Bacterial community changes in a paddy soil oxygen gradient, assessed by cultivation and mRNA expression profiling

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Submitted to the faculty of Biology
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By Pravin Malla Shrestha
from Kathmandu, Nepal.

Marburg/Lahn 2007

Pledge

I certify that the present thesis entitled:

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was carried out without any unlawful devices. I did not use any other than the described literature sources or technical devices. This work has never been submitted before in this or similar form to any other university and has not been used before any examination.

Marburg, 10.01.2007

Pravin Malla Shrestha

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I dedicate this work to:

- my wife,
- my parents,
- and those who will find it valuable.

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2) **Shrestha, P. M,** and W. Liesack**.** Bacterial community changes in a paddy soil oxygen gradient, assessed by mRNA expression profiling (in preparation).

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List of Abbreviations

Abbreviations	Full form
%……..	percentage
<……...	less than
>……...	greater than
≥……	greater than or equal to
≤……	less than or equal to
^{14}C……	radiocarbon
A……...	adenine
ANOVA……...	analysis of variance
AO……	acridine orange
ARB…….	Latin, "arbor" = tree
ARDRA……	amplified rDNA restriction analysis
C……..	cytosine
cDNA……...	copy DNA
CFU…….	colony forming unit
cm…….……	centimeter
CTC……..	5-cyano-2, 3-ditolyl tetrazolium chloride
DA……	day of appearance
DAPI……	4', 6-diamidino-2-phenylindole
DD……	double distilled
DFS…….....	differential stain
DGGE……...	denaturing gradient gel electrophoresis
DM.....	dimineralised water
DNA.....	deoxyribonucleic acid
EB.....	elution buffer
ES…….	early succession
FDA……..	fluorescent diacetate
Fig……	figure
FISH…….	fluorescent *in situ* hybridization
G……...	guanine

ha……..	hector
h…….....	hour/hours
i.e……..	that is
kb…….	kilo base
K-strategy…….	carrying capacity
LA.....	late succession
M……..	molar
min.....	minute/minutes
mg…….…	milligram
mm……...	millimeter
mM……...	millimolar
MPN…….	most probable number
NA……	nutrient agar
NCBI……	National Center for Biotechnology Information
ORF.....	open reading frame
rpm.....	revolutions per minute
RT-PCR ……...	reverse transcription-PCR
mRNA……..	messenger RNA
rrn……	ribosomal RNA operon
RBS.....	ribosomal binding site
s……...	second/seconds
SCDA……..	soybean casein digest agar
SLC…….	species-level clusters

Summary

In the first part of my Ph.D. project, I have studied the bacterial community shift in the oxic zone of a flooded, unplanted paddy soil oxygen gradient by using cultivation approach. The starting hypothesis was that the bacterial community succession corresponds to changes in the phylogenetic identity, growth response upon nutrient availability, and rRNA operon (*rrn*) copy number of culturable populations. This hypothesis was tested by comparing the bacterial fraction cultivable from the oxic zone of flooded, unplanted rice paddy soil microcosms after 1 day (early succession) and 70 days (late succession) incubation periods. The proportion of bacteria that were cultivable on solid media corresponded for early and late succession to 37-40% and 31-35% of total DAPI cell counts, which were $7.40(\pm0.36) \times 10^8$ and $5.54(\pm0.28) \times 10^8$ cells per gram of dry soil, respectively. Colony-forming curve analysis revealed a significant delay in the growth response of late successional bacteria compared to those from early succession. A total of 59 early and 66 late successional isolates were grouped into 19 and 30 species-level clusters (SLC), respectively. Except *Bacillus*-like spp., isolates from early succession always belonged to different SLC than those from late succession. *Beta*- and *Gammaproteobacteria* were most prevalent in early succession, while *Alphaproteobacteria* and *Actinobacteria* dominated late succession. Except two alphaproteobacterial SLC, isolates of 16 early successional SLC formed visible colonies within 1 (11 SLC) or 2 days (3 SLC), and exhibited an average *rrn* copy number >5. By contrast, isolates of 25 late successional SLC formed visible colonies only after 2 days (4 SLC), but mostly after 3 to 15 days (21 SLC) of incubation, and exhibited an average *rrn* copy number <2. Regardless of isolation from early (3 SLC) or late (5 SLC) succession, *Bacillus*-like isolates always showed a colony-forming time of 2 days and exhibited 9-11 *rrn* copies. Overall, phylogenetic identity, growth response time and *rrn* copy number were good indicators for successional changes in bacterial life strategy with the exception of *Bacillus*-like spp., presumably owing to their ability to form endospores.

In the second part of my Ph.D. project, I have studied the bacterial community shift in the flooded, unplanted paddy soil oxygen gradient by mRNA profiling. Initially, a protocol for the extraction of mRNA from the soil was developed which was then used for the direct

retrieval of mRNA transcript pools in order to study spatial and temporal changes in bacterial community structure and function in a paddy soil oxygen gradient. Following RT-PCR and generation of cDNA clone libraries, 417 clones were randomly selected for analysis. The vast majority of clones were derived from bacterial mRNA (88%). Taking into consideration only E-values more significant than e^{-10} in blastx analyses, early community development was dominated by transcripts of *Gammaproteobacteria* in the oxic zone, while activity of a phylogenetically highly diverse community was observed in the anoxic zone. Gene expression of mature communities was dominated by transcripts of *Alphaproteobacteria* in the oxic zone and *Deltaproteobacteria* in the anoxic zone. Overall, active genes were functionally assigned to metabolism (136 transcripts), information storage and processing (21), and cellular processes (43). A large number of transcripts were either conserved hypothetical (55) or predicted novel (114). Some of the transcripts could be linked to environmentally important processes such as denitrification (*nosZ*), propionate catabolism (*prpD*), and nitrate uptake (*nirB*). In conclusion, our random analysis of environmental transcripts provided a first insight into structural and functional changes during bacterial community succession in a paddy soil oxygen gradient.

Chapter I

Bacterial community changes in a paddy soil oxygen gradient, assessed by cultivation approach

1. Introduction

Soil is a structured, diverse, and discontinuous system generally poor in nutrients and energy sources, with microorganisms living in discrete microhabitats (Stotzky, 1997). The chemical, physical, and biological characteristics of these microhabitats change with time, resulting in successional shifts in microbial community composition. Therefore, an accurate estimation of bacterial abundance, biomass, and community structure in these habitats helps in assessing the roles of bacteria in food webs and biogeochemical cycles, as well as in understanding their population dynamics in nature.

Due to the progress in the molecular techniques, the study of microbial diversity and community structure in any environment is currently possible. The most common techniques that are used for examining microbial communities include direct counting (Bloem, 1995, Weinbauer *et al.,* 1998), fluorescent *in situ* hybridization (FISH) (Christensen *et al.*, 1999), 16S rRNA and 16S rRNA gene sequence analysis, construction and analysis of gene clone libraries, amplified rDNA restriction analysis (ARDRA) (Gich *et al.,* 2000), terminal restriction fragment length polymorphism (T-RFLP) analysis (Horz *et al.*, 2000), denaturing gradient gel electrophoresis (DGGE) (Heuer *et al.,* 2001), and phospholipid fatty acid (PLFA) analysis (Tunlid and White, 1992; Bossio and Scow, 1998; Zelles, 1999; Pankhurst *et al.,* 2001). Although all of these techniques allow us to gain an insight into native microbial communities present in the environmental samples, they are unable to tell us about the functional status comprising the nutrient uptake, energy flow, degradation of pollutants, diseases of the microbial communities.

One of the possibilities to overcome these problems is to use the traditional cultivation approach to study microbial communities. However, most of the bacteria are uncultivable because they have either selective nutritional requirements or are in a symbiotic relationship with their plant or animal host (Garland *et al*., 2001). Similarly, fast growing bacteria may exclude the slow growers on a solid media by depleting nutrients and by producing antibiotics (Balestra and Misaghi, 1997). There have been various attempts to increase the cultivation efficiency, including a careful use of growth substrate and choice of proper conditions to simulate the *in situ* condition (Sørheim *et al.*, 1989; Johnsen and Nielsen, 1999). Another important point appears to be the use of low concentrations of

growth-supporting nutrients (Mitsui *et al*., 1997; Liesack *et al.,* 1997). Janssen *et al.* (2002) and Davis *et al*. (2005) reported that a minor change in cultivation strategy (using a polymeric growth substrate, longer incubation times, and decreasing inoculum size) could result in higher cultivation recovery and isolation of globally distributed but previously uncultured phylogenetically novel soil bacteria. The use of a set of different media, as opposed to one or two media, increased the efficiency of plate counting method for estimating bacterial diversity (Balestra and Misaghi, 1997).

The following part of the introduction is a detailed literature review on bacterial community structure present in soil and their phylogenetic classification and methods to study microbial community structure.

1.1. Bacterial community structure in soil and their phylogenetic classification

Soil has a complex nutritional availability and is the natural habitat for highly diverse microbial flora. Torsvik *et al.* (1990) found that about 4,000 differently sized microbial genomes are present per gram of soil, representing roughly 13,000 different species. Obviously, these different types of bacteria are not present in equal number; instead, they may range from 1 cell to perhaps 10^8 cells g^{-1} of soil (Liesack *et al.*, 1997). The soil microbial community consists of members of all three major branches of life: a) *Bacteria*, b) *Archaea*, and c) *Eucarya*. Microbial biomass is large in a temperate grassland soil; the bacterial and fungal biomass amounted to 1-2 and 2-5 t ha^{-1}, respectively (Killham, 1994). Bacteria and archaea are dominant in waterlogged soils while fungi are more prevalent in aerobic soils (Shields *et al*., 1973; Alexander, 1977).

Benson *et al*. (2002) published a graphical representation of microbial diversity, which shows entries of 16S rRNA genes (obtained from both cultured isolates and environmental samples by cultivation-independent approaches) versus year of publication in GenBank (Fig. 1.1). The graph clearly shows that environmental gene clone sequences started to appear in large numbers in 1996, and by this time, the technology for recovering these sequences had become routine. Because seawater is easier to work with than soils, genes from marine systems dominated the entries in early years. However, by 2001 entries from soils are highest, probably because of their agricultural significance and because soils are

far more diverse (Rapp and Giovannoni, 2003). In 2002 alone, roughly 9500 environmental 16S rRNA gene clone sequences were deposited in GenBank (Fig. 1.1).

A 16S rRNA tree allows us to show the phylogenetic relationships within the domain *Bacteria*. The 12 original phyla, shown as black wedges (Fig. 1.2) were recognized in 1987; note that some of the phylogenetic nomenclature has more recently been changed. For example, the gram-positive bacteria are now recognized as two separate phyla, the *Firmicutes* (low GC) and *Actinobacteria* (high GC). Similarly, the *Proteobacteria* have been elevated to the rank of phylum and the subclasses α to ε have been elevated to the rank of classes, corresponding to the names *Alpha-*, *Beta-*, *Gamma-*, *Delta-*, and *Epsilon-proteobacteria* (Garrity, 2001). *Proteobacteria* are the classical gram-negative bacteria and, based on both cultivation and cultivation-independent approaches, are generally recognized as one of the predominant microbial groups on the planet (Zwart *et al*. 2002). In addition, the genera *Cytophaga*, *Bacteroides*, and *Flavobacterium* form a major lineage (Paster *et al*. 1985), now known as the *Bacteroidetes* phylum (Garrity, 2001).

Moreover, 14 phyla with cultivated representatives, shown as white wedges (Fig. 1.2), have been identified since 1987 (Hugenholtz, 2002; Hugenholtz *et al*., 1998; Pace, 1997) (Fig. 1.2). These groups include several phyla of predominantly thermophilic microorganisms such as *Aquificae*, *Thermodesulfobacteria*, *Dictyoglomi*, *Coprothermobacteria*, *Caldithrix*, and *Desulfurobacteria*. The *Verrucomicrobia* has been recognized as a separate phylum since 1995 (Hedlund *et al.*, 1997; Ward-Rainey *et al.*, 1995), but until now, only a few cultivated representatives have been taxonomically described such as *Verrucomicrobium vinosum*, *Prosthecobacter fusiformis*, *P. debontii*, *P. vanneervenii*, *P. dejongeii*, and *Opitutus terrae*. Ludwig *et al*. (1997) described the phylum *Acidobacteria* whose members are ubiquitously distributed and abundant in nature. Finally, 26 candidate phyla that are defined only by environmental sequences are shown as gray wedges (Fig. 1.2).

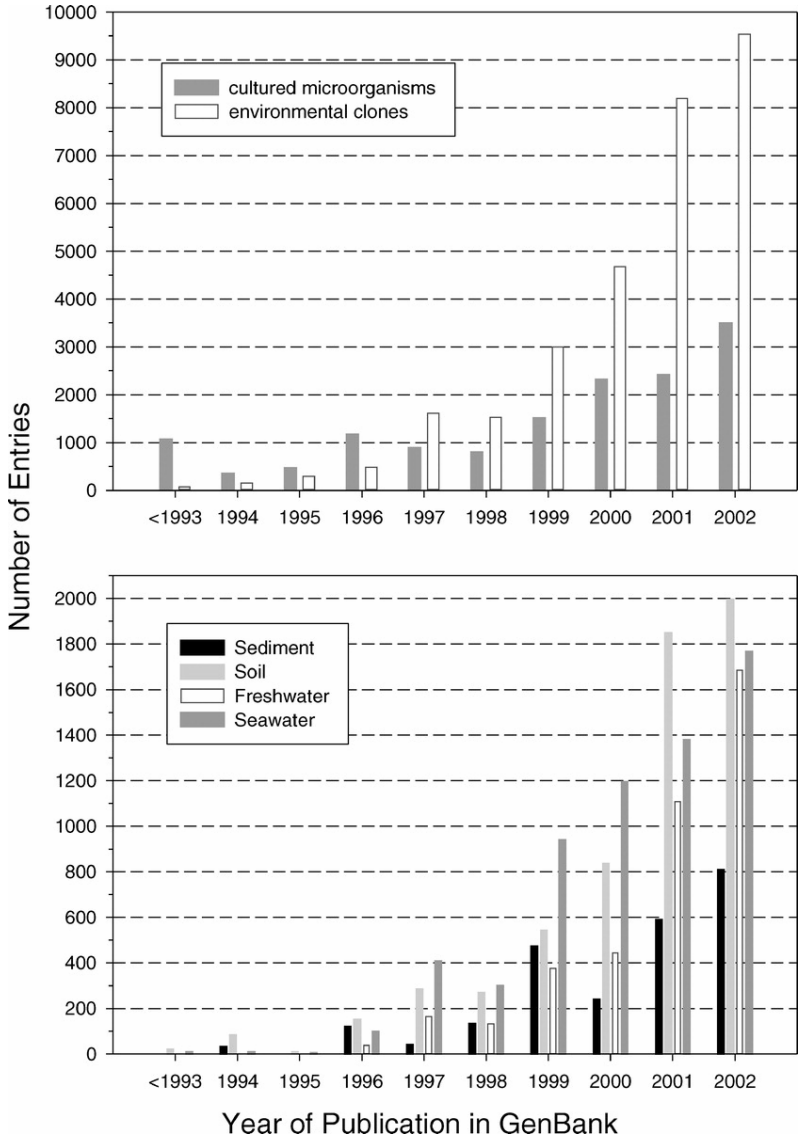

Fig. 1.1. Graph depicting the number of 16S rRNA gene sequences published in Gene Bank since 1993 (Benson *et al*., 2002). Above: total number of published 16S RNA gene sequences from cultivated *Bacteria* and *Archaea* (n=14,434) versus sequences derived from cultivation-independent studies (n=29,505) as a function of year. Below: total number of published environmental gene clone sequences obtained from sediment (n=2435), soil (n=6037), freshwater (n=3951), and seawater (n=6104) habitats as a function of year. All sequences published before 1993 are grouped in the first (<1993) column, whereas the "2002" column includes sequences published through November 19, 2002 (Rapp and Giovannoni, 2003).

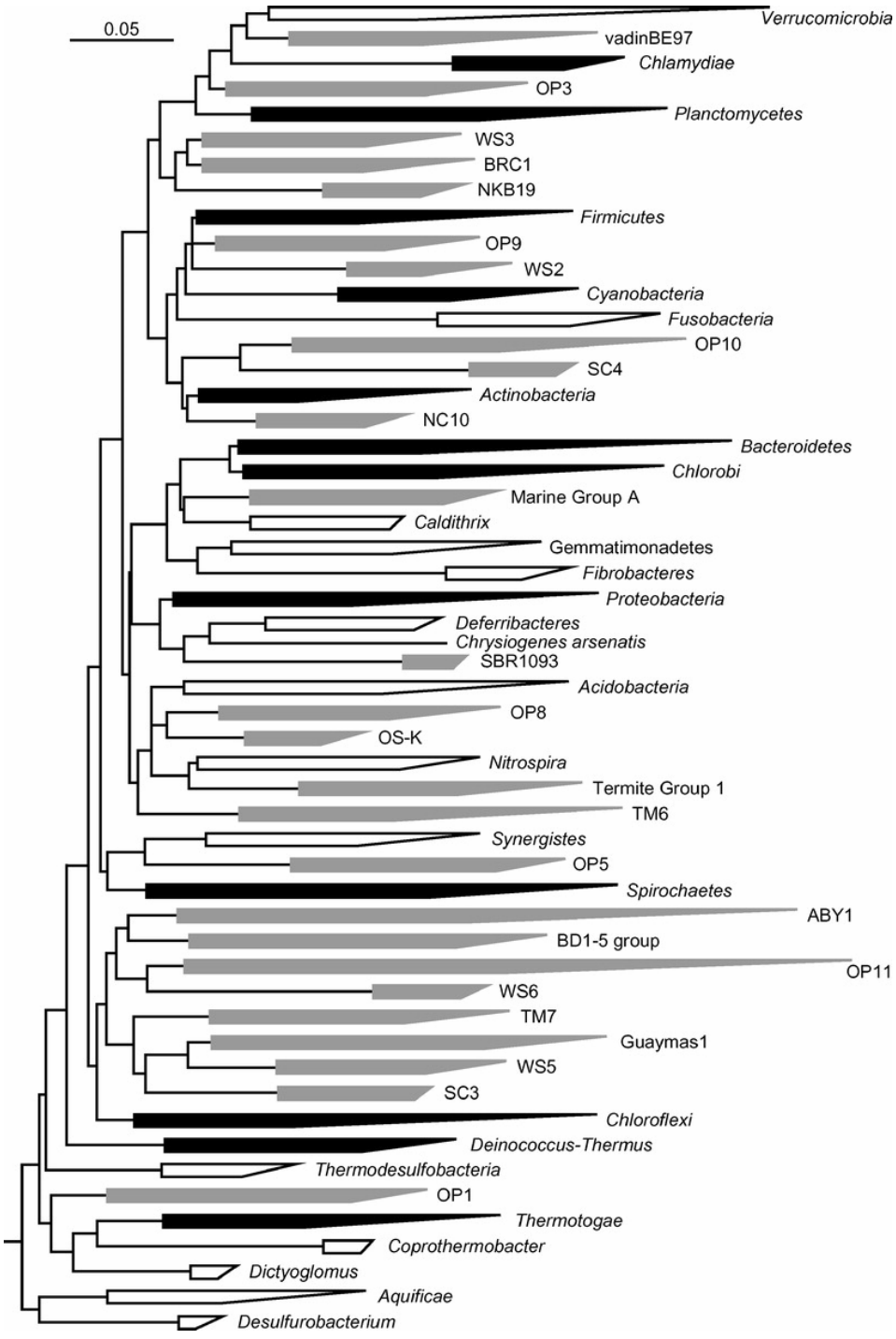

Fig. 1.2. Phylogenetic tree illustrating the major lineages (phyla) of the domain *Bacteria*. Wedges shown in black are the 12 original phyla, as described by Woese (1987); in white are the 14 phyla with cultivated representatives recognized since 1987; and in gray are the 26 candidate phyla that are defined only by environmental sequences (Rapp and Giovannoni, 2003).

1.2. Analysis of bacterial community structure

1.2.1. Cultivation-independent approach

Cultivation-independent approaches for the bacterial community analysis have now become increasingly routine because of its simplicity, accuracy, and reliability. Since the mid 1980s, the use of 16S rRNA-based techniques such as different fingerprinting techniques and cloning and sequencing have promoted the molecular identification of a wide variety of yet uncultivated microorganisms and novel isolates from various environments. Care is needed in interpreting the composition of microbial communities by molecular techniques because the method of extraction can bias the results of all fingerprinting techniques. Usually, an efficient extracting solution also solubilizes humic molecules, which inhibits the PCR, resulting in the need of extensive purification steps (Krsek and Wellington, 1999; Martin-Laurent *et al.*, 2001). Another problem while extracting DNA from soil is cell lysis. Many Gram-positive bacteria require harsh lysis conditions (Head *et al.*, 1998). However, harsh lysis methods should be avoided because they degrade DNA molecules to fragments below 1 kb, and short DNA fragments may lead to the generation of chimeric 16S rRNA after amplification (Liesack *et al.*, 1991). A summary of different fingerprinting methods that are applied to the microbial community analysis of environmental samples is listed below.

Table 1.1. List of fingerprinting methods commonly used for the study of microbial community structure.

SN	Methods	Environment studied	Reference
1	FISH	Soil, drinking water and lotic biofilms, oligotrophic lakes, marine systems, activated sludge	Hahn *et al.*, 1992; Kalmbach *et al.,* 1997a, 1997b; Manz *et al.,* 1993, 1994, 1996, 1998; Alfreider *et al.*, 1996; Grossart and Simon, 1993; Ramsing *et al.*,1996; Ravenschlag *et al.*, 2000
2	ARDRA	Marine systems	Dang and Lovell, 2000
3	PLFA	Soil	Tunlid and White, 1992; Bossio and Scow, 1998; Zelles, 1999; Pankhurst *et al.*, 2001
4	TRFLP	Soil	Horz *et al*., 2000
5	RADP	Soil	Yang *et al*., 2000
6	DGGE/ TGGE	Hydrothermal vents, hot springs, activated sludge, soil	Heuer *et al*., 2001; Muyzer and Smalla, 1998; Muyzer *et al*., 1993
7	RISA	Soil	Martin-Laurent *et al*., 2001

1.2.2. Cultivation approach

Microbial cell number can be quantified by counting techniques such as the plate count technique or the most probable number technique (Bakken, 1997; Johnsen *et al.*, 2001). Plate counts estimate only 1-10% of the overall soil microflora (Olsen and Bakken, 1987; Zarda *et al*., 1997). The low cultivation efficiency is a consequence of the interdependency of different organisms on each other (for example, the endosymbiotic bacteria in specific worms and mollusks), and the inability to create the environmental conditions during cultivation that microorganisms face in the soil environment and require for growth (Bakken, 1997; Muyzer and Smalla, 1998; Heuer *et al.*, 2001).

One of the most important factors that affect the culturability is suitable detachment of bacterial cells from the soil particles. A clumping of cells consisting of two or more species may result in a colony dominated only by the fast-growing species, such that the presence of the other species remains unrecognized once a pure culture is obtained (Janssen *et al*., 2002). Buesing and Gessner (2002) tested the effect of four detachment procedures (vortexing, ultrasonic cleaner, ultrasonic sonicator, and tissue homogenizer) on the release of bacteria associated with leaf litter, sediment, and epiphytic biofilms in a natural aquatic system. They noticed that ultrasonic cleaner, ultrasonic sonicator, and tissue homogenizer increased bacterial counts and biovolumes significantly compared to simple vortexing. In the cleaner and sonicator procedures, the longer dispersion time reflected the real size of bacterial number and was preferable for accurate estimation of mean bacterial biovolumes (Kuwae and Hosokawa, 1999). However, neither the detachment procedure nor the treatment time affected the composition of bacterial morphotypes (Buesing and Gessner, 2002).

The form of cell has also an effect in culturability; for example, small bacterial cells (dwarf cells or ultramicrobacteria) are difficult to culture as they rarely form colonies in agar plates (Nannipieri *et al*., 2003). By considering that larger cells are considered to account for about 80% of the total bacterial volume in soil, Bakken (1997) hypothesized that the culturable bacteria have a more important ecological significance in soil than would appear from their small numbers. Since the bulk (non-rhizospheric) soils seem to be an oligotrophic habitat, it is likely that a significant portion of the microbial community is viable but unculturable, as is found for sediment environments (Novitsky, 1987).

Researches have analyzed the changes in the number of specific taxonomic or functional groups by plating on agar media, and have precisely assessed the culturable diversity by isolating colonies from these media followed by identification using various methods such as 16S rRNA gene sequencing (Vandamme *et al.*, 1996). Ishikuri and Hattori (1985) observed the gross diversity of culturable microorganisms by plotting the different colonies identified on the medium against the incubation time. Similarly, Chin *et al.* (1999) performed most probable number counts for polysaccharolytic and saccharolytic fermenting bacteria in the anoxic bulk soil of flooded rice paddy soil. They found up to 2.5×10^8 cells per g of dry soil in a medium containing xylan, pectin, or a mixture of seven mono- and di- saccharides as the growth substrates. This is equivalent to about 50% or more of the total microscopic cell count (4.8×10^8 cells g^{-1}). The cultured populations belonged to the *Verrucomicrobia*, *Bacteroidetes*, *Actinobacteria*, clostridial cluster XIVa, clostridial cluster IX, and *Bacillus* spp. In addition, Janssen *et al.* (2002) reported that the culturability of bacteria from Australian pasture soil is as high as 14% of the total cell counts and many of these isolates represent the first known isolates of globally distributed groups of soil bacteria belonging to novel lineages within the phyla *Actinobacteria*, *Acidobacteria*, *Proteobacteria*, and *Verrucomicrobia*.

Chin *et al*. (1999) and Hengstmann *et al*. (1999) assessed the correspondence between culture methods and direct recovery of environmental 16S rRNA genes and observed that the isolates obtained are representative genotypes and phenotypes of predominant bacterial groups. These representatives accounted for 5 to 52% of total cells in the anoxic rice paddy soil studied. Thus, the authors concluded that a dual approach results in a more objective view of the structural and functional composition of a soil bacterial community than either cultivation or direct recovery of 16S rRNA gene sequences alone.

1.2.3. Direct bacterial counting approach

Direct counting by fluorescence microscopy can give 100-1000 times greater number than the numbers obtained by plate counting (Johnsen *et al.*, 2001). Strauss *et al.* (1995) used two fluorescent dyes [(5-cyano-2,3-ditolyl tetrazolium chloride (CTC) for actively respiring bacterial count and 4',6-diamidino-2-phenylindole (DAPI) for total bacterial counting)] and argued that both dyes can be used together for the same soil sample without

affecting counting results. Staining for 8 h with CTC and for 40 min with DAPI resulted in maximum numbers of stained cells. The optimal DAPI concentration for staining is 10 mg per liter and counts were significantly higher when sodium chloride was used (Strauss *et al.,* 1995). Several other stains specific to proteins or nucleic acids have also been used, including fluorescein isothiocyanate, acridine orange, and differential fluorescent stain. Bloem (1995) improved the direct counting method with a video camera on an epifluorescence microscope. However, these procedures do not allow one to count microbial cells only as there is a possibility to stain soil particles and dead cells also, giving false positive counting. The most widely used dyes for enumerating the bacterial cell number; their advantages and drawbacks are listed in table 1.2.

Table 1.2. List of most widely used dyes for the enumeration of soil bacteria.

S.N.	Dye	Molecules stained	Advantage	Disadvantage
1.	DAPI (Bloem, 1995) (Weinbauer *et al*.,1998) (Janssen *et al*., 2002)	DNA	Easy to stain	Background staining is present
2.	Differential fluorescent stain (Bloem, 1995)	DNA, RNA, cellulose, and polysaccharides	Europium stains DNA and RNA (red) while FB stains cellulose and polysaccharides (blue)	-
3.	Dichloro-triazinyl-amino-fluorescein (Bloem, 1995)	Binds covalently with neutral amino acid group proteins, especially on positively charged cell polymers	Less background staining	-
4.	Acridine orange (Scholefield *et al*., 1985)	Intercalates between the stacked bases of DNA and RNA but it also binds with other cellular constitutes, detritus and clay	Simple staining	Non specific binding, background staining
5.	Cybergreen II dye (Weinbauer *et al*.,1998)	DNA	Less back ground fluorescence	-
6.	SYTO 9 dye + Propidium Iodide (Janssen *et al*., 2002)			Sample should be very clean. If some particles are present, then high background fluorescence is present.

1.3. Conceptual approaches for studying microbial community structure

1.3.1. The species level concept vs. 16S rRNA gene sequencing

The golden standard in defining new species among prokaryotes is a DNA-DNA reassociation value of 70% in hybridization test of the total genomic DNA of two organisms. Below this threshold, two strains will be classified as two discrete species (Wayne *et al*., 1987). Later, Stackebrandt and Goebel (1994) compared 16S rRNA sequencing and DNA-DNA hybridization to test, if 16S rRNA gene sequence similarity could be used to assign new species. They observed that 16S rRNA gene sequence similarity value below 97% resulted in less than 70% of DNA-DNA reassociation value and thereby different species (Fig. 1.3). Importantly, values above 97% or even 100% 16S rRNA sequence similarity may or may not have more than 70% of DNA-DNA reassociation value.

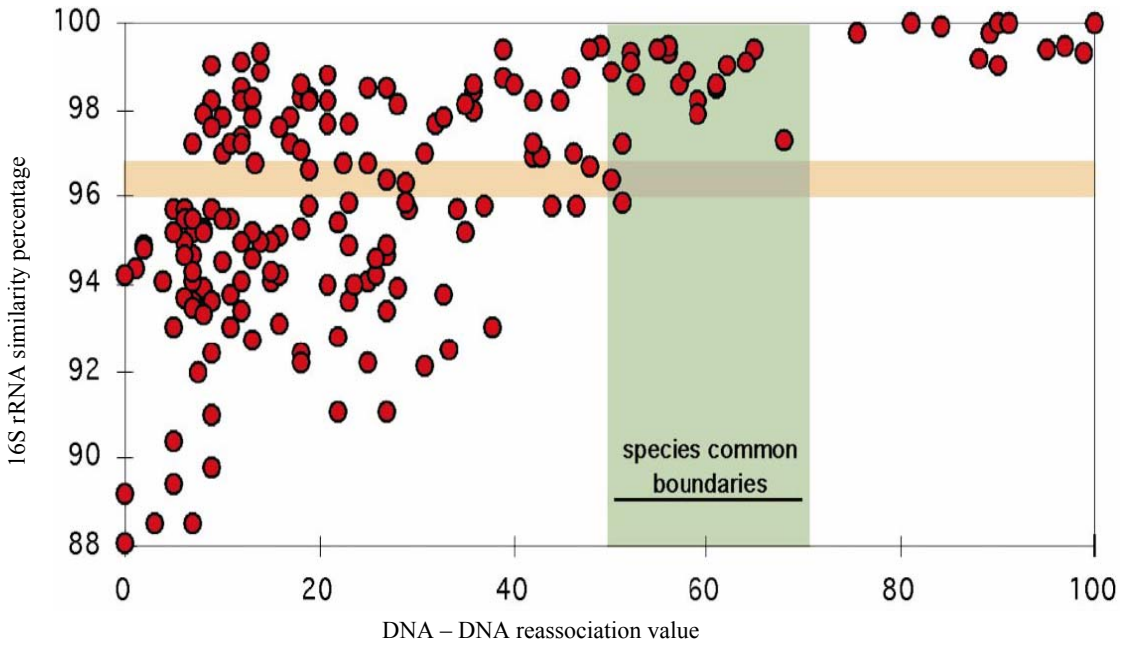

Fig. 1.3. Comparison of DNA–DNA reassociation value and percentage of 16S rRNA similarity (Roselló-Mora and Amann, 2001)

Species definition is made quasi-official [(American Society of Microbiology) (Vandamme *et al*., 1996; Stackebrandt *et al*., 2002)] by combining genomic, phylogenetic, and phenotypic approaches into a pragmatic and 'phylophenetic' (or 'polyphasic') taxonomic

framework. The species is then defined as a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminating phenotypic property' (Roselló-Mora and Amann, 2001). In practice, however, species definition relies on the 16S rRNA gene phylogeny (Young, 2001; Dijkshoorn *et al*., 2000), based on different ranges of 16S rRNA gene sequence similarity to known species (Table 1.3).

Table 1.3. List of 16S rRNA gene sequence similarity values that are commonly used for bacteria species definition.

SN	Species	Genus	Family	References
1	≥97%	-	-	Stakebrandt and Goebel, 1994
2	≥99%	<99% and ≥95%	<95%	Bosshard *et al*., 2003
3	≥99%	-	-	Hall *et al*., 2003; Kattar *et al*., 2001; Roth *et al*., 2003; Tortoli, 2003
4	≥98%	-	-	Dighe *et al*., 2004; Turenne *et al*., 2001

1.3.2. Microbial community succession, growth response time, and the *r*- / *K*- concept

The use of bacterial populations to study primary succession has several advantages over the use of macroorganisms. Torsvik *et al*. (1990) suggested that the presence of a large bacterial diversity in soil might display different patterns of diversity and activity that are useful for characterizing successional processes. In addition, sampling is simple as many samples can be easily collected, transported, and processed in a relatively short period of time. Furthermore, many bacteria may be subjected to analysis from a single sample, which in combination with an adequate sampling strategy increases the confidence in achieving a representative sample of the real bacterial population (Garland *et al*., 2001).

Characterizing an organism's metabolic status is an essential feature that assists in defining the different stages of primary succession (Pickett, 1976). Garland *et al*. (2001) showed that the ratio of opportunistic cells (those able to grow on a nonselective medium) to total cells (detected by microscopic cell counts) is higher in early than in late successional environments, indicating a propensity for cellular reproduction (*r*) over maintenance (*K*).

This observation reflects the theory of Tilman (1985) who suggested that those organisms that are able to reproduce and grow faster would have a competitive advantage over slower-growing organisms. In addition to metabolic status, bacterial community is also affected by successional stages (Dang and Lovell, 2000; Felske *et al*., 2000; Pennanen *et al*., 2001). Jackson *et al*. (2001) studied succession in biofilms revealing that early successional habitats supported an unorganized community with low species evenness and richness. Conversely, late succession is defined by increased habitat variation and resource availability which consequently led to increased bacterial community evenness and richness, a pattern also often noted in forefields plant succession (Matthews, 1992).

Alternative models of succession, including gradient-in-time (Pickett, 1976; Whittaker, 1953) and competitive sorting (Margalef, 1968; Margalef, 1963) predict that early successional communities will be dominated by species with broad niche width, rapid growth, and high investment in reproduction (i.e., opportunistic organisms). By contrast, late successional communities will be dominated by species with narrow niche width, slower growth, and low investment in reproduction (i.e., equilibrium organisms).

The terms *r*-strategist vs. *K*-strategist (MacAuthur and Wilson, 1967) have been used to distinguish opportunistic and equilibrium species (Gadgil and Solbrig, 1972), respectively, and have been applied to microbial ecology by Andrews and Harris (1986). *K*-strategists have a more efficient cell metabolism than *r*-strategists and are able to use recalcitrant substrates, such as lignin and cellulose. *K*-strategists are also thought to be less affected by toxins than *r*-strategists (De Leij *et al.*, 1993). Based on these descriptions, one could predict that microbial communities in an early successional stage would contain a higher proportion of opportunists (*r*-strategists), owing to their ability to grow faster on nonselective medium (no specialized growth needs). Conversely, an increasing proportion of equilibrium (*K*-strategists) types may be associated with late successional stage, since these types direct most energy into maintenance (Sigler and Zeyer, 2002). The fastidious nature of the equilibrium type may be a result of specific growth factor requirements, specialized use of a narrow range of carbon sources, or susceptibility to high nutrient concentrations.

Culturability and colony-forming time on solid media have been used to define successional stages of bacterial communities in various soil environments, including wheat

roots and soil (De Leij *et al*., 1993), deglaciated soils (Sigler and Zeyer, 2004), and spoil of brown coal colliery substrate (Kristufek *et al*., 2005). All of these results showed that early succession is predominated by fast-growing colonies (that form colonies within 48 h) whereas late succession is predominated by slow-growing colonies (that form colonies ≥72 h) in response to growth substrate. It is generally assumed that multiple copies of rRNA operons (*rrn*) in prokaryotic organisms are needed to achieve high growth rates (Bremer and Dennis, 1987). Klappenbach *et al*. (2000) provided some evidence that a high number of *rrn* copies (in average >5 copies) is more beneficial for soil bacterial isolates to rapidly form colonies on a nutritionally complex medium than strains bearing a low number of *rrn* copies (in average <2 copies) (Fig. 1.4). The rrndb (ribosomal RNA operon copy number database) web site is directly accessible at http://rrndb.cme.msu.edu/, which contains the latest annotated information on *rrn* copy number among prokaryotes.

1.4. Oxygen gradient system

One of the most significant challenges in microbial ecology is to understand the spatial and temporal variation of microbial communities in the environment (Torsvik *et al.*, 2002). The early consumption of oxygen by aerobic bacteria leads to a separation of aerobic and anaerobic processes and, as a result, to a spatial shift in the microbial community composition (Brune *et al.*, 2000; Liesack *et al.*, 2000) in an aquatic habitat.

The steepness of the oxygen gradient depends on the bioavailable organic matter in the surface layer and, as a result, on the biological oxygen demand (Reimers and Smith, 1986; Revsbech *et al.*, 1989). Apart from the content of degradable organic matter in the surface layer (Reimers and Smith, 1986; Revsbech *et al.*, 1989), the production of methane and its diffusion toward the oxic surface layer are known to cause an increase of the biological oxygen demand at the oxic-anoxic interface of such environments in which sulfate reduction is not a predominant process, e.g., flooded paddy soils, natural wetland soils, and freshwater sediments (Gilbert and Frenzel, 1995; Rothfuss *et al*., 1994; Sweerts *et al.*, 1991). Similarly, Zehnder and Stumm (1988) stated that due to the low solubility of molecular oxygen in water compared to the oxygen demand, all aquatic systems are characterized by oxic-anoxic interfaces. Such oxic-anoxic boundaries are defined by physical and chemical gradients that develop with time and have a significant impact on the growth and structure of the indigenous microbial community.

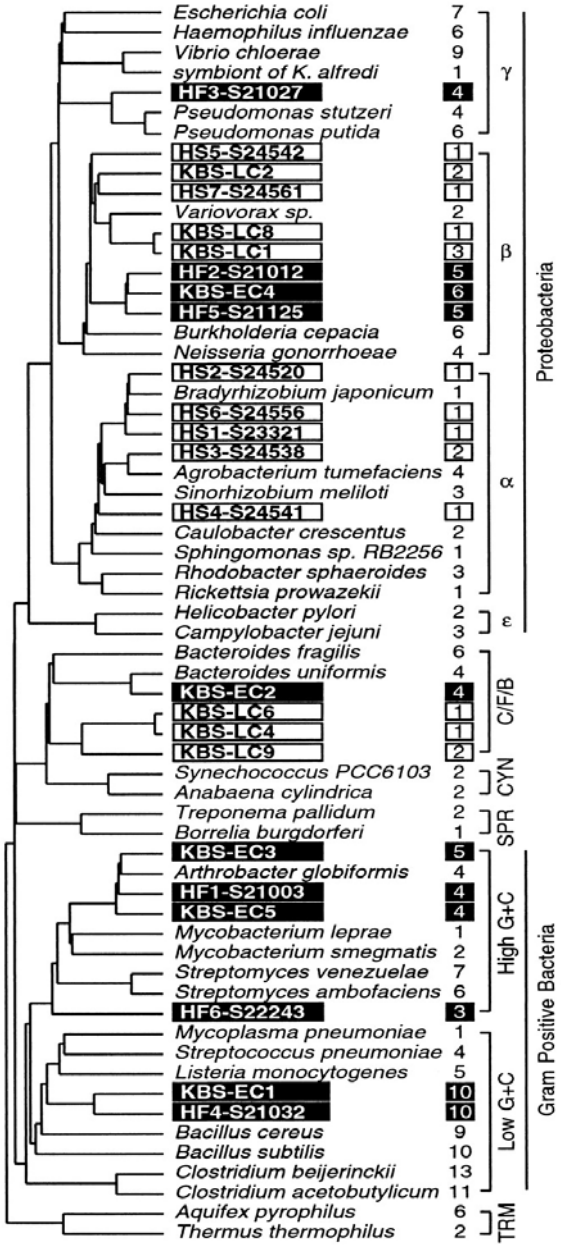

Fig. 1.4. Phylogenetic distribution of bacteria characterized for *rrn* copy number. Filled boxes indicate soil isolates that appeared early, while open boxes indicate isolates that appeared late. Isolates from conventional-tilled soils in Michigan (designated by prefix "KBS") and rice paddy soils in Japan (designated by prefix "HF" or "HS") are included. Values to the right of species' names indicate the number of *rrn* equivalents per chromosome. Major phyla are indicated on the far right with abbreviations as follows: C/F/B, *Cytophaga/Flexibacter/Bacteroides* (*Bacteroidetes*); CYN, cyanobacteria; SPR, spirochetes; TRM, thermopiles (Klappenbach *et al.*, 2000).

Noll *et al*. (2005) studied the spatial and temporal variation in microbial communities in flooded, unplanted rice paddy soil microcosms. They observed that within 6 h of flooding, oxygen starts to reduce from 200 µM at the floodwater-soil boundary to undetectable amounts at a soil depth of approximately 2 mm and below. The oxygen depletion in the oxic zone and upper transition zone was less pronounced at 84 days than at 6 h after flooding. Although the microelectrode measurements had detected no oxygen at depths corresponding to the lower transition zone and anoxic zone, the T-RFLP profiles obtained from these two soil depths revealed clear differences. Based on this observation, 2.9 mm soil depth was considered as a lower transition zone (Fig. 1.5) (Noll *et al.*, 2005).

Noll *et al*. (2005) analyzed microbial communities within the paddy soil oxygen gradient using T-RFLP fingerprinting method and indicated that the *Betaproteobacteria* are the abundant populations in the oxic zone of early succession whereas members of clostridial cluster I are predominant in the anoxic zone. Similarly, *Verrucomicrobia*, *Alphaproteobacteria* and *Nitrospira* (oxic zone), and the *Myxococcales* (anoxic zone) dominated late successional populations.

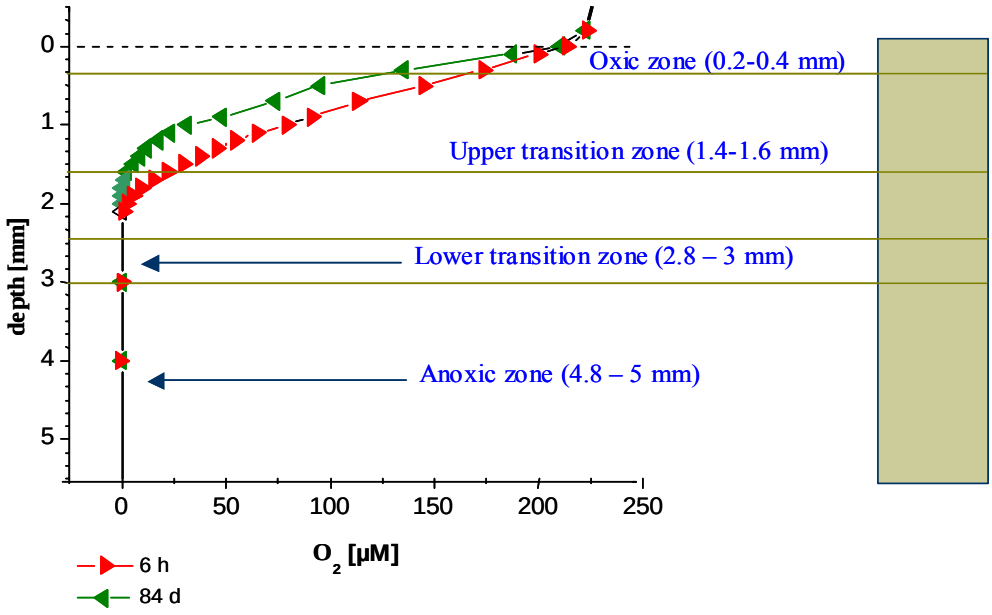

Fig. 1.5. Oxygen depth profiles determined for 6-h-old and 84-d-old flooded microcosms (Noll *et al.*, 2005).

1.5. Aim of the project

The main aim of this part of the Ph.D. project was to test the hypothesis that the bacterial community succession corresponds to a) changes in the phylogenetic identity, b) growth response time upon nutrient availability, and c) *rrn* copy number. This hypothesis was tested by comparing the bacterial fraction cultivable from the oxic zone of flooded, unplanted rice paddy soil microcosms incubated either for 1 day or 70 days. Following previous results (Noll *et al.*, 2005), the oxic-anoxic interfaces of flooded, unplanted rice paddy soil microcosms are colonized by early successional communities after 1 day of flooding and proceed into stable late successional communities within 70 days. Colony-forming curve (CFC) analyses on different solid media were used to monitor the growth response time of early and late successional isolates upon nutrient availability. The *rrn* copy number of early and late successional isolates was determined by the Southern blot hybridization analysis.

2. Methodology

2.1. Materials

2.1.1. Soil sample

Soil was taken from drained paddy fields of the Italian Rice Research Institute in Vercelli, Italy. The soil was air-dried and stored at room temperature. The soil characteristics have been described previously (Holzapfel-Pschorn *et al*., 1985).

2.1.2. Instruments

Soil was taken from drained paddy fields of the Italian Rice Research Institute in Vercelli, Italy. The soil was air-dried and stored at room temperature. The soil characteristics have been described previously (Holzapfel-Pschorn *et al*., 1985).

Items	Manufacturer
0.2 μm black poly-carbonated membrane filter	Whatman, UK
0.45 μm cellulose nitrate membrane filter	Whatman, UK
Autoclave HV-25	Hiraya Manufact. Corp. Japan
Axiophot epifluorescence microscope	Zeiss, Oberkochen, Germany
Bead beater dismembrator-S	Braun Biotech, Germany
Bio-analyzer	Agilent, England
DNA sequencer (ABI 310)	Applied Biosystems
DNA sequencer (ABI 373)	Applied Biosystems
Electronic balance	Mettler AT 261
Gel documentation	Intas
Gel electrophoresis unit	Bio Rad
Heating block	Techna DB-20, England
High speeds centrifuge	Eppendorf 5417R
Hot air oven	Heraeus, Instruments
Hybridization oven and hybridization bottle	Thermo Electronic
Incubator	Heraeus, Instruments

Laminar air flow cabinet H3244B	Heraeus, Instruments
Magnetic stand	Ambion
Micro-oven	Sharp, Japan
Micropipette	Bosch, Kirsch
Microtom- Kryostat HM-500	Germany
NanoDrop® ND-1000 UV-Vis spectrophotometer	NanoDrop Technologies, Inc. USA
Refrigerator (-80 °C and -20 °C)	Gilson, France
Sonicator	Transonic TS540, Germany
Thermal cycler (model 9600)	Applied Biosystems
Thermal cycler (model 9800)	Applied Biosystems
UV cabinet	Plas labs
UV cross linker	UV Stratalinker 1800
X-ray film cassette (8×10 cm)	Kodak, Japan

2.1.3. Growth media, chemicals, and reagents

All the growth media and reagents were prepared by using the standard protocol (Sambrook *et al*., 1989). Chemicals that were used to prepare reagents in the laboratory were purchased in the bulk from one of the following companies unless otherwise specified: BioRad, Munich; Biozym, Hess. Oldendorf; Boehringer Mannheim; New England Biolabs, Frankfurt; Fluka, Buchs, Switzerland; Gibco, Eggenstein; Merck, Darmstadt; MWG-Biotech, Ebersberg; Metabion, Martinsried; Perkin Elmer Applied Biosystems, Weiterstadt; Amersham Pharmacia, Freiburg; Biometra, Göttingen; Qiagen, Hilden; Stratagene, Heidelberg; Sigma, Germany.

Items	**Manufacturer/Supplier**
1/4th Ringer solution	
4’6-diamidino-2-phenylindole dihydrochloride	Sigma, Basel, Switzerland
Benzyl chloride	
Blocking solution	Roche, Germany
Chloroform-isoamyl alcohol [24:1 (v/v)]	
Deionized formamide	Applera, Darmstadt, Germany
Denaturation solution	

DEPC-pretreated water	
Depurination solution	
Detection buffer	
EB buffer	
Ethanol	
Hybridization solution	
Maleic acid buffer (10 × stock)	
Neutralization solution	
Nutrient agar	BD Diagnostic Systems, USA
PB buffer	
PE buffer	Sigma, Germany
Phenol-based lysis buffer	
Phenol–chloroform–isoamyl alcohol [25:24:1 (v/v/v)]	Sigma, Germany
Phosphate buffer saline (PBS buffer) pH 7.2	Sigma, Germany
R2A agar medium	BD Diagnostic Systems, USA
Soybean casein digest agar	BD Diagnostic Systems, USA
Sterile water	
TE buffer	Sigma, Germany
TMC buffer	Sigma, Germany
TPM buffer	Sigma, Germany
Transfer solution (20 × SSC)	
Washing buffer I	
Washing buffer II	
Washing buffer III	
Water-saturated phenol	

2.1.4. Enzymes and kits

Items	Manufacturers and suppliers
Access RT- PCR ®system	Promega, Germany
AutoSeq™G-50	GE Health Care Life Science, Germany
Big Dye™ Terminator V1.1/3.1 Cycle Sequencing kit	Applied Biosystems
DIG Wash and Block Buffer Set™	Roche Applied Science, Mannheim, Germany
DIG-High Prime™	Roche Applied Science, Mannheim, Germany
DNA Smart Ladder RNA™	Perkin Elmer Applied Biosystems
GoTaq® Flexi DNA Polymerase	Promega, Germany
Ladder Genescan-standard Rox-1000	Eurogentec, Searing, Belgium
Lysozyme from chicken egg	Promega, Germany
MICROB Express™ Bacterial mRNA Enrichment kit	Ambion
MspI, PstI, EcoRI, SmaI, PvuII	Promega, Germany
Nylon membrane, positively charged	Roche Applied Science, Mannheim, Germany
OPA13	MWG
OPA17	MWG
PCR Clean-up Kit™	Promega, Germany
Proteinase K	Promega, Germany
QIAamp™ DNA mini kit	Qiagen, Hilden, Germany
QIAquick™ purification kit	Sigma Aldrich, Deisenhofen, Germany
RNA later®	Ambion
RNase-free DNase	Promega, Germany
RNAsin®	Promega, Germany
RNeasy™ mini Kit	Qiagen, Germany
rRNA standard from *Escherichia coli,*	Roche, Indianapolis, USA
Shine Dalgarno specific primers (SD14)	MWG
TOPO TA cloning® Kit	Invitrogen, Germany

2.2.Methods

2.2.1. Model system

Immediately before use, the soil was passed through a 2 mm sieve, mixed with deionized water (DM) at a ratio of 2:1 (wt/vol) and then filled into microcosms with a diameter of 6.2 cm and a depth of 10 cm. Three replicate microcosms each were incubated with a 1 cm floodwater layer either for 1 day or for 70 days in the dark at 30 °C with constant aeration (Fig. 2.1). After incubation, the microcosms were carefully removed from the container. The remaining floodwater was removed from the microcosms using a sterile micropipette. Soil samples were collected from the upper 2 mm oxic zone of the microcosms using a sterile scalpel and were analyzed immediately for moisture content determination, microscopic cell count, and viable cell count.

Fig. 2.1. Model system (incubation of microcosms)

2.2.2. Moisture content determination

Approximately 1 g of soil was accurately weighed and then dried at 105 °C for 24 h (Janssen *et al.*, 2002). The samples were reweighed after they were allowed to cool in a desiccator to room temperature. The drying and cooling procedure was repeated until constant mass (± 0.005 mg) was obtained.

The moisture content was calculated using following formula:

$$\% \text{ moisture content} = \frac{(I_w - F_w)}{I_w} \times 100$$

where I_w= wet weight of the soil and F_w= dry weight of the soil

2.2.3. pH determination

1 g of soil sample was mixed in 10 ml of distilled water and pH was determined by a pH meter.

2.2.4. Microscopic cell count

An accurately weighed aliquot of soil (~1.5 g wet weight corresponding to ~1 g dry weight) was suspended in 10 ml of sterile one-quarter-strength Ringer solution (10^{-1} dilution step), vortexed for 5 min and then sonicated (Transonic TS540, Germany) for 30 sec (Janssen *et al*., 2002; Shayne *et al*., 2003). The suspension was then used to prepare a tenfold dilution series (10^{-2} to 10^{-9}) in the same solution. Aliquots (0.5 ml) of the 10^{-1} - 10^{-3} serial dilution steps were fixed at 4 °C for 24 h in filter-sterilized phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM Na_2HPO_4; pH 7.2–7.4) containing 4% (wt/vol) paraformaldehyde. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI; final concentration, 5mg/ml) (Sigma, Basel, Switzerland) for 15 min and then filtered through black polycarbonate Nuclepore membranes (pore size, 0.2 μm; diameter, 25 mm; Whatman, UK) at <10 kPa (Weinbauer *et al*., 1998). Cells were enumerated under UV, using an Axiophot epifluorescence microscope (Zeiss, Oberkochen, Germany). A total of ≥ 400 cells per filter were counted from 20 fields, each covering an area of 0.01 mm^2 (Buesing and Gessner, 2002). The total bacterial counts were calculated on dry weight basis using following equation:

Total number of bacteria = $N/X \times A/B \times 1/S$

where,

N= total number of bacteria counted

X= the number of viewed fields (grids counted)

A= the area of the slides covered by the sample, which should be checked microscopically

B= the area of the viewed fields, to be measured with an object stage micrometer

S= the amount of sample on the slide

2.2.5. Viable bacterial cell count (colony forming unit count)

The numbers of colony-forming units (CFU) were determined by spreading 100-µl aliquots of the 10^{-4} to 10^{-9} serial dilution steps onto plates containing nutrient agar (NA), R2A agar and soybean casein digest agar (SCDA) in full-strength (1:1) and diluted strength (1:100). All media were prepared according to the manufacturer's instructions (BD Diagnostic Systems, USA). The plates were incubated aerobically for up to 30 days at 30 °C. CFU were checked manually and counted when they became visible to the naked eye. Plates were randomly examined at low magnification (6×) for detection of microcolonies. Two sets of three microcosms incubated for either 1 or 70 days were analysed for viable cell numbers. For each microcosm of the two sets, serial tenfold dilution onto plates was carried out in triplicate for each of the six media compositions tested (NA, R2A, and SCDA in full-strength and diluted strength). In total, 576 agar plates were monitored for CFU analysis.

The CFU counts were calculated using following formula [(Bacteriological Analytical Manual, Edition 8, and Revision A, 1998, AOAC Official Methods of Analysis, sec. 966.23, with one procedural change (966.23C)].

$$N = \sum C / [(1\times n_1) + (0.1 \times n_2)] \times (d)$$

where,

N = Number of colonies g^{-1} of sample

$\sum C$ = Sum of all colonies on all plates counted

n_1 = Number of plates in first dilution counted, e.g. 3

n_2 = Number of plates in second dilution counted, e.g. 3

d = Dilution from which the first counts were obtained

2.2.6. Colony forming curve (CFC) analysis

Each day, the newly visible colonies were color-marked and enumerated. As per Koch (1994), Nairn *et al*. (2002), and Kristufek *et al*. (2005) plates showing 30 to 300 colonies were selected for enumeration. Colony appearance was monitored during total incubation time of up to 30 days. The bacterial colonies were classified based on when they appeared on the plates into the following incubation periods: 1, 2, 3-5, 6-10, 11-15, and 16-30 days.

The total numbers of culturable bacteria were calculated using the CFU counts of those plates that exhibited 30 to 300 colonies after 30 days of incubation. Since one-way analysis of variance (P=0.05) did not show any statistically significant difference among the replicates used for each of the six different media compositions, the nine replicates of each media composition were used to calculate mean values and standard deviations. In a separate approach, plates showing 10 to 30 colonies were also used for enumeration, but only for late succession. Here, the overall data of all three different media (NA, R2, SCDA) were used to calculate separate single colony-forming curves, separately for full and diluted strength.

2.2.7. Pure culture isolation

Colonies grown on plates of the terminal positive dilution steps were picked and replated for purification onto the respective medium and their phenotypic characteristics such as color, consistency, and day of colony appearance, were recorded (data not shown). Replating was continued until pure cultures were obtained. Pure culture status was concluded from the uniform size and morphology of single colony, phase-contrast microscopy, and 16S rRNA gene sequencing.

2.2.8. Extraction of genomic DNA for 16S rRNA gene sequencing

In order to sequence pure isolates, genomic DNA was prepared by two methods:

2.2.8.1. Rapid preparation of genomic DNA from bacterial cells

A simple protocol was used when the bacterial cell lysis was easily obtained, particularly suitable for gram-negative bacteria. The protocol used in this study was based on the method published by Holmes and Quigley (1981) for the preparation of plasmid DNA. Individual colonies were picked from an agar plate, using a sterile toothpick, and resuspended in 20 µl TE buffer. The cell suspension was incubated for 10 min in a 97 °C heating block and resulting cell lysate was centrifuged for 5 min at 13,000 × g. An aliquot (1 µl) of the supernatant was used directly for PCR amplification, without any additional purification steps.

2.2.8.2. Bead beating lysis method

Extraction of DNA, PCR amplification of the nearly complete 16S rRNA gene and sequencing were carried out using slightly modified standard protocol (Hengstmann *et al*., 1999). Individual colonies were scrapped out and were mixed with 200 µl of a one-quarter-strength Ringer solution and 100 µl of a 10% (wt/vol) solution of sodium dodecyl sulfate. Approximately 0.5 g of sterile glass beads (0.17- to 0.18-mm diameter) were added, and the suspension was shaken for 1 min at maximum speed in a bead beater (Dismembrator-S; B. Braun Biotech, Germany). Two freeze-thaw-cycles were performed by rapidly cooling in liquid nitrogen for 20 sec and then heating at 100 °C in heating block for 10 min. Cell debris was pelleted at 13,000 × *g* for 10 min at 4 °C, and the supernatant was treated with 0.5 volume of ammonium acetate buffer pH 7.2. Centrifugation was carried out at 13,000 × *g* for 5 min at 4 °C. The aqueous phase was treated with 2.5 volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate (pH 5.0), and then incubated at –80 °C for 60 min. DNA was then recovered by centrifuging at 13,000 × *g* for 30 min at 4 °C. DNA was washed once with 70% ethanol and dried in a vacuum dryer. Finally, the DNA was resuspended in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]).

2.2.9. Extraction of genomic DNA for Southern blot hybridization

Isolates were inoculated into 30 ml of full-strength R2A broth (Reasoner and Geldreich, 1985) and incubated at 30 °C with shaking (100 rpm) in a shaker until the cultures were turbid (OD_{600} of 0.1-0.5 for 10× diluted sample). The cells were then pelleted by centrifuging at 3,000 × *g* for 30 min at 4 °C. Finally, the high molecular weight genomic DNA was extracted by using one of the following three methods with a few modifications:

2.2.9.1. Silica gel membrane-based procedure

The procedure followed here was exactly as described in the protocol for QIAamp™ DNA Mini Kit (Qiagen, Germany). The detailed methodology can be found in http://www1.qiagen.com/literature/handbooks/PDF.

2.2.9.2. Phenol-chloroform extraction (Wilson, 1999 with slight modifications)

The cell pellets were washed by resuspending in 20 ml of TE buffer and centrifuging at 3,000 × *g* for 30 min. The pellets were then treated with 5 ml of lysozyme buffer and incubated at 37 °C for 30 min. 1 ml of 10% SDS, 22 µl RNase and 30 µl of 20 mg/ml Proteinase K (Qiagen, Germany) were added, mixed, and incubated at 55 °C for 30 min with regular mixing at 10 min intervals. The entire solution was then treated with an equal volume of phenol/chloroform/isoamyl alcohol, mixed by inverting the tube, and centrifuged at 13,000 × *g* for 10 min at 4 °C. The upper aqueous layer was transferred to a fresh tube and was incubated on ice for 5 min. Then 0.1 volume of 3 M sodium acetate and 0.6 volume of ice-cold iso-propanol were added and mixed gently until a stringy white DNA precipitate became clearly visible. The resulting pellets were transferred to a fresh tube containing 70% ethanol by hooking it onto the end of a pasture pipette that has been heat-sealed and bent in a Bunsen flame. If DNA precipitation was not clearly visible, the entire solution was incubated at –80 °C for 30 min. DNA was then precipitated by centrifuging at 13,000 × *g* for 30 min at 4 °C. DNA thus obtained was washed once with 70% ethanol and dried in a vacuum dryer. Finally, the DNA was resuspended in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]).

2.2.9.3. Benzyl chloride extraction (Zhu *et al*., 1993 with slight modification)

The cell pellets were washed by resuspending in 20 ml of TE buffer and centrifuging at 3,000 × *g* for 30 min. The pellets were then treated with 5 ml of lysozyme buffer and incubated at 37 ^{o}C for 30 min. 5 ml of extraction buffer (100 mM Tris-HCl, pH 9.0, 40 mM EDTA), 22 µl RNase, 30 µl of 20 mg/ml Proteinase K, 1 ml 10% SDS, and 3 ml benzyl chloride was added, and the tube was vortexed and incubated at 50 °C for 30 min with shaking or repeated vortexing at 5-min intervals to keep the two phases thoroughly mixed. Centrifugation was carried out at 3,000 × *g* for 30 min. The upper aqueous layer was transferred to a fresh tube and 3 ml of 3 M sodium acetate, pH 5.0 was added, and the tube was kept on ice for 15 min. After centrifugation at 3,000 × *g* for 15 min at 4 °C, the supernatant was collected, and 0.6 volume of precooled iso-propanol was added. The tube was shaken back and forth until a stringy white DNA precipitate became clearly visible. The pellets were transferred to a fresh tube containing 70% ethanol by hooking it onto the

end of a pasture pipette that was already been pre-heat-sealed and bent in a Bunsen flame. Finally, the DNA was resuspended in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]).

2.2.10. Determination of DNA concentration

The concentration of extracted DNA in suspension was estimated by spectrophotometric measurement at $A_{260.}$ For double-stranded DNA suspensions, an OD value at a wavelength of 260 nm and using a cuvette with 1 cm path length is equal to a concentration of 50 mg/ml. The quality of the DNA was evaluated by measurement of absorbance at 260, 280 and 230 nm. Ideally, the ratio of the A_{260}/A_{280} should be 1.8-2.0. A ratio less than 1.8 indicates protein or phenol contamination, whereas the ratio grater than 2.0 indicates the presence of RNA. The ratio of the A_{230}/A_{260} should be 0.3-0.9. Ratio greater than 0.9 indicate the presence of salts or humic acids.

2.2.11. Polymerase chain reaction (PCR) of bacterial 16S rRNA genes

PCR was carried out using the oligonucleotides primers 9f and 1492r (Lane, 1991), which amplify 16S rRNA genes of a wide range of members of the domain *Bacteria* from positions 28 through 1491 (*E. coli* numbering [Brosius *et al*., 1978]). The reaction mixture contained 1 µl of template DNA, 10 µl of 5 × reaction buffer (Promega, Germany), 3 µl of 25 mM $MgCl_2$ (Promega, Germany), 5 µl of 10 mM dNTP mix (Promega, Germany), 0.5 µl of 33 pmol (each) primer (MWG-Biotech, Ebensburg, Germany), and 2.5 U of Taq DNA polymerase (Promega, Germany). Finally, total volume was made to 50 µl with sterile water. Amplification was performed in 0.2 ml reaction tubes using a DNA thermal cycler (ABI 9600; PE Applied Biosystems). The thermal PCR profile was as follows: initial denaturation at 94 °C for 2 min; 30 cycles, consisting of denaturation at 94 °C for 45 s, primer annealing at 48 °C for 60 s, and elongation at 72 °C for 120 s. The final elongation step was extended to 12 min. Aliquots of the 16S rRNA gene amplicons (10 ml) were analyzed by electrophoresis on a 1% agarose gel.

The PCR products thus amplified were purified by using QIAquick™ purification kit (Qiagen, Germany) following the manufacturer's instructions. Purified PCR products were eluted from the purification columns by adding 50 µl 10 mM Tris-buffer (pH 8.0).

2.2.12. Cycle sequencing

For cycle sequencing, the BigDye™ Terminator v1.1 cycle sequencing kit (Applied Biosystem, Germany) was employed. The PCR reagent mix was prepared by combining the following reagents (on ice) in a 0.5 ml microcentrifuge tube: 2 µl ready reaction premix, 1 µl BigDye sequencing buffer, 3.2 pmol (f or r) primer, 60-80 ng (2 µl) of PCR product and distilled water was added to make the volume 10 µl. Tubes were placed in a thermal cycler (Applied Biosystems 9600) preheated to 104 ^{o}C. The program used was as follows: initial denaturation at 96 °C for 30 s; 25 cycles, consisting of denaturation at 94 °C for 10 s, primer annealing at 50 °C for 5 s, and final elongation at 60 °C for 4 min. The product was then stored at 4 °C until ready to purify the extension products. The following primers were used for the complete 16S rRNA gene sequencing:

Table 2.1. Primers used for the sequencing of 16S rRNA gene

Designation	**Sequence**	**References**
9f	GAG TTT GMT CCT GGC TCA G	Lane, 1991
315f	CAG ACT CCT ACG GGA GGC AGC AGT AGG GAA TC	Lane, 1991
519b	GTA TTA CCG CGG CTG CTGG	Stubner, 2002
907b	CCG TCA ATT C(A/C)T TT(A/G) AGT TT	Muyzer *et al*., 1993
1100b	AGG GTT GCG CTC GTT	Lane, 1991
1492b	ACG GYT ACC TTG TTA GGA CTT	Weisburg *et al*., 1991

The cycle-sequenced product was purified by using AutoSeq™G-50 (GE Health Care Life Science, Germany) columns as described in the manufacturer's protocol. Sequences were determined with an ABI-373 sequencer (Applied Biosystems, Germany) and analyzed with the sequence analysis software version 3.3 (Meixner sequencing service, Germany).

Phylogenetic analysis

Each of the 16S rRNA gene sequences had a length of at least 1300 nucleotides and was aligned to the ARB sequence database (Ludwig *et al.*, 2004). The resulting alignments were used for analyses without making changes of possible errors in the public-domain 16S rRNA gene sequences. Phylogenetic trees were constructed using Tree-Puzzle, a quartet maximum-likelihood method (Schmidt *et al*., 2002). The trees were constructed using a model of sequence evolution as suggested by Schoniger and von Haeseler (1994) and 10,000 puzzling steps.

2.2.13. Southern blot hybridization analysis

The copy number of *rrn* was determined by Southern blot hybridization analysis. First, genomic DNA from each isolate was digested by using three different restriction enzymes: EcoRI, PstI, PvuII (Promega, Germany). A survey of the ARB sequence database showed for the 16S rRNA genes of most isolates that these three enzymes did not have a recognition site within the probe-target region. However, if a recognition site was identified for one of the three enzymes, it was replaced with SmaI. For each restriction analysis, approximately 1 µg of genomic DNA was digested with the respective endonuclease using the manufacturer's protocol. The restricted genomic DNA's were separated on an agarose gel (0.8%) and transferred to a positively charged nylon membrane (Roche Applied Science, Germany) by capillary blotting (Sambrook *et al.,* 1989). A PvuII digest of *E. coli* genomic DNA was included on each Southern blot as a positive control. A digoxigenin (DIG)-labeled 16S rRNA gene hybridization probe complementary to the positions 9 to 519 of *E. coli* 16S rRNA (IUB nomenclature) was generated by PCR according to the supplier's instructions (Roche Applied Science, Germany). Southern blot hybridization and detection of DIG-labeled probes were carried out according to the protocol described in the DIG application manual for filter hybridization (Roche website: http://www.roche-applied-science.com). CDP-Star chemiluminescent substrate was used and signals were visualized on X-ray film (Kodak, Japan).

2.2.14. Nucleotide sequence accession numbers

The 16S rRNA gene sequences of all isolates from early and late succession have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. AM412116 to AM412174 and AM411905 to AM411970, respectively.

2.2.16. ANOVA and t-test

Significant differences in DAPI and CFU counts between early and late succession were tested by using *t*-test ($P<0.05$). One-way analysis of variance ($P<0.05$) was applied to test for significant differences between replicates of DAPI and CFU counts.

3. Results

3.1. Moisture content and pH determination

Moisture content of the soil from early and late succession was not significantly different (P>0.05), which was approximately 35% and 40% w/w respectively. This moisture content was of relevance for the total and cultivable bacterial count in dry weight basis. The pH of the soil sample was determined as approximately pH 6 for both early and late succession. Based on the pH of the soil, pH of the medium was adjusted to 6 with 1 M sodium hydroxide or 1 M hydrochloric acid.

3.2. Total and culturable community size

The DAPI counts were significantly different between early successional and late successional communities, while the total CFU counts did not differ significantly (Table 3.1). The proportion of bacteria that was culturable from early succession corresponded to approximately 40% (undiluted media) and 37% (diluted media) of the respective DAPI counts. Similarly, the recovery rate was 31% (undiluted media) and 35% (diluted media) for late succession (Table 3.1). DAPI-stained sample and colony morphology of culturable bacteria for early succession and late succession are shown in Fig. 3.1.

3.3. CFC analysis and successional stage

The growth-response time on the different media (NA, R2A, and SCDA in full and diluted strength) showed a similar trend for either early or late succession, except on full-strength SCDA medium in late succession. Overall, CFC analysis revealed a significant shift in colony-forming time between early and late succession (Fig. 3.2). In early succession greater than 90% of colonies became visible within 1 day and no colonies appeared after 8 days of incubation. In contrast, colony-forming time in late succession was between 2 and 15 days with the majority of colonies appearing after 2 days or, in case of full-strength SCDA medium in late succession, after 3-5 days of incubation.

The progression of the colony-forming curves indicative of late succession changed if plates showing 10-30 colonies were used for CFU enumeration, rather than those exhibiting 30-300 colonies (Fig. 3.2). Obviously, the average number of CFU that became visible with extended incubation time increased, particularly on the diluted media. However, the statistical significance of this observation was difficult to assess due to low absolute numbers of CFU to be enumerated and strong variations in CFU numbers between replicate plates. Thus, the CFU counts of all the plates (NA, R2A, and SCDA full and diluted strength media) exhibiting 10-30 colonies were combined to calculate a single CFC to represent an approximate trend of colony-forming time.

A total of 59 early successional and 66 late successional CFU were selected from the most terminal dilution plates for the isolation. The majority of early successional isolates were comprised of large and slimy colonies, while those from late succession were mostly small and non-slimy colonies and were difficult to disintegrate.

Table 3.1. Total bacterial counts (DAPI counts), and total viable cell counts from the oxic layer of flooded, unplanted rice paddy soil microcosms.

Microcosms	**Numbers of DAPI-stained cells and CFU ($N \times 10^8$ g^{-1} of dry soil)[1]**					
	1-day-old microcosms[2] (early succession)			70-day-old microcosms[2] (late succession)		
	DAPI	CFU[3] (1:1)	CFU[4] (1:100)	DAPI	CFU[3] (1:1)	CFU[4] (1:100)
M-1, M-4	7.30	2.66	2.09	5.87	1.53	1.75
M-2, M-5	7.60	3.50	3.25	5.39	2.32	2.33
M-3, M-6	7.30	2.73	3.04	5.37	1.45	1.80
Mean ± SD[5]	7.4 ± 0.6	2.9 ± 0.4	2.7 ± 0.6	5.5 ± 0.2	1.7 ± 0.4	1.9 ± 0.3

[1]For each microcosm, total DAPI and CFU counts are mean values of at least three replicates.

[2]CFU counts are mean values of numbers obtained for early succession (M-1, M-2, M-3) and late succession (M-4, M-5, M-6) on NA, R2A, and SCDA. Using ANOVA ($P<0.05$), no significant differences in the CFU numbers were observed among the media.

[3]CFU counts on undiluted (1:1) growth media.

[4]CFU counts on diluted (1:100) growth media.

[5]Mean values ± standard deviation (SD).

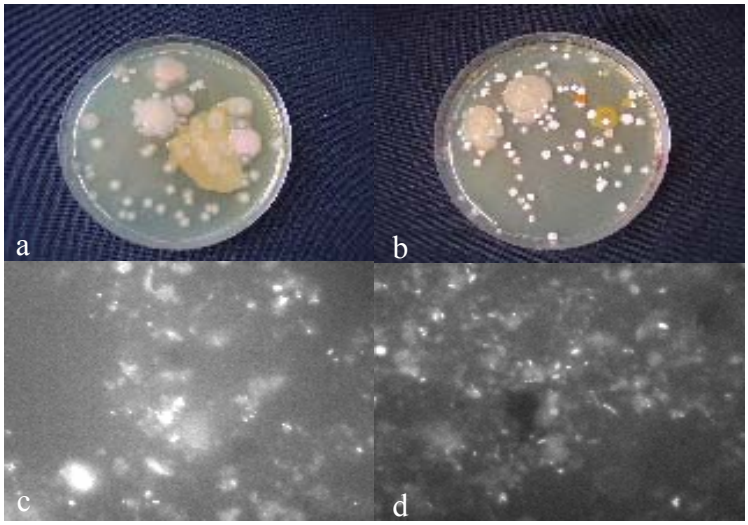

Fig. 3.1. Representative pictures showing DAPI-stained of total bacteria and colony morphology of culturable bacteria for early successional (a, c) and late successional (b, d) samples.

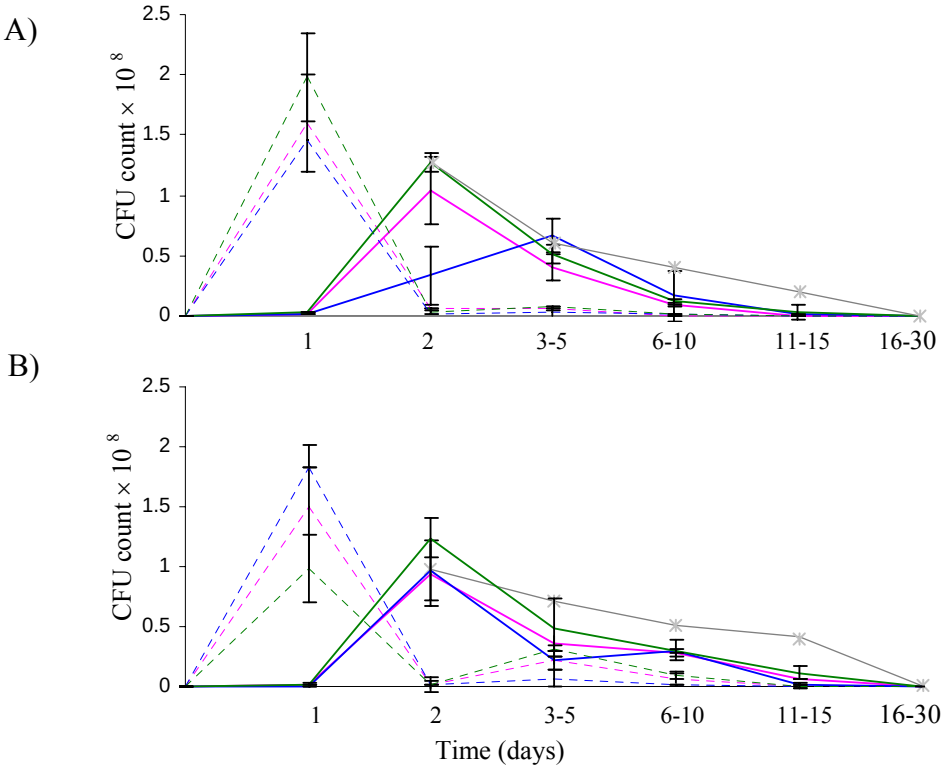

Fig. 3.2. Colony-forming curve analysis showing the number of CFU in relation to their appearance on full (A) and diluted (B) strength media (red, NA; blue, SCDA; green, R2A). Dotted lines show CFU counts from early succession and solid lines indicate those from late succession. Colony-forming curves were calculated using the CFU counts of plates

showing 30-300 colonies. Error bars represent standard deviation (n = 9 [triplicate plating for each of three replicate microcosms incubated for either 1 or 70 days]). Gray line indicates colony-forming curves that were calculated using plates showing 10-30 colonies. These curves represent an approximate for the increase in CFU counts on full-strength (1:1) and diluted (1:100) media between 2 and 30 days of incubation in late succession.

3.4. Phylogenetic classification and successional stage

Comparative 16S rRNA gene sequence analyses showed that all isolates grouped with one of the following six bacterial lineages: *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Bacilli*. The majority of early successional isolates belonged to the *Beta-* and *Gammaproteobacteria* (47 isolates), while the predominant groups among the late successional isolates were *Alphaproteobacteria* and *Actinobacteria* (49 isolates) (Figure 3.3). Isolates with an overall 16S rRNA gene sequence identity of ≥98% were considered to be highly related and to possibly form a single species (Stackebrandt and Goebel, 1994; Dighe *et al.*, 2004). Thus, they were grouped into species-level clusters (SLC) (Table 3.2). The number of isolates within the same SLC varied from 1 to 18. Without any exception, day of colony appearance of all isolates that belonged to the same SLC was identical in CFC analysis.

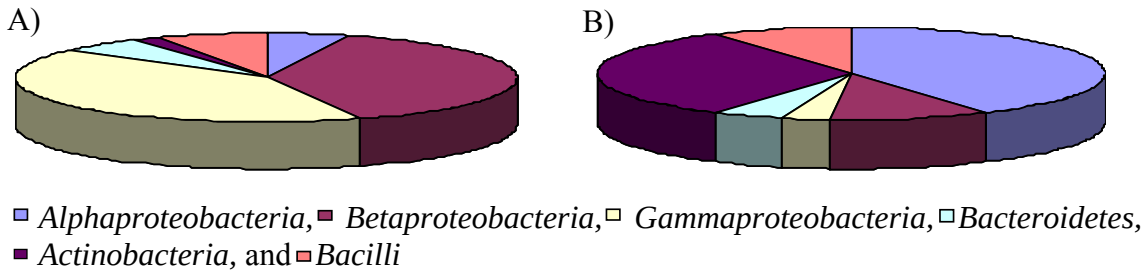

Fig. 3.3. Diagrams showing the phylogenetic distribution of species level clusters for A) early succession (total number of isolates = 59), and B) late succession (total number of isolates = 66). Phylogenetic assignment is based on nearly full-length 16S rRNA gene sequences (≥1300 nt).

All the betaproteobacterial SLC belonged to only two families (*Comamonadaceae* and *Oxalobacteriaceae*) while the alphaproteobacterial SLC were phylogenetically more diverse and affiliated with seven families, including *Beijerinckiaceae*, *Bradyrhizobiaceae,*

Caulobacteraceae, Hyphomicrobiaceae, Rhizobiaceae, Rhodospirillaceae, and *Sphingomonadaceae* (Fig. 3.5). Among *Gammaproteobacteria*, the four early successional SLC belonged to the *Moraxellaceae* and *Pseudomonadaceae*, while the two late successional SLC grouped with the *Xanthomonadaceae* (Fig. 3.5). Among *Bacteroidetes*, the single early successional SLC was assigned to the *Flavobacteriaceae*, while the two late successional SLC belonged to the *Flexibacteraceae* (Fig. 3.6a). The SLC that were assigned to *Actinobacteria,* grouped into the families *Gordoniaceae, Nocardiaceae, Microbacteriaceae, Micrococcaceae,* and *Mycobacteriaceae* (Fig. 3.6b) and the *Bacilli*-like SLC belonged to the *Bacillaceae and Paenibacillaceae* (Fig. 3.6c). Out of 49 SLC analyzed in total, 38 SLC exhibited 16S rRNA gene sequence similarities of ≥98% with taxonomically described species. Members of six SLC showed 16S rRNA gene sequence similarities between 95% and 97% with taxonomically described species. 16S rRNA gene sequence similarities below 95% with taxonomically described species were identified for members of five SLC, including alphaproteobacterial LS_SLC_10, LS_SLC_11, and LS_SLC_16 (Fig. 3.5), and two LS_SLC (_24, _25) assigned to *Bacteroidetes* (Fig. 3.6a).

3.5. Successional stage, growth response time, and *rrn* copy number

The *rrn* copy numbers were determined for 16 early and 26 late successional isolates, comprising a single representative of almost each SLC (Fig. 3.5, 3.6 a, b; Table 3.2). The only exception was SLC assigned to the *Bacilli*, whose analysis was limited to two SLC in order to confirm high *rrn* copy numbers (Fig. 3.6c). The homogeneity of the *rrn* copy numbers among isolates of the same SLC was tested by random analysis of two representatives from each of three early successional SLC and three late successional SLC. The *rrn* copy numbers between the two test strains of each SLC were identical. All the early successional SLC belonging to *Betaproteobacteria, Gammaproteobacteria, Bacteroidetes*, and *Actinobacteria* showed a perfect correspondence between successional stages, growth response time upon nutrient availability, and *rrn* copy number (Table 3.2; Figs. 3.5, 3.6 a, b). Their members formed visible colonies within one (11 SLC) or two days (6 SLC) of incubation and all early successional isolates tested possessed more than four *rrn* copies. By contrast, the two early successional SLC assigned to *Alphaproteobacteria* exhibited a colony-forming time of 6-10 days (Table 3.2; Fig. 3.5). However, their growth-response time corresponded well to the single *rrn* copy detected by Southern blot hybridization analysis.

Table 3.2. Total number of isolates and species-level clusters (SLC) assigned to *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Bacilli.*

Taxon	Early succession					Late succession				
	[1]Isolates	Total no. of SLC	[2]Time	SLC analyzed [2]	*rrn* copies	[1]Isolates	Total no. of SLC	[2]Time	SLC analyzed[2]	*rrn* copies
Alphaproteobacteria	3	2	3-5	NA	NA	**27**	**12**	**3-5**	**1**	**4**
								3-5	**3**	**1**
			6-10	1	1			**6-10**	**4**	**1**
								6-10	**2**	**2**
								11-15	**2**	**1**
Betaproteobacteria	**23**	**7**	**1**	**5**	**5**	6	3	3-5	1	2
				2	**4**			11-15	2	2
Gammaproteobacteria	**24**	**4**	**1**	**3**	**7**	2	1	6-10	1	3
			2	**1**	**5**					
Actinobacteria	2	2	2	1	4	**22**	**7**	**2**	**3**	**2**
								2	**1**	**3**
				1	4			**3-5**	**2**	**2**
								11-15	**1**	**2**
Bacteroidetes	3	1	1	1	7	3	2	11-15	1	1
								6-10	NA	NA
Bacilli	4	3	2	1	11	6	5	2	1	11
								2	1	9
Total	**59**	**19**		**16**		**66**	**30**		**26**	

[1] Total number of strains that were isolated from terminal positive dilution steps and phylogenetically analysed.
[2] Time (in days) needed for the isolates to form a visible colony on the solid agar medium.
[NA] Isolates whose *rrn* copy number could not be determined owing to very low biomass yield.

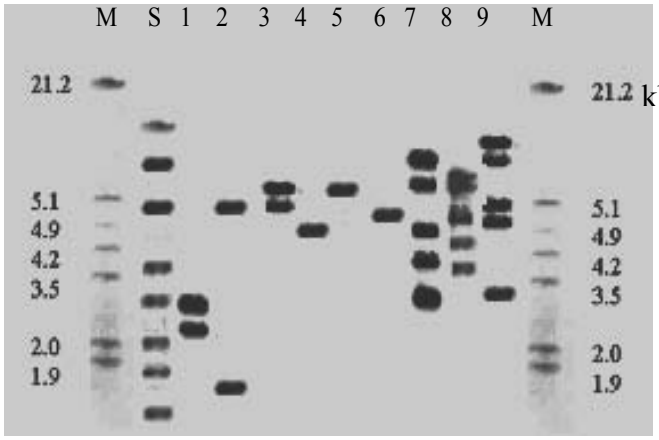

Fig. 3.4. Southern blot hybridization of genomic DNA from *E. coli* (lane S), P70-34 (lanes 1, 2, and 3), P70-23 (lanes 4, 5, and 6), and P1-188 (lanes 7, 8, and 9) cut with EcoRI (lane 1, 4, and 7), PstI (lane 2, 5, and 8), and PvuII (S, 3, 6, and 9). Lane 'M' represents the DNA size marker.

All the late successional SLC belonging to *Alphaproteobacteria*, *Betaproteobacteria, Gammaproteobacteria, Bacteroidetes*, and *Actinobacteria* also showed a strong correspondence between successional stages, growth response time, and *rrn* copy number (Table 3.2; Figs. 3.5, 3.6 a, b). Their members always exhibited a colony-forming time of 3 to 15 days (20 SLC), except four actinobacterial SLC that required only two days of incubation to form visible colonies (Fig. 3.6 b). All late successional isolates tested possessed ≤2 *rrn* copies with three exceptions: LS_SLC_9 (four *rrn* copies), LS_SLC_16 (three *rrn* copies) (Fig. 3.5), and LS_SLC_19 (three *rrn* copies) (Fig. 3.6 b). Among the five SLC assigned to the *Bacilli*, three SLC contained isolates from both early and late succession. Members of all *Bacilli*-like SLC showed a colony-forming time of 2 days and had more than eight *rrn* copies (Fig. 3.6 c).

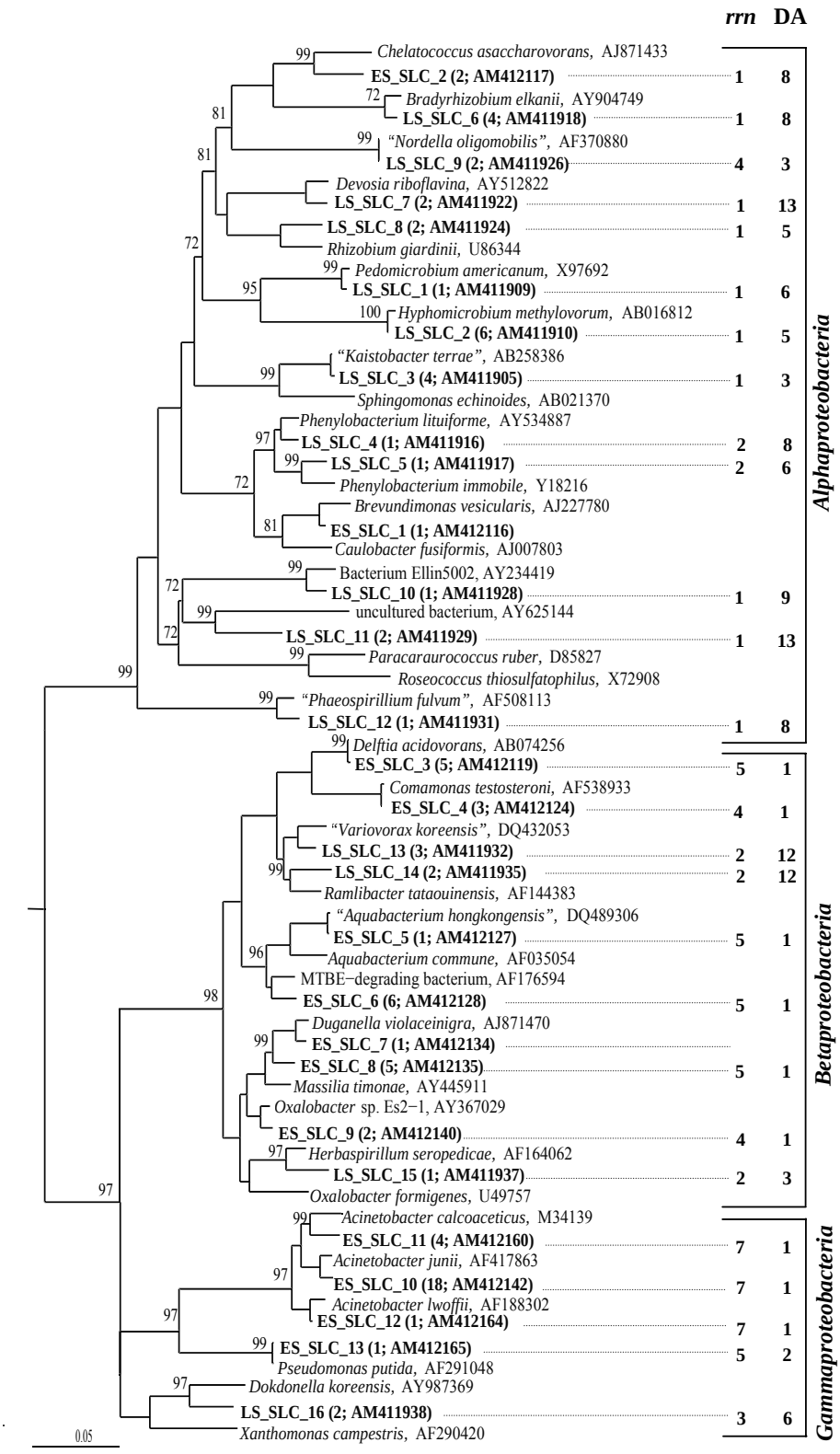

Fig. 3.5. Maximum likelihood tree showing the phylogenetic relationship between species-level clusters and representative members of the *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*. The prefixes ES_SLC and LS_SLC denote species-level

clusters from early and late succession, respectively. Number of isolates assigned to the respective SLC and the 16S rRNA gene accession number of a single representative are given in parentheses. The numbers in the columns indicate the *rrn* copy number (*rrn*, left column) and the day of colony appearance (DA, right column). The numbers at the branch points are tree puzzle values. Only values greater than 60 are shown. The scale bar represents 5% sequence divergence.

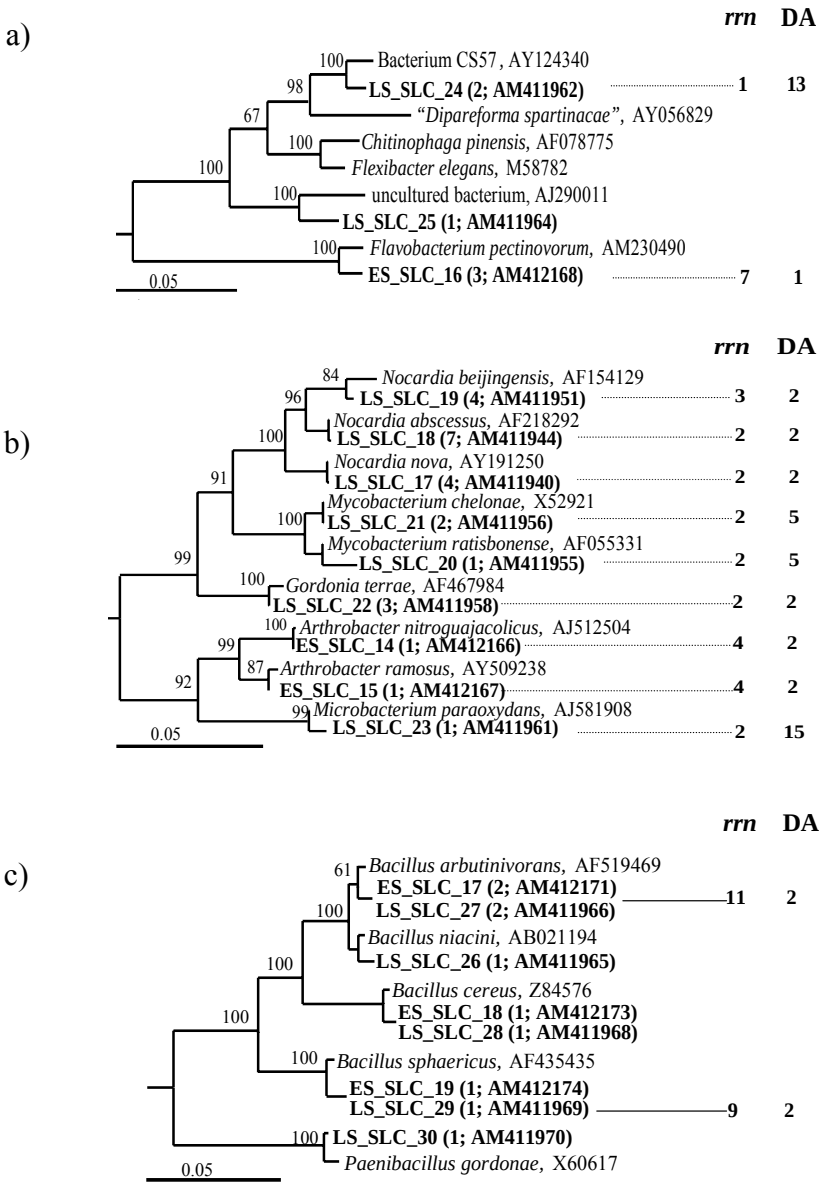

Fig. 3.6. Maximum likelihood trees showing the phylogenetic relationship between species-level clusters and representative members of the a) *Bacteroidetes* b) *Actinobacteria*, and c) *Bacilli*. For further details, see the legend to Fig. 3.5.

4. Discussions

4.1. Cultivation and CFC analyses

Regardless of the successional stage and the media used for cultivation, total culturable cell counts were found to be higher than in most other cultivation studies from soil (Olsen and Bakken, 1987; Zarda *et al*., 1999; Janssen *et al*., 2002) and similar to the cultivation studies by Chin *et al*. (1999) and Davis *et al*. (2005). Like in the study by Davis *et al*. (2005) who also used solid media to isolate bacteria from soil, microbial cells were detached from soil particles by vortexing coupled with ultrasonic treatment. It has been proposed that this approach is more efficient than simple vortexing (Buesing and Gessner, 2002; Kuwae and Hosokawa, 1999; McDaniel and Capone, 1985; Mott *et al*., 1998).

From all SLC tested, replating of isolates always resulted in the same colony-forming time, as originally observed in the isolation procedure. This finding provided evidence that the time required to form visible colonies was not related to the physiological state of the bacterial cells but, instead, was a stable characteristic of the isolates and thus of their life strategy. As a result, the CFC analysis enabled reliable classification of SLC into fast and slow growers, which corresponded well to successional stage (Table 3.2). A similar classification was also used by Sigler and Zeyer (2004) who studied bacterial community succession in deglaciated soil. Soils deglaciated for 10 years were colonized mainly by fast growers, whereas slow growers were prevalent in soils, which deglaciated for 100 or more years. In that study, fast growers formed visible colonies within 24 h, while slow growers were defined as those, which formed colonies after 36 h of incubation.

In my study, differentiation between fast and slow growers was more pronounced. Most of the early successional SLC (and isolates) had a colony-forming time of 1 day and were defined as fast growers. However, most of the late successional SLC required 3 to 15 days of incubation to form visible colonies and were classified as slow growers. Regardless of whether isolated from early (2 SLC) or late (4 SLC) succession, members of six actinobacterial SLC showed a colony-forming time of 2 days and may thus be considered intermediate growers. These intermediate growers, however, could be differentiated by their *rrn* copy numbers (Table 3.2). Except two *Bacillus*-like SLC, isolates from early

succession always belonged to different SLC than those from late succession. All our *Bacillus*-like isolates showed a colony-forming time of 2 days, irrespective of whether isolated from early (3 SLC) or late (5 SLC) succession. This finding may be due to the fact that a major portion of the *Bacilli* was present in the rice paddy soil as spores rather than as vegetative cells.

The species richness culturable from early succession was lower than that from late succession (19 vs. 30 SLC, Table 3.2). This trend towards increased species diversity in late succession agrees well with the theory that mature communities have a greater taxon richness and evenness than early communities (Andrews and Harris, 1986; Martiny *et al*., 2003). Similarly to my study, Garland *et al*. (2001) reported increasing species diversity with successional age for a bacterial rhizosphere community incubated in a bioreactor.

A good correspondence between number of SLC and CFC analysis was observed in early but not in late succession. In early succession, 11 SLC (60%) were detectable after 1 day of incubation, in correspondence to maximum numbers of CFU (Fig. 3.2). However, in late succession, only nine SLC (30%) were detectable after 2 days of incubation, although the maximum numbers of CFU were observed at this time point, for both NA and R2A media. Most of the late successional SLC showed a colony-forming time of 6-15 days (14 SLC, 46%), while seven SLC (24%) became visible after 3-5 days of incubation. To obtain statistically significant results, agar plates were used for CFU counts that contained between 30 and 300 colonies (mostly 100-200 CFU). Thus, a possible explanation for the discrepancy between CFC analysis and number of SLC isolated in late succession from the different incubation periods is that the high density of colonies growing within 2 days led to substrate depletion in the media and to inhibitory or competitive effects on more slowly growing populations (Balestra and Misaghi, 1997; Davis *et al*., 2005). As a consequence, slow growers may not have been able to form visible colonies on these already densely colonized plates, thereby resulting in biases towards overestimation of faster growing populations. This view is supported by the approximate trend of increased CFU numbers during later incubation periods, if plates showing 10-30 colonies were used in CFC analysis. This trend seems to be more pronounced for the 1:100-diluted media than for the full-strength media, which is in good correspondence with the prediction that late succession is characterized by oligotrophic (*K*-selected) bacteria. Notably, members of all

five late successional SLC that exhibited 16S rRNA gene sequence similarities below 95% to taxonomically described species and thus are novel at the genus level which required 6-15 days of incubation to form visible colonies. These findings corroborate the conclusion that isolation from terminal positive dilution steps and extended incubation times were the key factors for the increased species richness culturable from late succession.

4.2. Phylogenetic identity and successional stage

Overall, early succession in the oxic zone of the flooded rice paddy soil microcosms was characterized by fast-growing bacteria belonging to *Betaproteobacteria* and *Gammaproteobacteria*, whereas slow-growing bacteria of the *Alphaproteobacteria* and *Actinobacteria* were prevalent in late succession. Previous reports on successional changes in the phylogenetic composition of soil bacterial communities are rare, in particular on changes in the culturable fraction. Kristufek *et al*. (2005) used a cultivation approach to study the primary succession of heterotrophic bacterial populations in the surface and mineral layers of brown coal colliery spoil. The identification of isolates was achieved by phospholipid fatty acid (PLFA) analysis, having as a consequence some ambiguous taxonomic assignments. Despite these uncertainties in the phylogenetic identity of the strains isolated by Kristufek *et al*. (2005), the authors` overall findings agree to some extent with the results of my study. Fast-growing *Gammaproteobacteria*, in particular *Pseudomonas* spp., prevailed in early succession. In contrast, slow-growing *Actinobacteria* were typical of late succession, except *Arthrobacter* spp. that were isolated with high frequency from early succession, but also from late succession. Based on colony appearance within 1-2 days in CFC analysis from rice paddy soil, Mitsui *et al*. (1997) reported that *Arthrobacter*-related species are dominant fast growers. This observation corresponds well to the multiple *rrn* copies detected in members of the early successional SLC_14 and SLC_15 (Fig. 3.6.). Thus, in contrast to most other actinobacterial isolates, *Arthrobacter* spp. are typical representatives of early succession but may also be present in late succession, presumably owing to their ability to form dry-resistant cysts. The ubiquitous presence of *Bacillus* and *Paenibacillus* in all successional stages of soil bacterial communities seems to be a general phenomenon, as these organisms were also isolated by Kristufek *et al*. (2005) with high frequency from both early and late succession.

Remarkably, the overall microbial community pattern detected in ancient permafrost soils by a combination of cultivation and molecular techniques have some similarity to the composition of the late successional community in my model system. Permafrost soil can be considered as a *K*-selected environment in which the microbial community is characterized by adaptation to low-temperature life. Except spore formers, four major taxa were identified: *Sphingomonas* (*Alphaproteobacteria*), *Lysobacter* and *Psychrobacter* (*Gammaproteobacteria*), *Microbacteriaceae* (*Actinobacteria*), and *Sphingobacterium* (*Bacteroidetes*) (Vishnivetskaya *et al*., 2006). *Lysobacter* and *Psychrobacter* belong to the family *Xanthomonadaceae* (Fig. 3.5.). Members of this family are known to be slow growers possessing only 2 *rrn* copies; this in contrast to early successional *Gammaproteobacteria*. Similarly, *Sphingomonas* only harbors a single *rrn* copy and, accordingly, is well known as a slow grower (see below). In support with previous observations, the permafrost soil was also inhabited by *Bacillus*, *Paenibacillus*, and *Arthrobacter*.

4.3. *rrn* copy number and successional stage

In good agreement with previous reports that high numbers of rRNA genes enable fast growth response to resource availability (Klappenbach *et al*., 2000), the *rrn* copy number was almost perfectly correlated with the colony-forming time of isolates belonging to *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, and *Actinobacteria* and, as a consequence, also corresponded well to successional stage (Table 3.2).

It had previously been reported that the presence of multiple rrn copies supports high cell growth rates (Bremer et al., 1987; Tan et al., 2001). For example, Vibrio angustum and Escherichia coli show high growth rates in correspondence to their large set of multiple rrn copies. These two organisms possess 8-11 and 7 rrn copies, respectively (Wolfe and Haygood, 1993; Kiss et al., 1977). By contrast, slow-growing bacteria such as the marine Sphingomonas sp. strain RB2256 (Fegatella et al., 1998) and Mycobacterium spp. (Bercovier et al., 1986; Helguera-Repetto et al., 2004) have only one or two rrn copies.

Thus, I anticipated that the rrn copy number would be a suitable marker to corroborate that different positions of bacterial species in the r/K-continuum are due to adaptive differences

that correspond to genotypic features. This assumption was largely confirmed by the results. In early succession, all members of those SLC that showed colony-forming time of 1 day (11 SLC) possessed ≥4 rrn copies, whereas in late succession members of almost all 20 SLC that required more than 2 days for colony appearance (20 out of 22 SLC) exhibited ≤2 rrn copies. Most isolates characterized as intermediate growers belonged to the Actinobacteria. The Arthrobacter spp. isolated from early succession always showed 4 rrn copies (2 SLC), while the late successional isolates were related to Gordonia and Nocardia and possessed ≤3 rrn copies (4 SLC) (Table 3.2, Fig. 4.1).

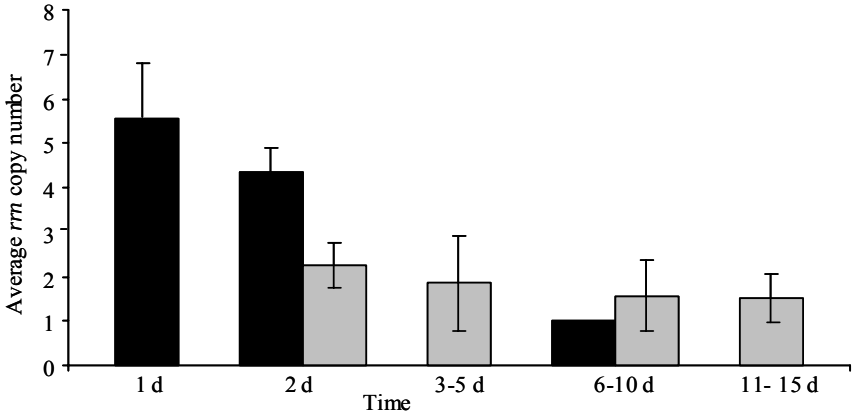

Fig. 4.1. Mean copy numbers of *rrn* determined for 15 early successional (black bars) and 24 late successional (light bars) species-level clusters. Each bar and error bar represents the mean value (n=3-11) and corresponding standard deviation for species-level clusters whose colonies appeared in colony-forming curve analysis after 1, 2, 3-5, 6-10, or 11-15 days. SLC assigned to *Bacilli* were not considered for calculation of the mean numbers.

The only exceptions are Bacillus-like species, which were isolated from both early and late succession with almost equal frequency. These organisms always contained ≥9 rrn copies. As already mentioned above, their isolation from both successional stages may be due to their ability to form spores.

4.4. Final remarks

I have addressed the issue of bacterial community succession in the oxic zone of flooded, unplanted rice paddy soil microcosms using cultivation techniques. The results revealed a clear shift in the bacterial composition cultivable from early and late succession. This finding was the starting point to demonstrate that phylogenetic identity, growth response

time and *rrn* copy number of heterotrophic isolates are useful markers for different stages in bacterial community succession. However, the degree of correlation among the three markers and to successional change varied. A high degree of correlation between growth response time and *rrn* copy number was given for almost all proteobacterial isolates, as exemplified by the alphaproteobacterial SLC ES_SLC_2. Despite the fact that the two strains of ES_SLC_2 were isolated from early succession, they possessed a single *rrn* copy and, accordingly, had a colony-forming time of 6-10 days. The only major exception were the two members of LS_SLC_9. These alphaproteobacterial isolates possessed 4 *rrn* copies, but required three days for colony appearance. The correlation between growth response time and *rrn* copy number was less pronounced for *Actinobacteria*, which corresponds to the fact that most intermediate growers belonged to this phylum.

The phylogenetic identity itself was a good marker for successional stage, because the vast majority of beta- and gammaproteobacterial isolates were obtained from early succession and, vice versa, almost all *Alphaproteobacteria* and *Actinobacteria* were isolated from late succession. However, major exceptions of this trend were observed for all three proteobacterial classes and for *Actinobacteria*. Examples are ES_SLC_1 assigned to the alphaproteobacterial *Brevundimonas vesicularis* and LS_SLC_13 assigned to the betaproteobacterial "*Variovorax koreesis*". Interestingly, in the study by Kristufek et al. (2005), *Brevundimonas vesicularis* and *Variovorax* paradoxon were also isolated as slow growers from early and late succession, respectively. These examples demonstrate that ecological strategies are not fully consistent with major taxonomic groups. Rather, adaptive processes towards changes in life strategy occur at the genus or even species level and thus evolutionarily in relatively short periods of time.

In principle, the growth strategy of pioneer organisms can be explained by the fact that these populations often possess increased tolerance to environmental extremes (Vitousek and White, 1981) despite the potentially high energetic cost of tolerating environmental stresses (Andersson and Levin, 1999). Such wasting of energy does not seem to have an effect on *r*-type organisms as it is regarded as a normal feature of early colonization strategy (Insam and Haselwandtner, 1989; Ohtonen *et al*., 1999). To further substantiate that, in my model system, early succession was defined by opportunistic type (*r*-strategist) whereas equilibrium type (*K*-strategist) prevailed in late succession, early and late successional isolates could be tested for their overall resistance to antibiotics. Previous

studies showed that opportunistic (*r*-selected) bacteria have a greater resistance to antibiotics than equilibrium (*K*-selected) bacteria (Andersson and Levin, 1999; Sigler and Zeyer, 2004).

Chapter II

Bacterial community changes in a paddy soil oxygen gradient, assessed by mRNA expression profiling

5. Introduction

It has been previously discussed in the first part of my Ph.D. work that the plate counts estimate only 1-10% of the overall soil microbial diversity (Olsen and Bakken, 1987; Zarda *et al*., 1999). However, recovery in my study was approximately 40% of the total count which was comparatively higher than most of the other studies. This does not mean that 40% of total bacterial diversity was cultured (for detail refer Chapter I). This showed that the majority of prokaryotic diversity is not represented in culture collections and hence remains unknown in terms of their phylogenetic and functional status. Although some preliminary information about the functional role of these uncharacterized groups can be revealed from *in situ* physiological studies such as MARFISH, this technique is cumbersome. Another possibility to study functional role includes study of gene expression (mRNA transcripts), thereby to identify genes and activities essential under varying conditions (Borneman and Triplett, 1997). Therefore, researchers are now focusing on the study of mRNA transcript pools, by which it should be possible to study the genes that are expressed by microbial communities, and to assign these genes both phylogenetically and functionally by comparing with the information available in the public-domain database. To monitor gene expression and to relate it to microbial activities that are observed in soil, it is necessary to extract purified RNA at a sufficient yield from environmental samples for subsequent analysis. Enzymatic inhibitors in soil such as humic acid can also impede post-extraction analysis (Tsai and Olson, 1992; Mendum *et al.*, 1998). A few studies (Pernthaler and Amann, 2004; Bürgmann *et al.*, 2003) have recorded gene expression in soil environments; they demonstrated that extraction of mRNA and RT-PCR analysis of transcripts could be a useful tool for the detailed analysis of activity and functional roles of microbial communities present in the environment. In a report by Bürgmann *et al.* (2003), *Azotobacter vinelandi*, grown in sterile sandy loam soil, served to examine *nif*H expression by RT-PCR. The following part of the introduction gives a detailed literature review on environmental transcriptome analysis and the environmental factors affecting the expression of the mRNA transcript pools.

5.1. Environmental transcriptome analysis

Due to the increasing number of whole genome sequences of microbes in public-domain database, there is a good possibility of identifying the microbial genetic diversity and

potential functional activity in soil (Torsvik and Øvreas, 2002). Some of the methods to assess potential activity, such as metagenomics, use high-molecular-weight DNA extractions directly from soil to create large-insert libraries of environmental microorganisms (Rondon *et al.*, 2000). Besides, the possibility to detect transcribed mRNA sequences directly in the environmental samples has permitted further insight into the functional activity. Several analytical procedures have been reported, most of them are restricted to isolating mRNA from a) pure cultures (Fleming *et al.*, 1998), b) soil amended with pure cultures (Tsai *et al.,* 1991), and c) soil for targeting specific transcripts by defined RT-PCR assays (Bürgmann *et al.*, 2003; Mendum *et al.*, 1998). Some of the most efficient methods currently available for the comparative analysis of mRNA transcript pools are differential display technique (Fislage *et al.*, 1997; Liang and Pardee, 1992; McClelland and Welsh, 1994; Wong and McClelland, 1994), poly (A) tailing (Grant *et al.*, 2006), and subtractive hybridization (Poretsky *et al.*, 2005). An alternative method for studying mRNA transcripts is to target the transcripts of specific metabolic activity *in situ*. For example, the *in situ* hybridization (ISH) of mRNA sequences has been used for studying gene expression in prokaryotic cells (Pernthaler and Amann, 2004) and eukaryotic cells and tissues (John *et al.*, 1969; Gerfen, 1989; Farquharson *et al.*, 1999; Morris *et al.*, 1990; Singer and Ward, 1982). Some of the approaches to quantify microbial gene expression in soil are given in Fig. 5.3.

5.1.1. Total RNA extraction

The starting point for the environmental transcriptome analysis is always the isolation of the total RNA pool. If the extraction method is generalized, the RNA isolation procedure can be divided into four steps: cell lysis, inactivation of nucleases, extraction of RNA from the environmental matrix, and purification of the RNA sample. The key step in this process is the complete lysis of microorganisms for intracellular RNA to be released (Ogram *et al.*, 1995). However, this step is subjected to the most variation among RNA extraction protocols. The most widely used cell lysis techniques for RNA extraction from soil are as follows: bead beating lysis of cells using glass or zirconium beads, solubilization of cell membranes by detergent, boiling or enzymatic degradation of the cell wall and membranes coupled with osmotic shock, usually with repeated freeze-thaw cycles (Borneman and Triplett, 1997; Bürgmann *et al.*, 2003; Hurt *et al.*, 2001; Ogram *et al.*, 1995). The method of choice for cell lysis can depend on the sample. The subsequent steps in RNA extractions

are standard between samples; inactivation of RNase activity to prevent losses of RNA, followed by the extraction of RNA, and purification of the RNA extract and removal of organic contaminants that co-extract with the nucleic acids (Ogram *et al.*, 1995). Each of these steps in the RNA isolation protocol causes a drop in RNA yield. Factors such as duration of treatment with nucleases, temperature of bead beating or types of lysis-denaturing solutions are varied to obtain maximum cell lysis, minimal RNA shearing, and optimal RNA yield and extraction efficiencies (Borneman and Triplett, 1997).

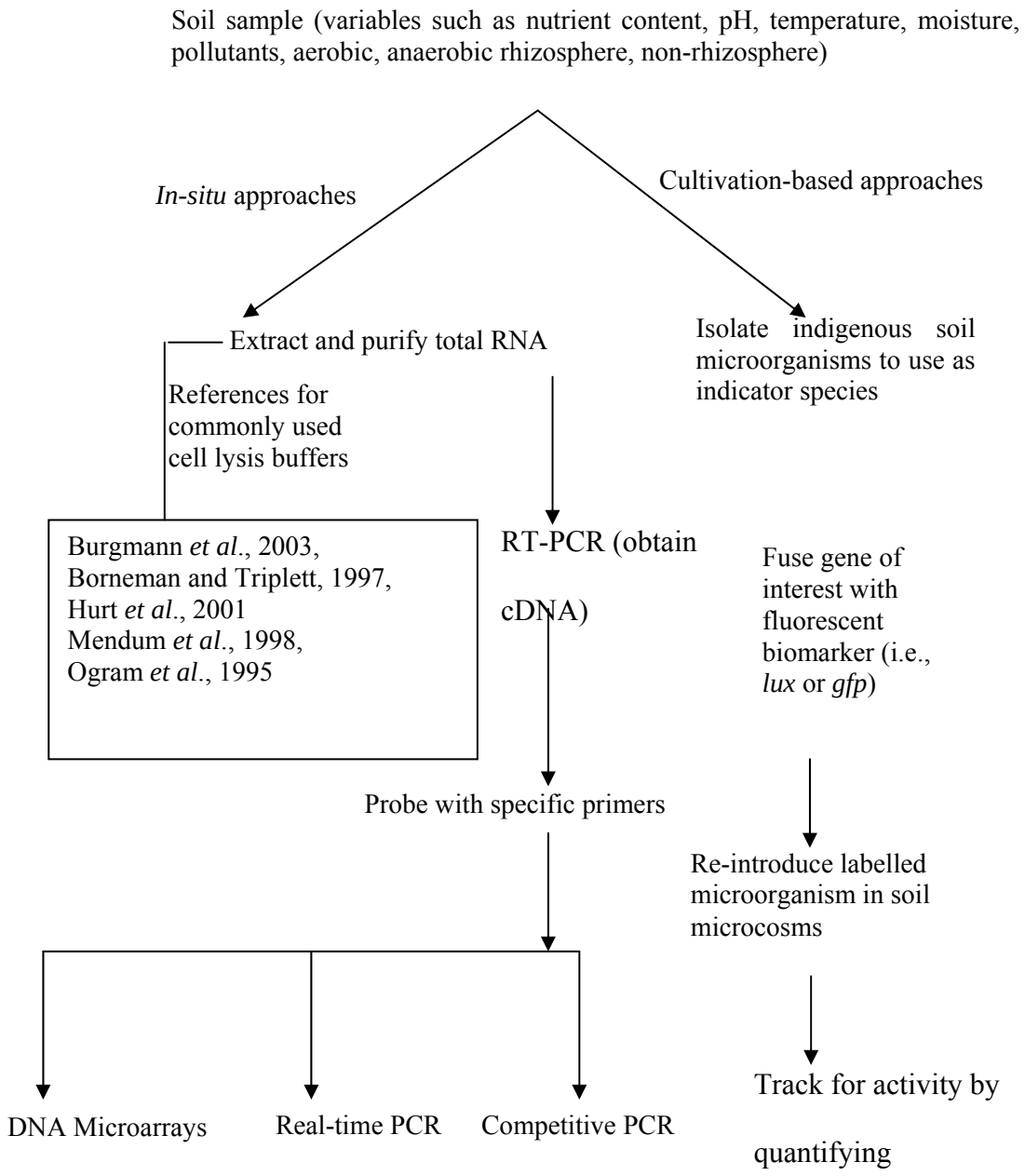

Fig. 5.1. Approaches to quantify microbial gene expression in soil samples (Saleh-Lakha *et al.,* 2005).

5.1.2. Total mRNA isolation difficulties

Difficulties in the extraction of total mRNA pools from microbial communities lies in the fact that prokaryotic mRNAs comprises less than 5% of total cellular RNA (Neidhardt and Umbarger, 1996). In addition, prokaryotic mRNA have very short (15-60 adenylate residue) unstable polyA tails (Liang and Pardee, 1992) and, as a consequence, prokaryotic mRNA has very short half-lives (Alifano *et al*., 1994; Belasco, 1993). In *Escherichia coli*, for example, mRNA half-lives range from several seconds to nearly 1 h, with an average lifetime of about 2 to 4 min (Donovan and Kushner, 1986; Emory and Belasco, 1990; Pedersen *et al.*, 1978). A key to explain these differences in decay rate is to identify the structural features of long or short-lived mRNA species that make them especially resistant or susceptible to degradation *in vivo*.

Among the most stable *E. coli* mRNA species are the transcripts of the *ompA* gene, which encodes a major outer membrane protein (OmpA). In cells rapidly growing at 30 °C, the half-life of this mRNA species is about 17 min (Emory and Belasco 1990; von Gabain *et al.*, 1983). Regulation of OmpA protein synthesis occurs mainly through modulation of the stability of *ompA* mRNA, whose half-life can fall by a factor of 4 in slowly growing cells (Emory and Belasco, 1990; Lundberg *et al.*, 1988; Nilsson *et al.*, 1984). The comparatively longer lifetime of the *E. coli ompA* transcript is not due to some unusual features of its 3'-terminal stem-loop structure (Belasco *et al.*, 1986), which, like the 3' hairpins of other bacterial mRNA species, is essential for protecting against 3'- exonuclease digestion *in vivo* (Chen *et al.*, 1988; Mott *et al.*, 1985; Newbury *et al.*, 1987). Instead, multiple lines of evidence suggest that the extraordinary longevity of the *ompA* transcript is attributable to its long (133-nucleotide) 5' untranslated region (UTR). This RNA segment works in *E. coli* as a growth rate-regulated mRNA stabilizer (Belasco *et al.*, 1986; Emory and Belasco, 1990). Some of the advantages and limitations of various methods for studying microbial gene expression in soil are listed in Table 5.1.

Table 5.1. Advantages and limitations of various methods for studying microbial gene expression in soil (Saleh-Lakha *et al.*, 2005).

Gene monitoring system	**Advantages**	**Limitations**	**Genes and organisms studied (references)**
Microarrays	–Specific, sensitive	–High specificity may exclude sequence divergent species	*nirS* (Cho and Tiedje, 2002); *nirS*, *nirK*, *pmoA* (Wu *et al*., 2001).
	–Low detection limit –Highly accurate –Wide applicability –Global gene expression analysis capability –Reproducible quantification of mRNAs or number of genes present in a sample	–Humic and clay substances can interfere with hybridization –Low mRNA extraction efficiencies from environmental samples	
Competitive RT-PCR	–Highly precise and accurate –Control of amplification efficiencies ensures accurate quantification	–Challenge to obtain highly pure, clean RNA with a sufficiently high yield to represent the soil microbial population –PCR inhibitors co-extracted interfere with the amplification and quantification process	*C. botulinum* E VH toxin gene expression in *Pseudomonas* sp. (McGrath *et al*., 2000). Legume-dependent rhizosphere effect on the diversity of *nirK* and *nirS* transcripts (Sharma *et al*., 2005).
Real-time PCR	–High sensitivity and precision –High-throughput –Specific and reproducible –Low detection limit (due to fluorescence technology) –Measures template abundance over six orders of magnitude –Allows for accurate quantification	–Specificity of primers are usually unable to capture sequence divergent species in environmental samples –Low mRNA extraction efficiencies do not accurately represent a typical soil microbial population –PCR inhibitors co-extracted with RNA interferes with quantification process –Non-specific binding	*Atz*ABCDEF in *Pseudomonas* sp. (Devers *et al*., 2004); *rpoH*, *groEL* and *tufA* gene expression in *Escherichia coli* (Sheridan *et al*., 1998).
Stable isotope probing (DNA)	–Allows *in situ* analysis of present and active microbial populations under the conditions tested	–Time involved in assimilation of the substrate	Genes expressed in ammonium fixation or methanogenesis in *Methylobacterium extroquens* (Radajewski *et al*., 2000).
		–Cross-feeding –Requires actively replicating cells at the time tested	
Stable isotope probing (RNA)	–RNA synthesis occurs more rapidly, shorter incubation periods –Amplification of the 16S rRNA for phylogenetic analysis –Does not require DNA synthesis or replication	–Necessitates heavy labelling, at close to 100% –Labelled substrate must be used for growth to overcome diluted label issues	Genes involved in syntrophic propionate oxidation in *Syntrophobacter* spp., *Smithella* spp. and *Pelotomaculum* spp. (Lueders *et al*., 2004).

5.1.3. Shine-Dalgarno (SD) sequence

It is well known that the process of prokaryotic translation initiation involves binding of the 16S rRNA and the initiator tRNA to the mRNA ribosomal binding site (RBS) on the mRNAs (Gold, 1988; Kozak, 1983). The RBS generally extends 20 nucleotides on either

side of the translation initiation codon (usually AUG) and contains, upstream from the AUG, a part or all of a polypurine sequence (UAAGGAGGU) known as the Shine-Dalgarno (SD) sequence (Gold, L., 1988; Steitz, J. A., 1969; Shine and Dalgarno, 1974). The SD sequences are complementary to a pyridine tract (the anti-SD or ASD region) in the 3'-end of the 16S rRNA and its role in translation initiation is well documented. The spacing between the SD sequence and the initiation codon varies considerably in natural mRNA species, with the average being 7 nucleotides (Gold, L., 1988; Kozak, M., 1983). Excessively long or short spacing between the SD and the initiation codon may be detrimental to efficient translation initiation (Roberts *et al.*, 1979). Ribosomal interaction with the mRNA occurs at two sites, the SD sequence, and the initiation codon. The former interaction is mediated by the ASD region, while the latter interaction involves fMet-tRNA in the ribosomal P-site (Gold, L. 1988). When both interactions occur, a minimal SD-AUG spacing is required, seemingly because the 16S rRNA and the fMet-tRNA must be kept a certain distance apart by configurational constraints (Fig. 5.4). Thus it is believed that SD-AUG spacing plays a significant role in the process of translation initiation and provides evidence that aligned spacing is the most appropriate measure of spacing. An optimally aligned spacing of 5 nt probably suits to the preferred spacing between the ASD region of the 16S rRNA and the fMet-tRNA (Chen *et al.*, 1994).

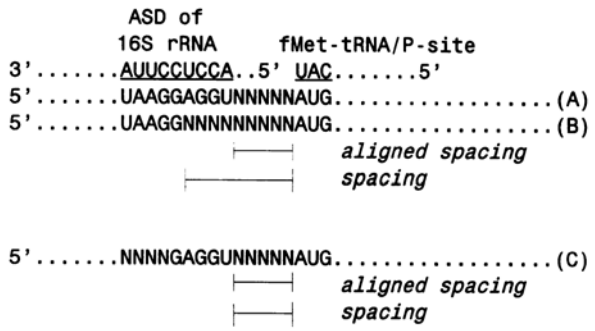

Fig. 5.2. Shine-Dalgarno region of messenger RNA (Chen *et al.*, 1994).

5.2. Environmental factors affecting gene expression in soil

Gene expression can be used to identify genes and activities essential for cellular role under varying conditions (Borneman and Triplett, 1997), but also to check *in situ* activities of genes coding for pollutant degradation (Fleming *et al.*, 1993). Although carbon is essential for microbial growth and survival, relatively few studies have been published on

how carbon substrates affect the expression of microbial genes in a soil environment. Tao *et al.* (1999) used DNA array technology to examine gene expression in *E. coli*, which could be extended for the study of various gene expressions that might be present in the soil systems. Although only a few studies have been carried out to monitor gene expression in soil environments, their combined results shows that extraction of mRNA and PCR analysis, as well as bioreporters, can be the useful tools for evaluating bioremediation technologies.

5.2.1. Oxygen status and pH effect

Certain proteins are expressed at higher activity levels under anaerobic versus aerobic conditions (Lynch and Lin, 1996). As an example, oxygen is likely a limiting factor in degrading naphthalene, and a key factor affecting the expression of the naphthalene-degrading pathway in *Pseudomonas fluorescens* HK44 (Ripp *et al.*, 2000). Baumann *et al.* (1996) studied the expression of mRNA encoding nitrate, nitrite and nitrous oxide reductase genes (*narH*-like gene, *nirS*-like gene, and *nosZ*-like gene, respectively) by using dot-blot hybridization. Anaerobic conditions, combined with the presence of N-oxides, were necessary for gene expression driving denitrification process. Soto *et al.* (2004) described the effects of pH and calcium on *nod* gene expression in *Sinorhizobium meliloti* LPU63 and the acid tolerant *Rhizobium* sp. LPU83. In *Rhizobium* sp. LPU83, the *nodC* gene expression was similar at pH of 7 or 5.6 with calcium concentrations of 0.7 or 6 mM. However, the *nodC* expression in *S. meliloti* LPU63 was adversely affected at the lower pH and in the presence of low calcium concentrations.

5.2.2. Soil moisture and temperature

Due to seasonal fluctuations, the possibility of changes in water content in soil is high, thereby directly affecting water availability in microhabitat (Torsvik and Ovreas, 2002). Water availability is critical to all physiological functions of the cell; thus, affecting microbial gene expression in soil. Studies on gene expression in response to changes in water availability are limited, and have not been conducted in soil environments (Saleh-Lakha *et al.*, 2005). Studies of temperature effect on microbial gene expression performed on pure cultures, for example *Listeria monocytogenes*, a mammalian pathogen, reveal that there is an up-regulation of virulence genes when its surroundings have reached the host's temperature of 37 °C. Temperature changes lead to altered expression of *prf*A, a transcript-

ional activator that in turn regulates virulence gene production (Johansson *et al*., 2002). At low temperatures, the mRNA encoding this protein forms a secondary structure that prevents translation initiation, most likely by sequestering the ribosomal binding site. Mutations that disrupted structure and exposed the ribosomal binding site led to increased expression. At 37 °C, base pairing within the secondary structure is disrupted in a manner that enables improved translation initiation. Similar temperature-sensing RNAs have been proposed to regulate phage lambda genes, *Escherichia coli* heat shock sigma factor, *rpoH*, as well as other genes (Altuvia *et al*., 1989; Narberhaus, 2002). Given that RNA structures can be considerably affected by temperature and by ionic conditions under which they fold, it seems that many more examples of these genes might be awaiting discovery.

5.3. Sequence annotation

For the phylogenetic and functional assignment of new sequences, the availability of homologous reference sequences in public-domain databases is mandatory. Thus, the presence of whole-genome sequences in public-domain databases plays an essential role in the putative assignment of the sequences that are retrieved from pure cultures or from environmental samples. A list of most commonly used public-domain databases for the phylogenetic and functional assignment is given below.

Table 5.2. Public-domain databases commonly used for the functional and phylogenetic assignment.

Database	**Webpage**
Gold™ Genomes Online Database	http://www.genomesonline.org/
The Institute for Genomic Research -Microbial Database (TIGR)	http://www.tigr.org/
National Center for Biotechnology Information -Microbial genome (NCBI)	http://www.ncbi.nlm.nih.gov/
The Wellcome Trust Sanger Institute - Microbial Genome	http://www.sanger.ac.uk/

The Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990) provided by NCBI (Table. 5.2) is one of the most commonly used tools for assigning nucleotide and amino acid sequences, both phylogenetically and functionally.

Table 5.3. Some of the commonly used blast tolls provided by the NCBI for the phylogenetic and functional assignment of sequences.

Tools	Purpose	Sequence format used	Database comparison
blastn	compares a nucleotide query sequence	FASTA, GenBank accession, or GI numbers	NCBI nucleotide databases
blastp	compares an amino acid query sequence	-do-	NCBI protein databases
blastx	compares a nucleotide query sequence translated in all ORFs	-do-	NCBI protein databases

During the putative phylogenetic or functional assignment of the sequences, E-value (Expect value) is considered as one of the most important parameters. It enables to make the decision whether assignment is statistically significant or not. The E-value is a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size. The lower the E-value or the closer it is to "0", the higher is the "significance" of the match. However, it is important to note that searches with short sequences can be virtually identical and have relatively high E-values as shorter sequences have a high probability of occurring in the database purely by chance. E-value of e^{-10} is commonly used value as a cutoff point for the putative phylogenetic and functional assignment of mRNA transcripts by blastx analysis (Poretsky *et al*., 2005).

5.4. Aim of the project

This part of the Ph.D. project was aimed to develop an efficient protocol for extracting prokaryotic mRNA transcript pools from soil. The newly developed protocol should be used to recover and analyze transcripts that were expressed in flooded, unplanted rice paddy soil microcosms in respect to oxic/anoxic zones and community successions. The methods involved were subtractive hybridization, reverse transcription PCR (RT-PCR), clone library generation, T-RFLP fingerprinting technique, and ultimately mRNA transcript annotation.

6. Methodology

6.1. Methods

6.1.1. Model system

Model system used is described in detail in the Method section 2.2.1. of Chapter I.

6.1.2. Sample preparation

After 1 day and 70 days of flooding, the whole microcosms were shock-frozen by dipping into liquid nitrogen. Soil of a single slice (approx. 500 mg of wet weight) was cut using the Microtom-Kryostat HM-500 (Germany) and mixed immediately with RNA*later*® (Ambion). After 24 h of incubation at 4 °C, samples were centrifuged at 5,000 × *g*, and then supernatant was removed. The precipitates were washed twice with one-quarter-strength Ringer solution in order to remove the remaining RNA*later*®. Then the extraction of total nucleic acids was performed. Before extraction of total nucleic acids from individual slices, all solutions and glassware were made RNase-free by treatment with diethyl pyrocarbonate (Noll *et al.*, 2005) and working areas with 2% Absolve™ (Perkin, USA).

6.1.3. Total nucleic acid extraction

Samples were mixed with 700 µl of pre-cooled TPM buffer [50mM Tris-HCl (pH 7.0), 1.7% (wt/vol) polyvinylpyrrolidon, 20 mM $MgCl_2$], and 0.5 g of glass beads (0.17- to 0.18 mm diameter). The mixture was shaken for 60 s at maximum speed in a bead beater (Dismembrator-S; Braun Biotech, Melsungen, Germany). Glass beads, cell debris, and soil particles were pelleted by centrifugation (at 13,000 × *g* for 5 min at 4 °C), and the supernatant was transferred to a new reaction tube. The pellet was resuspended in 700 µl of a phenol-based lysis buffer [5 mM Tris-HCl (pH 7.0), 5 mM Na_2EDTA; 0.1% (wt/vol) sodium dodecyl sulfate, 6% (v/v) water-saturated phenol], followed by a second round of bead beating. After centrifugation at 13,000 × *g*, the supernatants of the two bead-beating treatments were pooled and were extracted with 500 µl of water-saturated phenol, phenol-chloroform-isoamyl alcohol [25:24:1 (v/v/v)], and then with chloroform-isoamyl alcohol [24:1 (v/v)]. All the extraction procedures involved centrifugation at 13,000 × *g* for 5

minutes. The total nucleic acids were precipitated from the aqueous phase with absolute ethanol (three volumes total sample solute) and 3 M sodium acetate, pH 5.7 (1/10 volume of total sample solute) and cooling at –80 °C for 1 h followed by centrifugation at 13,000 × *g* for 1 h. Finally, total nucleic acid sample was washed twice with 70% ethanol, then air-dried, and was resuspended in 50 µl of TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)].

6.1.4. Total RNA isolation

For the removal of co-extracted DNA, total sample was treated with 5 U DNase (Promega, Germany), in combination with 10 U RNAsin (Promega, Germany) after adding 1× DNase buffer (Promega, Germany) and incubated at 37 °C for 30 min. Finally, total RNA was recovered by using RNeasy kit™ (Qiagen protocol). When the purified sample still contained humic substances, it was further purified by using column-containing Sephadex-50. The integrity of the 16S rRNA and 23S rRNA fragments was checked by electrophoresis on a 1% agarose gel and comparison to a rRNA standard from *Escherichia coli* (Roche Diagnostics, Germany) after ethidium bromide staining. If these two molecules were found intact than possibility of mRNA recovery should also be high.

6.1.5. Enrichment of mRNA

To enrich the mRNA, rRNA was removed by subtractive hybridization with capture oligonucleotides hybridized to magnetic beads (MICROBExpress™ Bacterial mRNA Enrichment kit, Ambion) following the protocol of the manufacturer. The enrichment was repeated second time to ensure the removal of rRNA as quantitatively as possible.

6.1.6. Quantification of total RNA and mRNA

Total RNA and enriched mRNA samples were quantified by measuring in Bio-analyzer 2100 (Agilent, England) and NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA) following the protocols of the manufacturer.

6.1.7. RT-PCR

RT-PCR was performed on 1 µl of mRNA sample using 10-mer random primers (OPA13, and OPA17; MWG) and SD14 primer (Table 6.1). SD14 was designed by Fleming et al. (1998), aimed to target the Shine-Dalgarno region of bacterial mRNAs. The reaction was performed in Applied Biosystems 9800 cycler using a one-step RT-PCR system (Access Quick™, Promega, Germany). The reaction mixture contained 12.5 µl of 1 × RT buffer mix, 0.5 µl of 33 pmol of forward and reverse primers each, 1 U of reverse transcriptase (Promega, Germany), and finally total volume was made up to 25 µl with sterile water. Reverse transcription step was carried out at 45 °C for 45 min, followed by PCR step consisting of an initial denaturation at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 45 s, and primer extension at 72 °C for 1 min. Final extension was carried out at 72 °C for 10 min. As negative control for DNA contaminants, reverse transcription reactions containing mRNA samples without reverse transcriptase enzyme were also included. RT-PCR products were checked by electrophoresis on a 1% agarose gel.

Table 6.1. List of random primers used for RT-PCR.

Designation	Sequence	References
OPA13	CAGCACCCAC	Poretsky *et al*., 2005
OPA17	GACCGCTTGT	Poretsky *et al*., 2005
SD14	GGGGAACGACGATG	Fleming *et al*., 1998; Poretsky et al., 2005

6.1.8. cDNA clone library generation

RT-PCR products were cloned into the pCR II TOPO vector using the TOPO TA cloning® kit (Invitrogen, Germany) following the protocol of the manufacturer. Positive clones (white clones) were randomly selected and transferred to fresh solid medium by using sterile toothpicks.

6.1.9. PCR of positive clones

Each of the colonies was picked up by using a sterile toothpick and suspended into 25 µl of sterile TE buffer (pH 8). The suspension was boiled in a heating block for 10 min and subsequently centrifuged at 13,000 × *g* for 15 sec. PCR was carried out using the oligonucleotides primers T7f and M13r. The reaction mixture contained 1 µl of supernatant solution; 5 µl of 5× reaction buffer (Promega, Germany), 1.5 µl of 25 mM $MgCl_2$ (Promega, Germany), 2.5 µl of 10 mM dNTP mix (Promega, Germany), 0.25 µl of 50 pmol (each) primer (MWG-Biotech, Germany), and 0.125 U of Taq DNA polymerase (Promega, Germany). Finally, total volume was made up to 25 µl with sterile water. The thermal PCR profile was as follows: initial denaturation at 94 °C for 3 min; 30 cycles consisting of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and elongation at 72 °C for 60 s. The final elongation step was extended to 5 min. Amplification was performed in a total volume of 25 µl in fast reaction tubes (Applied Biosystems, Germany) and a DNA thermal cycler (model 9800 fast; Applied Biosystems, Germany). Aliquots of the amplified products (5 µl) were checked by electrophoresis on a 1% agarose gel. The PCR amplicons were purified by using the QIAquick™ purification kit (Qiagen, Germany) following the instructions of the manufacturer. Purified PCR products were eluted from the purification columns by adding 40 µl of 10mM Tris buffer (pH 8.0).

6.1.10. Cycle sequencing

For cycle sequencing, the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Germany) was used. The PCR reagent mix was prepared by combining the following reagents (on ice) in a fast reaction tube: 2 µl ready reaction premix, 1 µl BigDye® sequencing buffer, 3.2 pmol (f or r) primer, 60-80 ng (2 µl) of PCR product and distilled water up to 10 µl. Tubes were placed in a thermal cycler preheated to 104 °C. The temperature program used was as follows: initial denaturation at 96 °C for 30 s; 25 cycles, consisting of denaturation at 94 °C for 10 s, primer annealing at 50 °C for 5 s, and final elongation at 60 °C for 4 min. The product was then stored at 4 °C until further processing. The cycle-sequenced product was purified by using AutoSeq™G-50 (GE Health Care Life Science, Germany) columns as described in the manufacturer's protocol. Sequences were generated with an ABI 310 sequencer (Applied Biosystems, Germany).

6.1.11. Sequence annotation

Sequences were analyzed using blastn, blastx, ORF finder, and blastp tools (http://www.ncbi.nlm.nih.gov/BLAST/). Blastn analysis served to identify rRNA sequences; blastx analysis was used for phylogenetic assignment based on best matches (E-value cutoff of e^{-10}). Functional gene assignments were based on blastx and blastp.

6.1.12. T-RFLP analysis of mRNA transcript pools

A terminal restriction fragment length polymorphism (T-RFLP) analysis of mRNA transcripts was carried out by using the RT-PCR protocol as detailed in the section 6.1.7, except that 5' FAM labelled SD14 primers (MWG Biotech, Ebersberg, Germany) was used during RT-PCR amplification of mRNA transcript pools. Purification of the RT-PCR product was done by using QIAquick® purification kit. Aliquots of the purified product (2 µl) were mixed with 12 µl deionized formamide (Applera, Darmstadt, Germany) and 0.2 µl of an internal DNA fragment length standard (X-Rhodamine MapMarker® 30-1000 bp; BioVentures, USA). Terminal restriction fragments (T-RFs) were separated with an automated DNA sequencer. The length of fluorescently labelled T-RFs was determined by comparison with the internal standard using GeneScan 3.71 software (Applied Biosystems, Germany).

7. Results

7.1. Optimization of mRNA extraction protocol

When soils are analyzed *ex situ*, there will be a significant time delay between soil sampling and analysis, thus results may not represent the gene expression that was present at the time of sampling. Therefore, to prevent degradation of the total RNA, soil samples were rapidly shock-frozen in liquid nitrogen and then soil slices were cut and stored in RNA*later*®. My personal experience revealed that reproducible results were obtained only when soils were mixed with RNA*later*® before further processing, instead of analyzing them immediately (Fig. 7.1a). The exact composition of RNA*later*® is not released by the manufacturer but it is expected to contain high salt concentration that prevents nuclease activity, thereby preserving total RNA.

Total RNA isolated from all the samples contained predominantly rRNA with two major bands. These corresponded to the 16S rRNA and the 23S rRNA of *E. coli* standard (Fig. 7.1b). mRNA separated from total RNA by using subtractive hybridization was found to be highly depleted in rRNAs as displayed by the Bioanalyzer and gel electrophoresis results (Fig.7.1d, e).

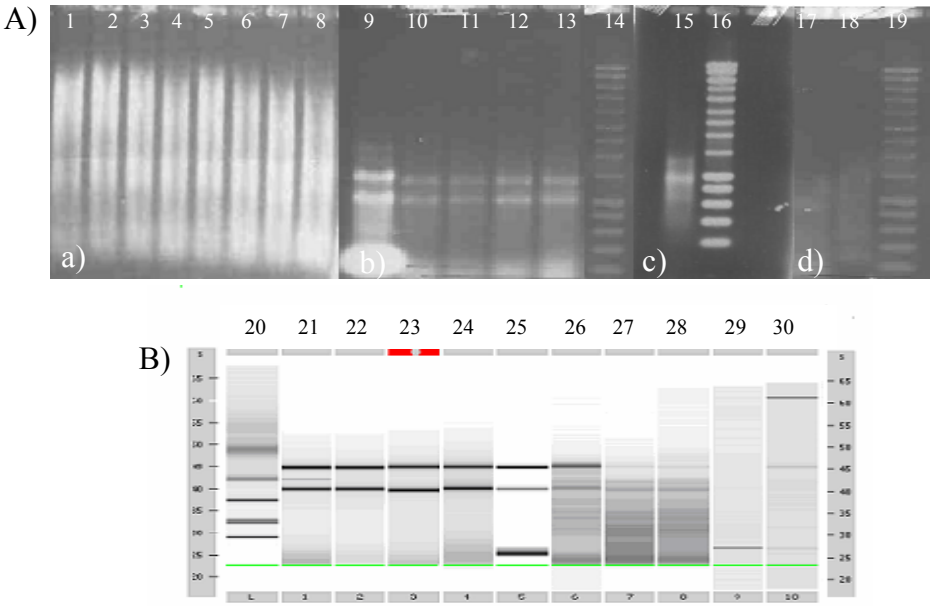

Fig. 7.1. A) Gel electrophoresis of a) total nucleic acids obtained from early succession (lanes 1-4) and late succession (lanes 5-8), b) RNA standard from *E. coli* (lane 9) and total

RNA after DNA digestion (lanes 10-13), c) 16S rRNA and 23S rRNA after removing 5S rRNA (lane 15), d) enriched mRNA after subtractive hybridization (lanes 17, 18), and smart ladder (lanes 14, 16 and 19), and B) total RNA (lanes 21-24) from the sample and standard (lane 25), and enriched mRNA from the sample (lanes 26-29) and *E. coli* total RNA standard (lane 30) as detected by Bioanalyzer 2100.

7.2. Development of the RT-PCR protocol

RT-PCR was carried out by using one-step reaction method. In the one-tube method, all the reverse-transcribed RNA (cDNA) is available for amplification, whereas in the two-tube method only a subsample is amplified (Sheridan *et al.*, 1998). The absence of contaminating genomic DNA was checked by performing a parallel RT-PCR reaction of mRNA samples without reverse transcriptase enzymes (Fig. 7.2a).

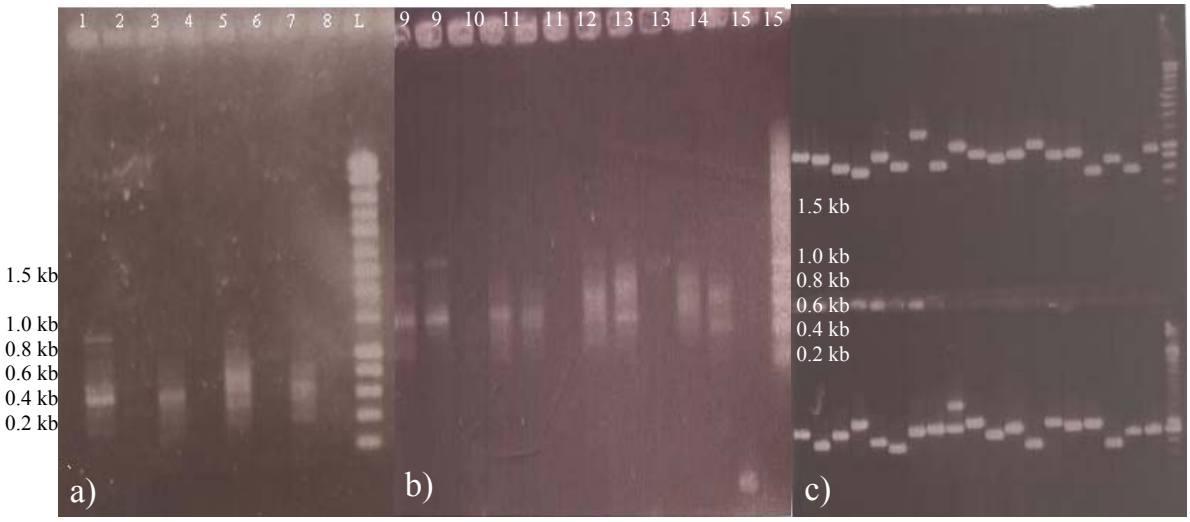

Fig. 7.2. RT-PCR products of mRNA transcripts obtained from different oxygen zones of early succession (a) and late succession (b). Lanes 1 (oxic zone), 3 (upper transition zone), 5 (lower transition zone), 7 (anoxic zone) represents the RT-PCR products of early succession whereas lanes 9 (oxic zone), 11 (upper transition), 13 (lower transition zone), and 15 (anoxic zone) represent the RT-PCR products of late succession. Lanes 2 (oxic zone), 4 (upper transition zone), 6 (lower transition zone), 8 (anoxic zone) and lanes 10 (oxic zone), 12 (upper transition zone), 14 (lower transition zone), and 16 (anoxic zone) represent the RT- products from control (without RT step) from early and late succession, respectively. In panel (c), PCR products of randomly selected cDNA clones from RT-PCR product of oxic zone sample are shown.

7.3. Environmental transcript libraries

Following RT-PCR and generation of cDNA clone libraries, 417 clones were randomly selected for analysis. Overall, 369 (88%) clones were derived from bacterial mRNA, 16 (4.0%) were from rRNA, and 32 (8.0%) were derived from eukaryotic mRNA. Putative phylogenetic assignments were based on the taxon of the most similar sequence by blastx analysis. Using E-values more significant than e^{-10} in blastx analyses, gene transcripts were assigned to major phylogenetic groups in both early and late succession, which are listed in Table 7.1. Among all transcripts, those affiliated to *Proteobacteria* were most abundant, covering 35% of total transcripts, followed by those affiliated to phylum *Acidobacteria* (5%), *Actinobacteria* (4%), and to 10 minor phyla that were also detected, covered only 14% of the total transcripts (Table 7.1).

Table 7.1. Phylogenetic assignment of transcripts retrieved from different oxic and anoxic zones of early and late succession based on blastx best hits.

Phylum / Class / Domain	**No. of cDNA clones**								**Total**
	Early succession				**Late succession**				
	Oxic	Upper transition	Lower transition	Anoxic	Oxic	Upper transition	Lower transition	Anoxic	
Alphaproteobacteria	4	4	7	3	10	12	5	4	**49**
Betaproteobacteria	2	2	7	1	7	7	4	1	**31**
Gammaproteobacteria	15	3	4	0	3	2	6	3	**36**
Deltaproteobacteria	2	2	3	3	3	5	4	7	**29**
Bacilliaceae	2	2	0	0	0	1	0	0	**5**
Clostrodiales	0	1	2	1	6	0	0	0	**10**
Actinobacteria	2	3	9	0	0	0	1	2	**17**
Bacteroidetes	2	0	1	0	6	1	0	0	**10**
Acidobacteria	1	5	5	2	2	2	1	2	**20**
Planctomycetes	1	1	0	0	0	0	2	1	**5**
Chlorobi	0	0	2	0	0	0	0	0	**2**
Chloroflexi	1	0	2	2	0	3	1	1	**10**
Cyanobacteria	0	3	0	0	2	0	0	0	**5**
Crenarchaeota	1	0	0	0	0	0	0	0	**1**
Euryarchaeota	0	1	5	2	0	0	0	4	**12**
Eukaryota	1	0	3	4	0	0	0	0	**8**
Predicated novel[1]	9	26	13	31	9	16	24	23	**151**
Total	**43**	**53**	**63**	**49**	**48**	**49**	**48**	**48**	**401**

[1] no reliable assignment due to E-value >e^{-10}

ORF finder and blastp were used for the functional assignment of the transcripts. However, if transcripts could not be assigned to any conserved putative domains by using ORF finder and blastp but exhibited E-values more significant than e^{-10} in blastx, then they were also assigned to the role categories based on the blastx best hit. In this manner, altogether 250 transcripts could be functionally assigned and these transcripts could be assigned to gene of metabolism (118 transcripts), information storage and processing (23 transcripts), cellular processes (31 transcripts), and conserved hypothetical (78 transcripts) (Table 7.3). The other 151 transcripts, which could not be assigned to any putative conserved domain in blastp and have E-value less significant than e^{-10} in blastx, were treated as predicted novel transcripts (hypothetical or unknown) (Table 7.3).

Based on the functional assignment, in total 147 different types of functional assignments were identified, among which 28 (<20%) transcripts were found twice or more often (Table 7.2). Similarity between nucleotide sequences of transcripts that were assigned to the same function were compared by using ClustalW program (www.ebi.ac.uk/clustalw/) and grouped into separate clusters when sequence similarity was <98% (Table 7.2).

Table 7.2. List of putative functional assignments that were detected in multiple numbers in oxic and anoxic zones of early and late succession.

Early succession	**Freq.**	**Clusters**	**Late succession**	**Freq.**	**Clusters**
Oxic zone			**Oxic zone**		
ClpA	2		*DUF1080*	5	2
Cytochrome c family	2		*FcbT1*	2	
Putative IcmL-like	8	3	*HemL*	2	
Upper transition zone			*LivK*	4	1
Predicted GTPase	4	1	*MM_CoA_mutase*	3	
Ubiquinol_Oxidase_I	2		*SgbH*	5	2
Lower transition zone			**Upper transition zone**		
CbiM protein	2		*Asd*	2	
LysU	3		*AsxRS_core*	2	
Predicted GTPase	6	1	*LivK*	3	1
QcrB	2		*Transposase OrfB*	2	
RibF	2		**Lower transition zone**		
Anoxic zone			*HemG*	2	
GNT-I	2		*PyrF*	2	
WW domain binding protein II	3		*Serine protease of the peptidase family S9A*	2	
Rho	2		*Conserved protein*	3	
			Anoxic zone		
			PrpD	12	2

Note: Transcripts whose sequence similarities were checked are highlighted with gray color.

Table 7.3. Functional assignment of transcripts retrieved from oxic, upper transition, lower transition, and anoxic zones. Transcripts were recovered from both early and late succession using blastx and blastp best hits.

SN	Role catagories	Oxic zone zone		Upper transition zone		Lower transition zone		Anoxic zone	
		ES[1]	LS[2]	ES[1]	LS[2]	ES[1]	LS	ES[1]	LS[2]
1.	**Information storage and processing**	**1**	**0**	**3**	**3**	**6**	**4**	**2**	**4**
	Translation, ribosomomal structure and biogenesis	1	-	-	2	4	2	-	4
	Transcription	-	-	2	-	-	1	2	-
	DNA replication and repair	-	-	1	1	2	1	-	-
2.	**Cellular processes**	**4**	**2**	**2**	**3**	**6**	**3**	**6**	**3**
	Cell cycle protein	-	-	1	-	-	-	-	-
	Regulatory protein	-	-	-	-	1	-	1	-
	Intracellular trafficking and secretions	-	-	-	-	1	-	-	-
	Signal transduction	-	2	-	2	2	1	2	-
	Defense mechanism	-	-	1	1	-	-		1
	Cell envelope biogenesis, outer membrane	-	-	-	-	-	1	1	-
	Cell motility secretion	-	-	-	-	1	1	-	1
	Post translational modification	4	-	-	-	-	-	-	1
	Protein synthesis	-	-	-	-	1	-	2	-
3.	**Metabolims**	**17**	**20**	**12**	**15**	**16**	**7**	**10**	**21**
	Energy production and conversion	1	-	4	2	3	2	2	3
	Carbohydrate transport and metabolism	2	5	1	2	3		2	-
	Amino acid transport and metabolism	4	5		4	4	2	3	1
	Nucleoid transport	-	-	1	-	-	1	-	1
	Sugar transporter and metabolism	-	-	-	-	-	-	-	1
	Phosphate transporter	-	-	-	1	-	-	-	-
	Inorganic ion transport and metabolism	2	1	2	1	-	1	-	1
	Lipid metabolism	-	-	1	-	-	-	-	1
	Co-enzyme metabolism	1	2	1	1	1	1	-	
	Catalytic function	5	5	2	3	4	-	3	12
	Periplasmic transporters	1	-	-	-	-	-	-	-
	Secondary metabolites biosynthesis, transport and catabolism	1	2	-	1	1	-	-	1
4.	**Conserved hypothetical**	**12**	**17**	**10**	**12**	**18**	**10**	**-**	**3**
5.	**Predicated novel**	**9**	**9**	**26**	**16**	**19**	**24**	**31**	**17**
	Total no. of transcripts	**43**	**48**	**53**	**49**	**63**	**48**	**49**	**48**

[1]ES = Early succession; [2] LS = Late succession

7.4. T-RFLP of mRNA transcript pools

Cloning results clearly showed that the use of subtractive hybridization alone did not remove all the 16S rRNA and 23S rRNA molecules. However, the cDNA clones generated from the RT-PCR products using SD14 primers coupled with subtractive hybridization resulted in a strong depletion of rRNAs. Therefore, it was decided to develop a protocol for T-RFLP fingerprinting of mRNA transcript pools by using SD14 primer. Firstly, reproducibility (tube-to-tube variation) was checked by generating triplicate T-RFLP fingerprints from a single mRNA extract. Although results showed reproducibility (±1 bp) among the triplicate samples in reference to presence and absence of major peaks detected there were strong variations when minor peaks were also considered (Fig.7.3). Besides, triplicate T-RFLP did not showed reproducibility in their relative abundance. This indicated that the method was still lacking reproducibility. Thus, the attempts to establish a reliable protocol for mRNA transcript pool fingerprinting by using T-RFLP was not continued.

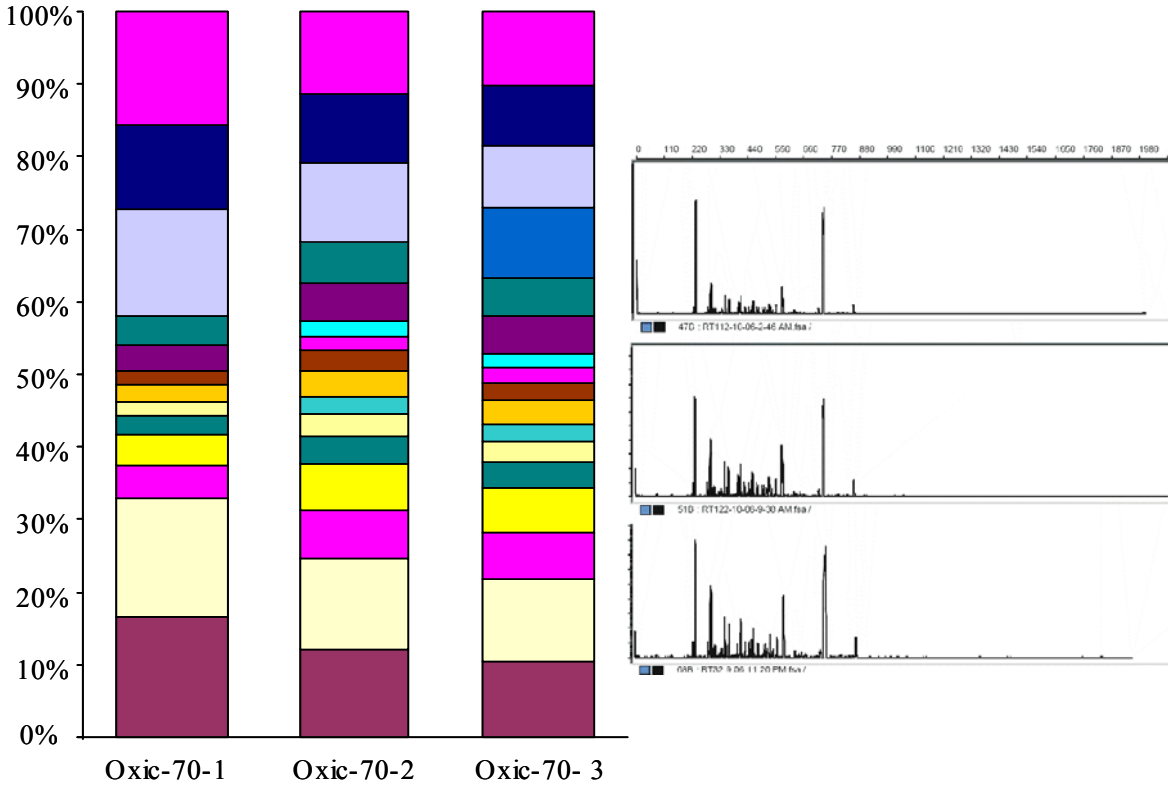

Fig. 7.3. Checking of tube-to-tube RT-PCR variation (reproducibility checking).

8. Discussions

The first section of this research was primarily focused on developing an efficient protocol for the extraction of total mRNA transcript pools from soil. The protocol was then used for extracting mRNA transcript pools from oxic and anoxic zones of flooded, unplanted rice paddy soil microcosms, incubated for either 1 or 70 days (early versus late succession). Subsequently, cDNA libraries were generated, in order to analyze mRNA transcript pools by phylogenetic and functional assignments of cloned cDNAs.

There were significant methodological obstacles in the extraction and analysis of environmental transcript pools. The following part of the discussion details the problems and how they were solved, if possible.

8.1. Subtractive hybridization

Since the aim was to construct cDNA clone libraries mainly comprising mRNA transcripts, rRNA was eliminated prior to RT-PCR using subtractive hybridization. In this method, oligonucleotides attached to magnetic beads were used to capture 16S and 23S RNA molecules. The magnetic beads, with 16S and 23S rRNAs molecules attached were pulled to the side of the tube with a magnet. Thus, supernatant contained enriched mRNA. However, performance of this method was weak, as concluded from initial analysis of various cDNA clone libraries generated by using different primer combinations (OPA13, OPA17, and SD14) in RT-PCR. Most cDNA clones (>70%) were derived from 16S and 23S rRNAs.

This finding may be explained by the fact that MICROB*Express*™ kit does not capture rRNAs of all the bacterial species; some of the examples are *Chloroflexus aurantiacus*, *Dehalococcoides ethenogenes*, *Deinococcus radiodurans*, and *Propionibacterium freundenreichii*. A list detailing the bacterial species, whose rRNAs are captured, partially captured or not captured at all by MICROB*Express* is available at http://www.ambion.com/techlib/misc/microbe.html.

In order to overcome the insufficient capture of rRNA molecules, the RT-PCR reaction was carried out only by using the SD14 primer. Notably, use of the SD14 primer in RT-

PCR after subtractive hybridization resulted in cDNA clone libraries, in which more than 95% of clones were derived from mRNA transcripts. Thus, all the cDNA clone libraries were generated using the SD14 primer in RT-PCR. This primer has also been used successfully in differential display analysis (Fleming *et al*., 1998) as well as in the reverse transcription PCR of mRNA transcripts retrieved from lake water samples (Poretsky *et al.*, 2005). The only methodological drawback of using SD14 primers is that RT-PCR appears to be biased towards the amplification of the 5' end of bacterial mRNA transcripts that possess a typical *Escherichia coli* like Shine-Dalgarno region (Ingraham *et al*., 1983). However, it has also been reported that SD14 primers, when used under low-stringent PCR conditions, do not necessarily target only the Shine-Dalgarno site (Poretsky *et al*., 2005). Since RT-PCR was carried out under low-stringent conditions, a bias towards retrieval of transcripts from bacteria related to *E. coli* (*Gammaproteobacteria*) is unlikely as indicated by the finding of low gammaproteobacterial transcript abundance in late succession, but cannot be fully excluded.

8.2. Cloning bias

In order to assess possible cloning bias, the size distribution of PCR-amplified cDNA inserts were examined by agarose gel electrophoresis. Average insert size ranged from 500 to 700 bp (Fig. 7.2b). Larger cDNA inserts (>1000 nt) were not found, presumeably because mRNA fragments between 500 and 700 nt are preferentially amplified in RT-PCR and cloned into the vector.

8.3. Short half-lives

The vast majority of mRNA sequences appeared to be transcribed from housekeeping genes, including genes encoding components of cell envelope, transport systems, and energy metabolism (Table 7.3). Studies on *E. coli* transcriptome suggest that transcripts of these three gene categories have longer half-lives than those of other gene categories (Bernstein *et al*., 2002). This might be the reason why cDNA libraries constructed in this study contained a high proportion of mRNA transcripts derived from these three above-mentioned gene categories.

8.4. Public-domain databases

One of the major problems in both phylogenetic and functional assignment is that the number of reference sequences in public-domain databases is still limited. It has already been reported that the accuracy of assignment is negatively affected by possible events of lateral gene transfer and positively correlated with the taxonomic coverage of the database for any given gene (Poretsky *et al*., 2005). Until the end of 2006, altogether 470 (397 bacterial, 44 eukaryal, and 29 archaeal) complete genome sequences and 62 metagenome sequences were available in Gold^{TM} Genomes Online Database (http://www.genomesonline.org/gold.cgi). Currently, the analysis of additional 1678 genome sequences is in progress. Out of 470 microbial species whose genome sequences were fully sequenced, 54% belong to the phylum *Proteobacteria*, 22% to the *Firmicutes*, 7% to the *Archaea,* and 17% to other major groups. This predominance of proteobacterial genome sequences in public-domain databases may explain why almost 35% of all the transcripts retrieved from the rice paddy soil microcosms were assigned to *Proteobacteria* with high significance (E-values $<e^{10}$). Also, those transcripts (37%) having either very high E-values ($>e^{-10}$) or having no matches at all in blastx analyses are most likely derived from phylogenetic groups, which are (strongly) underrepresented in public-domain databases. The phylogenetic assignment was also complicated by the fact that sometimes a single transcript was assigned to 2-3 different phylogenetic groups based on blastx best hit, which may be explained by events of lateral gene transfer as already outlined above (Table 8.1).

8.4.1. Phylogenetic assignment

Taking into account the above-mentioned findings, blastx searches resulting in E-values $<e^{-10}$ were considered of sufficient significance for putative assignment of transcripts. The putative transcript assignments thus suggested that the early community development was dominated by the activity of *Gammaproteobacteria* in the oxic zone, while a phylogenetically highly diverse community appeared to be active in the anoxic zone. Gene expression of mature communities was dominated by transcripts of *Alphaproteobacteria* in the oxic zone and *Deltaproteobacteria* in the anoxic zone. These findings were in good agreement with the first part of my Ph.D. study where fast-growing communities (mainly *Betaproteobacteria*, *Gammaproteobacteria*) were dominant in early succession, while

slow-growing communities (mainly *Alphaproteobacteria*) were prevalent in late succession. Transcripts of *Betaproteobacteria* and *Actinobacteria* were recovered from early and late succession with similar frequency, suggesting that they were active in both successional stages. Other phylogenetic groups were represented in the cDNA libraries by only a very few transcripts (Table 7.1).

Table 8.1. Phylogenetic and functional assignment of selected transcripts.

Clone	Description and closest match organism	Acc. no.	E-value
T-1.10	mandelate racemase / muconate lactonizing enzyme [*Sphingomonas* p. SKA58] / ***Alphaproteobacteria***	gi|94497565|	2e-51
	Galactokinase [*Saccharophagus degradans* 2-40]/ ***Gammaproteobacteria***	gi|90020711|	1e-50
	mandelate racemase/muconate lactonizing enzyme [*Polaromonas* sp. JS666] / ***Gammaproteobacteria***	gi|91789818|	2e-50
T-1.11	probable cytochrome C-type biogenesis protein [*Bdellovibrio bacteriovorus* HD100]/ ***Deltaproteobacteria***	gi|39575315|	2e-30
	robable cytochrome C-type biogenesis protein [*Bdellovibrio marina* DSM 3645] / ***Planctomycetes***	gi|87309290|	1e-26
	hypothetical protein [Parachlamydia sp. UWE25] / ***Chlamydiae***	gi|46400369|	1e-23
T-1.23	threonine aldolase [*Symbiobacterium thermophilum* IAM 14863]/ ***Actinobacteria***	gi|51857230|	1e-85
	Threonine aldolase [*Syntrophobacter fumaroxidans* MPOB] / ***Deltaproteobacteria***	gi|71546393|	1e-85
	Threonine aldolase [*Desulfotomaculum reducens* MI-1] / ***Clostridia***	gi|88946895|	9e-76
T-77	Succinyl-CoA synthetase, beta subunit [*Solibacter usitatus* Ellin6076] / ***Acidobacteria***	gi|67929956|	9e-35
	succinyl-CoA synthetase large subunit [*Hydrogenobacter thermophilus*] / ***Aquificales***	gi|46849523|	6e-34
	Succinyl-CoA synthetase, beta subunit [*Magnetococcus* sp. MC-1] / ***Proteobacteria***	gi|68245183|	3e-32
T-186	Glycosyl transferase, group 1 [*Solibacter usitatus Ellin6076*] / ***Acidobacteria***	gi|67929436|	8e-23
	WbnE [*Escherichia coli*] / ***Gammaproteobacteria***	gi|5739468|	6e-21
	putative galactosyltransferase WbgM [*Escherichia coli*] Length=364 / ***Gammaproteobacteria***	gi|18266398|	2e-18
T-188	Cytochrome-c oxidase [*Solibacter usitatus Ellin6076*] / ***Acidobacteria***	gi|67934493|	1e-18
	Cytochrome c oxidase polypeptide I [*Haloarcula marismortui ATCC 43049*]/ ***Euryarchaeota***	gi|55230797|	2e-18
	Cytochrome-c oxidase [*Shewanella sp. PV-4*] / ***Gammaproteobacteria***	gi|78366753|	2e-18
T-189	Cytochrome b/b6, N-terminal [*Chlorobium phaeobacteroides BS1*] / ***Chlorobi***	gi|67941709|	5e-37
	Cytochrome b/b6, N-terminal [*Solibacter usitatus Ellin6076*] ***Acidobacteria***	gi|67929232|	8e-32
	Cytochrome b/b6-like [*Geobacter metallireducens GS-15*7] / ***Deltaproteobacteria***	gi|78194581|	7e-31
T-1026	Methylmalonyl-CoA mutase, N-terminal [*Thermoanaerobacter ethanolicus* ATCC 33223] / ***Clostridia***	gi|76797288|	6e-53
	Methylmalonyl-CoA mutase, N-terminal domain/subunit [*T.tengcongensis* MB4] / ***Clostridia***	gi|20516219|	7e-53
	Methylmalonyl-CoA mutase subunit alpha [*Azoarcus* sp. EbN1] / ***Betaproteobacteria***	gi|56312619|	2e-52

8.4.2. Functional assignment

The functional assignment was often more ambiguous than the phylogenetic assignment, reflected by the high proportion of transcripts that had to be classified as predicted novel. However, this high percentage of unassignable transcripts is still in the range of that proportion of predicted novel genes in completely sequenced bacterial genomes. For example, *Pseudomonas aeruginosa* strain PA01 possesses 5,570 predicted ORFs of which 54.2% could be assigned to known functions. Of the other 45.8%, 13.2% were conserved hypothetical and 32% predicted novel, i.e. without any significant homology to reported sequences (Stover *et al*., 2000).

In addition to the findings discussed above, there were other interesting results as follows:

1. Acidobacterial mRNA transcripts were retrieved with high frequency from both early and late succession. Almost all of those were assigned to genes from *Solibacter usitatus* and *Acidobacteria bacterium* Ellin_345. These two organisms are the only representative isolates of the phylum *Acidobacteria*, whose full genome sequences are deposited in public-domain databases. This exemplarily shows how phylogenetic assignment might be skewed toward organisms for which a whole-genome sequence is available.

2. The retrieval of transcripts affiliated to *Myxococcus xanthus* from the oxic zone in early succession corresponds well to the phenotype of these bacteria, as they exhibit a strictly aerobic organotrophic metabolism and a rapid growth response to substrate availability (Dawid, 2000).

3. Similarly, the high frequency of transcripts of *Anaeromyxobacter*-like organisms in the lower transition and anoxic zone of late succession in comparison to early succession corresponds well to their facultative anaerobic metabolism.

4. Detection of a *nosZ* gene transcript assigned to *Anaeromyxobacter* in the lower transition zone of early succession may be explained by its comparatively long half-life, which was around 13 minutes when a pure culture of *Pseudomonas stutzeri* was studied (Nogales *et al*., 2002). *nosZ* encodes the enzyme nitrous oxide reductase, which catalyzes the final step in denitrification. Baumann *et al*. (1996) have shown that anaerobic

conditions, combined with the presence of N-oxides, were necessary for gene expression driving denitrification. *Anaeromyxobacter dehalogenans* uses a diverse range of alternative electron acceptors for anaerobic growth, including nitrate, Fe (III), and fumarate (Sanford *et al*., 2002).

5. Dissimilatory iron reduction is one of the predominant microbial processes within the oxygen gradients that develop in flooded rice paddy soils shortly after flooding (Yao *et al*., 1999). The high frequency with which transcripts related to *Geobacter* and *Anaeromyxobacter* spp. were detected in the upper and lower transition zones, and anoxic zone during late succession may suggest the presence of dissimilatory Fe(III)-reducing consortia. *Anaeromyxobacter dehalogenans* strain FAc12 was isolated from Vercelli rice paddy soil as a dissimilatory iron reducer (Treude *et al*., 2003). Representatives of this subgroup seem to be adapted to low substrate concentrations and to have low growth rates.

6. Assignment of transcripts to certain functions was observed, including putative IcmL-like type IV secretion system, LivK, PrpD (2-Methylcitrate dehydratase), NirB and predicted GTPase proteins (Table 7.2). Multiple assignments to the same function may suggest either an overwhelming importance of this function in the habitat from which the transcripts were recovered or biased retrieval towards these transcripts. The latter assumption is supported by the fact that transcripts assigned to the same function/protein showed exactly the same lengths of cloned cDNA inserts. Poretsky *et al*. (2005) also reported selective amplification of transcripts by PCR primers thought to be (almost) universal. Examples are *soxA* transcript amplification with OPA13 and OPA17 primers, and *aphA* transcript amplification with SD14 primer.

All the above findings indicate that successional changes in the phylogenetic composition of the rice paddy soil bacterial communities could be concluded with some level of confidence, but not significant changes in their functional state; the latter presumably due to the insufficient number of cDNA clones randomly sampled. However, in the future, high-throughput sequencing techniques and automated annotation tools should enable transcriptome of total microbial communities and their functional response to environmental changes.

References

Alexander, M. 1977. Introduction to soil microbiology, 2nd ed., Wiley, New York.

Alfreider, A., J. Pernthaler, R. Amann, B. Sattler, F. O. Glöckner, A. Wille, and R. Psenner. 1996. Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by *in situ* hybridization. Appl. Environ. Microbiol. **62:** 2138-2144.

Alifano, P., C. B. Bruni, and M. S. Carlomagno. 1994. Control of mRNA processing and decay in prokaryotes. Genetica **94:** 157-172.

Altschul, S. F., W. Gish, W. Miller, E. W. Meyers, and D. J. Lipman. 1990. Basic Local Alignment Search Tool. J. Mol. Biol. **215:** 403-410.

Andersson, D. I., and B. R. Levin. 1999. The biological cost of antibiotic resistance. Curr. Opin. Microbiol. **2:** 489-493.

Andrews, J. H., and R. F. Harris. 1986. *r*- and *K*-selection and microbial ecology. Adv. Microb. Ecol. **9:** 99-147.

Altuvia, S., D. Kornitzer, D. Teff, and A. B. Oppenheim. 1989. Alternative mRNA structures of the cIII gene of bacteriophage λ determine the rate of its translation initiation. J. Mol. Biol. **210:** 265-280.

Bakken, L. R. 1997. Culturable and nonculturable bacteria in soil. In: *Modern Soil Microbiology*
(eds J. D. van Elsas, J. T. Trevors and E. M. H. Wellington). Marcel Dekker, New York, pp. 47-61.

Baldani, J. I., B. Pot, G. Kirchhof, E. Falsen, V. L. D. Baldani, F. L. Olivares, B. Hoste, K. Kersters, A. Hartmann, M. Gillis, and J. Dobereiner. 1996. Emended description of *Herbaspirillum*; inclusion of [*Pseudomonas*] *rubrisubalbicans*, a mild pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov., and classification of a group of clinical isolates (EF group 1) as *Herbaspirillum* species 3. Int. J. Syst. Bacteriol. **46:** 802-810.

Balestra, G. M., and I. J. Misaghi. 1997. Increasing the efficiency of the plate counting method for estimating bacterial diversity. J. Microbiol. Meth. **30:** 111-117.

Baumann, B., M. Snozzi, A. J. B. Tehnder, and J. R. van der Meer. 1996. Dynamics of denitrification activity of *Paracoccus denitrificans* in continuous culture during aerobic-anaerobic changes. J. Bacteriol. **178:** 4367-4374.

Belasco, J. 1993**.** mRNA degradation in prokaryotic cells: an overview. In J. Belasco and G. Brawerman (ed.), *Control of messenger RNA stability*. Academic Press, Inc., San Diego, Calif, pp. 3-12.

Belasco, J. G., G. Nilsson, A. von Gabain, and S. N. Cohen. 1986. The stability of *E. coli* gene transcripts is dependent on determinants localized to specific mRNA segments. Cell **46:** 245-251.

Benson, D. A., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, B. A. Rapp, and D. L. Wheeler. 2002. GenBank. Nucl. Ac. Res. **30:** 17-20.

Bercovier, H., O. Kafri, and S. Sela. 1986. *Mycobacteria* possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. Biochem. Biophys. Res. Commun. **136:** 1136-1141.

Bernstein, J. A., A. B. Khodursky, P. H. Lin, S. Lin-Chao, and S. N. Cohen. 2002. Global analysis of mRNA decay and abundance in *Escherichia coli* at single-gene resolution using two-color fluorescent DNA microarrays. Proc. Natl. Acad. Sci. USA. **99:** 9697-9702.

Bloem, J. 1995. Fluorescent staining of microbes for total direct counts. In: Akkermans, A. D. L., van Elsas, J. D., de Brujin, F. J., editors; Akkermans, A. D. L., van Elsas, J. D., de Brujin, F. J., editors. *Molecular microbial ecology manual*. Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 1-12.

Borneman, J., and E. W. Triplett. 1997. Rapid and direct method for extraction of RNA from soil. Soil Biol. Biochem. **29:** 1621-1624.

Bosshard, P. P., S. Abels, R. Zbinden, E. C. Bottger, and M. Altwegg. 2003. Ribosomal DNA sequencing for identification of aerobic gram-positive rods in the clinical laboratory (an 18-month evaluation). J. Clin. Microbiol. **41:** 4134-4140.

Bossio, D. A., and K. M. Scow. 1998. Impacts of carbon and flooding on soil microbial communities: phospholipid fatty acid profiles and substrate utilization patterns. Microbial. Ecol. **35:** 265-278.

Boucher, Y., C. L. Nesbø, and W. F. Doolittle. 2001. Microbial genomes: dealing with diversity. Curr. Opin. Microbiol. **4:** 285-289.

Bowman, J. P., L. I. Sly, A. C. Hayward, Y. Spiegel, and E. Stackebrandt. 1993. *Telluria mixta* (*Pseudomonas mixta* Bowman, Sly, and Hayward 1988) gen. nov., comb. nov., and *Telluria chitinolytica*, sp. nov., soil-dwelling organisms which actively degrade polysaccharides. Int. J. Syst. Bacteriol. **43:** 120-124.

Bremer, H., and P. P. Dennis. 1987. Modulation of chemical composition and other parameters of the cell by growth rate. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: *cellular and molecular biology*, vol. 2. American Society for Microbiology, Washington, D.C. pp.1527-1542.

Brosius, J., J. J. Palmer, J. P. Kennedy, and H. F. Noller. 1978. Complete nucleotide sequence of a 16S ribosomal gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA. **75:** 4801-4805.

Brune, A., P. Frenzel, and H. Cypionka. 2000. Life at the oxic–anoxic interface: microbial activities and adaptations. FEMS Microbiol. Rev. **24:** 691-710.

Buesing, N., and M. O. Gessner. 2002. Comparison of detachment procedures for direct counts of bacteria associated with sediment particles, plant litter and epiphytic biofilms. Aquat. Microbiol. Ecol. **27:** 29-36.

Bürgmann, H., F. Widmer, W. V. Sigler, and J. Zeyer. 2003. mRNA extraction and reverse transcription-PCR protocol for detection of *nifH* gene expression by *Azotobacter vinelandii* in soil. Appl. Environ. Microbiol. **69:** 1928-1935.

Chen, C. Y. A., J. T. Beatty, S. N. Cohen, and J. G. Belasco. 1988. An intercistronic stem-loop structure functions as an mRNA decay terminator necessary but insufficient for puf mRNA stability. Cell **52:** 609-619.

Chen, H., M. Bjerknes, R. Kumar, and E. Jay. 1994. Determination of the optimal aligned spacing between the Shine-Dalgarno sequence and the translation initiation codon of *Escherichia coli* mRNAs. Nucl. Ac. Res. **22:** 4953-4957.

Chin, K. J., D. Hahn, U. Hengstmann, W. Liesack, and P. H. Janssen. 1999. Characterization and identification of numerically abundant culturable bacteria from the anoxic bulk soil of rice paddy microcosms. Appl. Environ. Microbiol. **11:** 5042-5049.

Cho, J. C., and J. M. Tiedje. 2002. Quantitative detection of microbial genes by using DNA microarrays. Appl. Environ. Microbiol. **68:** 1425-1430.

Christensen, H., M. Hansen, and J. Sørensen. 1999. Counting and size classification of active

soil bacteria by fluorescence *in situ* hybridization with an rRNA oligonucleotide probe. Appl. Environ. Microbiol. **65:** 1753-1761.

Dang, H., and C. R. Lovell. 2000. Bacterial primary colonization and early succession on surface in marine waters as determined by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes. Appl. Environ. Microbiol. **66:** 467-475.

Davis, K. E. R., S. J. Joseph, and P. 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.

Dawid, W. 2000. Biology and global distribution of *Myxobacteria* in soils. FEMS Microbiol. Rev. **24:** 403-427.

De Leij, F. A. A. M., J. M. Whipps, and J. M. Lynch. 1993. The use of colony development for the characterization of bacterial communities in soil and on roots. Microb. Ecol. **27:** 81-97.

Devers, M., G. Soulas, and F. Martin-Laurent. 2004. Real-time reverse transcription PCR analysis of expression of atrazine catabolism genes in two bacterial strains isolated from soil. J. Microbiol. Meth. **56:** 3-15.

Dighe, A. S., K. Jangid, J. M. Gonzalez, V. J. Pidiyar, M. S. Patole, D. R. Ranade, and Y. S. Shouche. 2004. Comparison of 16S rRNA gene sequences of genus *Methanobrevibacter*. BMC Microbiol. **4:** 20.

Dijkshoorn, L., B. M. Ursing, and J. B. Ursing. 2000. Strain, clone and species: comments on three basic concepts of bacteriology. J. Med. Microbiol. **49:** 397-401.

Donovan, W. P., and S. R. Kushner. 1986. Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA. **83:** 120-124.

Emory, S. A., and J. G. Belasco. 1990. The ompA 5' untranslated RNA segment functions in *Escherichia coli* as a growth-rate regulated mRNA stabilizer whose activity is unrelated to translational efficiency. J. Bacteriol. **172:** 4472-4481.

Farquharson, M., R. Harvie, and A. M. McNcol. 1999. Detection of messenger RNA using a digoxigenin end-labeled oligonucleotide probe. J. Clin. Pathol. **43:** 423-428.

Fegatella, F., J. Lim, S. Kjelleberg, and R. Cavicchioli. 1998. Implications of rRNA operon copy number and ribosome content in the marine oligotrophic ultramicrobacterium *Sphingomonas* sp. strain RB2256. Appl. Environ. Microbiol. **64:** 4433-4438.

Felske, A., A. Wolterink, R. van Lis, W. E. de Vos, and A. D. L. Akkermans. 2000. Response of a soil bacterial community to grassland succession as monitored by 16S rRNA levels of the predominant ribotypes. Appl. Environ. Microbiol. **66:** 3998-4003.

Fislage, R., M. Berceanu, Y. Humboldt, M. Wendt, and H. Oberender. 1997. Primer design for a prokaryotic differential display RT-PCR. Nucl. Ac. Res. **25:** 1830-1835.

Fleming, J. T., J. Sanseverino, and G. S. Sayler. 1993. Quantitative relationship between naphthalene catabolic gene frequency and expression in predicting PAH degradation in soils at town gas manufacturing sites. Environ. Sci. Technol. **27:** 1068-1074.

Fleming, J. T., W. H. Yao, and G. S. Sayler. 1998. Optimization of differential display of prokaryotic mRNA: application to pure culture and soil microcosms. Appl. Environ. Microbiol. **64:** 3698-3706.

Gadgil, M., and O. T. Solbrig. 1972. The concept of *r*- and *K*- selection: evidence from wildflowers and some theoretical considerations. Amer. Nat. **106:** 14-31.

Garland, J. L., K. L. Cook, J. L. Adams, and L. Kerkhof. 2001. Culturability as an indicator of succession in microbial communities. Microbiol. Ecol. **42:** 150-158.

Garrity, G. (Ed.). 2001. *Bergey's Manual of Systematic Bacteriology*, 2nd ed. Springer-Verlag New York, NY. **1:** 1-721.

Gerfen, C. R. 1989. Quantification of *in situ* hybridization histochemistry for analysis of brain function. In P. M. Conn (ed.), Methods in neuroscience. Academic Press, San Diego, Calif. 10. pp. 79-97.

Gich, F. B., E. Amer, J. B. Figueras, C. A. Abella, M. D. Balaguer, and M. Poch. 2000. Assessment of microbial community structure changes by amplified ribosomal DNA restriction analysis (ARDRA). Int. Microbiol. **3:** 103-106.

Gilbert, B., and P. Frenzel. 1995. Methanotrophic bacteria in the rhizosphere of rice microcosms and their effect on porewater methane concentration and methane emission. Biol. Fertil. Soils. **20:** 93-100.

Gold, L. 1988. Posttranscriptional regulatory mechanisms in *Escherichia coli*. Annu. Rev. Biochem. **57:** 199-233.

Grant, S., W. D. Grant, D. A. Cowan, B. E. Jones, Y. Ma, A. Ventosa, and S. Heaphy. 2006. Identification of eukaryotic open reading frames in metagenomic cDNA libraries made from environmental samples. Appl. Environ. Microbiol. **72:** 135-143.

Grossart, H. P., and M. Simon. 1993. Limnetic macroscopic organic aggregates (lake snow): occurrence, characteristics, and microbial dynamics in lake Constance. Limnol. Oceanogr. **38:** 532-546.

Hahn, D. A. R., W. Ludwig, A. D. L. Akkermans, and K. H. Schleifer. 1992. Detection of microorganisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. J. Gen. Microbiol. **138:** 879-887.

Hall, L., K. A. Doerr, L. S. Wohlfiel, and G. D. Roberts. 2003. Evaluation of the microseq system for identification of mycobacteria by 16S ribosomal DNA sequencing and

its integration into a routine clinical mycobacteriology laboratory. J. Clin. Microbiol. **41:** 1447-1453.

Head, I. M., J. R. Saunders, and R.W. Pickup. 1998. Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microbial. Ecol. **35:** 1-21.

Hedlund, B. P., J. J. Gosink, and J. T. Staley. 1997. *Verrucomicrobia* div. nov., a new division of the bacteria containing three new species of *Prosthecobacter*. Ant. van. Leeuwenh. **72**: 29-38.

Helguera-Repetto, C., R. A. Cox, J. L. Munoz-Sanchez, and J. A. Gonzalez-Y-Merchand. 2004. The pathogen *Mycobacterium marinum*, a faster growing close relative of *Mycobacterium tuberculosis*, has a single rRNA operon per genome. FEMS Microbiol. Lett. **235:** 281-288.

Hengstmann, U., K. J. Chin, P. H. Janssen, and W. Liesack. 1999. Comparative phylogenetic assignment of environmental sequences of genes encoding 16S rRNA and numerically abundant culturable bacteria from an anoxic rice paddy soil. Appl. Environ. Microbiol. **65:** 5050-5058.

Heuer, H., G. Wieland, J. Schonfeld, A. Schnwalder, N. C. M. Gomes, and K. Smalla. 2001. Bacterial community profiling using DGGE or TGGE analysis. In: Environmental Molecular Microbiology: Protocols and Applications (ed. P.A. Rochelle). Horizon Scientific Press, Wymondham, UK. pp. 177-190.

Holzapfel-Pschorn, A., R. Conrad, and W. Seiler. 1985. Production, oxidation and emission of methane in rice paddies. FEMS Microbiol. Ecol. **31:** 343-351.

Horz, H. P., J. H. Rotthauwe, T. Lukow, and W. Liesack. 2000. Identification of major subgroups of ammonia-oxidizing bacteria in environmental samples by T-RFLP analysis of *amoA* PCR products. J. Microbiol. Meth. **39:** 197-204.

Holmes, D. S., and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114:** 193-197.

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

Hugenholtz, P. 2002. Exploring prokaryotic diversity in the genomic era. Gen. Biol. **3:** 0003.1-0003.8.

Hurt, R. A., X. Qui, L. Wu, Y. Roh, A. V. Palumbo, J. M. Tiedje, and J. Zhou. 2001. Simultaneous recovery of RNA and DNA from soils and sediments. Appl. Environ. Microbiol. **67:** 4495-4503.

Ingraham, J. L., O. Maaloe, and F. C. Neidhardt. 1983. Growth of the bacterial cell. Sinauer Associates, Inc., Sunderland, Mass. pp 348-385.

Insam, H., and K. Haselwandtner. 1989. Metabolic quotient of the soil microflora in relation to plant succession. Oecologia **79:** 174-178.

Ishikuri, S., and T. Hattori. 1985. Formation of bacterial colonies in successive time intervals. Appl. Environ. Microbiol. **49:** 870-873.

Jackson, C. R, P. F. Churchill, and E. E. Roden. 2001. Successional changes in bacterial assemblage structure during epilithic biofilms development. Ecology **82:** 555-566.

Janssen, P. H., P. S. Yates, B. E. Grinton, P. M. Taylor, and M. Sait. 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.

Johansson, J., P. Mandin, A. Renzoni, C. Chiaruttini, M. Springer, and P. Cossart. 2002. An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. Cell **110:** 551-561.

John, H. A., M. L. Birnstiel, and K. W. Jones. 1969. RNA-DNA hybrids at the cytological level. Nature **223:** 582-587.

Johnsen, K., and P. Nielsen. 1999. Diversity of *Pseudomonas* strains isolated with King's B and Gould's S1 agar determined by repetitive extragenic palindromic-polymerase chain reaction, 16S rDNA sequencing and Fourier transform infrared spectroscopy characterization. FEMS Microbiol. Lett. **173:** 155-162.

Johnsen, K., C. S. Jacobsen, V. Torsvik, and J. Sørensen. 2001. Pesticide effects on bacterial diversity in agricultural soils – a review. Biol. Fert. Soils. **33:** 443-453.

Kalmbach, S., W. Manz, and U. Szewzyk. 1997a. Dynamics of biofilm formation in drinking water: phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and *in situ* hybridization. FEMS Microbiol. Ecol. **22:** 265-280.

Kalmbach, S., W. Manz, and U. Szewzyk. 1997b. Isolation of new bacterial species from drinking water biofilms and proof of their *in situ* dominance with highly specific 16S rRNA probes. Appl. Environ. Microbiol. **63:** 4164-4170.

Kämpfer, P., and R. Rosselló-Mora. 2004. The species concept for prokaryotic microorganisms - an obstacle for describing diversity? Poi. Pra. **3:** 62-72.

Kattar, M. M., J. F. Chavez, A. P. Limaye, S. L. Rassoulian-Barrett, S. L. Yarfitz, L. C. Carlson, Y. Houze, S. Swanzy, B. L. Wood, and B. T. Cookson. 2001. Application of 16S rRNA gene sequencing to identify *Bordetella hinzii* as the causative agent of fatal septicemia. J. Clin. Microbiol. **38:** 789-794.

Killham, K. 1994. Soil Ecology. Cambridge University Press, Cambridge.

Kiss, A., B. Sain, and P. Venitianer. 1977. The number of rRNA genes in *Escherichia coli*. FEBS Lett. **79:** 77-79.

Klappenbach, J. A., J. M. Dunbar, and T. M. Schmidt. 2000. rRNA operon copy number reflects ecological strategies of bacteria. Appl. Environ. Microbiol. **29:** 1328-1333.

Koch, A. L. 1994. Growth measurement. In P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C., pp. 248-277.

Koronelli, T. V., T. I. Komarova, and L. F. Tkebuchava. 2002. Bacterial succession on n-alkanes under the conditions of sulfate reduction. Appl. Biochem. Microbiol. **38:** 116-119.

Kozak, M. 1983. Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiol. Rev. **47:** 1-45.

Kristufek, V., D. Elhottova, A. Chronakova, I. Dostalkova, T. Picek, and J. Kalcik. 2005. Growth strategy of heterotrophic bacterial population along successional sequence on spoil of brown coal colliery substrate. Fol. Microbiol. **50:** 427-435.

Krsek, M., and E. M. H. Wellington. 1999. Comparison of different methods for the isolation and purification of total community DNA from soil. J. Microbiol. Meth. **39:** 1-16.

Kuwae, T., and Y. Hosokawa. 1999. Determination of abundance and biovolume of bacteria in sediments by dual staining with 4', 6-diamidino-2-phenylindole and acridine orange: relationship to dispersion treatment and sediment characteristics. Appl. Environ. Microbiol. **65:** 3407-3412.

Lan, R., and P. R. Reeves. 2001. When does a clone deserve a name? A perspective on bacterial species based on population genetics. Tren. Microbiol. **9:** 419-424.

Lane, D. J. 1991. 16S/23S rRNA sequencing. In E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics, John Wiley and Sons, New York, N.Y., pp. 115-175.

Leadbetter, J. R. 2003. Cultivation of recalcitrant microbes: cells are alive, well and revealing their secrets in the 21st century laboratory. Curr. Opin. Microbiol. **6:** 274-281.

Liang, P., and A. B. Pardee. 1992. Differential display of eucaryotic messenger RNA by means of the polymerase chain reaction. Science **257:** 967-971.

Liesack, W., H. Weyland, and E. Stackebrandt. 1991. Potential risks of gene amplification by PCR as determined by 16S ribosomal DNA analysis of a mixed-culture of strict barophilic bacteria. Microb. Ecol. **21:** 191-198.

Liesack, W., P. H. Janssen, F. A. Rainey, N. L. Ward-Raineyand, and E. **Stackebrandt.** 1997. Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques. In J. D. van Elsas, J. T. Trevors, and E. M. H. Wellington (ed.), Modern soil microbiology. Marcel Dekker, Inc., New York, N.Y., pp. 375-439.

Liesack, W., S. Schnell, and N. P. Revsbech. 2000. Microbiology of flooded rice paddies. FEMS Microbiol. Rev. **24:** 625-645.

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

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier *et al.* 2004. ARB: a software environment for sequence data. Nucl. Ac. Res. **32:** 1363-1371.

Ludwig, W., S. H. Bauer, M. Bauer, I. Held, and G. Kirchhof. 1997. Detection and *in situ* identification of representatives of a widely distributed new bacterial phylum. FEMS Microbiol. Lett. **153:** 181-190.

Lueders, T., B. Pommerenke, and M. W. Friedrich. 2004. Stable-isotope probing of microorganisms thriving at thermodynamic limits: syntrophic propionate oxidation in flooded soil. Appl. Environ. Microbiol. **70**: 5778-5786.

Lundberg, U., G. Nilsson, and A. von Gabain. 1988. The differential stability of the *Escherichia coli ompA* and *bla* mRNA at various growth rates is not correlated to the efficiency of translation. Gene **72:** 141-149.

Lynch, A. S., and E. C. C. Lin. 1996. Responses to molecular O_2. *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd edition. American Society of Microbiology, Washington, D.C. pp. 1527-1538.

MacArthur, R. H., and E. O. Wilson. 1967. The theory of island biogeography. Princeton, UK: Princeton University Press.

Manz, W., M. Eisenbrecher, T. R. Neu, and U. Szewzyk. 1998. Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by *in situ* probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol. Ecol. **25:** 43-61.

Manz, W., M. Wagner, R. Amann, and K. H. Schleifer. 1994. *In situ* characterization of the microbial consortia active in two wastewater treatment plants. Wat. Res. **28:** 1715-1723.

Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K. H. Schleifer. 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. Microbiology **142:** 1097-1106.

Manz, W., U. Szewzyk, P. Ericsson, R. Amann, K. H. Schleifer, and T. Stenström. 1993. *In situ* identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. Appl. Environ. Microbiol. **59:** 2293-2298.

Margalef, R. 1963. On certain unifying principles in ecology. Amer. Nat. **97:** 357-374.

Margalef, R. 1968. Perspective in ecological theory. University of Chicago Press, Chicago. pp.111.

Martin-Laurent, F., L. Philippot, S. Hallet, R. Chaussod, J. C. Germon, G. Soulas, and G. Catroux. 2001. DNA extraction from soils: old bias for new microbial diversity analysis methods. Appl. Environ. Microbiol. **67:** 2354-2359.

Martiny, A. C., T. M. Jorgensen, H. J. Albrechtsen, E. Arvin, and S. Molin. 2003. Long-term succession of structure and diversity of a biofilm formed in a model drinking water distribution system. Appl. Environ. Microbiol. **69:** 6899–6907

Matthews, J. A. 1992. The ecology of recently deglaciated terrain: A geological approach to glacier forelands and primary succession. Cambridge university press, Cambridge, UK.

McClelland, M., and J. W. Welsh. 1994. RNA fingerprinting by arbitrarily primed PCR. PCR Meth. Appl. **4:** S66-S81.

McDaniel, J. A., and D. G. Capone. 1985. A comparison of procedures for the separation of aquatic bacteria from sediments for subsequent direct enumeration. J. Microbiol. Meth. **3:** 291-302.

McGrath, S., J. S. G. Dooley, and R. W Haylock. 2000. Quantification of *Clostridium botulinum* toxin gene expression by competitive reverse transcription-PCR. Appl. Environ. Microbiol. **66:** 1423-1428.

Mendum, T. A., R. E. Sockett, and P. R Hirsch. 1998. The detection of gram-negative bacterial mRNA from soil by RT-PCR. FEMS Microbiol. Lett. **164:** 369-373.

Mitsui, H., K. Gorlach, H. Lee, R. Hattori, and T. Hattori. 1997. Incubation time and media requirements of culturable bacteria from different phylogenetic groups. J. Microbiol. Meth. **30:** 103-110.

Morris, R. G., M. J. Arends, P. E. Bishop, K. Sizer, E. Duvall, and C. C. Bird. 1990. Sensitivity of digoxigenin- and biotin-labeled probes for detection of human papillomavirus by *in situ* hybridization. J. Clin. Pathol. **43:** 800-805.

Mott, J. E., J. L. Galloway, and T. Platt. 1985. Maturation of *Escherichia coli* tryptophan operon mRNA: evidence for 3' exonucleolytic processing after *rho*-dependent termination. EMBO J. **4:** 1887-1891.

Mott, I. E. C., D. J. Stickler, W. T. Coakely, and T. R. Bott. 1998. The removal of bacterial biofilm from water–filled tubes using axially propagated ultrasound. J. Appl. Microbiol. **84:** 509-514.

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

Muyzer G., E. C. de Waal, and A. C. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes for 16S rRNA. Appl. Environ. Microbiol. **59:** 695-700.

Nairn, J. D., and C. P. Chanway. 2002. Temporary loss of antibiotic resistance by marked bacteria in the rhizosphere of spruce seedlings. FEMS Microbiol. Ecol. **40:** 167-170.

Nannipieri, P., J. Ascher, M. T. Ceccherini, L. Landi, G. Pietramellara, and G. Renella. 2003. Microbial diversity and soil functions. Eur. J. Soil Sci. **54:** 655-670.

Narberhaus, F. 2002. mRNA-mediated detection of environmental conditions. Arch. Microbiol.
178: 404-410.

Neidhardt, F. C., and H. E. Umbarger. 1996. In *Escherichia coli* and *Salmonella* (Neidhardt, F. C., Ed.). ASM Press, Washington, DC. **1:** 13-16.

Newbury, S. F., N. H. Smith, E. C. Robinson, I. D. Hiles, and C. F. Higgins. 1987. Stabilization of translationally active mRNA by prokaryotic REP sequences. Cell **48:** 297-310.

Nilsson, G., J. G. Belasco, S. N. Cohen, and A. von Gabain. 1984. Growth-rate dependent regulation of mRNA stability in *Escherichia coli*. Nature **312:** 75-77.

Nogales, B., K. Timmis, D. B. Nedwell, and A. M. Osborn. 2002. Detection and diversity of expressed denitrification genes in estuarine sediments after reverse transcription-PCR amplification from mRNA. Appl. Environ. Microbiol. **68:** 5017-5025.

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

Novitsky, J. A. 1987. Microbial growth rates and biomass production in a marine sediment: evidence for a very active by mostly non-growing community. Appl. Environ. Microbiol. **53:** 2368-2372.

Ogram, A., W. Sun, F. J. Brockman, and J. K. Fredrickson. 1995. Isolation and characterization of RNA from low-biomass deep-subsurface sediments. Appl. Environ. Microbiol. **61:** 763-768.

Ohtonen, R., H. Fritze, T. Pennanen, A. Jumpponen, and J. Trappe. 1999. Ecosystem properties and microbial community changes in primary succession on a glacier forefield. Oecologia **119:** 239-246.

Olsen, R. A., and L. R. Bakken. 1987. Viability of soil bacteria: Optimization of plate-counting technique and comparison between total counts and plate counts within different size groups. Microb. Ecol. **13:** 59-74.

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

Pankhurst, C. E., S. Yu, B. G. Hawke, and B. D. Harch. 2001. Capacity of fatty acid profiles and substrate utilization patterns to describe differences in soil microbial communities associated with increased salinity or alkalinity at three locations in South Australia. Biol. Fert. Soils. **33:** 204-217.

Paster, B. J., W. Ludwig, W. G. Weisburg, E. Stackebrandt, and R. B. Hespell. 1985. A phylogenetic grouping of the *Bacteroides*, *Cytophagas*, and certain *Flavobacteria*. Syst. Appl. Microbiol. **6:** 34-42.

Pedersen, S., S. Reeh, and J. D. Friesen. 1978. Functional mRNA half-lives in *E. coli*. Mol. Gen. Genet. **166:** 329-336.

Pennanen, T., R. Strommer, A. Markkola, and H. Fritze. 2001. Microbial and plant community structure across a primary succession gradient. Scand. J. For. Res. **16:** 37-43.

Pernthaler, A., and R. Amann. 2004. Simultaneous fluorescence *in situ* hybridization of mRNA and rRNA in environmental bacteria. Appl. Environ. Microbiol. **70:** 5426-5433.

Pickett, S. T. A. 1976. Succession: an evolutionary interpretation. Amer. Nat. **110:** 107-119.

Poretsky, R. S., N. Bano, A. Buchan, G. LeCleir, J. Kleikemper, M. Pickering, W. M. Pate, M. Ann Moran, and J. T. Hollibaugh. 2005. Analysis of microbial gene transcripts in environmental samples. Appl. Environ. Microbiol. **71:** 4121-4126.

Radajewski, S., P. Ineson, N. R. Parekh, and J. C. Murrell. 2000. Stable isotope probing as a tool in microbial ecology. Nature **403:** 646-649.

Ramsing, N. B., H. Fossing, T. G. Ferdelman, F. Andersen, and B. **Thamdrup.** 1996. Distribution of bacterial populations in a stratified fjord (Mariager Fjord, Denmark) quantified by *in situ* hybridization and related to chemical gradients in the water column. Appl. Environ. Microbiol. **62:** 1391-1404.

Rapp, M. S., and S. J. Giovannoni. 2003. The uncultured microbial majority. Annu. Rev. Microbiol. **57:** 369-394.

Ravenschlag, K., K. Sahm, and R. Amann. 2000. Quantitative molecular analysis of the microbial community in marine arctic sediments (Svalbard). Appl. Environ. Microbiol. **67:** 387-395.

Reasoner, D. J., and E. E. Geldreich. 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. **49:** 1-7.

Reimers, C. E., and K. L. Smith, Jr. 1986. Reconciling measured and predicted fluxes of oxygen across the deep sea sediment-water interface. Lim. Oceanogr. **31:** 305-318.

Revsbech, N. P. 1989. An oxygen microsensor with a guard cathode. Lim. Oceanogr. **34:** 474-478.

Ripp, S., D. E. Nivens, C. Werner, and G. S. Sayler. 2000. Bioluminescent most probable number monitoring of a genetically engineered bacterium during a long term contained field release. Appl. Microbiol. Biotechnol. **53:** 736-741.

Roberts, T. M., R. Kachich, and M. Ptashne. 1979. A general method for maximizing the expression of a cloned gene. Proc. Nat. Acad. Sci. USA. **76:** 760-764.

Rondon, M. R., P. R. August, A. D. Bettermann, S. F. Brady, T. H. Grossman *et al.* 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl. Environ. Microbiol. **66:** 2541-2547.

Rossello-Mora, R., and R. Amann. 2001. The species concept for prokaryotes. FEMS Microbiol. Rev. **25:** 39-67.

Rothfuss, F., P. Frenzel, and R. Conrad. 1994. Gas diffusion probe for measurement of CH_4 gradients. In Microbial Mats: Structure, Development and Environmental Significance. Stal, L. J., and Cumette, P. (eds). Berlin, Germany: Springer, pp. 167-172.

Roth, A., S. Andrees, R. M. Kroppenstedt, D. Harmsen, and H. Mauch. 2003. Phylogeny of the genus *Nocardia* based on reassessed 16S rRNA gene sequences reveals underspeciation and division of strains classified as *Nocardia asteroides* into three established species and two unnamed taxons. J. Clin. Microbiol. **41:** 851-856.

Saleh-Lakha, S., M. Millera, G. R. Campbell, K. Schneiderc, P. Elahimaneshd, M. M. Harta, and J. T. Trevorsa. 2005. Microbial gene expression in soil: methods, applications and challenges. J. Microbiol. Meth. **63:** 1-19.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, N.Y.

Sanford, R. A., J. R. Cole, and J. M. Tiedje. 2002. Characterization and description of *Anaeromyxobacter dehalogenans* gen. nov., sp. nov., an aryl-halorespiring facultative anaerobic myxobacterium. Appl. Environ. Microbiol. **68:** 893-900.

Schmidt, H. A., K. Strimmer, M. Vingron, and A. V. Haeseler. 2002. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics **18:** 502-504.

Scholefield, J., R. Manson, R. J. Johnston, and R. Scott. 1985. The use of acridine orange staining and image analysis to detect bacteriuria. Urol. Res. **13:**141-142.

Schoniger, M., and A. von Haeseler. 1994. A stochastic model for the evolution of autocorrelated DNA sequences. Mol. Phylogenet. Evol. **3:** 240-247.

Sharma, S., M. K. Aneja, J. Mayer, J. C. Munch, and M. Schloter. 2005. Diversity of transcripts of nitrite reductase genes (*nirK* and *nirS*) in rhizospheres of grain legumes. Appl. Environ. Microbiol. **71:** 2001-2007.

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

Sheridan, G. E. C., C. I. Masters, J. A. Shallcross, and B. M. Mackey. 1998. Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. Appl. Environ. Microbiol. **64:** 1313-1318.

Shields, J. A., E. A. Paul, W. E. Lowe, and D. Parkinson. 1973. Turnover of microbial tissue in soil under field conditions. Soil. Biol. Biochem. **5:** 753-764.

Shine, J., and L. Dalgarno. 1974. The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA. **71:** 1342-1346

Sigler, W. V., and J. Zeyer. 2002. Microbial diversity and activity along the forefields of two receding glaciers. Microb. Ecol. **43:** 397-407.

Sigler, W. V., and J. Zeyer. 2004. Colony-forming analysis of bacterial community succession in deglaciated soils indicates pioneer stress-tolerant opportunists. Microb. Ecol. **48:** 316-323.

Singer, R. H., and D. C. Ward. 1982. Actin gene expression visualized in chicken muscle tissue culture by using *in situ* hybridization with a biotinylated nucleotide analog. Proc. Natl. Acad. Sci. USA. **79:** 7331-7335.

Sørheim, R., V. L. Torsvik, and J. Goksøyr. 1989. Phenotypical divergences between populations of soil bacteria isolated on different media. Microb. Ecol. **17:** 181-192.

Soto, M. J., P. van Dillewijn, F. Martinez-Abarca, J. I. Jimenez-Zurdo, and N. Toro. 2004. Attachment to plant roots and nod gene expression are not affected by pH or calcium in the acid-tolerant alfalfa-nodulating bacteria *Rhizobium* sp. LPU83. FEMS Microbiol. Ecol. **48:** 71-77.

Stackebrandt, E., and B. M. Goebel. 1994. Taxonomic note; a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:** 846-849.

Stackebrandt, E., W. Frederisksen, G. M. Garrity *et al.* 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. **52:** 1043-1047.

Staley, J. T., and J. J. Gosink. 1999. Poles apart: biodiversity and biogeography of sea ice bacteria. Ann. Rev. Microbiol. **53:** 189-215.

Steege, D. A. 2000. Emerging features of mRNA decay in bacteria. RNA **6:** 1079-1090.

Steitz, J. A. 1969. Polypeptide chain initiation nucleotide sequences of the three ribosomal binding sites in bacteriophage R17 RNA. Nature **224:** 957-967.

Stotzky, G. 1997. Soil as an environment for microbial life. In: Modern Soil Microbiology (eds J. D. van Elsas, J. T. Trevors & E. M. H. Wellington). Marcel Dekker, New York, pp. 1-20.

Stover, C. K, **X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, et al.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:** 947-948.

Strauss, E. A., W. K. Dodds, M. K. Banks, J. Skalsky, and W. Yu. 1995. Optimal staining and sample storage time for direct microscopic enumeration of total and active bacteria in soil with two fluorescent dyes. Appl. Environ. Microbiol. **61:** 3367-3372.

Stubner, S. 2002. Enumeration of 16S rDNA of *Desulfotomaculum* lineage 1 in rice field soil by real-time PCR with SybrGreen detection. J. Microbiol. Meth. **50:** 155-164.

Sweerts, J. P. R. A., M. J. Bar-Gilissen, A. A. Cornelese, and T. E. Cappenberg. 1991. Oxygen-consuming processes at the profundal and littoral sediment water interface of a small meso-eutrophic lake (Lake Vechten, the Netherlands). Limnol. Oceanogr. **36:** 1124-1133.

Tan, Z., T. Hurek, P. Vinuesa, and P. Mu. 2001. Specific detection of *Bradyrhizobium* and *Rhizobium* strains colonizing rice (*Oryza sativa*) roots by 16S–23S ribosomal DNA intergenic spacer-targeted PCR, Appl. Environ. Microbiol. **67:** 3655-3664.

Tao, H., C. Bausch, C. Richmond, F. R. Blattner, and T. Conway. 1999. Functional genomics: expression analysis of *Escherichia coli* growing on minimal and rich media. J. Bacteriol. **181:** 6425-6440.

Tilman, D. 1985. The resource ratio hypothesis of succession. Amer. Nat. **125:** 827-852.

Tortoli, E. 2003. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin. Microbiol. Rev. **16:** 319-354.

Torsvik, V., and L. Øvreas. 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr. Opin. Microbiol. **5:** 240-245.

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

Treude, N., D. Rosencrantz, W. Liesack, and S. Schnell. 2003. Strain FAc12, a dissimilatory iron-reducing member of the *Anaeromyxobacter* subgroup of *Myxococcales*. FEMS Microbiol. Ecol. **44:** 261-269.

Tsai, Y. L., and B. H. Olson. 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for PCR. Appl. Environ. Microbiol. **58:** 2292-2295.

Tsai, Yu-li., M. J. Park, and B. H. Olson. 1991. Rapid method for direct extraction of mRNA from seeded soils. Appl. Environ. Microbiol. **57:** 765-768.

Tunlid, A., and D. C. White. 1992. Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil. In: Soil Biochemistry, (eds G. Stotzky & J.-M. Bollag) Marcel Dekker, New York, **7:** 229-262.

Turenne, C. Y., L. Tschetter, J. Wolfe, and A. Kabani. 2001. Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. J. Clin. Microbiol. **39:** 3637-3648.

Tyson, G. W., J. Chapman, P. Hugenholtz, E. E. Allen, R. J. Ram, P. M. Richardson, V. V. Solovyev, E. M. Rubin, D. S. Rokhsar, and J. F. Banfield. 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature **428:** 37-43.

Vandamme, P., B. Pot, M. Gillis, P. DeVos, K. Kersters, and S. J. Swing. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbial. Rev. **60:** 407-438.

Venter, J. C., K. Remington, J. F. Heidelberg, A. L., *et al*. 2004. Environmental genome shotgun sequencing of the Sargasso Sea. Science **304:** 66-74.

Vishnivetskaya, T. A., M. A. Petrova, J. Urbance, M. Ponder, C. L. Moyer, D. A. Gilichinsky, and J. M. Tiedje. 2006. Bacterial community in ancient Siberian permafrost as characterized by culture and culture-independent methods. Astrobiology. **6:** 400-414.

Vitousek, P. M, and P. S. White. 1981. Process studies in succession. In: West, D. C., Shugart, H. H., Botkin, D. B. (Eds.) Forest Succession: Concepts and Applications, Springer-Verlag, New York, pp. 267-276.

von Gabain, A., J. G. Belasco, J. L. Schottel, A. C. Y. Chang, and S. N. Cohen. 1983. Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. Proc. Natl. Acad. Sci. USA. **80:** 653-657.

Ward-Rainey, N., F. A. Rainey, H. Schlesner, and E. Stackebrandt. 1995. Assignment of hitherto unidentified 16S rDNA species to a main line of descent within the domain Bacteria. Microbiology **141:** 3247-3250.

Wayne, L. G., D. J. Brenner, R. R. Colwell, *et al*. 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37:** 463-464.

Weinbauer, M. G., C. Beckmann, and M. G. Höfle. 1998. Utility of green fluorescent nucleic acid dyes and aluminium oxide membrane filters for rapid epifluorescence enumeration of soil and sediment bacteria. Appl. Environ. Microbiol. **64:** 5000-5003.

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

Wen, A., M. Fegan, C. Hayward, S. Chakraborty, and L. I. Sly. 1999. Phylogenetic relationships among members of the *Comamonadaceae*, and description of *Delftia acidovorans* (den Dooren de Jong 1926 and Tamaoka *et al.* 1987) gen. nov., comb. nov. Int. J. Syst. Bact. **49:** 567-576.

Whittaker, R. H. 1953. A consideration of climax theory: the climax as a population and pattern. Ecol. Monogr. **23:** 41-78.

Wilson, K. 1999. Preparation of genomic DNA from bacteria. In *Current protocols in molecular biology*. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K. (ed). John Wiley & Sons, Inc., New York, N.Y. pp. 2.4.1-2.4.5.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. **51:** 221-271.

Wolfe, C. J., and M. G. Haygood. 1993. Bioluminescent symbionts of the caribbean flashlight fish (*Kryptophanaron alfredi*) have a single rRNA operon. Mol. Mar. Biol. Biotechnol. **2:** 189-197.

Wong, K. K., and M. McClelland. 1994. Stress-inducible gene of *Salmonella typhimurium* identified by arbitrarily primed PCR of RNA. Proc. Natl. Acad. Sci. USA. **91:** 639-643.

Wu, L., D. K. Thompson, G. Li, R. A. Hurt, J. M. Tiedje, and J. Zhou. 2001. Development and evaluation of functional gene arrays for detection of selected genes in the environment. Appl. Environ. Microbiol. **67:** 5780-5790.

Yang, Y.-H., J. Yao, S. Hu, and Y. Qi. 2000. Effects of agricultural chemicals on DNA sequence diversity of soil microbial community: A study with RAPD marker. Microb. Ecol. **39:** 72-79.

Yao, H., R. Conrad, R. Wassmann, and H. U. Neue. 1999. Effect of soil characteristics on sequential reduction and methane production in sixteen rice paddy soils from China, the Philippines, and Italy. Biogeochemistry **47:** 269-295.

Young, J. M. 2001. Implications of alternative classifications and horizontal gene transfer for bacterial taxonomy. Int. J. Syst. Evol. Microbiol. **51:** 945-953.

Zarda, B., D. Hahn, A. Chatzinotas, W. Schonhuber, A. Neef, R. I. Amann, and J. Zeyer. 1997. Analysis of bacterial community structure in bulk soil by *in situ* hybridization. Arch. Microbiol. **168:** 185-192.

Zehnder, A. J. B., and W. Stumm. 1988. Geochemistry and biogeochemistry of anaerobic habitats. In *Biology of Anaerobic Microorganisms.* Zehnder, A. J. B. (ed.). New York, USA: Wiley Interscience Publications, pp.1-38.

Zelles, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. Biol. Fert. Soils. **29:** 111-129.

Zhu, H., F. Qu, and L. H. Zhu. 1993. Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. Nucl. Ac. Res. **21:** 5279-5280.

Zwart, G., B. C. Crump, M. P. Kamst-van Agterveld, F. Hagen, and S. K. Han. 2002. Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat. Microb. Ecol. **28:** 141-55.

Curriculum vitae

Pravin Malla Shrestha, born on 12.03.1972

Citizenship: Nepali

Academic achievements

Jan. 2004 - Dec. 2006: Ph.D. work at the Max Planck Institute for Terrestrial Microbiology, Marburg under the supervision of PD Dr. Werner Liesack.

Jan. 2002 - Aug. 2003: M.Sc. in Bioprocess Technology with thesis entitled "Study of bacterial DNA compaction after slow lysis of spheroplast" from Asian Institute of Technology (AIT), Bangkok, Thailand.

1993 - 1995: M.Sc. in Microbiology with distinction in dissertation entitled "Improvement of nutritional value of soybean by fermentation using *Aspergillus oryzae*" from Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal.

1991 - 1992: B.Sc. (Microbiology/Chemistry/Botany), Tri-Chandra Multiple Campus, Kathmandu, Nepal.

1988 - 1989: I.Sc. (Biology), Tri-Chandra Multiple Campus, Kathmandu, Nepal.

1986: School Leaving Certificate, Tribhuvan Adarsha Madyamik Vidyalaya, Pharping, Kathmandu, Nepal.

Working experiences

Aug. 1996 - Dec. 2001: Worked as a Quality assurance executive/ Senior microbiologist in Nepal Pharmaceuticals Laboratories Pvt. Ltd., Birgunj, Nepal.

Jan. 1995 - Aug. 1996: Worked as a Science teacher in Tribhuvan Adarsha Secondary School, Pharping, Kathmandu, Nepal.

Rewards and scholarships

Jan. 2004 - Jan. 2006: Stipendium from International Max Planck Research School to pursue Ph.D. in Max Planck Institute for Terrestrial Microbiology, Marburg, Germany.

Jan. 2002 - Aug. 2003: Austrian government scholarship to pursue M.Sc. in Bioprocess technology at Asian Institute of Technology (AIT), Thailand.

Publications

1) **Shrestha, P. M.**, M. Noll, and W. Liesack. **2007.** Phylogenetic identity, growth-response time and rRNA operon copy number of soil bacteria indicate different stages of community succession. Environ. Microbiol. **9:**2464-2474.

2) Shrestha, M., Abraham W.R., **Shrestha, P.M.,** Noll, M., and R. Conrad. **2007.** Activity and composition of methanotrophic bacterial communities in planted rice soil studied by flux measurements, analyses of *pmoA* gene and stable isotope probing of phospholipid fatty acids. Environ. Microbiol. *In press*.