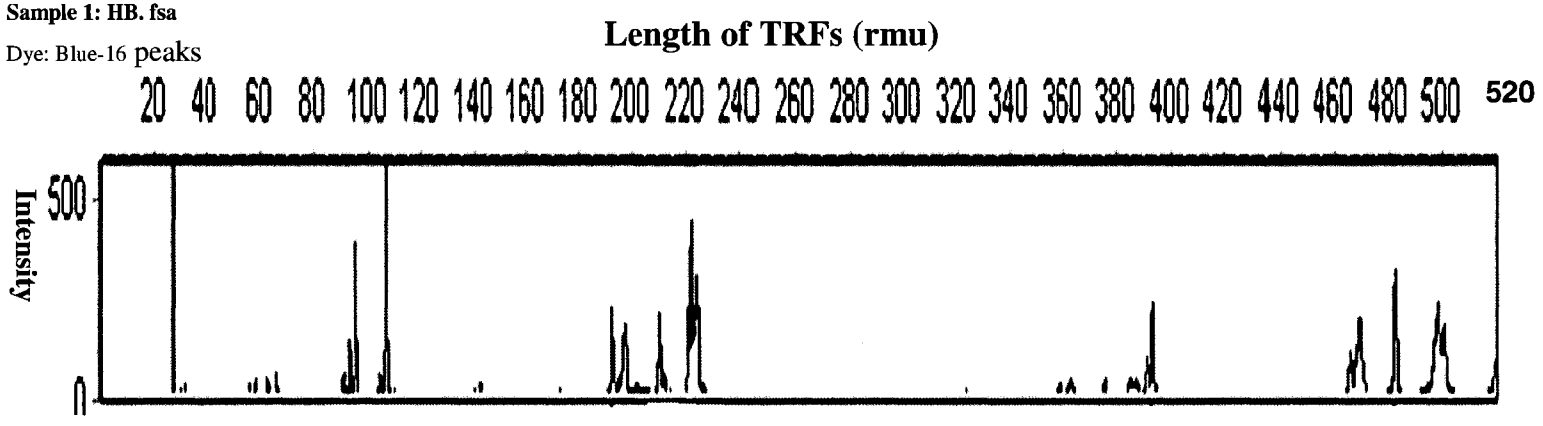


Figure 16. BstUI-generated profiles



Sample 1: HB. fsa

Dye: Blue-16 peaks



4.4 Speculation that the Coenzyme F420 Dependent Hydrogenase as the Possible Hydrogenase in Actinobacteria

4.4.1 Hydrogenases found in the phylum Actinobacteria

Immunogold localization has demonstrated the presence of a hydrogenase in the hyphae of ammonia-grown cells and in the hyphae and vesicles of N_2-grown cells (Lindblad and Sellstedt, 1990). Although hydrogen-oxidizing bacteria are neither ammonia-grown nor N_2-grown cells, yet because the *Actinobacteria* also possess hyphae, the fact that hydrogenases can be found in hyphae of other groups made it possible they could also be found in the hyphae of the *Actinobacteria*. Different forms of hydrogenase enzymes have been subsequently discovered in members of the *Actinobacteria*: including *Mycobacterium gordonae* (Park and DeCicco, 1974); *Nocardia opaca* 1b (Reh and Schlegel, 1981; Goodfellow, 1992); *Frankia sp.* (Sellstedt, 1989; Murry and Lopez, 1989) and *Streptomyces thermoautotrophicus* (Gadkari et al., 1990).

4.4.2 Coenzyme F420---a two-electron transferase found in bacteria

What is more, the coenzyme F420, a two-electron transfer coenzyme first discovered in *Methanogenic archaea* (Eirich *et al.*, 1979), has been subsequently found to be widely distributed in *Mycobacteria, Nocardia and Streptomyces* species (McCormick and Morton, 1982; Naraoka *et al.*, 1984; Daniels *et al.*, 1985; Kuo *et al.*, 1989; Purwantini and Daniels, 1996; Purwantini et al., 1997). The identification in

Nocardioides simplex FJ2-1A strain of a hydride-transferring enzyme composed of a NADPH-dependent F420 oxidoreductase and a hydride transferase indicated the presence of F420 in this *Nocardioides simplex* FJ2-1A strain and the possible existence of F420 in other *Nocardioides* species (Ebert *et al.*, 1999). Although to this author's knowledge, there have not been any reports of a F420-dependent hydrogenase present in the above listed genera of the *Actinobacteria*; the widespread existence of a F420-dependent dehydrogenase such as the F420-dependent glucose-6-phosphate dehydrogenase (FGD) has been demonstrated in *Mycobacterium* and *Nocardia* species (Purwantini and Daniels, 1996; Purwantini *et al.*, 1997). Furthermore, researchers have found a F420-dependent hydrogenase present in methanogenic bacteria, a F420-dependent NADP-linked hydrogenase system has been found to play an important role in electron transfer reactions in the energy metabolism of methanogenic bacteria (Tzeng *et al.*, 1974) and coenzyme F420 is essential for the reduction of NADP (Nicotinamide adenine dinucleotide phosphate) in the presence of a hydrogen phase (Tzeng *et al.*, 1974). Further to, and consistent with this, Jacobson and his colleagues showed that the coenzyme F420 served as a two-electron acceptor in reaction with H_2 in *Methanobacterium thermoautotrophicum* (Jacobson *et al.*, 1981).

4.4.3 Reasons for speculating F420 dependent hydrogenase as the possible hydrogenase in the Actinobacteria

As illustrated above, the hydrogenases found in all presently known aerobic

hydrogen-oxidizing bacteria belong to the [NiFe] group of hydrogenase (Schwartz and Friedrich, 2006). A major characteristic of one group of [NiFe] hydrogenases is that the hydrogenase module is strongly associated with a second subunit which harbors the binding sites for coenzymes like F420, NAD or NADP, which functions as two-electron acceptors (Schwartz and Friedrich, 2006).

Along with previous analyses, it may shed some light on the possible role of the coenzyme F420 found in the *Mycobacteria, Streptomycete, Nocardia and Nocardioides* species. Molecular analysis of genes encoding F420 dependent glucose-6-phosphate dehydrogenase (FGD) in *Mycobacteria smegmatis* has shown a marked similarity with the genes of at least eight unknown proteins from Mycobacteria tuberculosis and to the lmbY gene from *Streptomyces lincolnensis* (Purwantini and Daniels, 1998). Purwantini and Daniels have hypothesized that these unknown hypothetical proteins in M. tuberculosis interact with F420 and that the study of the possible functions of F420 might lead to the discovery of novel enzymes like F420-dependent hydrogenase, formate dehydrogenase, methylene-tetrahydromethanopterin dehydrogenase or photolyase in *Mycobacteria* (Purwantini and Daniels, 1998).

Thus, although this is not a final proof, it is tempting to speculate that the hydrogen oxidation catalyzed by F420 dependent hydrogenases may also occur in the phylum *Actinobacteria.* However, support for this hypothesis will require a rigorous and considerable biochemical examination of members of the *Actinobacteria.*

4.5TRFLP Genetic Profiling Method for Reflecting the Structure and Diversity of Numerically Dominant Members of the Microbial Community

4.5.1 Limitations of T-RFLP fingerprinting method

As both PCR amplification and downstream analyses (restriction enzyme cutting and capillary electrophoresis) seem subject to biases (Egert and Friedrich, 2003; Lueders and Friedrich, 2003; Curtis and Sloan, 2004), the level of resolution of the T-RFLP fingerprinting method to reflect real community composition and diversity of the soil microbial community has been controversially discussed in the literature (Dunbar *et al.*, 2000; Osborn *et al.*, 2000; Lueders and Friedrich, 2003; Hartmann and Widmer, 2008). The discussion has mainly focused on to what extent the method may be subject to biases. A maximum bias factor of 14 was reported in the final T-RFLP profiles introduced by PCR amplification, downstream restriction enzyme cutting and capillary electrophoresis (Hartmann and Widmer, 2008).

T-RFLP community profiles showed higher degrees of similarity among the four soil samples studied than RFLP analysis of the four 16S rDNA clone libraries; the phylogenetic richness of each soil sample detected by T-RFLP analysis is not consistent with that documented by RFLP analysis of 16S rDNA clone libraries and among different restriction enzymes, and the community evenness index calculated from community TRF profiles of two soil DNA samples contradicts the results using the Shannon-Weiner diversity index as well as that from RFLP data (Dunbar *et al.*,

2000). Dunbar thus warns of the limitations of the method to provide reliable information on the relative diversity and community structure of highly complex communities (Dunbar *et al.*, 2000). Whether this conclusion is correct or not cannot be resolved at this time, yet a careful investigation of the data in the paper exposed several problems.

To begin with, it is unconvincing and of little significance to compare data generated from the same sample by different restriction enzymes; instead they should be compared with data derived from another sample cut with the same restriction enzyme. It is a commonly approved practice to combine data from more than one restriction enzyme when assessing microbial community diversity as information from one enzyme may be biased or strongly limited.

Furthermore, the data, particularly the community evenness value calculated from the total number of distinct TRF sizes in the community TRF profile is not reliable as the soil DNA may have suffered from serious degradation as a result of 4 years' storage.

4.5.2 T-RFLP---document diversity of dominant bacterial groups

Comparison of community level T-RFLP profiles with results deduced from 16S rRNA gene clone library analysis allows for the assessment of the expressiveness of the T-RFLP fingerprinting technique (Graff and Conrad, 2005; Noll *et al.*, 2005). It has been widely reported that community T-RFLP profiles generated from environmental samples give information in general accordance with analysis of clone

libraries derived from the same samples, mostly targeting 16S rRNA genes (Braker *et al.*, 2000; Dunbar *et al.*, 2000; Ludemann *et al.*, 2000; Hackl *et al.*, 2004; Graff and Conrad, 2005; Hartmann and Widmer, 2006).

When it comes to the present data, Figure 13-16 shows that for any of the four restriction enzymes, the TRF_{seq} profile (The number of clones versus. fragment sizes) has almost the same pattern and distributions of peaks as the corresponding community TRF profile (scales in T-RFLP profiles were adjusted individually for ease of comparison). For instance, the type of 16S rRNA genes dominate in the T-RFLP profiles are also the ones mostly found in the cloning library, although some discrepancies do exist (see above), indicating the validity of the data obtained. This is in accordance with the presumption that direct PCR-based analysis of 16S rRNA genes should theoretically reflect the composition of the dominant bacterial groups in the sample (Diviacco *et al.*, 1992; Siebert and Larrick, 1992).

Along with previous analysis of 16S rDNA clones, it is concluded that the T-RFLP profiling analysis of hydrogen treated soil samples did reflect the composition and diversity of numerically dominant bacterial groups in the samples. This is in agreement with the report that T-RFLP offers a semi-quantitative measure of relative abundance of dominant members within a microbial community (Liu *et al.*, 1997). Moreover, it has been found that template DNA ratios in an artificial community of a four-membered pure methanogen culture was precisely reflected by the relative abundance of T-RFs in T-RFLP profiles; T-RFLP analysis of amplified 16S rRNA genes has also been found to be stable with increasing PCR cycles and

independent of PCR annealing temperature (Lueders and Friedrich, 2003). Last but not least, gradual changes in bacterial community structure directly corresponding to gradient depletion of oxygen with depth were reflected in a series of T-RFLP fingerprinting patterns and clones assigned to the predominant T-RFs fitted well with each zones in this gradient system (Ludemann *et al.*, 2000).

Thus, although T-RFLP fingerprinting method may not be able to detect minor bacterial populations that are possibly highly diverse (Liu *et al.*, 1997; Hackl *et al.*, 2004), yet at least it can be said that the T-RFLP measure of microbial community is not as strongly affected by PCR-inherent biases as indicated in some of the literature (Ludemann *et al.*, 2000; Hartmann and Widmer, 2008).

4.5.3 Reasons explaining the general consistency observed between community T-RFLP Profile of hydrogen-treated soil and in silico digestion of 16S rDNA library

Even though it cannot be stated outright that the T-RFLP molecular fingerprinting technique is an exception to the rule that 'each physical, chemical and biological step involved in the molecular analysis of an environment is a source of bias which will lead to a distorted view of the real world' (Wintzingerode *et al.*, 1997), it is suggested that with careful planning and optimal experimental protocol, a series of biases that may affect the accuracy of T-RFLP measurement can be kept under relatively tight control. This may be helpful in explaining the consistency observed between the community T-RFLP analysis of hydrogen-treated soil samples and the in

silico T-RFLP analysis of 16S rDNA clones derived from the same samples.

Firstly, DNA extraction, PCR and restriction digestion protocols were optimized to the extent at which biases are reduced to the minimum. For instance, replicate DNA extractions were pooled together to reduce random biases and 35 cycles of PCR amplification is considered optimal to ensure the generation of a board community T-RFLP profile, not a limited one (Osborn *et al.*, 2000). The bacterial universal primers were used in the PCR to hybridize to as many sequences as possible in the microbial community and DNA template concentrations of 10 to 20ng each per 50-μl reaction mixture (with sufficient PCR reagents) help avoid stochastic fluctuations that can occur in PCR (Chandler *et al.*, 1997). Targeting the amplicon length at about 500bp in the present study facilitates robust PCR amplification as longer amplicons have been reported to induce biases (Bustin, 2000; Fortin *et al.*, 2001; Giulietti *et al.*, 2001) and sufficient amount of restriction enzymes were applied to prevent the partial digestion of PCR products (Bruce, 1997; Clement *et al.*, 1998). Master mixes of reagents were used both in all replicate PCR amplification and restriction digestions in order to maximally reduce as much as possible any possible bias resulting from pipetting inaccuracies.

Secondly, the two approaches we adopted to characterize the diversity of hydrogen-treated soil bacterial communities both have biases, the biases operated uniformly and thus experienced mutual counteraction. As both T-RFLP profiles and 16S rDNA clones were derived from the same PCR products amplified from the same DNA preparations, the biases inherent in the PCR amplification would be introduced

to the same extent in both approaches.

Altogether, the general consistency found between the T-RFLP fingerprinting method and T-RFLP analysis of 16S rDNA clones allows for the conclusion that T-RFLP genetic profiling of microbial communities can be used to document the number and abundance of numerically dominant bacterial populations within the community with careful optimization of PCR and downstream restriction digestion protocols.

4.6 Speculation on Clones from Air-treated Soil

The four hydrogen-TRF_{seq} clone graphs (the number of clones versus T-RF sizes) were also compared with the increased intensity variation graphs (intensity variation of the total area *versus* T-RF sizes; Figure 17-20) In fact, the intensity variation graph is only supposed to be compared with the clone variation graph, if only because the 16S rDNA clone library from air-treated soil samples was not generated in the current study. The clone variation graph describes the composition of the library generated from hydrogen-treated soil samples from which clones derived from air-treated soil samples are excluded based on the fact that hydrogen treatment increased bacterial populations in the soil as reflected by figure 4-7.

Nonetheless, it is interesting to make a comparison and this revealed that there are more peaks in hydrogen-TRF_{seq} clone graph than in the corresponding intensity variation graph, indicating the possible presence of peaks of similar T-RFs with the same intensity in the air-treated soil clone graph if generated, which is quite reasonable. With this exception, most of the peaks appearing in the clone graph were

also present in the increased intensity variation graph, indicating that bacteria of similar phylogenetic groups as those found in the 16 S rRNA gene library derived from hydrogen-treated soil might also be found in the library from air-treated soil samples.

Thus prediction can be made that there may be clones affiliated with the same phylogenetic groups in the air-treated soil 16S rDNA clone library as in the hydrogen-treated soil 16S rDNA clone library. The difference in these two libraries mainly lie in the abundance of certain specific phylogenetic group. This may be indicative that hydrogen treatment influences the soil community at a lower phylogenetic level rather than changing the abundance at the phylum level.

In consistence with this, 21 libraries of 16S rRNA or 16S rRNA genes generated from a variety of different soil samples are generally contributed by the same phyla, the dominant phyla in almost all soils: Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes, and Firmicutes, although differing in abundance in different libraries (Janssen, 2006).

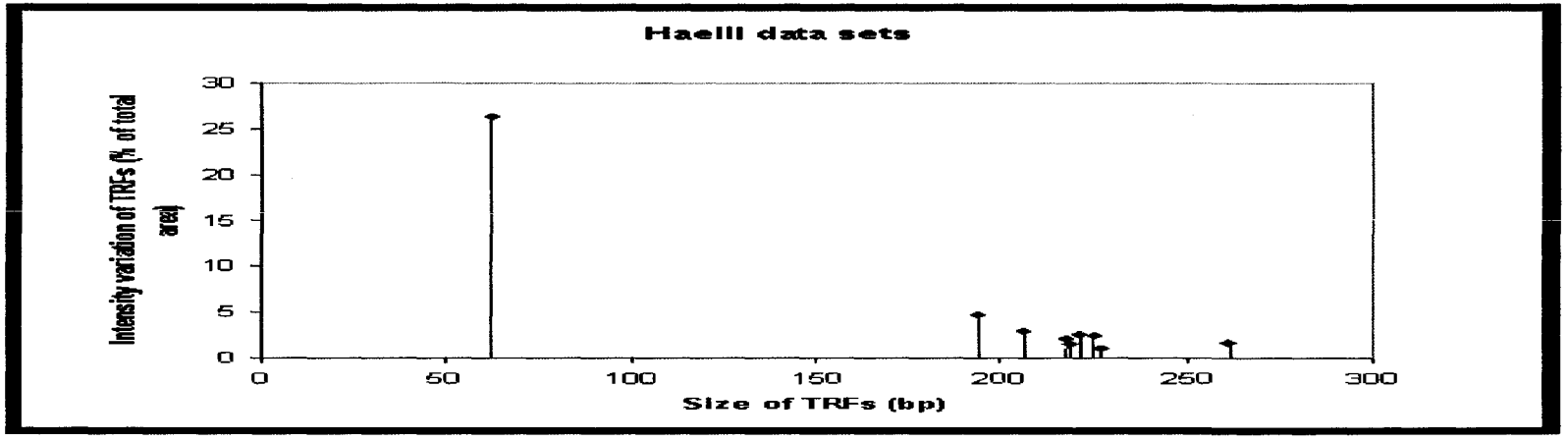
Besides, three soil bacterial 16S rRNA gene libraries derived from three different agricultural treatments: BIODYN, CONFYM, and NOFERT share similar bacterial phyla and similar distributions and abundances of the different phyla (Hartmann and Widmer, 2006)

Furthermore, phylogenetic analysis of 16S rRNA gene sequences showed there were no significant differences between two soils differing in both fertilization

regimen and plant species in terms of diversity and occurrence of particular phylogenetic groups (McCaig *et al.*, 1999).

Figure 17-20: Comparison of TRFseq clone graphs (the number of clones *versus* T-RF sizes) and increased intensity variation graphs (intensity variation of the total area *versus* T-RF sizes) (HaeIII-, HinfI-, MspI- and BstUI-generated profiles).

Figure 17. HaeIII-generated profiles



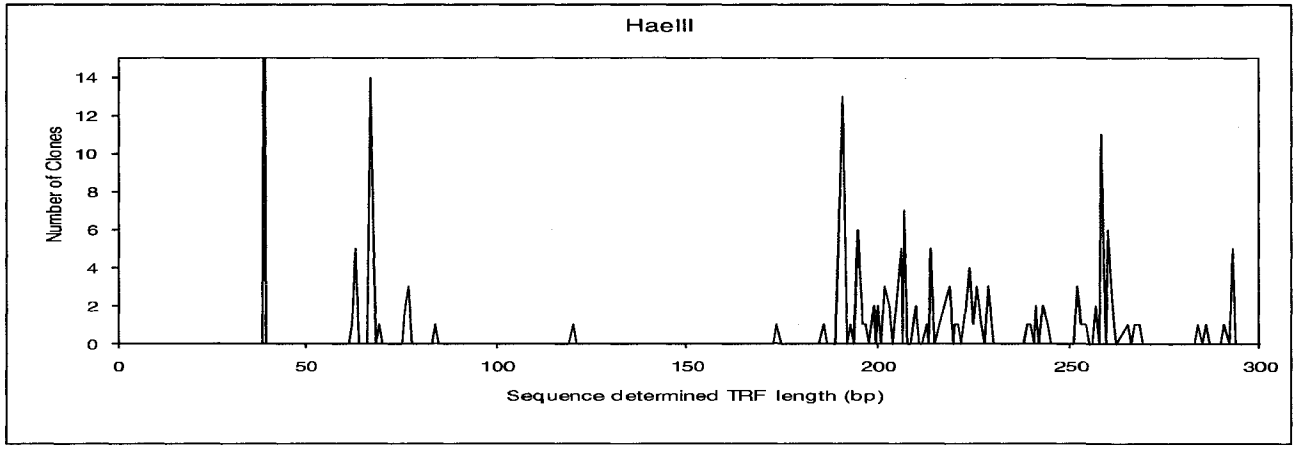
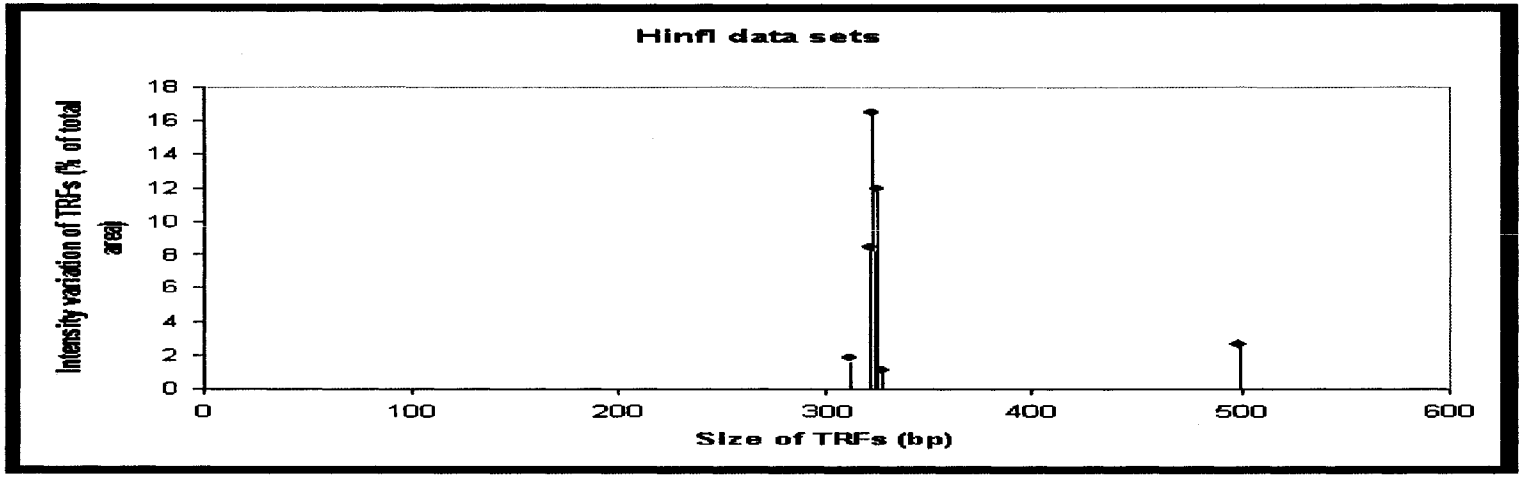


Figure 18. HinfI-generated profiles



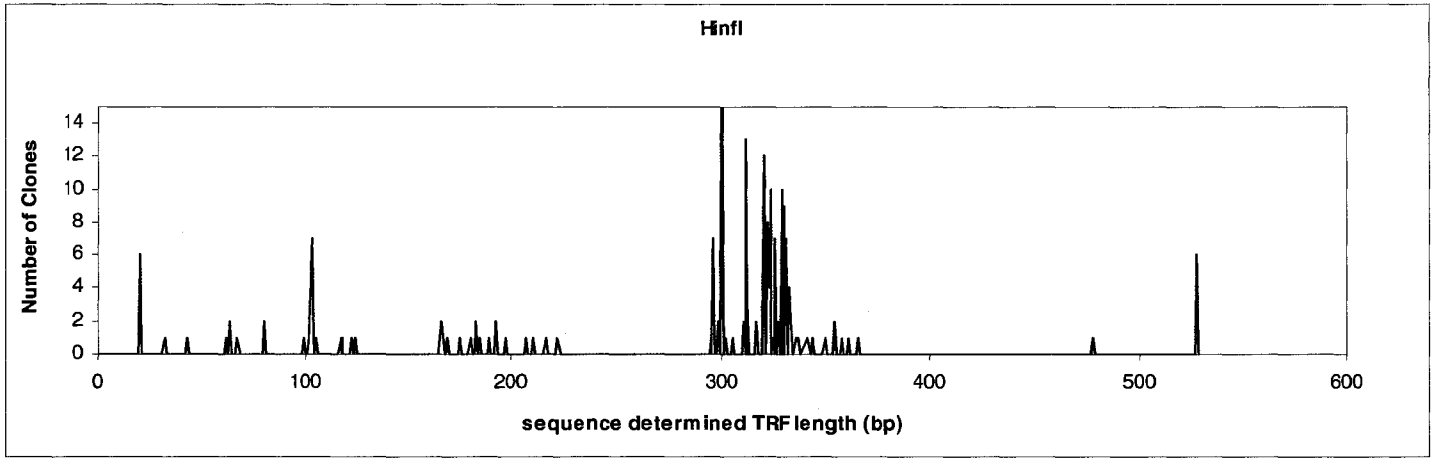


Figure 19. MspI-generated profiles

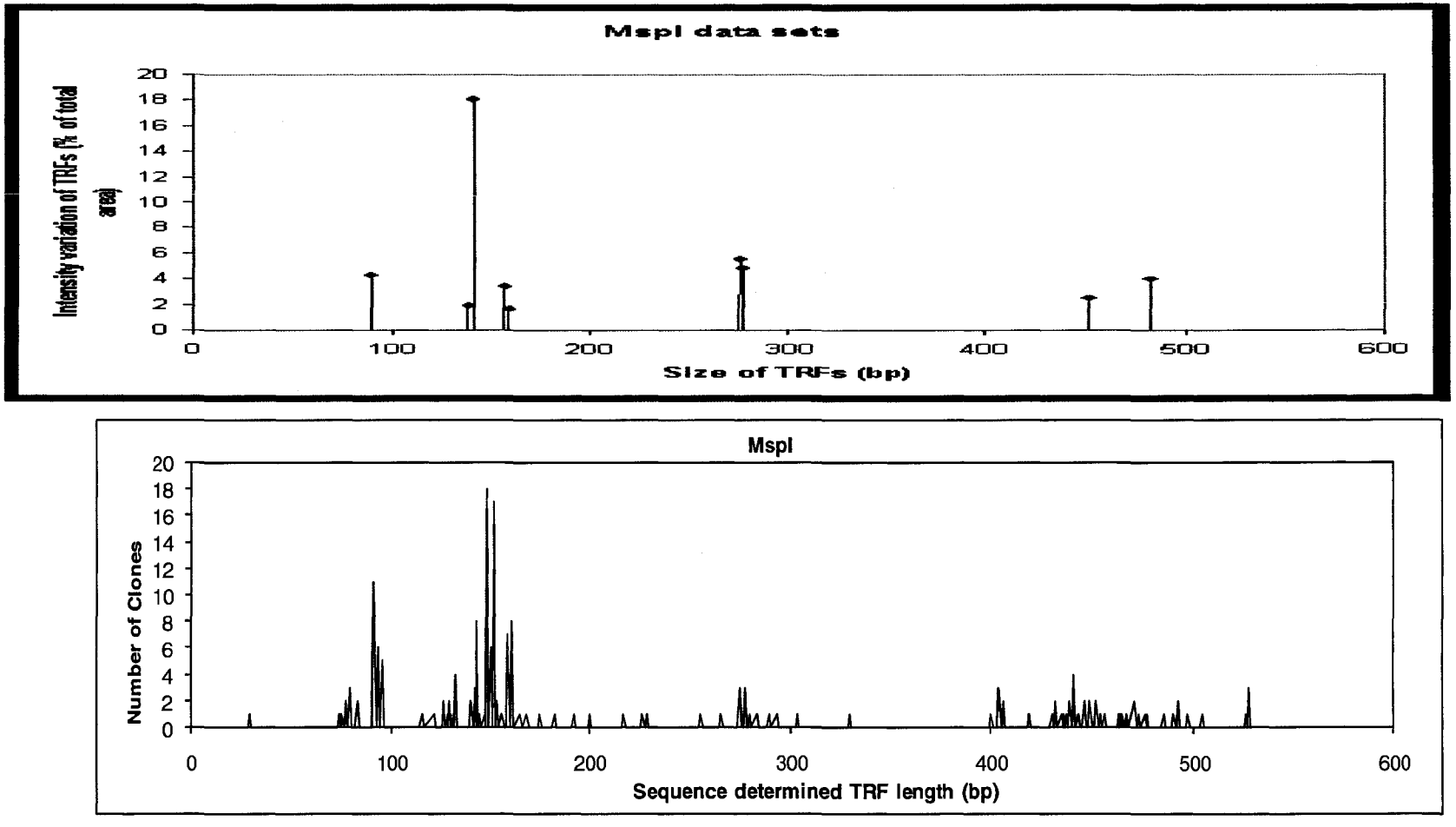
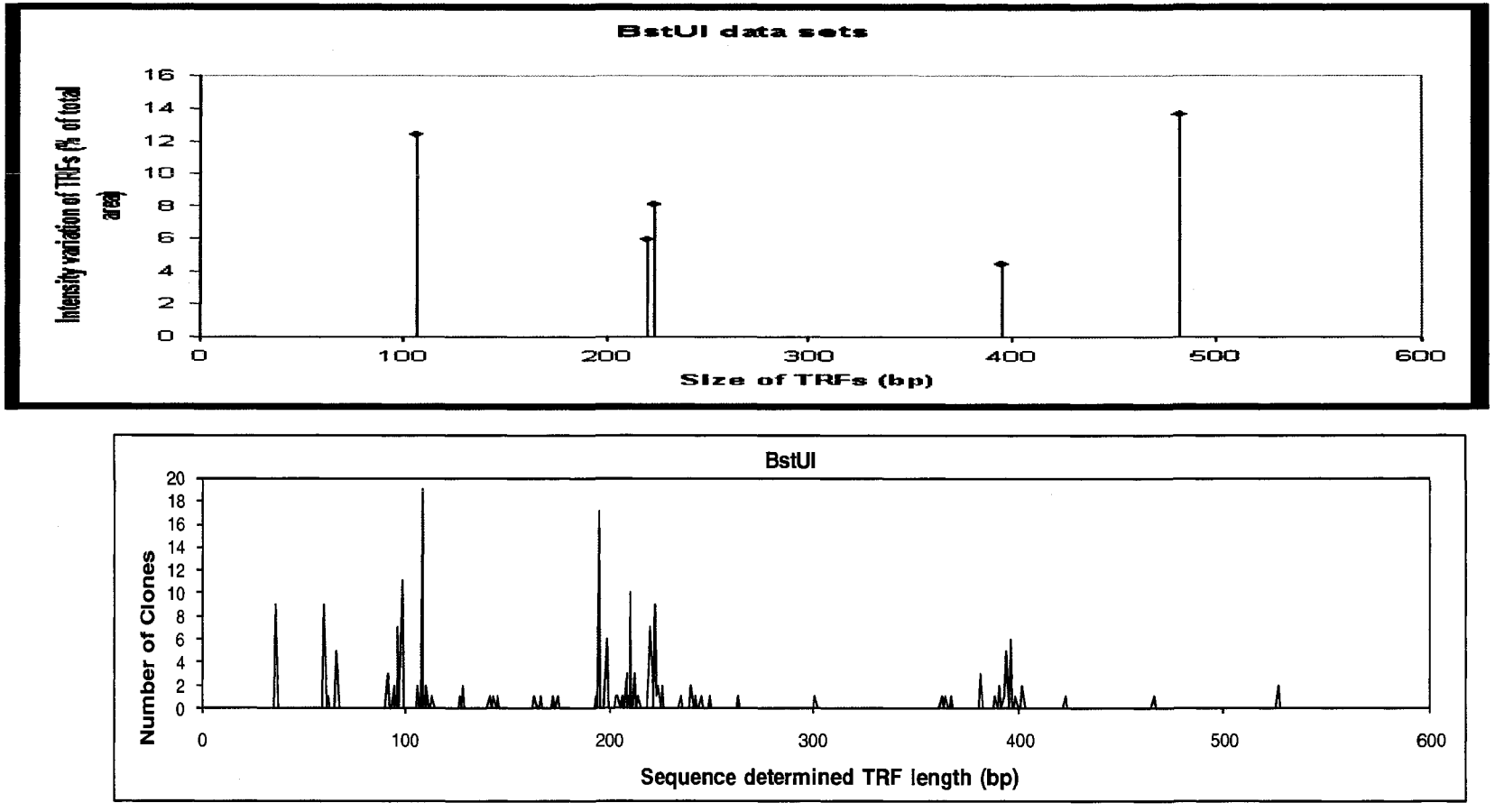


Figure 20. BstUI-generated profiles



5. General conclusions

The present study focused on identification of bacteria responsible for the major increased T-RFs in T-RFLP profiles derived from hydrogen-treated soil samples compared with air-treated soil samples.

The T-RFLP profiles from soils treated by H2 are significantly different from T-RFLP profiles of soil treated by air under the same conditions in the laboratory. It was concluded that hydrogen metabolism resulted in pronounced variation of bacterial community structure in hydrogen-treated soil samples compared to air-treated soil samples.

A comparison between community T-RFLP profiles of hydrogen-treated soil samples and in silico T-RFLP profiles of the same soil samples revealed very similar patterns and distributions of predominant peaks, indicating that community T-RFLP analysis manages to capture real soil microbial community structures and diversities. It can be inferred that the T-RFLP fingerprinting method does have the ability to reflect the diversity and structure of numerically dominant bacterial populations within microbial communities.

Sequencing of the 16S rRNA gene library derived from hydrogen-treated soil samples revealed that clones affiliated with the Actinobacteria division are the most abundant, comprising about 19% of all clones. This suggests that the Actinobacteria group becomes an important member of the soil bacterial community following hydrogen treatment.

Computer-simulated T-RFLP analysis of each clone sequence from the 16S rRNA gene library and BLAST showed most clones associated with the major increased T-RFs in community T-RFLP profiles of hydrogen-treated soil compared with air-treated soil to be members of the Actinobacteria. It was concluded that the predominant bacterial genera contributing to the major increased T-RFs in T-RFLP profiles of hydrogen-treated soil might belong to the Actinobacteria. Further studies into the mechanisms of hydrogen uptake in the Actinobacteria and their potential effects on plant growth promotion may prove promising in the search for non-chemical fertilizers in the future.

6. References cited

Alikhani, H. A., Saleh-Rastin, N. and Antoun, H. 2006. Phosphate solubilisation activity of rhizobia native to Iranian soils. Plant Soil. 287: 35-41.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403– 410.

Alvey, S., Yang, C-H., Buerkert, A, Crowley, D. E. 2003. Cereal/legume rotation effects on rhizosphere bacterial community structure in west african soils. Biol Fertil Soils.37: 73-82

Alexander, M. 1977. Introduction to soil microbiology, 2nd ed. John Wiley and Sons, New York, N.Y.

Aragno M and Schlegel H. G.1981. The hydrogen-oxidizing bacteria. In: Starr MP, Stolp H, Trüper H.G., Balows A., Schlegel H.G. (eds) The prokaryotes, a handbook on habitats, isolation, and identification of bacteria. Springer, Berlin Heidelberg New York, pp 865–893

Aragno, M. and Schlegel, H. G.1992. The mesophilic hydrogenoxidizing (Knallgas) bacteria. In The Prokaryotes, 2nd edn. Edited by A. Balows, H. G. Triiper, M. Dworkin, W.Harder & K.-H. Schleifer. New York: Springer Verlag, pp 344-384.

Avaniss-Aghajani, E., Jones, K., Holtzman, A., Aronson,T., Glover, N., Boian, M., Froman, S. and Brunk, C. F. 1996. Molecular technique for rapid identification of mycobacteria. J. Clin. Microbiol. 34: 98–102.

Bagayoko, M., Buerkert, A., Lung, G., Bationo, A. and Rmheld, V. 2000b. Cereal/legume rotation effects on cereal growth in Sudano-Sahelian West Africa: soil mineral

nitrogen, mycorrhizae and nematodes. Plant Soil 218:103–116

Baker, D. and Selig, E. 1984. Frankia: new light on an actinomycete symbiont. In Biological, Biochemical and Biomedical Aspects of Actinomycetes .Edited by L. Ortiz-Ortiz, L. Bojalil & V. Yakoleff. New York: Academic Press. pp. 563-574.

Baldock, J. O., Higgs R. L., Paulson W. H., Jackobs J. A. and Shrader W. D. 1981. Legume and mineral N effects on crop yields in several crop sequences in the Upper Mississippi Valley. Agron. J. 73: 885–890

Barea, J. M., Azcon, R.and Azcon-Aguilar, C. 2004. Mycorrhizosphere fungi and plant growth promoting rhizobacterial. In: Plant surface microbiology. Varma, A., Abbott, L, Werner, D., Hampp, R. eds. Springer-Verlag. pp: 351-371

Bardgett, R. D. 2002. Causes and consequences of biological diversity in soil. Zoology 105: 367–374.

Bashan, Y. and Holguin, G. 1998. Proposal for the division of plant growth promoting rhizobacterial into two classifications: biocontrol-PGPR and PGPR. Soil Biol Biochem 30: 1225-1228.

Bengtsson, J. 1998. Which species? What kind of diversity? Which ecosystem function? Some problems in studies of relations between biodiversity and ecosystem function. Appl. Soil Ecol. 10: 191–199.

Benhamou, N., Belanger, R. R. and Paulitz, T. C. 1996. Induction of differential host responses by Pseudomonas fluorescens in Ri T-DNA-transformed pea roots after challenge with Fusarium oxysporum f. sp. pisi and Pythium ultimum. Phytopathology 86: 114-178.

Blackwood, C. B., Marsh, T., Kim, S-H. and Paul, E. A. 2003. Terminal Restriction Fragment Length Polymorphism Data Analysis for Quantitative Comparison of Microbial Communities. Appl. Environ. Microbiol. 69(2): 926-932.

Bloemberg, G. V. and Lugtenberg, B. J. 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr Opin Plant Biol 4: 343–350

Boddington, C. L. and Dodd, J. C. 2000. The effect of agricultural practices on the development of indigenous arbuscular mycorrhizal fungi. I. Field studies in an Indonesian ultisol. Plant Soil. 218:137–144.

Bollon, H. J., Frederickson, J. K. and Elliot, L. F.1993. Microbial ecology of the rhizosphere. In: Soil Microbial Ecology. Metting, F.B.J., Ed., Marcel Dekker, New York, pp27-63

Bond, P. L., Hugenholtz, P., Keller, J. and Blackall, L. L. 1995. Bacterial community structures of phosphate-removing and non-phosphate-removing activated sludge from sequencing batch reactors. Appl. Environ. Microbiol. 61:1910–1916.

Bowen, G. D. and Rovira, A. D. 1999. The rhizosphere and its management to improve

plant growth. Advance in Agronomy . 66: 1-102

Bowien, B.and Schlegel, H.G. 1981. Physiology and Biochemistry of Aerobic Hydrogen-Oxidizing Bacteria. Annu. Rev. Micro. 35: 405-452

Braker, G., Ayala-del-Rio, H. L., Devol, A. H., Fesefeldt, A. and Tiedje, J. M., 2001. Community structure of denitrifiers, bacteria, and archaea along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment analysis of amplified nitrite reductase (nirS) and 16S rRNA genes. Appl. Environ. Microbiol. 67: 1893–1901.

Brock, T. D. 1987. The study of micro-organisms in situ: progress and problems, Symposium of the Society for General Microbiology 41: 1–17.

Bruce, K.D. 1997. Analysis of mer gene subclasses within bacterial communities in soils and sediment resolved by fluorescent- PCR-restriction fragment length polymorphism profiling. Appl. Environ. Microbiol.63: 4914–4919

Brunk, C. F., Avaniss-Aghajani, E. and Brunk, C.A. 1996. A computer analysis of primer and probe hybridization potential with bacterial small-subunit rRNA sequences. Appl. Environ. Microbiol. 62: 872-879.

Buerkert, A., Piepho, H. –P. and Bationo, A. 2002. Multi-site time-trend analysis of soil fertility management effects on crop production in sub-Saharan West Africa. Experimental Agriculture. 38: 163-183

Bullock, D. G.1992. Crop Rotation. Crit. Rev. Plant Sci. 11(4): 309-326

Bustin, S.A. 2000. Absolute quantification of mRNA using real time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25: 169–193

Buyer, J. S., Roberts, D. P. and Russek-Cohen, E. 1999. Microbial community structure and function in the spermosphere as affected by soil and seed type. Can. J. Microbiol. 45: 138–144.

Cammack, R., Fernandez, V. M. and Hatchikian, E. C. 1994. Nickel-iron hydrogenase. Methods Enzymol. 243: 43-68

Cammack, R., Frey, M., and Robson, R. 2001. Hydrogen as a fuel. Taylor & Francis, London and New York

Cariello, N. F., Thilly, W. G., Swenberg, J. A. and Skopek, T. R. 1991. Deletion mutagenesis during polymerase chain reaction: dependence on DNA polymerase. Gene 99: 105-108.

Carrillo-Castañeda, G., Juárez Muños, J., Peralta-Videa, J.R., Gomez, E., Tiemannb, K. J., Duarte-Gardea, M. and Gardea-Torresdey, J. L. 2002. Alfalfa growth promotion by bacteria grown under iron limiting conditions. Adv. Environ. Res. 6: 391–399

Chakraborty, U., Chakraborty, B.and Basnet, M. 2006. Plant growth promotion and induction of resistance in Camellia sinensis by Bacillus megaterium. J. Basic

Microbiol. 3: 186-195.

Chandler, D.P., Li, S., Spadoni, C.M., Drake, G.R., Balkwill, D.L., Fredrickson, J. K.and Brockman, F. J. 1997. A molecular comparison of culturable aerobic heterotrophic bacteria and 16S rDNA clones derived from a deep subsurface sediment. FEMS Microbiol. Ecol. 23:131-144.

Chen, C., Bélanger, R. R., Benhamou, N. and Paulitz, T. C. 2000. Defense enzymes induced in cucumber roots by treatment with plant growth-promoting rhizobacteria (PGPR) and Pythium aphanidermatum. Physiol. Mol. Plant Pathol. 56:13-23.

Clement, B.G., Kehl, L.E., DeBord, K.L. and Kitts, C.L. 1998. Terminal restriction fragment patterns [TRFPs], a rapid, PCR-based method for the comparison of complex bacterial communities, J Microbiol Methods 31: 135–142.

Clement, B.and.Kitts, C.L. 2000. Isolating PCR quality DNA from human feces with a soil DNA kit. BioTechniques 28: 640-646

Conrad, R. and Seiler, W. 1979. The role of hydrogen bacteria during the decomposition of hydrogen by soil FEMS Microbiol. Lett., 6: 143–145

Conrad, R. 1988. Biogeochemistry and ecophysiology of atmospheric CO and H2. Adv Microb Ecol 10:231–384

Cunningham,S. D., Kapulnik, Y., Phillips, D. A. 1986. Distribution of hydrogen-metabolizing bacteria in alfalfa field soil. Appl Environ Microbiol 52:1091–1095

Curci, M., Pizzigallo, M. D. R., Crecchio, C., Mininni, R. and Ruggiero, P. 1997. Effects of conventional tillage on biochemical properties of soils. Biol Fertil Soils 25: 1–6.

Curtis, T. P., Sloan, W. T. and Scannell, J. C. 2002. Estimating prokaryotic diversity and its limits. Proc. Natl. Acad. Sci. USA 99:10494-10499.

Curtis, T.P. and Sloan, W.T. 2004. Prokaryotic diversity and its limits: microbial community structure in nature and implifications for microbial ecology. Curr. Opin Micro. 7: 221-226

Dandurand, L. M. and Knudsen, G. R. 1993. Influence of Pseudomonas fluorescens on hyphal growth and biocontrol activity of Trichoderma harzianum in the spermosphere and rhizosphere of pea. Phytopathology 83: 265–270

Daniels, L., Bakhiet, N. and Harmon, K. 1985. Evidence for the widespread distribution in Streptomyces and related organisms of the electron transferring cofactor, 5-deaza£avin.Syst. Appl. Microbiol. 6: 12-17.

Danovaro, R., Luna, G. M., Dell'anno, A.and Pietrangeli, B. 2006. Comparison of two fingerprinting techniques, terminal restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis, for determination of bacterial diversity in aquatic environments. Appl. Environ. Microbol. 72: 5982–5989

Davis, K. E. R., Joseph, S. J. and Janssen, P. H. 2005. Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. Appl. Environ. Microbiol. 71: 826-834.

Défago, G. 1993. 2,4-Diacetylphloroglucinol, a promising compound in biocontrol. Plant Pathol. 42: 311-312

Dey, R.,Pal, K. K., Bhatt, D. M.and Chauhan, S. M. 2004. Growth promotion and yield enhancement of peanut (Arachis hypogaea L.) by application of plant growth-promoting rhizobacteria. Microbiol. Res. 1(59): 371-394.

Di Cello, F., Bevivino, L., Chiarini, R., Fani, R., and Paffetti, D. 1997. Biodiversity of a Burkhilderia cepacia population isolated from the maize rhizosphere at different plant growth stages. Appl. Environ. Microbiol. 63: 4485-4493.

Diviacco, S., Norio, P., Zentilin, L., Menzo, S., Clememti, M., Biamonti, G., Riva, S., Falaschi, A. and Giacca , M. 1992. A novel procedure for quantitative polymerase chain reaction by coamplification of competitive templates. Gene 122: 313–320.

Dixon, R. O. D. 1967. Hydrogen uptake and exchange by pea root nodules. Ann. Bot. N.S. 31: 179–188.

Dixon, R. O. D. 1968. Hydrogenase in pea root nodule bacteroids. Arch. Mikrobiol. 62: 272–283.

Domenech, J., Ramos, S. B., Probanza, A., Lucas, G. J. A. and Gutierrez, M. F. J. 2007. Elicitation of systemic resistance and growth promotion of Arabidopsis thaliana by PGPRs from Nicotiana glauca: a study of the putative induction pathway. Plant Soil. 290: 43-50

Dong, Z. and Layzell, D. B. 2001. H_2 oxidation, O_2 uptake and CO_2 fixation in hydrogen treated soils. Plant Soil. 229: 1-12.

Dong, Z. and Layzell, D.B. 2002. Why do legume nodules evolve hydrogen gas? In Nitrogen Fixation Global Perspectives ed.

Dong, Z., Wu, L., Kettlewell, B., Caldwell, C. D. and. Layzell, D. B. 2003. Hydrogen fertilization of soils – is this a benefit of legumes in rotation? Plant Cell Environ. 26: 1875-1879.

Doran, J. W. and Zeiss, M. R. 2000. Soil health and sustainability: managing the biotic component of soil quality. Appl. Soil. Ecol. 15: 3-11

Duffy, B. K. 2001. Competition. In O. C. Maloy and T. D. Murray (ed.), Encyclopedia of plant pathology. John Wiley & Sons, Inc., New York, N.Y. p. 243-244.

Dugnani, L, Wyrsch, I, Gandolla, M and Aragno, M. 1986. Biological oxidation of hydrogen in soils flushed with a mixture of H2, CO_2, O_2, and N_2. FEMS Microbiol Lett 38: 347–351.

Duijff, B. J., Gianinazzi-Pearson, V.and Lemanceau, P. 1997. Involvment of the outer

membrane lipopolysaccharides in the endophytic colonization of tomato root by biocontrol Pseudomonas fluorescens strain WC417r, New Phytol.135: 325-334.

Dunbar, J., Barns, S. M., Ticknor, O. L. and Kuske, C. R. 2002. Empirical and theoretical bacterial diversity in four Arizona soils. Appl. Environ. Microbiol. 68:3035–3045.

Dunbar, J., Ticknor, L. O. and Kuske, C. R. 2000. Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl. Environ. Microbiol. 66:2943-2950.

Ebert, S., Rieger, P. G. and Knackmuss, H. J. 1999. Function of coenzyme F420 in aerobic catabolism of 2,4,6-trinitrophenol and 2,4-dinitrophenol by Nocardioides simplex FJ2-1A. J. Bacteriol. 181: 2669-2674

Egert, M. and Friedrich, M. W. 2003. Formation of pseudoterminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. Appl. Environ. Microbiol. 69: 2555–2562.

Elliott, L.F. and Scott, D.E. 1997. Influence of no-till cropping systems on microbial relationships. Advances in Agronomy. 60: 121-147

Eirich, L. D., Vogels, G. D. and Wolfe, R. S. 1979. Distribution of coenzyme F420 and properties of its hydrolytic fragments. J. Bacteriol. 140: 20–27.

Eisbrenner, G., and Evans, H. J. 1983. Aspects of hydrogen metabolism in nitrogen-fixing legumes and other plant-microbe associations. Annu. Rev. Plant Physiol. 34:105-137.

Erdman, L. W., Johnson, H. W. and Clark, F. 1956. A bacterial-induced chlorosis in the Lee soybean, Plant Dis Rep 40: 646.

Esitken, A., Pirlak, L., Turan, M. and Sahin, F. 2006. Effects of floral and foliar application of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrition of sweet cherry, Sci. Hort. 110: 324–327.

Evans, H. J., Purohit, K., Cantrell, M. A., Eisbrenner, G.and Russel, S. A. 1981. Hydrogen losses and hydrogenases in nitrogen-fixing organisms, In A. H.Gibson and W. H.Newton (ed.), Current Perspectives in Nitrogen Fixation. Elsevier/North Holland, New York. p.84-96.

Farag, M. A., Ryu, G. M., Sumner, L. W. and Paré, P. W. 2006. GC-MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. Phytochemistry. 67: 2262-2268.

Farrelly, V., Rainey, F.A and Stackebrandt, E. 1995. Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl. Environ. Microbiol. 61:2798–2801.

Fauque, G., Peck, H. D., Jr., Moura, J. J. G., Huynh, B. H., Berlier, Y., DerVartanian, D. V., Teixeira, M., Przybyla, A. E., Lespinat, P. A., Moura, I. and LeGall, J. 1988. The three classes of hydrogenases from sulfatereducing bacteria of the genus

Desulfovibrio. FEMS Microbiol. Rev. 4: 299-344.

Fortin, N.Y, Mulchandani, A and Chen, W. 2001. Use of real-time polymerase chain reaction and molecular beacons for the detection of Escherichia coli O157: H7. Anal. Biochem. 289: 281–288.

Friedrich, C.G., Friedrich. B. and Bowien, B. 1981. Formation of enzymes of autotrophic metabolism during heterotrophic growth of Alcaligenes eutrophus. J. Gen. Microbiol.122: 69-78.

Frostegard, A., Courtois, S.,Ramissse, V., Clerc, S., Bernillon, D., Le Gall, F., Jeannin, P., Nesme, X. and Simonet, P. 1999. Quantification of bias related to the extraction of DNA from soils, Appl. Environ. Microbiol. 65: 5409–5420.

Fyson, A and Oaks, A. 1987. Physical factors involved in the formation of soil sheaths on corn seedling roots. Can. J. Soil Sci. 67: 591–600.

Gadkari, D., Schricker, K., Acker, G., Kroppenstedt, R. M. and Meyer, O. 1990. Streptomyces thermoautotrophicus sp. nov., a thermophilic CO- and H_2-oxidizing obligate chemolithoautotroph. Appl. Environ. Microbiol. 56: 3727–3734.

Garbeva, P., van Veen, J. A.and van Elsas, J. D. 2004. Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annu. Rev. Phytopathol. 42: 243-270.

Gelfand, D.H. 1992. Taq DNA polymerase, PCR technology.In: Principles and Applications for DNA Amplication (Erlich, H.A., Ed.), 17-22. Freeman and Company, New York.

Gerhardson, B. 2002. Biological substitutes for pesticides. Trends Biotechnol. 20: 338-343.

Giri, B., Giang, P. H., Kumari, R., Prasad, R.and Varma, A. 2005. Microbial diversity in soil. In: Microorganisms in soils: roles in genesis and functions. Buscot, F., Varma, S. (eds). Springer-Verlag. pp: 195-212.

Girvan, M. S., Bullimore, J., Pretty, J. N., Osborn, A.M.and Andrew, S. B. 2003. Soil Type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Appl. Environ. Microbiol. 69:1800-1809.

Giulietti, A, Overbergh, L, Valckx, D, Decallonne, B, Bouillon, R and Mathieu, C 2001. An overview of real-time quantitative PCR applications to quantify cytokine gene expression. Methods 25: 386–401.

Glick, B.R. 1999. Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria. Glick, B.R., Patten, C.N., Holguin, G., Penrose, D.M., editors. London: Imperial College Press; pp. 1–13.

Goodfellow, M. 1992. The family Nocardiaceae. In: The Prokaryotes (Balows. A., Triiper. H.G., Dworkin, M.. Harder. W. and Schleifer. K.-H., Eds.), 2nd Edn.. Springer

Verlag, New York.2: 1188-1213.

Graff, A., and Conrad, R. 2005. Impact of flooding on soil bacterial communities associated with poplar (Populus sp.) trees. FEMS Microbiol Ecol 53: 401–415.

Granastein, D. M., Bezdicek, D. F, Cochran, V. L., Elliott, L. F. and Hammel, J. 1987. Long-term tillage and rotation effects on soil microbial biomass, carbon, and nitrogen. Biol Fertil Soils 5: 265–270.

Gras, N.S.B. 1925. History of Agriculture. F.S.Crofts Publisher, New York, USA.

Grayston, S. J., Wang, S., Campbell, C.D., Edwards, A.C. 1998. Selective influence of plant species on microbial diversity in rhizosphere. Soil Biol. Biochem. 30: 369-378.

Groffman, P. M., Howard, G., Gold, A. J. and Nelson, W. M., 1996. Microbial nitrate processing in shallow groundwater in a riparian forest. J. Environ.l Qual. 25:1309–1316.

Grüter, D., Schmid, B. and Brandl, H. 2006. Influence of plant diversity and elevated atmospheric carbon dioxide levels on belowground bacterial diversity. BMC Microbiol. 6:68.

Gyamfi, S., Pfeifer, U., Stierschneider, M., and Sessitch, A. 2002. Effects of transgenic glucosinate-tolerant oilseed rape and the associated herbicide application on eubacterial and Pseudomonas communities in rhizosphere. FEMS Microbiol. Ecol. 41: 181-190.

Hackl, E., Zechmeister-Boltenstern, S., Bodrossy, L. and Sessitsch, A. 2004. Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. Appl. Environ. Microbiol. 70:5057-5065.

Harrison, N.G., Allan, J.D., Colwell, R.K., Futuyma, D.J. and Howell, J. 1968. The relationship between species diversity and stability: an experimental approach with protozoa and bacteria. Ecology 1091–101.

Hartmann, M., Frey, B., Kölliker, R.and Widmer, F. 2005. Semi-automated genetic analyses of soil microbial communities: comparison of T-RFLP and RISA based on descriptive and discriminative statistical approaches, J Microbiol Metheods 63: 349–360.

Hartmann, M., and Widmer, F. 2006. Community structure analyses are more sensitive to differences in soil bacterial communities than anonymous diversity indices. Appl. Environ. Microbiol.

Hartmann, M. and Widmer, F. 2008. Reliability for detecting composition and changes of microbial communities by T-RFLP genetic profiling, FEMS Microbiol. Ecol. 63: 249–260.

Hesterman, O.B., Sheaffer, C.C., Barnes, D.K., Lueschen, W.E. and Ford, J.H. 1986. Alfalfa dry matter and nitrogen production, and fertilizer nitrogen response in

legume-corn rotations. Agron. J. 78: 19–23.

Hoch, G. E., Little, H. N.and Burris, R. H. 1957. H_2 evolution from soybean root nodules. Nature (London) 179: 430-431.

Hontzeas, N., Hontzeas, C. E. and Glick, B. R. 2006. Reaction mechanisms of thebacterial enzyme 1-aminocyclopropane-1-carboxylate deaminase. Biotech. Adv.24: 420-426.

Hill, G. T., Mitkowski, N. A., Aldeich-Wolfe, L., Emele, L. R., Jurkonie, D. D. 2000. Methods for assessing the composition and diversity of soil microbial communities. Appl. Soil Ecol. 15: 25–36.

Hugenholtz, P.,Goebel, B.M and Pace, N.R. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180: 4765–4774.

Hugenholtz, P., Tyson, G. W., Webb, R. I., Wagner, A. M. and Blackall, L. L. 2001. Investigation of candidate division TM7, a recently recognized major lineage of the domain bacteria with no known pure culture representatives. Appl. Environ. Microbiol. 67:411-419.

Hunt, S and Layzell, D.B. 1993. Gas exchange of legume nodules and the regulation of nitrogenase activity. Annu Rev Plant Physiol Mol Biol 44: 483-511.

Hunter, W. J. 1993. Ethylene production by root nodules and effect of ethylene on nodulation in glycine max. Appl. Environ. Microbiol. 59(6): 1947-1950.

Ishii, K. and Fukui, M. 2001.Optimization of Annealing Temperature To Reduce Bias Caused by a Primer Mismatch in Multitemplate PCR. Appl. Environ. Microbiol. 67(8): 3753-3755.

Jacobson, F. S., Daniels, L., Fox, J., Walsh, C. T. and Orme-Johnson, W. H. 1981. Purification and properties of an 8-hydroxy-5-deaza£avin-reducing hydrogenase from Methanobacterium thermoautotrophicum. J. Biol. Chem. 257: 3385-3388.

Jaeger, C. H., Lindow, S. E., Miller, W., Clark, E. and Firestone, M. 1999. Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. Appl. Environ. Microbiol. 65:2685-2690.

Janssen, P.H. 2006. Identifying the Dominant Soil Bacterial Taxa in Libraries of 16S rRNA and 16S rRNA Genes. Appl and Environ Microbiol. 72:1719-1728.

Janssen, P.H.,Yates, P.S., Grinton, B.E.,Taylor, P.M.and Sait, M. 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. Appl. Environ. Microbiol. 68: 2391–2396.

Jensen, V.1962. Studies on the microflora of Danish beech forest soils. I. The dilution plate count technique for the enumeration of bacteria and fungi in soil. Zentbl. Bakteriol. Parasitenkd. Abt. 2 116: 13–32.

Jensen, V. 1968. The plate count technique. In T. R. G. Gray and D. Parkinson (ed.), The ecology of soil bacteria. Liverpool University Press, Liverpool, United Kingdom. pp:158–170.

Johnsen, K., Samuelson, L., Teskey, R., McNulty, S. and Fox, T. 2001. Process models as tools in forestry research and management. For. Sci. 47: 2–8.

Johnson, P.R. and Ecker, J.R. 1998. The ethylene gas signal transduction pathway: a molecular perspective. Ann. Rev. Genetics: 32: 227-254.

Joseph, S.J., Hugenholtz, P., Sangwan, P., Osborne, C.A. and Janssen, P.H. 2003. Laboratory cultivation of widespread and previously uncultured soil bacteria. Appl. Environ. Microbiol. 69: 7210–7215.

Kaiser, O., Puhler, A., and Selbitschka, W. 2001. Phylogenetic analysis of microbial diversity in the rhizoplane of oilseed rape employing cultivation-dependent and cultivation-independent approaches. Micro. Ecol. 42: 136-49.

Kathryn, E. R. Davis, Shayne J. Joseph, and Peter H. Janssen. 2005. Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. Appl. Environ. Microbiol. 71: 826-834.

Kaplan, C.W., Astaire, J.C., Sanders, M.E., Reddy, B.S. and Kitts, C.L., 2001. 16S Ribosomal DNA terminal restriction fragment pattern analysis of bacterial communities in feces of rats fed Lactobacillus acidophilus NCFM. Appl. Environ. Microbiol. 67:1935–1939.

Kaplan, C.W. and Kitts, C.L. 2003. Variation between observed and true Terminal Restriction Fragment length is dependent on true TRF length and purine content.J. Microbiol. Meth. 54: 121-125.

Kennedy, N., Brodie, E., Connolly, J. and Clipson, N. 2004. Impact of lime, nitrogen and plant species on bacterial community structure in grassland microcosms. Environ. Microbiol. 6: 1070-1080.

Kennedy, A.C. and Smith, K.L. 1995. Soil microbial diversity and the sustainability of agricultural soils. Plant and Soil. 170: 75-86.

Keyser, H., Weber, D. F. and Uratsu, S. L. 1984. Rhizobium japonicum serogroup and hydrogenase phenotype distribution in 12 states. Appl. Environ. Microbiol. 47: 613-615.

Kieber, J. J. 2002. Cytokinins. In CR Somerville, EM Meyerowitz, eds, [www.aspb.org/publications/arabidopsis/ The Arabidopsis Book]. American Society of Plant Biologists, Rockville, MD, doi: 10.1199/tab.0009.

Kim, B. S., Moon, S. S., and Hwang, B. K. 1999. Isolation, identification and antifungal activity of a macrolide antibiotic, oligomycin A, produced by Streptomyces libani. Can. J. Bot. 77: 850-858.

Kirk, J. L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee, H. and Trevors, J. T. 2004. Methods of studying soil microbial diversity. J. Microbiol Meth.58: 169-188.

Kitts, C.L., 2001. Terminal restriction fragment patterns, a tool for comparing microbial communities and assessing community dynamics. Curr. Issues Intest. Microbiol. 2: 17–25.

Kloepper, J. W., Zablotowick, R. M., Tipping, E. M., and Lifshitz, R. 1991. Plant growth promotion mediated by bacterial rhizosphere colonizers. In: The rhizosphere and plant growth. Keister, D.L., Cregan, P.B., (eds). Dordrecht, Netherlands: Kluwer Academic Publishers, 315-326.

Kloepper, J.W. 1994. Plant growth promoting rhizobacterial (other system). In: Azospirillum/plant associations. Y. Okon, (eds.). Boca Raton, FL. USA: CRC Press, 111-118.

Kloepper, J. W., Ryu, C. M.and Zhang, S.A. 2004. Induced systemic resistance and promotion of plant growth by Bacillus spp. Phytopathology 94: 1259-1266.

Kluber, H. D., Lechner, S. and Conrad, R. 1995. Characterization of populations of aerobic hydrogen-oxidizing soil bacteria. FEMS Microbiology Ecology. 16:167-176.

Knox, C. M., Cevellos, V., Margolis, T. P. and Dean, D. 1999. Identification of bacterial pathogens in patients with endophthalmitis by 16S ribosomal DNA typing. Am. J. Ophthalmol. 128: 511-512.

Klüber, H. D., Lechner, S. and Conrad, R. 1994. Characterization of populations of aerobic hydrogen-oxidizing soil bacteria. FEMS Micro. Ecol.16: 167-176.

Krassilnikov, N. A. 1938. Ray fungi and related organisms, Actinomycetales. Akademiya Nauk SSSR, Moscow.

Kruske, C. R., Barn, S. M., and Busch, J. D. 1997. Divers uncultivated bacterial groups from soils of arid southwestern United States that are present in many geographic regions. Appl. Eviron. Microbiol. 63: 3614-3621.

Kuo, M.-S., Yurek, D. A., Coats, J. H. and Li, G. P. 1989. Isolation and identification of 7,8-didemethyl-8-hydroxy-5-deazaribo£avin, an unusual cosynthetic factor from Streptomyces lincolnensis. J. Antibiotics 42: 475-478.

Kuske, R. C., Ticknor, L. O., Miller, M. E. and Dunbar, J. M. 2002. Comparison of soil bacterial communities in rhizospheres of three plant species and the interspaces in an arid grassland. Appl. Environ. Microbiol. 68:1854-1863.

La Favre, J. S. and Focht, D. D. 1983. Conservation in soil of H_2 liberated from N_2 fixation by HUP- nodules. Appl Environ Microbiol. 46:304-311 .

Lechner, S. and Conrad, R. 1997. Detection in soil of aerobic hydrogen-oxidizing bacteria related to Alcaligenes eutrophus by PCR and hybridization assays targeting the gene

of the membrane-bound (NiFe) hydrogenase. FEMS Microbiol Ecol. 22: 193-206.

Liesack, W., Janssen, P. H., Rainey, F. A., Ward-Rainey, N. L. and Stackebrandt, E. 1997. Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques, p. 375–439.

Lindblad P and Sellstedt A. 1989. Immunogold localization of hydrogenase in free-living Frankia CpI1. FEMS Microbiol Lett 60: 311-316.

Liu, W.T., Marsh, T.L., Cheng, H., and Forney, L.J. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S r RNA. Appl. Environ. Microbiol. 63: 4516-4522.

Liu, W.T, Marsh, T. L. and Forney, L. J. 1998. Determination of the microbial diversity of anaerobic-aerobic activated sludge by a novel molecular biological technique. Water Sci. Technol. 37: 417-422.

Loper, J. E.and Henkels, M. D. 1999. Utilization of heterologous siderophores enhances levels of iron available to Pseudomonas putida in the rhizosphere. Appl. Environ. Microbiol. 65:5357–5363.

Loper, J. E., Schroth, M. N. 1986. Phytopathology. 76: 386–389.

Ludemann, H., Arth, I. and Liesack, W. 2000. Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy soil cores. Appl. Environ. Microbiol. 66:754-762.

Lueders, T., and Friedrich, M. 2000. Archaeal population dynamics during sequential reduction processes in rice field soil. Appl. Environ.Microbiol. 66: 2732-2742.

Lueders, T. and Friedrich, M. W. 2003. Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and mcrA genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. Appl. Environ. Microbiol. 69:320-326.

Lugtenberg, B.J.J., de Weger, L.A. and Bennett, J.W. 1991. Microbial stimulation of plant growth and protection from disease. Current Opinion in Biotechnology. 2:457-464.

Lupwai, N.Z.; W.A. 1998. Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. 1998. Soil Biol Biochem. 30:1733-1741.

Lynch, J. M. 1990. Microbial metabolites. In The Rhizosphere. Ed. J MLynch. pp 177–206. John Wiley, Chichester.

MacDonald, E. M. S, Powell, G. K., Regier, D.A., Glass, N. L., Roberto, F., Kosuge, T., Morris, R. O. 1986. Secretion of Zeatin, Ribosylzeatin, and Ribosyl-1" -Methylzeatin by Pseudomonas savastanoi: Plasmid-Coded Cytokinin Biosynthesis Plant Physiol. 82:742–747.

Macdonald, S. E., Reid, D. M. and Chinnappa, C. C.1986. Studies on the Stellaria longipes complex: phenotypic plasticity. II Gibberellins, abscisic acid, and stem

elongation. Can. J. Bot. 64: 2617–2621.

Maier, R.J., Campbell, N.E.R., Hanus, F.J., Simpson, F.B.,Russell, S.A. and Evans, H.J. 1978. Expression of hydogenase activity in free-living Rhizobium japonicum. Proc. Natl. Acad. Sci. USA 75, 32583262.

Maimaiti, J., Zhang, Y., Yang, J., Cen, Y.-P., Layzell, D. B., Peoples, M.and Dong, Z. 2007. Isolation and characterization of hydrogen-oxidizing bacteria induced following exposure of soil to hydrogen gas and their impact on plant growth. Environ. Microbiol. 9(2): 435-444.

Maloney, P.E., Van Bruggen, A.H.C., and Hu, S. 1997. Bacterial community structure in relation to the carbon environments in lettuce and tomato rhizospheres and bulk soil. Micro. Ecol. 34: 109-117.

Marcel G, Van Der Heijden, A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, R., Wiemken, A., Sanders, I.R. 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396: 69–72.

Marschner, P., Kandeler, E. and Marschner, B. 2003. Structure and function of the soil microbial community in a long-term fertilizer experiment, Soil Biol Biochem. 35: 453–461.

Marsh, T.L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Curr. Opin. Microbiol. 2: 323-7.

Matsumoto, M., Sakamoto, M., Hayashi, H., and Benno, Y. 2005. Novel phylogenetic assignment database for terminal-restriction fragment length polymorphism analysis of human colonic microbiota. J. Microbiol. Methods 61: 305-319.

McCaig, A. E., Glover, L. A. and Prosser, J. I. 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl. Environ. Microbiol. 65:1721–1730.

McCormick, J. R. D., and Morton, G. O. 1982. Identity of cosynthetic factor 1 of Streptomyces aureofaciens and fragment FO from coenzyme F420 of Methanobacterium sp. J. Am. Chem. Soc. 104: 4014–4015.

McLearn, N. and Dong, Z. 2002. Microbial nature of the hydrogen-oxidizing agent in hydrogen treated soil. Biol. Fertil. Soil. 35: 465-469.

Miller, R. W.and Sirois, J. C. 1982. Relative efficacy of different alfalfa cultivar Rhizobiuim meliloti strain combinations of symbiotic nitrogen fixation. Appl. Environ. Microbiol. 43:764-768.

Milner, J. L., Raffel, S. J., Lethbridge, B. J. and Handelsman, J. 1995. Culture conditions that influence accumulation of zwittermicin A by Bacillus cereus UW85. Appl.

Microbiol. Biotechnol. 43:685-691.

Moeseneder, M. M., Arrieta, J. M., Muyzer, G.,Winter, C. and Herndl, G. J. 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 65:3518–3525.

Molina, L., Constantinescu, F., Reimmann, C., Duffy, B. and Défago, G. 2003. Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. FEMS Microbiol. Ecol. 45:71-81.

Moyer, C. L., Dobbs, F. C. and Karl, D. M. 1994. Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. Appl. Environ. Microbiol. 60:871–879.

Murry, M.A., and Lopez, M.F. 1989. Interaction between hydrogenase, nitrogenase and respiratory activities in a Frankia isolate from Alnus rubra. Can. J. Microbiol. 35: 636–641.

Naraoka, T., Momoi, K., Fukasawa, K. and Goto, M. 1984. Isolation and identification of a naturally occurring 7, 8-dide- methyl-8-hydroxy-5-deazaribo£avin derivative from Mycobacterium avium. Biochim. Biophys. Acta. 797: 377-380.

Noll, M., Matthies, D., Frenzel, P., Derakshani, M. and Liesack, W. 2005. Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. Environ. Microbiol. 7:382–395.

Nordström, A. 2004. Auxin regulation of cytokinin biosynthesis in Arabidopsis thaliana: A factor of potential importance for auxin–cytokinin-regulated development. PNAS 101:8039–8044.

Olsen, R. A., and Bakken, L. R. 1987. Viability of soil bacteria: optimization of plate-counting techniques and comparisons between total counts and plate counts within different size groups. Microb. Ecol. 13:59–74.

Osborn, A. M., Moore, E. R. B. and Timmis, K. N. 1999. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ. Microbiol. 2: 39-50.

Osborne, C. A., Rees, G. N., Bernstein, Y. and Janssen, P. H. 2006. New Threshold and Confidence Estimates for Terminal Restriction Length Polymorphism Analysis of Complex Bacterial Communities. Appl. Environ. Microbiol. 72:1270-1278.

Palleroni, N. J. 1997. Prokaryotic diversity and the importance of culturing. Antonie van Leeuwenhoek 72: 3–19.

Pankhurst, C.E., Ophel-Keller, K., Doube, B. and Gupta, V.V.S.R. 1995. Biodiversity of soil microbial communities in agricultural systems. Biodiversity and Conservation.

Springer Netherlands 5:197-209.

Park, S. S. and DeCicco, B. T. 1974. Autotrophic growth with hydrogen of Mycobacterium gordonae and another scoto-chromogenic mycobacterium. Int. J. Syst. Bact. 24: 338-345.

Paul, E. A., and Clark, F. E. 1996. Soil microbiology and biochemistry, 2nd ed. Academic Press, San Diego, Calif.

Peixoto, R.S., Coutinho, H.L.C., Madari, B., Machado, P.L.O.A., Rumjanek, N.G., Elsas, J.D., Seldin, L. and Rosado, A.S. 2006. Soil aggregation and bacterial community structure as affected by tillage and cover cropping in the Brazilian Cerrados, Soil Till. Res. 90: 16–28.

Persello-Cartieaux, F., Nussaume, L.and Robaglia, C. 2003. Tales from the underground: molecular plant-rhizobacteria interations. Plant, Cell and Environment 26: 189-199.

Phelps, C. D., Kerkhof, L. J., and Young, L. Y. 1998. Molecular characterization of a sulfate-reducing consortium which mineralization benzene. FEMS Microbiol. Ecol. 27: 269-279.

Pierce, F. J. and Rice, C. W. 1988. Crop rotation and its impact on efficiency of water and nitrogen use. In Cropping Strategies for Efficient use of Water and Nitrogen. Ed. WL Hargrove. pp 21–42. ASA Special Publication Number 51. ASA-CSSA-SSSA, Madison, WI. USA.

Polz, M. F. and Cavanaugh, C. M. 1998. Bias in Template-to-Product Ratios in Multitemplate PCR. Appl. Environ. Microbiol. 64: 3724-3730.

Popelier, F., Liessens, J., and Verstraete, W. 1985. Soil hydrogen uptake in relation to soil properties and rhizobial hydrogen production. Plant and Soil. 85: 85-96.

Punvantini, E.and Daniels, L. 1996. Purification of a novel coenzyme F420-dependent glucose-6-phosphate dehydrogenase from Mycohacterium smeginatis, J. Bacteriol. 178: 2861 -2866.

Purwantini, E., and Daniels, L. 1998. Molecular analysis of the gene encoding F420-dependent glucose-6-phosphate dehydrogenase from Mycobacterium smegmatis. J. Bacteriol. 180:2212-2219.

Purwantini, E., Gillis, T. and Daniels, L. 1997. Presence of F420-dependent glucose-6-phosphate dehydrogenase in Mycobacterium and Nocardia species, but absence in Streptomyces and Corynebacterium species and methanogenic Archaea. FEMS Microbiol Lett 146:129–134.

Qiu, X.Y., Wu, L.Y., Huang, H.S., McDonel, P.E., Palumbo, A.V., Tiedje, J.M. and Zhou, J.Z. 2001. Evaluation of PCR-generated chimeras: mutations, and heteroduplexes with 16S rRNA gene-based cloning, Appl. Environ. Microbiol. 67 : 880–887.

Rainey, F.A., Ward, N., Sly, L.I. and Stackebrandt, E. 1994. Dependence on the taxon

composition of clone libraries for PCR amplified, naturally occurring 16S rDNA, on the primer pair and the cloning system used. Experientia 50:796-797.

Rangel-Castro, J. I., Killham, K., Ostle, N., Nicol, G. W., Anderson, I. C., Scrimgeour, C. M., Ineson, P., Meharg, A. and Prosser, J. 2005. Stable isotope probing analysis of the influence of liming on root exudate utilization by soil microorganisms. Environ. Microbiol.7:828-838.

Reh, M. and Schlegel, H. G. 1981. Hydrogen autotrophy as a transferable genetic character of Nocardia opaca lb. J. Gen. Microbial. 126: 327-336.

Roselló-Mora R, Amann R. 2001. The species concept for prokaryotes. FEMS Microbiol Rev 25:36–67.

Ruiz-Argueso, T., Hanus, J. and Evans, H. J. 1978. Hydrogen production and uptake by pea nodules as affected by strains of Rhizobium leguminosarum. Arch. Microbiol. 116:113-119.

Sait, M., Hugenholtz, P. and Janssen, P. H. 2002. Cultivation of globallydistributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. Environ. Microbiol. 4:654–666.

Saravanakumar, D., Vijayakumar, C., Kumar, N.and Samiyappan, R. 2007. PGPR-induced defense responses in the tea plant against blister blight disease. Crop. Prot.26: 556-565.

Schink, B and Schlegel, H. G. 1978. Hydrogen metabolism in aerobic hydrogen-oxidizing bacteria.Biochimie 60: 297–305

Schink, B. and Schlegel, H. G. 1979. The membrane-bound hydrogenase ofAlcaligenes eutrophus. I.Solubilization, purifcation, and biochemical properties. -- Biochim. Biophys. Acta 567:315-324.

Schippers, B., Bakker, A.W., and Bakker, P.A.H.M. 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Annu Rev Phytopathol 25: 339–358.

Schlegel, H.G. and Mayer, M. 1985. Isolation of hydrogenase regulatory mutants of hydrogen-oxidizing bacteria by a colony-screening method. Archives of Microbiology. 141: 377-383.

Schmitt-Wagner, D., Friedrich, M. W., Wagner, B. and Brune, A. 2003. Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (Cubitermes spp.). Appl. Environ. Microbiol. 69:6007-6017.

Schneider, J. and Schlegel, H. G. 1976. Purification and properties of soluble hydrogenase from A lcaligenes eutrophus H 16. Biochim. Biophys. Acta. 452:66-80.

Schubert, K. R and Evans, H. J. 1976. Hydrogen evolution: A major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc. Nat. Acad. Sci.USA.

73: 1207-1211.

Schuler, S. and Conrad, R. 1990. Soil contain two different activities for oxidation of hydrogen. FEMS Microbiology. 73: 77-84.

Schuler, S and Conrad, R. 1991. Hydrogen oxidation activities in soil as influenced by pH, temperature, moisture, and season. Biol Fertil Soils 12:127–130.

Schwartz, E and Friedrich, B. 2006. The H_2-Metabolizing Prokaryotes. Prokaryotes 2:496-563.

Seiler W. 1978. The influence of the biosphere on the atmospheric CO and H_2 cycles, in Environmental Biogeochemistry and Geomicrobiology Ann Arbor Science 3: 773–810.

Seldin, L., Rosado, A. S., da Cruz, D.W., Nobrega, A. and van Elsas, J. D. 1998. Comparison of Paenibacillus azotofixans strains isolated from rhizoplane, rhizosphere, non-associated soil from maize planted in two different Brazilian soils. Appl. Environ. Microbiol. 64: 3860-3868.

Sellstedt, A. and Lindblad, P. 1990. Activities, occurrence, and localization of hydrogenase in free-living and symbiotic Frankia. Plant Physiol 92:809-815.

Semenov, A.M., Van Bruggen, A.H.C., and Zelenev, V.V. 1999. Moving waves of bacterial populations and total organic carbon along roots of wheat. Microb. Ecol. 37: 116-128.

Shah, S., Li, J., Moffatt, B.A. and Glick, B.R. 1998. Isolation and characterization of ACC deaminase genes from two different plant growth-promoting rhizobacteria. Can. J. Microbiol. 44: 833-843.

Sharma, S.B.and MacDonald, D. 1990. Global status of nematode problems of groundnut, pigeonpea, chickpea, sorghum and pearl millet, and suggestions for future work. Crop protection 9: 453-458.

Siebert, P.D. and Larrick, J.W. 1992. Competitive PCR. Nature 359: 557-558.

Smith, S.E. and Read, D.J. 1997. In: Mycorrhizal Symbiosis, Academic Press, London, p605.

Song, B., Kerkhof, L. J.and Haggblom, M. M. 2002. Characterization of bacterial consortia capable of degrading 4-chlorobenzoate and 4-bromobenzoate under denitrifying conditions. FEMS Microbiol. Lett. 213:183-188.

Sugawara, M., Okazaki, S., Nukui, N., Ezura, H., Mitsui, H.and Minamisawa, K. 2006. Rhizobitoxine modulates plant-microbe interactions by ethylene inhibition. Biotech. Adv. 24: 382-388.

Sun, H., Xu, J.,Yang, S., Liu, G., Dai, S. 2004. Plant uptake of aldicarb from contaminated soil and its enhanced degradation in the rhizosphere. Chemosphere. 54:569–574.

Suzuki, M.T. and Giovannoni, S.J. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62: 625-630.

Suzuki, M. T., Rappe, M. S., Haimberger, Z. W., Winfield, H., Adair, N., Strobel, J. and Giovannoni, S. J. 1997. Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Appl. Environ. Microbiol. 63:983-989.

Taiz, L. and Zeiger, E. 1991. Plant Physiology. Benjamin/Cummings Publishing Company, Inc., Redwood City, CA, pp565.

Tilak, K.V.B.R., Ranganayaki, N., Pal, K.K., De, R., Saxena, A.K., Shekhar Nautiyal, C., Shilpi Mittal, Tripathi, A.K., and Johri, B.N. 2005. Diversity of plant growth and soil health supporting bacteria. Current Science. 89: 136-150.

Timmusk, S., Nicander, B., Granhall, U., Tillberg, E. 1999. Cytokinin production by Paenibacillus polymyxa Soil Biol Biochem.31:1847–1852.

Tjepkema, J. D. and Torrey, J. G. 1979. Symbiotic nitrogen fixation in actinomycete-nodulated plants. Bot. Gaz. (Chicargo) Suppl. 140: i-ii.

Torsvik, V., Goksøyr, J. and Daae, F. L. 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. 56:782-787.

Trevors, J. T. 1985. Appl. Microbiol. Biotech. 23:152.

Tzeng, S. F., Wolfe, R. S. and Bryant, M. P. 1975. Factor 420-dependent pyridine nucleotide-linked hydrogenase system of Methanobacterium ruminantium. J. Bacteriol. 121:184-191.

Ueda, Y., Yamamoto, M., Urasaki, T., Arai, H., Ishii, M and Igarashi, Y. 2007. Sequencing and reverse transcription-polymerase chain reaction (RT-PCR) analysis of four hydrogenase gene clusters from an obligately autotrophic hydrogen-oxidizing bacterium, hydrogenobacter thermophilus TK-6. J. biosci. bioeng. 104: 470-475.

Uratsu, S. L., Keyser, H., Weber, D. F. and Lim, S. T. 1982. Hydrogen uptake activity of Rhizobium japonicum from major U.S. soybean production areas. Crop Sci. 22: 600-602.

Uroz, S., D'Angelo-Picard,C., Carlier, A., Elasri, M., Sicot, C., Petit, A., Oger, P., Faure, D. and Dessaux, Y. 2003. Novel bacteria degrading N-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. Microbiology 149:1981-1989.

van Veen, J. A., van Overbeek, L. S., and van Elsas, J. D. 1997. Fate and activity of microorganisms introduced into soil. Mcriobiol. Mol. Bio. Rev. 61: 121-135.

Venter, C. J., Remington, K., Heidelberg, J. F., Halpern, A. L., Rusch, D., Eisen, J. A., Wu, D., Paulsen, I., Nelson, K. E., Nelson, W., Fouts, D. E., Levy, S., Knap, A. H. ,

Lomas, M. W., Nealson, K. H., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., C., P., Rogers, Y.-H. and Smith, H. O. 2004. Environmental genome shotgun sequencing of the Sargasso Sea. Science 304:66-74.

Vignais, P. M., Billoud, B. and Meyer, J. 2001. Classification and phylogeny of hydrogenases. FEMS Microbiol. Rev. 25:455–501.

Vn Elsas J. D. and Trevors J. T. 1997. Modern Soil Microbiology. New York: Marcel Dekker.

von Bodman, S. B., Bauer, W. D. and Coplin, D. L. 2003. Quorum sensing in plant-pathogenic bacteria. Annu. Rev. Phytopathol. 41:455-482.

Wang, M., Ahrne, S., Antonsson, M. and Molin. G. 2004. T-RFLP combined with principal component analysis and 16S rRNA gene sequencing: an effective strategy for comparison of fecal microbiota in infants of different ages. J. Microbiol. Methods 59:53-69.

Wang, G. C.-Y., and Wang, Y. 1996. The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. Microbiology 142:1107–1114.

Wang, G. C.-Y., and Wang, Y. 1997. Frequency of formation of chimeric molecules as a consequence of PCR coamplification of 16S rRNA genes from mixed bacterial genomes. Appl. Environ. Microbiol. 63: 4645–4650.

Whipps, J. M. 2001. Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52:487–511.

Wilson, K. H. and Blitchington, R. B. 1996. Human colonic biota studied by ribosomal DNA sequence analysis. Appl. Environ. Microbiol. 62:2273-2278.

Wintzingerode, F. V., Go¨bel, U. B. and Stackebrandt, E. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microb. Rev. 21:213–229.

Wolf, R. 1977. Organic Farming: Yesterday's and Tomorrow's Agriculture Rodale Press, Emmaus, PA, USA.

Wong, T. Y., Graham, L., O'Hara, E., Maier, R. J. 1986. Enrichment for hydrogen-oxidizing Acinetobacter spp. in the rhizosphere of hydrogen evolving soybean root nodules. Appl Environ Microbiol 52: 1008–1013.

Zhang, Y. 2006. Mechanisms of Isolated Hydrogen-Oxidizing Bacteria in Plant Growth Promotion and Effects of Hydrogen Metabolism on Rhizobacterial Community Structure. M.Sc. Thesis, Saint Mary's University, Halifax, Nova Scotia, Canada.