Kings without crowns:

Analysis of abundant bacilli in different soil ecosystems

Vesela A. Tzeneva

Promotor:	**Prof. dr. W. M. de Vos** Hoogleraar Microbiologie Wageningen Universiteit
Co-promotoren:	**Dr. H. Smidt** Universitair Docent, Laboratorium voor Microbiologie Wageningen Universiteit
	Dr. A. D. L. Akkermans Universitair Hoofddocent, Laboratorium voor Microbiologie Wageningen Universiteit
Promotiecommissie:	**Prof. Dr. P. de Vos** Universiteit Gent, België
	Prof. Dr. J. D. van Elsas Rijksuniversiteit Groningen
	Prof. Dr. A. H. C. van Bruggen Wageningen Universiteit
	Dr. Ir. J. Dolfing Globalviewmanagement, Deventer

Dit onderzoek is uitgevoerd binnen de onderzoekschool SENSE

Kings without Crowns:

Analysis of abundant bacilli in different soil ecosystems

Vesela A. Tzeneva

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. M.J. Kropff,
in het openbaar te verdedigen
op maandag 9 oktober 2006
des namiddags te 16.00 uur in de Aula.

Vesela A. Tzeneva – Kings without crowns: Analysis of abundant bacilli in different soil ecosystems – 2006

Thesis Wageningen University, Wageningen, The Netherlands – With summary in Dutch
ISBN – 90-9020989-1

Посвещавам на Татко

Dedicated to my Father

Content

Abstract

This study describes culture-dependent as well as culture-independent strategies to monitor diversity and abundance of *Bacillus benzoevorans*-related soil bacteria. These bacteria are wide spread around the world, inhabiting a variety of terrestrial environments. A distinguishing feature of the family *Bacillaceae* is their ability to form endospores. This capacity makes the genus *Bacillus* amenable to retrospective and biogeographical studies, as their endospores provide the means for survival under environmental conditions of stress, allowing their detection later on. For the rapid detection of *B. benzoevorans*-related populations in soil samples selective cultivation media and group-specific primers and probes were developed. Using these techniques the global distribution of this group was demonstrated and indicated their adaptive capacity to diverse soil ecosystems. A unique soil sample archive provided insight in the microbiological impact of land reclamation and flooding. Using multivariate statistical approaches were used to assess the microbial community dynamics over time and in response to the changing environmental conditions.

Found in a variety of soil ecosystems, microorganisms related to *B. benzoevorans* seem to be able to populate a broad range of niches, which indicates a high degree of metabolic versatility and strong adaptive capability. Moreover, they account for a significant part of the total bacterial community (up to 30 %). Based on our exploratory study the importance of their role is just indicated, but not acknowledged yet. Therefore, it is proposed to regard *B. benzoevorans* relatives as ‘kings without crowns’; as this group of bacteria deserves more scientific attention in future studies aiming to unravel their eco-physiology and functionality as major players of the soil microbiota.

CHAPTER 1

INTRODUCTION

General introduction

A single shovel full of rich garden soil contains more species of organisms than can be found seen aboveground in the entire Amazon rain forest. The soil ecosystem is known to provide ecological niches for an extremely high number of microorganisms (94). The relationship between the diversity of living organisms and the ecosystem functions has become an important issue of modern microbial ecology. Microbial communities in the soil are involved in major processes, essential for the existence and sustainability of any terrestrial ecosystem, i.e. decomposition of organic matter (27) and mineralization of nutrients in soil (8). Alongside with this important ecosystem-defining function, soil microorganisms, mainly bacteria and fungi, provide an essential contribution to a wealth of other processes, including nitrogen fixation, solubilization of parent rock minerals, synthesis of plant-growth-promoting and other physiologically active substances, etc. Moreover, microorganisms take part in the decomposition of toxic inorganic and organic pollutants in the soil (13, 25, 82, 83, 85).

When excluding plants, soil organisms can be largely divided into bacteria, archaea and eukarya (fungi, protozoa, nematodes, arthropods and earthworms). Among these, bacteria, archaea and fungi comprise the majority of the soil microbiota and are functionally important communities for soil fertility. In agricultural and grassland soils bacteria often are the predominant group (42).

Soil microbiota

The genus *Bacillus*

The genus *Bacillus*, belonging to the family *Bacillaceae* within the *Firmicutes*, was first discovered by Ferdinand Cohn in 1872 with *Bacillus subtilis* as the first member. A distinguishing feature of the family is their ability to form endospores. Endospores are structures formed within bacterial cells that enable the cell to resist environmental conditions of stress. The members of the genus *Bacillus* are furthermore characterized as Gram-positive, rod-shaped, and aerobic or facultatively anaerobic (70).

In Bergey's Manual of Systematic Bacteriology (1st ed. 1986), the G+C content of known species of *Bacillus* is listed to range from 32 to 69% (46), illustrating the genomic heterogeneity of the genus. Variations are observed from species to species, and sometimes

profound differences in G+C content are even found among strains within a species. For example, the G+C content of the *B. megaterium* group ranges from 36 to 45%.

According to the "Bacterial Nomenclature up-to-date" (DSMZ -Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, http://www.dsmz.de/bactnom/bactname.htm), there are currently 188 species and 6 subspecies recognized in the genus *Bacillus* (available data in May 2006). A large number of valid species have been designated and novel species are discovered regularly, as evidenced by the fact that only since 2005, 29 novel species have been described.

The genus *Bacillus* is both taxonomically and metabolically diverse. The primary habitat is the soil and associated plants or rivers, although some species are pathogenic for mammals (e.g. *B. anthracis*) and insects (e.g. *B. sphaericus, B. thuringensis*) (103). Bacilli form an important part of the microbiota in many soils, and many species are of considerable practical importance. Some *Bacillus* spp., including those that are thermophilic, are crucial for biotechnological applications as they are sources of enzymes and other products of industrial interest (7). Other *Bacillus* spp., such as *B. thuringensis*, *B. subtilis*, *B. cereus* and *B. sphaericus*, play important roles as biological control agents of different phytopathogenic organisms (24, 75, 86). Some members of the genus *Bacillus* are able to produce antibiotics. The majority of these antibiotics are low-molecular-weight peptides with different biological activities, including antimicrobial, antiviral, and antitumor activities (41), and several *Bacillus* isolates obtained from soil and plants have recently been reported to be capable of enzymatic inactivation of N-acyl homoserine lactone quorum sensing signals (19). The ability of *Bacillus* to form highly resistant endospores imparts an enormous competitive advantage in environments such as soil, where long periods of drought and nutrient deprivation are common (103). At least 4% of the 4.2 Mbp large *B. subtilis* genome, comprising 4100 protein-encoding genes (51), is dedicated to the processes of sporulation, germination and out-growth. Bacteria in temperate soils must be able to adapt rapidly to changes in ambient temperatures. For this purpose the *B. subtilis* genome encodes numerous stress proteins, including the chaperones (44). The ability of *Bacillus* spp. to secrete a wide variety of extracellular depolymerases is a major factor contributing to their colonisation in soil. Genes encoding secreted amylases, arabinases, chitonases, mannanases, cellulases and xylanases are

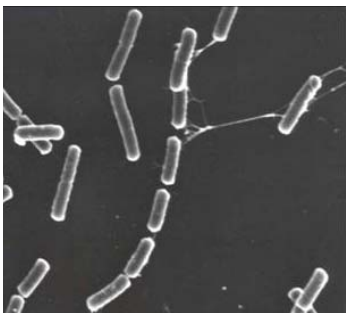


Figure 1. Electron micrograph of *B. cereus* ATCC 10987 (T. Lindbäck, http://mlstoslo.uio.no/bacillus.html, accession date: 13 June 2006).

evident in the genome sequence. Proteases are also frequently encountered, both intracellular and extracellular, the latter allowing for the use of protein as sources of both carbon and nitrogen (103). To date, the complete genomes of 12 strains of *Bacillus* have been elucidated, which all showed to be relatively large, ranging from 4.2 Mbp (*B. halodurans* C-125) to 5.6 Mbp (*B anthracis* AMES 0581) (http://genomesonline.org, accession date: 18 March 2006). This might reflect the ability of bacilli to survive and adapt to frequent changes in the physico-chemical conditions in the soil and to be abundant in different environments. A simular observation was made for strains of *Lactobacillus plantarum* by Molenaar *et al* (62).

Ecological role in soil

Aerobic endospore-forming bacteria are ubiquitous in agricultural systems (40). Common physiological traits important to their survival include production of a multilayered cell wall structure, formation of stress-resistant endospores, and secretion of peptide antibiotics, peptide signal molecules, and extracellular enzymes. However, significant variation exists in other key traits, including nutrient utilization, motility, and physicochemical growth optima. Quantitative and qualitative variations in these traits allow for these bacteria to inhabit diverse ecological niches (76). Their microscopic size and omnipresence in soils facilitates their colonization of plants and animals, but the degree of niche localization of most species has not been thoroughly studied. Indeed, the ecological significance of the genotypic and phenotypic diversity of *Bacillus* and related genera remains largely unknown. Recently, our insight has been improved in understanding of the ecology of *Bacillus* and how different subpopulations of this genus can promote crop health. Native populations occur abundantly in most agricultural soils, while plant tissues are differentially colonized by distinct subpopulations. Multiple *Bacillus* spp. can promote crop health (40). Among these, some populations suppress plant pathogens and pests by producing antibiotic metabolites, while others may directly stimulate plant host defenses prior to infection. Some strains can also stimulate nutrient uptake by plants, either by promoting rhizobial and mycorrhizal symbioses or by fixing atmospheric nitrogen directly.

***Bacillus benzoevorans*- relatives**

Bacillus benzoevorans was described as an organism able to degrade various aromatic acids and phenols (73, 74). Recently, investigations in Dutch Drentse A grassland soil have demonstrated the abundance of 16S rRNA gene sequences that originated from microbial populations related to *B. benzoevorans* (28, 32). Analysis of a clone library based on direct

recovery of 16S rRNA genes from this soil indicated that 32 % of the bacterial sequences belonged to this novel phylogenetic lineage, which includes *B. benzoevorans* (74), *B. niacini* (68), *B. pseudomegaterium*, *B. jeotgali* (104), five recently isolated species *B. novalis*, *B. vireti*, *B. soli*, *B. bataviae* and *B. drentensis* (45), the uncultured Drentse A bacterium DA001, and their relatives (31). Their homogeneous distribution and activity over extended distances has been demonstrated previously in the Dutch Drentse A grasslands (29). Uncultured members of this lineage were also reported to be present as the predominant group of *Bacillus* spp. in soil samples from other countries, including the United States (Wisconsin) (10), Amazonia (11), The Netherlands (30, 39, 97), United Kingdom (57), Portugal and Pakistan (Himalaya) (97). So far only a fraction of the bacteria from this cluster has been cultivated (33, 48). *B. benzoevorans* relatives were detected as abundant populations in air-dry soil samples collected over a period of 44 years from Wieringermeer polder in The Netherlands immediately after the reclamation of the polder (97).

The protozoa

Protozoa are unicellular, eukaryotic organisms, which were first surveyed and described by Anthonie van Leeuwenhoek (1632-1723) who called them animalicules or "little animals" (98). These microorganisms, ranging in size from 2 μm (nanoflagellates) to over 6000 μm (some *Sacrodina* species), are the smallest but most numerous of all animals. Free-living heterotrophic protozoa can be found in virtually all ecosystems in the world. Those generally found in aerated soil include flagellates, naked amoeba, and ciliates. The protozoa biomass is estimated to be 5-10 μg C x g^{-1} while the total microbial biomass in agricultural soil is ranging from 300- 500 μg C x g^{-1} (81). Although the total biomass of protozoa is 10 to 100 times lower than that of the microbial biomass, the importance of the protozoa in soil should not be underestimated. They play an important role in the soil nutrient economy where they account for a high rate of biomass production. The most important food source of free living heterotrophic protozoa is bacteria. This predation by protozoa removes bacteria and a decrease in bacterial activity and consequently in the decomposition of organic matter and mineralization of nutrients might be expected. However, the opposite effect has been observed many times and the stimulating effect of protozoa on the bacterial activity was already shown long time ago (6, 49). Upon predation by protozoa the growth rate of bacteria increased, even though the bacterial biomass was reduced. This was a response to a higher level of available carbon, nitrogen and phosphorus upon predation.

Monitoring the impact of global change on structure and functioning of the various ecosystems is one of the major challenges for contemporary biology. While the study of communities of macroscopic, multicellular organisms is progressing using well established approaches, analyses of the microbial domain of the various ecosystems are still rather fragmentary. In particular, our knowledge on the role and the dynamics of the microbial eukaryotic communities in soil is very limited (4, 9, 23), although remarkable advances have been made recently using culture- independent, molecular approaches in aquatic environments (17, 21, 55, 63, 65, 106). Not a single molecular approach addressing eukaryotic biodiversity has been published so far for historical soil samples, which potentially could reveal changes of the eukaryotic soil microbiota in time. The eukaryotic biodiversity in historical air-dried samples of Dutch agricultural soil has been assessed by random sequencing of an 18S ribosomal RNA library and by denaturing gradient gel electrophoresis (DGGE). Representatives of nearly all taxa of eukaryotic soil microbes could be identified demonstrating that it is possible to study eukaryotic microbiota in samples from soil archives that were stored for more than 30 years (64).

Environments studied in this thesis

In this thesis the total bacterial and *B. benzoevorans* related communities were studied in soil samples all over the world. However, several studies focussed on two main environments: Dutch polder, where agricultural soil was investigated; and grassland soil, collected from the Rothamsed experimental station (UK).

The polder environment

A polder is an area where land was reclaimed from shallow sea or lake water. Naturally the area is below the surrounding water level, and a network of artificial canals is necessary to drain the soils. One or more pumps remove the water from the area into the surrounding water. These pumps and inlets allow regulation of the soil water level. There are about 3000 polders located throughout The Netherlands, rendering these one of the most characteristic environments in this country.

Wieringermeer polder

In 1932 the Zuider Zee was cut off from the North Sea in The Netherlands by an enclosing dam, resulting in the formation of the IJsselmeer (lake IJssel) that gradually changed into a fresh water environment (99) (Fig. 2). During reclamation of the polders, a series of processes occurred by which the soft, water-saturated and unaerated soil turned into typical arable soil. This successive development has been documented extensively and was characterized by physical (84), chemical (109) and microbiological (99) changes. The first period following the recession of the water was characterized by an increase in the number of cultivable aerobic soil bacteria, which initially was small. This increase was roughly proportional to the decrease in moisture and salt content, and the improved aeration of the soils.

Figure 2. The IJssel lake polders (3 polders reclaimed, 1 polder in progress, 1 projected polder.) (99)

Cultivation techniques were applied to isolate several bacterial groups, such as nitrifying bacteria, as well as nitrogen fixers including *Azotobacter* and *Rhizobium* spp., from Wieringermeer polder soil samples (99). Recently, we applied cultivation-independent molecular techniques and detected the presence of the *B. benzoevorans*- relatives (97). The development of the *B. benzoevorans* related community was followed during the period 1942-1986, using historical samples from the TAGA archive of air-dried soil samples (ALTERRA BV, Wageningen, The Netherlands). Interesting changes in the diversity of the *B. benzoevorans* related community were detected in the period between 1942 and 1950 which could be explained by the fact that in 1945 the Wieringermeer polder was re-flooded, leading to a de novo succession after renewed reclamation (91).

After the polder was reclaimed the land was used for agricultural purposes and different crops were grown. These areas are characterized by environmental conditions strictly controlled by man, leading to changes in the soil bacterial community compared with natural environments (93). Crop rotation, agricultural management as well as other soil parameters, such as soil type and seasonal changes, have found to be crucial factors for the microbial diversity (2, 38).

Rothamsted experimental grassland station

Grassland soil microbial communities are among the most intensively studied systems in the context of soil management. Different culture dependent and independent techniques were used to disclose structure and function of these communities (12, 15, 33, 60).

Rothamsted experimental station was started between 1843 and 1856. The Park Grass experiment, laid down in 1856, is the oldest of its type in Great Britain (1, 16, 50). The field had been pasture for at least a century when the experiment was started. It demonstrates in a unique way how continued application of a variety of different fertilizers affects both the botanical composition and the yield of a mixed population of grasses, clovers and other herbs. After more than 130 years, the boundaries of the plots are still clearly defined; the transition between adjacent treatments occupies 30 cm or less, showing that there is little sideways movement of nutrients in flat undisturbed soil.

The park grass was separated into experimental plots where different fertilizer and pH values were applied. These treatments led to differences in the grass cover and allowed for the development of unique ecosystems. Moreover, periodically samples were taken and stored providing a valuable archive of soil samples with traceable history.

Biogeography of soil bacterial communities

Although microorganisms are one of the most diverse (95, 100) and abundant (102) types of organism on Earth, the distribution of microbial diversity at continental scales is poorly understood. Biogeography is the study of the distribution of biodiversity over space and time (59). It aims to reveal where the organisms live, at what abundance, and why. Since the eighteenth century, biologists have investigated the geographic distribution of plant and animal diversity. More recently the geographic distribution of microorganisms has been examined. Genetic methodology has revealed that conventional culture-based studies missed most microbial diversity (35, 71, 101). With the advent of ribosomal RNA-based methods that permit the characterisation of bacterial communities without culturing (34), it is now possible to examine the full extend of microbial diversity and describe the biogeographical patterns exhibited by microorganisms at large spatial scales.

It has been known for some time that many host-associated microorganisms exhibit patterns of genetic, morphological and functional differentiation that are related to the

distribution of their host (5, 26, 72, 107). Recent studies showed that free-living microorganisms also vary in abundance, distribution and diversity over various taxonomic and spatial scales (59). One of the main conclusions of microbial biogeographical studies has been that microbial composition across a landscape is non-random. For example, Cho and Tiedje (14) showed that genetic distance between fluorescent pseudomonads was related to geographic distance. However, few studies have attempted to specify, which environmental factors exert the strongest influence on microbial communities in nature (34).

The understanding of the biogeography of soil bacteria is particularly limited, although the diversity and composition of soil bacterial communities can have a direct effect on a wide range of ecosystem processes. Now we know that soil bacterial diversity is huge (20, 96) and that the compositions of soil bacterial communities can be influenced by a wide range of biotic and abiotic factors. Fierer et al. (34) showed that the structure of soil bacterial communities is not random at the continental scale and that the diversity and composition of soil bacterial communities at large spatial scales can largely be predicted with a single variable. It has been suggested that to some degree these large-scale biogeographical patterns observed in soil microorganisms are fundamentally distinct from those observed for well studied plants and animal taxa. Although the biogeography of microorganisms remains poorly understood, and many questions are still unanswered, studying the microbial ecology can provide more comprehensive understanding of the factors influencing biodiversity and biochemistry.

Open the black boxes: methods for assessing microbial communities

Most taxonomic methods currently used to describe members of soil microbial communities usually require cultivation techniques, which are often biased and do not reflect the actual diversity of soil microorganisms *in situ*. In this way, a significant part of the soil microbiota remains inaccessible to detailed studies due to inadequate media and methods for cultivation (3, 10, 105). Recently developed biomolecular techniques to detect the diversity of nucleic acid molecules, extracted directly from soil, allow us to circumvent the need for cultivation and to make considerable progress in investigating soil microorganisms, including those problematic for cultivation (92). Molecular fingerprinting methods targeting 16S rRNA or the corresponding genes, such as denaturing or temperature gradient gel electrophoresis

(DGGE or TGGE) can be used for the rapid evaluation and comparison of composition and activity of complex microbial assemblages at moderately high temporal and spatial resolution (66).

Both, cultivation and molecular techniques have their advantages and disadvantages. While culturing allows for the recovery of microbial isolates, pivotal for more detailed taxonomic, physiological and biochemical characterization, these techniques are not representative, often very laborious, and currently catch only a small part of the soil microbiota. In contrast, culture independent techniques allow for detection of a big part of the bacterial community, including those populations inaccessible to cultivation, while isolates are not recovered. Hence, strategies that integrate both approaches provide unique opportunities for comprehensive monitoring of the soil microbiota, or selected populations of interest.

Microbial communities are pivotal to development and function of practically any environment on and even beyond the planet earth. The astounding development of molecular microbial ecological approaches targeting a variety of cellular biomarkers now allows for monitoring of community composition and functionality independent of the ability to cultivate microorganisms (43, 52). Targeted biomarkers can be any biological component that indicates the presence of a particular group of microorganisms at different levels of taxonomic resolution, and include cellular components such as surface structures, proteins, lipids, DNA or RNA. These molecules can be detected even when the cell is not alive. This enables us to study microbial communities and to follow their development under changing environmental conditions, even from historical samples, archived as air-dried specimen in the past for future analyses, or retrieved from the paleome, completely inaccessible to cultivation-based efforts (18, 47, 97). Convenient for microbial ecology is the application of small subunit ribosomal RNA (SSU rRNA; 16S rRNA for Bacteria and Archaea, 18S rRNA for Eukarya) and the corresponding genes as biomarkers. Targeting these molecules, a variety of complementary molecular techniques can be applied for monitoring of the total bacterial, archaeal or eukaryal communities, as well as specific groups of interest, in different environments. Molecular fingerprinting methods, mostly targeting the SSU rRNA gene as a universal phylogenetic marker, are frequently used in molecular microbial ecology to rapidly monitor differences in microbial community composition in space and/or time. In combination with statistical analyses, these techniques are powerful tools to investigate how changing environmental factors can influence microbial community composition (22, 69). The construction of clone

libraries from DNA or RNA directly isolated from the environmental samples can elucidate the actual microbial diversity detected by diverse fingerprinting techniques.

Gradient gel electrophoresis (DGGE, TGGE) for community profiling

Several types of gradient gel electrophoresis have been validated for the description of microbial communities in environmental samples. Most commonly used are denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE), but recently also temporal temperature gradient gel electrophoresis (TTGE). The first application of DGGE in microbial ecology was used for the profiling of bacterial diversity in a marine ecosystem (66). Gradient gel electrophoresis techniques are useful for rapidly comparing microbial communities from different environments and monitoring changes in the composition of abundant members of a specific community over time.

DGGE allows for separation of DNA fragment mixtures of equal length depending on their sequence (67). The separation is based on their sequence-specific melting point in a polyacrylamide gel with a gradient of a denaturant chemical (generally urea and formamide). Complete denaturation of the DNA fragments is prevented by addition of a so-called GC-clamp to the 5'-end of the molecule. This GC-clamp is 30- 50 bp long, and is added to one of the primers used for PCR amplification of the target gene fragment prior to DGGE analysis. In principle, a single base pair difference at any given position throughout the amplified fragment can be separated for PCR products not longer that 500 bp. Fragments with different sequences will stop migration at different positions in the denaturing gradient.

DGGE analysis of environmental samples often results in multiple band fingerprints. To identify the origin of the DGGE bands cloning and sequencing are commonly used techniques. Construction of environmental clone libraries and further sequencing, or direct sequencing of the DGGE bands can lead to actual identification of species present in the studied sample. For the screening of the different clones restriction enzyme, as well as PCR-DGGE techniques could be used.

For complete understanding of the DGGE fingerprints, as well as linking those to the environmental factors, multivariate statistical analysis is an appropriate investigation tool.

Approaches for quantitative description of soil microbial communities

- **Real Time PCR**

Real-time PCR specifically quantifies the initial amount of a target sequence, which can be either DNA, or RNA when combined with reverse transcription (often termed RT-RT-

PCR). It is a preferable alternative to other forms of quantitative PCR approaches, where the amount of the amplified product is detected at the end-point (36, 77, 89). In real-time PCR, the amplification progress is followed by measuring the increase in fluorescence emission during the reaction. This is an indicator of amplicon production during each PCR cycle as compared to the end point detection. While the real-time progress of the reaction can be viewed, the method does not allow for the detection of amplicon size. Real-time PCR offers a wide dynamic range of quantitative detection of up to 10^7-fold (compared to 1000-fold in conventional PCR), which implies that even small amounts of target can be quantified with good sensitivity and specificity.

- **Dot Blot hybridisation**

Hybridization is a technique based on binding of complementary pairs of single-stranded DNA or RNA molecules, and allows for specific and quantitative detection of target molecules, including ribosomal RNA sequences (87). Denatured DNA or RNA is transferred and attached to a sheet of nitrocellulose or derivatized nylon where the hybridization with a specific probe occurs. When the immobilized single stranded nucleic acid molecules match with the labeled probe, the newly formed double stranded molecules can be detected after removal of unbound probe by more or less stringent washing. From the intensity of the observed signal (e.g. radioactivity or fluorescence, depending on the label used) the amount of initially spotted DNA or RNA can be quantified.

Whole Genome DNA microarrays

Recent developments aiming at miniaturization and multiplexing of hybridization-based approaches have led to the development of DNA microarrays. This technology allows fast analysis of abundance and homology of a large number of genes in one single experiment, providing unprecedented insight in microbial function and diversity (54, 79, 108). Using genomic fragment-targeted probes attached to a surface, e.g. a glass-slide, one is able to detect similarities or differences in gene expression profiles under different experimental conditions, and in the genetic content between species. Depending on the size of the spotted probes, the DNA microarrays can generally be grouped in oligonucleotide-based and PCR product-based arrays. Microarray approaches comprise a high-throughput technology based on dot blot hybridization. The experiment includes preparation of the array slide, probe preparation, hybridization and data analysis. Microarray technology provides advantages in

the fast genetic and functional screening of the microbial diversity. In this way it is possible to target, study and exploit different functional genes for relevant environmental processes (54).

For phylogenetic analysis of microbial communities, primarily oligonucleotide microarrays are used (108). The oligonucleotide probes can be designed in a phylogenetic framework to survey different levels of sequence conservation, from broad taxonomic level to genus- and species-level groupings. Recently such phylogenetic microarrays have entered the field of microbial ecology as microbial diagnostic microarrays, and were primarily applied in environmental and industrial microbiology to obtain a picture of the structure of the microbial community being analysed (56). With such diversity arrays parallel detection of many microorganisms at the level of species, genus or a higher taxon is possible and they offer the potential for some level of quantification. The Human Intestinal Tract Chip (HIT Chip) is an example of the new generation diversity array containing probes for more than 1000 species. This microarray allows, in a single experiment, to identify the majority of bacterial species present in a sample. The HIT Chip also offers a possibility to follow a microorganism (or taxonomic group) during a trial and to relatively quantify the observed changes. As diversity microarray, the HIT Chip allows for extremely high throughput data analysis, and can practically be seen as a combination of different techniques, such as DGGE, cloning, sequencing and FISH (78).

Multivariate analysis as a tool for microbial diversity investigations

Multivariate analysis has widely been used in ecological research, and in the past few years it has been also applied by microbial ecologists for the description of microbial community dynamics (90). The main advantage of this statistical technique is that a large number of species and environmental factors can be evaluated together. This provides a possibility to assess changes of complex community structures corresponding to factors such as fertilisation, soil history etc. (88). Although multivariate analysis can be applied to any method that provides information on species distribution per collected sample, only recently it has been applied as a statistical tool to interpret DGGE profiles as an alternative to more traditional ways to analyse DGGE fingerprints by clustering analysis using UPGMA (unweighted pair group method with mathematical averages) (37, 58, 61).

The goal of multivariate analysis as an ordination technique is to arrange sample points in a space with reduced dimensionality (in most cases 2 dimensions) in such a way that the axes used represent the greatest variability in the community structure. The distribution of

sample points is then visualised using an ordination diagram, which is interpreted following the basic assumption that graphical proximity means close similarity (53).

In this thesis, CANOCO software was chosen (CANOCO 4.5; Biometris, Wageningen, The Netherlands) to perform the statistical analysis of DGGE profiles versus environmental variables. Redundancy analysis (37) explains the structure of the "species" data table (in this case, band intensities) by environmental variables, assuming a linear distribution of species, while canonical correspondence analysis (CCA) is used when a unimodal distribution of species can be assumed (80, 90).

Aim and outline of the thesis

Previous research has recently identified *Bacillus* spp. closely related to *B. benzoevorans* as major components of grassland microbial communities. However, knowledge on geographical distribution, as well as diversity, abundance and ecological relevance of this novel group in different soil environments were largely unknown when this study was initiated as part of a project funded by the European Union (*Exploration of Genomic and Metabolite Diversity of a Novel Group of Abundant Soil Bacteria*, BACREX-project QLK3-2000-01678). The aim of this research was therefore the monitoring of microbial communities in soils from several geographical locations, with a specific focus on qualitative and quantitative aspects of the total bacterial and the predominant *Bacillus benzoevorans* related communities.

The current chapter (**Chapter 1**) provides a state of the art description of general features of the genus *Bacillus* and particularly *B. benzoevorans* as most predominant soil bacteria as well as some soil environments which were used as study area in this research. Some general research approaches are described as well.

Chapter 2 describes cultivation approaches for *B. niacini*, member of the cluster belonging to *B. benzoevorans* relatives. Media were developed suitable for isolation of new strains.

Chapter 3 focuses on monitoring of *B. benzoevorans* relatives in different soil environments. A culture-independent PCR-DGGE approach was developed for comparing the total bacterial with the *B. benzoevorans* related community.

In **Chapter 4,** quantitative and qualitative approaches are described for comparing the total bacterial and the *B. benzoevorans* related community in historical soil samples collected from the Dutch Wieringermeer polder covering a period of 26 years.

Chapter 5 describes the results of a study on the soil eukaryotic diversity in Wieringermeer polder during the period 1942- 1975. A PCR-DGGE analysis of samples collected between 1950 and 1975 revealed significant changes in the composition of the eukaryotic microbiota, which was assigned to fungi and a variety of aerobic and anaerobic protists such as cercozoans, ciliates, xanthophytes (stramenopiles), heteroloboseans, or amoebozoans.

Two independent studies, described in **Chapter 6,** focused on the effect of soil drying and long time storage on the bacterial community. In this study soil samples from Rothamsted experimental grassland field (United Kingdom) and Wageningen experimental field (The Netherlands) were used. The effect of pH and fertilizer on the total bacterial and *B. benzoevorans* related communities was investigated as well.

Chapter 7 represents a microarray approach as high-throughput technology for the fast genetic and functional screening of the microbial diversity. Hybridization of genomic DNA of four representative strains of the group of *B. benzoevorans*- relatives, which have 16S rRNA sequence identities of 93-95 % with *B. subtilis*, yielded common and strain-specific positive signals for 1.0 to 5.6 % of ORFs targeted on the array, including those encoding proteins of yet unknown function.

Chapter 8 summarizes and discusses the general conclusions from the research described in this thesis.

References

1. 1991. Rothamsted experimental station; Guide to the classical field experiments. Lawes Agricultural Trust, Herts.
2. **Alvey, S., C.-H. Yang, A. Buerkert, and D. E. Crowley.** 2003. Cereal/legume rotation effects on rhizospere bacterial community structure in west african soils. Biol. Fertil. Soils. **37:**73-82.
3. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143-169.
4. **Anderson, I. C., and J. W. G. Cairney.** 2004. Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. Environ. Microbiol. **6:** 769-779.
5. **Bala, A., P. Murphy, and K. E. Giller.** 2003. Distribution and diversity of rhizobia nodulating agroforestry legumes in soils from three continents in the tropics. Molecular Ecology **12:**917-929.
6. **Barsdate, R. J., R. T. Prentki, and T. Fenchel.** 1974. Phosphorus cycle of model ecosystem: significance for decomposer food chains and effect of bacterial grazers. Oikos **25:**239-251.
7. **Bergquist, P. L., and H. W. Morgan.** 1992. The molecular genetics and biotechnological application of enzymes from extremely thermophilic eubacteria, p. 44–75. *In* R. A. Herbert and R. J. Sharp (ed.), Molecular Biology and Biotechnology of Extremophiles. Chapman & Hall, New York.
8. **Blume, E., M. Bischoff, T. B. Moorman, and R. F. Turco.** 2004. Degradation and binding of atrazine in surface and subsurface soils. J. Agric. Food. Chem. **52:**7382- 7388.
9. **Bonkowski, M.** 2004. Protozoa and plant growth: the microbial loop in soil revisited. New Phytologist **162:**617-631.
10. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:**1935-1943.
11. **Borneman, J., and E. W. Triplett.** 1997. Molecular microbial diversity in soils from Eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Environ. Microbiol. **63:**2647-2653.
12. **Buckley, D. H., and T. M. Schmidt.** 2003. Diversity and dynamics of microbial communities in soils from agro-ecosystems. Environ. Microbiol. **5:**441-452.
13. **Chaudhry, Q., M. Blom-Zandstra, S. Gupta, and E. J. Joner.** 2005. Utilising the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment. Environ. Sci. Pollut. Res. Int. **12:**34-48.
14. **Cho, J. C., and J. M. Tiedje.** 2000. Biogeography and degree of endemicity of fluorescent *Pseudomonas* strains in soil. Appl. Environ. Microbiol. **66:**5448-5456.
15. **Clegg, C. D., R. D. L. Lovell, and P. J. Hobbs.** 2003. The impact of grassland management regime on the community

structure of selected bacterial groups in soils. FEMS Microbiol. Ecol. **43:**263-270.

16. **Crawley, M. J., A. E. Johnston, J. Silvertown, M. Dodd, C. de Mazancourt, M. S. Heard, D. F. Henman, and G. R. Edwards.** 2005. Determinants of species richness in the park grass experiment. Am. Nat. **165:**179-192.
17. **Dawson, S. C., and N. R. Pace.** 2002. Novel kingdom-level eukaryotic diversity in anoxic environments. Proc. Nat. Acad. Sci. USA **99:**8324-8329.
18. **Dolfing, J., A. Vos, J. Bloem, P. A. I. Ehlert, N. B. Naumova, and P. J. Kuikman.** 2004. Microbial diversity in archived soils. Science **306:**813.
19. **Dong, Y. H., A. R. Gusti, Q. Zhang, J. L. Xu, and L. H. Zhang.** 2002. Identification of quorum-quenching N-acyl homoserine lactone enzymes from *Bacillus* species. Appl. Environ. Microbiol. **68:**1754-1759.
20. **Dunbar, J., S. M. Barns, L. O. Ticknor, and C. R. Kuske.** 2002. Empirical and theoretical bacterial diversity in four Arizona soils. Appl. Environ. Microbiol. **68:**3035-3045.
21. **Edgcomb, V. P., D. T. Kysela, A. Teske, A. de Vera Gomez, and M. L. Sogin.** 2002. Benthic eukaryotic diversity in the Guaymas Basin hydrothermal vent environment. Proc.Nat. Acad. Sci. USA **99:**7658-7662.
22. **Egert, M., S. Marhan, B. Wagner, S. Scheu, and M. W. Friedrich.** 2004. Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* L. (Oligochaeta: *Lumbricidae*). FEMS Microbiol. Ecol. **48:**187-197.
23. **Ekelund, F., and R. Rønn.** 1994. Notes on protozoa in agricultural soil with emphasis on heterotrophic flagellates and naked amoebae and their ecology. FEMS Microbiol. Rev. **15:**321-353.
24. **Emmert, E. A. B., and J. Handelsman.** 1999. Biocontrol of plant disease: a (Gram-) positive perspective. FEMS Microbiol. Lett. **171:**1-9.
25. **Eyers, L., I. George, L. Schuler, B. Stenuit, S. N. Agathos, and S. E. Fantroussi.** 2004. Environmental genomics: exploring the unmined richness of microbes to degrade xenobiotics. Appl. Microbiol. Biotechnol. **66:**123-130.
26. **Falush, D., T. Wirth, B. Linz, J. K. Pritchard, M. Stephens, M. Kidd, M. J. Blaser, D. Y. Graham, S. Vacher, G. I. Perez-Perez, Y. Yamaoka, F. Mégraud, K. Otto, U. Reichard, E. Katzowitsch, X. Wang, M. Achtman, and S. Suerbaum.** 2003. Traces of human migrations in Helicobacter pylori populations. Science **299:**1582-1585.
27. **Fang, C., P. Smith, J. B. Moncrieff, and J. U. Smith.** 2005. Similar response of labile and resistant soil organic matter pools to changes in temperature. Nature **433:**57- 59.
28. **Felske, A.** 1999. Reviewing the DA001-files: A 16S rRNA chase on suspect #X99967, a *Bacillus* and Dutch underground activist. J. Microbiol. Meth. **36:**77-93.
29. **Felske, A., and A. D. L. Akkermans.** 1998. Spatial homogeneity of the most abundant bacterial 16S rRNA molecules in grassland soils. Microbiol. Ecol. **36:**31-36.

30. **Felske, A., A. D. L. Akkermans, and W. M. de Vos.** 1998. In situ detection of an uncultured predominant *Bacillus* in Dutch grassland soils. Appl. Environ. Microbiol. **64:**4588-4590.

31. **Felske, A., A. Wolterink, R. van Lis, and A. D. L. Akkermans.** 1998. Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). Appl. Environ. Microbiol. **64:**871-879.

32. **Felske, A., A. Wolterink, R. van Lis, W. M. de Vos, and A. D. L. Akkermans.** 1999. Searching for predominant soil bacteria: 16S rDNA cloning versus strain cultivation. FEMS Microbiol. Ecol. **30:**137-145.

33. **Felske, A. D. M., V. A. Tzeneva, J. Heyrman, M. A. Langeveld, A. D. L. Akkermans, and P. De Vos.** 2004. Isolation and biodiversity of hitherto undescribed soil bacteria related to *Bacillus niacini*. Microbiol. Ecol. **48:**111-119.

34. **Fierer, N., and R. B. Jackson.** 2006. The diversity and biogeography of soil bacterial communities. Proc. Natl. Acad. Sci. USA **103:**626-631.

35. **Floyd, M. M., J. Tang, M. Kane, and D. Emerson.** 2005. Captured diversity in a culture collection: case study of the geographic and habitat distributions of environmental isolates held at the American type culture collection. Appl. Environ. Microbiol. **71:**2813-2823.

36. **Freeman, W. M., S. J. Walker, and V. K. E.** 1999. Quantitative RT-PCR:pitfalls and potential. Biotechniques **26:**112-122.

37. **Fromin, N., J. Hamelin, S. Tarnawski, D. Roesti, K. Jourdain-Miserez, N. Forestier, S. Teyssier-Cuvelle, F. Gillet, M. Aragno, and P. Rossi.** 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ. Microbiol. **4:**634-643.

38. **Garbeva, P., J. A. van Veen, and J. D. van Elsas.** 2004. Microbial diversity in soil: Selection of microbial populations by plant and soil type and implications for disease supperssiveness. Ann. Rev. Phytopath. **42:**243-270.

39. **Garbeva, P., J. A. van Veen, and J. D. van Elsas.** 2003. Predominant *Bacillus* spp. in agricultural soil under different management regimes detected via PCR-DGGE. Microbial Ecology **45:**302-316.

40. **Gardener, B. B. M.** 2004. The nature and application of biocontrol microbes: *Bacillus* spp.- Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems. Phytopathology **94:**1252-1257.

41. **Giacomodonato, M. N., M. J. Pettinari, I. S. Guadalupe, S. M. Beatriz, and N. I. Lopez.** 2001. A PCR-based method for the screening of bacterial strains with antifungal activity in suppressive soybean rhizosphere. World Journal of Microbiology and Biotechnology **17:**51-55.

42. **Grayston, S. J., C. D. Campbell, R. D. Bardgett, J. L. Mawdsley, C. D. Clegg, K. Ritz, B. S. Griffiths, J. S. Rodwell, S. J. Edwards, and W. J. Davies.** 2004. Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques. Applied Soil Ecology **25:**63.

43. **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. Microbiol. Ecol. **35:**1.

44. **Hecker, M., W. Schumann, and U. Volker.** 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol. Microbiol. **19:**417-428.

45. **Heyrman, J., B. Vanparys, N. A. Logan, A. Balcaen, M. Rodriguez-Diaz, A. Felske, and P. De Vos.** 2004. *Bacillus novalis sp.* nov., *Bacillus vireti sp.* nov., *Bacillus soli sp.* nov., *Bacillus bataviae sp.* nov. and *Bacillus drentensis sp.* nov., five new species isolated from the Drentse A grasslands. Int. J. Syst. Evol. Microbiol. **54:**47-57.

46. **Holt, J. G.** 1986. Bergey's manual of systematic bacteriology, vol. 2. Williams & Wilkins, Baltimore.

47. **Inagaki, F., H. Okada, A. I. Tsapin, and K. H. Nealson.** 2005. Microbial survival: the paleome: a sedimentary genetic record of past microbial communities. Astrobiology **5:**141-153.

48. **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.

49. **Johannes, R. E.** 1965. Influence of marine protozoa on nutrient regeneration. Limnology and Oceanography **10:**434-442.

50. **Kunin, W. E.** 1998. Biodiversity at the edge: a test of the importance of spatial "mass effects" in the Rothamsted Park Grass experiments. Proc. Natl. Acad. Sci. USA **95:**207-212.

51. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, N. J. Cummings, R. A. Daniel, F. Denizot, K. M. Devine, A. Dusterhoft, S. D. Ehrlich, P. T. Emmerson, K. D. Entian, J. Errington, C. Fabret, E. Ferrari, D. Foulger, C. Fritz, M. Fujita, Y. Fujita, S. Fuma, A. Galizzi, N. Galleron, S. Y. Ghim, P. Glaser, A. Goffeau, E. J. Golightly, G. Grandi, G. Guiseppi, B. J. Guy, K. Haga, J. Haiech, C. R. Harwood, A. Henaut, H. Hilbert, S. Holsappel, S. Hosono, M. F. Hullo, M. Itaya, L. Jones, B. Joris, D. Karamata, Y. Kasahara, M. Klaerr-Blanchard, C. Klein, Y. Kobayashi, P. Koetter, G. Koningstein, S. Krogh, M. Kumano, K. Kurita, A. Lapidus, S. Lardinois, J. Lauber, V. Lazarevic, S. M. Lee, A. Levine, H. Liu, S. Masuda, C. Mauel, C. Medigue, N. Medina, R. P. Mellado, M. Mizuno, D. Moestl, S. Nakai, M. Noback, D. Noone, M. O'Reilly, K. Ogawa, A. Ogiwara, B. Oudega, S. H. Park, V. Parro, T. M. Pohl, D. Portetelle, S. Porwollik, A. M. Prescott, E. Presecan, P. Pujic, B. Purnelle, et al.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249-256.

52. **La Duc, M. T., R. Kern, and K. Venkateswaran.** 2004. Microbial

monitoring of spacecraft and associated environments. Microbiol. Ecol. **47:**150.

53. **Leps, J., and P. Smilauer.** 2003. Multivariate analysis of ecological data using CANOCO. University Press, Cambridge.

54. **Liu, W.-T., and l. Zhu.** 2005. Environmental microbiology-on-a-chip and its future impacts. TIBTECH **23:**174-179.

55. **Lopez-Garcia, P., F. Rodriguez-Valera, C. Pedros-Alio, and D. Moreira.** 2001. Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature **409:**603-607.

56. **Loy, A., and L. Bodrossy.** 2006. Highly parallel microbial diagnostics using oligonucleotide microarrays. Clinica Chimica Acta **363:**106-119.

57. **Macrae, A., C. M. M. Lucon, D. L. Rimmer, and A. G. O'Donnell.** 2001. Sampling DNA from the rhizosphere of *Brassica napus* to investigate rhizobacterial community structure. Plant Soil **233:**223-230.

58. **Marschner, P., and K. Baumann.** 2003. Changes in bacterial community structure induced by mycorrhizal colonisation in split-root maize. Plant Soil **251:**279-289.

59. **Martiny, J. B. H., B. J. M. Bohannan, J. H. Brown, R. K. Colwell, J. A. Fuhrman, J. L. Green, M. C. Horner-Devine, M. Kane, J. A. Krumins, C. R. Kuske, P. J. Morin, S. Naeem, L. Øvreås, A.-L. Reysenbach, S. V. H., and J. T. Staley.** 2006. Microbial biogeography: putting microorganisms on the map. Nature Reviews Microbiology **4:**102-112.

60. **McCaig, A. E., L. A. Glover, and J. I. Prosser.** 2001. Numerical analysis of grassland bacterial community structure under different land management regimes by using 16S ribosomal DNA sequences data and denaturing gradient gel electrophoresis banding patterns. Appl. Environ. Microbiol. **67:**4554-4559.

61. **McCaig, A. E., S. J. Graystone, J. I. Prosser, and L. A. Glover.** 2001. Impact of cultivation on characterisation of species composition of soil bacterial communities. FEMS Microbiol. Ecol. **35:**37-48.

62. **Molenaar, D., F. Bringel, F. H. Schuren, W. M. de Vos, R. J. Siezen, and M. Kleerebezem.** 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. Journal of Bacteriology **187:**6119-6127.

63. **Moon-van der Staay, S. Y., R. de Wachter, and D. Vaulot.** 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nat. Biotechnol. **409:**607-610.

64. **Moon-van der Staay, S. Y., V. A. Tzeneva, G. W. M. van der Staay, W. M. de Vos, H. Smidt, and J. H. P. Hackstein.** 2006. Eukaryotic diversity in historical soil samples. FEMS Microbiol. Ecol. **in press**.

65. **Moon-van der Staay, S. Y., G. W. M. van der Staay, L. Guillou, H. Claustre, L. K. Medlin, and D. Vaulot.** 2000. Abundance and diversity of Prymnesiophyceae in the picoplankton community from the equatorial Pacific ocean inferred from 18S rDNA sequences. Limnol. Oceanogr. **45:**98-109.

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

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

68. **Nagel, M., and J. R. Andreesen.** 1991. *Bacillus niacini* sp. nov., a nicotinate-metabolizing mesophile isolated from soil. Int. J. Syst. Bacteriol. **41:**134-139.

69. **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. Microbiol. **7:**382-395.

70. **Norris, J. R., R. C. W. Berkeley, N. A. Logan, and A. G. O'Donnell.** 1981. The genera *Bacillus* and *Sporolactobacillus*, p. 1711-1742. *In* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria, vol. 2. Springer-Verlag, Berlin, Heidelberg, New-York, Tokyo.

71. **Øvreås, L.** 2000. Population and community level approaches for analysing microbial diversity in natural environments. Ecology Letters **3:**236-251.

72. **Papke, R. T., and D. M. Ward.** 2004. The importance of physical isolation to microbial diversification. FEMS Microbiol. Ecol. **48:**293-303.

73. **Pichinoty, F., and J. Asselineau.** 1984. Morphology and cytology of *Bacillus benzoevorans*, a sheated mesophilic species which degrades various aromatic acids and phenols. Annual Microbiology **135:**199-207.

74. **Pichinoty, F., J. Asselineau, and M. Mandel.** 1984. Biochemical characterization of *Bacillus benzoevorans sp.* nov., a new filamentous, sheated mesophilic species which degrades various aromatic acids and phenols. Annual Microbiology **135:**209-217.

75. **Pleban, S., and J. Sörensen.** 1996. Multi-target and medium- independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. FEMS Microbiol. Ecol. **22:**183-192.

76. **Radnedge, L., P. G. Agron, K. K. Hill, P. J. Jackson, L. O. Ticknor, P. Keim, and G. L. Andersen.** 2003. Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. Appl. Environ. Microbiol. **69:**2755-2764.

77. **Raeymaekers, L.** 2000. Basic principles of quantitative PCR. Molecular Biotechnology **15:**115-122.

78. **Rajlilić-Stojanović, M., H. G. Heilig, D. Molenaar, H. Smidt, E. G. Zoetendal, and W. M. de Vos.** HITChip - the microarray for profiling and quantification of the Human Intestinal Tract microbiota. **in preparation**.

79. **Rick, W. Y., L. Wang, L. Bedzyk, and K. Croker.** 2001. Application of DNA microarrays in microbial systems. J. Microbiol. Meth. **47:**257-272.

80. **Salles, J. F., J. A. van Veen, and J. D. van Elsas.** 2004. Multivariate analyses of *Burkholderia* species in soil: effect of crop and land use history. Appl. Environ. Microbiol. **70:**4012-4020.

81. **Schnurer, J., M. Clarholm, S. Bostrom, and T. Rosswall.** 1986. Effect of moisture on soil microorganisms and nematodes: a field experiment. Microbiol. Ecol. **12:**217-230.

82. **Smidt, H., A. D. L. Akkermans, J. van der Oost, and W. M. de Vos.** 2000. Halorespiring bacteria-molecular characterization and detection. Enzyme Microbiol. Technol. **27:**812.

83. **Smidt, H., and W. M. de Vos.** 2004. Anaerobic microbial dehalogenation. Annu. Rev. Microbiol. **58:**43-73.

84. **Smits, H.** 1962. De fysische rijping der gronden in de IJsselmeerpolders (The physical ripening of the soils in the IJssel-lake polders). Van Zee Ld. **32 (7)**.

85. **Somers, E., J. Vanderleyden, and M. Srinivasan.** 2004. Rhizosphere bacterial signalling: a love parade beneath our feet. Crit. Rev. Microbiol. **30:**205-240.

86. **Spharga, B. M., and G. D. Lyon.** 1998. *Bacillus subtilis* BS107 as an antagonist of potato blackleg and soft rot bacteria. Can. J. Micro. **44:**777-783.

87. **Stahl, D. A.** 1995. Application of phylogenetically-based probes to microbial ecology. Molecular Ecology **4:**535-542.

88. **Steenwerth, K. L., L. E. Jackson, F. J. Calderon, M. R. Stromberg, and K. M. Scow.** 2002. Soil microbial community composition and land use history in cultivated and grassland ecosystems of coastal California. Soil Biol. Biochem. **34:**1599-1611.

89. **Suzuki, M. T., L. T. Taylor, and E. F. DeLong.** 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. Appl. Environ. Microbiol. **66:**4605-4614.

90. **ter Braak, C. J. F.** 1987. The analysis of vegetation-environment relationships by canonical correspondence analysis. Vegetatio **69:**69-77.

91. **Terpstra, P.** 1979. 50 jaar Wieringermeer. M. A. van Seijen-Leeuwarden, The Netherlands.

92. **Tiedje, J. M., S. Asuming-Brempong, K. Nusslein, T. L. Marsh, and S. J. Flynn.** 1999. Opening the black box of soil microbial diversity. Applied Soil Ecology **13:**109-122.

93. **Torsvik, V., F. L. Daae, R. A. Sandaa, and L. Ovreas.** 1998. Novel techniques for analysing microbial diversity in natural and perturbed environments. J Biotechnol **64:**53-62.

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

95. **Torsvik, V., L. Øvreås, and T. F. Thingstad.** 2002. Prokaryotic diversity-magnitude, dynamics, and controlling factors. Science **296:**1064-1066.

96. **Tringe, S. G., C. von Mering, A. Kobayashi, A. A. Salamov, K. Chen, H. W. Chang, M. Podar, J. M. Short, E. J. Mathur, J. C. Detter, P. Bork, P. Hugenholtz, and E. M. Rubin.** 2005. Comparative metagenomics of microbial communities. Science **308:**554-557.

97. **Tzeneva, V. A., Y. Li, A. D. M. Felske, W. M. de Vos, A. D. L. Akkermans, E. E. Vaughan, and H. Smidt.** 2004. Development and application of a selective PCR-Denaturing Gradient Gel Electrophoresis approach to detect a recently cultivated *Bacillus* group predominant in soil. Appl. Environ. Microbiol. **70:**5801-5809.

98. **van Leeuwenhoek, A.** 1677. Philosophical Transactions.

99. **van Schreven, D. A., and G. W. Harmsen.** 1968. Soil bacteria in relation to the development of polders in the region of the former Zuider zee. Liverpool Univerity Press, United Kingdom.

100. **Venter, J. C., K. Remington, J. F. Heidelberg, A. L. Halpern, D. Rusch, J. A. Eisen, D. Wu, I. Paulsen, K. E. Nelson, W. Nelson, D. E. Fouts, S. Levy, A. H. Knap, M. W. Lomas, K. Nealson, O. White, J. Peterson, J. Hoffman, R. Parsons, H. Baden-Tillson, C. Pfannkoch, Y.-H. Rogers, and H. O. Smith.** 2004. Environmental genome shotgun sequencing of the Sargasso sea. Science **304:**66-74.

101. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S ribosomal-RNA sequences reveal numerous uncultured microorganisms in a natural community. Nature **345**.

102. **Whitman, W. B., D. C. Coleman, and W. J. Wiebe.** 1998. Prokaryotes: The unseen majority. Proc. Natl. Acad. Sci. USA **95:**6578-6583.

103. **Wipat, A., and C. R. Harwood.** 1999. The Bacillus subtilis genome sequence: the molecular blueprint of a soil bacterium. FEMS Microbial Ecology **28:**1-9.

104. **Yoon, J.-H., S.-S. Kang, K.-C. Lee, Y. H. Kho, S. H. Choi, and K. H. P. Kang, Y.-H.** 2001. *Bacillus jeotgali* sp. nov., isolated from jeotgal, Korean traditional fermented seafood. Int. J. Syst. Evol. Microbiol. **51:**1087-1092.

105. **Zengler, K., G. Toledo, M. Rappe, J. Elkins, E. J. Mathur, J. M. Short, and M. Keller.** 2002. Cultivating the uncultured. Proc. Natl. Acad. Sci. USA **99:**15681-15686.

106. **Zettler, L. A., F. Gómez, E. Zettler, B. G. Keenan, R. Amils, and M. L. Sogin.** 2002. Eukaryotic diversity in Spain's River of Fire. Nature **417:**137.

107. **Zhang, N., and M. Blackwell.** 2002. Population structure of dogwood anthracnose fungus. Phytopathology **92:**1276-1283.

108. **Zhou, J.** 2003. Microarrays for bacterial detection and microbial community analysis. Curr. Opin. Microbiol. **6:**288-294.

109. **Zuur, A. J.** 1962. De chemische rijping der gronden in de IJsselmeerpolders (The chemical ripening of the soils in the IJssel-lake polders). Van Zee Ld. **32:**24-30.

CHAPTER 2

ISOLATION AND BIODIVERSITY OF HITHERTO UNDESCRIBED SOIL BACTERIA RELATED TO *BACILLUS NIACINI*

Andreas D.M. Felske, Vesela A. Tzeneva, Jeroen Heyrman, Maaike A. Langeveld, Antoon D.L. Akkermans and Paul De Vos

Molecular Ecology (2004) vol. 48 p. 111-119

Abstract

The hitherto largely not described phylogenetic neighborhood of *Bacillus niacini* has been explored by a comprehensive cultivation experiment and genomic variety studies. Previous culture-independent studies demonstrated that 15 % of all *Bacillus* 16S rDNA directly extracted from soils worldwide was affiliated to *B. niacini*. Seven different media were inoculated with soil suspensions in serial dilutions and incubated at different temperatures. Then, bacterial colonies were picked and analyzed by sequencing. A mineral medium with acetate as carbon source yielded a *B. niacini* rate of >3 % of all picked colonies. Other media were less efficient but also successful. Applying this culturing approach, we succeeded in obtaining 64 isolates from different Dutch soils. The isolates turned out to be diverse, although closely related to *B. niacini* as revealed by 16S rDNA sequencing. Close matches with environmental clones were also found, thus demonstrating much more diversity beyond previously known 16S rDNA sequences. The rep-PCR fingerprinting method revealed a high genomic variety, redundancy could not be observed among our isolates. Hence, the hitherto neglected *B. niacini* lineage, apparently among the most abundant soil Bacillus, was accessible to our cultivation approach.

Introduction

Isolation of the hitherto uncultured predominant soil bacteria has become a major concern in soil microbiology. The prominent appearance of hitherto neglected lineages like *Acidobacteria* (20) in culture-independent approaches indicated an extreme bias between the strains deposited in culture collections and the truly abundant environmental bacteria. Even the genus *Bacillus*, one of the best-studied soil bacteria taxa, still harbors a considerable amount of not yet explored lineages, including the most abundant soil *Bacillus* members. The main target of this study was a hitherto uncultured abundant *Bacillus* cluster of the rRNA group I (2), related to *B. niacini*. This cluster was first recognized by Hengstmann et al. (16) and at that time called the ‘‘*Bacillus pseudomegaterium*’’ cluster (referring to the neither validly described nor further studied strain ATCC 49866). However, the recently sequenced 16S rDNA (14) of the species *Bacillus niacini* (23) joined this cluster and represented therefore the first validly described species within, and consequently this species was used here to name this cluster. A few dozen other 16S rDNA sequences of uncultured bacteria from Europe and America also joined this group (Table 1). Nowadays, this cluster accounts for 15 % of all uncultured *Bacillus* 16S rDNA clones from directly extracted soil DNA. This apparent relative abundance of the *B. niacini* relatives in soil was also confirmed by a more specific culture-independent survey on agricultural soils focusing on *Bacillus* (12). Recently, closely related isolates from very different environments became public, giving a first hint of the ecological variety within this cluster. Ando et al. (1) isolated a hitherto unknown species from a Japanese mangrove swamp (‘‘*B. aestuarii*’’), while Logan et al. (19) described the species *B. fumarioli* from geothermal environments in Antarctica. Janssen et al. (18) could grow one member of this group (their isolate Ellin411 scored 96.5% 16S rDNA sequence similarity with *B. niacini*) from agricultural soil by improving the efficacy of their cultivation approach, apparently getting much closer to growing the truly abundant soil bacteria.

Soil DNA-based study	*16S rDNA sequences*			*Reference*
	Bacteria	*Bacillus*	*B. niacini*	
Clover- grass pasture, Wisconsin	180	31	7	(4)
Four aired soil, Arizona	203	12	4	(7)
Rice paddy soil microcosms, Germany	57	13	8	(16)
Brassica napus rhizosphere, UK	73	20	7	(21)
Anoxic paddy soil, Germany	31	3	2	(38)
Agricultural fields, California	100	23	5	(34)

Table 1. Abundance of *Bacillus niacini* relatives in several culture-independent 16S rDNA-based studies on microbial communities in soil.

Here we report on our approach to grow a more comprehensive number of isolates from this cluster in pure culture. The starting point was the Drentse A area, a grassland with a soil bacteria community that previously proved to harbor a large fraction of hitherto uncultured *Bacillus* lineages (10). The applied media were chosen following the experiences of a former, not exactly successful cultivation attempt of hitherto uncultured bacteria in the Drentse A grasslands (11), and from the studies published by Mitsui et al. (22) and Janssen et al. (18). The premise of our strategy was to try various medium compositions and growth temperatures and to recover all growing colonies from petri dishes (15). Consequently, an extensive enrichment experiment and subsequent analysis of thousands of isolates was used to access this hitherto neglected group of abundant soil *Bacillus* spp.

Materials and Methods

Soil sampling and sites

The soil samples for this study can be divided in two main groups: (i) fresh soil samples recently collected and stored at 4 °C, and (ii) air-dried samples collected many years ago from different locations. The locations and soil characteristics of fresh soil samples were as follows: Drentse A agricultural research area in the Netherlands (06410E, 53030N), representing a 1.5 km stretch of grassland along the Anlooër Diepje Brook (details of the soil properties have been published elsewhere; (32)), and Hoeksma and Sikkema grassland farms, Friesland, The Netherlands. Soil cores of 50 g were taken with a drill from the upper 25 cm and transferred into sterile sample bags. Samples from each plot were pooled to representative samples by sieving and mixing equal amounts.

The air-dried soil samples were Wageningen University experimental field (05580E,

52010N), (sandy soil, 0–25 cm) and samples from the TAGA archive of air-dried soil samples (ALTERRA B.V., Wageningen, The Netherland). Of the collection, samples were investigated from the top layer (0–25 cm) of non fertilized areas of an agricultural field in the Wieringermeer polder, The Netherlands (05010E, 52810N). Land reclamation by drainage of this former sea bottom was started in 1930 and completed in 1940, followed by transformation into agricultural lands (35). Samples from no different sites were analyzed that were taken in 1942, 1950, 1951, 1966, 1973, and 1975.

Enrichment of soil bacteria

Soil material (1 g) was suspended in sterile PBS buffer and diluted in 10-fold steps. Different agar media were inoculated with 100 µl of these suspensions, corresponding to 10^{3} –10^{7} g soil per plate. The two different dilute nutrient agar of Mitsui et al. (22) were used for enrichment: DNB (0.08 g Difco nutrient broth; BD Diagnostic Systems, Sparks, MD) and medium BYPcng (0.5 g each of Bacto beef extract, Bacto yeast extract, Bacto peptone, 0.2 g casamino acids, 0.1 g NaCl, and 0.4 g glucose, pH 7.0). All amounts are per 1 l. The mineral media (27) were composed as follows: 3.575 g $Na_2HPO_4.12H_2O$, 0.98 g KH_2PO_4, 0.5 g NH_4Cl, 0.03 g $MgSO_4.7H_2O$, 200 µl trace element solution (50 g EDTA, 2.2 g $ZnSO_4.7H_2O$, 5.54 g $CaCl_2$, 5.06 g $MnCl_2.4H_2O$, 4.79 g $FeSO_4.7H2O$, 1.1 g $NH_4MO_7O_{24}.4H_2O$, 1.57 g $CuSO_4.5H_2O$, 1.6 g $CoCl_2.6H_2O$, 0.05 g H_3BO_3; distilled water, adjusted to pH 6.0 with KOH), plus additional defined carbon sources, i.e., 2 g acetate or benzoate, plus 1 g yeast extract. An additional version of the acetate medium was prepared without yeast extract. All agar media contained 15 g agar and 50 mg cycloheximide to reduce fungal growth and had a pH of 7. The inoculated petri dishes were incubated for several weeks at 4 °C, room temperature (21 °C), 30 °C, 37 °C, or 40 °C in tin boxes together with a beaker of water (to prevent drying of the agar).

Sampling and screening of colonies and further cultivation

After incubation for 2 weeks, 4 weeks, and 6 weeks, colonies from the agar plates inoculated with soil suspensions were picked with sterile toothpicks. Only tiny bits of biomass (1 mm^{3} or less) were transferred to 96-well microtiter plates loaded with 100 µl sterile distilled water in each well. The toothpick tips with the picked colonies were scratched to the walls of the wells and then they were pricked a few times into 25-compartiment petri dishes filled with DSMZ1 agar medium. These petri dishes were placed at room temperature for intermediate storage. All picked colonies and their according subcultures were named

‘‘IDA’’ plus a four-digit serial number (‘‘IDA’’ is a code for our culture collection; its original meaning is ‘‘Isolates Drentse A’’). All the further subculture was done with the rich nutrient broth DSMZ medium 1, a standard medium for Bacillus cultures (6). The microtiter plates with the colony samples in water were frozen at 20 ºC for cell lysis and melted again before use. These microtiter plates with cell lysates were used for a multiplex PCR screening as described previously (8). Positive samples were forwarded to sequencing.

Sequence analysis of PCR products from isolates

Amplification of 16S rDNA sequences was performed with a GeneAmp PCR System 9700 thermocycler (PerkinElmer-Cetus, Norwalk, CT) using 35 cycles of 94 °C for 10 s, 50 °C for 20 s, and 68 °C for 90 s. The PCR reactions (50 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 1.5% polyvinylpyrrolidone K25, 150 µM each of dATP, dCTP, dGTP, and dTTP, 100 pmol each of universal bacteria primers t-BACf and t-UNI1493r (9), 2.5 units of Taq DNA polymerase (Qiagen, Hilden, Germany), and 2 µl cell lysate (see above). PCR products were purified and concentrated (from 50 to 30 µl) with glass fiber spin columns following the manufacturer’s instructions (NucleoSpin kit: Macherey-Nagel, Du¨ren, Germany). Purified DNA was eluted from the columns with 30 µl deionized water. The sequencing was done externally via the services of GATC (Konstanz, Germany). Phylogenetic analysis of the sequences was performed with the ARB software (33). The ARB package is a combination of alignment and dendrogram tools, allowing alignments to a comprehensive SSU rDNA database and detailed phylogenetic analysis. A maximum likelihood tree was generated with the implemented ARB tools to generate a basic tree with almost complete 16S rDNA sequences from the few proposed species among the *B. niacini* relatives and some more almost complete sequences of high quality from not yet described isolates and culture-independent surveys. A second tree had been constructed in the same way, now including all sequences from this study and also short sequences from other authors. A few sequences from culture-independent surveys had to be neglected due to their very short length or bad quality. The secondary structure of the *B. niacini* 16S rRNA has been reconstructed according to the structure proposed by Stern et al. (31). Comparing the sequences of all *B. niacini* relatives, the variable nucleobase positions among them were identified. Comparing this consensus result to other *Bacillus* spp. sequences, the nucleobase positions conserved among all *B. niacini* relatives but variable among other *Bacillus* spp. were identified. These conserved *B. niacini* positions were used to generate short sequences of 10–15 bp and checked back with the ARB probe match tool against a collection of 1000

16S rDNA sequences from other *Bacillus* spp. and a collection of 2231 non-*Bacillus* prokaryote 16S rDNA sequences, respectively. The number of hits was used to estimate the dissemination of these nucleobase variations among the genus *Bacillus* and prokaryotes in general. The sequences of our 64 isolates can be found in the EMBL database by their accession numbers AJ542505–AJ542515 and AJ563486– AJ563538.

Rep-PCR genomic fingerprinting

The Rep-PCR was performed with the (GTG)5 primer (37) using the PCR-conditions as previously described by Rademaker and de Bruijn (28). For each strain, 6 µl PCR product mixed with 2 µl loading buffer (28) was used for electrophoresis in a 1.5 % (w/v) agarose gel and TAE buffer (1.21 g l^{-1} Tris 2-amino-2-(hydroxymethyl)-1,3 propanediol, 0.2 ml l^{-1} 0.5 M EDTA, pH 8) for 15 h at a constant 55 V and 4 °C. The first and every sixth lane were loaded with 6 µl of the molecular ruler [45.5 % (v/v) 100bp ruler (BioRad), 36.5 % (v/v) 500-bp ruler (BioRad), and 18 % (v/v) loading buffer]. After staining with ethidium bromide (0.5 µg ml^{-1}), the patterns were digitalized and a Pearson correlation of the resulting band patterns was performed using the BioNumerics 2.0 Software (Applied Maths, Sint-Martens-Latem, Belgium).

Results

Enrichment and cultivation of strains

The enrichment of novel *B. niacini* relatives was performed on five different agar media compositions and all succeeded (Fig. 1). The average total cfu yield of the amended

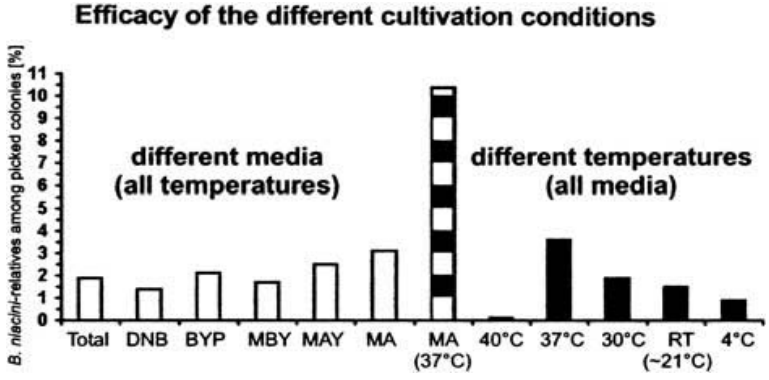


Figure 1. Influence of different media and cultivation tempera-tures on the enrichment efficacy for B. niacini relatives. Abbrevi-ations for the media, DNB: dilute nutrient broth; BYP: the other dilute nutrient broth BYPcng; MBY: mineral medium plus ben-zoate and yeast extract; MAY: mineral medium plus acetate and yeast extract; MA: mineral medium plus acetate.

mineral media was in the range of 10^6 cfu g^{-1} soil; the dilute complex media reached 10^7 cfu g^{-1} soil. On average, almost 2 % of all picked colonies were revealed to be a close *B. niacini*-relative. However, the carbon source acetate yielded the highest rates, up to >3 % for the mineral medium with acetate but without yeast extract. Of the five different growth temperatures, enrichment at 37 °C was the most effective one. Hence, the enrichment on mineral medium with acetate at 37 °C should be the most promising combination. Indeed, the rate was increased to 10.4 % *B. niacini* relatives among all picked colonies. Positive colonies were picked at different sampling times, indicating different lag times and growth rates among our *B. niacini* relatives. However, most strains grew easily and fast on standard nutrient broth DSMZ1 at room temperature. Here, from all 64 identified strains, 30 formed colonies of >1 mm diameter within 2 days, 22 required up to 1 week, and 12 required >1 week. Only one strain failed to grow in subculture; another one was lost to contamination. Growth speed often increased during further subculture. Additional testing of the strains (17) showed that they are all facultative anaerobes and have a pH optimum between 7 and 8. Gram stain and cell morphology is variable among the strains.

The 16S rDNA analysis

The identified *B. niacini* relatives constituted a well-defined cluster within the 16S rDNA phylogeny of *Bacillus*. They belong to the *Bacillus* 16S rDNA group I (2) and group in the proximity of *B. niacini* and *B. fumarioli*. Quite a number of the strains mentioned in this study could already be used to delineate and describe five species (17): *B. novalis*, *B. vireti*, *B. soli*, *B. bataviensis*, and *B. drentensis*. They constituted the clustering standard for 16S rDNA sequence analysis (Fig. 2a) of a group which is possibly much more diverse and rich in species numbers than indicated by our or any previous study. In a FASTA search (26), the type strains of these five newly described species showed 16S rDNA sequence similarities to *B. niacini* (AB021194) of 97.8– 98.2 % and to *B. fumarioli* (AJ210056) of 96.7– 97.4 %. Most of the remaining strains could be affiliated with the five newly described species, but 10 strains still clustered separately without clear affiliation (Fig. 2b). By comparison with literature data, the drentensis group (Fig. 2b, Table 2) also contained the abundant soil bacterium Ellin411, recently isolated by Janssen et al. (18).

B. niacini-related groups (according to Fig. 2)	*Tags (Fig. 4)*	*Cloned 16S rDNAs-uncultured*	*Other strains (not described)*	*Isolates (this work)*	*Genome variants-different species?*
BSV subgroup		11	0	0	N.D.
B. niacini		3	*B. niacini* (2)	0	N.D.
Novalis subgroup	N	0	0	13	7
B. soli	S	0	0	5	1
Vireti subgroup	V	0	0	6	4
Bataviensis subgroup	B	5	(4)	12	5
Drentensis subgroup	D	6	(2)	18	9
Affiliation unclear	?	0	0	10	8

Table 2. Subgroups within the *Bacillus niacini* relatives according to 16S rDNA sequence analysis and compared to genome variability.

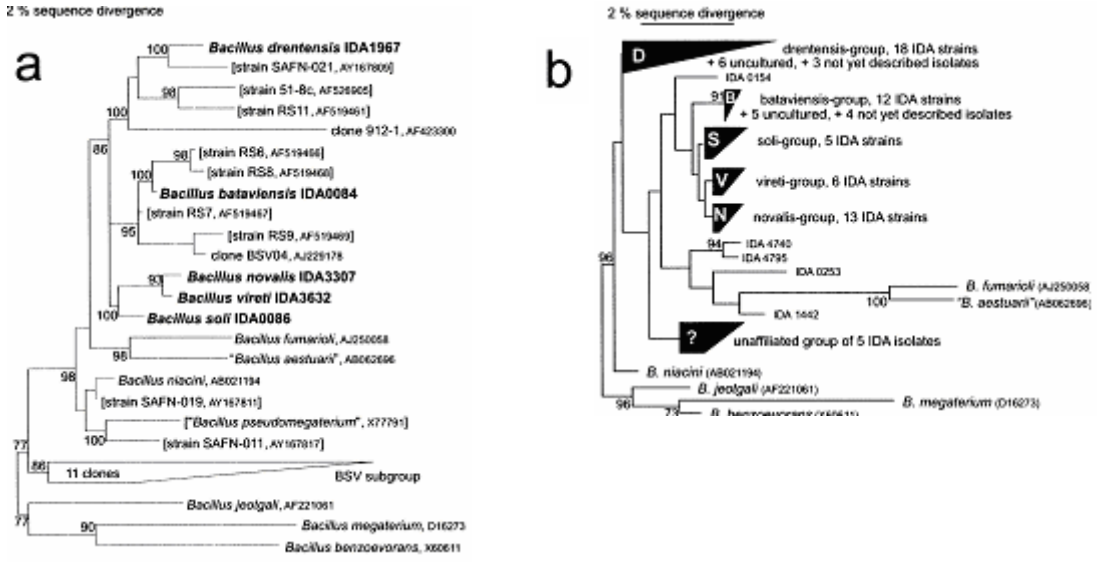


Figure 2. Phylogenetic tree of 16S rDNA sequences from *B. niacini* relatives. (a) This maximum likelihood tree is based only on almost complete sequences from the EMBL database plus five new species that already emerged from this study (printed in bold). All strains from our study were called ''IDA'' plus a four-digit serial number. The strains mentioned in brackets have not been published yet in scientific papers. The bootstrap values are indicated (if > 70). (b) The second tree was generated to reveal the subclustering tendencies among all other IDA sequences of this study and also some short sequences of lower quality from the EMBL database. Since the latter was jeopardizing the stability, the grouping of the added sequences should only be considered as preliminary, espe-cially the drentensis cluster, which is no longer reliable. Eight of the *B. niacini* relatives mentioned in Table 1 could not be used here because the lack of proper size and/or quality.

Further, novel sequences of not yet published strains recently appeared in the public databases. Garcia et al. (unpublished data) isolated strains of denitrifying *Bacillus* from tropical rice soils, where five belonged to the *B. niacini* neighborhood. Four affiliated with the bataviensis-group (RS-strains, Fig. 2a), and one with the drentensis-group. Venkateswaran et al. (unpublished data) investigated the microbial diversity of spacecraft assembly facilities (USA) and isolated four *B. niacini* relatives, two affiliated with the drentensis group and two with *B. niacini* (SAFN-strains and 51-8c, Fig. 2a). It has to be mentioned that, based on 16S rDNA, the drentensis group is the worst defined one, and there might well be some overlap with other groups. This heterogeneity and thus reduced reliability of the drentensis cluster was also reflected by the genome variability data (next paragraph). The approach to identify suitable targets for group-specific primers and probes within the 16S rRNA molecule of the *B. niacini* relatives did not reveal promising sites (Fig. 3). Hence, the consequent plan to design oligonucleotides for 16S rRNA/ DNA quantification via quantitative PCR or hybridization with directly extracted soil RNA/ DNA samples was abandoned.

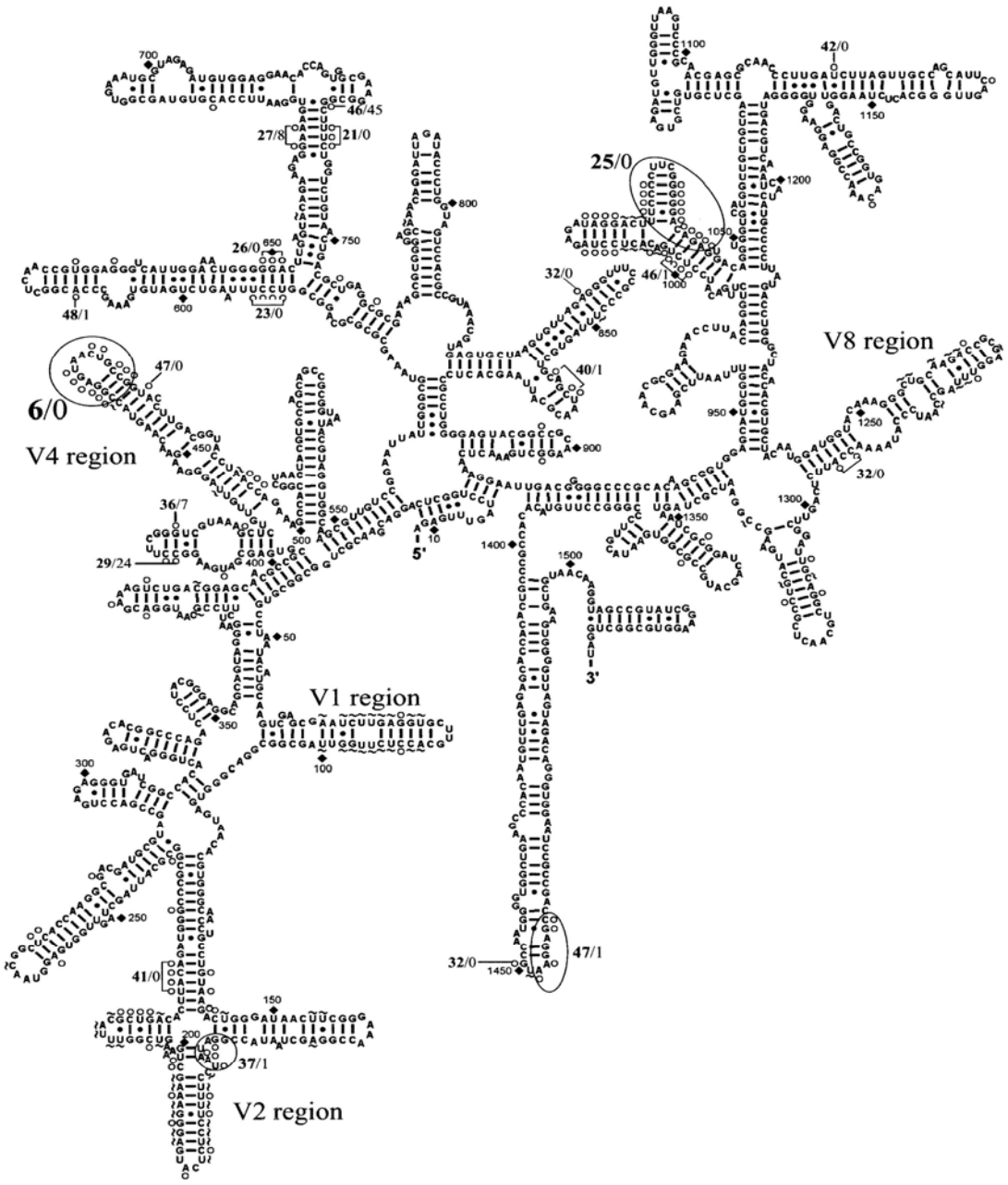


Figure 3. Hypothetical secondary structure of the *B. niacini* 16S rRNA and potential signature nucleobases. The nucleobases labeled with ''~'' are variable among the hitherto identified *B. niacini* relatives and highly variable among *Bacillus* spp. in general. The nucleobases labeled with ''○'' are conserved among the hitherto identified *B. niacini*-relatives, but variable among other *Bacillus* spp. Such sites were investigated for their specificity as visualized by the attached values: ''27/8'' for instance, means 27 % of all other *Bacillus* spp. share this sequence feature and 8 % of all other prokaryotes as well. These 22 highlighted sequence features may be single nucleobases or an entire stretch, as indicated by frames. The nucleobase positions labeled with ''○'' but without attached value are shared by > 50 % of other *Bacillus* spp. Some nucleobase positions labeled with ''○'' in the regions V1, V2, and V8 were excluded, because they were accompanied by highly variable nucleobases. This analysis was based on a subset of 1000 sequences of other *Bacillus* spp. and 2231 sequences of other prokaryotes, respectively. The nucleobases labeled with ■ and a number indicate the nucleobase numbering according to Brosius et al. (5).

Detection of genome variability among our isolates

The identification of taxonomic relations via comparative analysis of 16S rDNA sequences is problematic (13). Therefore, we used a genomic fingerprinting approach to identify highly related genomes that would likely represent the same species. For this purpose rep-PCR fingerprinting was applied, since it was shown previously that it is generally in close agreement with DNA–DNA relatedness studies (25, 29). The (GTG)5 primer was used, since the experience of the Laboratory of Microbiology (Ghent University) has shown that this primer gives the best results for various Bacillus strains (data not shown). (GTG)5-PCR showed that the genomic diversity among the investigated strains was very high (Fig. 4). Of the 64 investigated strains, 17 showed a unique pattern (Pearson correlation < 50 % to all other patterns). The remaining strains were divided over 14 small groups (Fig. 4, shaded branches), mostly only containing two or three strains. Thus, 31 different pattern types could be observed. In addition, only five clusters containing highly similar strains could be delineated (with Pearson correlation ≥80 %).

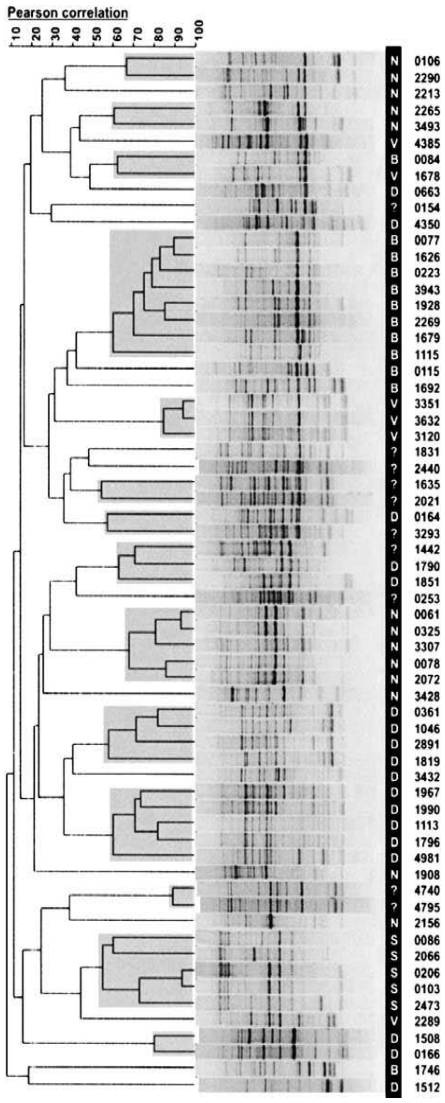


Figure 4. Grouping based on (GTG)5 fingerprints of the *B. niacini* related isolates of this study. The shaded areas are indicating the groups of related fingerprints with the regard to the 50 % threshold. The numbers of the right panel indicated the strains in the IDA collection; the black text nest to it refers to the 16S rDNA clustering outlined in figure 2b and table2.

The clustering by genomic fingerprinting revealed a less coherent distribution of several members of the drentensis cluster, while other clusters showed a much more coherent grouping. This observation is in accordance with the low reliability of the drentensis cluster found by the 16S rDNA analysis (Fig. 2b).

Discussion

Enrichment media for *B. niacini* relatives

Obviously, classic bacterial cultivation approaches neglected the *B. niacini* relatives somehow. More selective media and/or high-throughout screening of large numbers of strains were required to isolate them. A variety of media was used in this enrichment campaign, and many different strains of this cluster could be isolated, especially by using mineral medium amended with acetate. This reflected a previously described concept for growing recalcitrant bacteria based on the use of the passively cell-wall-penetrating carbon source acetate (5). The results suggested that mineral medium plus acetate at 37 °C was the best culture condition to enrich *B. niacini* relatives on agar plates inoculated with soil suspensions. This variant demonstrated efficacy, since 1 of 10 picked colonies was a *B. niacini* relative. Given the highly complex source community, this yield was considerable. The mineral medium with acetate and yeast extract showed reduced efficacy. At first glance, the apparently negative impact of yeast extract may be surprising, since the new strains usually grew very well in pure culture on complex media such as DSMZ1. However, the addition of yeast extract notably promoted the growth of rapidly spreading colonies such as *B. mycoides*. Hence, this yeast extract problem was a question of competition. Furthermore, the spreading bacteria hampered the picking of single colonies and caused subsequent contamination problems. Apart from the best media, it should be remembered that the other media also yielded *B. niacini* relatives. In light of the very low redundancy among all the isolates, it could be assumed that the high variety among our isolates was at least in part caused by the use of different media. Hence, we would hesitate to say that the isolation of *B. niacini*– related bacteria should always be done with acetate medium. The various defined subgroups (Fig. 4) could not be clearly affiliated to a certain medium type. There was no indication for a medium selectivity within this *B. niacini* group. Subculturing almost always succeeded on standard nutrient agar such as DSMZ1. After a few rounds of transfer the majority of strains could even be regarded as fast growing. It was apparently not some limited or unusual metabolic potential that could explain their absence in previous cultivation surveys.

Also, the most carefully studied member, *B. niacini*, did not show any restrained utilization of C-sources; it is even one of the most versatile utilizers of organic and amino acids (24). If this ability would be similar to the *B. niacini*-related bacteria discussed here, it

would nicely fit to the high presence of these bacteria in soils as indicated by the molecular data (Table 1).

The 16S rDNA as a marker for *B. niacini* relatives

Considering the history of this cluster, first recognized in 16S rDNA clone libraries as abundant but yet uncultured soil bacteria (16), it is obvious that the 16S rDNA sequence is the prime identification feature for this group. Hence, this molecule deserved a closer look to describe its distinct properties. First, the basic properties and conserved sequence stretches do not deviate from other *Bacillus* spp., and the highly variable region V1 is also in this case the region of highest variation. The overall sequence variation in the highly variable regions was lower than that in the entire genus *Bacillus*, which was not surprising since we were looking only on a tiny subset of *Bacillus* diversity. Of utmost interest was to identify those 16S rDNA stretches stable among *B. niacini* relatives but variable or even completely different compared to other *Bacillus* spp. or bacteria. Such regions would be promising for probe and primer design and subsequent straightforward molecular detection and quantification. Only one region was revealed to be possibly promising: the terminal loop of the V4 region (16S rRNA pos. 460–474, Fig. 3). Approximately 6% of all other *Bacillus* spp. matched with this sequence stretch.

Among these 6 % were *B. jeotgali* and relatives and, unfortunately, some *B. benzoevorans* relatives. Interference of the latter would be very critical, since they were also known to be highly abundant in our soil environment (10). To make things worse, Behrens et al. (3) identified this terminal loop of the V4 region as one of the least promising hybridization probe targets present in the 16S rRNA. Hence, this single almost specific sequence stretch alone would constitute a very poor template for probe and primer design. In other words, the specificity of the 16S rRNA sequence of *B. niacini* relatives is not based on unique nucleobase positions but much more on a particular combination of features, each shared with one Bacillus lineage or another, of more or less distant relationship.

Genomic variability of *B. niacini* relatives as an indicator for hitherto unknown species

The problems on the level of 16S rDNA analysis, as apparent in the probe site search and the tree construction, indicated that we were approaching the resolution limit of the 16S rDNA approach for bacteria distinction. The genus *Bacillus* is known to be highly diverse, or it may be better to say that the *Bacillus* species richness is just better explored than most other bacterial taxa. Several *Bacillus* species are closely related at the level of 16S rDNA, so

it is better to look at their genomes to differentiate species (30). A routinely used rep-PCR fingerprinting was applied, since it was demonstrated previously that it is generally in close agreement with DNA–DNA reassociation kinetics. If we found highly similar patterns for different isolates (Pearson correlation >80 %), these isolates would most likely belong to a single species. However, the isolates showed very diverse (GTG)5 patterns: 31 completely different pattern types could be observed (when considering a Pearson correlation threshold of 50 %). Hence, it is possible that many more species in our culture collection await description. Yet much further research is needed to elaborate the true picture behind this preliminary assessment. Considering the vast bacteria species richness in soil, our culture collection may still be a minute subset of present *B. niacini*– related species.

In general, the various 16S rDNA-based subgroups showed a quite coherent grouping. The drentensis group showed the highest number of strains displaying unique, dissimilar rep patterns. Thus, the genomic fingerprinting further undermined the consistency of the drentensis cluster, which was already jeopardized by the additional 16S rDNA sequences found in this and other studies. It revealed that this cluster attracted a large number of related yet not identical strains. This makes the group so highly diverse that the minimal 16S rDNA sequence variations were probably no longer useful to define a distinct cluster or to describe the variation within. The high genomic (and species) variability within the *B. niacini* neighborhood, as suggested by our data, may well be in line with the results of previous culture-independent surveys. If this particular *Bacillus* lineage, just a tiny fraction of the known *Bacillus* 16S rRNA phylogeny, is indeed so highly abundant in nature, we must consider it as an extremely successful soil inhabitant. Therefore, it should have been able to conquer quite a range of various niches and, by this means, segregate into countless specialized populations and finally evolve into numerous different species. We have not yet been able to provide single determining features of these species to be described from our culture collection. Hence, the differentiation of our newly described species had still to rely completely on the classic polyphasic approach, which is the actual concept of bacterial taxonomy work (36).

Conclusion

It remains unclear which ecological function this *Bacillus* lineage may occupy in soil, but the apparent diversity of isolates may indicate that there will be no single straightforward answer. Also the various sources and metabolic capabilities of recently studied strains closely related to this group hint to a remarkable functional heterogeneity. We just have begun to explore the enormous species richness present in this group and our culture collection. The classic polyphasic approach to bacterial taxonomy is at the moment the only way to explore the variability in this group. The first output of this tedious taxonomic work of species description has already yielded five newly described species from our culture collection, and many more may follow. Nevertheless, single distinct features characterizing this group or its subgroups may be discovered one day. The other quest is to track these abundant soil bacteria in the environment. At the moment, we still have to rely on cloning and sequencing of 16S rDNAs from directly extracted environmental DNA to detect these organisms with molecular tools since they do not provide useful single, distinct nucleotide positions (‘‘signatures’’) for straightforward PCR and hybridization approaches. Nevertheless, this study proves the cumulative value of the culture-independent approach linked to and directing the isolation of novel bacterial lineages. In this way, the disturbing discrepancy between the 16S rDNA sequence databases packed with yet uncultured but predominant soil bacteria and the species present in public strain collections will be reduced step by step.

Acknowledgements

This work was supported by a grant from the European Communities EC project ‘‘Exploration of Genomic and Metabolite Diversity of a Novel Group of Abundant Soil Bacteria’’ (BACREX project QLK3-2000-01678, http:// www.bacrex.com). We thank the Dutch State Forestry Commission, who allowed us access to the nature reserve.

References

1. **Ando, Y., N. Mitsugi, K. Yano, and I. Karube.** 2001. Isolation of a bacterium from mangrove soil for degradation of sea sludge. Appl. Biochem. Biotech. **95:**175-182.
2. **Ash, C., J. A. E. Farrow, S. Wallbanks, and M. D. Collins.** 1991. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. Lett. Appl. Microbiol. **13:**202–206.
3. **Behrens, S., C. Ruhland, J. Inacio, H. Huber, A. Fonseca, I. Spencer-Martins, B. M. Fuchs, and R. Amann.** 2003. In situ accessibility of small-subunit rRNA of members of the domains Bacteria, Archaea, and Eucarya to Cy3-labeled oligonucleotide probes. Appl. Environ. Microbiol. **69:**1748-1758.
4. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:**1935-1943.
5. **Button, D. K., B. R. Robertson, P. W. Lepp, and T. M. Schmidt.** 1998. A small, dilute-cytoplasm, high-affinity, novel bacterium isolated by extinction culture and having kinetic constants compatible with growth at ambient concentrations of dissolved nutrients in sea-water. Appl. Environ. Microbiol. **64:**4467-4476.
6. **DSMZ.** 2001. Catalogue of Strains 2001, 7th ed. DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.
7. **Dunbar, J., S. Takala, S. M. Barns, J. A. Davis, and C. R. Kuske.** 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl. Environ. Microbiol. **65:**1662-1669.
8. **Felske, A., J. Heyrman, A. Balcaen, and P. De Vos.** 2003. Multiplex PCR screening of soil isolates for novel *Bacillus*-related lineages. J. Microbiol. Meth. **55:**447-458.
9. **Felske, A., and R. Weller.** 2000. Cloning 16S rRNA genes and utilization to type bacterial communities. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular Microbial Ecology Manual, Supplement 5, chapter 3.3.3, vol. Kluwer Academic Publishers, Dordrecht.
10. **Felske, A., A. Wolterink, R. van Lis, and A. D. L. Akkermans.** 1998. Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). Appl. Environ. Microbiol. **64:**871-879.
11. **Felske, A., A. Wolterink, R. van Lis, W. M. de Vos, and A. D. L. Akkermans.** 1999. Searching for predominant soil bacteria: 16S rDNA cloning versus strain cultivation. FEMS Microbiol. Ecol. **30:**137-145.
12. **Garbeva, P., J. A. van Veen, and J. D. van Elsas.** 2004. Microbial diversity in soil: Selection of microbial populations by plant and soil type and implications for disease supperssiveness. Ann. Rev. Phytopath. **42:**243-270.
13. **Gest, H., and J. Favinger.** 2001. Taxonomic ambiguities: a case history. Int. J. Syst. Evol. Microbiol. **51:**707-710.

14. **Goto, K., T. Omura, Y. Hara, and Y. Sadaie.** 2000. Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. J Gen Appl Microbiol **46:**1-8.

15. **Hattori, T., H. Mitsui, H. Haga, N. Wakao, S. Shikano, K. Gorlach, Y. Kasahara, A. el-Beltagy, and R. Hattori.** 1997. Advances in soil microbial ecology and the biodiversity. Antonie Leeuwenhoek **72:**21-28.

16. **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.

17. **Heyrman, J., B. Vanparys, N. A. Logan, A. Balcaen, M. Rodriguez-Diaz, A. Felske, and P. De Vos.** 2004. *Bacillus novalis sp.* nov., *Bacillus vireti sp.* nov., *Bacillus soli sp.* nov., *Bacillus bataviae sp.* nov. and *Bacillus drentensis sp.* nov., five new species isolated from the Drentse A grasslands. Int. J. Syst. Evol. Microbiol. **54:**47-57.

18. **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.

19. **Logan, N. A., L. Lebbe, B. Hoste, J. Goris, G. Forsyth, M. Heyndrickx, B. L. Murray, N. Syme, D. D. Wynn-Williams, and P. De Vos.** 2000. Aerobic endospore-forming bacteria from geothermal environments in northern Victoria Land, Antarctica, and Candlemas Island, South Sandwich archipelago, with the proposal of *Bacillus fumarioli* sp. nov. Int. J. Syst. Evol. Microbiol. **50:**1741-1753.

20. **Ludwig, W., S. H. Bauer, M. Bauer, I. Held, G. Kirchhof, R. Schulze, I. Huber, S. Spring, A. Hartmann, and K.-H. Schleifer.** 1997. Detection and in situ identification of representatives of a widely distributed new bacterial phylum. FEMS Microbiol. Lett. **153:**181–190.

21. **Macrae, A., D. L. Rimmer, and A. G. O'Donnell.** 2000. Novel bacterial diversity recovered from the rhizosphere of oilseed rape (*Brassica napus*) determined by the analysis of 16S ribosomal DNA. Antonie Leeuwenhoek **78:**13-21.

22. **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.

23. **Nagel, M., and J. R. Andreesen.** 1991. *Bacillus niacini* sp. nov., a nicotinate-metabolizing mesophile isolated from soil. Int. J. Syst. Bacteriol. **41:**134-139.

24. **Nagel, M., and J. R. Andreesen.** 1992. Utilization of organic acids and amino acides by species of the genus *Bacillus*: a useful means in taxonomy. J Basic Microbiol **32:**91-98.

25. **Nick, G., M. Jussila, B. Hoste, R. M. Niemi, S. Kaijalainen, P. de Lajudie, M. Gillis, F. J. de Bruijn, and K. Lindstro¨m.** 1999. Rhizobia isolated from root nodules of tropical leguminous trees characterized using DNA-DNA dot-blot

hybridization and rep-PCR genomic fingerprinting. System Appl Microbiol **22:**287-299.

26. **Pearson, W. R., and D. J. Lipman.** 1998. Improved tools for biological sequence comparison. Proc Natl Acad Sci USA **85:**2444-2448.
27. **Pichinoty, F., M. Mandel, B. Greenway, and J.-L. Garcia.** 1977. Isolation and properties of a denitrifying bacterium related to *Pseudomonas lemignei*. Int. J. Syst. Bacteriol. **27:**346-348.
28. **Rademaker, J. L. W., and F. J. de Bruijn.** 1997. Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer assisted pattern analysis, p. 151–171. *In* G. Gaetano-Analle´s and P. M. Gresshoof (ed.), DNA Markers: Protocols, Applications and Overviews. John Wiley, New York.
29. **Rademaker, J. L. W., B. Hoste, F. J. Louws, K. Kersters, J. Swings, L. Vauterin, P. Vauterin, and F. J. de Bruijn.** 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. Int. J. Syst. Evol. Microbiol. **50:**665-677.
30. **Radnedge, L., P. G. Agron, K. K. Hill, P. J. Jackson, L. O. Ticknor, P. Keim, and G. L. Andersen.** 2003. Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. Appl. Environ. Microbiol. **69:**2755-2764.
31. **Stern, S., B. Weiser, and H. F. Noller.** 1988. Model for the three-dimensional folding of 16S ribosomal RNA. J. Mol. Biol. **204:**447- 481.
32. **Stienstra, A. W., P. Klein Gunnewiek, and H. J. Laanbroek.** 1994. Repression of nitrification in soils under climax grassland vegetation. FEMS Microbiol. Ecol. **14:**45-52.
33. **Strunk, O., and W. Ludwig.** 1995. ARB-a software environment for sequence data. http://www.mikro.biologie.tu-muenchen.de/pub/ ARB/(Internet). Department of Microbiology, Technical University of Munich, Munich, Germany.
34. **Valinsky, L., G. Della Vedova, A. J. Scupham, S. Alvey, A. Figueroa, B. Yin, R. J. Hartin, M. Chrobak, D. E. Crowley, T. Jiang, and J. Borneman.** 2002. Analysis of bacterial community composition by oligonucleotide fingerprinting of rRNA genes. Appl. Environ. Microbiol. **68:**3243-3250.
35. **van Schreven, D. A., and G. W. Harmsen.** 1968. Soil bacteria in relation to the development of polders in the region of the former Zuider zee. Liverpool Univerity Press, United Kingdom.
36. **Vandamme, P., B. Pot, M. Gillis, P. De Vos, K. Kersters, and J. Swings.** 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. **60:**407-438.
37. **Versalovic, J., M. Schneider, F. J. de Bruijn, and J. R. Lupksi.** 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Meth. Mol. Cel. Biol. **5:**25-40.
38. **Weber, S., S. Stubner, and R. Conrad.** 2001. Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. Appl. Environ. Microbiol. **67:**1318–1327.

CHAPTER 3

DEVELOPMENT AND APPLICATION OF A SELECTIVE PCR DGGE APPROACH TO DETECT A RECENTLY CULTIVATED *BACILLUS*-GROUP PREDOMINANT IN THE SOIL

Vesela A. Tzeneva, Youguo Li, Andreas D. M. Felske, Willem M. de Vos, Antoon D. L. Akkermans, Elaine E. Vaughan, and Hauke Smidt

Applied and Environmental Microbiology (2004) vol. 70 pp. 5801-5809

Abstract

The worldwide presence of a hitherto non-described group of predominant soil microorganisms related to *Bacillus benzoevorans* was analyzed after development of two sets of selective primers targeting 16S ribosomal RNA genes (rRNA genes) in combination with denaturing gradient gel electrophoresis (DGGE). Their high abundance and cultivability of at least some representatives makes them an appropriate object for studies on their biogeographical dissemination and diversity. As cultivability can vary significantly with the physiological state and even between closely related strains, we developed a culture-independent 16S rRNA gene-targeted DGGE fingerprinting protocol for detection of these bacteria from soil samples. The composition of the *Bacillus benzoevorans*-relatives in the soil samples from The Netherlands, Bulgaria, Russia, Pakistan and Portugal showed remarkable differences between the different countries. Differences in the DGGE profiles of these communities in archived soil samples from the Dutch Wieringermeer polder were observed over time during which a shift from anaerobic to aerobic and from saline to fresh water conditions occurred. To complement the molecular methods, we additionally cultivated *Bacillus benzoevorans*-related strains from all the soil samples. The highest number of *B. benzoevorans* relatives was found in the soils from the northern part of The Netherlands. The present study contributes to our knowledge on the diversity and abundance of this interesting group of microbes in soils throughout the world.

Intoduction

The soil ecosystem is known to provide ecological niches for an extremely high number of microorganisms (33). Investigations in Drentse A grassland soils in The Netherlands have demonstrated the abundance of 16S ribosomal RNA gene (rRNA gene) sequences that originated from a group related to *Bacillus benzoevorans* (6). Analysis of a clone library based on direct recovery of 16S rRNA genes from this Dutch soil indicated that a substantial proportion (32 %) of the bacterial sequences belonged to this novel phylogenetic lineage, which includes *B. benzoevorans, B. niacini, B. pseudomegaterium, B. jeotgali* (36), five recently isolated species comprising *B. novalis , B. vireti , B. soli , B. bataviae* and *B. drentensis* (15), uncultured bacterium DA001, and their relatives (10) (DA= Drentse A). Their quite homogeneous distribution and activity over kilometer distances has been demonstrated previously in the Dutch Drentse A grasslands (7). Hence, it is feasible to broaden the focus for a comparison of more distant landscapes. Following its discovery in Dutch soils more uncultured members of this lineage were reported to be present as the predominant group of *Bacillus* spp. in soil samples from other countries including Wisconsin (USA) (2), Amazonia (3), The Netherlands (8), and United Kingdom (20).

Since the *B. benzoevorans*-related bacteria are so abundant, they provide an attractive object for evolutionary studies and the opportunity to examine the microdiversity of one of the most dominant microbial groups in the grassland soil biosphere. Moreover, only a fraction of the bacteria from this cluster has been cultivated so far (12, 16). Although new approaches continue to be developed it is well recognised that the larger part of the soil community remains inaccessible to detailed studies due to inadequate media and methods for cultivation (1, 2, 37).

The purpose of this study was to develop tools for the fast and sensitive cultivation-independent detection of *B. benzoevorans*-related bacteria from soil samples. Molecular fingerprinting methods targeting 16S rRNA genes and rRNA, such as denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) can be used for the rapid evaluation of composition and activity of complex microbial assemblages at moderately high temporal and spatial resolution (22). In the present study, the design and validation of 16S rRNA gene-targeted primer-sets specific for the cluster of *B. benzoevorans* relatives for use in combination with DGGE are presented. These primers allow for the monitoring of the distribution and succession of these bacteria over time and space. This molecular approach

was complemented with cultivation of isolates from several European locations that revealed the presence and, sometimes remarkable, abundance of these bacteria.

Materials and Methods

Bacterial strains, growth conditions and soil samples

Reference strains, used for validation of the newly developed primers for detection of the predominant *B. benzoevorans* relatives, are listed in Table 1 with their respective sources and growth media. The primers were further tested and validated using pure culture isolates that were isolated and identified by 16S rRNA gene sequence analysis (Table 1).

The soil samples in this study can be divided in two main groups: fresh- stored at 4 ºC for 6-12 months, and air-dried- collected during different years and from different locations. The locations and soil characteristics of the fresh soils from The Netherlands were: Drentse A, where *B. benzoevorans* relatives were discovered, an agricultural research area (06^{o}41'E, 53 ^{o}03'N) with high bacterial diversity (10) representing a 1.5 km stretch of grasslands along the Anlooër Diepje river (11), 0-25 cm sampling depth; Hoeksma and Sikkema farms, Friesland 0-25 cm; Wageningen University experimental field, sandy soil, 0-25 cm; shore of the river Rhine, Wageningen (The Netherlands), wet clay soil, 0-10 cm; root nodule surface of *Alnus glutinosa* and the surrounding bulk soil, 0-10 cm (35). Additional fresh samples were obtained from various locations including a river site, grassland, pine-tree forest, fruit-tree garden and vegetable garden within the Provinzende de Cima, Portugal; and from a vegetable garden in Sofia, Bulgaria. Air-dried samples originated from soil under *Coriaria nepalensis* plants from the Murree area, Himalayan Mountains in Pakistan (collected in 1993)(21), and from the Russian tundra (collected in 2001).

In addition, air-dried soil samples were used that had been collected from the top 0-25 cm layer of non-fertilized areas of an agricultural field in the Wieringermeer polder, The Netherlands. These samples were taken from an extensive archive for technical information and soil samples (TAGA) (ALTERRA B.V., Wageningen, The Netherlands). The collection contains the results of field experiments, performed in the period between 1879 and 1998 in The Netherlands (5). Land reclamation by drainage of this former sea bottom was started in 1930 and completed in 1940, followed by transformation into agricultural lands (34). To describe the changes in the total bacterial and *B. benzoevorans*-related community after land

reclamation, samples of years 1942, 1950, 1951, 1966, 1973 and 1975 were analyzed. Two non-soil samples were also analyzed: sludge from an anaerobic wastewater treatment system (Eerbeek, The Netherlands)(27), and piglet feces (17).

Species[a] and isolates[b]	Medium	Collection[a] and GenBank accession[b] number	Primer sets	
			REX460f / REX1466r	REX576f / REX1446r
A) Reference Species				
***B. benzoevorans* Relatives**				
Bacillus benzoevorans	1[c]	LMG15526	+[f]	+
Bacillus pseudomegaterium	1	LMG18519	+	+
Bacillus niacini	1	LMG16677^T	+	+
Bacillus jeotgali	Marine broth	JCM10885	+	+
NON-*B. benzoevorans* Relatives				
Paenibacillus polymixa	51[d]	LMG6319^T	-[g]	
Bacillus methanolicus	CM0129[e]	NCIMB13113	-	
Bacillus cereus	1	LMG6923^T	-	
Bacillus simplex	1	LMG11160^T	+	-
Bacillus megaterium	1	LMG7127^T	+	-
Bacillus mycoides	1	LMG7128^T	-	
Bacillus subtilis	1	LMG7135^T	-	
Escherichia coli	1	LMG18221	-	
Staphylococcus hominis	1	LMG13348^T	-	
Streptomyces murinus	78	LMG10475^T	-	
Sporosarcina globispora	1 or 51	LMG6928^T	+	-

Species[a] and isolates[b]	Medium	Collection[a] and GenBank accession[b] number	Primer sets	
			REX460f/ REX1466r	REX576f/ REX1446r
B) New isolates[h]				
***B. benzoevorans* Relatives**				
Bacillus benzoevorans IDA[i]4715 (97%)[j]	Na-Benzoate	AY289495	+	+
Bacillus benzoevorans IDA4919 (98%)	Na-Benzoate	AY289496	+	+
Bacillus bataviensis IDA4789 (95%)	Na-Benzoate	AY289499	+	+
Bacillus bataviensis IDA4730 (99%)	Na-Benzoate	AY289497	+	+
Bacillus bataviensis IDA4740 (97%)	Na-Benzoate	AY289498	+	+
uncultured bacterium DA001 IDA4921 (97%)	Na-Benzoate	AY289500	+	+
NON-*B. benzoevorans* Relatives				
Paenibacillus sp. IDA4508 (96%)	Na-Acetate	AY289501	-	
Paenibacillus sp. IDA5358 (99%)	Na-Acetate	AY289507	-	
Bacillus mycoides IDA4575 (99%)	Na-Acetate	AY289502	-	
Bacillus megaterium IDA4770 (99%)	Na-Benzoate	AY289503	+	-
Bacillus simplex IDA4917 (98%)	Na-Benzoate	AY289504	+	-
Bacillus macroides IDA5330 (99%)	Na-Benzoate	AY289506	+	-
Bacillus megaterium IDA5367 (99%)	Na-Acetate	AY289508	+	-
Streptomyces mirabilis IDA5203 (99%)	Na-Acetate	AY289505	-	

Table 1. Reference strains (A), and pure cultures (B) of *Bacillus benzoevorans*-relatives isolated and characterized in this study, growth conditions and sources, used for validation of the new *Bacillus benzoevorans* cluster-specific primers (REX460f/REX1466r and REX576f/REX1446r).

[a]reference strains from strain collections: LMG, Laboratory of Microbiology, University of Gent, B-9000 Gent, Belgium; NCIMB, National Collections of Industrial, Food and Marine bacteria, NCIMB Ltd, Aberdeen AB24 3RY, United Kingdom; JCM, Japan Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research), Japan; [b] isolates from Wageningen University; [c](19); [d](19) amended with glucose 1g/l; [e]CM0129. Tryptone Soya Broth (Oxoid, Haarlem, The Netherlands); [f]+ PCR products generated with respective primer pair; [g]- no PCR products generated with respective primer pair. After a negative PCR result with REX460f/REX1466r, the subsequent REX576f/REX1466r nested PCR was not performed (empty cells in the table); [h]- the cultivation temperature of all reference species and new isolates was 30 °C, except *Bacillus methanolicus* NCIMB13113 (45 °C) and *Sporosarcina globispora* LMG6928^T (15 °C); [i]IDA= isolate from Drentse A; [j]percentage similarity to closest cultured relative.

Strain isolation procedure

Two types of mineral media were used for strain isolation. Briefly, they contained (per liter of distilled water): sodium acetate or sodium benzoate, 2 g; yeast extract, 2 g; P-medium, 50 ml; oligo-element solution (26), 200 µl. Agar (BBL®)(Becton, Dickinson and Company, 38800 Le Pontde Claix, France) was added at 20 g per liter for solidification where needed. P-

medium comprised per liter of distilled water: $Na_2HPO_4.2H_2O$, 37.75 g; KH_2PO_4, 19.6 g; $MgSO_4.H_2O$, 0.36 g; NH_4Cl, 2 g. The pH was adjusted to 7.0, and media were autoclaved for 20 min at 121 ^oC. Cells were isolated from 0.1 g of soil via vertical shaking for 60 min at room temperature in 950 µl 1xPBS buffer (29). Cell suspensions were diluted to 10^{-5}, and 100 µl was plated on two different media. The plates were incubated for 5-7 days at 20 ^oC. 96 colonies were randomly chosen from each medium and from each sample for further investigation.

Platewash

After the colonies were picked the plates were washed with 1ml 1x PBS buffer.

DNA isolation, primer design and PCR conditions

DNA was isolated from pure cultures (1 ml) and directly from soils (1 g) using the Fast DNA®SPIN®Kit (For Soil) (Q BIOgene, Cambs, United Kingdom) according to the manufacturers instructions. Preliminary experiments showed that using the kit-based extraction gave the best DNA isolation results (data not shown). DNA from *B. novalis, B. vireti , B. soli , B. bataviae* and *B. drentensis* was kindly provided by Jeroen Heyrman.

All primers used in this study are listed in Table 2. For primer design 16S rRNA gene sequences of phylogenetically closely related species were retrieved from GenBank at www.ncbi.nlm.nih.gov and aligned using CLUSTAL W (32). Two sets of primers (REX primers) were developed for specific detection of *B. benzoevorans*-related strains. The first primer set REX460f/REX1466r excludes *Paenibacillus, B. methanolicus*, *B. infernus, Streptococcus mirabilis,* and *B. macroides*. The second primer set REX576f/REX1446r excludes *B. mycoides, B. cereus, B. megaterium, B. simplex, B. macroides, B. subtilis, B. methanolicus, Paenibacillus, S. mirabilis*, *B. infernus*, *and B. globisporus* (Table 3).

The specificity of the primers was checked *in silico* by submitting the sequence to the Check Probe program of the Ribosomal Database Project II (4, 14) and to BLAST search (www.ncbi.nlm.nih.gov/BLAST/). The decision on specificity if the primers was made based on the number, type and position of mismatches with non-target sequences.

Primers	Sequence	Reference
27f	5'- GTT TGA TCC TGG CTC AG-3'	(18)
1492r	5'- CGG CTA CCT TGT TAC GAC-3'	(18)
REX460f	5'-GAG TAA CTG C(T/C)(C/G) GTA CC-3'	This study
REX1466r	5'-CCA ATC ATC TGT CCC ACC TTA-3'	This study
REX576f	5'-AAG CGC GCG CAG GCG GTC CT-3'	This study
REX1446r	5'-CCT TAG GCG GCT GGC TCC TTA-3'	This study
GC-0968f	5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA CCT TAC-3'	(24)
1401r	5'- GCG TGT GTA CAA GAC CC-3'	(24)
SP6	5'- ATT TAG GTG ACA CTA TAG-3'	Promega
T7	5'- AAT ACG ACT CAC TAT AGG-3'	Promega
519r	5'- G(A/T)A TTA CCG CGG C(G/T)G CTG-3'	(18)
BACREXf	5'- GATCCTGGCTCAGGAYSAACGCTGGCGGC-3'	(9)
LGb1513r	5'- GGTGATCCAGCCGCACCTTCCGA-3'	(9)
ACB1409r	5'- CARCCSRCTTTCGTGAT-3'	(9)
VER877r	5'- GCGACCGTACTYCCCAGGCGGC-3'	(9)
Bmeg180r	5'- CATCTCCCATGAAGGAGAAGA-3'	(9)
PAE835f	5'- GGGGTTTCGATACCCTTGGTGC-3'	(9)
REX1426f	5'- CACCCGAAGTCGGTGGGGTAACCGYA-3'	(9)

Table 2. Sequences of PCR primers used in this study

Primers were purchased from MWG Biotech AG (Ebersberg, Germany) and were used in a nested approach of two successive specific PCR`s. PCR products that were obtained after amplification with primers REX460f/REX1466r, were used as a template for PCR with primer set REX576f/REX1446r at a concentration of 1.0 to 2.6 ng of DNA per 50 µl reaction. Subsequently REX576f/REX1446r amplicons were used as template for DGGE-PCR with primers 0968-GCf and 1401r (24).

PCR was performed with *Taq* polymerase (Life Technologies, Gaithersburg, Md.). DNA was amplified in a Whatman Biometra Thermocycler (Göttingen, Germany) using the following conditions: REX460f/REX1466r, 94 °C for 1 min, 35 cycles of 94 °C for 10 sec, 56 °C for 20 sec, and 68 °C for 1 min, and finally 68 °C for 7 min; REX576f/REX1446r 94 °C for 5 min, 25 cycles of 94°C for 1 min, 71 °C for 20 sec, and 72 °C for 1 min, and finally 72 °C for 3 min. PCR with primers 0968GCf/1401r was performed as previously described (24). Multiplex PCR was used in this study to differentiate between *B. benzoevorans*-related and non-*B. benzoevorans*-related bacterial species.

Species	REX460f 5'-GAGTAACTGCYSGTACC'-3	REX1466r 5'-CCAATCATCTGTCCCACCTTA-3'
Bacillus benzoevorans[a)]	GAGTAACTGCTCGTACC	3'-GGTTAGTAGA**T**AGGGTGGAAT-5'
Bacillus pseudomegaterium[a)]	GAGTAACTG**GG**CGTACC	----------AGGGTGGAAT
Bacillus niacini[a)]	GAGTAACTGCCGGTACC	GGTTAGTAGACAGGGTGGAAT
Bacillus jeotgali[a)]	GAGTAACTGCCGGTACC	GGTTAGTAGACAGGGTGGAAT
Uncultured bacterium-DA032[a)]	GAGTAACTGCTGGT**G**CC	GGTTAGTAGACAGGGTGGAAT
Bacillus mycoides	G**TTG**AA**TAAG**C**T**G**GC**AC	GGTTAGTAGACAGGGTGGAAT
Bacillus cereus-AH-527	G**TTG**AA**TAAG**C**T**G**GC**AC	GGTTAGTAGACAGGGTGGAAT
Bacillus megaterium	GAGTAACTGST**T**GTACC	GGTTAGTAGACAGGGTGGAAT
Bacillus macroides	GAGTAACTGCTGGTACC	----------GGAA**G**
Bacillus subtilis	**TC**G**AATAG**G**G**CGGTACC	----------
Bacillus methanolicus	**TC**G**AATAG**G**G**CGGTAC**T**	GGTTAGTAGACAGGGTGGAAT
Paenibacillus spp.	GAGTAACTGCTC**CATAG**	----------

Species	REX576f 5'-AAGCGCGCGCAGGCGGTCCT-3'	REX1446r 5'-CCTTAGGCGGCTGGCTCCTTA-3'
Bacillus benzoevorans[a)]	AAGCGCGCGCAGGCGGTCCT	3'-GGAATCCGCCGACCGAGGA**T**T-5'
Bacillus pseudomegaterium[a)]	AAGCGCGCGCAGGCGGTCCT	GGAATCCGCCGACCGAGGAAT
Bacillus niacini[a)]	AAGCGCGCGCAGGCGGTCCT	GGAATCCGCCGACCGAGGAAT
Bacillus jeotgali[a)]	AAGCGCGCGCAGGCGGTCCT	GGAATCCGCCGACCGAGG**TT**T
Uncultured bacterium-DA032[a)]	AAGCGCGCGCAGGCGGTCCT	GGAATCCGCCGACCG**T**GG**T**AT
Bacillus mycoides	AAGCGCGCGCAGG**T**GGT**TTC**	GGAATCCGCCGACCGAGG**T**AT
Bacillus cereus-AH-527	AAGCGCGCGCAGG**T**GGT**TTC**	GGAATCCGCCGACCGAGG**T**AT
Bacillus megaterium	AAGCGCGCGCAGGCGGT**TTC**	GGAATCCGCCGA**T**CGAGGAAT
Bacillus macroides	AAGCGCGCGCAGG**T**GGT**TC**C	GGAA**G**CCGCCGACCGAGG**T**A**C**
Bacillus subtilis	AAG**G**GC**T**CGCAGGCGGT**TTC**	----------
Bacillus methanolicus	AAGCGCGCGCAGGCGGT**T**C**C**	GGAATCCGCCGACCGAGGAAT
Paenibacillus spp.	AAGCGCGCGCAGGCGG**CTAA**	----------A**A**CG**CCC**AAT

Table 3. Alignment of primer sets REX460f/REX1466r and REX576f/REX1446r with the respective 16S rRNA gene target and non-targeted sequences from different *Bacillus* spp.

[a)]- *Bacillus* species, belonging to the cluster of *B. benzoevorans* relatives.
Mismatching bases are shown in bold and underlined. Incomplete sequence information is indicated by hyphen.

Seven primers (BACREXf, LGb1513r, ACB1409r, VER877r, Bmeg189r, PE835f and REX1426f) were used to amplify different organisms. The sequences of the primers are listed in Table 2. The multiplex PCR was performed in 96-well microtiter plates according to a previously published procedure (9). PCR products from multiplex positive isolates were sequenced with primer 519r (Table 2), yielding approximately 500 bp of sequence information.

PCR conditions were optimized for specificity using genomic DNA extracted from reference strains as well as pure cultures isolates obtained at Wageningen University (Table 1). The primers were further tested using cloned 16S rRNA genes, isolated from soil samples that had been previously demonstrated to contain high numbers of *B. benzoevorans*-related species (6).

Analysis of PCR products by DGGE and sequencing

The PCR products obtained after three nested PCR reactions with primer sets, as follows REX460f/REX1466r, REX576f/REX1446r and 0968-GCf/1401r, were separated by DGGE (22, 23). 16S rRNA genes targeted PCR-DGGE fingerprinting was used to analyze the profiles of the *B. benzoevorans* relatives from different soil samples and also to identify different clones from clone libraries. A gradient of 38-50% of the denaturing chemicals (urea and formamide) was used, and gel electrophoresis was performed according to Heilig *et* al. (14),the gels were stained with $AgNO_3$ according to the method of Sanguinetti *et* al. (30), scanned at 400 dpi, and analyzed using the software Molecular Analyst 1.12 (Biorad). The similarity between the DGGE profiles was determined by calculating similarity indices of the densitometric curves of the profiles compared using the Pearson product-moment correlation (13), (38). The UPGMA algorithm was used as implemented in the analysis software for the construction of dendrograms.

PCR amplicons after performing nested PCR with primer sets REX460f/REX1466r and REX576f/REX1446r were purified with Qiaquick PCR purification kit according to the manufacturer's instructions and cloned into *E. coli* JM109 using the Promega pGEM-T vector system (Promega, Madison, Wis.) as previously described (14). The size of the inserts was confirmed by PCR with specific vector-primers T7 and Sp6 (Table 2). Clones were screened by Restriction Fragment Length Polymorphism (RFLP) with restriction enzymes *Msp*I and *Cfo*I. Plasmids containing unique inserts were further purified and inserts were sequenced. Sequencing reactions were performed with the Sequenase (T7) sequencing kit (Amersham Life Sciences, Slough, United Kingdom) as previously described (14). Sequences were automatically analyzed on a LI-COR DNA sequencer 4000L (Lincoln, Nabr.) and corrected manually.

The GenBank Accession numbers of the 16S rRNA gene sequences of isolates obtained in this study are provided in Table 1.

Statistical analysis

Student's t-test was used for statistical analysis of comparison between similarity indices from the DGGE profiles.

Results

Cultivation and identification of *B. benzoevorans* relatives and strain isolation

Samples from Drentse A, Hoeksma and Sikkema farms, Wageningen University experimental field, *Alnus glutinosa*-associated rhizosphere nodules and bulk soil, soil from Bulgaria, five different Portuguese soils, Russian tundra soil, and six Wieringermeer soils were used for cultivation and isolation of potential *B. benzoevorans* relatives. All soils yielded about 10^4-10^5 colony forming units (CFU) per gram of soil on either of the two mineral media, except for the Wieringermeer samples, from which only about 103 CFU.g-1 could be retrieved.

Multiplex PCR was used to tentatively identify B. benzoevorans relatives. 192 CFU from each Dutch soil sample were screened, except Wieringermeer samples where the possible maximum of CFU was picked, and 48 CFU from the Portuguese, Bulgarian and Russian soils were analyzed. In general, approximately 20% of all screened colonies retrieved from Dutch soil samples (except Wieringermeer samples) tested multiplex positive, in contrast to the isolates from Portuguese (2.5%), Bulgarian (no positives),and Alnus glutinosa-associated (no positives) samples. B. benzoevorans relatives could not be detected in the Russian tundra, Bulgarian vegetable garden and Portuguese river site, grassland and vegetable garden soil samples nor in the Wageningen Alnus glutinosa rhizosphere soil. Single colony isolates that were identified as potentially B. benzoevorans-related by multiplex PCR were subjected to 16S rRNA gene sequence analysis for further identification.

The highest number of B. benzoevorans relatives was found in the soils from the northern part of The Netherlands. About 9% of all 384 screened isolates obtained from the Drentse A and Friesland samples were identified as B. benzoevorans relatives, while the remaining multiplex-positive isolates belonged to other Bacillus spp. The fraction of B. benzoevorans-related isolates that could be retrieved from Wageningen and Portuguese soil samples was significantly lower (respectively, 3.5% of the screened 96 CFU and about 1.7% of the screened 240 CFU). The length of the sequences was about 0.5 kb, and a threshold of

97% sequence similarity was used to consider strains as B. benzoevorans related (data not shown).

The isolates used for validation of the new B. benzoevorans cluster-specific primers are shown in Table 1. They were chosen to represent a range of B. benzoevorans related and unrelated microorganisms. Since the 16S rRNA gene sequence similarity of several isolates was lower than 97%, some potentially novel B. benzoevorans relatives were cultivated.

Design and validation of new group specific primers for detection of B. benzoevorans relatives

Primer sets REX460f/REX1466r and REX576f /REX1446r for the specific detection of B. benzoevorans relatives were designed based on a multiple alignment of the complete 16S rRNA gene sequences of B. benzoevorans relatives as well as non-B. benzoevorans-related species (Table 3).

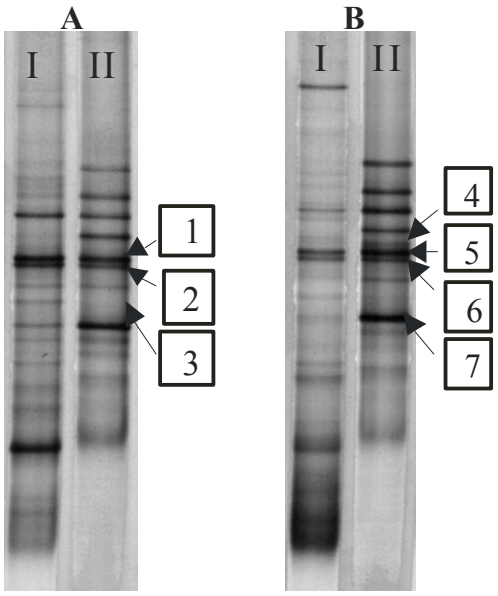
The optimal conditions for PCR amplification were experimentally determined using genomic DNA from 29 bacterial strains as the template (Table 1). The optimum for REX460f/REX1466r was annealing at 56°C and 35 cycles, and for REX576f/REX1446f 71°C and 25 cycles. In agreement with the multiple alignment, the primers discriminated against different *Bacillus* species, which do not belong to the cluster of *B. benzoevorans* relatives (Table 3). These primer sets, when applied in a nested approach, specifically amplified 16S rRNA gene fragments of *Bacillus benzoevorans*, *B. niacini*, *B. pseudomegaterium, B. jeotgali* and uncultured bacterium DA001 as expected (Table 1).

The diversity of *B. benzoevorans* relatives judged by DGGE was compared between DNA isolated directly form the soil and after platewashing (data not shown). The DGGE fingerprints sowed different profiles.

Specific DGGE patterns of *B. benzoevorans* relatives from the Dutch soil samples

The applicability of the REX-primers in combination with DGGE for monitoring of the *B. benzoevorans*-related community was initially tested using DNA isolated from soil samples from Drentse A and Friesian farms in The Netherlands (Fig. 1, lane II), since these grassland fields contain high numbers of *B. benzoevorans*-related isolates. PCR products generated by the two-step nested specific REX PCR were used as the template for PCR with

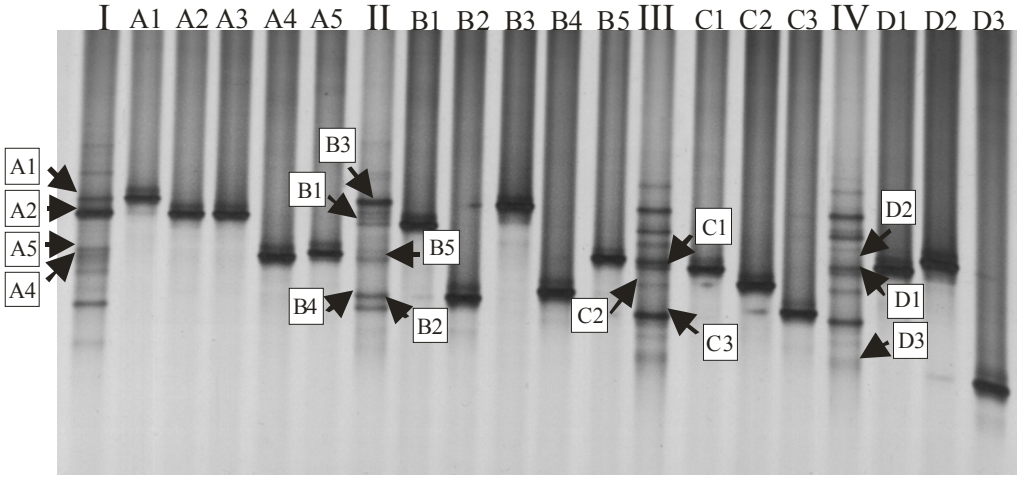
universal primers, 0968-GCf/1401r, and products were separated by DGGE. The resulting profiles of *B. benzoevorans* relatives were compared to total bacterial community profiles of the two Dutch soil samples from Drentse A and Friesland (lane I). The results revealed that the predominant bands of the upper half of the bacterial DGGE patterns corresponded to bands in the DGGE fingerprint of B. benzoevorans relatives.The DGGE profiles of B. benzoevorans-related microorganisms were compared in five soil samples (Figs. 1 and 2) which demonstrated differences in the diversity of *B. benzoevorans* relatives in the different



No	Closest relative	Length (bp)	Identity (%)	GenBank accession number
1	*Bacillus benzoevorans*	891	97	AY289513
2	*Bacillus benzoevorans*	916	98	AY289515
3	Uncultured bacterium DA001	884	97	AY289514
4	*Bacillus benzoevorans*	889	97	AY289511
5	Uncultured bacterium DA001	916	98	AY289509
6	Uncultured bacterium DA001	891	98	AY289510
7	Uncultured bacterium DA001	962	99	AY289512

Figure 1. DGGE profiles of the total bacterial and *B. benzoevorans*-related communities in Drentse A (A) and Friesian (B) soil samples. Identified bands are indicated with numbers and explained in the table below. Lane I- Total bacterial fingerprint (universal DGGE primers GC-0968f/1401r); lane II- *B. benzoevorans*-related fingerprint (specific primers REX460f/REX1466r and REX576f/REX1446r, and universal DGGE primers GC-0968f/1401r).

soils. In order to confirm the specificity of the newly developed nested REX PCR approach, cloning and sequence analysis was performed for PCR products that were obtained from several soil samples, including Drentse A and Friesland (Fig. 1), Wageningen University experimental field, river Rhine, and Himalaya (Fig. 2). These analyses showed that all of the unique clones, selected after Restriction Fragment Length Polymorphism (RFLP) analysis, belonged to the cluster *B. benzoevorans* relatives. The DGGE bands of 20 out of 22 studied clones (Fig. 1 and Fig. 2, lanes A1-A5, B1-B5, C1-C3, and D1-D3) from all tested samples



No	Closest relative	Length (bp)	Identity (%)	GenBank accession number
A1	Uncultured bacterium DA001	901	97	AY289516
A2	*Bacillus benzoevorans*	901	97	AY289517
A4	Uncultured bacterium DA001	902	97	AY289518
A5	Uncultured bacterium DA001	901	99	AY289519
B1	*Bacillus benzoevorans*	901	98	AY289520
B2	*Bacillus benzoevorans*	906	98	AY289521
B3	*Bacillus benzoevorans*	907	97	AY289522
B4	*Bacillus benzoevorans*	901	97	AY289523
B5	Uncultured bacterium DA001	901	99	AY289524
C1	*Bacillus batavensis*	911	97	AY289525
C2	*Bacillus benzoevorans*	902	97	AY289526
C3	*Bacillus benzoevorans*	901	96	AY289527
D1	*Bacillus benzoevorans*	901	98	AY289528
D2	*Bacillus benzoevorans*	891	98	AY289529
D3	*Bacillus benzoevorans*	901	97	AY289530

Figure 2. DGGE analysis of the *B. benzoevorans* related communities in 4 different soil samples. Identified bands are indicated with numbers and explained in the table below. Lane I- Wageningen University experimental field; lane II- river Rhine shore; lane III- Himalaya, bulk soil; lane IV- rhizosphere soil sample from *Coriaria nepalensis*, Himalaya; A1-A5- clones from DNA extracted from sample I; B1-B5- clones from DNA extracted from sample II; C1-C3- clones from DNA extracted from sample III; D1-D3- clones from DNA extracted from sample IV.

corresponded to the predominant bands in the fingerprint of the *B. benzoevorans* relatives. The majority of sequences retrieved from the clone libraries were most closely related with clones from uncultured bacteria, also related to *B. benzoevorans*, detected in Drentse A grassland soils in a previous study (10).

Comparison of the diversity of *B. benzoevorans* relatives in different places

A remarkable richness of *B. benzoevorans* relatives in different places was observed based on DGGE profiles (Fig. 3). No *B. benzoevorans* relatives could be detected in the non-soil samples, i.e. sludge from an anaerobic wastewater treatment reactor and piglet intestine, nor in the Bulgarian and Russian tundra soil samples (data not shown). In contrast, the other 13 locations showed some similar but also different DGGE profiles. To determine whether the *B. benzoevorans*-related communities from different soil samples were significantly different or similar, similarity indices of the DGGE profiles were calculated. Both Himalayan rhizosphere soil samples showed almost identical *B. benzoevorans*-related communities (similarity index of 95). Remarkably, they showed significant similarity (similarity indices from 82.5 to 88.8) with the *B. benzoevorans* cluster-specific DGGE fingerprints of Dutch soil samples. *B. benzoevorans*-related communities in the soils from Portugal and also in the samples from *Alnus glutinosa*-nodules and surrounding soil, were less diverse as compared with the Dutch and Himalayan soils (Fig. 3). The *B. benzoevorans* cluster-specific DGGE banding patterns observed for the different Portuguese samples were significantly less similar, and were also distinct from those obtained from the Dutch and Himalayan soils, based on the different positions of the bands in the profiles (Fig. 3b). The similarity indices between the Portuguese and the Himalayan rhizosphere samples varied from 22.2 to 69.7. The DGGE profiles of the samples from the river site and vegetable garden in Portugal showed only 2 dominant bands. In contrast to other Dutch soil samples, low-complexity *B. benzoevorans*-related communities were found on *Alnus glutinosa* nodules and the surrounding soil where only one predominant species and possibly some species present in lower numbers could be detected. Student's t-test revealed that there was a significant difference between the two separately clustering groups shown on the dendrogram (Fig. 3), since the lowest $P_{2\text{-}tail}$ observed was 0.199776×10^{-7} (d.f.=18).

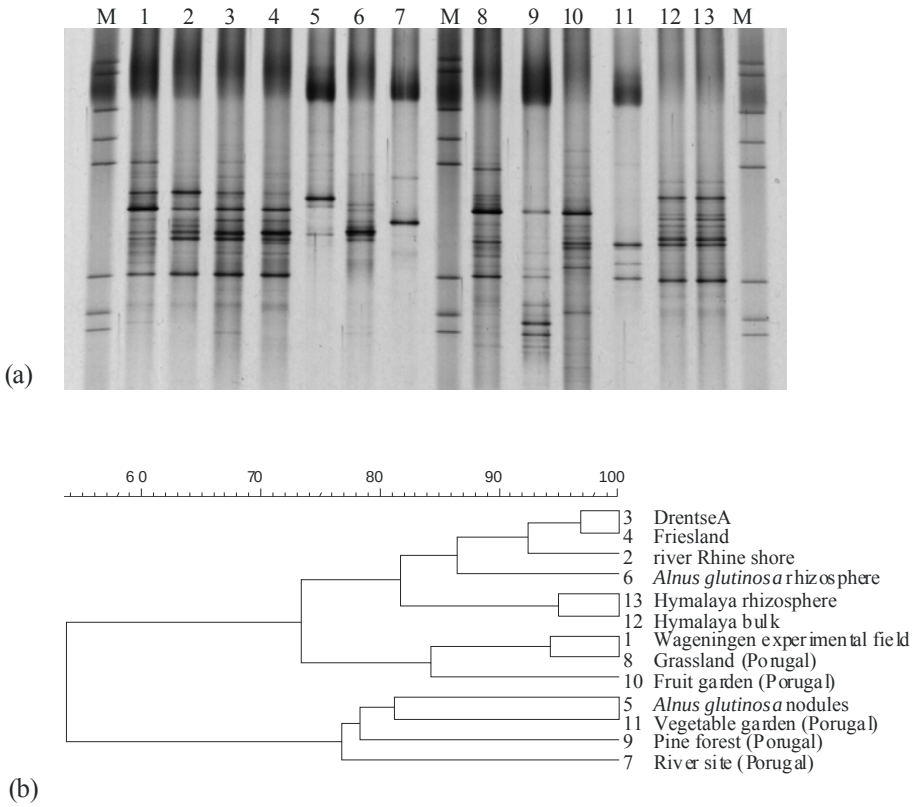
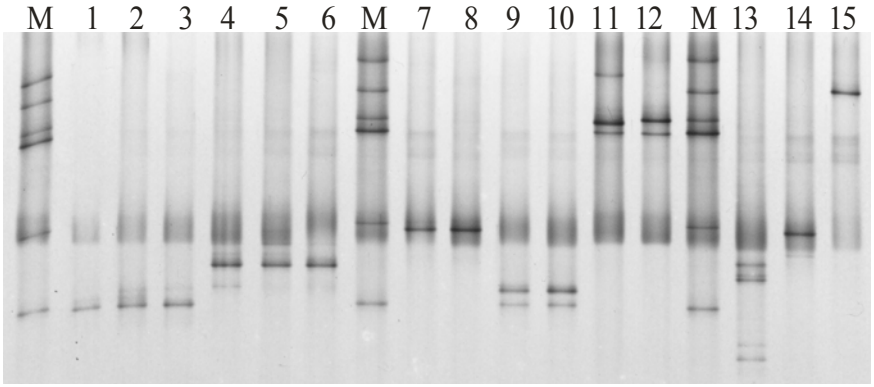


Figure 3. DGGE profile of the *B. benzoevorans* related community in soil samples from different places (a) and corresponding dendogram (UPGMA clustering)(b). M- Marker; 1- Wageningen University experimental field (The Netherlands); 2- river Rhine shore (The Netherlands); 3- Drentse A (The Netherlands); 4- Friesland (The Netherlands); 5- *Alnus glutinosa* nodules (The Netherlands); 6- *Alnus glutinosa* rhizosphere (The Netherlands); 7- river site (Portugal); 8- grassland (Portugal); 9- pine forest (Portugal); 10- Fruit garden (Portugal); 11- Vegetable garden (Portugal); 12- Himalaya, bulk soil; 13- Himalaya, rhizosphere soil from *Coriaria nepalensis*.

Heterogeneity of the 16S rRNA genes of *B. benzoevorans* relatives

The heterogeneity of the 16S rRNA genes was studied to determine if each single band from the DGGE profiles of *B. benzoevorans*-related members belongs to a different sequence. DGGE fingerprints of 12 pure cultures were compared (Fig. 4). Most of the *B. benzoevorans*-related isolates showed a single dominant band, often with one to two fainter bands, except *B. benzoevorans* IDA4919, which had four bands. This indicates that at least some of the specied have multiple, heterogeneous 16S rRNA gene alleles. Tb addition, the positions of the predominant bands obtained for some of the isolates were similar. These findings suggest that the diversity of the *B. benzoevorans* relatives can be either slightly over- or underestimated, depending on the populatuons present in an environmental sample.



No	Closest relative	GenBank accession number
1	*Bacillus novalis* IDA 0078	AJ542511
2	*Bacillus novalis* IDA 0106	AJ542510
3	*Bacillus vireti* IDA 3632	AJ542509
4	*Bacillus soli* IDA 0086	AJ542513
5	*Bacillus soli* IDA 2066	AJ542515
6	*Bacillus soli* IDA 2473	AJ542514
7	*Bacillus batavensis* IDA 0084	AJ542507
8	*Bacillus batavensis* IDA 1115	AJ542508
9	*Bacillus drentensis* IDA 1113	AJ542505
10	*Bacillus drentensis* IDA 1967	AJ542506
11	Uncultured bacterium DA001 IDA 1527	AJ544783
12	Uncultured bacterium DA001 IDA 3504	AJ544784
13	*Bacillus benzoevorans* IDA 4919	AY289496
14	*Bacillus batavensis* IDA 4730	AY289597
15	Uncultured bacterium DA001 IDA 4921	AY289500

Figure 4. DGGE fingerprint and list of 15 pure culture of *B. benzoevorans* related isolates, showing heterogeneity of their rRNA. M-Marker. IDA= Isolates from Drentse A. a)- cultured strain shows identical 16S rRNA gene sequence as uncultured bacterium DA001.

Development and succession of *B. benzoevorans*-related communities in Wieringermeer Polder soil samples

Changes in the *B. benzoevorans*-related communities over a period of 33 years were investigated in soil samples from an experimental field in the Wieringermeer polder (The Netherlands) with year serial 1942, 1950, 1951, 1966, 1973 and 1975. The soil environment changed from anaerobic to aerobic during the reclamation process (34). *B. benzoevorans*-specific PCR-DGGE was applied to determine if *B. benzoevorans*-related communities were present in these soils and if changes occurred over the years (Fig. 5).

In the 1942 soil sample, only two major bands were detected, while the number of the bands had increased by 1950 to at least four strong bands, indicating an increase in the

diversity during these years. No major changes in the diversity of *B. benzoevorans*-related communities in the period between 1950 and 1975 were detected.

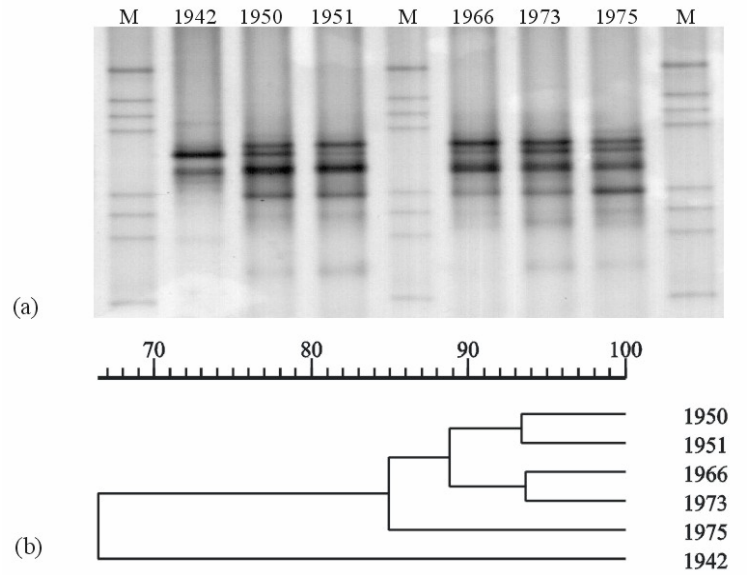


Figure 5. DGGE profile of the *B. benzoevorans* related community in soil samples from different years (Wieringermeer polder) (a) and corresponding dendogram (UPGMA clustering) (b). M- Marker; 1942, 1950, 1951, 1966, 1973, 1975 indicate the year of sampling.

Discussion

In this study, both culture-dependent and independent strategies were developed to monitor B. benzoevorans-related soil bacteria that are surprisingly predominant in some soils around the world. Specific primers for the group were developed and validated which allowed their rapid detection in environmental samples. The validity of the primers was demonstrated by following the distribution of the group in different geographical locations and also diversity over time in various soil samples.

A large number of B. benzoevorans isolates could be retrieved from variety of soil samples using mineral media supplemented with Na-acetate or Na-benzoate as substrates. It was possible to obtain and keep the bacteria viable on the mineral media developed in this study, which will allow for further morphological and physiological characterization of isolates. The majority of *B. benzoevorans* relatives were isolated from the sodium benzoate medium, whereas sodium acetate amended media proved less suitable for the isolation of *B. benzoevorans* relatives. Probably the species belonging to this cluster have better metabolic potential to utilize benzoate than acetate, as a substrate (25). The isolation of a substantial

collection of cultured representatives of this novel lineage of *Bacillus* now offers a solid basis to gain more insight in the physiology of these microorganisms. In this context it is noteworthy, that recently obtained pure cultures isolates (e.g. IDA1527 and IDA3504) (Fig. 4) of the *B. benzoevorans* group share an identical 16S rRNA gene sequence (similarities 99.5 and 99.9%, respectively) with clones of the highly abundant uncultured bacterium DA001 (Jeroen Heyrman, personal communication).

Nevertheless, the cultivation procedure followed here was rather time consuming, and the yield of cultured stains was still low. Less than 7% of all isolates obtained in this study were *B. benzoevorans* relatives. Moreover, it is likely that not all members of this lineage can be cultivated with similar efficiency, as it is widely accepted that most of the soil microorganisms (estimated 99.5 to 99.9%) have not yet been isolated and cultivated on laboratory media (1, 2, 28, 33). To compensate for this well-recognized inability to comprehensively address microbial diversity solely by cultivation, we developed a fast and sensitive method for qualitative analysis of the *B. benzoevorans* relatives in the soil based on a group-specific PCR and DGGE. Since this study focused on a fast and easy detection of *B. benzoevorans*-related bacteria in different soil samples these two primer sets were constructed to consecutively exclude non-target *Bacillus* species. Because different *Bacillus* species showed too high 16S rRNA sequence similarities it was not possible to develop only one primer pair that would be sufficiently specific to amplify only target sequences from *B. benzoevorans* relatives. Therefore, a two steps nested PCR approach was necessary for molecular detection of the *B. benzoevorans*-relatives. Indeed the DGGE profiles showed that most of the predominant *B. benzoevorans* related microorganisms detected by these primers could not be cultivated.

The products of REX576f/REX1446r PCR were subsequently used as a template for DGGE-PCR. We did not use a GC-clamp for either of these primers because the products would have a size of about 900 bp, which is not optimal for separation by DGGE (23). We also observed that the high annealing temperature, required for specificity (71 ^{o}C), negatively influenced the amplification efficiency of the GC-clamp primer.

The specificity of the REX primers for *B. benzoevorans* relatives was demonstrated by cloning and sequencing PCR products obtained with DNA isolated directly from different soil samples. All of the sequenced clones belonged to the *B. benzoevorans*-related cluster. In most cases, highest sequence similarity was found with the DA (Drentse A) clones corresponding

to uncultured relatives of *B. benzoevorans*, that were retrieved from a Dutch Drentse A soil 16S rRNA gene clone library (10, 11).

The REX primers provide a convenient and fast method not only for detection of *B. benzoevorans* relative populations in the soil samples, but also for monitoring of this part of the bacterial community over time and its distribution in different places. We studied the presence of these microorganisms in 15 soil samples and two non-soil samples (anaerobic sludge and gut content). In neither of the two non-soil environments *B. benzoevorans* relatives were detected, which was an expected result as those ecosystems are not among the preferred habitats for characterized *Bacillus* species belonging to this cluster. Specifically, the anaerobic conditions in the two environments are probably a limiting factor for the aerobic *B. benzoevorans* relatives, although the genus *Bacillus* includes some facultative anaerobic members. In the soil samples, *B. benzoevorans* relatives were present in 19 from all 21 studied soil samples from the different countries. Although *B. benzoevorans* relatives could be found worldwide (8, 10), they were not detectable in all the samples from the different countries, and thus, do not comprise the predominant soil bacteria everywhere. In the Russian and Bulgarian samples they are, if at all, present in numbers that are below the detection threshold of the PCR-DGGE method. The highest predominance of the *Bacillus* species from this cluster was demonstrated in the Dutch and Himalayan soil samples. In all of the Portuguese habitats *B. benzoevorans* relatives were present but not as the predominant population. When soil samples from similar environments but from different countries were compared (e.g. river sites in The Netherlands and Portugal) it was found that the *B. benzoevorans*-related community was also different. Significant differences in the *B. benzoevorans*-related communities were found between samples from the different countries, but also between soils collected from different habitats in the same country.

In the period 1942-1950 significant differences in the DGGE profiles of *B. benzoevorans* related community in the soil samples from Wieringermeer polder were observed. These changes in the diversity of the *B. benzoevorans*-relatives can be explained with the fact that 1945, the Wieringermeer polder was flooded during the war (31). It was subsequently reclaimed and at this time agricultural activities were also developed which can be another reason for the observed increase in the bacterial diversity of the reclaimed land. From 1950 onwards, the richness did not alter, but the intensity of the DGGE bands slightly varied, suggesting changing relative abundance of the populations within this bacterial community.

The strategy that combines the *B. benzoevorans* relatives-specific PCR with DGGE is widely applicable for monitoring of the temporal and spatial diversity of *B. benzoevorans*-related bacilli and their abundance in the bacterial community. It can be suggested from their abundance that these broadly distributed and at certain places predominant microorganisms have an important role in the soil ecosystems, which will be addressed in further studies.

Acknowledgements

This work was supported by a grand from the European Communities EC project "Exploration of Genomic and Metabolite Diversity of a Novel Group of Abundant Soil Bacteria" (BACREX-project QLK3-2000-01678). We would like to thank Phillip Ehlert, TAGA (ALTERRA B.V., Wageningen, The Netherlands), who allowed us access to the soil collection. Special thanks to Jeroen Heyrman (Ghent University, Belgium) for the kindly providing DNA samples. Carla Belo is especially acknowledged for the help with the experimental part of the work. Netherlands Organization for International Cooperation in Higher Education (NUFFIC) is acknowledged for the financial support offered to Youguo Li.

References

1. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143-169.
2. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:**1935-1943.
3. **Borneman, J., and E. W. Triplett.** 1997. Molecular microbial diversity in soils from Eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Environ. Microbiol. **63:**2647-2653.
4. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje.** 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. **31:**442-443.
5. **De Willigen, P., P. F. A. M. Römkens, and P. A. I. Ehlert.** 2001. Data mining TAGA for background levels of heavy metals. (In Dutch.) Rep. 261.Alterra, Wageningen, the Netherlands.
6. **Felske, A.** 1999. Reviewing the DA001-files: A 16S rRNA chase on suspect #X99967, a *Bacillus* and Dutch underground activist. J. Microbiol. Meth. **36:**77-93.
7. **Felske, A., and A. D. L. Akkermans.** 1998. Spatial homogeneity of the most abundant bacterial 16S rRNA molecules in grassland soils. Microbiol. Ecol. **36:**31-36.
8. **Felske, A., A. D. L. Akkermans, and W. M. de Vos.** 1998. In situ detection of an uncultured predominant *Bacillus* in Dutch grassland soils. Appl. Environ. Microbiol. **64:**4588-4590.
9. **Felske, A., J. Heyrman, A. Balcaen, and P. De Vos.** 2003. Multiplex PCR screening of soil isolates for novel *Bacillus*-related lineages. J. Microbiol. Meth. **55:**447-458.
10. **Felske, A., A. Wolterink, R. van Lis, and A. D. L. Akkermans.** 1998. Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). Appl. Environ. Microbiol. **64:**871-879.
11. **Felske, A., A. Wolterink, R. van Lis, W. M. de Vos, and A. D. L. Akkermans.** 1999. Searching for predominant soil bacteria: 16S rDNA cloning versus strain cultivation. FEMS Microbiol. Ecol. **30:**137-145.
12. **Felske, A. D. M., V. A. Tzeneva, J. Heyrman, and P. De Vos.** 2004. Isolation and biodiversity of hitherto not described soil bacteria related to *Bacillus niacini*. Microbiol. Ecol. **48:**111-119.
13. **Häne, B. G., K. Jäger, and H. Drexler.** 1993. The pearson product-moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. Electrophoresis. **14:**967-972.
14. **Heilig, H. G. H. J., E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. L. Akkermans, and W. M. de Vos.** 2002. Molecular diversity of *Lactobacillus spp.*

and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ. Microbiol. **68:**114-123.

15. **Heyrman, J., B. Vanparys, N. A. Logan, A. Balcaen, M. Rodriguez-Diaz, A. Felske, and P. De Vos.** 2003. *Bacillus novalis sp.* nov., *Bacillus vireti sp.* nov., *Bacillus soli sp.* nov., *Bacillus bataviae sp.* nov. and *Bacillus drentensis sp.* nov., five new species isolated from the Drentse A grasslands. Int. J. Syst. Evol. Microbiol. **54:**47-57.
16. **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.
17. **Konstantinov, S. R., W.-Y. Zhu, B. A. Williams, S. Tamminga, W. M. de Vos, and A. D. L. Akkermans.** 2003. Effect of fermentable carbohydrates on piglet fecal bacterial communities as revealed by denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA. FEMS Microbiol. Ecol. **43:**225-235.
18. **Lane, D. J.** 1991. 16S/23S rRNA sequencing. John Wiley & sons Ltd, UK.
19. **Lapage, S. P., J. E. Shelton, and T. G. Mitchell** 1970. Nutrient agar, p. 116. *In* J. R. Norris, and D. W. Ribbons (eds), Methods Microbiology, vol. 3A. Acad. Press, London, UK.
20. **Macrae, A., C. M. M. Lucon, D. L. Rimmer, and A. G. O'Donnell.** 2001. Sampling DNA from the rhizosphere of *Brassica napus* to investigate rhizobacterial community structure. Plant Soil. **233:**223-230.
21. **Mirza, M. S., W. M. Akkermans, and A. D. L. Akkermans.** 1994. PCR-amplified 16S rRNA sequence analysis to confirm nodulation of *Datisca cannabina* L. by the endophyte of *Coriaria nepalensis* Wall. Plant Soil. **160:**147-152.
22. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695-700.
23. **Muyzer, G., and K. Smalla.** 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Leeuwenhoek. **73:**127-141.
24. **Nübel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig, and H. Backhaus.** 1996. Sequence hetereogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis of 16S rDNA fragments. J. Bacteriol. **178:**5636-5643.
25. **Pichinoty, F., J. Asselineau, and M. Mandel.** 1984. Biochemical characterization of *Bacillus benzoevorans sp.* nov., a new filamentous, sheated mesophilic species which degrades various aromatic acids and phenols. Ann. Microbiol. **135:**209-217.
26. **Pichinoty, F., M. Mandel, B. Greenway, and J.-L. Garcia.** 1977. Isolation and properties of a denitrifying bacterium

related to *Pseudomonas lemignei*. Int. J. Syst. Bacteriol. **27:**346-348.

27. **Roest, K., H. G. H. J. Heilig, H. Smidt, W. M. de Vos, A. J. M. Stams, and A. D. L. Akkermans.** 2005. Community analysis of a full-scale anaerobic bioreactor treating paper mill wastewater. System. Appl. Microbiol. **28:**175-185.
28. **Rosswall, T., and E. Kvillner.** 1978. Principal-components and factor analysis for the description of microbial populations. Adv. Microbiol. Ecol. **2:**1-48.
29. **Sambrook, J., E. F. Fritsche, and T. Maniatis.** 1989. Molecular Cloning a laboratory manual., vol. 3. Cold Spring Harbor Laboratory Press, USA.
30. **Sanguinetti, C. J., D. Neto, and A. J. G. Simpson.** 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. BioTechniques. **17:**915-919.
31. **Terpstra, P.** 1979. 50 jaar Wieringermeer. Uitgeverij M. A. van Seijen-Leeuwarden, The Netherlands.
32. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673-4680.
33. **Torsvik, V., J. Goksøyr, and F. L. Daae.** 1990. High diversity of DNA of soil bacteria. Appl. Environ. Microbiol. **56:**782-787.
34. **van Schreven, D. A., and G. W. Harmsen.** 1968. Soil bacteria in relation to the development of polders in the region of the former Zuider zee. Liverpool Univerity Press, United Kingdom.
35. **Wolters, D. J., A. D. L. Akkermans, and C. van Dijk.** 1997. Ineffective *Frankia* strains in wet stands of *Alnus glutinosa* L. Gaertn. in The Netherlands. Soil Biol. Biochem. **29:**1707-1712.
36. **Yoon, J.-H., S.-S. Kang, K.-C. Lee, Y. H. Kho, S. H. Choi, and K. H. P. Kang, Y.-H.** 2001. *Bacillus jeotgali* sp. nov., isolated from jeotgal, Korean traditional fermented seafood. Int. J. Syst. Evol. Microbiol. **51:**1087-1092.
37. **Zengler, K., G. Toledo, M. Rappe, J. Elkins, E. J. Mathur, J. M. Short, and M. Keller.** 2002. Cultivating the uncultured. Proc. Natl. Acad. Sci. USA. **99:**15681-15686.
38. **Zoetendal, E. G., A. D. L. Akkermans, W. M. Akkermans - van Vliet, J. A. G. M. de Visser, and W. M. de Vos.** 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Microbial Ecol. Health Dis. **13:**129-134.

CHAPTER 4

RIDING THE ECOLOGICAL TIME MACHINE: SUCCESSION OF ABUNDANT SOIL BACTERIA IN RECLAIMED LAND

Vesela A. Tzeneva, Phillip A. I. Ehlert, Willem M. de Vos, Antoon D. L. Akkermans, and Hauke Smidt

Abstract

Unique archived samples from a young Dutch polder vague soil, covering a period of 26-years, were used as soil genesis model, focusing on diversity and abundance of predominant populations related to *Bacillus benzoevorans*. 16S rRNA gene-targeted DGGE fingerprinting showed differences in the composition of *B.benzoevorans*-related and total bacterial communities with time and cropping history. Complementary, we quantified the relative abundance of *B.benzoevorans* relatives by specific dot blot hybridization revealing that they accounted for up to 40% of the total bacterial community. We demonstrate the potential of archived soil samples to study the development, distribution and diversity of microbial communities during the process of soil genesis.

Introduction

In 1932 the Dutch cut off the Zuider Zee from the North Sea by an enclosing dam, resulting in the formation of the IJsselmeer (lake IJssel) that gradually changed into a fresh water ecosystem (30). Reclamation of the so-called polders turned the soft, water-saturated and unaerated sediment into typical arable soil. The polder soil environment is in fact a reset ecosystem where bacteria re-start their colonization due to the drastic changes in the environmental conditions. From a microbiologist's perspective such ecosystem has just been born, in an environment with fit nature allowing for de novo bacterial succession. The induced physical (25), chemical (33) and microbiological (30) changes have been documented extensively. In earlier studies, cultivation techniques were used to isolate bacteria involved in nitrogen cycling (30). Recently, we applied cultivation-independent techniques and detected an abundant spore forming group related to *Bacillus benzoevorans* (28). *B. benzoevorans* relatives were previously identified as predominant microorganisms (up to 32%) in Dutch Drentse A soil samples (5, 7), but also in soil samples from other soil environments throughout The Netherlands and in other countries (1, 2, 17, 28). Using archived air-dried soil samples (TAGA collection, ALTERRA BV, Wageningen, The Netherlands), we previously followed their succession in a developing polder vague soil during the period 1942-1975 by 16S rRNA gene-based denaturing gradient gel electrophoresis (DGGE) profiling, showing their potential as bio-indicator for soil succession (28).

Materials and Methods

Soil samples

The soil samples used in this study were collected during different years from different plots from the top 0-25 cm layer of an experimental field Wieringerwerf in the Wieringermeer polder (The Netherlands). Samples were air-dried at 30- 40 ºC and further stored at room temperature. They were provided from the TAGA collection, an archive of soil, crop and manure samples that is maintained by Alterra B.V., Wageningen, The Netherlands. The archive contains about 250.000 samples from previous experiments, starting in 1879, plus data and information on the experiments from which the samples were taken. Land reclamation by drainage of the former sea bottom in the Wieringermeer region was

started in 1930 and completed in 1940, followed by transformation into agricultural land (30). To describe the changes in the total bacterial and *B. benzoevorans*-related community after land reclamation, samples of years 1960, 1962, 1965, 1968, 1971, 1973, 1975, 1981, 1984 and 1986 were analyzed. Each year was presented with three samples collected from plots where different crops were planted. For 1986, only two samples could be analyzed. The crop rotation started in 1954 and the first experimental year was 1955. The crop rotation of the examined samples is shown in Table 1. Samples from experimental field Wieringerwerf in the Wieringermeer polder before 1960 were unfortunately not available in the soil collection.

DNA isolation, primers and PCR conditions

DNA was isolated directly from soils (1 g) using the Fast DNA®SPIN®Kit For Soil (Q BIOgene, Cambridge, UK) according to the manufacturers' instructions. The kit has been designed for efficient lysis of all microorganisms, including Gram-positive bacteria, yeast, algae, nematodes and fungi, as well as bacterial spores and endospores. DNA isolation was performed in triplicate, and DNA extracts were checked with universal primers 27f and 1492r for sufficient material and purity for 16S rRNA gene-targeted PCR amplification (14).

All primers and probes used in this study are listed in Table 2. Primers for specific amplification of *B. benzoevorans* relatives 16S rRNA genes were used in a nested approach of two successive specific PCR`s (28). PCR products that were obtained after amplification with primers REX460f/REX1466r were used as a template for PCR with primer set REX576f/REX1446r at a concentration of 1.0 to 2.6 ng of DNA per 50 µl reaction. Subsequently REX576f/REX1446r amplicons were used as template for DGGE-PCR with primers 0968-GCf and 1401r (21).

Primers	Sequence	Reference
27f	5'- GTT TGA TCC TGG CTC AG-3'	(14)
1492r	5'- CGG CTA CCT TGT TAC GAC-3'	(14)
REX460f	5'-GAG TAA CTG C(T/C)(C/G) GTA CC-3'	(28)
REX1466r	5'-CCA ATC ATC TGT CCC ACC TTA-3'	(28)
REX576f	5'-AAG CGC GCG CAG GCG GTC CT-3'	(28)
REX1446r	5'-CCT TAG GCG GCT GGC TCC TTA-3'	(28)
GC-0968f	5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA CCT TAC-3'	(21)
1401r	5'- GCG TGT GTA CAA GAC CC-3'	(21)
0968f	5'- AAC GCG AAG AAC CTT AC-3'	(14)
F0985PTO	5'- AAC GCG AAG AAC CTT ACC-3'	(11)
R1046PTO	5'- ACA GCC ATG CAG CAC CT-3'	(11)
Univ1390	5'- GAC GGG CGG TGT CTA CAA-3'	(31)

Table 2. DNA oligonucleotide primers and probes used in this study.

PCR was performed with *Taq* polymerase (Invitrogen, Carlsbad, USA) in a Whatman Biometra Thermocycler (Göttingen, Germany), using the conditions of the *B. benzoevorans* relatives specific PCR as described previously (28). PCR with primers 0968GCf/1401r was performed as described by Nübel *et al.* (21). PCR with universal primers 27f and 1492r was performed with the following conditions: 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 48 °C for 20 sec, and 68 °C for 40 sec, and finally 68 °C for 7 min.

For preparation of the *B. benzoevorans*-relatives specific V6 probe, PCR with F985PTO and R1046PTO was performed (11). As template for this reaction, the products after PCR with REX576f/REX1446r were used. The PCR conditions were: 94 °C for 2 min, 40 cycles of 94 °C for 20 sec, 50 °C for 40 sec, and 72 °C for 40 sec, and 72 °C for 5 min.

Analysis of PCR products by DGGE

The PCR products obtained after three nested PCR reactions with primer sets, as follows REX460f/REX1466r, REX576f/REX1446r and 0968-GCf/1401r, and those after PCR with universal DGGE primers 0968-GCf/1401r, were separated by DGGE (19, 20). 16S rRNA gene-targeted PCR-DGGE fingerprinting was used to analyze the profiles of the *B. benzoevorans* relatives from different soil samples. A gradient of 38-50% of the denaturing chemicals (100% denaturing conditions are defined as 7 M urea and 40% formamide) was used and gel electrophoresis was performed according to Heilig *et* al. (10). The gels were stained with $AgNO_3$ according to the method of Sanguinetti *et al.* (24), scanned at 400dpi, and

analyzed using the software Bionumerics 4.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). The similarity between DGGE profiles was determined by calculating similarity indices of the densitometric curves of the profiles compared using the Pearson product-moment correlation (9, 32). The UPGMA algorithm was used as implemented in the analysis software for the construction of dendrograms.

B. benzoevorans-relatives specific probe and universal bacterial oligonucleotide probe

A specific probe for *B. benzoevorans*-relatives was generated by amplifying the V6 region of the 16S rRNA gene in a nested approach as follows. First, *B. benzoevorans*-relatives specific amplification was performed with primer sets REX460f/REX1466r and REX576f/REX1446r (28). PCR amplicons from the nested PCR were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and the concentration of the purified *B. benzoevorans*-relatives specific PCR products was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). The 16S rRNA gene fragments amplified from the different soil samples were mixed in even aliquots to yield a stock solution of 15 ng/µl. One µl of this stock solution was used as a template for amplification with universal F985PTO/R1046PTO primers as described above. The PCR amplicons were purified, using the QIAEX II kit (Qiagen, Hilden, Germany), and subsequent enzymatic removal of the primer sites from the PCR products up to the phosphorothioate bond was performed as described by Heuer *et al.* (11). Their concentration was measured as described above and 25 ng was used as a *B. benzoevorans*-relatives-specific V6 probe.

The universal oligonucleotide probe S-*-Univ-1390-a-A-18 was used in this study to allow for calculation of relative abundance of *B. benzoevorans*-relatives (31).

Dot blot and Southern blot hybridization

DNA from each soil sample was used as a template for amplification with universal primers (27f/1492r). Hundred nanograms of PCR product was denatured using 7 µl formaldehyde, 21 µl formamide, 2 µl 20× SSC and heat (10 min at 68 ºC), and blotted in duplicate onto a Hybond-N+ membrane (Amersham BioSciences, UK). For a standard dilution series, serial diliutions (100, 50, 25, 10 and 5 ng per dot) of PCR products obtained after nested PCR with primer sets REX460f/REX1466r and REX576f/REX1446r were used. The membrane was briefly rinsed in 0.5× TBE buffer, treated with 0.4 N NaOH for 10 min,

and rinsed with 2× SSC for 10 min. Finally, DNA was UV-cross-linked to the membrane for 1 min at 120 mJ in a UV Stratalinker 2400 (Stratagene, La Jolla, USA).

DGGE gels for Southern blot hybridization were stained using ethidium bromide. The gel was destained by shaking for 15 min in 0.5× TBE buffer (23). A sandwich from gel-sized Whatman filter paper pieces and one sheet of Hybond-N+ (Amersham BioSciences, Piscataway, NJ), directly placed on the gel was assembled into a TransBlot SD Electrophoretic Transfer Cell (Bio-Rad, Veenendaal, The Netherlands), and a current of 400 mA was applied for 1 h. After transfer, the membrane was separated from the gel and rinsed briefly in 0.5× TBE buffer. The DNA was denatured with 0.4 N NaOH for 10 min. Subsequently the membrane was rinsed in 2× SSC for 10 min and baked at 80 ºC for 1 h.

The *B. benzoevorans*-specific V6 probe was labeled with 50 µCi [α-^{32}P]dCTP (Amersham BioSciences) using DNA polymerase I, Large (Klenow) fragment. The labeling reaction was incubated for 1h at room temperature in a total volume of 50 µl, using the Prime-a-Gene labeling system (Promega, Leiden, The Netherlands), and the labeling was terminated by heating at 95-100 ºC after adding 100 µl of 10 mg/ml salmon sperm DNA solution. The hybridization was performed over night at 68 ºC in 30 ml preheated QuickHyb hybridization buffer (Stratagene, Cambridge, UK). The membrane was washed with 2× SSC+ 0.1% SDS two times for 15 min at room temperature and once with preheated 0.2× SSC + 0.1% SDS for 30 min at 68 ºC.

The oligonucleotide probe S-*-Univ-1390-a-A-18 was 5'-end labeled with 50 µCi [γ-^{32}P]dATP (Amersham BioSciences) using T4-polynucleotide kinase and Promega 5'-labeling kit. The labeling reaction was incubated at 37 ºC for 90 min in a total volume of 20 µl. The hybridization was performed over night at 42 ºC in 30 ml preheated QuickHyb hybridization buffer (Stratagene). The washing was performed twice for 30 min with preheated 1× SSC + 1% SDS at 44 ºC.

The washed membranes were kept over night in Molecular Dynamics phosphor screens (Amersham BioSciences), scanned using a Molecular Dynamics STORM system, and the signals were quantified using Image Quant (Version 2003.01).

The hybridizations were performed in duplicate and average values of signal intensity were calculated. As a measure for the relative abundance of *B. benzoevorans*-relatives, the ratio of signals obtained with V6 specific probe and S-*-Univ-1390-a-A-18 was used. To account for differences in relative signal intensity between the two probes the ratio was corrected with a factor of 1/3.1. The correction factor was determined by normalization of ratios measured for the standard curve to 1.

Statistical analysis

Student's t-test was used to assess statistical significance of clustering based on similarity indices calculated from the DGGE profiles.

To assess to what extend environmental variables can explain the variation in soil microbiota diversity, canonical correspondence analysis (CCA) was chosen, as it explains the structure of the data in the "species" table (in this case, band intensities) by environmental variables, assuming a unimodal distribution of species (22, 27). For that purpose, community structure based on the surface and position of the bands per lane were analyzed performing CCA as implemented in the CANOCO 4.5 software package (Biometris, Wageningen, The Netherlands). Community similarities were visualized by using ordination plots with scaling focused on inter-sample difference (18). The ordination plot of species and environmental variables is characterized by a biplot that approximates the weighed averages of each species with respect to each of the environmental variables. Thus, the ordination diagram represents not only a pattern of community distribution, but also the main features of the distribution of species along the environmental variables (27). The classes of the nominal environmental variables, such as crop and plot, are symbolized by centroids (the weight average). Sampling time, relative abundance data based on dot blot quantification, as well as meteorological data were considered as quantitative environmental variables and are represented as vectors. The length and the slope of the vector are significant parameters. Long vectors forming smaller angle with an ordination axis are more strongly correlated with that axis (26). The angle between the vectors provides an approximation of their correlation. Vectors pointing in the same direction (angle $< 90°$) are positively correlated and those in opposite directions are negatively correlated (15, 27). The position of the centroids representing nominal variables determines the relationship of these variables with either of the ordination axes (26).

In order to check the homogeneity of the data set, detrending correspondence analysis (DCA) was applied, using detrending by segments as the selected method. As the gradient length with the data set analyzed here was 3.826, CCA was chosen as the appropriate method (15).

To test the significance of the relationship of community response with environmental variables, a Monte Carlo permutation test was performed with 499 random permutations, and a significance level (p) of 0.05 was chosen. The permutation was unrestricted.

The meteorological data were extracted from the public available information of the Royal Dutch Meteorological Institute (Koninklijk Nederlands Meteorologisch Instituut (KNMI) http://www.knmi.nl/).

Results and Discussion

The present study for the first time explored the succession of a bacterial community over a long time sequence in young soils reclaimed from the sea. Succession was assessed by analyzing the developments in diversity and relative abundance of *B. benzoevorans*-related as well as total bacteria. Well documented samples were collected during a period of 26 years in the framework of a long term field experiment, which assessed the agronomic effects of several crop rotations. This allowed us to weigh the impact of time on succession as well as that of other parameters, such as climate, plot location and crop regime. The samples were up to 44 years old and air-dried. It has recently been shown that such soil samples can be used for assessing microbial community structure using biomolecular techniques (3, 28). Similar strategies were suitable to explore microbial community dynamics after reclaiming land from sea, and the effect of agricultural practice on the microbial communities shortly after reclamation; processes that are not yet fully understood. To assess the diversity of the *B. benzoevorans* related and total bacterial communities, 16S rRNA gene-targeted DGGE fingerprinting was used, while a lineage-specific auto-probing dot blot hybridization approach was developed for quantitative detection of *B. benzoevorans* relatives.

A total of 29 archived soil samples were taken from the Wieringerwerf experimental field (Wieringermeer polder, The Netherlands), and were collected in 1960, 1962, 1965, 1968, 1971, 1973, 1975, 1981, 198, and 1986, after which the field experiment was terminated. To measure the relative abundance of *B. benzoevorans* related bacteria in the studied soil samples, a V6 probe specific for this group was developed (Fig. 1).

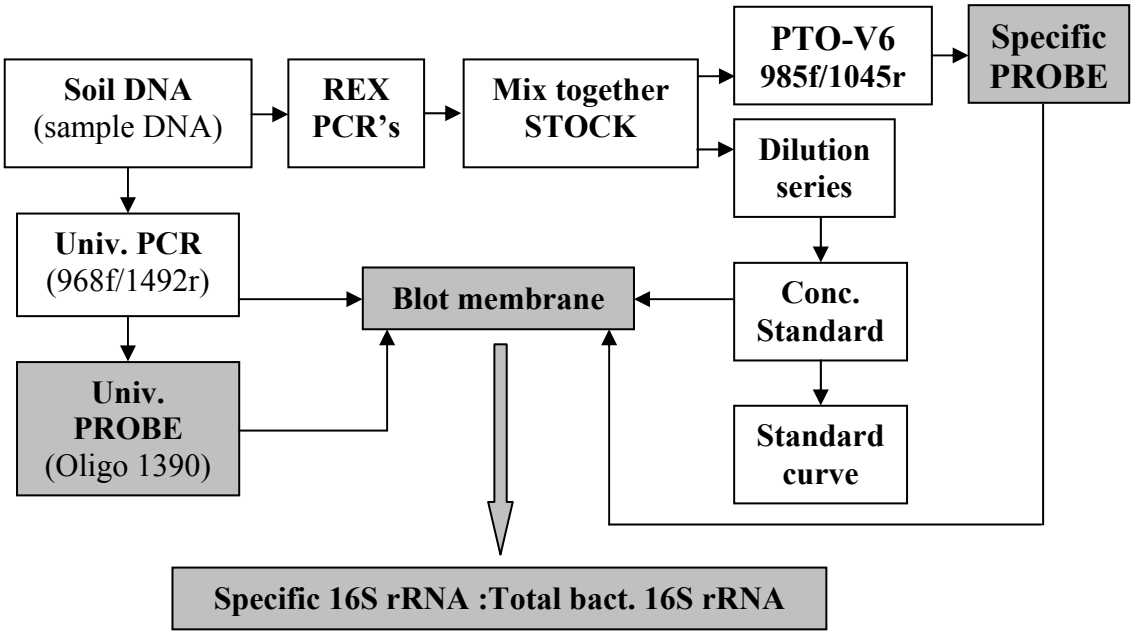


Figure 1. Scheme of the quantitative dot-blot hybridization method. Stars (*) indicate steps where the DNA concentration was measured.

16S rRNA genes were amplified from all samples with a nested approach specific for *B. benzoevorans*-relatives (28) and afterwards pooled in equal amounts. This yielded a mix of *B. benzoevorans* related DNA, representative and specific for the studied set of soils. This pool was used as a template for V6-targeted PCR amplification using PTO-modified primers for the generation of the group-specific probe. As such primers allow for the post-amplification removal of universal primer sequences, the chance for subsequent cross-hybridization is minimal. Yield of DNA directly extracted from the samples was low, as could be expected for air-dried samples of up to 44 years of age. 16S rRNA genes were first amplified with universal primers, immobilized on nylon membranes, and subsequently hybridized with specific and universal probes.

On average about 21 % of the 16S rRNA gene pool amplified from the studied soil samples belonged to *B. benzoevorans*-relatives. In each of the three plots, the amount of *B. benzoevorans*-relatives was fluctuating but in general the amount increased with time (Fig. 2).

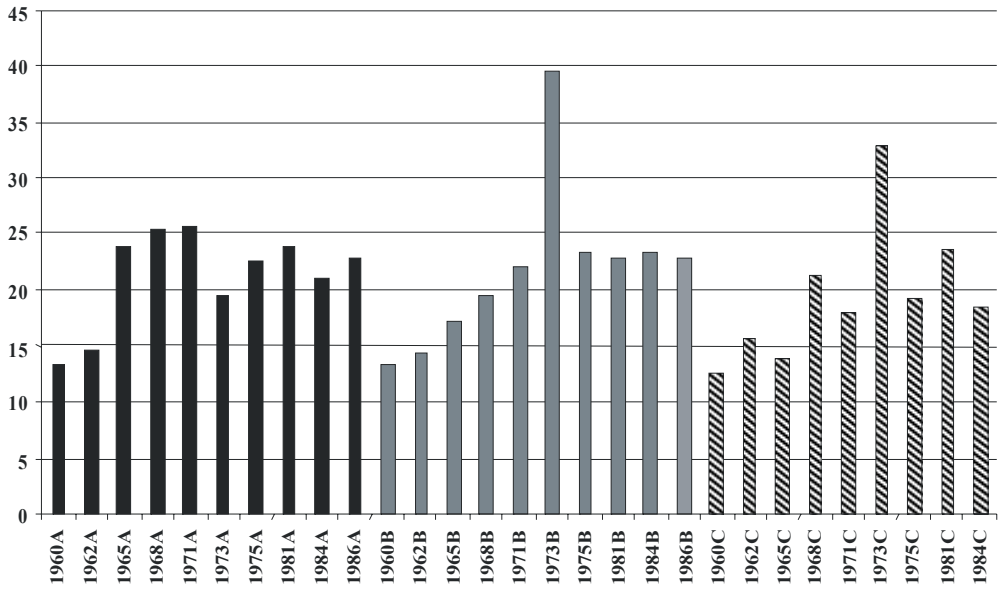


Figure 2. Relative abundance of *B. benzoevorans* relatives in air-dried soil samples from Wieringermeer polder as analyzed by 16S rRNA gene-targeted dot blot hybridization. X- axis, year of sampling; A-C, experimental plot; Y-axis, relative abundance (%) of *B. benzoevorans* relatives.

Their relative abundance increased from 13 % to ca. 20 % in the sixties, where it stabilized at that level. Only samples 1973B and C showed a significantly higher abundance of *B. benzoevorans*- relatives (>30%). The specificity of the developed auto-probing was confirmed by Southern blot hybridization of a DGGE gel containing total bacterial and *B. benzoevorans*-specific profiles from three soil samples (Fig. 3).

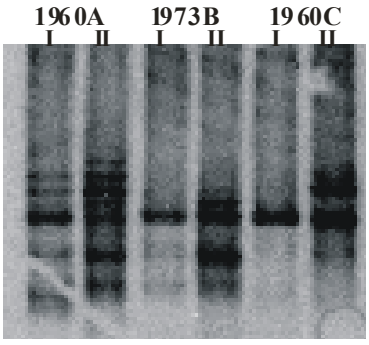


Figure 3. Southern blot hybridization of DGGE gel. 1960 and 1973 indicate the year of sampling. A, B and C indicate the experimental plot. I- total bacterial profile, II- *B. benzoevorans* related profile.
Hybridization was repeated with duplicate gels, yielding identical results (not shown).

Total bacterial- and *B. benzoevorans*-relatives-specific 16S rRNA gene targeted PCR-DGGE analysis was used to determine whether the respective communities changed over time

and with cropping regime. Cropping regime was specified by annual rotation of winter wheat, oat and potato (respectively plots A, B and C) (Table 1).

Year	Plot A	Plot B	Plot C
1960	Winter wheat	Potato	Oats
1962	Potato	Oats	Winter wheat
1965	Potato	Oats	Winter wheat
1968	Potato	Oats	Winter wheat
1971	Potato	Oats	Winter wheat
1973	Oats	Winter wheat	Potato
1975	Winter wheat	Potato	Oats
1981	Winter wheat	Potato	Oats
1984	Winter wheat	Potato	Oats
1986	Oats	Winter wheat	---

Table 1. Sampling years and planted crop per year and per experimental plot from Wieringermeer polder (experimental field Wieringerwerf).

During the entire period, the DGGE profiles obtained from soil samples collected from the three different plots showed differences in the *B. benzoevorans*-related (Fig. 4) and total bacterial community (Fig. 5) with respect to number and position of bands. For several soil samples, all bands observed in total bacterial profiles matched with those of the *B. benzoevorans*-relatives (i.g. 1973A, 1981A, 1984A, 1986A, 1984C). In samples taken before 1973A and for almost all samples from plots B and C, additional dominant bands were observed besides those related to *B. benzoevorans*. Overall, this dominance of *B. benzoevorans*-related bands confirmed their high relative abundance observed by dot blot hybridization. The fact that for some samples no additional bands were observed while dot blot hybridization indicated a relative abundance of only 15-30 % suggests that in these samples, a large number of populations not related to *B. benzoevorans* were present at low individual abundance thus not detectable by DGGE.

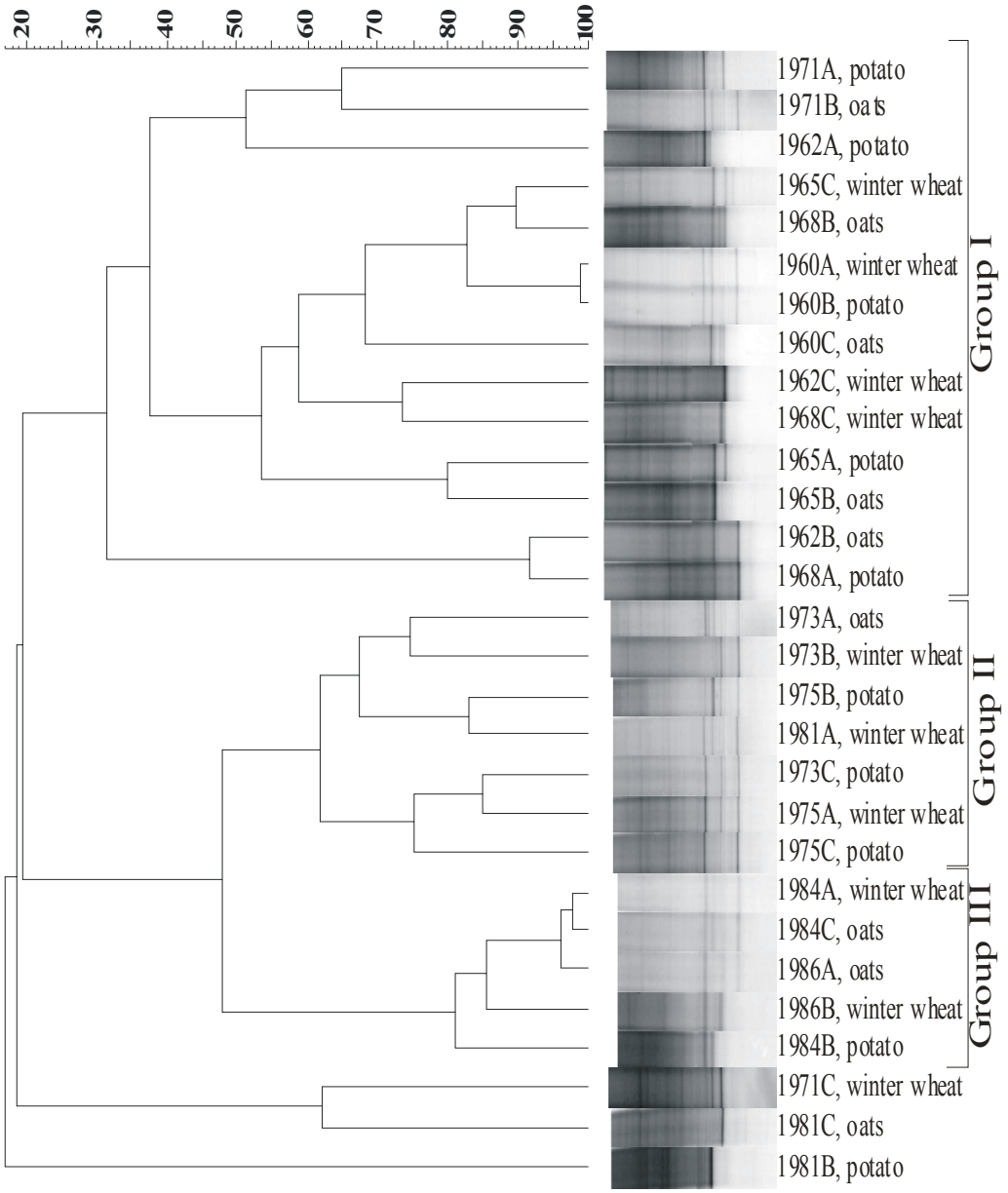


Figure 4. DGGE profiles of the *B. benzoevorans* related community in Wieringermeer polder soil samples from different years, and corresponding dendogram (UPGMA clustering). 1960-1986, year of sampling; A-C, experimental plot. Groups I-III cluster the soil samples collected during the 1960s the '70s and the '80s, respectively.

To determine the relationship between the bacterial communities isolated from soil samples from different years and different crops, similarity indices of the DGGE profiles were calculated, and used for cluster analysis. With few exceptions, *B. benzoevorans* relatives clustered into groups, depending mainly on the time of sampling, i.e. group I (sixties), group II (seventies) and group III (eighties) (Fig. 4). The significance of group separation was confirmed by Student's t-test (data not shown). Similarity indices among samples within the different groups were high, and varied between 50 and 99 %. No grouping of samples by crop

could be found, which can possibly be attributed to the fact that bulk soil was analyzed rather than rhizosphere material (13).

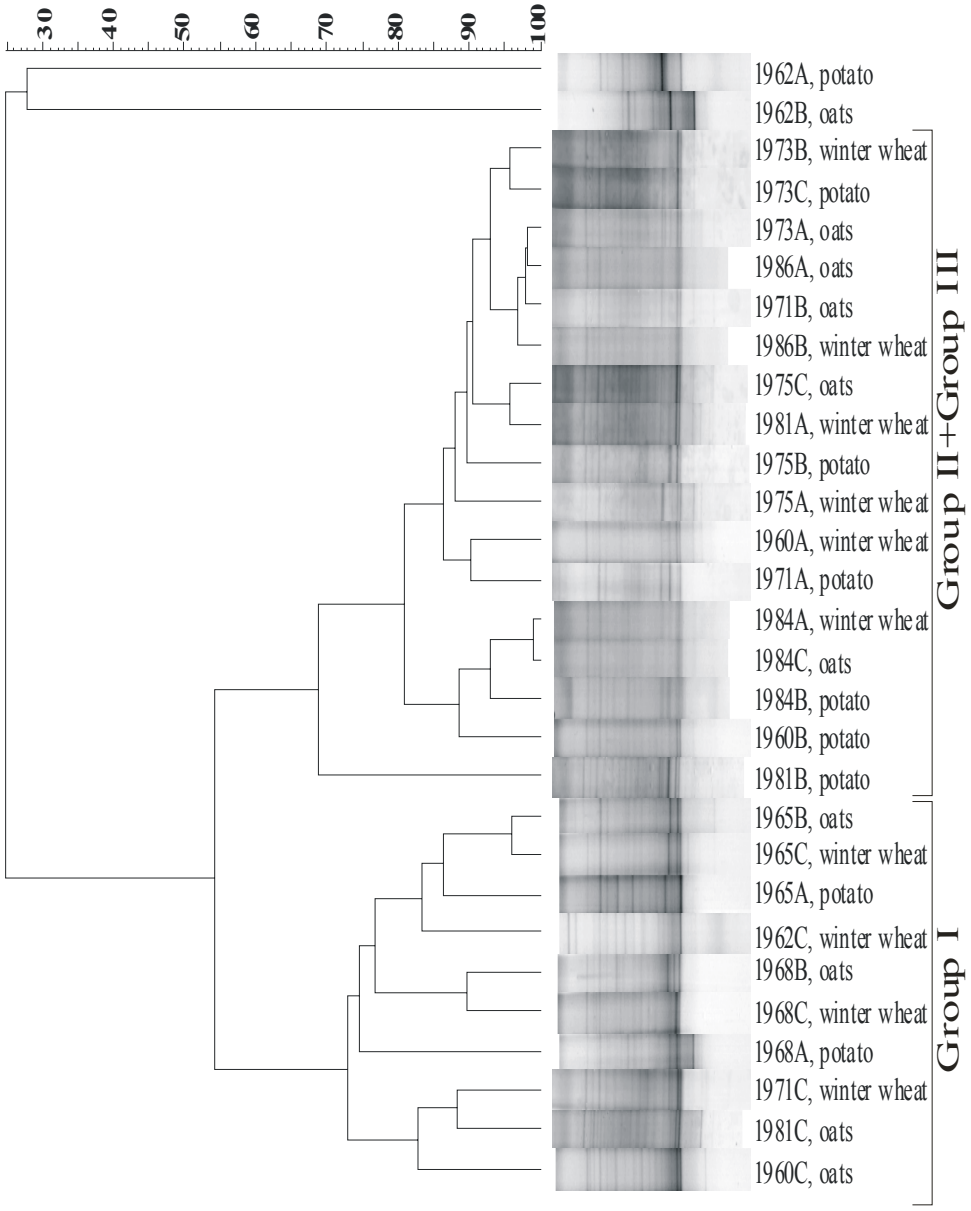


Figure 5. DGGE profiles of the total bacterial community in Wieringermeer polder soil samples from different years, and corresponding dendogram (UPGMA clustering). 1960-1986, year of sampling; A-C, experimental plot. Group I clusters the soil samples collected during the '60s. Group II + Group III clusters the soil samples collected during the '70s and '80s.

Similar effects were found for the total bacterial profiles, although the grouping of the samples was not as clear (Fig. 5). Group I was still separated, while groups II and III clustered together. The similarity indices among the samples were again high (between 68 and 96 %).

Using canonical correspondence analysis (CCA) we could visualize the effect of environmental variables (time, crop, plot, average summer temperature and average summer

precipitation) on the *B. benzoevorans* related community in the studied soil samples based on DGGE fingerprints (Fig. 6).

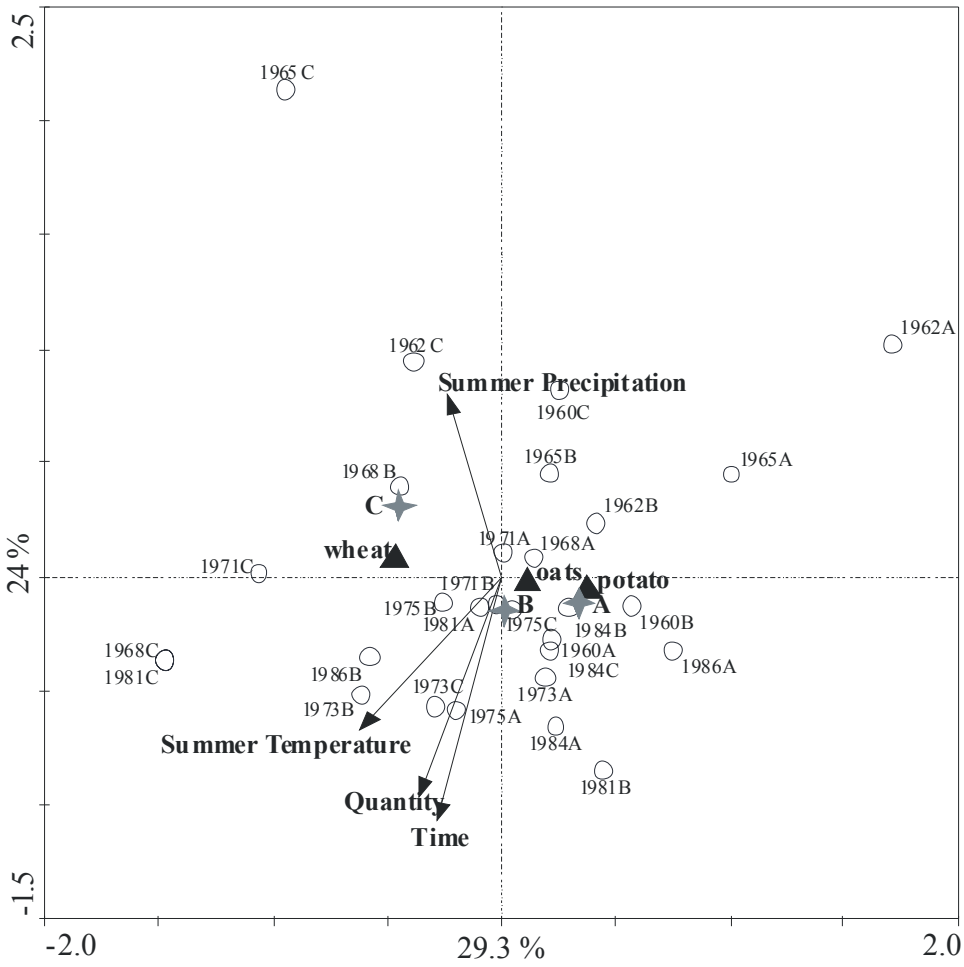


Figure 6. Ordination plot of *B. benzoevorans* related community in Dutch polder soil samples collected over a period of 26 years under different crop rotation. Open circles (○) visualize the samples; A-C, different experimental plots with crop rotation; 1960-1986, year of sampling; vectors (→), quantitative environmental variables (time, relative abundance of *B. benzoevorans* relatives, average summer temperature and average summer precipitation); filled triangles (▲), crops as nominal environmental variables; filled stars (✦), plots as nominal environmental variables.

Though no significant effect (P>0.1) of either of the studied environmental factors was found, samples generally clustered according to the time (i.e. year of sampling), rather than to plot (A, B, C) or crop. Remarkably, the relative abundance of the *B. benzoevorans*- related community showed a positive correlation with time and average summer temperatures. Furthermore, plot C showed to be more homogeneous than plots A and B. A clear distinction between oat and winter wheat could also be observed. While the quantitative environmental variables time, relative abundance of *B. benzoevorans*-relatives and average summer temperature showed strong negative correlation with the second canonical axis in the CCA

biplot, which explains 24 % of the variation, summer precipitation showed an opposite correlation.

The DGGE profiles of the *B. benzoevorans* relatives revealed low richness, as could be expected from previous studies on the same polder (28, 30). Nevertheless, our methods were instrumental to identify fluctuations in the community composition of the *B. benzoevorans* relatives even in archived samples stored for more than 40 years. All bands from the *B. benzoevorans*-related profiles could be detected as dominant bands in the total bacterial DGGE fingerprints. This suggested that *B. benzoevorans* relatives constituted a predominant group of bacteria in the Wieringermeer polder during the studied period, in line with findings from previous studies for other soils (4, 8, 28). In that respect, it was remarkable that for several samples the dominant fraction of the total bacterial community was even almost identical to that of the *B. benzoevorans* relatives as could be visualized by DGGE analysis.

Possible explanations for the observed low overall bacterial richness could be that the soil samples were collected from an agricultural area, where the soil conditions were strictly controlled by farming practices (16, 22, 29). The fact that dot-blot hybridization indicated that the relative abundance of *B. benzoevorans* relatives increased from 12.5 to 39.6 % in time (Fig. 2), suggested that the prolonged storage most probably did not significantly biased the detected bacterial community structure towards these spore-forming bacteria. It can, however, not be excluded that the soil microbiota was characterized by a high number of populations with low relative abundance (<1 %) that would elude detection by DGGE fingerprinting. This could also explain the discrepancy with the quantification by dot blot hybridization, which indicated an overall relative abundance of non-*B. benzoevorans* relatives between 60 and 87%.

Crop rotation did not significantly affect community dynamics. As in this study bulk surface soils were analyzed, this observation is in line with previous studies, which showed that the crop only affected the rhizosphere community rather than the bulk soil community (12, 13). The collection of soil samples analyzed here was a unique source of information, for the first time providing us with the opportunity to explore in detail the microbial dynamics in Dutch polder lands shortly after their reclamation.

We developed and applied qualitative and quantitative approaches to monitor *B. benzoevorans*-related soil bacteria that are remarkably predominant in soils around the world, in relation to the total bacterial community.

Until now, the yet-greatly unexplored but highly abundant *B. benzoevorans* related community was quantitatively estimated based on clone libraries (7) or using reverse-transcript PCR in combination with DGGE (6), both time- and labor- consuming methods. The comprehensive set of qualitative and quantitative approaches for monitoring of *B. benzoevorans*-related bacteria in different soil samples described here is, in comparison, fast, specific and applicable directly to environmental samples. Archived soil samples offer the unique possibility to follow the behavior of soil microbiota over a long period of time. We could show for Dutch polder samples that the time has a major effect on the development of the *B. benzoevorans*-related as well as total community. The present study for first time allowed for retrospective analysis of diversity and dynamics of this abundant group of microbes in archived soils during a long period of time in a unique and successively changing environment.

Acknowledgements

This work was supported by a grant from the European Communities EC project "Exploration of Genomic and Metabolite Diversity of a Novel Group of Abundant Soil Bacteria" (BACREX-project QLK3-2000-01678). We are grateful to Joana Salles and Petr Šmilauer for their valuable help with multivariate analysis using CANOCO. The Royal Netherlands Meteorological Institute is acknowledged.

References

1. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:**1935-1943.
2. **Borneman, J., and E. W. Triplett.** 1997. Molecular microbial diversity in soils from Eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Environ. Microbiol. **63:**2647-2653.
3. **Dolfing, J., A. Vos, J. Bloem, P. A. I. Ehlert, N. B. Naumova, and P. J. Kuikman.** 2004. Microbial diversity in archived soils. Science **306:**813.
4. **Felske, A.** 1999. Reviewing the DA001-files: A 16S rRNA chase on suspect #X99967, a *Bacillus* and Dutch underground activist. J. Microbiol. Meth. **36:**77-93.
5. **Felske, A., and A. D. L. Akkermans.** 1998. Spatial homogeneity of the most abundant bacterial 16S rRNA molecules in grassland soils. Microbiol. Ecol. **36:**31-36.
6. **Felske, A., A. D. L. Akkermans, and W. M. de Vos.** 1998. Quantification of 16S rRNAs in complex bacterial communities by multiple competitive reverse transcription-PCR in temperature gradient gel electrophoresis fingerprints. Appl. Environ. Microbiol. **64:**4581-4587.
7. **Felske, A., A. Wolterink, R. van Lis, and A. D. L. Akkermans.** 1998. Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). Appl. Environ. Microbiol. **64:**871-879.
8. **Felske, A., A. Wolterink, R. van Lis, W. M. de Vos, and A. D. L. Akkermans.** 1999. Searching for predominant soil bacteria: 16S rDNA cloning versus strain cultivation. FEMS Microbiol. Ecol. **30:**137-145.
9. **Häne, B. G., K. Jäger, and H. Drexler.** 1993. The pearson product-moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. Electrophoresis **14:**967-972.
10. **Heilig, H. G. H. J., E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. L. Akkermans, and W. M. de Vos.** 2002. Molecular diversity of *Lactobacillus spp.* and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ. Microbiol. **68:**114-123.
11. **Heuer, H., K. Hartung, G. Wieland, I. Kramer, and K. Smalla.** 1999. Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community fingerprints. Appl. Environ. Microbiol. **65:**1045-1049.
12. **Horner-Devine, M. C., M. Lage, J. B. Hughes, and B. J. M. Bohannan.** 2004. A taxa-area relationship for bacteria. Nature **432:**750-753.
13. **Kowalchuk, G. A., D. S. Buma, W. de Boer, P. G. Klinkhamer, and J. A. van Veen.** 2002. Effects of above-ground plant species composition and diversity on the

diversity of soil-borne microorganisms. Antonie Leeuwenhoek **81:**509-520.

14. **Lane, D. J.** 1991. 16S/23S rRNA sequencing. John Wiley & sons Ltd, UK.
15. **Leps, J., and P. Smilauer.** 2003. Multivariate analysis of ecological data using CANOCO. University Press, Cambridge.
16. **Lin, X. G., R. Yin, H. Y. Zhang, J. F. Huang, R. R. Chen, and Z. H. Cao.** 2004. Changes of soil microbiological properties caused by land use changing from rice-wheat rotation to vegetable cultivation. Environ. Geochem. Health **26:**119-128.
17. **Lloyd-Jones, G., and P. C. Lau.** 1998. A molecular view of microbial diversity in a dynamic landfill in Quebec. FEMS Microbiol. Lett. **162:**219-226.
18. **Marschner, P., and K. Baumann.** 2003. Changes in bacterial community structure induced by mycorrhizal colonisation in split-root maize. Plant Soil **251:**279-289.
19. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695-700.
20. **Muyzer, G., and K. Smalla.** 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Leeuwenhoek **73:**127-141.
21. **Nübel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig, and H. Backhaus.** 1996. Sequence hetereogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis of 16S rDNA fragments. J. Bacteriol. **178:**5636-5643.
22. **Salles, J. F., J. A. van Veen, and J. D. van Elsas.** 2004. Multivariate analyses of *Burkholderia* species in soil: effect of crop and land use history. Appl. Environ. Microbiol. **70:**4012-4020.
23. **Sambrook, J., E. F. Fritsche, and T. Maniatis.** 1989. Molecular Cloning a laboratory manual., vol. 3. Cold Spring Harbor Laboratory Press, USA.
24. **Sanguinetti, C. J., D. Neto, and A. J. G. Simpson.** 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques **17:**915-919.
25. **Smits, H.** 1962. De fysische rijping der gronden in de IJsselmeerpolders (The physical ripening of the soils in the IJssel-lake polders). Van Zee Ld. **32:**7-13.
26. **Steenwerth, K. L., L. E. Jackson, F. J. Calderon, M. R. Stromberg, and K. M. Scow.** 2002. Soil microbial community composition and land use history in cultivated and grassland ecosystems of coastal California. Soil Biol. Biochem. **34:**1599-1611.
27. **ter Braak, C. J. F.** 1987. The analysis of vegetation-environment relationships by canonical correspondence analysis. Vegetatio **69:**69-77.
28. **Tzeneva, V. A., Y. Li, A. D. M. Felske, W. M. de Vos, A. D. L. Akkermans, E. E. Vaughan, and H. Smidt.** 2004. Development and application of a selective PCR-Denaturing Gradient Gel Electrophoresis approach to detect a recently cultivated *Bacillus* group

predominant in soil. Appl. Environ. Microbiol. **70:**5801-5809.

29. **van Elsas, J. D., P. Garbeva, and J. Salles.** 2002. Effects of agronomical measures on the microbial diversity of soils as related to the suppression of soil-borne plant pathogens. Biodegradation **13:**29-40.

30. **van Schreven, D. A., and G. W. Harmsen.** 1968. Soil bacteria in relation to the development of polders in the region of the former Zuider zee. Liverpool Univerity Press, United Kingdom.

31. **Zheng, D., E. Alm, D. Stahl, and L. Raskin.** 1996. Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl. Environ. Microbiol. **62:**4504-4513.

32. **Zoetendal, E. G., A. D. L. Akkermans, W. M. Akkermans - van Vliet, J. A. G. M. de Visser, and W. M. de Vos.** 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Microbial Ecol. Health Dis. **13:**129-134.

33. **Zuur, A. J.** 1962. De chemische rijping der gronden in de IJsselmeerpolders (The chemical ripening of the soils in the IJssel-lake polders). Van Zee Ld. **32:**24-30.

CHAPTER 5

EUKARYOTIC DIVERSITY IN HISTORICAL SOIL SAMPLES

Seung Yeo Moon-van der Staay, Vesela A. Tzeneva, Georg W. M. van der Staay, Willem M. de Vos, Hauke Smidt, and Johannes H. P. Hackstein

FEMS Microbiology Ecology (2006) in press

Abstract

The eukaryotic biodiversity in historical air-dried samples of Dutch agricultural soil has been assessed by random sequencing of an 18S ribosomal RNA gene library and by denaturing gradient gel electrophoresis (DGGE). Representatives of nearly all taxa of eukaryotic soil microbes could be identified demonstrating that it is possible to study eukaryotic microbiota in samples from soil archives that were stored for more than 30 years at room temperature. In a pilot study, 41 sequences were retrieved that could be assigned to fungi and a variety of aerobic and anaerobic protists such as cercozoans, ciliates, xanthophytes (stramenopiles), heteroloboseans, or amoebozoans. A PCR-DGGE analysis of samples collected between 1950 and 1975 revealed significant changes in the composition of the eukaryotic microbiota.

Introduction

Monitoring the impact of global change on the various ecosystems is one of the major challenges for contemporary biology. While the study of communities of macroscopic, multicellular organisms is progressing using well established approaches, analyses of the microbial domain of the various ecosystems are still rather fragmentary. In particular, our knowledge about the role and the dynamics of the microbial eukaryotic communities in soil is very limited (3, 5, 19), although remarkable advances have been made recently using culture-independent, molecular approaches in aquatic environments (2, 14, 17, 35, 37, 38).

Not a single molecular approach addressing eukaryotic biodiversity has been published so far for historical, archivated soil samples, which potentially could reveal changes of the eukaryotic soil microbiota in time. Such historical samples are available, for example, through the TAGA archive of soil, crop and manure samples that is maintained by Alterra B.V., Wageningen, The Netherlands. It has been shown previously that these samples can be used for molecular analyses of prokaryotic diversity (16), in particular of the spore-forming *Bacillus*-group (55). However, since the samples had been air-dried at 42 ^{o}C, homogenized and stored at room temperature for several decades, it remained unclear as to whether any intact eukaryotic DNA could persist in these samples. Here we describe a first attempt to analyze the molecular diversity of microbial eukaryotes in air-dried soil samples that document the land reclamation by drainage of a former sea bottom (Wieringermeer polder, The Netherlands) and its subsequent agricultural use over the years 1950-1975 (53, 56). In this study, a clone library of eukaryotic SSU rRNA genes from a soil sample collected in 1975 was generated and analyzed phylogenetically. Nearly the whole spectrum of eukaryotic soil microorganisms was represented in this sample suggesting that it might be feasible to monitor changes in the composition of the eukaryotic soil microbiota over extended periods of time. A preliminary DGGE analysis of eukaryotic 18S rRNA (SSU rRNA) gene fragments amplified from DNA extracted from soil samples collected between 1950 and 1975 demonstrates that it is possible to assess changes in the composition of the eukaryotic microbiota using air-dried soil samples that were collected decades ago.

Materials and methods

Sample collection and DNA extraction

The soil samples were from the top 0-25 cm layer of non-fertilized areas of an agricultural field in the Wieringermeer polder, The Netherlands, collected between 1942 and 1975. The samples were taken on 15-04-1942 (no eukaryotic amplification products obtained), 28-11-1950, 12-12-1951, 19-03-1966, 05-12-1973, and 16-10-1975. They were collected from the plough layer with an auger. Per soil sample 40 cores were collected. The samples were dried at 30-40 °C, crushed, and sieved over 2 mm to remove plant residues (stubbles, roots), course grit and shells. The samples were stored in the dark at ambient temperatures (~ 15 °C).

The samples were provided from the TAGA archive. TAGA is an archive of soil, crop and manure samples that is maintained by Alterra B.V., Wageningen, The Netherlands (Contact information: Philip A.I. Ehlert, Alterra B.V., P.O. Box 47, 6700 AA Wageningen, The Netherlands. Email: Philip.Ehlert@wur.nl). The archive contains about 250.000 soil samples from experiments performed in the period 1879-1998, plus data and information on the experiments from which the samples were taken. Land reclamation by drainage of the Wieringermeer former sea bottom was set up in 1930 and completed in 1940, followed by transformation into agricultural lands (53), interrupted by a flooding at the end of World War 2. Genomic DNA was isolated directly from 1 g soil after bead-beating using the Fast DNA SPIN Kit (Q BIOgene, Cambs, United Kingdom) according to the manufacturer's instructions. Usually 2-5 micrograms of DNA were recovered from 1g of dried soil sample. If not otherwise mentioned, repeated PCR amplifications and DGGE runs have been performed.

Gene amplification, sequencing, and phylogenetic analysis

For the construction of clone libraries, the eukaryotic 18S (SSU) rRNA genes were amplified by PCR with the oligonucleotide primers targeting the conserved sequences close to the 5' and 3' termini of eukaryotic 18S rRNA genes (38) using DNA from the 1975 sample as template. The PCR product was inserted into pGEM-T Easy plasmid vector (Promega), and *E. coli* XL1 blue cells were transformed with the ligation mixture. 42 recombinant clones from the 18S rRNA gene library were selected randomly for sequencing. The initial comparison of the partial environmental sequences with those from Genbank using FASTA (46) and BLAST searches (1) revealed a high phylogenetic diversity of the clones. All

selected clones were sequenced completely. The CHECK_CHIMERA program of the Ribosomal Database Project (9), the BLAST searches and phylogenetic analyses of separate sequence domains identified one potential chimerical gene artefact, which was excluded from further phylogenetic analyses. Thus, only the non-chimeric 41 recombinant clones were used for the phylogenetic analyses.

Nucleotide sequences were aligned with sequences obtained from the GenBank database using the Clustal X program (31) and refined manually with the aid of the BioEdit program (27). The program Gblocks (6) was used to identify regions of defined sequence conservation. The number of nucleotide positions used in the phylogenetic analyses is shown in the figure legends. A global eukaryotic phylogenetic tree was inferred by the neighbour-joining method with the PHYLIP package (22). Evolutionary distances were calculated with the Kimura two-parameter model, with a transition/transversion ratio of 2.0. The monophyly of the clusters was assessed by using bootstrap replicates. Bayesian analyses that evaluate posterior likelihood probabilities of clades were also performed for the detailed phylogenetic analyses of subgroups with the MRBAYES program version 3.1.1 (30) using the GTR+I+G model (with four gamma-distributed rate categories plus invariant positions) that was selected based on the Akaike Information Criteria (AIC) using Modeltest 3.06 (47). Markov chain Monte Carlo from a random starting tree was initiated and run until the standard deviation of split frequencies reached below 0.01, and trees were sampled every 100 generations. The first 30% of the samples were discarded as 'burnin', and the rest of the samples, after the chain reached apparent stationarity, was used for inferring a Bayesian tree.

Clones with more than 99% sequence identity were considered to be identical, since it cannot be excluded that PCR errors (32) and minor differences between the individual repeats of reiterated or amplified rRNA genes might be responsible for such variations.

Denaturing gradient gel electrophoresis (DGGE)

18S rRNA gene targeted PCR-DGGE fingerprinting was used to analyze the profiles of the eukaryotic community from Wieringermeer soil samples. PCR was performed with *Taq* polymerase (Life Technologies, Gaithersburg, Md.). DNA was amplified in a Whatman Biometra Thermocycler (Göttingen, Germany) with eukaryotic primers Euk516-GC-forward and Euk 1A-reverse using the conditions for specific eukaryotic DGGE PCR as described earlier (15). The PCR products were separated by DGGE (40, 41). Electrophoresis was performed with 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 37.5:1) at

60°C. A gradient of 15-45% of the denaturing chemicals (100% denaturing condition was defined as 7 M urea and 40% formamide) was used, gel electrophoresis was performed according to Heilig *et al.* (29), and the gels were stained with $AgNO_3$ according to the method of Sanguinetti *et al.* (49). Gels were scanned at 400dpi and further analyzed using the software Bionumerics 3.0 (Applied Maths BVBA, Belgium). The similarity between DGGE profiles was determined by calculating similarity indices of the densitometric curves of the profiles compared using the Pearson product-moment correlation (28, 57). The UPGMA algorithm was used as implemented in the analysis software for the construction of dendrograms.

Results and Discussion

DNA from an air-dried soil sample collected in 1975 was used to generate an 18S (SSU) rRNA gene library. After DNA sequencing of 41 randomly chosen clones, the data were analyzed phylogenetically as described in Materials and Methods. A total of 24 different clades, which represent the majority of all microbial eukaryotic taxa known to be present in soil, could be identified. Eight of these clades matched with at least 99% sequence identity to published sequences of known eukaryotic microorganisms: two from cercozoans (WIM2, WIM57), four from fungi (WIM52, WIM38, WIM14, WIM108), and two from green algae (WIM12, WIM107).

The eukaryotic diversity in the soil sample from 1975 is displayed in a phylogenetic tree (Fig. 1). In addition to the above-mentioned clones, WIM103 showed a best (but lower than 99 %) match with known sequences from xanthophytes. Only one (novel) ciliate sequence (WIM26) was found, which differed for more than 1 % from the 18S rRNA sequences from known ciliates. This might correctly reflect the low abundance and diversity of ciliates in certain agricultural soils described earlier (18, 48). However, since a remarkable diversity of ciliates has been described for a variety of soils using a *culture-dependent* approach allowing the recovery of living ciliates from resting cysts (24), the low number of different ciliate species identified here might be a consequence of the failure to extract DNA from resting ciliate cysts. A PCR-bias against the amplification of ciliate sequences is unlikely, since we know from extended studies (25, 26, 39) that our primers are well suited to amplify ciliate rRNA genes. Of course, we cannot exclude odd losses of protist DNA due to

the treatment and storage of the samples. However, the recovery of 14 fungal clones argues against such losses, since our results from the 30 year old sample are comparable to those obtained with "native" soil samples (3, 33, 50). Nine of our clones, represented by WIM14, WIM38, WIM52, WIM108, matched with known fungal sequences of Ascomycetes or Basidiomycetes, respectively. Notably, also two novel clades (WIM27, WIM48, represented by six clones), were clustering within the fungal lineage. They showed only sequence identities of 87 – 89 % to known eukaryotic 18S rRNA gene sequences. Since many novel fungal large subunit rRNA gene sequences were described recently by Schadt et al. (50), as well as novel 18S rRNA gene sequences from freshwater sediments (34, 36), the molecular characterization of the diversity of fungal lineages in both contemporary and ancient soil samples is a challenging task for future analyses. Nine 18S rRNA clones, comprising six different clades, could be assigned to cercozoans. These flagellates are of major ecological importance in soil environments (19, 20, 23) but also in marine and freshwater habitats. They constitute a morphologically very diverse taxon, which was only recently recognized to be monophyletic (8). Recent culture-independent approaches have reported many novel cercozoan sequences (4, 14, 52), suggesting that the diversity of this important group of protists could be much higher than anticipated previously. A more detailed phylogenetic analysis of our clones (not shown) suggested that the clones WIM44, WIM47, and WIM71 belonged to the Heteromitidae. Clone WIM11 appeared to represent a sister clade to a cluster comprising *Pseudodifflugia* cf. *gracilis* and the environmental clone LKM48. One clone, WIM57, matched with the sequence of *Polymyxa betae* (Phytomyxea), a well-known plant pathogen. Therefore, the recovery of six very diverse cercozoan clades out of the total of 41 clones analyzed in this pilot study, suggests that it will be feasible to monitor complex changes within the cercozoan community in time or in response to crop management and different fertilization regimes – even in historical samples of air-dried soil. As many as 12 sequences belonging to 7 different clades were derived from unikont amoebozoans, which, however, could not be placed unequivocally in our global 18S rRNA tree (Fig. 1). Therefore, we performed an additional phylogenetic analysis using Bayesian inference allowing among-site rate variation for these groups, which exhibit largely different evolutionary rates. These phylogenetic analyses revealed the phylogenetic positions of the clades WIM30, WIM1, WIM5, WIM80, WIM81, WIM16, WIM53 within the unikont amoebozoans (Fig. 2).

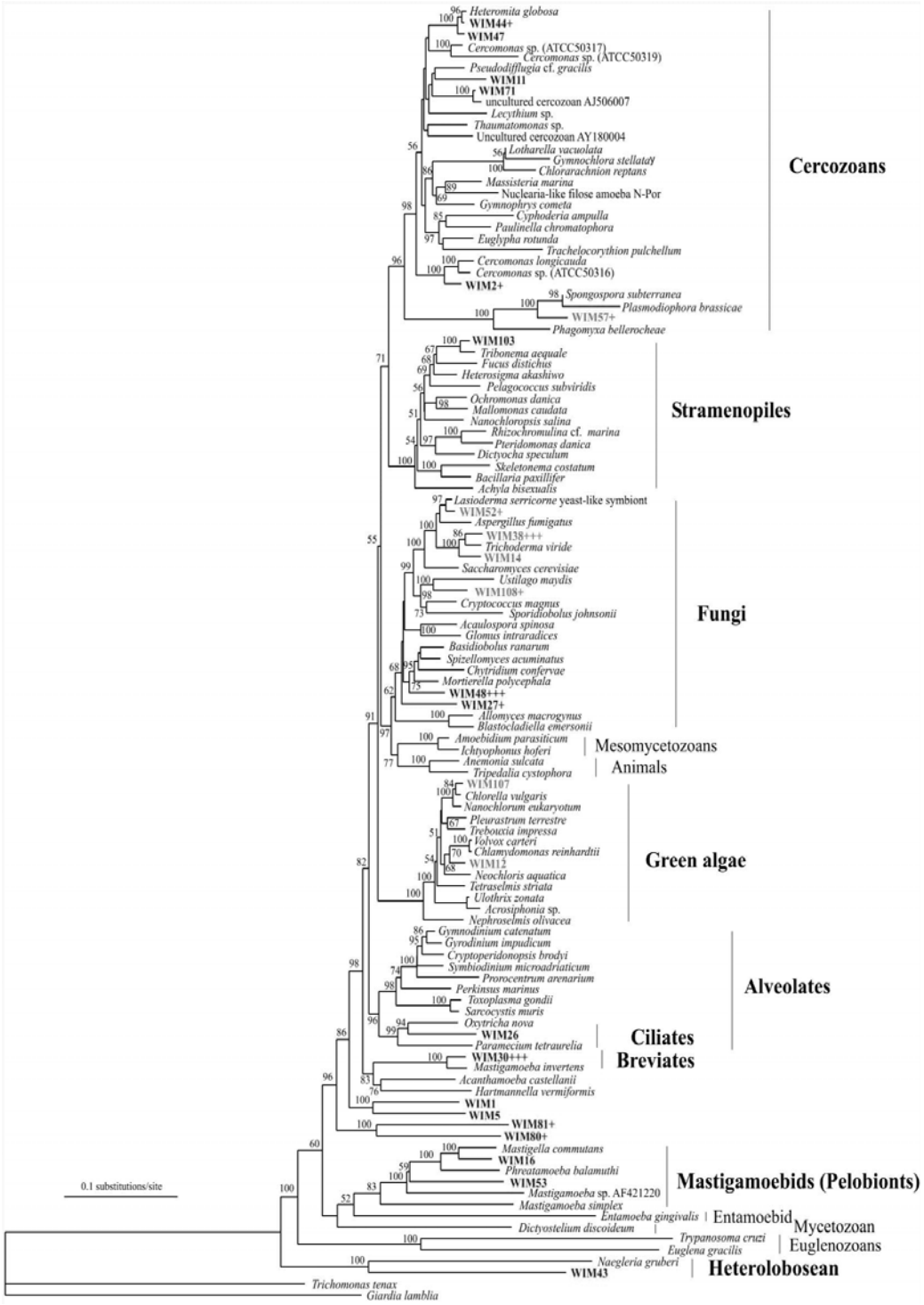


Figure 1. Neighbour joining tree based on nuclear 18S (SSU) rRNA gene sequences using 1615 positions. Sequences beginning with 'WIM' correspond to those retrieved from the Wieringermeer polder soil sample. The sequences matching known sequences with less than 99% sequence identity are marked with an asterisks. Closely related clones from our library with more than 99% sequence identity are indicated as plus (+) next to the clone identifyer. The number of plus' (+) indicates the number of the closely related sequences. Numbers at nodes represent the bootstrap percentages from 100 replicates. Values below 50% are not shown.

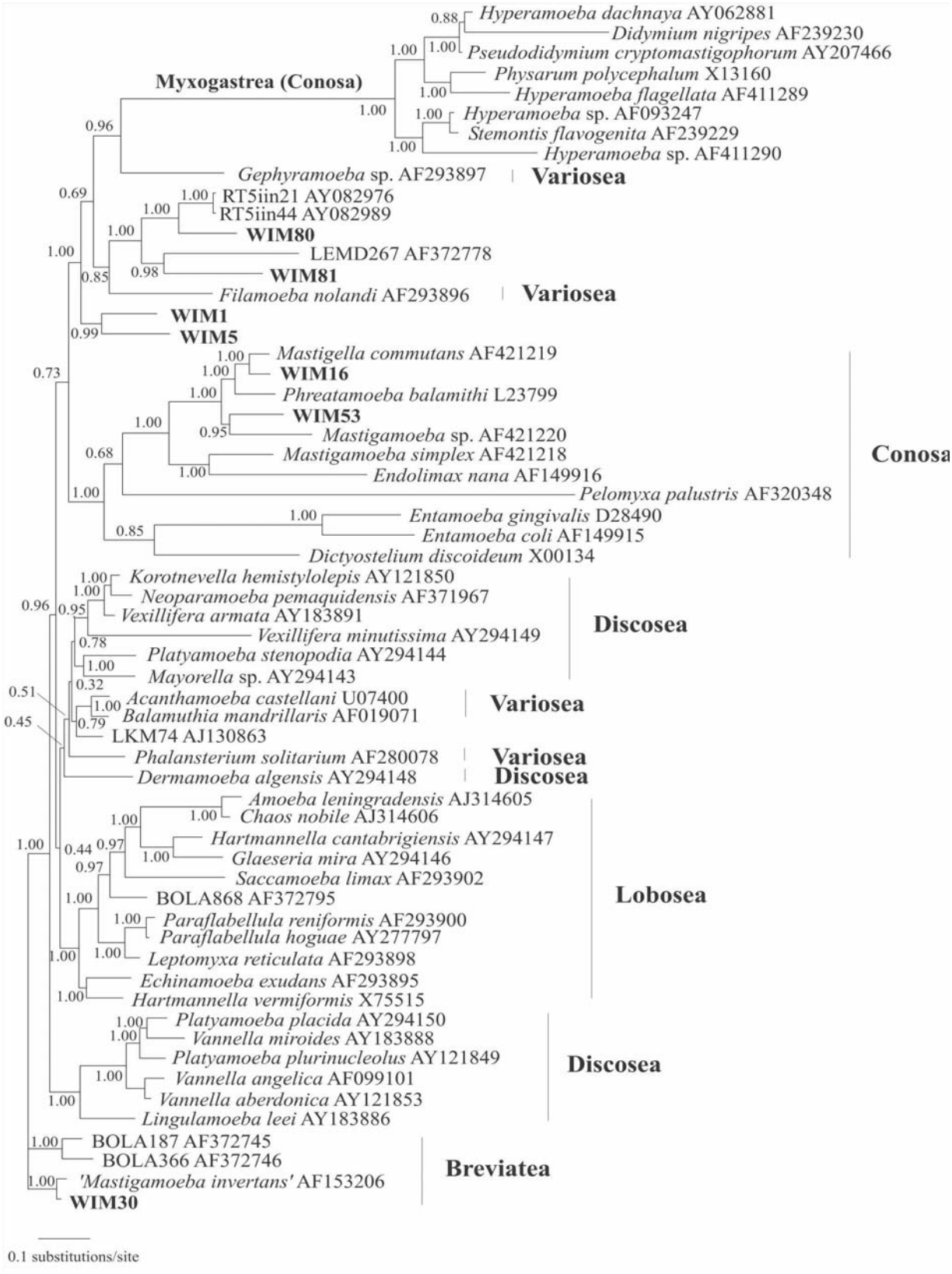


Figure 2. Bayesian tree of amoebozoans based on nuclear SSU rRNA gene sequences using 1510 positions. The classification is based on the most recently revised system (7). Numbers at nodes represent the posterior probability.

Clone WIM30 clusters with *Mastigamoeba invertens*, but it is more closely related to *M. invertens* than the clade consisting of the known environmental sequences BOLA187 and BOLA366 (Berney *et al*., 2004; Cavalier-Smith, 2004). Clone WIM80 clusters with the environmental clones RT5iin21 and RT5iin44 (2), and clone WIM81 clusters with the environmental clone LEMD267 (14), suggesting a sister-group relationship with *Filamoeba nolandi.* The clones (RT5iin21, RT5iin44) were recovered from an extremely acidic river and clone LEMD267 from anoxic freshwater sediments. The discovery of the closely related clones (WIM80 and WIM81) in agricultural soil suggests that this lineage of microbial eukaryotes has a much broader ecological distribution than anticipated. Clones WIM1 and WIM5 appear as sister group to the lineages comprising the myxogastrids, the *Gephyramoeba* sp., and the *Filamoeba nolandi*. Moreover, our analyses revealed two novel sequences (WIM16, WIM53) that could be assigned to the pelobiont mastigamoebids (17, 45). The sequences of these clones are very long, about 2.5 kb and 2.6 kb, as characteristic for pelobionts. Clones WIM16 and WIM53 cluster with *Mastigella commutans* and *Mastigamoeba simplex*, respectively.

Lastly, we identified a clone, WIM43, which clusters with *Stachyamoeba* sp. (Fig. 3).

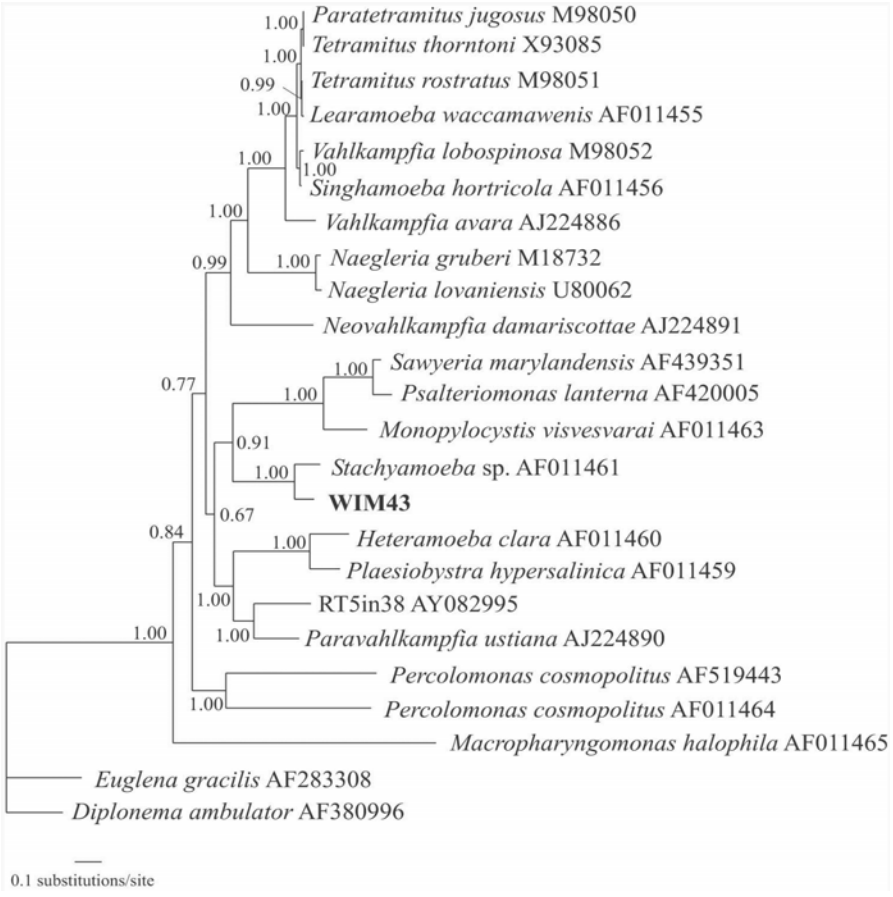


Figure 3. Bayesian tree of heteroloboseans based on nuclear SSU rRNA gene sequences using 1698 positions. Numbers at nodes represent the posterior probability.

Although the sequence identity between the two sequences is only about 85%, WIM43 clusters significantly with sequences from the Heterolobosea (Discicristata, Excavata), which are known to be common in soil (44).

Thus, it is possible to identify a broad spectrum of representatives of all major microbial eukaryotes in samples of 30 year old soil that has been heated to 42°C, air-dried and stored at room temperature. In a previous study, both culture-dependent and culture-independent (DGGE) studies had revealed substantial changes in the bacterial communities of the *Bacillus*-group from 1942 to 1975 (55). Using the same DNA as a template for PCR with approved primers for eukaryotic SSU rRNA genes, we obtained PCR products from all samples besides the oldest one, which was collected in 1942. Separating these DNAs with the aid of DGGE revealed considerable variations of the pattern of bands over the years (Fig. 4A). DGGE with amplicons of representative clones from the 1975 SSU rRNA gene library, which has been analyzed in some detail and described above, strongly suggested that many of them match with major bands of the corresponding DGGE gel (Fig. 4A, lane 1975).

Currently, it must remain unclear as to whether these changes in the DGGE patterns reflect actual changes in the composition of the eukaryotic microbiota or merely "noise" due to the inherent limitations in the sampling and sample conservation of the TAGA archive. The previous analysis of the DGGE profiles of the prokaryotic *Bacillus* group, however, argued in favour of authentical changes in time (55). Calculating similarity indices of the densitometric curves of the profiles of the eukaryotic DGGE patterns displayed in Fig 4A also support the interpretation that the observed variations in time are not the consequence of random deviations caused by sampling problems or storage effects (Fig. 4B). Therefore, it is likely that a broad spectrum of eukaryotic rRNA gene fragments can survive in dried, archivated soil samples for decades.

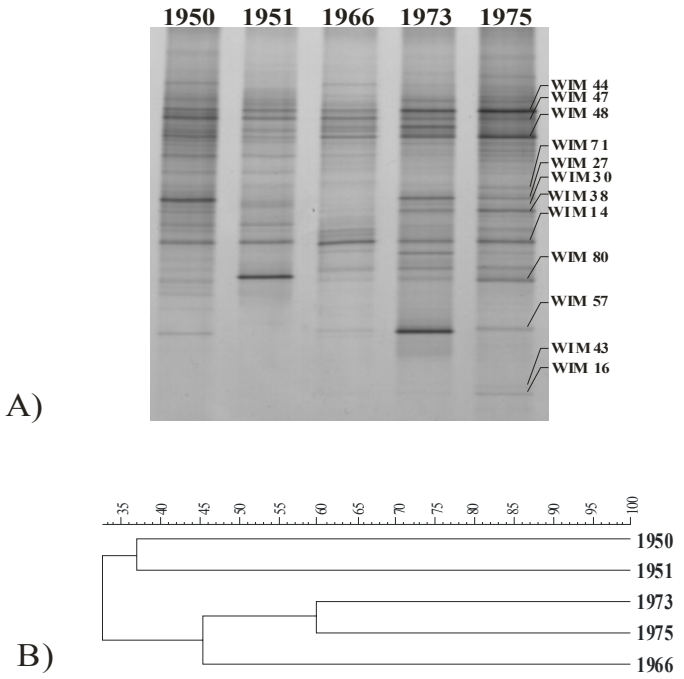


Figure 4. A: SSU rRNA gene-targeted PCR-DGGE profile of the eukaryotic community in air-dried soil samples from different years originating from Wieringermeer polder. 1950, 1951, 1966, 1973, 1975 indicate the year of sampling. The numbered bands on 1975 profile are indicating matching clones from the corresponding rRNA library. B: UPGMA cluster analysis of the similarity indexes calculated for the different DGGE profiles.

It has been shown earlier that eukaryotic DNA can survive in antarctical holocene sediments for prolonged periods of time (11). Notably, also environmental DNA bound to marine sands allowed the construction of highly complex shotgun and PCR-based 18S rRNA gene libraries. Approximately 10% of the clones contained inserts of eukaryotic origin (42). In general, DNA bound to aquatic sediments is present in much higher concentrations than in the water column (12). Soil can contain as much as 1,9 microgram/gram dried sample of free DNA (43), which is in good agreement with the 2-5 micrograms of DNA per 1g of dried soil sample recovered in our study. Notabene, our samples had been treated by bead-beating before DNA isolation allowing also the recovery of DNA from resting cysts, which had been demonstrated in a previous study focussed on the recovery of DNA from spore-forming *Bacillus*-species (55). Therefore, we cannot discriminate between free DNA and DNA derived from cysts. Notwithstanding, there is a wealth of evidence that DNA bound to soil particles is much better protected against degradation than DNA in solution (10, 13, 21, 54). The TAGA samples of the present study had been air-dried at 30-40°C, crushed, sieved, and stored at ambient temperatures over extended periods of time. Obviously, such a treatment does allow

the survival of eukaryotic DNA. Because our samples had been homogenized, sieved and thoroughly mixed, the recovered DNA is likely to be representative for the sampling site. Indeed, the spectrum of organisms identified in our study reasonably reflects the protists diversity known from recent studies on freshwater eukaryotes (51).

In conclusion, we could show that it is possible and feasible to analyze the composition of eukaryotic soil microbiota even in historical samples of air-dried and homogenized soil, which were collected over extended periods of time and stored for more than 30 years at room temperature. Even the moderate number of clones analyzed so far allowed the identification of representatives of nearly all taxa of eukaryotic soil microbes suggesting that the collection and the treatment of the soil samples did not destroy all the eukaryotic DNA in these samples. Although we cannot exclude odd losses of DNA or biases in PCR amplification of the various protists, the diversity of different soil microbes clearly shows that it is possible to analyze ancient soil communities using historical samples. Even our limited, explorative approach revealed the presence of quite a number of hitherto unknown protist rRNA sequences. It seems feasible that real time PCR using primers directed against a variety of genes will allow the quantification of particular protists in such samples. In fresh samples, FISH using specific probes derived from the historical libraries of soil samples will facilitate the unequivocal identification of hitherto unknown protists, or, alternatively, the absence of well-known "indicator" species , which might be indicative of local or global environmental changes.

Acknowledgements

This work was partly supported by a grant to the Laboratory of Microbiology, Wageningen University, from the European Communities EC project “Exploration of Genomic and Metabolite Diversity of a Novel Group of Abundant Soil Bacteria” (BACREX-project QLK3-2000-01678). We would like to thank Phillip Ehlert, TAGA (ALTERRA B.V., Wageningen, The Netherlands), who provided access to the soil collection, and detailed information on sample background that was essential for sample selection.

References

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. H. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-3402.
2. **Amaral Zettler, L. A., F. Gomez, E. Zettler, B. G. Keenan, R. Amils, and M. L. Sogin.** 2002. Eukaryotic diversity in Spain's River of Fire. Nature **417:**137.
3. **Anderson, I. C., and J. W. G. Cairney.** 2004. Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. Environ. Microbiol. **6:** 769-779.
4. **Bass, D., and T. Cavalier-Smith.** 2004. Phylum-specific environmental DNA analysis reveals remarkably high global biodiversity of Cercozoa (Protozoa). Int. J. Syst. Evol. Microbiol. **54:**2393-2404.
5. **Bonkowski, M.** 2004. Protozoa and plant growth: the microbial loop in soil revisited. New Phytologist **162:**617-631.
6. **Castresana, J.** 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. **17:**540-552.
7. **Cavalier-Smith, T.** 2003. Protist phylogeny and the high-level classification of protozoa. Eur. J. Protistol. **39:**338-348.
8. **Cavalier-Smith, T.** 1998. A revised six-kingdom system of life. Biological reviews of the Cambridge Philosophical Society **73:**203-266.
9. **Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje.** 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. **33:**D294-296.
10. **Coolen, M. J., and J. Overmann.** 1998. Analysis of subfossil molecular remains of purple sulfur bacteria in a lake sediment. Appl. Environ. Microbiol. **64:**4513-4521.
11. **Coolen, M. J. L., G. Muyzer, W. I. C. Rijpstra, S. Schouten, J. K. Volkman, and J. S. Damste.** 2004. Combined DNA and lipid analyses of sediments reveal changes in Holocene haptophyte and diatom populations in an Antarctic lake. Earth and Planetary Science Letters **223:**225-239.
12. **Corinaldesi, C., R. Canovaro, and A. Dell'Anno.** 2005. Simultaneous recovery of extracellular and intracellular DNA suitable for molecular studies from marine sediments. Appl. Environ. Microbiol. **71:**46-50.
13. **Crecchio, C., P. Ruggiero, M. Curci, C. Colombo, G. Palumbo, and G. Stotzky.** 2005. Binding of DNA from *Bacillus subtilis* on montmorillonite-humic acids-aluminum or iron hydroxypolymers: Effects on transformation and protection against DNase. Soil Science Society of America Journal **69:**834-841.
14. **Dawson, S. C., and N. R. Pace.** 2002. Novel kingdom-level eukaryotic diversity in anoxic environments. Proc. Nat. Acad. Sci. USA **99:**8324-8329.
15. **Diez, B., C. Pedros-Alio, T. L. Marsh, and R. Massana.** 2001. Application of denaturing gradient gel electrophoresis

(DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl. Environ. Microbiol. **67:**2942-2951.

16. **Dolfing, J., A. Vos, J. Bloem, P. A. I. Ehlert, N. B. Naumova, and P. J. Kuikman.** 2004. Microbial diversity in archived soils. Science **306:**813.
17. **Edgcomb, V. P., A. G. Simpson, L. A. Amaral Zettler, T. A. Nerad, D. J. Patterson, M. E. Holder, and M. L. Sogin.** 2002. Pelobionts are degenerate protists: insights from molecules and morphology. Mol. Biol. Evol. **19:**978-982.
18. **Ekelund, F., H. B. Frederiksen, and R. Rønn.** 2002. Population dynamics of active and total ciliate populations in arable soil amended with wheat. Appl. Environ. Microbiol. **68:**1096-1101.
19. **Ekelund, F., and R. Rønn.** 1994. Notes on protozoa in agricultural soil with emphasis on heterotrophic flagellates and naked amoebae and their ecology. FEMS Microbiol. Rev. **15:**321-353.
20. **Ekelund, F., R. Rønn, and B. S. Griffiths.** 2001. Quantitative estimation of flagellate community structure and diversity in soil samples. Protist **152:**301-314.
21. **England, L. S., S. B. Holmes, and J. T. Trevors.** 1998. Persistence of viruses and DNA in soil. World Journal of Microbiology & Biotechnology **14:**163-169.
22. **Felsenstein, J.** 2002. PHYLIP (Phylogeny Inference Package) version 3.6a3. Distributed by the author Department of Genome Sciences, University of Washington, Seattle.
23. **Foissner, W.** 1991. Diversity and ecology of soil flagellates, vol. 45. Systematics Association, Clarendon Press, Oxford.
24. **Foissner, W.** 1999. Soil protozoa as bioindicators: Pros and cons, methods, diversity, representative examples. Agric. Ecosyst. Environ. **74:**95-112.
25. **Foissner, W., S. Y. Moon-van der Staay, G. W. M. van der Staay, J. H. P. Hackstein, K. W-D., and H. Berger.** 2004. Reconciling classical and molecular phylogenies in the stichotrichines (Ciliophora, Spirotrichea), including new sequences from some rare species. Eur. J. Protistol. **40:**265-281.
26. **Foissner, W., M. Strüder-Kypke, G. W. M. van der Staay, S. Y. Moon-van der Staay, and J. H. P. Hackstein.** 2003. Endemic ciliates (Protozoa, Ciliophora) from tank bromeliads (*Bromeliaceae*): a combined morphological, molecular and ecological study. Eur. J. Protistol. **39:**365-372.
27. **Hall, T. A.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:**95-98.
28. **Häne, B. G., K. Jäger, and H. Drexler.** 1993. The pearson product-moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. Electrophoresis **14:**967-972.
29. **Heilig, H. G. H. J., E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. L. Akkermans, and W. M. de Vos.** 2002. Molecular diversity of *Lactobacillus spp.* and other lactic acid bacteria in the human intestine as determined by specific

amplification of 16S ribosomal DNA. Appl. Environ. Microbiol. **68:**114-123.

30. **Huelsenbeck, J. P., and F. Ronquist.** 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics **17:**754-755.
31. **Jeanmougin, F., J. D. Thompson, M. Gouym, D. G. Higgins, and T. J. Gibson.** 1998. Multiple sequence alignment with Clustal X. Trends Biochem. Sci. **23:**403-405.
32. **Kwiatowsky, J., D. Skarecky, S. Hernandez, D. Pham, F. Quijas, and F. J. Ayala.** 1991. High fidelity of the polymerase chain reaction. Mol. Biol. Evol. **8:**884-887.
33. **Lawley, B., S. Ripley, P. Bridge, and P. Convey.** 2004. Molecular analysis of geographic patterns of eukaryotic diversity in Antarctic soils. Appl. Environ. Microbiol. **70:**5963-5972.
34. **Lefranc, M., A. Thenot, C. Lepere, and D. Debroas.** 2005. Genetic diversity of small eukaryotes in lakes differing by their trophic status. Appl. Environ. Microbiol. **71:**5935-5942.
35. **Lopez-Garcia, P., F. Rodriguez-Valera, C. Pedros-Alio, and D. Moreira.** 2001. Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature **409:**603-607.
36. **Luo, Q., L. R. Krumholz, F. Z. Najar, A. D. Peacock, B. A. Roe, D. C. White, and M. S. Elshahed.** 2005. Diversity of the microeukaryotic community in sulfide-rich Zodletone Spring (Oklahoma). Appl. Environ. Microbiol. **71:**6175-6184.
37. **Moon-van der Staay, S. Y., R. de Wachter, and D. Vaulot.** 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nat. Biotechnol. **409:**607-610.
38. **Moon-van der Staay, S. Y., G. W. M. van der Staay, L. Guillou, H. Claustre, L. K. Medlin, and D. Vaulot.** 2000. Abundance and diversity of Prymnesiophyceae in the picoplankton community from the equatorial Pacific ocean inferred from 18S rDNA sequences. Limnol. Oceanogr. **45:**98-109.
39. **Moon-van der Staay, S. Y., G. W. M. van der Staay, and P. Javorský.** 2002. Diversity of rumen ciliates revealed by 18S ribosomal DNA analysis. Reprod. Nutr. Dev. **42 (Suppl. 1):**76.
40. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695-700.
41. **Muyzer, G., and K. Smalla.** 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Leeuwenhoek **73:**127-141.
42. **Naviaux, R. K., B. Good, J. D. McPherson, D. L. Steffen, D. Markusic, B. Ransom, and J. Corbeil.** 2005. Sand DNA - a genetic library of life at the water's edge. Mar. Ecol. Prog. Ser. **301:**9-22.
43. **Niemeyer, J., and F. Gessler.** 2002. Determination of free DNA in soils. J. Plant. Nutr. Soil. Sc. **165:**121-124.
44. **O'Kelly, C. J., J. D. Silberman, L. A. Amaral Zettler, T. A. Nerad, and M. L. Sogin.** 2003. *Monopylocystis visvesvarai*

n. gen., n. sp. and *Sawyeria marylandensis* n. gen., n. sp.: two new amitochondrial heterolobosean amoebae from anoxic environments. Protist **154:**281-290.

45. **Patterson, D. J.** 1999. The diversity of Eukaryotes. Am. Nat. **154:**S96-S124.
46. **Pearson, W. R., and D. J. Lipman.** 1998. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444-2448.
47. **Posada, D., and K. A. Crandall.** 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14:**817-818.
48. **Robinson, B. S., S. S. Bamforth, and P. J. Dobson.** 2002. Density and diversity of protozoa in some arid Australian soils. J. Eukaryot. Microbiol. **49:**449-453.
49. **Sanguinetti, C. J., D. Neto, and A. J. G. Simpson.** 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques **17:**915-919.
50. **Schadt, C. W., A. P. Martin, D. A. Lipson, and S. K. Schmidt.** 2003. Seasonal dynamics of previously unknown fungal lineages in tundra soils. Science **301:**1359-1361.
51. **Slapeta, J., D. Moreira, and P. P. R. S. B.-B. S. Lopez-Garcia.** 2005. The extent of protist diversity: insights from molecular ecology of freshwater eukaryotes. P. Roy. Soc. B-Biol. Sci. **272:**2073-2081.
52. **Stoeck, T., and S. Epstein.** 2003. Novel eukaryotic lineages inferred from small-subunit rRNA analyses of oxygen-depleted marine environments. Appl. Environ. Microbiol. **69:**2657-2663.
53. **Terpstra, P.** 1979. 50 jaar Wieringermeer. M. A. van Seijen-Leeuwarden, The Netherlands.
54. **Trevors, J. T.** 1996. DNA in soil: Adsorption, genetic transformation, molecular evolution and genetic microchip. Antonie Leeuwenhoek **70:**1-10.
55. **Tzeneva, V. A., Y. Li, A. D. M. Felske, W. M. de Vos, A. D. L. Akkermans, E. E. Vaughan, and H. Smidt.** 2004. Development and application of a selective PCR-Denaturing Gradient Gel Electrophoresis approach to detect a recently cultivated *Bacillus* group predominant in soil. Appl. Environ. Microbiol. **70:**5801-5809.
56. **van Schreven, D. A., and G. W. Harmsen.** 1968. Soil bacteria in relation to the development of polders in the region of the former Zuider zee. Liverpool Univerity Press, United Kingdom.
57. **Zoetendal, E. G., A. D. L. Akkermans, W. M. Akkermans - van Vliet, J. A. G. M. de Visser, and W. M. de Vos.** 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Microbial Ecol. Health Dis. **13:**129-134.

CHAPTER 6

EFFECT OF SOIL DRYING AND STORAGE ON THE BACTERIAL AND EUKARYOTIC DIVERSITY MONITORED BY DGGE

Vesela A. Tzeneva, Joana F. Salles, Natalia Naumova, Willem M. de Vos, Peter J. Kuikman, Jan Dolfing and Hauke Smidt

Abstract

Archived soil samples provide a valuable source for retrospective ecological studies, and their recent analysis by molecular ecological approaches has drawn significant attention in the scientific community. However, the effect of drying and extended storage on the detectable bacterial and eukaryal microbiota in the soil has not yet been evaluated. To achieve that, soil samples collected from two long-term grassland experiments in the United Kingdom and The Netherlands were subjected to air-drying at 40-42 ºC and storage at room temperature. The total bacterial, *Bacillus benzoevorans*- related and eukaryotic communities associated with these soil samples were analyzed by small subunit ribosomal RNA (SSU rRNA) gene-targeted PCR-DGGE fingerprinting. Changes in microbial community structure due to drying and storage were evaluated using multivariate statistics. This analysis demonstrated that neither drying nor storage has a strong effected on the detectable part of the pro- and eukaryotic communities. With this knowledge, the effect of specific soil parameters addressed in the long term grassland experiments, including fertilization, pH and soil type, could be confidently addressed, indicating that none of the variables strongly influenced bacterial community structure in the analyzed bulk soil samples.

Introduction

The soil ecosystem is known to provide ecological niches for an extremely high number of microorganisms (29). The relationship between the diversity of living organisms and ecosystem functioning has become an important issue of modern microbial ecology, especially with the advent of molecular approaches to reveal the hidden majority of not yet cultured microorganisms, whose function is still largely unknown (32). The soil microbiota is the driver of major processes, essential for the existence and sustainability of any terrestrial ecosystem, i.e. decomposition of organic matter (10) and mineralization of nutrients in soil (23). Alongside with this important ecosystem-defining function, soil microorganisms, mainly bacteria and fungi, perform a wealth of other processes, such as nitrogen fixation, solubilization of parent rock minerals (2), decomposition of toxic inorganic and organic pollutants, and synthesis of plant-growth-promoting and other physiologically active substances (5, 9, 24, 25).

Taxonomical methods used to describe members of soil microbial communities usually require cultivation techniques, which are often biased and do not reflect the actual diversity of soil microorganisms *in situ*. Although new approaches for cultivation continue to be developed, the majority of the soil microbiota remains inaccessible to detailed studies due to inadequate media and methods for cultivation (1, 3, 34). Molecular approaches to detect the diversity of nucleic acids, extracted directly from soil, allow us to circumvent such cultivation biases, and have largely contributed to improve our ability to investigate soil microorganisms, including those problematic for cultivation (28). Molecular fingerprinting methods targeting ribosomal RNA and the corresponding genes, such as denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) can be used for the rapid evaluation of composition and activity of complex microbial assemblages at moderately high temporal and spatial resolution (18).

Recently, with intensification of land use and agriculture, soil microbiologists and ecologists have become increasingly aware that such practices may result, or have already resulted, in losses in soil microbial diversity (4). This may result in changes of soil ecosystem functioning, and in the long run may have significant impact on the future of terrestrial ecosystems. Archived soil collections represent a vast pool of samples, for which detailed records of sampling conditions and land use are available. These collections contain agricultural soil samples collected during many years and from different soil treatments.

Analyses of the microbial communities present in archived soil samples using molecular techniques may enable to estimate changes in microbial diversity due to factors such as land use or climate change on a long term basis. Recently Dolfing and co- workers could show that the application of molecular techniques to air-dried soils is feasible, generating meaningful information on bacterial diversity changes due to agricultural practice (8). By analyzing samples taken after the land reclamation of Wieringermeer polder (The Netherlands) over a period of 33 years of agricultural land use, we could also retrospectively follow the changes of a group of novel *Bacillus benzoevorans*-related microorganisms, as well as the eukaryotic community (17, 30).

Given the high scientific value of archived soil samples, which represent a unique source of information about the microbial communities over prolonged periods of time, the aim of this study was to evaluate the effect of drying and storing conditions on the detectable bacterial and eukaryal microbiota in the soil. To achieve that, samples collected from two long term grassland experiments in the UK and The Netherlands were subjected to air-drying and subsequent storage. Microbial communities associated with these samples were analyzed by small subunit ribosomal RNA (SSU rRNA) gene-targeted PCR-DGGE fingerprinting. Changes in microbial community structure due to drying and storage were studied by comparison to DGGE patterns obtained from fresh soil samples. The DGGE analysis showed that changes in pro- and eukaryotic communities due to drying and storage of the soil samples did not affect our ability to study the effect of changing environmental conditions and agricultural practice.

Materials and Methods

Field experiments and soil samples

Soil samples used in this study were collected from two independent studies long-term grassland experiments conducted at Rothamsted (UK) and Wageningen (The Netherlands).

Rothamsted: samples were obtained in July 2003 from the Park Grass experiment at Rothamsted (UK). The hay experiment, laid down in 1856, is the oldest in Great Britain. The field is divided in experimental plots where different pH and fertilizer have been applied. The pH has been maintained by liming. Some soil properties and vegetation characteristics of the

studied plots are shown in Table 1. The samples were transported to The Netherlands in sterile tubes at a temperature between 4 and 10 °C for 16 hours, and then stored at -80 °C.

Plot		Soil pH	Grass type	Fertilizer
3	a	7	red clover (*Trifolium pratense*), broad leaves species	NONE
	b	6	red clover (*Trifolium pratense*), broad leaves species	
	c	5	red clover (*Trifolium pratense*), broad leaves species	
	d	5 (Not adj.)	red clover (*Trifolium pratense*), broad leaves species	
7	a	7	meadow vetchling (*Lathyrus pratensis*), red clover, False oat-grass (*Arrhenatherum elatius*), Buttercups (*Ranunculus sp*), Dandelion (*Taraxacum spp*), Hogweed (*Heracelum sphondylium*)	PKNaMg
	b	6	meadow vetchling (*Lathyrus pratensis*), red clover, False oat-grass (*Arrhenatherum elatius*), Buttercups (*Ranunculus sp*), Dandelion (*Taraxacum spp*), Hogweed (*Heracelum sphondylium*)	
	c	5	meadow vetchling (*Lathyrus pratensis*), red clover, False oat-grass (*Arrhenatherum elatius*), Buttercups (*Ranunculus sp*), Dandelion (*Taraxacum spp*), Hogweed (*Heracelum sphondylium*)	
	d	5 (Not adj.)	meadow vetchling (*Lathyrus pratensis*), red clover, False oat-grass (*Arrhenatherum elatius*), Buttercups (*Ranunculus sp*), Dandelion (*Taraxacum spp*), Hogweed (*Heracelum sphondylium*)	
9/2	a	7	sweet vernal-grass (*Anthoxanthum odoratum*), False oat-grass, Meadow foxtail (*Alopecurus pratensis*)	(NH_3)PKNaMg
	b	6	sweet vernal-grass (*Anthoxanthum odoratum*), False oat-grass, Meadow foxtail (*Alopecurus pratensis*)	
	c	5	sweet vernal-grass (*Anthoxanthum odoratum*), False oat-grass, Meadow foxtail (*Alopecurus pratensis*)	
	d	3.5 (Not adj.)	sweet vernal-grass (*Anthoxanthum odoratum*), False oat-grass, Meadow foxtail (*Alopecurus pratensis*)	
14/2	a	7	False oat-grass, Meadow foxtail	(NO_2)PKNaMg
	b	6	False oat-grass, Meadow foxtail	
	c	6	False oat-grass, Meadow foxtail	
	d	6 (Not adj.)	False oat-grass, Meadow foxtail	

Not adj.- pH not adjusted

Table 1. Soil properties and vegetation characteristics of the study sites at the Park Grass experiment at Rothamsted (Great Britain).

Wageningen: soil samples were collected in February 1999 from two long-term grassland experimental fields of the Institute of Agrobiology and Soil Fertility (Wageningen, The Netherlands). Some soil properties and vegetation characteristics are shown in Table 2.

Soil texture	Land use	Soil organic matter, %	Soil Ntot, %	Soil pH (H_2O)
Clay	Pasture	22.4	0.909	5.1
Clay	Hayfield	20.4	0.710	5.0
Sandy	Hayfield	n.a.	n.a.	n.a.

n.a.- data not available

Table 2. Vegetation characteristics and soil properties of long-term grassland experimental fields of the Institute of Agrobiology and Soil Fertility (Wageningen, The Netherlands).

Soil monoliths collected from both experiments were cut into small (1-2 cm) pieces; big animals and fragments of plant material were removed by hand, and soil was thoroughly mixed. A small portion of these soil samples was stored at -80 °C and considered as fresh soil samples. The remaining soil was air-dried at 42° C for 48 hours, yielding air-dried soil samples. Subsequently, aliquots of the air-dried soils were stored at +20 °C for 90 days or +15 °C for 120 days, for Rothamsted and Wageningen soil samples, respectively.

DNA extraction

As samples were processed in the framework of two independent studies, two different DNA extraction methods were used as described below.

Direct DNA isolation from Rothamsted soil samples was performed in duplicate using the Fast DNA®SPIN®Kit For Soil (Q BIOgene, Cambridge, United Kingdom) according to the manufacturers' instructions, using 0.5 g of soil. DNA extracts were checked with universal primers 27f and 1492r for sufficient material and purity for 16S rRNA gene-targeted PCR amplification (14).

Extraction of DNA from Wageningen soil samples was performed in triplicate according to the protocol designed by van Elsas and Smalla (31) with minor modifications. Briefly, 2 g of soil and 3 g glass beads (90 μm in diameter) were resuspended in a 10 ml polypropylene tube in 3 ml of 120 mM phosphate buffer (pH 8), containing 5mg/ml of lysozyme. Suspensions were incubated for 15 min at 37°C and further chilled on ice for 15 min. The suspensions were homogenized 3 times for 90 s with 10 s intervals in a bead beater (MSK cell homogenizer B.Braun Diessel Biotech Melsungen, Gernmany) at 4000 beats·min^{-1}.

After bead-beating, 180 µl of 20% sodium dodecyl sulphate were added to the soil slurry and carefully mixed manually and incubated for 15 min at room temperature to enhance lysis. Three ml of Tris-buffered phenol (pH 8.0) were added, mixed manually, and the tubes were centrifuged for 15 min at 3000 g at room temperature. The upper aqueous phase was recovered into a new polypropylene tube, and the phenol-soil slurry was back-extracted with 1 ml of phosphate buffer, with a resultant aqueous phase pooled together with the one obtained at the previous step. The pooled aqueous phase was extracted with 3 ml of chloroform-isoamylalcohol mixture (24:1) and centrifuged for 5 min at 1000 g at room temperature. The upper aqueous phase was recovered into a new polypropylene tube, and 0.3 ml of 5 M NaCl and 6 ml of 96 % ice-cold ethanol were added. The tubes were centrifuged at 3000 g for 15 min; the supernatant was discarded and the pellet washed with ice-cold 70 % ethanol. The pellet was air-dried for 1 hour at 4 °C and afterwards resuspended in 1 ml of sterile Tris-EDTA buffer (pH 8.0, 10 mM Tris-HCl, 1 mM EDTA). Crude DNA extract (150 µl) was mixed with CsCl (150 mg) and incubated for 1.5 hours at room temperature. The mixture was centrifuged for 20 min at the maximal speed 16000 g. The resultant supernatant was recovered into a new 1.5 ml Eppendorf tube, mixed with 0.6 ml of deionized water and 0.4 ml of isopropanol and incubated for 5 min at room temperature. After centrifugation at 10000 g for 15 min, supernatant was discarded, the pellet re-suspended in 150 µl TE buffer (pH 8.0) and transferred into a new Eppendorf tube. In the case of clay soils, no further purification was needed, and the DNA extracts obtained after precipitation with CsCl, were used as templates for PCR amplification. In case of sandy soils, another step of purification with the Wizard DNA Clean-UP System (Promega, Madison, WI, USA) was needed as extracts were coloured, probably due to residual humic substances. This purification step was performed according to the protocol supplied by manufacturer.

Primers and Polymerase Chain Reaction (PCR)

All primers used in this study are listed in Table 3. Primers for specific amplification of 16S rRNA genes of *B. benzoevorans* relatives, were used in a nested approach of two successive specific PCRs as described previously (30). Subsequently, specific products were used as template for DGGE-PCR with primers 0968-GCf and 1401r (20). Total bacterial communities were analyzed with primer set GC-357f/518r (33) and GC-341f/543r for Rothamsted and Wageningen samples, respectively. The eukaryotic community was analyzed

by 18S rRNA-targeted PCR with primer pair Euk1A/ Euk516r-GC and the conditions described by Diez et al. (7).

Primers	Sequence	Reference
27f	5'- GTT TGA TCC TGG CTC AG-3'	(14)
1492r	5'- CGG CTA CCT TGT TAC GAC-3'	(14)
REX460f	5'-GAG TAA CTG C(T/C)(C/G) GTA CC-3'	(30)
REX1466r	5'-CCA ATC ATC TGT CCC ACC TTA-3'	(30)
REX576f	5'-AAG CGC GCG CAG GCG GTC CT-3'	(30)
REX1446r	5'-CCT TAG GCG GCT GGC TCC TTA-3'	(30)
GC-0968f	5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA CCT TAC-3'	(20)
1401r	5'- GCG TGT GTA CAA GAC CC-3'	(20)
GC-357f	5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCC TAC GGG AGG CAG CAG -3'	(33)
518r	5'- ATT ACC GCG GCT GCT GG-3'	(33)
Euk1A	5'- CTG GTT GAT CCT GCC AG-3'	(7)
Euk516r-GC	5'- ACG CCC GGG GCG CGC CCC GGG CGG GGC GGG GGC ACG GGG GGA CCA GAC TTG CCC TCC-3'	(7)

Table 3. PCR primers used in this study

For Rothamsted samples, PCR was performed with *Taq* polymerase (Invitrogen Co., Breda, The Netherlands), using a Whatman Biometra Thermocycler (Göttingen, Germany). *B. benzoevorans* relatives specific PCR was done according to Tzeneva et al. (30). PCR with primers 0968GCf/1401r and GC-357f/518r was performed as previously described (20). PCR with universal primers 27f and 1492r was performed with the following conditions: 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 48 °C for 20 sec, and 68 °C for 40 sec, and finally 68 °C for 7 min.

PCR amplification from Wageningen samples with primers GC-341f and 543r was performed with a DNA thermal Cycler 480 (PerkinElmer, Wellesley, MA) according the following protocol: the reaction mixture consisted of 1µl of template DNA (ca. 10 ng), 5 µl of 10×PCR buffer $MgCl_2$, 10 µl of equimolar mixture of deoxyribonucleoside triphosphate, 1 µl of each of the primers (10 pmol), 2 µl of bovine serum albumin (5 mg/ ml) to prevent inhibition of the reaction, and 29.25 µl of sterile milli-Q water. The reaction mixture was overlaid with a drop of sterile mineral oil. After 5 min of denaturation at 94 °C, the reaction mixture was cooled to 80°C, and during subsequent 5 min incubation at this temperature 0.75 µl (2.6 U) of DNA polymerase (Expand High-Fidelity PCR System, Boehringer Mannheim

GmbH, Germany) was added. This hot start was necessary to prevent non-specific annealing of the primers to non-target DNA (6). Then polymerization during 40 cycles followed, each consisting of 1 min at 94 °C, 1min at 55 °C, 3min at 72 °C , 10 min at 72 °C (18).

Analysis of PCR products by DGGE

The PCR products for the *B. benzoevorans* relatives, total bacteria and eukaryotes from DNA isolated from Rothamsted and Wageningen soil samples, were separated by DGGE (18, 19). Different gradients of the denaturing chemicals (100% denaturing acrylamide was defined as 7 M urea and 40% formamide) were used for the different groups. For Rothamsted and Wageningen total bacterial community, the 16S rRNA V3 region was amplified and respectively separated on 10 % gel with gradient of 30-60 % and 8 % gel with 45-75 % gradient. For *B. benzoevorans* relatives, an 8 % gel with a 30-60 % gradient was used. For the eukaryotic community the 18S rRNA V1 region was amplified and separated on a 6 % gel with a 15-45 % gradient. DGGE electrophoresis of samples derived from Rothamsted samples was performed according to Heilig *et* al. (13), the gels were subsequently stained with $AgNO_3$ according to the method of Sanguinetti *et* al. (22), and scanned at 400 dpi.

DGGE electrophoresis of PCR products from Wageningen soil samples was performed according to Muyzer at al.(18). Bands were visualized by incubating the gel for 30 min in 0.5 x TAE buffer, containing ethidium bromide (5 $mg \cdot l^{-1}$). The gels were rinsed for 5 min with demineralized water and photographed in a UV-transilluminator at 302 nm with a video image processor P67E (Mitsubishi Co., Japan).

DGGE gels images were analyzed using Bionumerics 4.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). The similarity between DGGE profiles was determined by calculating similarity indices of the densitometric curves of the profiles compared using the Pearson product-moment correlation (12, 35). The UPGMA algorithm was used as implemented in the analysis software for the construction of dendrograms.

Multivariate statistical analysis

Multivariate statistical analysis was performed using CANOCO 4.5 software (Biometris, Wageningen, The Netherlands) to assess to what extend soil characteristics and preservation (further referred to as environmental variables) affected the soil microbiota as measured by DGGE profiling. Redundancy analysis (11) was chosen, as it explains the structure of the “species” data table (in this case, band position and relative intensity) by

environmental variables, assuming a linear distribution of species (21, 27). Community similarities were graphed by using ordination plots with scaling focused on inter-samples difference (16). The ordination plot of species and environmental variables is characterized by biplots that approximate the weighed averages of each species with respect to each of the environmental variables. Thus, the ordination diagram represents not only a pattern of community distribution, but also the main features of the distribution of species along the environmental variables (27). The classes of the nominal environmental variables, such as soil type, fertilizer type, vegetation etc., are symbolized by centroids (the weighted average). The pH was considered as quantitative environmental variable and is represented as vector, with length and slope of the vector as significant parameters. Long vectors forming a smaller angle with an ordination axis are more strongly correlated with that axis (26). The angle between the vectors provides an approximation of their correlation. Vectors pointing in the same direction (angle < 90°) are positively correlated while those in opposite directions are negatively correlated (15, 27). The position of the centroids representing nominal variables determines the relationship of these variables with either of the ordination axes (26).

In the analyzed samples from Rothamsted experimental station, all soil treatments: fresh, dried, stored, pH (pH 7, 6, 5 and 3.5), type of fertilizer ((NH_3)PKNaMg, (NO_2)PKNaMg, PKNaMg and not fertilized (further referred to as NONE)), and type of vegetation were considered as environmental variables. The vegetation type was correlated with the type of fertilizer and for simplification of figures was not included in the final analysis. For Wageningen soil samples the following soil parameters were selected as environmental variables: fresh, dried, stored, soil type (sandy, clay), and vegetation (pasture, hayfield). All environmental variables were presented as not nominal (vectors).

To test the significance of the relationship of community response with environmental variables, unrestricted Monte Carlo permutation tests were performed with 499 random permutations and a significance level (p) of 0.1.

Results

Effect of drying and storage

In this chapter we discuss the effect of drying and storage of fresh soil samples on detectable bacterial and eukaryotic communities judged by DGGE analyses of SSU rDNA amplicons.

Rothamsted soil samples. Changes in the total bacterial and *Bacillus benzoevorans*-related communities were investigated in 16 soil samples collected from plots with different treatment within the Park Grass experiment at Rothamsted (Table 1). DNA was isolated from fresh soil samples, after drying at 42 °C, and after storage of dried samples for 3 months, and subsequently analyzed by rRNA gene-targeted PCR-DGGE. Multivariate statistical analysis as implemented in the CANOCO software package was used to assess the impact of drying and storing, as well as the effect of different soil treatments (type fertilizer, pH, soil type) on microbial community structure (Figs. 1 and 2).

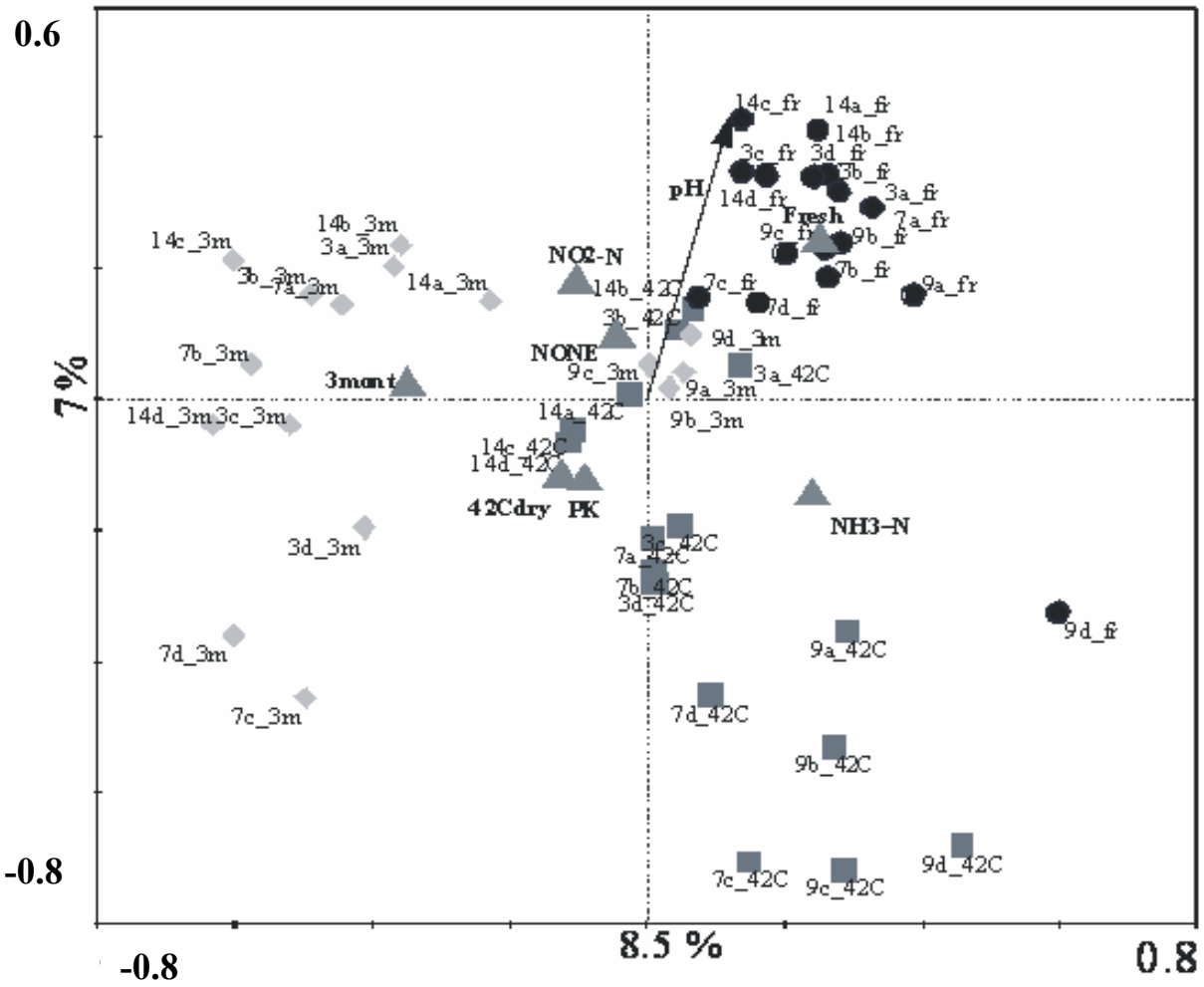


Figure 1. Redundancy analysis (RDA) ordination plot of total bacterial community in Rothamsted soil samples. Multivariate statistical analysis was used to assess the impact of drying and storing, as well as the effect of different soil treatments (type fertilizer, pH, soil type) on microbial community structure as measured by rRNA-based molecular approaches. 3- samples collected from soil with no fertilizer; 7- PKNaMg as a fertilizer; 9- (NH3)PKNaMg as fertilizer; 14- (NO2)PKNaMg as fertilizer; a- soil pH=7; b- soil pH=6; c- soil pH=5; d- not adjusted soil; ●- fresh soil samples; ■- soil samples dried at 42°C; ♦- dried and stored soil samples; ▲- type of fertilizer as nominal environmental variables; → the vectors represent the quantitative environmental variable pH.

Redundancy analysis (11) was performed using different settings with respect to environmental variables for both total bacterial and *B. benzoevorans* related communities. In the first analysis all environmental variables were taken into consideration. The effect of environmental variables was significant for the total bacterial community (P_{PKNaMg}=0.01, all other variables P=0.002; highest significance possible at 499 permutations), but not always as high or even not significant for the *B. benzoevorans* related community ($P_{(fresh, dry, stored)}$=0.002, P_{NONE}=0.028, $P_{(all\ other\ env.var.)}$>0.1). The results showed that 29.6 % and 29.3 % of the variation in total bacterial and *B. benzoevorans* relatives community structure, respectively, could be explained by all canonical environmental variables analyzed together (Table 4). This analysis,

however, did not allow for assessing the importance of drying and storage relative to other soil parameters on the studied community.

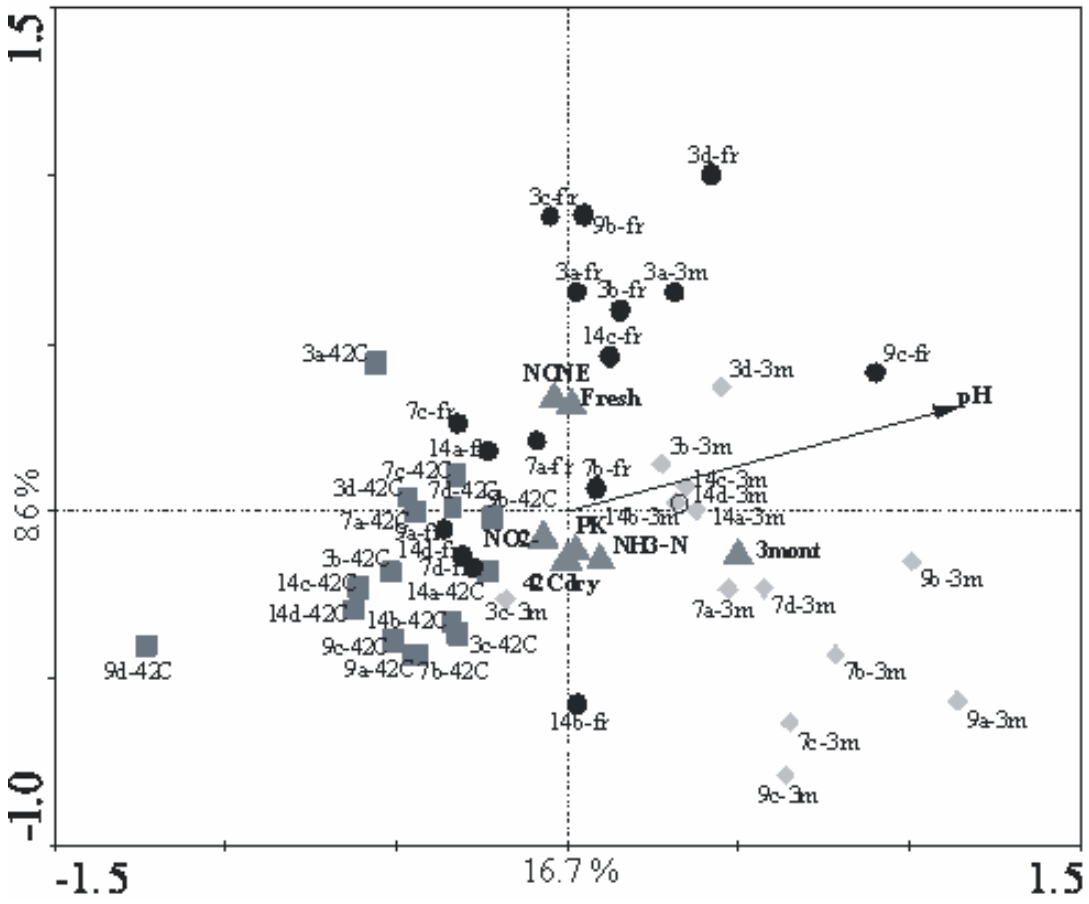


Figure 2. Redundancy analysis (RDA) ordination plot of the *B. benzoevorans* related community in Rothamsted soil samples. Multivariate statistical analysis was used to assess the impact of drying and storing, as well as the effect of different soil treatments (type fertilizer, pH, soil type) on microbial community structure as measured by rRNA-based molecular approaches. 3- samples collected from soil with no fertilizer; 7- PKNaMg as a fertilizer; 9- (NH3)PKNaMg as fertilizer; 14- (NO2)PKNaMg as fertilizer; a- soil pH=7; b- soil pH=6; c- soil pH=5; d- not adjusted soil; ●- fresh soil samples; ■- soil samples dried at 42°C; ♦- dried and stored soil samples; ▲- type of fertilizer as nominal environmental variables; → the vectors represent the quantitative environmental variable pH.

To address the issue of drying and storage, the different sample states (fresh, dry and storage) were used as environmental variables, while fertilizer and pH were treated as covariables. The effect of environmental variables was significant for both total bacterial and *B. benzoevorans* related community (P=0.002), and could explain 13 and 21.1 % of the total variability in bacterial and *B. benzoevorans* relatives community profiles, respectively (Table 4). This indicated that indeed sample conservation by drying and further storage at room temperature had a measurable effect on detectable microbiota.

Community	Grassland station	Sum of all env. variables (%)**	Effect of drying and storage (%)	Effect of other soil parameters (%)*
Total bacterial	Rothamsted	29.6	13	16.6
B. benzoevorans related	Rothamsted	29.3	21.1	8.2
Total bacterial	Wageningen	76.3	20.5	55.8
Eukaryotic	Rothamsted	58	14.1	43.9

**The environmental variables explained by the first two canonical axes in RDA analysis.

Table 4. The effect of the environmental variables on the pro- and eukaryotic soil communities in grassland experimental fields in Rothamsted (UK) and Wageningen (The Netherlands)

To complement this analysis, we also assessed the effect of fertilizer and pH, while using the samples state as covariables. While this effect was again significant for the total bacterial community (P=0.002), this was not the case for the *B. benzoevorans* related community, except for NONE fertilizer (P=0.018). This was in accordance with the fact that 16.6 and 8.2 % of variability in total bacterial and *B. benzoevorans* community profiles could be explained by environmental variables. Thus, while in case of *B. benzoevorans*-relatives, only drying and sample storage significantly affected community profiles, the effect of fertilizer and pH values was stronger for the total bacterial community in comparison with the effect of the drying and storing of the soil (16.6 % v/s 13 %) (Table 4).

Wageningen soil samples. Similar analyses were performed for the soil samples from long-term grassland experimental fields in Wageningen. In the first analysis all environmental variables were taken into consideration (Fig. 3). The effect of environmental variables was not as highly significant as observed for the previously described Rothamsted experiment. In this case significant variables included clay (P=0.014) and four months storage of the dried soil (P=0.016). Overall, 76.3 % of variability in bacterial community profiles could be explained by all environmental variables analyzed together. When considering soil types and vegetation as covariables, only storage of dried samples significantly affected detectable community profiles ($P_{(4\ months\ stored)}$=0.062). The complementary analysis (soil type and vegetation as environmental variables) revealed a significant effect for clay and sandy soil (P=0.014), but not for pasture (P=0.592). Overall, 55.8 % of the total variation in bacterial community profiles could be explained by these environmental variables, while only 20.5 % was accounted by drying and storage (Table 4).

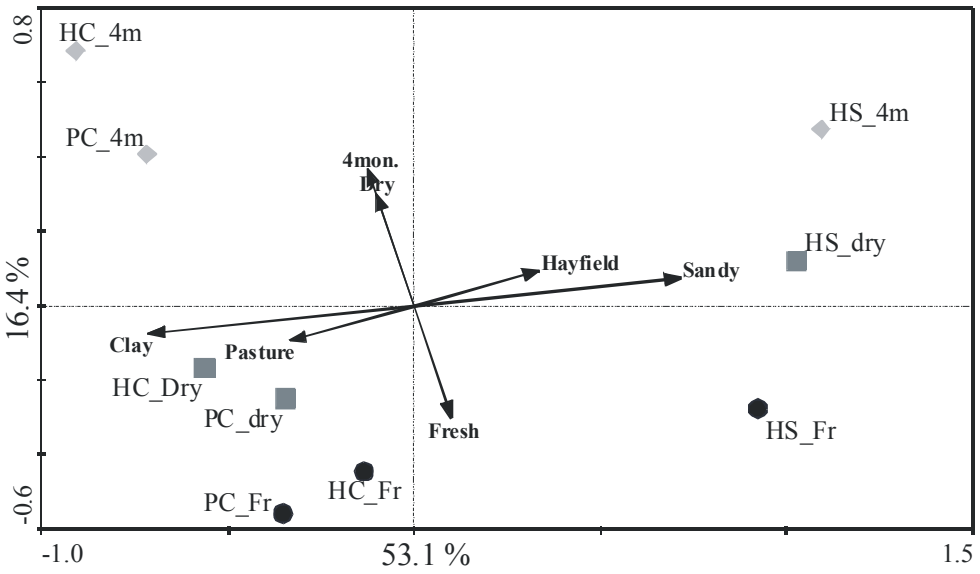


Figure 3. Redundancy analysis (RDA) ordination plot of the total bacterial community in Wageningen soil samples. Multivariate statistical analysis was used to assess the impact of drying and storing, as well as the effect of different soil type and vegetation on microbial community structure as measured by rRNA-based molecular approaches. H- samples collected from hayfield; P- samples collected from pasture; C- clay soil; S- sandy soil; ●- fresh soil samples; ■- soil samples dried at 42°C; ♦- dried and stored soil samples; → the vectors represent the quantitative environmental variables.

Eukaryotic community in Rothamsted soil samples. Changes in the eukaryotic community were investigated in selected soil samples from the Rothamsted soil collection, namely from plots with different fertilizer treatment at pH 7. When taking all environmental variables into consideration for RDA analysis, the only significant environmental variables were NONE fertilizer (P=0.008) and fertilizer (NO_2)PKNaMg (P=0.002). Overall, 58 % of the total variability of eukaryotic community profiles could be explained. When RDA was performed with fertilizer types as covariables, none of the environmental variables (i.e. samples preservation) significantly affected community profiles ($P_{(fresh)}$=0.384 and $P_{(3\ months\ stored)}$=0.620), and only 14.1 % of the total variability was accounted for these environmental variables. This indicated that drying and subsequent storage of soil samples at room temperature did not significantly affect the eukaryotic community. Using drying and storage of the soil as covariables, significant environmental variables were NONE fertilized (P=0.018) and fertilization with (NO_2)PKNaMg (P=0.018), with 43.9 % of the variability in DGGE profiles being explained (Table 4).

Effect of fertilizer and pH on the total bacterial and *B. benzoevorans* related communities in fresh, dry and stored soil samples

To assess whether preservation of soil samples by drying and prolonged storage at room temperature adversely affects our ability to identify effects of e.g. different soil management regimes on detectable soil microbiota, changes in the total bacterial and *B. benzoevorans*-related communities were investigated separately in 16 fresh, dried, and dried and stored soil samples collected from plots with different treatment within the Park Grass experiment at Rothamsted (Table 1). Overall, total bacterial DGGE fingerprints in the fresh, dried and stored soil samples showed highly complex profiles. *B. benzoevorans*-related profiles, however, were simpler and no major changes could be observed among the different experimental plots, as evidenced by the fact that similarities of almost all *B. benzoevorans* group profiles were above 65 %. As example the DGGE profiles of the total bacterial and *B. benzoevorans* related communities in fresh soils are provided (Fig. 4b, d).

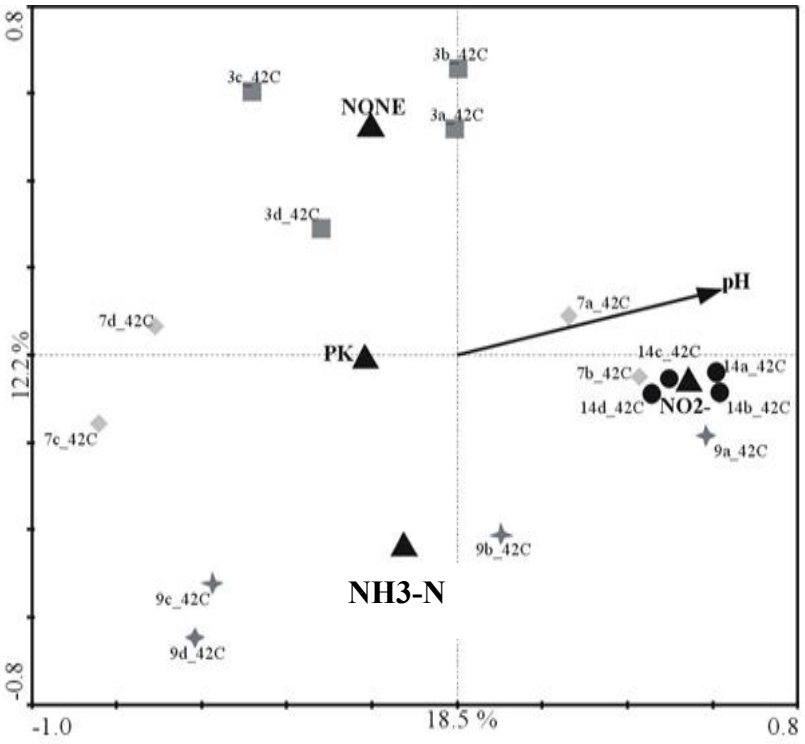
We used RDA to analyze and visualize the effect of different soil treatments (type fertilizer, pH, vegetation etc.) (Fig. 4a, c). The effect of environmental variables on the total bacterial and *B. benzoevorans* related communities in fresh, dried and stored soils was evaluated and the significance of the environmental variables (P value) was considered (Table 5). Most of the environmental parameters influencing the total bacterial community showed significant effect (P< 0.1). However, the opposite was observed for the *B. benzoevorans* related community, where often P> 0.1, indicating that the influence of the environmental parameters was statistically not significant.

Soil samples	Explained variability (%)**		pH		(NH_3)PKNaMg		(NO_2)PKNaMg		PKNaMg		NONE	
	T	B	T	B	T	B	T	B	T	B	T	B
Fresh	46.5	23.4	**0.024***	0.7	**0.006**	0.554	**0.006**	N.A.	0.35	0.956	N.A.	0.12
Dried	40.5	24	**0.022**	0.55	0.202	0.356	**0.028**	0.428	N.A.	0.79	**0.018**	N.A.
Stored	54.5	45	**0.002**	0.33	**0.01**	**0.04**	**0.006**	N.A.	**0.05**	**0.062**	N.A.	0.378

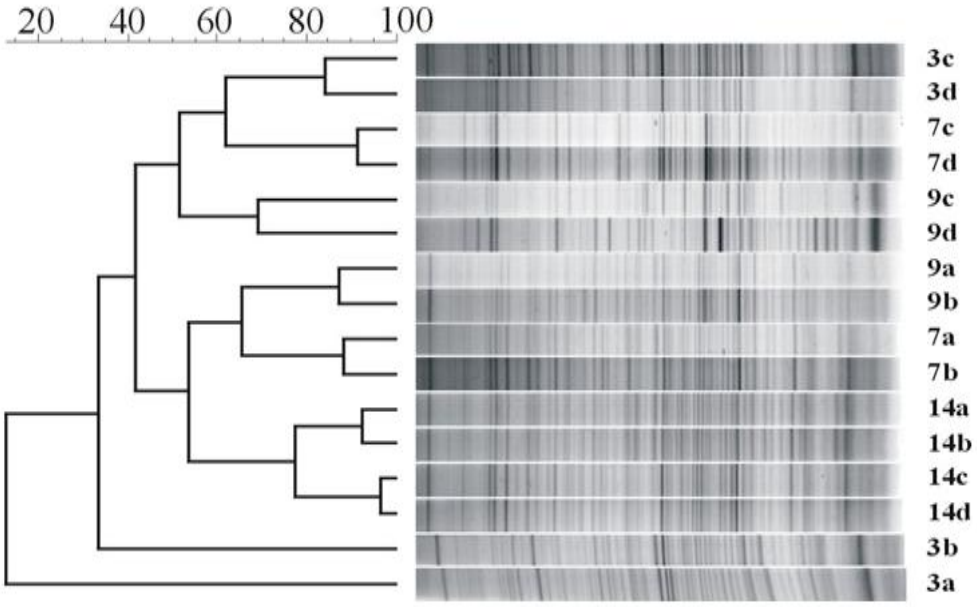
N.A.- data not available; *- numbers in "**Bold**" indicate significant P-values; **Explained variability indicates the variation in community composition that could be explained by all canonical axes in RDA analysis.

Table 5. Community variations and effect of the environmental variables (pH and fertilizer type) and their significance (P-values) on the total bacterial (T) and *B. benzoevorans* related (B) communities in fresh, dried and stored soil samples from the Park Grass experiment at Rothamsted.

A)


0.8
-0.8
12.2 %
-1.0
18.5 %
0.8
3b_42C
3c_42C
NONE
3a_42C
3d_42C
pH
7a_42C
7d_42C
PK
14c_42C
7b_42C
14a_42C
14d_42C
NO2-
14b_42C
7c_42C
9a_42C
9b_42C
NH3-N
9c_42C
9d_42C

B)


20
40
60
80
100
3c
3d
7c
7d
9c
9d
9a
9b
7a
7b
14a
14b
14c
14d
3b
3a

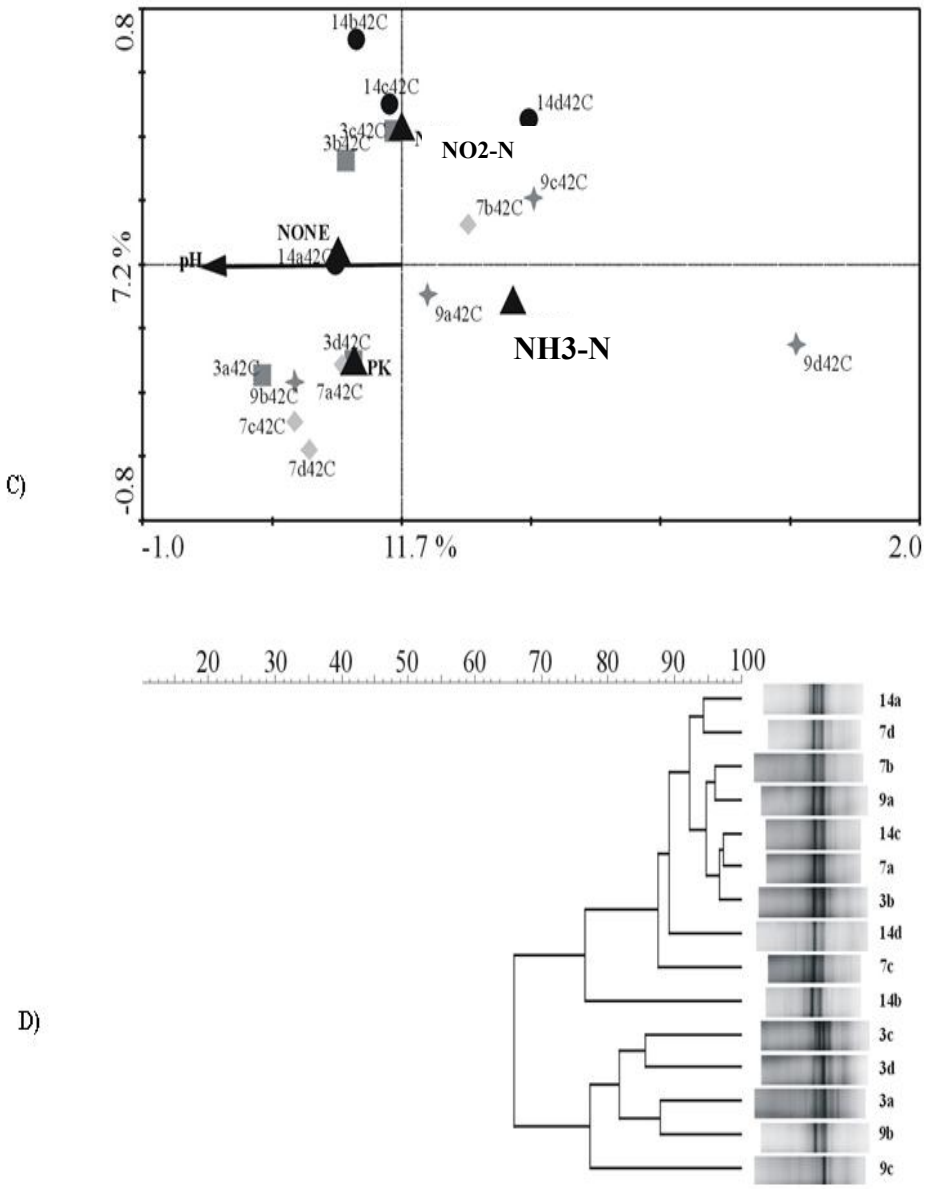


Figure 4. Redundancy analysis (RDA) ordination plot of the total bacterial (a) and *B. benzoevorans* related (b) communities Rothamsted fresh soil samples. Multivariate statistical analysis was used to assess the impact of drying and storing, as well as the effect of different soil treatments (type fertilizer, pH, soil type) on microbial community structure as measured by rRNA-based molecular approaches. ■3- samples collected from soil with no fertilizer (NONE); ♦7- PKNaMg as a fertilizer (PK); ✦9- (NH3)PKNaMg as fertilizer (NH3-N); ●14- (NO2)PKNaMg as fertilizer (NO2-N); a- soil pH=7; b- soil pH=6; c- soil pH=5; d- not adjusted soil; ▲- type of fertilizer as nominal environmental variables; → the vectors represent the quantitative environmental variable pH. The correlation tree based on the DGGE profiles of the total bacterial (b) and and B. benzoevorans related (d) communities are present.

Discussion

It has recently been shown that the application of molecular techniques to soil samples preserved by air-drying and long term storage could be used to identify differences in soil microbiota (prokaryotic as well as eukaryotic) along temporal and spatial gradients, as well as in response to different soil management strategies (8, 17, 30). However the impact of drying and storage of the soil have not been systematically addressed. In this study this effect on the pro- and eukaryotic soil communities was analyzed and compared in its magnitude to that of other soil characteristics such as fertilizer, pH, soil type and vegetation. The investigations were performed in two independent studies, using soil samples from the Park Grass experiment at Rothamsted as well as long-term grassland experimental fields in Wageningen. The changes in the pro- and eukaryotic soil communities were analyzed using SSU rRNA gene targeted PCR-DGGE, and the fingerprints were analyzed by multivariate statistics, using redundancy analysis (11) as implemented in the CANOCO software package. In order to assess the impact of soil preservation on detectable community profiles relative to the effect of other changes in environmental conditions (such as pH, type of fertilizer, type of soil and vegetation), the statistical analysis was performed in three steps. In this analysis either all soil parameters were chosen as environmental variables, or alternatively some soil parameters were chosen to be environmental variables and others- covariables (15). In the analysis of the total bacterial community in Rothamsted and Wageningen soil samples, and eukaryotic community (Rothamsted soil), most environmental variables showed to be significant ($P<0.1$). In this case, a large fraction of the observed differences in microbial community structure could be explained by the soil environmental parameters pH, fertilizer or soil type, while the influence of drying and storage on the bacterial community was smaller albeit still significant (Table 4). Hence, even though we observed a significant effect of drying and storage on bacterial community profiles, it did not interfere with our ability to identity changes in response to differences in environmental conditions, including agricultural practice. Zooming into a specific microbial group, namely that of *B. benzoevorans* related populations in the Rothamsted soil samples, however, a relatively stronger effect of sample preservation as compared to other environmental parameters was shown. Nevertheless, the *B. benzoevorans* related community showed relatively low diversity as judged by DGGE. In such case presence or absence of one band in the DGGE profile or even differences in the relative intensity of two bands could lead to significant statistical differences in the diversity,

which might be the cause of the observed stronger soil preservation effect. Such statistical differences do not necessarily correspond to significant biological changes in the community.

The total bacterial and *B. benzoevorans* related communities were studied in corresponding sets of fresh, dried and 3 months stored soils samples. While none of the environmental variables in the fresh and dried soil samples showed significant influence on the *B. benzoevorans* related community, fertilizer types were significant in samples stored for three months. Nevertheless, tendencies of stronger fertilizer effect could be also observed for the fresh and dried samples. These results were in line with those obtained for total bacterial community profiles.

The present study shows the results of two independent studies that demonstrated the influence of conservation of soil samples by drying and prolonged storage on detectable soil microbiota. Since soil samples archives can be seen as a treasure chest for ecological studies, and such are present not only at Wageningen University and Rothamsted experimental station but also in many other institutes, the findings reported here provide additional confidence that biomolecular approaches can indeed be used to tap into this rich resource.

Acknowledgements

This work was supported by a grand from the European Communities EC project "Exploration of Genomic and Metabolite Diversity of a Novel Group of Abundant Soil Bacteria" (BACREX-project QLK3-2000-01678). We specially acknowledge Antoon Akkermans for critical reading and stimulating discussions. We thank Paul Poulton, Rothamsted experimental station, and the Soil Science Center of Wageningen University and Research Center for kindly providing soil samples.

References

1. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143-169.
2. **Baskar, S., R. Baskar, and A. Kaushik.** 2003. Role of micro-organisms in weathering of the Konkan–Goa laterite formations. Current Science **85:**1129-1134.
3. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:**1935-1943.
4. **Bossio, D. A., M. S. Girvan, L. Verchot, J. Bullimore, T. Borelli, A. Albrecht, K. M. Scow, A. S. Ball, J. N. Pretty, and A. M. Osborn.** 2005. Soil microbial community response to land use change in an agricultural landscape of Western Kenya. Microbical Ecology **on line**.
5. **Chaudhry, Q., M. Blom-Zandstra, S. Gupta, and E. J. Joner.** 2005. Utilising the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment. Environ Sci Pollut Res Int. **12:**34-48.
6. **Chou, Q., M. Russel, D. Birch, J. Raymond, and W. Bloch.** 1992. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy number amplifications. Nucleic Acids Res. **20:**1717-1723.
7. **Diez, B., C. Pedros-Alio, T. L. Marsh, and R. Massana.** 2001. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl. Environ. Microbiol. **67:**2942-2951.
8. **Dolfing, J., A. Vos, J. Bloem, P. A. I. Ehlert, N. B. Naumova, and P. J. Kuikman.** 2004. Microbial diversity in archived soils. Science **306:**813.
9. **Eyers, L., I. George, L. Schuler, B. Stenuit, S. N. Agathos, and S. E. Fantroussi.** 2004. Environmental genomics: exploring the unmined richness of microbes to degrade xenobiotics. Appl. Microbiol. Biotechnol. **66:**123-130.
10. **Fang, C., P. Smith, J. B. Moncrieff, and J. U. Smith.** 2005. Similar response of labile and resistant soil organic matter pools to changes in temperature. Nature **433:**57- 59.
11. **Fromin, N., J. Hamelin, S. Tarnawski, D. Roesti, K. Jourdain-Miserez, N. Forestier, S. Teyssier-Cuvelle, F. Gillet, M. Aragno, and P. Rossi.** 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ. Microbiol. **4:**634-643.
12. **Häne, B. G., K. Jäger, and H. Drexler.** 1993. The pearson product-moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. Electrophoresis **14:**967-972.
13. **Heilig, H. G. H. J., E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. L.**

Akkermans, and W. M. de Vos. 2002. Molecular diversity of *Lactobacillus spp.* and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ. Microbiol. **68:**114-123.

14. **Lane, D. J.** 1991. 16S/23S rRNA sequencing. John Wiley & sons Ltd, UK.
15. **Leps, J., and P. Smilauer.** 2003. Multivariate analysis of ecological data using CANOCO. University Press, Cambridge.
16. **Marschner, P., and K. Baumann.** 2003. Changes in bacterial community structure induced by mycorrhizal colonisation in split-root maize. Plant and Soil **251:**279-289.
17. **Moon-van der Staay, S. Y., V. A. Tzeneva, G. W. M. van der Staay, W. M. de Vos, H. Smidt, and J. H. P. Hackstein.** 2006. Eukaryotic diversity in historical soil samples. FEMS Microbiol. Ecol. **in press**.
18. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695-700.
19. **Muyzer, G., and K. Smalla.** 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Leeuwenhoek **73:**127-141.
20. **Nübel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig, and H. Backhaus.** 1996. Sequence hetereogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis of 16S rDNA fragments. J. Bacteriol. **178:**5636-5643.
21. **Salles, J. F., J. A. van Veen, and J. D. van Elsas.** 2004. Multivariate analyses of *Burkholderia* species in soil: effect of crop and land use history. Appl. Environ. Microbiol. **70:**4012-4020.
22. **Sanguinetti, C. J., D. Neto, and A. J. G. Simpson.** 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques **17:**915-919.
23. **Semple, K. T., N. M. Dew, K. J. Doick, and A. H. Rhodes.** 2006. Can microbial mineralization be used to estimate microbial availability of organic contaminants in soil? Environ. Pollut. **104:**164-172.
24. **Smidt, H., and W. M. de Vos.** 2004. Anaerobic microbial dehalogenation. Annu. Rev. Microbiol. **58:**43-73.
25. **Somers, E., J. Vanderleyden, and M. Srinivasan.** 2004. Rhizosphere bacterial signalling: a love parade beneath our feet. Crit Rev Microbiol. **30:**205-240.
26. **Steenwerth, K. L., L. E. Jackson, F. J. Calderon, M. R. Stromberg, and K. M. Scow.** 2002. Soil microbial community composition and land use history in cultivated and grassland ecosystems of coastal California. Soil Biol. Biochem. **34:**1599-1611.
27. **ter Braak, C. J. F.** 1987. The analysis of vegetation-environment relationships by canonical correspondence analysis. Vegetatio **69:**69-77.
28. **Tiedje, J. M., S. Asuming-Brempong, K. Nusslein, T. L. Marsh, and S. J. Flynn.**

1999. Opening the black box of soil microbial diversity. Applied Soil Ecology **13:**109-122.

29. **Torsvik, V., J. Goksøyr, and F. L. Daae.** 1990. High diversity of DNA of soil bacteria. Appl. Environ. Microbiol. **56:**782-787.
30. **Tzeneva, V. A., Y. Li, A. D. M. Felske, W. M. de Vos, A. D. L. Akkermans, E. E. Vaughan, and H. Smidt.** 2004. Development and application of a selective PCR-Denaturing Gradient Gel Electrophoresis approach to detect a recently cultivated *Bacillus* group predominant in soil. Appl. Environ. Microbiol. **70:**5801-5809.
31. **van Elsas, J. D., and K. Smalla.** 1995. Extraction of microbial community DNA from soil. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular Microbial Ecology Manual, chapter 1.3.3. Kluwer Academic Publisher.
32. **Wagner, M.** 2004. Deciphering the function of uncultured microorganisms. ASM News **70:**63-70.
33. **Yu, Z., and M. Morrison.** 2004. Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-Denaturing Gradient Gel Electrophoresis. Appl. Environ. Microbiol. **70:**4800-4806.
34. **Zengler, K., G. Toledo, M. Rappe, J. Elkins, E. J. Mathur, J. M. Short, and M. Keller.** 2002. Cultivating the uncultured. Proc. Natl. Acad. Sci. USA **99:**15681-15686.
35. **Zoetendal, E. G., A. D. L. Akkermans, W. M. Akkermans - van Vliet, J. A. G. M. de Visser, and W. M. de Vos.** 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Microbial Ecol. Health Dis. **13:**129-134.

CHAPTER 7

COMPARATIVE GENOMICS OF *BACILLUS BENZOEVORANS* RELATED STRAINS USING A *BACILLUS SUBTILIS* MICROARRAY

Vesela A. Tzeneva, Kathrin Fritsche, Willem M. de Vos and Hauke Smidt

Abstract

While it was previously demonstrated that bacterial strains closely related to *Bacillus benzoevorans* constitute a significant part of soil microbiota, hardly any information is available on the genomic content of this group. In this study, we present a comparative genomics analysis of four strains from the group of *B. benzoevorans*-relatives using a *B. subtilis* whole genome microarray. Hybridization with reference sequences of known sequence identity with *B. subtilis* genes targeted on the array showed that sequences with 70 % identity could not be detected even under conditions of low stringency, concomitant with a high degree of unspecific cross-hybridization. Hybridization of genomic DNA of four representative strains of the group of *B. benzoevorans*- relatives, which have 16S rRNA sequence identities of 93-95 % with *B. subtilis*, yielded positive signals for 1.0 to 5.6 % of open reading frames (ORFs) targeted on the array, using a threshold of 50 % of the respective signal obtained with *B. subtilis* genomic DNA. Detected genes included mostly house keeping genes within most major functional classes, but also ORFs predicted to encode for proteins of yet unknown function. In addition to genes detected in all four strains tested in this study, mostly involved in protein synthesis, each strain showed also specific hybridization signals, which to a large extend, i.e. 33 – 50%, corresponded to genes within the categories "hypothetical proteins", "unclassified" or "unknown function".

Introduction

Previous studies could demonstrate that bacterial populations closely related to *Bacillus benzoevorans* constituted a predominant microbial group (up to 32%) in Dutch Drentse A soil samples (9, 10). In other studies they were detected in soil samples from other parts of The Netherlands, as well as in other countries, such as Portugal and Pakistan (the area of Himalaya), and the American continent (2, 3, 16, 24, 25). This novel phylogenetic lineage includes *B. benzoevorans, B. niacini, B. pseudomegaterium, B. jeotgali* (27), five recently isolated novel species: *B. novalis, B. vireti, B. soli, B. bataviae* and *B. drentensis* (13), uncultured bacterium DA001 (DA= Drentse A), and their relatives (7, 10).

While analysis of diversity and relative abundance of *B. benzoevorans* relatives was performed in several studies (11, 24-26), the functional and genomic diversity of the group remains largely unknown. Since recently, microbial function and diversity can be determined by the design and application of DNA microarrays (15, 23, 28). This technology allows fast analysis of abundance and similarity of genes, and their expression at the genomic scale in one experiment. In addition, microarrays have been designed for the simultaneous analysis of comprehensive sets of functional genes key to relevant environmental processes (15). Several studies describe the application of whole genome microarrays for the genomic comparison of different bacterial strains, species and even genera (4, 6, 18, 19). Read *et al.* used *B. anthracis* microarrays to test 19 *B. cereus* strains (16S rRNA identity 99.5 %) (22). In another study Dong *et al.* successfully hybridized *Klebsiella pneumoniae* DNA to a DNA-chip of *E. coli* K12 (16S rRNA identity 97%). Here we describe a comparison of the genomic content of four strains from the *B. benzoevorans*- related group using a *B. subtilis* whole genome microarray.

Materials and Methods

Pure cultures, DNA isolation, referent genes, primers and PCR conditions

Four pure cultures of *B. benzoevorans* (LMG15526), *B. drentensis* (LMG21830), *B. vireti* (LMG21834), *B. soli* (LMG21838) (provided by J. Heyrman, Ghent University, Belgium) (with 16S rRNA gene similarities with the reference organism *B. subtilis* of 93.0 %, 94.4 %, 93.6 %, and 93.8 %, respectively) and *B. subtilis* 168 were grown in liquid medium as recommended by the culture collection (BCCM/LMG medium 1; beef extract 1 g/l, yeast

extract 2 g/l, peptone 5 g/l, NaCl 5 g/l, pH 7,4) and DNA was extracted from 1 ml of fully grown culture. DNA was isolated directly using the Fast DNA®SPIN®Kit (For Soil) (Q BIOgene, Cambridge, United Kingdom) according to the manufacturers' instructions. DNA extracts were checked on a 1.2 % agarose gel, and nucleic acid concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). In order to control the specificity of the hybridization, reference genes with 70 % and 100 % similarity to *B. subtilis* genes were used. The 70 % similar genes were identified from genomic DNA of *B. benzoevorans*-related strains using representational difference analysis (8, 12), and corresponding fragments, cloned in pGEM-T plasmid vector, were kindly provided by Andreas Felske (Table 1).

Nr	Gene	% nucleotide identity to *B. subtilis*
1	*yusE*	70.5
2	*pyrAB*	70.1
3	*yfkN*	69.6
4	*rpsL*	75.1
5	*yabM*	71
6	*yqjI*	67.7
7	*ilvC*	68
8	*yqhB*	70.7

Table 1. Genomic fragments retrieved from *B. benzoevorans*-related strains by RDA with known similarity to *B. subtilis* genes used in this study for controls of the hybridization.

As a consequence of RDA procedures, cloned fragments are flanked by SP6 primer binding sites on either end, and hence could be amplified with primer SP6 (5'- ATT TAG GTG ACA CTA TAG- 3') (Promega, Leiden, The Netherlands). PCR was performed with *Taq* polymerase (Invitrogen, Carlsbad, CA) in a Whatman Biometra Thermocycler (Göttingen, Germany) using the following conditions: 68 °C for 4 min, 4 °C for 3 min, 45 cycles of 94 °C for 15 sec, 44 °C for 30 sec, and 68 °C for 1 min 50 sec, and finally 68 °C for 4 min 50 sec.

As 100 % similarity reference sequences, eight *B. subtilis* genes, namely *yau, yobI, yobO, yocE, yodJ, yojK, yojO* and *yomR*, were amplified with gene specific primers (provided by S. Holsappel, Groningen University, The Netherlands) using the following conditions: 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 48 °C for 20 sec, and 68 °C for 40 sec, and finally 68 °C for 7 min.

The amplified genes were purified with a QIAquick PCR purification Kit (Qiagen, Hilden, Germany) using manufacturer's instructions, the concentration of the genes was determined using a NanoDrop ND-1000 Spectrophotometer and the products were mixed in equimolar concentrations.

B. subtilis microarray

For comparative genome hybridization of DNA of *B. benzoevorans* related strains, a *B. subtilis*168 microarray was used. The chip was commercially produced by Eurogentech (Seraing, Belgium). *B. subtilis* microarrays were manufactured by printing PCR amplicons suspended in optimized buffer onto aldehyde coated glass slides. 8192 primers were designed and synthesized to amplify *B. subtilis* 4096 ORFs from the genome. The primers were designed to amplify each ORF beginning at the start codon and ending at the stop codon. PCR products were spotted in duplicates on an area of 1.8x1.8 cm. Each spot had a diameter of 100 micron and consisted of 0.6nl of DNA solution.

Labeling, hybridization and washing conditions

1 µg of genomic DNA was labelled with a random priming protocol based on the BioPrime DNA labeling kit (Invitrogen, Carlsbad, CA). DNA of the tested strains was labelled with Cy-5, while DNA of *B. subtilis* 168, used as reference strain, was labelled with Cy-3. The labeling reaction was incubated for 1.5 h at 37 ^{o}C. Removal of unincorporated dye molecules was performed using AutoSeq G50 columns (Amersham Biosciences, Piscataway, NJ). Hybridization was performed overnight at 37 or 42 ^{o}C (standard conditions) using the hybridization buffer EasyHyb (Roche, Basel, Switzerland) in a water bath. Washing of the slides was performed at 37 ^{o}C by 10 sec shaking in 1×SSC, 0.2% SDS, 10 sec shaking in 0.5×SSC, followed by two times 10 min shaking in 0.2×SSC at 37 ^{o}C on a rotary shaker at 300 rpm. The washed slides were dried under a flow of N_2 and scanned.

Scanning and data analysis

Slides were scanned using an Agilent DNA Microarray Scanner BA (Agilent Technologies Inc., Palo Alto, CA). The data was analyzed using the grid provided by Eurogentech. Mean signals were corrected by subtraction of the background signal from the mean value per hybridized spot. Signals were considered positive when the ratio of signal to background was >2. The ratio between background-corrected Cy-5 (tester) and Cy3 (*B. subtilis* control) signals was used to call presence or absence of the gene in the tested strain, using a threshold of Cy5 / Cy3 of either ≥ 0,1 or ≥ 0,5.

Assignment of ORFs to functional categories was done according to data provided through the Comprehensive Microbial Resource of The Institute for Genomic Research (TIGR-CMR) (20).

Results and Discussion

The goal of this study was to assess the possibility for comparative genome hybridization of *B. benzoevorans* related strains using a microarray designed based on the whole genome of *B. subtilis* strain 168. Microarray technology for the first time provides the means for comprehensive and fast analysis of abundance and similarity of a large number of genes in one single experiment, providing unprecedented insight in microbial function and diversity (15, 17, 23, 28). Using genomic fragment-targeted probes attached to a surface, e.g. a glass-slide, one is able to detect similarities or differences in gene expression profiles under different experimental conditions and in the genetic content between strains. The application of DNA microarrays for genomic comparison has been reported in previous studies. Although most of the studies compared strains belonging to the same species, some inter-species microarray hybridizations have been described, comparing genomes of microorganisms with 16S rRNA identities of ≥ 97 % (4, 6, 19, 22). In our study, however, the 16S rRNA gene similarity between *B. subtilis* 168 and *B. benzoevorans* relatives was less than 95 %. This fact required some optimisation in the hybridizing conditions. For this aim we used genes with known (i.e. 70 and 100 %) similarity to *B. subtilis* genes targeted on the microarray. Hybridization was performed at two different stringencies, namely at 37 and 42 ºC. Hybridization of the reference genes with approximately 70 % identity to *B. subtilis* genes was not observed at any of the tested hybridization conditions. However, while hybridization at 37 ºC resulted in a high degree of unspecific cross-hybridization and thus a large number of false positives, this was not observed at the recommended 42 ºC, concomitant with specific signals for genes with 100% identity. Hence, these conditions were further used for the analysis of *B. benzoevorans* related species.

For quality control of the hybridization and as a reference for strain comparison, each array was hybridized with *B. subtilis* 168 genomic DNA labelled with Cy3, resulting in positive signals for 99 % of all ORFs targeted on the array. Hybridization with Cy5-labelled genomic DNA of tested strains yielded positive signals for 11.7 to 35.1 % of all ORFs targeted on the chip, when a threshold of 10 % of the corresponding signal obtained for *B. subtilis* 168 was used (Table 2).

***B. benzoevorans* related strains**	***B. subtilis* 168 16S rRNA**	**10 % signal**	**50 % signal**
B. benzoevorans (LMG15526)	93.0	35.1	1.0
B. drentensis (LMG21830)	94.3	11.7	5.6
B. soli (LMG21838)	93.8	12.8	5.0
B. vireti (LMG21834)	93.6	19.2	2.5

Table 2. 16S rRNA sequence identity of different *B. benzoevorans* related strains compared with *B. subtilis* 168, and percentage of ORFs with positive hybridization at two different threshold levels (10 % or 50 % of corresponding *B. subtilis* 168 signals).

However, only 1.0 to 5.6 % positive signals were observed when the threshold was set to 50 %. ORFs detected at this cut-off represented all major functional classes, with the exception of signal transduction (Table 3). Detected genes included mainly house keeping genes, such as genes encoding ribosomal proteins, ATP synthase, amino acyl tRNA synthetases, RNA polymerase, pyruvate dehydrogenase, and genes coding for polypeptides involved in amino acid and sugar metabolism, and transcription regulation. At a threshold of 50 %, but not at 10 %, the extend of genomic similarity of *B. benzoevorans*-related strains and *B. subtilis* strain 168 was in agreement with 16S rRNA sequence identity, with *B. benzoevorans* and *B. drentensis* showing the lowest and highest number of positive hybridization events, respectively (Fig. 1).

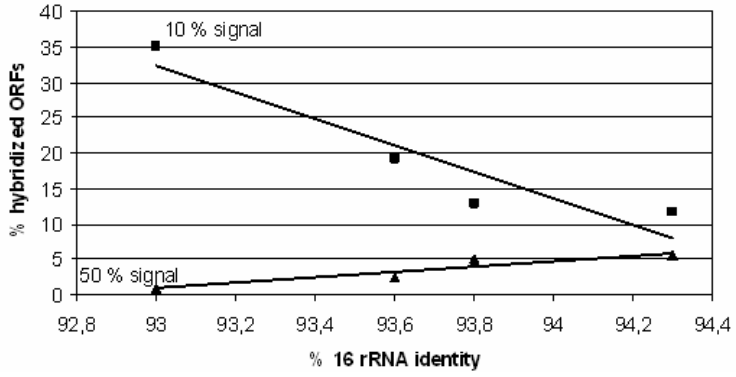


Figure 1: Correlation of 16S rRNA sequence identity between *B. subtilis* strain 168 and four *B. benzoevorans*-related strains (see Table 2 for details) and fraction of ORFs with hybridization signals passing a threshold of 10% (■) or 50% (▲)when compared to signals obtained for *B. subtilis* strain 168.

Interestingly, *B. benzoevorans* showed the highest fraction of genes hybridized at a threshold of 10 % (i.e. 35.1 % of all targeted ORFs), indicating that this cut-off is less well suited for the detection of real positive signals. Overall ribosomal protein-encoding genes were the most frequently found positive signals in the four tested strains, ranging from 9.7 to 48.7 % of all detected ORFs (Table 3). As expected, this fraction was highest for strains with lower overall

similarity with *B. subtilis*. Moreover, the functional category "protein synthesis" comprised 75 % (15/20) of genes with positive hybridization (at 50 % threshold) to all four strains (Table 4). Other predicted products of genes shared by all strains included an enolase involved in energy metabolism, heat shock protein GroEL, RNA polymerase, and a protein with unknown function, YpzE. All of these, with the exception of *ypzE* and ribosomal protein L11-encoding *rplK*, were also found to be essential for *B. subtilis* in a systematic genome-wide gene-inactivation effort (14). Interestingly, each strain showed also strain-specific hybridization signals, which to a large extend, i.e. 33 – 50 %, corresponded to genes within the categories "hypothetical proteins", "unclassified" or "unknown function" (Table 4). None of these genes were found or predicted to be essential in *B. subtilis*, suggesting that the different strains, even though closely related, share different, non-essential genes with the more distantly related *B. subtilis*.

Functional classes	***B.b.***	***B.d.***	***B.so.***	***B.v.***
Amino acid biosynthesis	1 (2.6)	8 (3.5)	6 (2.9)	1 (1.0)
Biosynthesis of cofactors, prosthetic groups, and carriers	0	4 (1.7)	6 (2.9)	0
Cell envelope	0	3 (1.3)	1 (0.5)	0
Cellular processes	0	10 (4.3)	11 (5.3)	5 (4.8)
Central intermediary metabolism	0	3 (1.3)	3 (1.4)	1 (1.0)
DNA metabolism	0	12 (5.2)	8 (3.9)	0
Energy metabolism	3 (7.7)	35 (15.2)	19 (9.2)	12 (11.5)
Fatty acid and phospholipid metabolism	1 (2.6)	2 (0.9)	9 (4.3)	1 (1.0)
Hypothetical proteins	1 (2.6)	9 (3.9)	4 (1.9)	2 (1.9)
Mobile and extrachromosomal element functions	0	2 (0.9)	2 (1)	0
Protein fate	1 (2.6)	10 (4.3)	13 (6.3)	6 (5.8)
Protein synthesis	21(53.8)	51 (22.1)	27 (1)	45 (43.3)
Ribosomal proteins	19 (48.7)	32 (13.8)	20 (9.7)	42 (40.4)
Purines, pyrimidines, nucleosides, and nucleotides	0	10 (4.3)	1 (0.5)	2 (1.9)
Regulatory functions	2 (5.1)	7 (3.0)	8 (3.9)	8 (7.7)
Signal transduction	0	0	0	0
Transcription	4 (10.3)	10 (4.3)	5 (2.4)	6 (5.8)
Transport and binding proteins	0	10 (4.3)	17 (8.2)	2 (1.9)
Unclassified	5 (12.8)	38 (16.5)	59 (28.5)	12 (11.5)
Unknown function	0	7 (3.0)	8 (3.9)	1 (1.0)
TOTAL	**39**	**231**	**207**	**104**

Table 3. Distribution of positive signals (50 % threshold) over functional classes. For the assignment of ORFs to functional classes, data provided by TIGR-CMR were used (20). *B.b.*, *B. benzoevorans*; *B.d.*, *B. drentensis*; *B.so.*, *B. soli*; *B.v.*, *B. vireti*. Values in parentheses are given as percentages of the total number of detected ORFs per strain.

Functional classes	**All**	***B.b.***	***B.d.***	***B.so.***	***B.v.***
Amino acid biosynthesis	0	0	0	1	0
Biosynthesis of cofactors, prosthetic groups, and carriers	0	0	0	2	0
Cell envelope	0	0	0	0	0
Cellular processes	0	0	2	7	2
Central intermediary metabolism	0	0	0	2	1
DNA metabolism	0	0	0	5	0
Energy metabolism	1	0	1	9	0
Fatty acid and phospholipid metabolism	0	1	0	1	1
Hypothetical proteins	0	0	1	2	2
Mobile and extrachromosomal element functions	0	0	1	1	0
Protein fate	1	0	0	6	0
Protein synthesis	15	0	1	1	2
Purines, pyrimidines, nucleosides, and nucleotides	0	0	1	0	0
Regulatory functions	0	1	1	3	4
Signal transduction	0	0	0	0	0
Transcription	2	0	0	0	0
Transport and binding proteins	0	0	3	8	2
Unclassified	1	2	7	35	3
Unknown function	0	0	1	6	1
TOTAL	**20**	**4**	**19**	**89**	**18**

Table 4. Functional class distribution of genes showing positive hybridization signals with genomic DNA of all or single *B. benzoevorans*-related strains at a threshold of 50 % signal intensity compared to *B. subtilis* strain 168. *B.b.*, *B. benzoevorans*; *B.d.*, *B. drentensis*; *B.so.*, *B. soli*; *B.v.*, *B. vireti*. Assignment to functional classes was according to data provided at TIGR-CMR.

Using a *B. subtilis* whole genome microarray we were able to perform comparative genomics among different strains belonging to the group of *B. benzoevorans* relatives, albeit only a limited number of genes targeted on the array showed positive hybridization due to low overall genomic similarity. While different studies demonstrated that genome hybridization at the inter-species level is possible, in our case sequence similarity between the tested *B. benzoevorans*-related strains *B. subtilis* was obviously at the high end of allowable genomic distance. Nevertheless, we were able to detect several, mainly house keeping genes within different functional classes, but also a significant number of genes predicted to encode for proteins of yet unknown function, providing leads for future studies aiming at elucidation of their functional role, and their contribution to eco-physiological differentiation of closely related strains within the group of *B. benzoevorans* relatives. Hence, while this method is expected to be useful for the development of novel taxonomic approaches, different strategies might be expected to yield higher discriminatory power, such as random genomic fragment- or clone-based arrays constructed from a number of reference strains within the group of *B. benzoevorans* relatives (5, 21, 28).

Acknowledgements

This work was supported by a grant from the European Communities EC project "Exploration of Genomic and Metabolite Diversity of a Novel Group of Abundant Soil Bacteria" (BACREX-project QLK3-2000-01678). We thank Erwin Zoetendal for the valuable discussions and Andreas Felske for providing the RDA fragments.

References

1. **Ash, C., J. A. E. Farrow, S. Wallbanks, and M. D. Collins.** 1991. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. Lett. Appl. Microbiol. **13:**202–206.
2. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:**1935-1943.
3. **Borneman, J., and E. W. Triplett.** 1997. Molecular microbial diversity in soils from Eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Environ. Microbiol. **63:**2647-2653.
4. **Chan, K., S. Baker, C. C. Kim, C. S. Detweiler, G. Dougan, and S. Falkow.** 2003. Genomic comparison of *Salmonella enterica* serovars and *Salmonella bongori* by use of an *S. enterica* serovar typhimurium DNA microarray. J. Bacteriol. **185:**553-563.
5. **Cho, J.-C., and J. M. Tiedje.** 2001. Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays. Appl. Environ. Microbiol. **67:**3677-3682.
6. **Dong, Y., J. D. Glasner, F. R. Blattner, and E. W. Triplett.** 2001. Genomic interspecies microarray hybridization: rapid discovery of three thousand genes in the mize endophyte, *Klebsiella pneumoniae* 342, by microarray hybridization with *Escherichia coli* K-12 open reading frames. Appl. Environ. Microbiol. **67:**1911-1921.
7. **Felske, A.** 1999. Reviewing the DA001-files: A 16S rRNA chase on suspect #X99967, a *Bacillus* and Dutch underground activist. J. Microbiol. Meth. **36:**77-93.
8. **Felske, A.** 2002. Streamlined representational difference analysis for comprehensive studies of numerous genomes. J. Microbiol. Meth. **50:**305-311.
9. **Felske, A., and A. D. L. Akkermans.** 1998. Spatial homogeneity of the most abundant bacterial 16S rRNA molecules in grassland soils. Microbiol. Ecol. **36:**31-36.
10. **Felske, A., A. Wolterink, R. van Lis, and A. D. L. Akkermans.** 1998. Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). Appl. Environ. Microbiol. **64:**871-879.
11. **Felske, A. D. M., V. A. Tzeneva, J. Heyrman, M. A. Langeveld, A. D. L. Akkermans, and P. De Vos.** 2004. Isolation and biodiversity of hitherto undescribed soil bacteria related to *Bacillus niacini*. Microbiol. Ecol. **48:**111-119.
12. **Fromin, N., J. Hamelin, S. Tarnawski, D. Roesti, K. Jourdain-Miserez, N. Forestier, S. Teyssier-Cuvelle, F. Gillet, M. Aragno, and P. Rossi.** 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ. Microbiol. **4:**634-643.
13. **Heyrman, J., B. Vanparys, N. A. Logan, A. Balcaen, M. Rodriguez-Diaz, A. Felske, and P. De Vos.** 2004. *Bacillus*

novalis sp. nov., *Bacillus vireti sp.* nov., *Bacillus soli sp.* nov., *Bacillus bataviae sp.* nov. and *Bacillus drentensis sp.* nov., five new species isolated from the Drentse A grasslands. Int. J. Syst. Evol. Microbiol. **54:**47-57.

14. **Kobayashi, K., S. D. Ehrlich, A. Albertini, G. Amati, K. K. Andersen, M. Arnaud, K. Asai, S. Ashikaga, S. Aymerich, P. Bessieres, F. Boland, S. C. Brignell, S. Bron, K. Bunai, J. Chapuis, L. C. Christiansen, A. Danchin, M. Debarbouille, E. Dervyn, E. Deuerling, K. Devine, S. K. Devine, O. Dreesen, J. Errington, S. Fillinger, S. J. Foster, Y. Fujita, A. Galizzi, R. Gardan, C. Eschevins, T. Fukushima, K. Haga, C. R. Harwood, M. Hecker, D. Hosoya, M. F. Hullo, H. Kakeshita, D. Karamata, Y. Kasahara, F. Kawamura, K. Koga, P. Koski, R. Kuwana, D. Imamura, M. Ishimaru, S. Ishikawa, I. Ishio, D. Le Coq, A. Masson, C. Mauel, R. Meima, R. P. Mellado, A. Moir, S. Moriya, E. Nagakawa, H. Nanamiya, S. Nakai, P. Nygaard, M. Ogura, T. Ohanan, M. O'Reilly, M. O'Rourke, Z. Pragai, H. M. Pooley, G. Rapoport, J. P. Rawlins, L. A. Rivas, C. Rivolta, A. Sadaie, Y. Sadaie, M. Sarvas, T. Sato, H. H. Saxild, E. Scanlan, W. Schumann, J. F. M. L. Seegers, J. Sekiguchi, A. Sekowska, S. J. Seror, M. Simon, P. Stragier, R. Studer, H. Takamatsu, T. Tanaka, M. Takeuchi, H. B. Thomaides, V. Vagner, J. M. van Dijl, K. Watabe, A. Wipat, H. Yamamoto, M. Yamamoto, Y. Yamamoto, K. Yamane, K. Yata, K. Yoshida, H. Yoshikawa, U. Zuber, and N. Ogasawara.** 2003. Essential *Bacillus subtilis* genes. Proc. Natl. Acad. Sci. USA **100:**4678-4683.

15. **Liu, W.-T., and l. Zhu.** 2005. Environmental microbiology-on-a-chip and its future impacts. TIBTECH **23:**174-179.

16. **Lloyd-Jones, G., and P. C. Lau.** 1998. A molecular view of microbial diversity in a dynamic landfill in Quebec. FEMS Microbiol. Lett. **162:**219-226.

17. **Loy, A., and L. Bodrossy.** 2006. Highly parallel microbial diagnostics using oligonucleotide microarrays. Clinica Chimica Acta **363:**106-119.

18. **Molenaar, D., F. Bringel, F. H. Schuren, W. M. de Vos, R. J. Siezen, and M. Kleerebezem.** 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J. Bacteriol. **187:**6119-6127.

19. **Murray, A. E., D. Lies, G. Li, K. Nealson, J. Zhou, and J. M. Tiedje.** 2001. DNA/DNA hybridization to microarrays reveals gene-specific differences between closely related microbial genomes. Proc. Natl. Acad. Sci. USA **98:**9853-9858.

20. **Peterson, J. D., L. A. Umayam, T. M. Dickinson, E. K. Hickey, and O. White.** 2001. The Comprehensive Microbial Resource. Nucleic Acids Res. **29:**123-125.

21. **Pieterse, B., E. J. Quirijns, F. H. Schuren, and M. J. van der Werf.** 2005. Mathematical design of prokaryotic clone-based microarrays. BMC Bioinformatics **6:**238.

22. **Read, T. D., S. N. Peterson, N. Tourasse, L. W. Baillie, I. T. Paulsen, K. E. Nelson, H. Tettelin, D. E. Fouts, J. A. Eisen, S. R. Gill, E. K. Holtzapple, O. A.**

Okstad, E. Helgason, J. Rilstone, M. Wu, J. F. Kolonay, M. J. Beanan, R. J. Dodson, L. M. Brinkac, M. Gwinn, R. T. DeBoy, R. Madpu, S. C. Daugherty, A. S. Durkin, D. H. Haft, W. C. Nelson, J. D. Peterson, M. Pop, H. M. Khouri, D. Radune, J. L. Benton, Y. Mahamoud, L. Jiang, I. R. Hance, J. F. Weidman, K. J. Berry, R. D. Plaut, A. M. Wolf, K. L. Watkins, W. C. Nierman, A. Hazen, R. Cline, C. Redmond, J. E. Thwaite, O. White, S. L. Salzberg, B. Thomason, A. M. Friedlander, T. M. Koehler, P. C. Hanna, A.-B. Kolsto, and C. M. Fraser. 2003. The genome sequence of *Bacillus anthracis* ames and comparison to closely related bacteria. Nature **423:**81.

23. **Rick, W. Y., L. Wang, L. Bedzyk, and K. Croker.** 2001. Application of DNA microarrays in microbial systems. J. Microbiol. Meth. **47:**257-272.

24. **Tzeneva, V. A., P. A. I. Ehlert, W. M. de Vos, A. D. L. Akkermans, and H. Smidt.** 2006. Riding ecological time- machine: succession of abundant soil bacteria in reclaimed land. Science **to be submitted**.

25. **Tzeneva, V. A., Y. Li, A. D. M. Felske, W. M. de Vos, A. D. L. Akkermans, E. E. Vaughan, and H. Smidt.** 2004. Development and application of a selective PCR-Denaturing Gradient Gel Electrophoresis approach to detect a recently cultivated *Bacillus* group predominant in soil. Appl. Environ. Microbiol. **70:**5801-5809.

26. **Tzeneva, V. A., J. F. Salles, N. Naumova, W. M. de Vos, P. J. Kuikman, J. Dolfing, and H. Smidt.** 2006. Effect of drying and long term storage of soil samples on the bacterial and eukaryotic diversity monitored by DGGE. FEMS Microbial Ecology **to be submitted**.

27. **Yoon, J.-H., S.-S. Kang, K.-C. Lee, Y. H. Kho, S. H. Choi, and K. H. P. Kang, Y.-H.** 2001. *Bacillus jeotgali* sp. nov., isolated from jeotgal, Korean traditional fermented seafood. Int. J. Syst. Evol. Microbiol. **51:**1087-1092.

28. **Zhou, J.** 2003. Microarrays for bacterial detection and microbial community analysis. Curr. Opin. Microbiol. **6:**288-294.

CHAPTER 8

DISCUSSION

General Disussion

Spore-forming bacteria of the genus Bacillus are ubiquitous in various environments and their spores represents one of the hardiest and longest-lived cells on Earth (36, 37). Spores were found not only in soil environments, but e. g. also in mined amber (7), halite crystals (58), and marine manganese nodules (35). Recently, granite rock was found to contain large numbers of cultivable Bacillus spores, including some members belonging to the group of B. benzoevorans relatives (15).

In this thesis, both culture-dependent and independent strategies are described to monitor B. benzoevorans-related soil bacteria that are predominant in a variety of terrestrial environments around the world. Selective cultivation media and group-specific primers and probes were developed allowing rapid detection of their diversity and relative abundance in environmental samples. Using these techniques we demonstrated the distribution of this intriguing group in different geographical locations, and monitored their dynamics over time and in response to changing environmental conditions in various soil samples.

Media for the enrichment of **B. benzoevorans** *relatives*

Until now classic bacterial cultivation approaches were not sufficient for detection and isolation of bacteria related to *B. benzoevorans*. Media with high selectivity in combination with high-throughout screening of colony forming units were required for their isolation. In an enrichment campaign we used a variety of mineral media, and especially those amended with acetate resulted in the isolation of a large number of different strains related to *B. niacini*, one of the sub-clusters belonging to the group of *B. benzoevorans* relatives. This was in line with a previously described concept for growing recalcitrant bacteria based on the use of the passively cell-wall-penetrating carbon source acetate (6). Among the conditions tested here, incubation on mineral medium plus acetate at 37 ºC was the best approach to enrich *B. niacini* relatives on agar plates inoculated with soil suspensions. This variant demonstrated highest efficacy, as 1 of 10 picked colonies was a *B. niacini* relative, which was a considerable yield in such a highly complex source community. Nevertheless, also other media yielded significant numbers of *B. niacini* related isolates.

Besides the successful cultivation of *B. niacini* relatives, we were also able to retrieve a large number of other *B. benzoevorans*- related isolates from a variety of soil samples using mineral media supplemented with acetate or benzoate as substrates. The majority of isolates was obtained using sodium benzoate amended mineral medium, whereas sodium acetate media proved less suitable for the isolation of *B. benzoevorans* relatives (except *B. niacini*). This indicated that most probably the majority of species belonging to the *B. benzoevorans* related cluster have better metabolic potential to utilize benzoate than acetate, as a substrate (39).

It can be assumed that the very low redundancy among all the isolates was at least in part caused by the use of different media. This indicated that for the isolation of the largest possible diversity of *B. benzoevorans*– related bacteria, the application of an array of different media will be more efficient than the use of a single, optimized medium. This is in line with observations made by other innovative high-throughput cultivation campaigns, where a larger diversity was observed when using complementary media (27, 49). Interestingly, we observed that the majority of strains could be regarded as fast growing after a few consecutive transfers. This observation suggested that their absence in previous cultivation surveys was apparently not caused by limited or unusual metabolic potential. Also, the most thoroughly studied member, *B. niacini*, did not show any restrained utilization of C-sources. Rather, it is even one of the most versatile utilizers of organic and amino acids (34).

The group of *B. benzoevorans* related bacteria was first recognized in 16S rRNA gene clone libraries as abundant but yet uncultured soil bacteria (22). The 16S rRNA gene sequence is the prime identification feature for this group. Moreover, the success of the cultivation study underlines the cumulative value of culture-independent approaches when they are linked to and directing the isolation of novel bacterial lineages (43). The isolation of a substantial collection of cultured representatives of this novel lineage of *Bacillus* now offers a solid basis to gain more insight in the eco-physiology of these microorganisms.

Molecular tools for monitoring of B. benzoevorans- *relatives*

Even though a large number of novel isolates of the *B. benzoevorans* relatives could be obtained, not all members of this lineage can be cultivated with similar efficiency, in line with the general assumption that most members of the soil microbiota (estimated 99.5 to

99.9%) have not yet been isolated and cultivated on laboratory media (1, 4, 44, 52, 59). To compensate for this obvious shortcoming, we developed fast and sensitive methods for qualitative and quantitative analysis of the *B. benzoevorans* relatives in the soil based on group-specific molecular approaches (PCR, DGGE, dot-blot hybridization). Focusing on a fast and easy detection of *B. benzoevorans*-related bacteria in different soil samples we designed two primer sets, called REX primers, to consecutively exclude non-target *Bacillus* species. Comparison of DGGE profiles generated from DNA directly isolated from soil samples with fingerprints of populations that could be cultivated from the same environments, showed that most of the predominant *B. benzoevorans* related microorganisms detected by these primers could actually not be cultivated under the applied conditions (54). The REX primers provide a convenient and fast method not only for detection of *B. benzoevorans* relative populations in the soil samples, but also for monitoring their dynamics over time and space, including extended biogeographic surveys using soil samples from different countries around the globe.

Although the specific PCR-DGGE approach has proven to be helpful to understand qualitative changes in composition and diversity of the bacterial community, no or only little quantitative information was obtained. To complement for this limitation inherent to PCR-DGGE analysis, we developed and applied quantitative approaches to monitor relative abundance of *B. benzoevorans*-relatives in the soil. This method, based on dot-blot hybridization with group specific auto-probing, can be used for estimation of relative abundance of *B. benzoevorans* relatives in the soil. A real time PCR approach was tested as a possible alternative, however, different *Bacillus* species showed too high 16S rRNA sequence similarities and it was not possible to develop a practical number of primer pairs that would be sufficiently specific to amplify only target sequences from *B. benzoevorans* relatives. Until now the *B. benzoevorans* related community was quantitatively estimated based on clone libraries (19) or using reverse-transcript PCR in combination with DGGE (18), both time- and labor- consuming methods. Approaches for qualitative and quantitative monitoring of *B. benzoevorans*-related bacteria in different soil samples described in this thesis are fast, specific and applicable directly to environmental samples. This allowed us to provide comprehensive datasets on their distribution, diversity and abundance as a function of time, space, as well as changing environmental conditions.

We applied multivariate statistical analyses as implemented in CANOCO software to assess the significance of the differences observed between qualitative and quantitative biomolecular data obtained for different samples, and to correlate these differences with

environmental parameters, such as fertilization, sampling time, and above-ground vegetation. Since CANOCO software was developed by biologists and its focus is to be applied for biological studies, it is a convenient statistical tool for observing the relationship between the soil bacterial community and environmental parameters (50).

The last decade has witnessed the development of numerous microarray-based approaches for fast and comprehensive genetic and functional screening of microorganisms. This high-throughput technology provides the opportunity to target, study and exploit functional genes for relevant environmental processes at the genomic and metagenomic scales (30). Furthermore, microarray technology has also been exploited for the genomic comparison between microorganisms of different bacterial species and even genera (8, 12, 33, 45), as well as for diversity profiling of microbial communities (31, 60). A recent example of such a diversity microarray is the Human Intestinal Tract Chip (HITChip), which allows for the high through-put detection, identification and relative quantification of more than 1000 species present in the human intestinal tract microbiota (42). In our study, we compared different members of the *B. benzoevorans* relatives using a microarray based on the *B. subtilis* strain 168 genome. While 16S rRNA gene similarity between *B. subtilis* strain 168 and four *B. benzoevorans* relatives tested in this study was below 95 %, hybridization was observed with a significant number of genes targeted by the array. These included ORFs common to all strains tested, but also strain-specific genes, which to a large extend corresponded to genes predicted to encode for proteins of yet unknown function.

Changing environments shape soil microbial communities

The polder soil as developing environment for bacterial community succession

The polder soil environment, created by man, is in fact a reset ecosystem in which bacteria re-start their colonization due to the drastic changes in environmental conditions. From the microbiologists' perspective such ecosystem has just been born, in an environment with fit nurture, allowing for de novo bacterial succession.

Wieringermeer polder soil samples were collected during the period 1942- 1975, air-dried and stored for several decades. Significant differences in the DGGE profiles of the *B. benzoevorans* related community in these soil samples were observed in the period from 1942 to 1950. These changes could be explained, on one hand, with the fact that in 1945 the

Wieringermeer polder was flooded during the war (51). On the other hand, the polder was subsequently reclaimed and at this time agricultural activities were also developed which can be another reason for the observed increase in the bacterial diversity in the later period of the reclaimed land. From 1950 onwards, the richness did not alter, but the intensity of the DGGE bands slightly varied, suggesting changing relative abundance of the populations within this bacterial community. The *B. benzoevorans*-related community in another set of 29 soil samples from a second field trial within the Wieringermeer polder revealed similarly low richness with respect to the *B. benzoevorans* related as well as the total bacterial community. Because of the soil type and in analogy to findings from previous studies (54, 57), such simple fingerprints could be expected. The samples were collected from an agricultural area, where the soil conditions were strictly controlled by farming practices, which can explain the low total bacterial diversity (29, 46, 56). While another possible explanation of the observed low richness could be the fact that the soil samples were air-dried and stored for a period of up to 44 years, systematic analysis of this effect using samples from two different soil environments did not reveal significant reduction in diversity upon drying and storage for up to 4 months, rendering this explanation less plausible (55).

Remarkably, for several samples the total bacterial community was almost identical to that of the *B. benzoevorans* relatives, as judged by DGGE. Moreover, all bands from the *B. benzoevorans* related profiles could be detected as dominant bands in the total bacterial DGGE fingerprints. This suggested that, as observed in previous studies for other soils (16, 20, 54) they were a predominant group of bacteria in the Wieringermeer polder during the studied period. To further substantiate this, a novel approach to measure relative abundance of the group of *B. benzoevorans* relatives was developed in the framework of this thesis. Applying this method to the microbial community in the 29 Wieringermeer polder soil samples, we were able to quantitatively follow the succession of the *B. benzoevorans* related community in the polder soil confirming that they comprised a significant part (up to 40 %) of the total DNA isolated from these soil samples. Interestingly, a gradual increase of relative abundance in time could be observed.

Our experimental observations were confirmed by applying multivariate statistical analyses using Canonical Correspondence Analysis (CCA), showing that time significantly contributed to the explanation of variation between fingerprints. In contrast, we could not identify a significant influence of the above-ground vegetation on the microbial communities. The crop rotation showed no significant effect, which was expected for bulk surface soils where the rhizosphere is not well developed (24, 28). Archived soil samples collected from

Dutch polder environments provided us with a unique possibility to follow the behavior of the soil communities over a long period of time. In fact, with this study we could deepen our understanding of the microbial dynamics in the Dutch polder lands shortly after their reclamation.

Eukaryotic community in Wieringermeer polder soil samples

It is generally accepted that, although the protozoa biomass in agricultural soil is estimated to be 10 to 100 times lower than the total microbial biomass (47), their presence positively affects the bacterial community, as previously evidenced by an increase of microbial activity (3, 26). Therefore, eukaryotic 18S rRNA gene-targeted PCR-DGGE analysis was used in combination with cloning and sequencing to study the eukaryotic microbial community in the same Wieringermeer soil samples. Although these soil samples were collected over extended periods of time and stored for more than 30 years at room temperature, it was possible to analyze the composition of eukaryotic soil microbiota. Even the moderate number of clones analyzed here allowed for the identification of representatives of nearly all taxa of eukaryotic soil microbes suggesting that the collection and the treatment of the soil samples did not significantly affect the integrity of eukaryotic DNA in these samples. Although we cannot exclude losses of DNA or biases in PCR amplification the diversity of different soil eukaryptes clearly shows that it is possible to analyze ancient soil communities using archived samples. Even our limited, explorative approach revealed the presence of quite a number of hitherto unknown protist rRNA sequences.

How does the drying of the soil affect the microbial community?

Recent studies, including those described in this thesis, have demonstrated that biomolecular techniques can be applied soil samples that were air-dried and stored for extended period of time, using them as a “time machine” and giving us a unique glimpse of pro- and eukaryotic community development back in time (11, 32, 54). However, the potential impact of drying and storing of the soil was not clear. Hence, this we investigated by experiments performed in two independent studies on soil samples from Park Grass experiment at Rothamsted and long-term grassland experimental fields in Wageningen. The effect of soil drying and storing on the diversity of pro- and eukaryotic communities was compared with that of some other soil characteristics such as fertilizer, pH and soil type. The changes in the microbial communities were analyzed using SSU rRNA gene targeted PCR-DGGE, and the fingerprints were analyzed by multivariate analysis using redundancy analysis

(RDA). This revealed that, while most environmental variables significantly affected microbial community structure (P< 0.1), the influence of the drying and storing of the soils on the bacterial community was smaller than that of the soil environmental parameters pH, fertilizer and soil type. A somewhat different picture was obtained with regard to the *B. benzoevorans*- related community in the Rothamsted soil samples. Here, the environmental variables showed lower and even no significance, while drying and storage explained the majority of the variance in detectable community profiles. The *B. benzoevorans* related community showed relatively low diversity as judged by DGGE. In such case presence or absence of one band in the DGGE profile or even differences in the relative intensity of two bands could lead to significant statistical differences in the diversity, which might be the cause of the observed stronger soil preservation effect. Hence, such statistical differences do not necessarily correspond to significant biological changes in the community.

The results from two independent studies demonstrated that no dramatic changes were observed in neither of the studied microbial communities in the selected soil samples upon the different treatments for sample conservation. This gives empirical and statistical confidence to recent reports on the bacterial and eukaryotic diversity in archived soils.

Global distribution of B. benzoevorans *relatives in terrestrial environments*

Initially detected as abundant and predominant in the northern part of The Netherlands, the *B. benzoevorans*-related group of soil bacteria has recently drawn the attention of microbial ecologists. Several studies showed independently that microorganisms belonging to this group are indeed predominant in soils around the globe. They were reported to be present in agricultural soil in Wisconsin (USA) (4), eastern Amazonia (5), Dutch Drentse A area (17), other Dutch agricultural soils in Friesland and grasslands around Wageningen (The Netherlands), Portuguese soils and soil samples from Himalaya (54), Thailand (unpublished data) and English grassland samples (Fig. 1). They could be followed during an extended period in archived Dutch polder soil samples (53, 54) and could be used as reporter organisms for studying the behavior of the bacterial community in time.

Surprisingly, a large variety of *Bacillus* spp. were found not only in soil but also in more unusual environments such as granite rock (15). It was recently reported that granite rock contains 500 cultivable *Bacillus* spores and 10^4 total cultivable bacteria per gram, and many of the *Bacillus* isolates produced a previously unreported diffusible blue fluorescent compound. Two strains of eight tested exhibited increased spore UV resistance (related to a standard *Bacillus subtilis* UV biodosimetry). Applying 16S rRNA gene-based analysis several of the granite isolates were found to be most closely related to *B. drentensis* and *B. niacini* and thus belonging the group of *B. benzoevorans* relatives. Some granite isolates were closely related to a limited number of *Bacillus* spp. previously found to inhabit endolithic sites and other extreme environments. Thus, their occurrence in unusual environments is not accidental. These environments create unique niches to which a limited number of *Bacillus* spp. are specifically adapted.

Figure 1. Geographical locations where the *B. benzoevorans* relatives were found in this and other studies.

Of particular recent interest has been the potential role that endolithic microbes may play in the transfer of life between terrestrial planets. Because of their extreme resistance properties, bacterial spores have been the model system of choice for testing the theory that viable microbes could be successfully transferred through space between the environments of terrestrial planets such as Earth and Mars (37, 38). An important but untested aspect is that microbes situated on or within meteorites could survive hypervelocity entry from space through Earth's atmosphere (14). Granite samples permeated with spores of *Bacillus subtilis* strain WN511 were attached to the exterior telemetry module of a sounding rocket and launched into space. The surfaces of the post-flight granite samples were swabbed and tested for recovery and survival of WN511 spores. Spore survivors were isolated at high frequency, ranging from 1.2% to 4.4% compared with ground controls. Sporulation-defective mutants were noted among the spaceflight survivors at high frequency (4 %). These experiments constitute the first report of spore survival to hypervelocity atmospheric transit.

Kings without crowns

The group of *Bacillus benzoevorans* relatives are predominant and widely distributed. Found in environments as diverse as Himalayan meadows and Dutch polder soils, these microorganisms seem to be able to populate a broad range of niches, indicating a strong adaptive capability and eco-physiological versatility. Besides, they constitute a significant part of the total bacterial community of up to 40 %. Although it is yet unknown wich specific role the bacterial cluster might have, it seems that due to their abundace and adaptability, their ecological role might be of great importance to the bacterial world. Based on our exploratory study this role is just indicated, but not acknowledged yet. Therefore we state that *B. benzoevorans* relatives are kings without crowns; they are a group of bacteria that deserve further scientific attention aiming to understand their functional role in soil ecosystems.

The apparent diversity of isolates belonging to this lineage of *B. benzoevorans*, however, may indicate that there will be no single straightforward answer. Also, the various sources and metabolic capabilities of recently studied strains closely related to this group point towards a remarkable functional heterogeneity. With the studies described here, we have just begun to explore the enormous species richness present in this group and our culture collection. Polyphasic taxonomical approaches have proven to be useful to explore the variability in this group. The first output of this taxonomic work of species description has already yielded five newly described species (23), and many more may follow. Nevertheless, single distinct features characterizing this group or its subgroups may be discovered, using more specific approaches for the detection of genetic and/or physiologic differences between phylogenetically closely related isolates (25). These also include novel approaches of comparative and functional genomics.

Using a *B. subtilis* microarray we were able to perform comparative genomics among different strains belonging to the group of *B. benzoevorans* relatives, albeit only a limited number of genes targeted on the array showed positive hybridization due to low overall genomic similarity. While different studies demonstrated that genome hybridization at the inter-species level is possible, in our case sequence similarity between the tested *B. benzoevorans* related strains *B. subtilis* was obviously at the high end of allowable genomic distance. Nevertheless, we were able to detect several, mainly house keeping genes within different functional classes. Moreover, *B. benzoevorans*-strain specific genes that are in common with *B. subtilis* were detected, mostly corresponding to open reading frames within

the categories “hypothetical proteins”, “unclassified” or “unknown function”. Hence, this method is expected to be useful for the development of novel taxonomic approaches, the more if combined with different strategies, such as random genomic fragment- or clone-based arrays constructed from a number of reference strains within the group of *B. benzoevorans* relatives (9, 40, 60).

The role and function of a large number of *Bacillus* species was already elucidated in different studies. Fajardo-Cavazos *et al.* (15) described high UV resistance of several bacilli, isolated from a granite rock, including some members of *B. benzoevorans*- relatives and Bias *et al*. demonstrated the use of *B. subtilis* 6051 for biocontrol against infection of *Arabidopsis* roots by *Pseudomonas syringae* (2). They showed that upon the root colonization *B. subtilis* 6051 forms a stable, extensive biofilm and secretes surfactin, which act together to protect plants against attack by pathogenic bacteria. Other bacilli applied for biological control are *B. thuringiensis*, *B. subtilis*, *B. cereus*, and *B. sphaericus* (13, 41, 48). The production of low-molecular-weight peptides, used as antibiotic contributes to modern medicine (21). For biotechnological application several *Bacillus* species provide sources of thermostable enzymes and other products of industrial interest (10).

From the studies described in this thesis, including techniques for monitoring of the temporal and spatial diversity of *B. benzoevorans*-related bacilli and their abundance in a variety of soil environments, it can be suggested that these broadly distributed and predominant microorganisms have a royal role in terrestrial ecosystems, and deserve more future attention.

References

1. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143-169.
2. **Bais, H. P., R. Fall, and J. M. Vivanco.** 2004. Biocontrol of *Bacillus subtilis* against infection of arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiology **134:**307-319.
3. **Barsdate, R. J., R. T. Prentki, and T. Fenchel.** 1974. Phosphorus cycle of model ecosystem: significance for decomposer food chains and effect of bacterial grazers. Oikos **25:**239-251.
4. **Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62:**1935-1943.
5. **Borneman, J., and E. W. Triplett.** 1997. Molecular microbial diversity in soils from Eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Environ. Microbiol. **63:**2647-2653.
6. **Button, D. K., B. R. Robertson, P. W. Lepp, and T. M. Schmidt.** 1998. A small, dilute-cytoplasm, high-affinity, novel bacterium isolated by extinction culture and having kinetic constants compatible with growth at ambient concentrations of dissolved nutrients in sea-water. Appl. Environ. Microbiol. **64:**4467-4476.
7. **Cano, R. J., and M. K. Borucki.** 1995. Revival and identification of bacterial spores in 25- to 40-million-year-old Domican amber. Science **268:**1060-1064.
8. **Chan, K., S. Baker, C. C. Kim, C. S. Detweiler, G. Dougan, and S. Falkow.** 2003. Genomic comparison of *Salmonella enterica* serovars and *Salmonella bongori* by use of an *S. enterica* serovar typhimurium DNA microarray. J. Bacteriol. **185:**553-563.
9. **Cho, J.-C., and J. M. Tiedje.** 2001. Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays. Appl. Environ. Microbiol. **67:**3677-3682.
10. **CM0129.** Tryptone Soya Broth (Oxoid, Haarlem, The Netherlands).
11. **Dolfing, J., A. Vos, J. Bloem, P. A. I. Ehlert, N. B. Naumova, and P. J. Kuikman.** 2004. Microbial diversity in archived soils. Science **306:**813.
12. **Dong, Y., J. D. Glasner, F. R. Blattner, and E. W. Triplett.** 2001. Genomic interspecies microarray hybridization: rapid discovery of three thousand genes in the mize endophyte, *Klebsiella pneumoniae* 342, by microarray hybridization with *Escherichia coli* K-12 open reading frames. Appl. Environ. Microbiol. **67:**1911-1921.
13. **Emmert, E. A. B., and J. Handelsman.** 1999. Biocontrol of plant disease: a (Gram-) positive perspective. FEMS Microbiol. Lett. **171:**1-9.
14. **Fajardo-Cavazos, P., L. Link, H. J. Melosh, and W. L. Nicholson.** 2005. *Bacillus subtilis* spores on artificial

meteorites survive hypervelocity atmospheric entry: implications for lithopanspermia. Astrobiology **5:**726-736.

15. **Fajardo-Cavazos, P., and W. Nicholson.** 2006. *Bacillus* endospores isolated from granite: close molecular relationships to globally distributed *Bacillus* spp. from endolithic and extreme environments. Appl. Environ. Microbiol. **72:**2856-2863.
16. **Felske, A.** 1999. Reviewing the DA001-files: A 16S rRNA chase on suspect #X99967, a *Bacillus* and Dutch underground activist. J. Microbiol. Meth. **36:**77-93.
17. **Felske, A., A. D. L. Akkermans, and W. M. de Vos.** 1998. In situ detection of an uncultured predominant *Bacillus* in Dutch grassland soils. Appl. Environ. Microbiol. **64:**4588-4590.
18. **Felske, A., A. D. L. Akkermans, and W. M. de Vos.** 1998. Quantification of 16S rRNAs in complex bacterial communities by multiple competitive reverse transcription-PCR in temperature gradient gel electrophoresis fingerprints. Appl. Environ. Microbiol. **64:**4581-4587.
19. **Felske, A., A. Wolterink, R. van Lis, and A. D. L. Akkermans.** 1998. Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). Appl. Environ. Microbiol. **64:**871-879.
20. **Felske, A., A. Wolterink, R. van Lis, W. M. de Vos, and A. D. L. Akkermans.** 1999. Searching for predominant soil bacteria: 16S rDNA cloning versus strain cultivation. FEMS Microbiol. Ecol. **30:**137-145.
21. **Giacomodonato, M. N., M. J. Pettinari, I. S. Guadalupe, S. M. Beatriz, and N. I. Lopez.** 2001. A PCR-based method for the screening of bacterial strains with antifungal activity in suppressive soybean rhizosphere. World Journal of Microbiology and Biotechnology **17:**51-55.
22. **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.
23. **Heyrman, J., B. Vanparys, N. A. Logan, A. Balcaen, M. Rodriguez-Diaz, A. Felske, and P. De Vos.** 2004. *Bacillus novalis sp.* nov., *Bacillus vireti sp.* nov., *Bacillus soli sp.* nov., *Bacillus bataviae sp.* nov. and *Bacillus drentensis sp.* nov., five new species isolated from the Drentse A grasslands. Int. J. Syst. Evol. Microbiol. **54:**47-57.
24. **Horner-Devine, M. C., M. Lage, J. B. Hughes, and B. J. M. Bohannan.** 2004. A taxa-area relationship for bacteria. Nature **432:**750-753.
25. **Jaspers, E., and J. Overmann.** 2004. Ecological significance of microdiversity: identical 16S rRNA gene sequences can be found in bacteria with highly divergent genomes and ecophysiologies. Appl. Environ. Microbiol. **70:**4831-4839.
26. **Johannes, R. E.** 1965. Influence of marine protozoa on nutrient regeneration. Limnology and Oceanography **10:**434-442.
27. **Joseph, S. 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.

28. **Kowalchuk, G. A., D. S. Buma, W. de Boer, P. G. Klinkhamer, and J. A. van Veen.** 2002. Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. Antonie Leeuwenhoek **81:**509-520.
29. **Lin, X. G., R. Yin, H. Y. Zhang, J. F. Huang, R. R. Chen, and Z. H. Cao.** 2004. Changes of soil microbiological properties caused by land use changing from rice-wheat rotation to vegetable cultivation. Environ. Geochem. Health **26:**119-128.
30. **Liu, W.-T., and l. Zhu.** 2005. Environmental microbiology-on-a-chip and its future impacts. TIBTECH **23:**174-179.
31. **Loy, A., and L. Bodrossy.** 2006. Highly parallel microbial diagnostics using oligonucleotide microarrays. Clinica Chimica Acta **363:**106-119.
32. **Moon-van der Staay, S. Y., V. A. Tzeneva, G. W. M. van der Staay, W. M. de Vos, H. Smidt, and J. H. P. Hackstein.** 2006. Eukaryotic diversity in historical soil samples. FEMS Microbiol. Ecol. **in press**.
33. **Murray, A. E., D. Lies, G. Li, K. Nealson, J. Zhou, and J. M. Tiedje.** 2001. DNA/DNA hybridization to microarrays reveals gene-specific differences between closely related microbial genomes. Proc. Natl. Acad. Sci. USA **98:**9853-9858.
34. **Nagel, M., and J. R. Andreesen.** 1992. Utilization of organic acids and amino acides by species of the genus *Bacillus*: a useful means in taxonomy. J. Basic Microbiol. **32:**91-98.
35. **Nealson, K. H., and J. Ford.** 1980. Surface enhancement of bacterial manganese oxidation: implications for aquatic environments. Geomicrobiology Journal **2:**21-37.
36. **Nicholson, W. L.** 2002. Roles of Bacillus spores in the environment. Cellular and Molecular Life Sciences **59:**410-416.
37. **Nicholson, W. L., N. Munakata, G. Horneck, H. J. Melosh, and P. Setlow.** 2000. Resistance of bacterial endospores to extreme terrestrial and extraterrestrial environments. Microbiology and Molecular Biology Review **64:**548-572.
38. **Nicholson, W. L., A. C. Schuerger, and P. Setlow.** 2005. The solar UV environment and bacterial spore UV resistance: considerations for Earth-to-Mars transport by natural processes and human spaceflight. Mutation Research **571:**249-264.
39. **Pichinoty, F., M. Mandel, B. Greenway, and J.-L. Garcia.** 1977. Isolation and properties of a denitrifying bacterium related to *Pseudomonas lemignei*. Int. J. Syst. Bacteriol. **27:**346-348.
40. **Pieterse, B., E. J. Quirijns, F. H. Schuren, and M. J. van der Werf.** 2005. Mathematical design of prokaryotic clone-based microarrays. BMC Bioinformatics **6:**238.
41. **Pleban, S., and J. Sörensen.** 1996. Multi-target and medium- independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. FEMS Microbiol. Ecol. **22:**183-192.

42. **Rajlilić-Stojanović, M., H. G. Heilig, D. Molenaar, H. Smidt, E. Zoetendal, and W. M. de Vos.** HITChip- the microarray for profiling and quantification of the Human Intestinal Tract microbiota. **in preparation**.

43. **Rappe, M. S., S. A. Connon, K. L. Vergin, and S. J. Giovannoni.** 2002. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature **418:**630-633.

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

45. **Read, T. D., S. N. Peterson, N. Tourasse, L. W. Baillie, I. T. Paulsen, K. E. Nelson, H. Tettelin, D. E. Fouts, J. A. Eisen, S. R. Gill, E. K. Holtzapple, O. A. Okstad, E. Helgason, J. Rilstone, M. Wu, J. F. Kolonay, M. J. Beanan, R. J. Dodson, L. M. Brinkac, M. Gwinn, R. T. DeBoy, R. Madpu, S. C. Daugherty, A. S. Durkin, D. H. Haft, W. C. Nelson, J. D. Peterson, M. Pop, H. M. Khouri, D. Radune, J. L. Benton, Y. Mahamoud, L. Jiang, I. R. Hance, J. F. Weidman, K. J. Berry, R. D. Plaut, A. M. Wolf, K. L. Watkins, W. C. Nierman, A. Hazen, R. Cline, C. Redmond, J. E. Thwaite, O. White, S. L. Salzberg, B. Thomason, A. M. Friedlander, T. M. Koehler, P. C. Hanna, A.-B. Kolsto, and C. M. Fraser.** 2003. The genome sequence of *Bacillus anthracis* ames and comparison to closely related bacteria. Nature **423:**81.

46. **Salles, J. F., J. A. van Veen, and J. D. van Elsas.** 2004. Multivariate analyses of *Burkholderia* species in soil: effect of crop and land use history. Appl. Environ. Microbiol. **70:**4012-4020.

47. **Schnurer, J., M. Clarholm, S. Bostrom, and T. Rosswall.** 1986. Effect of moisture on soil microorganisms and nematodes: a field experiment. Microbiol. Ecol. **12:**217-230.

48. **Spharga, B. M., and G. D. Lyon.** 1998. *Bacillus subtilis* BS107 as an antagonist of potato blackleg and soft rot bacteria. Can. J. Micro. **44:**777-783.

49. **Stevenson, B. S., S. A. Eichorst, J. T. Wertz, T. M. Schmidt, and J. A. Breznak.** 2004. New strategies for cultivation and detection of previously uncultured microbes. Appl. Environ. Microbiol. **70:**4748-4755.

50. **ter Braak, C. J. F., and P. Smilauer.** 2002. CANOCO reference manual and CanoDraw for Windows user's guide. Biometris, Wageningen.

51. **Terpstra, P.** 1979. 50 jaar Wieringermeer. M. A. van Seijen-Leeuwarden, The Netherlands.

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

53. **Tzeneva, V. A., P. A. I. Ehlert, W. M. de Vos, A. D. L. Akkermans, and H. Smidt.** 2006. Riding ecological time- machine: succession of abundant soil bacteria in reclaimed land. Science **to be submitted**.

54. **Tzeneva, V. A., Y. Li, A. D. M. Felske, W. M. de Vos, A. D. L. Akkermans, E. E. Vaughan, and H. Smidt.** 2004. Development and application of a selective PCR-Denaturing Gradient Gel Electrophoresis approach to detect a recently cultivated *Bacillus* group predominant in soil. Appl. Environ. Microbiol. **70:**5801-5809.

55. **Tzeneva, V. A., J. F. Salles, N. Naumova, W. M. de Vos, P. J. Kuikman, J. Dolfing, and H. Smidt.** 2006. Effect of drying and long term storage of soil samples on the bacterial and eukaryotic diversity monitored by DGGE. FEMS Microbial Ecology **to be submitted**.

56. **van Elsas, J. D., P. Garbeva, and J. Salles.** 2002. Effects of agronomical measures on the microbial diversity of soils as related to the suppression of soil-borne plant pathogens. Biodegradation **13:**29-40.

57. **van Schreven, D. A., and G. W. Harmsen.** 1968. Soil bacteria in relation to the development of polders in the region of the former Zuider zee. Liverpool Univerity Press, United Kingdom.

58. **Vreeland, R. H., W. D. Rosenzweig, and D. W. Powers.** 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. Nat. Biotechnol. **407:**897-900.

59. **Wolters, D. J., A. D. L. Akkermans, and C. van Dijk.** 1997. Ineffective *Frankia* strains in wet stands of *Alnus glutinosa* L. Gaertn. in The Netherlands. Soil Biol. Biochem. **29:**1707-1712.

60. **Zhou, J.** 2003. Microarrays for bacterial detection and microbial community analysis. Curr. Opin. Microbiol. **6:**288-294.

SUMMARY

The soil ecosystem provides ecological niches for an extremely high number of microorganisms. Hence, the relationship between the diversity of living organisms and the ecosystem functions has become an important issue of modern microbial ecology. The genus *Bacillus* represents a large group within the soil microbiota. We studied the global distribution of a group of *Bacillus benzoevorans* related predominant soil bacteria in different soil environments. To this end, we developed specific integrated cultivation-dependent and biomolecular approaches. Our study revealed a high abundance of the *B. benzoevorans* related group in the studied soil environments. Their partial cultivability makes them an appropriate object for biogeographical dissemination and diversity studies (**Chapter 1**).

In an extensive cultivation campaign we developed mineral media which were suitable for the isolation of not yet cultured *B. benzoevorans* relatives. As carbon source acetate or benzoate was used. The media yielded a culture collection of *B. benzoevorans* relatives isolated from different Dutch soil samples (**Chapter 2**). However, the cultivation approach limits us to reveal only the cultivable bacteria. To compensate this methodological restriction and to be able to screen and compare the total bacterial and *B. benzoevorans* related community in soil, we developed two sets of selective primers targeting 16S ribosomal RNA genes (rRNA genes) and used them in combination with denaturing gradient gel electrophoresis (DGGE). This approach allowed for monitoring the *B. benzoevorans* related community in various soil environments. Soil samples from The Netherlands, Bulgaria, Russia, Pakistan and Portugal showed remarkable differences in the diversity of *B. benzoevorans* relatives. Our study also included unique archived soil samples that were collected from a young Dutch polder (Wieringermeer) from the moment of reclamation on. During reclamation, the ecosystem was shifted from anaerobic to aerobic and from saline to fresh water conditions. In 1945 the polder, however, was flooded again. With our techniques we could demonstrate the impact of both of these environmental changes (**Chapter 3**).

In recent years, soil archives have re-drawn scientific attention. Covering a period of 26-years, the archived Dutch polder vague soil samples analyzed in the framework of this thesis provided a unique source for retrospective studies on soil genesis. 16S rRNA gene-targeted DGGE fingerprinting showed that the composition of *B. benzoevorans*-related and total bacterial communities varied with time and cropping history. We quantified the relative abundance of *B. benzoevorans* relatives by specific dot blot hybridization revealing that they accounted for up to 40 % of the total bacterial community, which ones again confirmed the predominance of the group in the soil (**Chapter 4**).

In the same air-dried samples the eukaryotic biodiversity has been assessed by random sequencing of an 18S ribosomal RNA gene library and by DGGE. Representatives of nearly all taxa of eukaryotic soil microbes could be identified. This demonstrates that even in archived soil samples stored for more than 30 years, it is possible to study eukaryotic microbiota (**Chapter 5**). Nevertheless, the effect of drying and extended storage on the detectable bacterial and eukaryal microbiota in the soil has not yet been evaluated. To achieve that, we exposed fresh soil samples collected from two long-term grassland experiments to air-drying and storage, and determined the impact on total bacterial, *B. benzoevorans*- related and eukaryotic communities associated with these soils using molecular techniques. Changes in microbial community structure due to the drying and storage were evaluated using multivariate statistics. It was demonstrated that neither drying nor storage had an effect on the detectable part of the pro- and eukaryotic communities strong enough to impair our ability to detect changes in response to changing environmental conditions (**Chapter 6**).

Since there was no information available on the genomic content of the *B. benzoevorans*-related group we performed a comparative genomics analysis of four strains from the *B. benzoevorans*-relatives using a *B. subtilis* whole genome microarray. Hybridization of genomic DNA of the representative strains of the group of *B. benzoevorans*-relatives showed 93-95 % identity with the 16S rRNA sequence of *B. subtilis*. The positive signals were 1.0 to 5.6 % of ORFs targeted on the array. Detected genes included mostly house keeping genes within different functional classes, but also genes predicted to encode for proteins of yet unknown function. The identifying of the genes was not possible because of the low similarity of the genomes of both *Bacillus* groups (**Chapter 7**).

Although predominant, widely distributed and partly cultivable, it remains unclear which ecological function the group of *B. benzoevorans* relatives may occupy in soil. There is no single straightforward answer. The various sources and metabolic capabilities of recently studied strains closely related to this group point towards a remarkable functional heterogeneity (**Chapter 8**).

With the described studies, we have just begun to explore the enormous species richness present in this group and our culture collection. Found in a variety soil ecosystems, phylotypes belonging to the *B. benzoevorans* relatives seem to be able to populate a broad range of niches, indicating metabolic versatility and/or strong adaptive capability. Besides, they constitute a significant part of the total bacterial community. Based on our exploratory study their role is just indicated, but not acknowledged yet. Therefore we state that *B. benzoevorans* relatives are kings without crowns; they are an important group of bacteria that

deserve additional scientific attention aiming to provide further insight in their eco-physiology and importance for soil ecosystem functioning.

SAMENVATTING

Het bodemecosysteem biedt ecologische niches aan een enorm aantal micro-organismen. De relatie tussen de diversiteit van de levende organismen en de functies van het ecosysteem is dan ook een belangrijk vraagstuk voor microbieel georiënteerde ecologen.

Het genus *Bacillus* vertegenwoordigt een grote groep binnen de microbiota van de bodem. Wij bestudeerden de wereldwijde distributie van een groep *Bacillus benzoevorans*-gerelateerde bodembacteriën, welke overheersend aanwezig zijn in verschillende bodemmilieus. Daartoe ontwikkelden we specifieke, geïntegreerde kweekafhankelijke en biomoleculaire onderzoeksstrategieën. Onze studie toonde de grote verspreiding van de *B. benzoevorans* gerelateerde groep in de bestudeerde bodemmilieus. Aangezien deze bacteriën gedeeltelijk gekweekt kunnen worden, zijn ze geschikt als studieobject binnen biogeographische disseminatie- en diversiteitsstudies (**Hoofdstuk 1**).

Middels een extensieve kweekopstelling ontwikkelden we een mineraal medium dat geschikt was voor de isolatie van tot nog toe niet gekweekte *B. benzoevorans* gerelateerde bacteriën. Als koolstofbron werd acetaat of benzoaat gebruikt. Ons medium bracht een kweekcollectie op, van uit Nederlandse bodemmonsters geïsoleerde *B. benzoevorans* gerelateerde bacteriën (**Hoofdstuk 2**). Een nadeel van kweektechnieken is echter dat het ons beperkt tot het kweekbare bacteriespectrum. Om deze methodologische tekortkoming te compenseren, en het mogelijk te maken om de *B. benzoevorans* gerelateerde gemeenschap te bepalen en te vergelijken de totale bacteriële gemeenschap, ontwikkelden we twee sets selectieve primers die zich richten op 16S ribosomale RNA-genen (rRNA-genen) en gebruikten deze in combinatie met denaturerende gradiënt gel electroforese (DGGE). Deze techniek maakte het mogelijk om de *B. benzoevorans* gerelateerde gemeenschap te monitoren in uiteenlopende bodemmilieus. Bodemmonsters uit Nederland, Bulgarije, Rusland, Pakistan en Portugal toonden een opmerkelijk verschil in diversiteit wat betreft de aanwezigheid van *B. benzoevorans* gerelateerden.

Ons onderzoek maakte tevens gebruik van een uniek bodemarchief, bestaande uit monsters van jonge poldergrond uit de Wieringermeer, Noord-Holland, die verzameld werden vanaf de drooglegging. Door de inpoldering van de bodem, veranderde het ecosysteem van anaëroob naar aëroob en werd het zoutwater verdrongen door zoetwater. In 1945 werd de polder tijdelijk weer overstroomd. Onze bovengenoemde onderzoekstechnieken stelden ons in staat de invloeden van beide milieuveranderingen inzichtelijk te maken (**Hoofdstuk 3**).

Recentelijk zijn bodemarchieven weer in wetenschappelijke belangstelling komen te staan. Onze gebruikte bodemarchief met poldergrond representeert een tijdspanne van 26 jaar en is een unieke bron voor retrospectief onderzoek naar de microbiële onstaansgeschiedenis

van de bodem. De 16S *rRNA gene-targeted DGGE fingerprinting* technieken demonstreerden dat de compositie van de *B. benzoevorans*-gerelateerde gemeenschap en de totale bacteriële gemeenschappen varieerde als gevolg van de het voortschrijden van de tijd en het landbouwgebruik (i.e. gewasverbouwing). We kwantificeerden de relatieve abondantie van de *B. benzoevorans* gerelateerden door specifieke *dot blot* hybridisatie, en vonden dat deze groep tot 40 % van de totale bacteriële gemeenschap uitmaakt, hetgeen nogmaals de overheersende aanwezigheid van deze bacteriën in de bodem bevestigt. (**Hoofdstuk 4**).

In dezelfde luchtgedroogde monsters bekeken we de eukaryotische biodiversiteit door willekeurige sequeneren van een 18S ribosomale RNA-gene *library* en door middel van DGGE. Daarbij konden vertegenwoordigers van bijna alle taxa van eukaryotische bodemmicroben worden geïdentificeerd. Dit toont aan dat zelfs gearchiveerde monsters die meer dan 30 jaar opgeslagen zijn, gebruikt kunnen worden om eukaryotische microbiota te bestuderen. (**Hoofdstuk 5**). Echter, het specifieke effect van droging en lange opslag op de detecteerbare bacteriën en het eukaryotische microbiota in de bodems was daarbij nog niet geëvalueerd. Daarom stelden we vervolgens verse bodemmonsters bloot aan luchtdroging en opslag en bepaalden het effect. We kozen voor monsters die werden verzameld in een langlopend onderzoek naar graslanden en bepaalden met moleculaire technieken de invloed van droging en opslag op de totale bacteriële, de *B. benzoevorans*- gerelateerde en de eukaryotische gemeenschappen die normaliter in dit soort gronden voorkomen. Veranderingen in de microbiële gemeenschap en structuur tengevolge van droging en opslag werden geëvalueerd met behulp van multivariate statistiek. Op basis van deze analyse bleek dat het effect van zowel opslag als droging op het detecteerbare deel van de pro- and eukaryotische gemeenschappen dusdanig klein is, dat het geen nadelige invloed heeft op het detecteren van de microbiologische respons ten gevolge van veranderingen in milieucondities. (**Hoofdstuk 6**).

Omdat er nog geen informatie beschikbaar is over de genetische achtergrond van de *B. benzoevorans*-gerelateerde groep bacteriën voerden we een *comparative genomics analysis* uit op vier stammen van de *B. benzoevorans*-gerelateerden. Daarbij maakten we gebruik van een *microarray* en werd het gehele genoom van *B. subtilis* geanalyseerd. Hybridisatie van genomic DNA van de vertegenwoordigende stammen van de groep *B. benzoevorans*-gerelateerden toonde 93-95 % overeenkomst met de 16S rRNA sequentie van *B. subtilis*. De positieve signalen kwamen overeen met 1.0 to 5.6 % van de ORFs de die werden aangegrepen op de *array*. De gedetecteerde genen waren voor het grootste deel *house keeping genes* binnen

verschillende functionele klassen, maar ook genen die coderen voor eiwitten en nog onbekende functies. (**Hoofdstuk 7**).

Hoewel de distributie van *B. benzoevorans* gerelateerde bacteriën enorm is, zij in grote aantallen voorkomen en ze deels kweekbaar zijn, is de ecologische rol van deze groep bacteriën in de bodem nog grotendeels onbekend. Er is geen eensluidend antwoord op deze kwestie. De verschillende bronnen en metabolische vermogens van de bestudeerde groep duidt op een opmerkelijke functionele heterogeniteit. (**Hoofdstuk 8**).

Met de in hier beschreven onderzoeken is slechts een start gemaakt met het verkennen van de enorme soortenrijkdom binnen deze groep en slechts binnen onze kweekverzameling. Aangezien deze bacteriën in een grote variëteit aan bodemecosystemen voorkomen, lijken deze phylotypen van de *B. benzoevorans* gerelateerden zich te kunnen aanpassen aan een breed scala niches, wat duidt op een metabolische veelzijdigheid en/of een sterk aanpassingsvermogen. Bovendien maken zij een aanzienlijk deel van de bacteriële gemeenschap in de bodem uit. Ons verkennend onderzoek geeft de indruk dat deze groep bacteriën een belangrijke ecologische rol vervult, welke hen echter nog niet is toegekend. We beschouwen de *B. benzoevorans* gerelateerden daarom vooralsnog als de ongekroonde koningen van het microbiologische bodemleven; een belangrijke groep bacteriën die verdere wetenschappelijke aandacht verdient teneinde meer inzicht te verschaffen in hun ecofysiologie en hun rol in het bodemecosysteem.

Acknowledgements

Everything started in the year 2000 when for the first time I came to Wageningen. Then I did not suspect that this would become a crucial time for my future. I have met people that directed new paths in my life. These people I would like to thank now.

First of all I thank my co-promotors Antoon Akkermans and Hauke Smidt and my promotor Willem de Vos. Antoon, you offered me your help and experience from the moment we met. You have the skill of managing the lab professionally with a gentle and respected style, which is still very inspiring for me. Big part of the research presented in this thesis was due to your inspiration and involvement. Thanks for everything you have done for me as a researcher, but also for your support in my private life.

Hauke, you came in the project later but in very short time you were completely involved in my work. I thank you for the nice discussion we had and for the support during the difficult and disappointing moments (the ones, of course, all PhD students have). You were always enthusiastic and positive. Your great ideas and accuracy are still inspiring for me. I thank you for all your help and involvement.

Willem, thank you for the support and the freedom you gave me during this research. Your comments added always the missing link to my work. Our meetings were encouraging and stimulated me to strife higher. Thank you for all inspiration and trust. It is nice that our cooperation is still ongoing.

For paranimfs I invited two members of the lab's "Balkan room", Mirjana and Neslihan. Mirjana and Neslihan, I was very glad when both of you accepted this role with enthusiasm. The time we shared was very pleasant; we had a very "gezellig" office. I want to thank both of you for the discussions that we had and for the time that I was in need of support and pep talk. To Mirjana I want to thank also for the help by painting my apartment (although you spent quite some paint on decorating your self).

I would like to thank all members of the Molecular Ecology group for the great time I had during week days but also during our two wonderful lab trips in the weekend; to Texel and Zeeland. Thanks to all who travelled more than 2000 km just to attend my wedding in Sofia. I appreciate this very much. I certainly will never forget the cheerful time I had with of all of you; Hans, Kees, Wilma, Ineke, Mark, Maaike, Meta, Elaine, Alcina, Carien, David, Muriel, Petja, Farai, Eline, Diana, Erwin, Hoa, Prapha, Jordan, Jana, Kauther, Mahmut, Markus, Marianna, Odette, Reetta, Rosio, Toshio, Sergey, Anna and Cincia. All Micro's I would like to thank for the great time. Some other collegues I would like to mention by name: Gosse, Reneé, Ans, Caroline, Bernd, Wim, Francis, Jannie, Nees. Miriam, you probably do not realize that your strong mentality was of great help for me at several difficult moments. And ofcourse I would like to thank the students and researchers involved in my project: Kathrin, Youguo, Maaike, Carla, Mafalda.

I want to express my gratitude to our colleagues with which we had a fruitful collaboration: Phillip Ehlert and Jan Dolfing, who kindly offered us access to TAGA soil collection. Phillip and Jan thanks for your critical reading of my manuscripts. Your comments were very contributing. I would like to thank the people involved in the BACREX project: Andreas Felske, Paul de Vos**,** Jeroen Heyrman and Corina Koch. Thanks to Joana Sales, Pert Smilauer and Anna Castioni for their help with the CANOCO software. We had a very nice collaboration with our collegues from Nijmegen University, Johannes Hackstein and Seung Yeo Moon-van der Staay. It was a pleasure for me to work with eukaryotic microorganisms which resulted in an article.

A word of thanks also to all my friends in privat life, without their friendship and support my integration and naturalization ('een goed gelukte inburgering') in The Netherlands would not have happened. Jullie wil ik daarom bedanken voor jullie warmte en geduld met mij. Ik kan voorstellen dat het niet erg leuk was, vanwege mij, lang tijd alleen Engels te spreken en later wel Nederlands, maar op een kinderniveau. Ik heb een geweldige tijd in Nederland en dat is voor een groot deel dankzij jullie vriendschap: Marcel, Erik, Jacobien - en inmiddels kleine Noah - Joost, Jappe, Jelle, Rieks, Nico, Niels, Harrold, Bianca, Sandra, Willem en Anneke, Gerko, Petra en Mike. Ik heb zeker een leuke, en soms ook gekke, tijd met jullie gehad.

There are also other friends that have helped me or we have just enjoyed together. Thanks a lot to Hans, Sergey, Pavlina and Meta for helping us with the moving from one place to another. Also many thanks to Radoslava, Elena and Aernout, Petja and Walter, Mirjana, Mladjan and the small Gavrilo, Hans, Kees for the nice evenings and weekends together.

A special place in this text I would like to devote to my family:

Marcus, I want to thank you for the support and help during the last 5 years and especially in the last couple of months. They were very difficult and busy for me and you never complained for that. You were even deeply involved in my work and did everything to make it easier for me. I appreciate this so much! Thanks for the complete support during the whole time we know each other. You are my great husband.

To my mother and sister: Нана и Мама, до сега никога не съм ви казвала колко много съм ви благодарна, че не отрязахте крилата ми и ми позволихте да летя на кадето поискам. За всички нас беше толкова трудно да се разделим, но вие не помислихте за себе си, а само за мойто добро. Благодаря ви за това! Много ви обичам! Все пак тази работа аз посвещавам на Татко, който щеше да е много щастлив и горд сега. Благодаря на чичо Кольо и леля Дора за милото приятелство и вечната отзивчивост при добро и зло . Благодарности и на всички приятели и братовчед, които още си мислят за мен Милена, Поли и Жоро.

Ik wil ook graag Tini en Hary bedanken, voor hun de begrip, steun en alle hulp.

List of publications

Groudeva, V., Tzeneva, V. A. 2001. Biodiversity of sulfate reducing bacteria (SRB) in anaerobic reactor for removal of heavy metals polluted water. In: Vocational training in biotechnological innovation and environmental protection, Module 2, Environmental protection and biotechnology (innovative aspects), Pilot Project European Commission DG XXI- Education, Training and Youth

Felske, A. D. M., V. A. Tzeneva, Heyrman, J., Langeveld, M. A., Akkermans, A. D. L., De Vos, P. 2004. Isolation of biodiversity of hitherto undescribed soil bacteria related to *Bacillus niacini*. Microbial Ecology 48(1): 111-119.

Tzeneva, V. A., Y. Li, Felske, A. D. M., de Vos, W. M., Akkermans, A. D. L., Vaughan, E. E., Smidt, H. 2004. Development and application of a selective PCR-Denaturing Gradient Gel Electrophoresis approach to detect a recently cultivated Bacillus group predominant in soil. Appl Environ Microbiol 70(10): 5801-5809.

van Houten BH, Roest K, Tzeneva VA, Dijkman H, Smidt H, Stams AJ. 2006. Occurrence of methanogenesis during start-up of a full-scale synthesis gas-fed reactor treating sulfate and metal-rich wastewater. Water Res. 40(3):553-60.

Moon-van der Staay, S. Y., Tzeneva, V. A., van der Staay, G. W. M., de Vos, W. M., Smidt, H., and Hackstein, J. H. P. 2006. Eukaryotic diversity in historical soil samples. FEMS Microbiology Ecology, available on line.

Tzeneva, V. A., Heilig, H., Akkermans - van Vlieg, W., de Vos, W. M., Smidt, H. (2006). Section 3: Ecological diversity, Chapter 17. 16S gene target DGGE fingerprinting., Methods in Molecular Biology – Environmental Genomics- *in press*

Tzeneva V. A., Ehlert, de Vos, W. M., Akkermans, A. D. L., Smidt, H. 2006. Riding the ecological time-machine: analyzing abundant bacteria in archived polder soils. *submitted for publication*

Tzeneva, V. A., Salles, J., Naumova, N. B. , de Vos, W. M., Kuikman, P. J., Dolfing, J., and Smidt, H. 2006. Effect of drying, grinding and long term storage of soil samples on the bacterial and eukaryotic diversity monitored by DGGE. *submitted for publication*

Castioni, A. and Tzeneva, V. A., Hoa, P. T. K., Jansen, P., Rademaker, J., Starrenburg, M., Torriani, S., Hugenholtz, J., de Vos, W. M., Smidt, H., Zoetendal, E., Molenaar, D., van Hylckama Vlieg, J. E. T. Application of genomic and functional diversity of *Lactobacillus plantarum. manuscript in preparation*

About the author

Vesela Alexandrova Tzeneva was born on February 10, 1975, in Sofia, Bulgaria. In 1994 she completed her secondary school and received a diploma for machine technician. From 1995 to 2000 she studied in the University of Sofia, Faculty of Biology, where she graduated with completing two MSc programs. Her MSc thesis entitled "Analysis of sulfate-reducing bacteria in different habitats and the possibility for practical use" was defended in the department of Ecology and Environmental Protection at the University of Sofia. The second MSc thesis was performed in the frame of the TEMPUS project in collaboration with Wageningen University and was entitled "Characterization and comparison of the microbial diversity in two different types of sludge samples- Budel Zinc and Kennecott".

In 2001 Tzeneva continued practicing science as a PhD student in the Laboratory of Microbiology at Wageningen University and Research Centre (The Netherlands) under the supervision of Dr. Antoon D. L. Akkermans, Dr. Hauke Smidt and prof. Willem M. de Vos. This research was part of the European Union Project "Exploration of genomic and metabolite diversity of a novel group of abundant soil bacteria" performed in collaboration with Gesellschaft für Biotechnologische Forschung - Braunschweig, bioLeads- Heidelberg, Rijksuniversiteit Gent. The result of the project is presented in this thesis. Since October 2005 she has been appointed as a post-doctoral researcher at Wageningen Center for Food Science.

Activities in the frame of SENSE research school

Course	Year
Safe handeling with radioactive materials and sources	2002
Course of written English	2002
Advanced Course on Bioinformation Technology-1	2003
Techniques for Writing and Presenting Scientific Papers	2003
Career orientation	2004
Environmental Research in Context	2005
Research Context Project	2006

Printed by Universal Press-Veenendaal
Cover picture by Marc Zitzen