Stellingen

1. Estimation of bacterial community changes as numeric values (diversity indices) rather than banding patterns, may facilitate comparisons and prediction of trends.

   This thesis.

2. "Everything is everywhere, the environment selects", Beijerinck proposed this paradigm nearly one century ago, but it can still be used today to explain the finding of phylogenetically related symbionts and co-symbionts in geographically distant actinorhizae from the Rhamnaceae.

3. Pheromone peptides including the "resuscitation-promoting factor" described by Mukamolova *et al.* play an important role in intercellular signaling among the high G+C Gram-positive bacteria. Similar types of molecules may open the door for culturing many hitherto uncultured *Frankia* strains.

   G.V. Mukamolova, A.S. Kaprelyants, D.I. Young, M. Young and D.B. Kell (1998). Proc. Natl. Acad. Sci. **95**:8916-8921

4. The poorly defined species concept in Microbiology has complicated the evaluation of bacterial diversity. The problem will continue as long as we consider bacterial species as separate entities, rather than a range of more or less phylogenetically related organisms.

5. Dominance of global (Earth) metabolism falls squarely within the realm of those organisms which neither form species nor go extinct: the metabolically diverse prokaryotes.

   Lynn Margulis (1993), Microbial Communities as Units of Selection

6. Some referees and reviewers must be more consistent with the maximum parsimony principle on keeping changes to a minimum.

7. There should be plenty of jobs for microbiologists in the future, as in the last 130 years we have studied at most 1% of the bacteria in nature.

8. Hoe laat is het? Dutch is the only european language that assumes it's already late when asking for the time; maybe that is the secret for the strict dutch timing.

9. "...when we don't possess things we interpret signs, and signs from signs." William de Baskerville explaining to his apprentice, on how he could describe a horse he had not got seen. However, this quote is certainly applicable to bacteria and 16S rRNA as well.

   Umberto Eco, The Name of the Rose.

10. Many "stellingen" (like this one) are born out of need.

Stellingen behorende bij het proefschrift "Molecular Ecology of *Frankia* and other Soil Bacteria under Natural and Chlorobenzoate-stressed Conditions".

Hugo César Ramírez Saad
Wageningen, NL. 14 juni 1999

MOLECULAR ECOLOGY OF *FRANKIA* AND OTHER SOIL BACTERIA UNDER NATURAL AND CHLOROBENZOATE-STRESSED CONDITIONS

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Hugo César Ramírez Saad

Molecular Ecology of *Frankia* and other Soil Bacteria under Natural and Chlorobenzoate-stressed Conditions

PROEFSCHRIFT

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op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op maandag 14 juni 1999
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CHAPTER 1

GENERAL INTRODUCTION

MICROBIAL ECOLOGY OF SOIL BACTERIA

Natural microbial communities carry out crucial processes required for sustaining the biosphere on a global scale, supporting the life of most of the other organisms, and destroying many of the waste products of human society. The sustained activity and interaction of several microbial populations or communities are required to perform these processes. Characterising the structure and function of both populations and communities in relation with their environment is one of the major goals of microbial ecology. As depicted in Figure 1.1, primary knowledge about microbial communities is a key point for understanding other processes ranging from basic ones, like microbial evolution and biodiversity, to the very applied ones, such as biodegradation and bioremediation. Furthermore, analyses of microbial populations from extreme environments allow understanding their unique physiology and may result in further biotechnological applications.

Many of the questions regarding microbial communities and their environment have been in the interest of microbiologists for several decades. However, Microbial Ecology is a relatively young science. One of the first symposia devoted to this field was held in London in 1957 (Society for General Microbiology 1957), while the first textbook entitled Principles of Microbial Ecology appeared only in 1966 (Brock 1966).

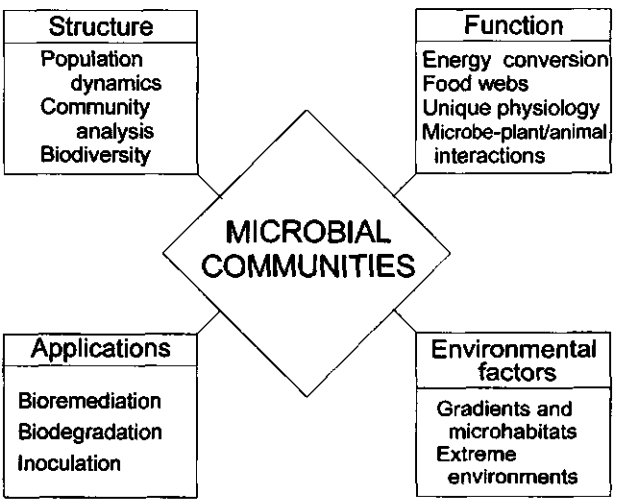

Figure 1.1. Some of the major aspects of microbial ecology dependent upon an adequate description of specific microbial communities (modified from Marshall 1993).

Compared to macroecologists, the microbial ecologists are faced with the following fundamental difficulties: 1) the very large number of individuals in a sample (i.e. 10^9 bacteria g^{-1} soil), 2) the difficulty of easily distinguishing among the different populations, 3) the very high species diversity at a small scale (i.e. $>10^3$ species g^{-1} soil), and 4) the difficulty in defining a prokaryote species or another taxonomic unit that captures the appropriate level of diversity (Klug and Tiedje 1993).

MOLECULAR TOOLS TO STUDY SOIL BACTERIA

Molecular Ecology is an emerging discipline that is benefiting from recent developments in other fields such as the improvement of analytical procedures that allow a more sensitive detection of specific biomolecules known as biomarkers. This term is given to any biological component that can be used to indicate a useful feature of a particular microbial group. As desirable characteristics these compounds have to provide a means of specific detection, and they should be rapidly decomposed after cell death, allowing their use as estimators of living-cell populations. Biomarkers could therefore be cell components such as DNA, RNA, lipids, proteins, or even the cells themselves (Morgan and Winstanley 1997).

Biomarker molecules present in the cell envelope have been widely used for analysing isolates or environmental samples. Pioneering work on the detection of bacterial surface components was based on immunological techniques. Fluorescently labelled polyclonal antibodies were used to monitor the fate of bacteria released into soil or rhizosphere (Bohlool and Schmidt 1970, Reyes and Schmidt 1979). In those cases the biomarker molecules were not specifically selected, but in general the antibodies were recognising lipopolysaccharides, lipoproteins or outer membrane proteins. The advent of monoclonal antibody technology increased substantially the specificity, however, there are still drawbacks in the microscopic detection that relate to background fluorescence and time requirements. As an alternative to face the background problems the confocal scanning laser microscopy has been proposed (Caldwell *et al.* 1992). Moreover, automated flow cytometry detection has proven to be a fast and effective way of detecting, counting and sorting cells in mixed populations (Page and Burns 1991, Porter *et al.* 1993).

Phospholipids are major components of most cellular membranes. Chromatographic analysis of their constituent fatty acids gives a specific fingerprint that can be used for identification purposes even at the sub-species level (Guckert *et al.* 1991, Chapter 3 this thesis). A commercial typing system based on this fingerprinting is available (Microbial ID, Inc. Newark, Canada). Microbial biomass and community structure has also been assessed by this means in different environments (Cavigelli *et al.* 1995, Lindahl *et al.* 1997, Sundh *et al.* 1997). Evaluation of impact on the microbial community of halogen-polluted sites (Napolitano *et al.* 1994, Zelles *et al.* 1997) and monitoring of bioremediation processes (White *et al.* 1998) have been addressed with this approach as well. However, due to the complexity of the community fingerprints obtained, it is difficult to assess less predominant populations or uncultured

bacteria, such as many nodule-forming *Frankia* strains, whose specific fatty acid patterns are unknown.

Non-fatty acid lipids present in cell envelopes have also been used as biomarkers. Quantification of the polycyclic sterol ergosterol has been utilised as means to evaluate fungal biomass in soil (Hart and Brookes 1996, Stahl and Parkin 1996). Analysis of respiratory quinones (Hedrick and White 1986, Lipski *et al.*1992) and polyamines (White and Tabor 1985) has been utilised more as a taxonomic tool, since their utility in microbial ecology is somehow more restricted. The utilisation of other molecules such as lipopolysaccharides and lipoproteines, or microbial cells (i.e. *Escherichia coli*, spore-formers) as biomarkers has been reviewed by Morgan and Winstanley (1997).

NUCLEIC ACIDS AS BIOMARKERS

Due to their unique properties nucleic acids can be regarded as the ultimate biomarker. Their common ubiquity in all living forms and the differences in the primary structure sequence can be used for highly specialised detection, even in complex environments. Either physiologically or phylogenetically related groups of bacteria can be readily detected by means of functional genes (i.e. genes involved in nitrogen fixation or xenobiotic degradation), or by using phylogenetic markers. Among these, the ribosomal RNA's and particularly the small subunit (SSU) or 16S rRNA (Figure 1.2) and its encoding gene (16S rDNA), have been by far the preferred evolutionary markers used in microbial ecology studies (Liesack *et al.* 1997).

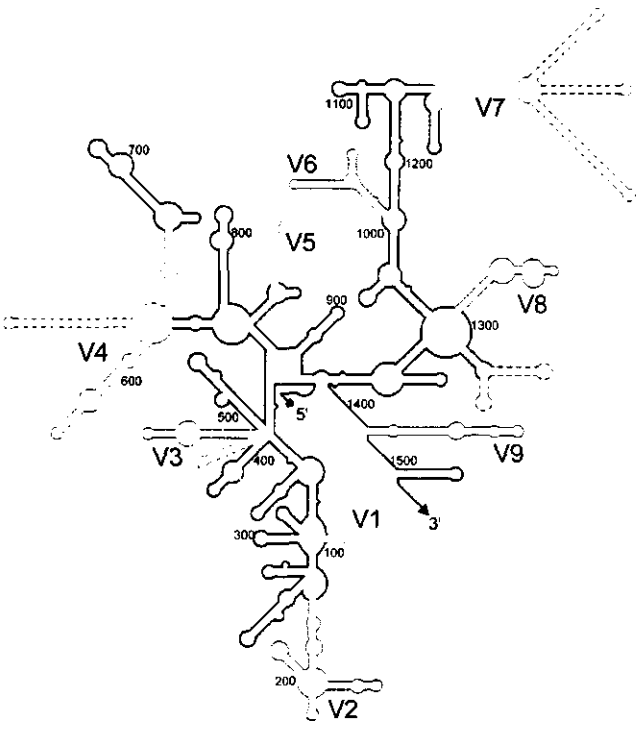

Figure 1.2. Secundary structure model for prokaryotic 16S rRNA. Bold lines represent regions with highly conserved structure. Nine variable regions (numbered V1 to V9) are drawn in thin lines, their approximate position is given according to the *Escherichia coli* numbering system (Brosius *et al* 1987). Helices drawn in broken lines are present only exceptionally (modified from de Rijk *et al.* 1992).

Several reasons support the suitability of rRNA's (small and large sub-units) to be used as both biomarkers and evolutionary chronometers: a) they are present in all organisms performing the same functions, b) their overall secundary and tertiary structure is highly conserved, c) the primary structure consists of alternating conserved and variable regions (Figure 1.2), offering unique possibilities for detection, amplification and sequencing, d) the amount of sequence information present in both molecules is enough to perform statistically significant comparisons, and e) the rRNA genes seem to be free from artifacts of lateral transfer between contemporary organisms. Thus, relationships established by sequence comparisons represent true phylgenetic relationships (Pace *et al.* 1986).

Woese and Fox (1977), and Woese (1987) have already postulated the profound evolutionary implications of rRNAs. Since then, the analysis of natural microbial populations could be addressed with a phylogenetic and evolutionary approach (Amann *et al.* 1995, Hugenholtz *et al.* 1998, Pace *et al.* 1986, Ward *et al.* 1992). Currently there is a wealth of knowledge produced after more than one decade of molecular microbial ecology studies based on rRNA analysis. The major contributions arising from the application of rRNA-based molecular tools in microbiology and microbial ecology have, in summary, resulted in: 1) providing the missing phylogenetic framework for bacterial systematics, 2) giving new insight into the natural microbial diversity, 3) allowing characterisation of microorganisms by culture-independent approaches. The latter argument is particularly relevant to soil bacteria and *Frankia* in root nodules of actinorhizal plants, since many of these bacteria have never been obtained in pure culture. The most important achievements of the culture-independent approaches have been reviewed by Amann *et al.* (1995), Head *et al.* (1998), Hugenholtz and Pace (1996), and Pace (1997), including major drawbacks of its use (Liesack *et al.* 1997, Witzingenrode *et al.* 1997). Accordingly with this progress, the number of available techniques has also increased enormously (Akkermans *et al.* 1998, Trevors and van Elsas 1995).

A simplified flow chart is presented in Figure 1.3 showing the basic steps to analyse soil bacterial communities by the rDNA/rRNA methodology. A general initial step is the extraction of nucleic acids that can be followed by direct hybridisation or by cloning. However, *in vitro* amplification of the rRNA genes by the polymerase chain reaction (PCR) is currently mostly used enhancing the possibilities for detection. This approach will require systems to separate the complex mixture of amplicons obtained after PCR. One group of techniques will provide community fingerprints, which can be used to elucidate the structure of the community. Denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.* 1993), and the equivalent temperature gradient gel electrophoresis (TGGE) (Felske *et al.* 1996) are good examples of such community profiling techniques. Cloning and sequencing represent the other group of techniques offering possibilities for phylogenetic identification and probe design. Based on sequence analysis and database comparison, oligonucleotide probes complementary to unique or very particular sequences can be designed. Probe labelling with radioactive or fluorescent reporter groups is essential for further detection. As both groups of approaches (i.e. community profiling and cloning-sequencing) provide complementary information, their simultaneous application is commonly used.

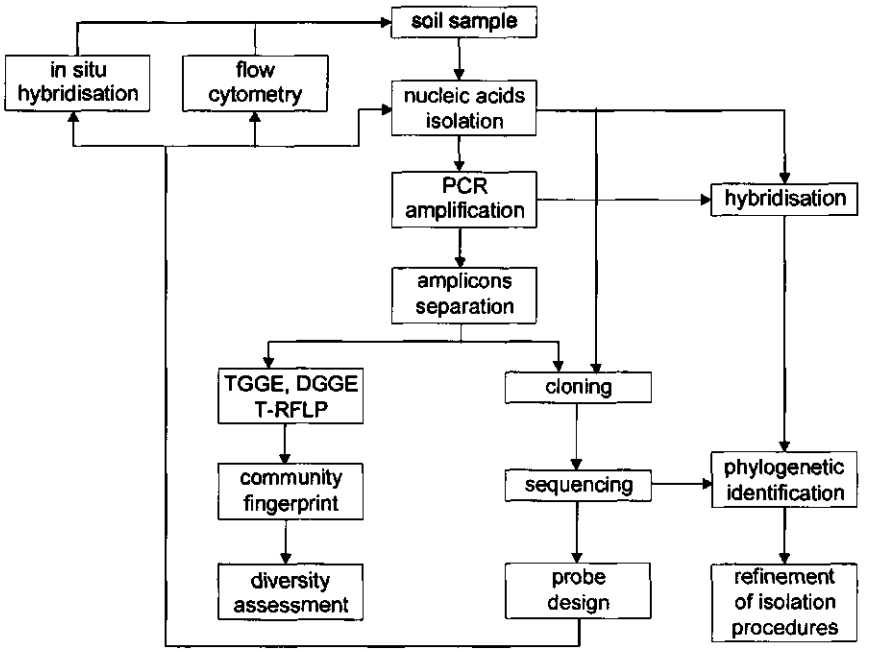

Figure 1.3. Flow chart with the principal phases of the rRNA/rDNA approach for culture-independent studies of soil microbial communities

Application of the designed probes to the original soil sample may lead to a better understanding of the population structure of specific bacteria in their natural environment. Valuable information can be obtained from two approaches: Awidely used one is *in situ* hybridisation with fluorescently labelled probes (FISH) which aims to visualise the distribution and abundance of microorganisms in soil samples as undisturbed as possible. The other one is flow cytometry that is mainly applicable to soil-extracted cells and provides a fast way of counting and even sorting those cells reacting with the fluorescent probe. Hybridisation of the extracted nucleic acids with the radioactively or fluorescently labelled probe may also offer some means of quantification, which are not easily translatable into cell numbers. So far, physiological aspects of specific bacterial groups are mainly obtained upon cultivation. Detection and phylogenetic identification of recalcitrant organisms in their natural environment may help on designing refined isolation procedures. As pointed out by Liesack *et al.* (1997), only polyphasic approaches including on the one hand biochemical, morphological and physiological data derived from cultivation-based techniques as well as molecular characterisations may provide a complete view of structure and function of microbial communities.

MOLECULAR ECOLOGY OF *FRANKIA*

The actinomycetes of the genus *Frankia* are characterised by their particular morphology that can differentiate into three cell types: a) the hyphae or vegetative growing type, b) the multilocular sporangia containing the reproductive, non-motile spores, and c) the spherical-shaped vesicles, which are specialised terminal-cell structures where nitrogen fixation takes place. In addition, *Frankia* is also able to form N_2-fixing root nodules (or actinorhiza) with a number of vascular plants, generically called actinorhizal plants (AP) (Benson and Silvester 1993). Taxonomically the AP are spread over 8 families of dicotyledoneous plants, comprising 25 genera and more than 200 different species. Most AP have major ecological importance in their respective environments and some of them are also economically important (Tjepkema and Schwintzer 1990). Because of this capacity, substantial attention has been paid to the ecological aspects of *Frankia* in relation with its host plants. A considerable amount of information is available on strains isolated from or within root nodules, regarded as an important ecological niche and a natural *Frankia*-enrichment (Benson and Silvester 1993). Studies on the behaviour and distribution of *Frankia* in soil, its other ecological niche, have been hampered in the past by problems encountered with isolation, specific detection and enumeration of this recalcitrant organism. The application of molecular techniques has provided means to tackle the last two problems.

Considerable efforts have been made to elucidate the structure of the genus *Frankia*, and a number of diagnostic molecules have been proposed (i.e. 2-*O*-methyl-D-mannose, bacteriohopanotetreol), in addition to host specificity, morphological, physiological, and nucleic acid-based criteria (reviewed by Lechevalier 1994). The most comprehensive taxonomic approaches based mainly on genotypic characteristics were made first by Fernandez *et al.* (1989, 1991) who applied the DNA-DNA reassociation technique defining 9 genomic species within the genus. Later on, Normand *et al.* (1996) postulated the current taxonomic status of the family Frankiaceae, based on analysis of full-length 16S rDNA sequences from a number of cultured and uncultured frankiae. With this phylogenetic approach they have divided the genus *Frankia* in 4 clusters that, in addition, were more or less coherent with the host plant from which the strains or sequences were obtained. Although these studies already pointed to some phylogenetically-based division within the genus, there is still little agreement on the species definition. As proposed by an *ad hoc* committee (Murray *et al.* 1990), current species definitions should arise from polyphasic approaches that incorporate morphologic, chemotaxonomic and phylogenetic data. However, there is a lack of consensus on the number and types of strains to be used, which must reflect the large variability within the *Frankia* group including cultured and recalcitrant strains.

The first reports on the use of 16S rRNA in *Frankia* molecular ecology studies were published by Hahn *et al.* (1989, 1990a,b), who designed a set of oligonucleotide-probes targeting variable regions of the 16S rRNA. By applying the *Frankia* probe (FP) and the effective *Frankia* probe (EFP) to rRNA extracted from soil and *Alnus* nodules, they could detect *Frankia* without culturing, and discriminate between two strains in nodulation

experiments. However, problems regarding the specificity of the probes and quantification of the obtained signals had to be solved. In addition to the 16S rRNA molecule, the *nif* genes coding for the nitrogenase could also be exploited as biomarkers in *Frankia*, with the extra advantage that within the nodules, such genes are only found in the N_2-fixing (Fix^+) symbionts (Akkermans *et al.* 1991). Using this approach, Simonet *et al.* (1990) also addressed the problem of strain identification in experimentally induced nodules. PCR products obtained with primers targeting conserved regions of the *nif*H gene were further hybridised with strain-specific probes. However, the variability within the short amplified fragment (213 bp) did not offer enough possibilities for further use with other strains. In a later paper, Simonet *et al.* (1991) explored the high variability offered by the intergenic spacers (IGS) found between the 16S-23S rDNA and the *nif*H-*nif*D genes, for a more specific PCR amplification and detection. With this approach they could discriminate a contaminating strain isolated from *A. nepalensis* nodules from the real endophyte inhabiting the actinorhiza. Even though the results were encouraging, there remain still problems for the detection of differences at the strain level.

Different *Frankia* endophytes and Fix^-/Nod^- strains (i.e. strains unable to fix N_2, and to reinfect their original host plant) were characterised at the molecular level, by using specific biomarkers. Special attention was given to symbionts from actinorhizal plants of the following genera (family): *Coriaria* (Coriariaceae), *Datisca* (Datiscaceae), *Ceanothus, Colletia, Retanilla, Trevoa* (Rhamnaceae), *Purshia* and *Dryas* (Rosaceae), which in most cases had resisted isolation. Nick *et al.* (1992) attempted to evaluate the global diversity of *Coriaria* endophytes from nodules collected in geographically distant areas. Using partial 16S rDNA sequences comprising 274 nucleotides around the V7-V8 regions, they demonstrated that the endophytes originating from New Zealand, France and Mexico form a distinct lineage within the *Frankia*. This finding was also supported by analysis of the IGS region flanked by the *nif*H and *nif*D genes. These results suggested a depauperate diversity among the *Coriaria* uncultured endophytes.

Different attempts to isolate the endophyte of *Coriaria nepalensis* yielded several Nod^-/Fix^- strains that were first characterised based upon fatty acid analysis (Mirza *et al.* 1991), morphological features, and partial 16S rDNA sequence analysis (Mirza *et al.* 1992). These *Frankia*-related strains formed a cluster together with similar Nod^-/Fix^- isolates obtained from *Datisca cannabina* nodules collected in Pakistan. Remarkably, the uncultured effective endophytes from both actinorhizae were also phylogenetically related at the level of both the *nif*H and the 16S rDNA (regions V2 to V6) (Mirza *et al.* 1994a). Unfortunately, the results obtained by both groups could not be compared as the sequences they have obtained hardly overlap, stressing the need of determining full sequences for reliable and comparable phylogenetic inferences. Additional work on the genetic diversity of *D. cannabina* and *C. nepalensis* endophytes from Pakistani soils pointed to some degree of heterogeneity found at the V2 region of the 16S rRNA, although these endophytes remained as nearest phylogenetic neighbours (Mirza *et al.* 1994b). Their close relatedness was also supported by effective nodulation after cross-inoculation experiments using crushed nodules of both plants. Further 16S rDNA sequence analysis confirmed that the endophyte of *C. nepalensis* could nodulate *D. cannabina* as well (Mirza *et al.* 1994c).

An evaluation of the diversity within uncultured endophytes of the Rhamnaceae, the largest AP family, was conducted in root nodules collected from 6 species of *Ceanothus* (Murry *et al.* 1997). A combination of genomic fingerprinting (so-called rep-PCR) and partial 16S rDNA sequence analysis revealed a high-resolution capacity of the former technique. Uncultured endophytes with identical 16S rDNA partial sequences showed different rep-PCR patterns. Phylogenetic inferences were possible, as the sequenced fragments were comparable to previously determined sequences within the Frankiaceae. The sequenced *Ceanothus* endophytes formed a very compact cluster and were related to *Elaeagnus*-infective strains. However, the partial 16S rDNA sequence data used in this and earlier analyses (Nazaret *et al.* 1991, Nick *et al.* 1992) showed only few informative nucleotide positions preventing the creation of stronger phylogenetic dendrograms.

Benson *et al.* (1996) carried out a comprehensive approach to assess the diversity and phylogenetic relations of uncultured *Frankia* symbionts, with greenhouse- and field-collected nodules from *Ceanothus griseus, Coriaria arborea, Coriaria plumosa, Discaria toumatou* and *Purshia tridentata*. By analising the V1 and V2 variable regions in the 16S rDNA, they could demonstrate the close relatedness of these endophytes, which with exception of the one in *D. toumatou* formed two neighbouring and very compact clades. One clade contained exclusively sequences of the symbionts of *Coriaria*, and the other was formed by the symbionts of *P. tridentata, Dryas drummondii* (Rosaceae) and *Ceanothus griseus* (Rhamnaceae) that surprisingly presented identical sequences in the determined stretch. All the uncultured endophytes from both clades were included in the *Dryas* cluster (Figure 1.4). The sequence obtained from *Discaria toumatou* (Rosaceae) actinorhiza was most related to the *Frankia* strains infective in *Elaeagnus*. Clawson *et al.* (1998) obtained results supporting the grouping of *Frankia* isolates obtained from host plants of the Rhamnaceae (*Colletia, Discaria, Retanilla, Talgenea* and *Trevoa*), and non-cultured nodule endophytes from this family and *Ceanothus*. Based on full-length and partial 16S rDNA sequence analyses, they concluded that the region proximal to the 5′ terminus containing the hypervariable regions V1 and V2 (*Escherichia coli* positions 28 to 419), provide sufficient information for reliable phylogenetic analysis of *Frankia*. Only the uncultured *Ceanothus* symbiont grouped in the *Dryas* cluster, while all the other isolates from the Rhamnaceae were positioned in the *Elaeagnus* cluster, indicating that AP within the same family may contain phylogenetically distinct *Frankia* symbionts.

Evidence obtained from cross-inoculation assays, morphological observations and phylogenetic analysis indicated that the *Elaeagnus* cluster may harbour *Frankia* strains able to infect hosts from several, even unrelated, plant families. Navarro *et al.* (1997) discovered that *Elaeagnus*-infective strains are indeed the microsymbionts in *Gymnostoma* actinorhiza, which is an AP genus included in the Casuarinaceae. This plant family was thought to have a very restricted *Frankia* diversity, since isolates from different geographic origins presented almost identical 16S rDNA partial sequences (Nazaret *et al.* 1989), and similar restriction fragment patterns from the spacer region between the16S and the 23S rDNA (Maggia *et al.* 1992). However, using this latter technique, so-called RFLP, Rouvier *et al.* (1996) found a higher genetic diversity of Casuarinaceae-compatible *Frankia* in Australia, regarded as the place of origin of this plant family. Furthermore, nitrogen-fixing actinomycetes isolated from *Casuarina*

equisetifolia, which were unable to elicit root nodules in the original host, and in few other promiscuous AP, showed to be in the Frankiaceae but out of the main *Frankia* group (Niner *et al.* 1996).

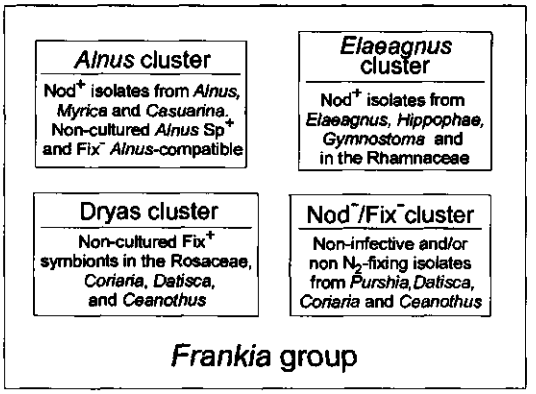

Figure 1.4. Schematic representation of the current clustering of the *Frankia* group. Cluster definition was based mainly on 16S rDNA sequence analysis. The main groups of strains or uncultured endophytes as well as the nodulation and N_2-fixing abilities are indicated.

The 23S rRNA and its encoding gene have also been explored as possible target for *Frankia* characterisation. Being almost twice the size as the 16S rDNA it offers more possibilities for specific targeting. Special attention has been paid to the insertion sequence of about 100 bp found in the domain III of the 23S rRNA of high DNA G+C content Gram-positive bacteria (Roller *et al.* 1992). An hypervariable region within that intervening sequence was successfully used to characterise uncultured and cultured *Alnus*-compatible frankiae (Hönerlage *et al.* 1994). In addition, they proved that the topology of phylogenetic dendrograms generated with 16S rDNA sequences was basically the same as the one based on the 23S rDNA intervening sequence. Zepp *et al.* (1997) developed a series of oligonucleotides targeting this insertion to characterise *Frankia* strains in *Alnus* spp. root nodules, and used fluorescently- and digoxigenin-labelled probes in whole cell hybridisation experiments. Although the whole-cell hybridisation methodology and the 23S rDNA as target offer good possibilities to be further exploited, problems relating to cell permeabilisation have to be solved, while the reduced number of available 23S rDNA sequences is still hampering its use on a more general basis.

Molecular characterisations have been used to assess morphological and functional *Frankia* types. Van Dijk and Merkus (1976) originally described the spore positive (Sp^+) and spore negative (Sp^-) types of *Alnus*-infective *Frankia*, refering to the presence or absence of sporangia in the actinorhiza. Although some lines of evidence pointed to the co-existence of both spore types inside one nodule (reviewed by Schwintzer 1990), further proof was still needed. Simonet *et al.* (1994) tackled the problem by screening few hundred nodules and soil from a natural *Alnus viridis* stand. By applying a combination of DNA-DNA hybridisation with *nif* genes and PCR targeting the 16S rDNA with spore type-specific primers, they could demonstrate that both the Sp^+ and Sp^- strain types could be present in the same nodule, and that each strain type was genetically very homogenous. These results were contrasting those of

Zepp *et al.* (1994), who could only detect one strain type per nodule by using whole cell hybridisation and 23S rRNA as target. The results regarding the distribution of both spore types in soil were less conclusive, although the strain type Sp^+ was detected more often than the Sp^- type.

Van Dijk and Sluimer-Stolk (1990) described another functional type of *Alnus*-compatible *Frankia* based on the ability to form generally small root nodules that are ineffective in nitrogen fixation (these endophytes are referred as Fix^- *Alnus*-compatible in Figure 1.3). This ineffective type of *Frankia* was reported to occur throughout the Netherlands, in relatively undisturbed *Alnus glutinosa* stands where the soil is waterlogged most of the year (Wolters *et al.* 1997a, Wolters 1998). Phylogenetic characterisation of the uncultured, ineffective microsymbionts proved that they formed a very homogenous group, but different from few already obtained ineffective isolates from *A. glutinosa* (Wolters *et al.* 1997b).

The use of molecular techniques has proven to be very effective on providing information about the diversity of *Frankia* symbionts. As long as the isolation problems are not resolved for a number of actinorhizae, this is the only available means to establish the phylogenetic relations. Despite the significant progress made in rDNA analysis of *Frankia*, this approach still have to be extended to a larger number of endophytes particularly from Rhamnaceae and Rosaceae.

MOLECULAR TOOLS TO STUDY THE BIOREMEDIATION OF CHLORINATED BENZENES AND BENZOATES IN SOIL

Chlorinated benzenes (CB's) are a group of compounds consisting of an aromatic ring of which each carbon atom may be substituted with a chlorine atom. Addition of different substituents (i.e. carboxyl or methyl groups) to the central benzene ring has been done in order to modify their physico-chemical properties, among others the solubility in water. In general, all these compounds are used as industrial solvents, insect repellents, fungicides, herbicides, dielectric fluids, odorisers and intermediates in the manufacturing of various chemicals (Middeldorp 1997). CB's are also formed as dead-end products of polychlorinated biphenyls (PCB) degradation, and therefore they could also be present in sites contaminated with these compounds (Furukawa *et al.* 1979). Due to the patterns of use of CB's and PCB's, a fraction of the total production is released into the environment causing dispersed pollution, characterised by a low concentration of pollutant in a widespread form. A different type of contamination, called point source pollution, may result during the industrial synthesis of these chemicals, via losses and wastes from production sites. In this case the concentration of the pollutant is high and more localised (Reineke and Knackmuss 1988).

Soil is the main repository for CB's and many other xenobiotic compounds, and contamination of ground and surface water occurs primarily by washing or leakage from this source. The fate of xenobiotic compounds greatly depends on their own chemo-dynamic properties and the physicochemical properties of the soil. These interactions will be important factors in the degradation processes mediated by the biological components residing in the soil (Figure 1.5). The general effects of xenobiotics on the microbial community have been

primarily assessed by measuring soil microbiological processes, like respiration or single carbon- and nitrogen-source transformations. However, these evaluations are either non-specific or insufficient to give an indication of stimulation or suppression on particular bacterial groups (Hicks *et al.* 1990).

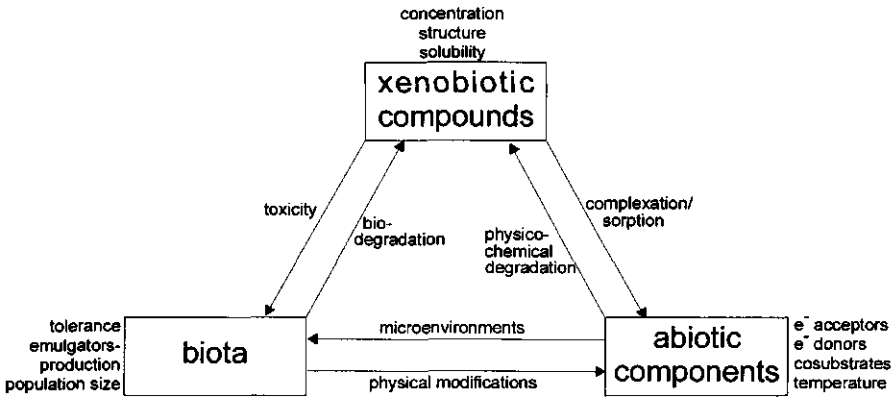

Figure 1.5. Interactions of xenobiotic chemicals with biotic and abiotic components from soil. Important features influencing the interactions of the biota, xenobiotic and abiotic components are indicated (modified from Hicks *et al.* 1990).

The majority of the bacterial isolates able to degrade mono- and di-chlorobenzenes aerobically belong to the β subclass of Proteobacteria (Dorn *et al.* 1974, Pettigrew *et al.* 1991, Babu *et al.* 1995, Krooneman *et al.* 1996). Degradation of CB's with up to four chlorine substituents has also been reported (Sander *et al.* 1991, Hernández *et al.* 1991). So far, reductive dechlorination is the only biotransformation process observed for penta- and hexachlorobenzenes (Dolfing and Beurskens 1995). Microbial degradation of CB's can take place under aerobic and anaerobic circumstances, and the metabolic pathways involved have been abundantly described (Reineke and Knackmuss 1988, Spain 1990, Schlömann *et al.* 1990, Mohn and Tiedje 1992, Dolfing and Beurskens 1995, Selkov 1996).

Most CB's-degrading bacterial cultures have been obtained from water and sediments, and their isolation from soil has been less frequently reported. Nevertheless, Fulthorpe *et al.* (1996) described evidence for the global occurrence of microorganisms able to degrade 3-chlorobenzoate (3CBA) or the related compound 2,4-dichlorophenoxyacetate (2,4D), since several pristine soils collected from various locations around the world could mineralise one or both substances. In addition, naturally occurring microbial degradation of CB's occurring in river sediments has been reported for highly chlorinated benzenes (Beurskens, 1995), and for mono- and di-chlorobenzenes (Clover *et al.* 1998, Peel and Wyndham 1997). This so-called passive bioremediation or natural attenuation plays an important role on the mineralization of

other chlorinated compounds, mainly aliphatic solvents found in soil and sediments (Beeman *et al.* 1994, Bosma, *et al.* 1998, Burdick *et al.* 1998, Harkness *et al.* 1998, Lorah and Olsen 1998).

The use of CB's-degraders in large-scale bioremediation processes has been scarce (Folsom *et al.* 1995). Most reports about microbial degradation of CB's in soil have been performed under lab or pilot conditions (Scheunert and Korte 1986, van der Meer *et al.* 1987, Brunsbach and Reineke 1993, Ramanand *et al.* 1994, Guiot *et al.* 1995, Peck *et al.* 1995). The approaches for remediation of heavily polluted soils in general involve removal and *ex situ* treatment, while disperse contamination has to be approached by *in situ* remediation processes. Soil inoculation of microorganisms with degrading abilities has been one of the preferred bioremediation technologies. Since the final goal is the removal of the soil pollutant, the overall process can be purely evaluated by the dissapearance of the xenobiotic compound. However, such an approach does not take into account the fate of the pollutants in the environment, and does not contribute to the understanding of the occurring microbiological processes, which determine to a large extent the final output (Heitzer 1998).

Evaluation and monitoring of the processes conducting to bioremediation of toxic compounds in the environment have recently been approached with molecular techniques focussing on the microbiological processes (White *et al.* 1998), toxicological aspects (Power *et al.* 1998), and site assessment (Stapleton *et al.* 1998, Höhener *et al.* 1998). Nucleic acid probes for about 100 different genes involved in biodegradation of over 40 contaminants are available for assessment of different aspects of the bioremediation process (Sayles *et al.* 1995).

The application of molecular approaches for assessment and characterisation of particular CB-degrading populations in soil or model systems has also been addressed. Specific detection of the anaerobic CB-degraders *Desulfomonile tiedje* and *Desulfitobacterium dehalogenans* in soil model systems has been achieved by nested-PCR targeting the 16S rDNA (Fantroussi *et al.* 1997). Detection of the former bacteria has also been intended in granular sludge applying immuno-fluorescent techniques (Ahring *et al.* 1992), and by fatty acid analysis in sediment incubations (Ringelberg *et al.* 1992). In all cases the presence of bacteria was related to the disappearance of CB's. Becker *et al.* (1998) studied the anaerobic microbial community from 3CBA-degrading sediment by using a combination of DGGE and sequence analysis. They have identified predominant bacteria in the community that may have similar functions and syntrophic interactions as a previously described methanogenic consortium which also metabolise 3CBA (Dolfing and Tiedje 1986). Despite the occurrence of reports on the degradation of CB's and CBA's, remarkably little attention has been given on the effect of these compounds on non-cultured soil bacteria such as *Frankia*.

The various molecular approaches described above have proven to be valuable when addressing changes in bacterial populations involved in biodegradation processes. However, special attention has to be paid to techniques providing bacterial community fingerprints, which try to reflect complex interactions and changes on several populations, including the non-cultured bacteria. The resulting patterns are in general difficult to interpret and some means to evaluate these have to be developed. Although plant and animal ecologists do not have to face culturability problems when analysing such communities, they have developed models and community parameters that can be adapted to fulfil the needs of microbial ecologists. Diversity

and equitability have been already applied in microbial ecology studies of cultivable community fractions (Bej *et al.* 1991, Marilley *et al.* 1998). What remains is their application on community fingerprints, as they can be used as descriptors of community changes in response to environmental conditions.

OUTLINE OF THE THESIS

The aim of this work was to assess selected bacterial groups under natural and stressed soil conditions using a combination of molecular and traditional microbiology techniques. Special attention was given to bacterial groups that are difficult to culture or still remain uncultured, such as many *Frankia* strains. Chlorobenzoates were used as model compounds to induce stress in the soil bacterial community, and their effect was addressed with a similar combined approach.

The first part of the thesis presents molecular tools that were used to characterise microbial populations in undisturbed environments, in particular the actinorhizal root nodules. Chapter 2 describes a method to efficiently extract DNA from different actinorhiza, which can be applied even to milligram amounts of nodule tissue. The method emphasises the use of diverse cell lysis procedures and purification steps to obtain an enriched fraction of *Frankia* DNA. This methodology has been applied to characterise an uncultured *Frankia* endophyte present in the Mexican actinorhizal plant *Ceanothus caeruleus*. Morphological and physiological features, together with molecular tools were used to further characterise *Frankia*-related isolates obtained from the same nodules. The results of this study are reported in Chapter 3.

The second part of the thesis is focussing on the assessment of bacterial communities in chlorobenzoate-stressed environments. A peat-forest soil was utilised in microcosm experiments as model system to address the effect of chlorobenzoates. Population changes in the stressed bacterial communities were detected, and included the enrichment in soil of uncultured *Burkholderia*-related bacteria. These results and the characterisation of the latter bacterial group are presented in Chapter 4. Furthermore, an approach based on estimation of diversity and equitability indices was applied to evaluate these parameters in the uncultured bacterial communities from the microcosm systems. In addition, fluorescent pseudomonads were isolated from the stressed and the unstressed microcosms, and the respective changes in diversity and equitability were assessed using the same indices (Chapter 5). The population changes induced by the chlorinated benzoates in the native *Frankia* were evaluated using a most probable number approach, which included both the total uncultured population as well as the fraction that was able to nodulate *Alnus glutinosa* (reported in Chapter 6). Chapter 7 describes the effect of 3-chlorobenzoate on the development of *A. glutinosa*, and the influence of the plants on the dechlorination activity of the 3CBA-degrader *Pseudomonas* sp. strain B13. Chapter 8 includes the concluding remarks section, where the main findings of this thesis are summarised and discussed.

CHAPTER 2

*DNA EXTRACTION FROM ACTINORHIZAL NODULES

Hugo Ramírez-Saad, Wilma L. Akkermans and Antoon D.L. Akkermans

* Molecular Microbial Ecology Manual, Chapter 1.4.4, pp 1-11. *Edited by:* A.D.L. Akkermans, J.D. van Elsas and F.J. de Bruijn. Kluwer Academic Publishers, Dordrecht, The Netherlands (1996).

INTRODUCTION

The nitrogen-fixing actinomycete *Frankia* has its main ecological niche in the root nodules that are formed with a wide taxonomic range of host plants called by this fact actinorhizal plants. This generic name comprises more than 200 species of vascular dicotyledonous plants, distributed in 20 genera and eight families (Benson and Silvester 1993).

Frankia root nodules, also known as actinorhiza can be regarded as natural enrichments of *Frankia* populations, from where almost all existing isolates have derived. However, in spite of the numerous *in vitro* cultures obtained from actinorhiza of many different host plants, there are still some recalcitrant endophytes that have resisted isolation, hence their characterisation had to be addressed under different approaches.

The application of molecular techniques has proven effective in microbial ecology studies (Akkermans *et al.* 1991, 1994). Molecular markers such as ribosomal RNA/DNA and *nif* genes, have been widely preferred (Hennecke *et al.* 1985, Normand and Bousquet 1989, Olsen *et al.* 1986), due to their known properties as conserved and ubiquitous molecules.

These approaches applied in *Frankia* research, have given new insights in identification and characterisation (Hahn *et al.* 1990a, b, Hönerlage *et al.* 1994, Simonet *et al.* 1990), phylogeny (Bosco *et al.* 1993, Mirza *et al.* 1994b, Nick *et al.* 1992) and ecology (Simonet *et al.* 1994), of both uncultured and cultured *Frankia* strains *in planta*, circumventing the problematic step of cultivation.

All these studies require efficient protocols to extract nucleic acids from nodules. A general scheme for this purpose has the following basic steps: a) Plant cell lysis and release of *Frankia* clusters, commonly done by crushing in mortar and pestle, aided with a freezing agent. b) *Frankia* cell wall lysis, approached in several ways such as: enzymatic lysis (Hönerlage *et al.* 1994, Simonet *et al.* 1990), sonication (Hahn *et al.* 1990b, Mirza *et al.* 1994a), hot detergent (Baker and Mullin 1994), bead beating (Mirza *et al.* 1994b), or a combination of these plus microwave shock (McEwan *et al.* 1994, Simonet *et al.* 1994). c) DNA precipitation and purification: phenol-chloroform extraction followed by ethanol or isopropanol precipitation are the most commonly used techniques, but Elutip D column purification (Simonet *et al.* 1994) or even a crude cell lysate (Simonet *et al.* 1990) have been tried as well. Physical lysis methods alone or combined are preferred for recalcitrant microorganisms (Johnson 1991).

Detection approaches are based either on direct hybridisation with labelled probes, of specific target sequences from total DNA/RNA extracts (Baker and Mullin 1994, Hahn *et al.* 1990b, Hahn *et al.* 1993, Simonet *et al.* 1988), or by PCR-assisted amplification and retrieval, which is by far the most widely used approach (Bosco *et al.* 1993, Hameed *et al.* 1994, Hönerlage *et al.* 1994, McEwan *et al.* 1994, Mirza *et al.* 1994a, b, c, Nick *et al.* 1992, Simonet *et al.* 1990, 1994).

In this chapter we describe a simple DNA extraction protocol developed in our laboratory, which has been successfully applied to a variety of woody actinorhiza.

EXPERIMENTAL APPROACH

In most DNA extraction procedures there is a compromise between yield, purity and size of the final product. In this protocol we have opted for a high yield and good purity, sacrificing the need for high molecular weight DNA. The final product can be used as a target for PCR amplification or directly in dot blot or Southern blot hybridisations.

The final yield of nucleic acids is affected by different factors such as nodule source, age, and way of preservation or season. Seasonal variations in nodule and endophyte activity are well documented for temperate region actinorhizal plants (Huss-Danell 1990, Schwintzer *et al.* 1982). The amount of vesicles and active hyphae inside infected cells is lower during the colder seasons, whereas nodules collected in late spring and summer have the highest amount of newly infected cells containing active symbionts.

Young freshly collected nodules give the best results, but nodules frozen shortly after collection or fixed nodules as described by Hahn *et al.* (1993)[1] are suitable for the procedure.

With large, older nodules, care must be taken with the selection of lobes and lobe tips, avoiding senescent material that is rich in phenolic compounds and poor in active *Frankia* clusters.

Peeling of nodule lobes to avoid possible contaminant microorganisms is quite laborious; even though it considerably reduces the possibility of contamination, does not ensure the absence of other soil microorganisms. Nodules with remaining soil material attached must be washed with a liquid detergent solution and then rinsed throughout with sterile water.

Direct extraction of DNA from nodules results in a mixture of plant and *Frankia* DNAs. Since the DNA is generally used for direct hybridisation or PCR amplification of specific sequences, there is no need to separate plant and bacterial DNA. Nevertheless separation of both DNAs can be achieved by using buoyant density differences in CsCl (Mullin *et al.* 1983). An alternative approach (Akkermans *et al.* 1981, McEwan *et al.* 1994) is based on two filtration steps of nodule homogenates, to collect a *Frankia*-enriched fraction and then proceed with DNA extraction. However, these procedures require samples significantly larger than those needed for direct extraction.

PROCEDURE

The procedure combines on the one hand, the capacity of the non-ionic detergent cetyltrimethylammonium bromide (CTAB), to form complexes with nucleic acids, while

[1] Clean nodules are fixed at 4°C for 3 to 16 h in fixation buffer (4% paraformaldehyde in PBS [0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; pH 7.2 in water]). Washed in PBS (3 times) and stored at -20°C in 50% ethanol in PBS until used.

contaminant substances such as proteins and polysaccharides are efficiently removed by chloroform-isoamyl alcohol extraction. The addition of polyvinylpirrolidone (PVP) in the CTAB buffer helps in absorbing polyphenolic compounds, strong inhibitors of DNA polymerase commonly present in nodules. On the other hand, violent shaking in the presence of glass or zirconium beads, known as bead beating, provides the powerful cell disrupter capacity required to break or disrupt the resistant *Frankia* cell wall.

This protocol is based essentially on the CTAB extraction methods described by Rogers and Bendich (1985) and Rogers *et al.* (1989) for extraction of DNA from plant tissues and basidiomycetes respectively, and the bead beating approach applied to actinorhizal nodules, previously described by Mirza *et al.* (1994c).

CTAB-bead beating extraction

The procedure is described for 100 mg fresh weight nodule material, which represents an amount enough to allow small losses during the process; however, the procedure can be easily scaled down to 20 mg nodule material and still produce sufficient *Frankia* DNA that can be specifically amplified by PCR. Scaling down should be done by applying proportional reductions in the amounts of other components as beads, buffers and other solutions. Amounts larger than 250 mg nodules may require not only proportional increases in reagents used but also different grinding vessels (e.g. the mortar and pestle or bead beating machine).

Steps in the procedure

1. Weigh approximately 100 mg of fresh nodules, lobes or lobe tips. To facilitate grinding, cut them aseptically in small pieces. Transfer cut pieces to a grinding plastic tube with round bottom, freeze by addition of liquid nitrogen[2] and grind to a fine powder with a sterile micropestle. Merck micropestles (cat No 0030120.973) and 2 ml Safelock grinding tubes (Merck cat No 0030120.094) are very suitable for this purpose.
2. Transfer the ground frozen sample to a 2 ml mini bead-beater tube, containing approximately 300 mg of zirconium beads (0.5 mm ∅) and 250 mg of smaller zirconium beads (0.07 to 0.11 mm ∅)[3]. Add 350 µl of 2% CTAB extraction buffer, heated to 65°C.

[2] Handling liquid nitrogen is somewhat problematic, however small volumes are generally enough to freeze the nodule samples. Those can be pipetted using an automatic pipette with blue tip, as follows. Set the volume above 600 µl, press the push button and immerse for a few seconds in the liquid nitrogen about 5-8 mm of the tip. Depress the push button partially and bring the small volume of liquid nitrogen immediately into the grinding tube. The liquid comes out by itself after one or two seconds; do not press the push button to release it. Try to pour the liquid nitrogen against the tube wall, to avoid the sample being expelled from the tube. Even though the volumes handled are very small, for protection it is advisable to wear vinyl or latex gloves

[3] The use of zirconium beads of two sizes ensures lysis of both plant and microbial cells. Beads must be baked overnight at 160°C prior to use, to destroy DNAses and RNAses.

The buffer can first be used to rinse the grinding tube and recover remainings of nodule material in it.

3. Homogenise 7 times for 30 s in a mini bead beater (BioSpec Products) at 5,000 rpm, with intervals of about 1 min. Due to the detergent, a lot of foam is produced but most of it disappears during the next step.
4. Place the tubes in a 65°C water bath for 60 min with occasional mixing of the content.
5. Add 150 µl of 1% CTAB extraction buffer and mix thoroughly.
6. Add 500 µl chloroform-isoamyl alcohol (24:1), vortex to form an emulsion and centrifuge at 10,800 x *g* (11,000 rpm on a bench centrifuge) for 5 min.
7. Transfer the water (upper) phase to an Eppendorf tube, determine the volume (ca 500 µl) and add 0.1 volume of hot (65°C) 10% CTAB buffer, mix well.
8. Add one volume of chloroform-isoamyl alcohol and proceed as in steps 1.6 and 1.7, until no precipitated protein is seen at the interface (usually one or two repetitions are sufficient).
9. Precipitate DNA by adding 0.1 volume 3M sodium acetate (pH 5.2) and one volume ice-cold isopropanol, mix well and store at -20°C for at least 30 min.
10. Centrifuge at 15,100 xg (13,000 rpm on a bench centrifuge) for 15 min, discard isopropanol and wash pellet in 0.5 ml of cold 70% ethanol, centrifuge again for 5 min.
11. Carefully remove ethanol and air-dry or vacuum-dry the DNA pellet until no traces of ethanol are left.
12. Resuspend the pellet in 50 µl or a suitable volume of sterile TE buffer (pH 8.0). If resuspension is difficult, warm to 40-45°C for a few minutes.
13. Electrophorese 1/5 to 1/10 volume of the final solution in TAE-0.8% agarose gel. Before pouring the gel, add ethidium bromide stock solution (10 mg/ml) to a final concentration of 0.5 µg/ml.

Polyvinylpirrolidone purification

If the final DNA solution has a brownish colour, this is indicative of the presence of polyphenolic compounds that may interfere with further uses. Extra purification can be done by using a PVP containing buffer, as described below.

Steps in the procedure

1. Add enough sterile TE buffer to bring the DNA solution to 100 µl, then add 100 µl TPN buffer, incubate 5 min at room temperature under continuos mixing.
2. Add 200 µl phenol-chloroform-isoamyl alcohol (25:24:1), vortex shortly and centrifuge 5 min. at 15,100 x *g*.
3. Recover as much as possible of the water phase and precipitate DNA as above.
4. Resuspend the pellet in the smallest possible volume of TE, since some loss of product is expected.
5. The initial addition of TE buffer might be omitted, if the DNA solution is very dilute. Instead, add an equal volume of TPN buffer and proceed with incubation.

Microwave lysis protocol

For very tiny nodules and amounts smaller than 10 mg, alternative lysis methods such as sonication (Hahn *et al.* 1990, Mirza *et al.* 1994a) or microwave shock (McEwan *et al.* 1994, Simonet *et al.* 1994) should be tried, in combination with CTAB extraction. Although these physical cell disrupting methods are less powerful than bead beating, they have the advantage that lysis can be performed in small volume samples, which is not possible with bead beating. An alternative protocol based on the use of microwave heating (McEwan *et al.* 1994, Simonet *et al.* 1994) is described below.

Steps in the procedure

1. Grind the sample as described in 1.1.
2. Collect all ground material in the bottom of the grinding tube by rinsing the micropestle and tube wall with 100-150 µl sterile TE, then centrifuge at 3,200 x *g* (±6,000 rpm on a bench centrifuge) and discard supernatant.
3. Resuspend the pellet in the same tube with 10 µl of 2% CTAB extraction buffer.
4. Wrap the tube loosely with Saran wrap, to avoid cross contamination if samples are expelled while microwaving, but leave the lid open.
5. Put the tubes inside a microwave oven and operate it at 400 W for 6 times, 10 s each. Let the samples cool down between treatments.
6. Unwrap the tubes, add 10 µl of 1% CTAB extraction buffer and put them, with the lid closed, in a 65°C water bath for 1 h, with occasional mixing, trying to keep all components at the bottom of the tube.
7. Add 30 µl chloroform-isoamyl alcohol (24:1), mix thoroughly (do not vortex) and centrifuge at 10,800 x *g* for 5 min.
8. Transfer the water (upper) phase to an Eppendorf tube, determine the volume and add 0.1 volume of hot (65°C) 10% CTAB buffer, mix well.
9. Proceed as in steps 1.8 and 1.9.
10. Centrifuge at 15, 100 x *g* (13,000 rpm) for 15 min, discard isopropanol and wash pellet in 100 µl of cold 70% ethanol, centrifuge again for 5 min.
11. Carefully remove ethanol and air-dry or vacuum-dry the DNA pellet until no traces of ethanol are left.
12. Resuspend the DNA pellet in 10 µl of sterile TE buffer (pH 8.0).

SOLUTIONS

- 2% CTAB extraction buffer
  - 2% (w/v) CTAB (cetyltrimethylammonium bromide)
  - 100 mM Tris-HCl (pH 8.0)
  - 1 mM EDTA
  - 1.4 M NaCl
  - 1% (w/v) PVP 40 (polyvinylpirrolidone 40)
- 1% CTAB extraction buffer
  - 2% CTAB extraction buffer diluted (1:1) in distilled water
- 10% CTAB extraction buffer (very viscous solution, warming at 65°C makes pipetting easier)
  - 10% (w/v) CTAB
  - 0.7 M NaCl
- TE buffer (pH 8.0)
  - 10 mM Tris-HCl (pH 8.0)
  - 1 mM EDTA
- TPN buffer (pH 8.0)
  - 10 mM Tris-HCl (pH 8.0)
  - 2% (w/v) PVP 40
  - 150 mM NaCl
- TAE buffer
  - 40 mM Tris-acetate
  - 1 mM EDTA

NOTES

The final yield values of DNAs shown in Fig. 2.1, extracted under procedure 1, ranged from 18 ng to 170 ng total DNA-RNA/mg nodule, these amounts of nucleic acids may be sufficient for dot blot or Southern blot detection. It is difficult to establish typical yields, as they may have a large variation. Though, actinorhiza-extracted nucleic acids are a mixture from plant and endophyte origin, Mullin *et al* (1983) established that about 56% of the DNA extracted from *Alnus glutinosa* nodules belongs to *Frankia*. However, the final ratio can be affected by several factors, among which the extraction procedure itself.

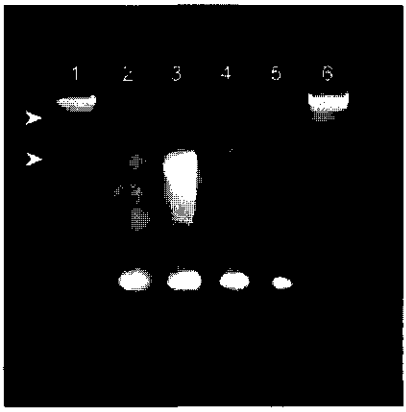

Figure 2.1. Agarose gel electrophoresis of DNA/RNA extracted from nodules of different host plants that were preserved under different conditions: lanes 1 and 6, λ/*Hin*dIII DNA, the lower arrow points to the 2.0 and 2.3 kb bands, the upper arrow points to the 9.4 kb band; lane 2, *Alnus glutinosa* young fresh nodule, from a greenhouse-grown plant; lane 3, *Ceanothus caeruleus* fixed nodule, field collected; lane 4, *Casuarina glauca* young frozen nodule, from a greenhouse-grown plant; lane 5, *A. glutinosa* young fixed nodule (stored for more than 2 years), field collected.

Due to the bead beating steps the DNA obtained is sheared. In Fig. 2.1, DNA extracted from fresh or freshly frozen nodules (lanes 2 and 4) gave the larger size molecules from up to 8 Kb. Whereas fixed nodules yielded shorter DNA fragments, being the larger molecules of about 2.5-3 Kb. This is probably due to an effect of the fixative on the nodule tissue, that makes it easier to lyse during bead beating, on such fixed samples, a smaller amount of bead beating repetitions may be used, if there is a need for larger size DNA. However, any of these or similarly obtained DNAs can be used as template for almost any PCR mediated amplification.

In most cases, DNA obtained under the conditions described must be diluted ten or one hundred fold, prior to its use as target for PCR. This has the advantage that possible inhibitors are also diluted. When PVP purification is required, a ten-fold dilution or undiluted DNA solutions may be used as template.

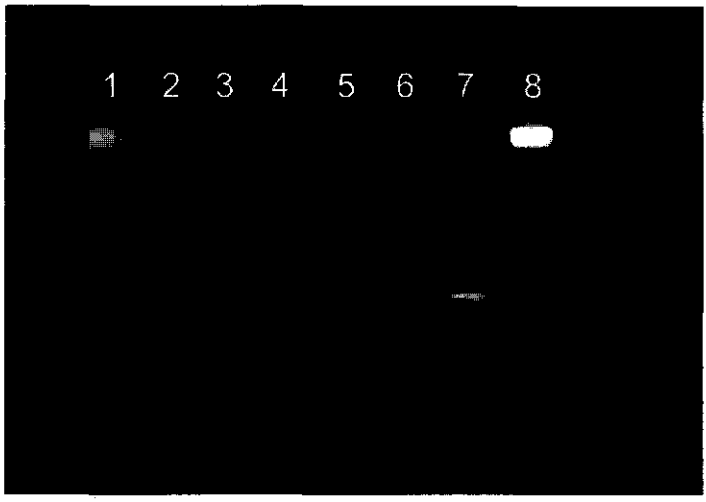

Figure 2.2. Agarose gel electrophoresis of PCR products from partial 23S rDNA sequences, the upper bands correspond to *Frankia* and lower bands are of plant-plastid origin. Template DNAs for PCR were diluted tenfold before use. Lanes 1 and 8, 123 bp molecular weight marker ladder (Life Technologies); lane 2, *C. glauca* young fixed nodule; lane 3, *A. glutinosa* young fresh nodule; lane 4, *C. glauca* young frozen nodule; lane 5, *C. glauca* young frozen nodule (template DNA after PVP purification); lane 6, *A. glutinosa* leaves, showing only the lower band; lane 7, *Frankia* ORS020608 pure culture, showing the upper band only.

Fig. 2.2 shows an example of differential PCR amplification, by using the primers designed by Roller *et al* (1992), to amplify the insertion element present within the 23S rRNA gene of high G+C content bacteria (not present in plant plastids). Two PCR products are synthesised, one of about 280 bp presumably from plant chloroplast/mitochondria and a second one of 375 bp that corresponds to *Frankia*. This region has been used as discriminating target to characterise uncultured and cultured *Frankia* (Hönerlage *et al.* 1994). The PCR amplifications on Fig. 2.2 were performed in 50 µl/reaction, using a PCR kit of Life Technologies (Gaithersburg,MD) under the following conditions: 10X PCR buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 5 µl; 10 mM dNTP mixture, 1 µl; 50 mM $MgCl_2$, 2 µl; primers (Roller *et al.* 1992) 23INSv (400 ng/µl) and 23INSr (400 ng/µl), 0.5 µl each; W-1 detergent (1% solution), 1 µl; *Taq* polymerase (5 U/µl), 0.25 µl; target DNA, 1 µl of ten fold diluted solutions. Thermal cycling was as follows: initial denaturation step of 5 min at 94°C, then 40 cycles of 45 s at 94°C, 45 s at 46°C, 1 min at 72°C finished by one post-elongation step of 7 min at 72°C.

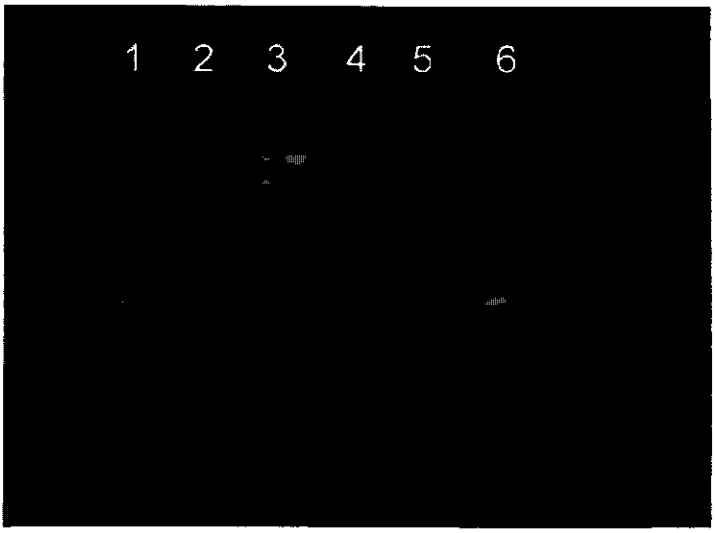

Figure 2.3. Agarose gel electrophoresis of PCR products from partial *nif*H genes, different template DNAs for PCR were diluted tenfold before use. Lane 1, *C. caeruleus* fixed nodule; lane 2, *C. glauca* young frozen nodule (template DNA after PVP purification); lane 3, 123 bp molecular weight marker ladder (Life Technologies); lane 4, *C. glauca* young frozen nodule; lane 5, *C. glauca* young fixed nodule; lane 6, *Frankia* ORS020608 pure culture.

Genes for nitrogen fixation are expected only in the endophyte fraction of the extracted DNA. The structural *nif* genes as well as the intergenic spacers have been used as PCR target to characterise *Frankia* strains (Mirza *et al.* 1994b, Simonet *et al.* 1994). In Fig. 2.3, a fraction of about 650 bp of the *nif*H gene was PCR amplified in 50 µl reactions (PCR kit of Life Technologies), under the following conditions: 10X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 5 µl; 10 mM dNTP mixture, 1 µl; 50 mM $MgCl_2$, 1.5 µl; primers (Mirza *et al.* 1994b) *nif*H_F (100 ng/µl) and *nif*H_R (100ng/µl), 1 µl each; W-1 detergent (1% solution), 1 µl; *Taq* polymerase (5 U/µl), 0.25 µl; template DNA 1 µl of ten fold diluted solutions. Thermal cycling was as follows: initial denaturation step of 5 min at 94°C, then 40 cycles of 45 s at 94°C, 45 s at 52°C, 1 min at 72°C finished by one post-elongation step of 7 min at 72°C.

CHAPTER 3

*ROOT NODULES OF *CEANOTHUS CAERULEUS* CONTAIN BOTH THE N_2-FIXING *FRANKIA* ENDOPHYTE AND PHYLOGENETICALLY RELATED NOD$^-$/FIX$^-$ ACTINOMYCETES

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SUMMARY

Attempts to isolate the N_2-fixing endophyte of *Ceanothus caeruleus* (Rhamnaceae) root nodules, led to the isolation of nine actinomycetous strains. Due to their inability to fix nitrogen (Fix^-) and nodulate (Nod^-), they could not be regarded as the effective endophyte. Characterisation was done based on morphological and physiological features and 16S rDNA sequence analysis. The effective *Frankia* endophyte was characterised without cultivation by amplification, cloning, and sequencing of nearly full length 16S rDNA and partial *nif*H genes. Phylogenetic analysis based on 16S rDNA revealed that both the effective endophyte and the isolated actinomycetes belong to two different but well-defined lineages within the family Frankiaceae. One lineage is formed mainly by "uncultured endophytes" that so far have resisted isolation, and the other includes only Fix^-/Nod^- isolates. Application of Temperature Gradient Gel Electrophoresis (TGGE) techniques to actinorhizal nodules allowed us to detect and identify 16S rDNA sequences from both the Fix^+ and the Fix^- nodule inhabitants. Interestingly, these same two sequences were detected on *Hippophae rhamnoides* nodules obtained after inoculation with *C. caeruleus* nodule suspensions. The isolates were located in the outer layers of the nodule.

INTRODUCTION

Actinomycetes within the genus *Frankia* are able to induce nitrogen-fixing root nodules with a wide taxonomic group of vascular plants distributed in 8 families, which are designated as actinorhizal plants (AP). *Frankia* strains have been obtained from 20 out of 24 AP genera, although in several instances reinfection of the original host has not been achieved. This is particularly true for isolates from AP belonging to the families Rhamnaceae, Coriariaceae, Datiscaceae and Rosaceae (Benson and Silvester 1993).

Ceanothus is the largest of seven AP genera in the Rhamnaceae. This genus comprises more than 50 species of shrubs native to North America, mostly found as pioneers in disturbed sites or naturally occurring in chaparral and xerophytic areas at high altitude (Baker and Schwintzer 1990). Due to their tolerance to drought, temperature fluctuations, and poor soils, they are frequently utilised for low-maintenance landscape and revegetation (Benoit and Berry 1990).

Many attempts have been carried out to obtain and characterise *Frankia* strains from AP within the Rhamnaceae (Akkermans *et al.* 1984, Lechevalier and Ruan 1984, Baker 1987, Carú 1993, Carrasco *et al.* 1995), Coriariaceae (Chaudhary and Mirza 1987, Mirza *et al.* 1992) and Datiscaceae (Hafeez 1983). So far, isolation of infective (Nod^+) and effective N_2-fixing (Fix^+) frankiae from these AP families has only been reported by Carrasco *et al.* (1995), who isolated two strains from *Trevoa trinervis*. Another *Frankia* strain, designated Cc1.17, isolated from *Colletia cruciata* (Akkermans *et al.* 1984), was later found to be infective on its original host and on *Hippophae rhamnoides* (Akkermans, unpublished results). All other attempts have led to the formerly called "atypical isolates", referring to strains that lack at least one of the distinguishing morphological and/or physiological features of the genus *Frankia* (Akkermans and Hirsch 1997).

Characterisation of those endophytes that resisted isolation has been achieved by using molecular techniques. In general, PCR assisted retrieval of partial 16S rDNA genes, followed by cloning and sequencing have proved effective in characterising uncultured endophytes from Coriariaceae, (Mirza *et al.* 1992, Nick *et al.* 1992), Datiscaceae (Mirza *et al.* 1994b, c), and have recently been applied to a wider range of AP families (Benson *et al.* 1996). Trials to isolate effective endophytes from surface-sterilised root nodules of these recalcitrant AP have only resulted in the isolation of Nod^-/Fix^- strains (Hafeez 1983, Mirza *et al.* 1992, 1994b).

In the present paper we describe the occurrence and characterisation of various types of *Frankia* strains present in the root nodules of *Ceanothus caeruleus*, with emphasis on the phylogenetic position of the uncultured endophyte and isolated Nod^-/Fix^- strains, and the possible localisation of the latter within the nodule tissue.

MATERIALS AND METHODS

Source of organisms and soil

Actinorhizal root nodules and rhizospheric soil were collected from a *C. caeruleus* natural stand growing in a pine-oak forest at 2500 m altitude, in Santo Tomás Atzinco, Estado de México. Nodules for isolation trials and rhizospheric soil samples were kept at 4°C and used as soon as possible, whereas nodules for DNA extraction were frozen (-20°C) until needed.

Isolation and culture conditions

Root nodules were surface-sterilised with 1% (w/v) sodium hypochlorite for 15 min, then washed several times with sterile water. Isolation was performed by the sucrose-gradient method (Baker and O'Keefe 1984). After 5-10 weeks of culturing in P+N agar (Meesters *et al.* 1985), actinomycetous microcolonies developed and single colonies were transferred to liquid P+N medium. Purity was frequently assessed by light microscopy and also checked by transfer into Qmod medium (Lalonde and Calvert 1979). The isolated strains were routinely subcultured on liquid P+N medium at 28°C.

DNA extraction

DNA/RNA used for hybridisation or as template in PCR reactions was extracted from young nodules and from pure cultures. Nodules were washed with a detergent solution (Decondi 118, Otares, Enschede, The Netherlands), and rinsed profusely with sterile water. Nodule lobes weighing as much as 1 - 3 mg were excised aseptically, and DNA from single lobes was extracted as described previously (Ramírez-Saad *et al.* 1996). When needed, nodule lobes were carefully peeled under a dissecting microscope using sterile forceps and scalpel. DNA from pure cultures was obtained by bead beating followed by phenol-chloroform extraction (Mirza *et al.* 1994c).

Hybridisation

Total DNA/RNA extracted from the isolates or PCR products from the 16S rDNA-cloned fragments were used as target in dot blot hybridisations with a radioactively labelled *Frankia* specific probe (FP) (Hahn *et al.* 1990). Probe labelling was done following standard procedures (Sambrook *et al.* 1989), and hybridisation was performed according to Hahn *et al.* (1990).

Total cell Fatty Acid Methyl Ester analysis (FAME)

Typing of isolates was done essentially as described by Mirza *et al.* (1991). Resulting fatty acid profiles were compared to similar profiles present in the created Plant Protection Service (PD) library by using the Microbial Identification System (Microbial ID, Newark, DE,

USA). Comparison of the profiles was done by principal component analysis with options available in the same system.

DAP analysis

Determination of the diaminopimelic acid (DAP) isomer present in the peptidoglycan of the isolates was performed by subjecting crude cell lysates to a thin layer chromatography-based technique described by Trujillo (1994).

Nodulation tests

Nodulation ability of isolates Cea1.3 and Cea5.1 was assayed essentially as described by Selim and Schwencke (1995). Syringe-homogenised cell suspensions of the isolates were inoculated on the roots of *Ceanothus prostratus* and *C. griseus* (Clyde Robin Seed Co., Hayward, CA, USA) plantlets. *Hippophae rhamnoides* (Pelgrum Vink Materialen, Westervoort, The Netherlands), a promiscuous AP, was also inoculated (Table 1). Plants were grown in modified Leonard's jars using gravel-vermiculite (1:1 v/v) as support and N-free Hoagland nutrient solution at 1/2 strength (Hoagland and Arnon 1950). Additionally, *H. rhamnoides* seedlings were inoculated with suspensions of *C. caeruleus* crushed nodules or rhizospheric soil (Table 3.1). Inoculated plants were kept in a growth chamber with a thermoperiod of 14/23°C and a photoperiod of 16/8 h (day/night).

Table 3.1. Inoculation trials using different inoculum source and actinorhizal plants.

	Number of inoculated / nodulated plants		
Inoculum source	***C. griseus***	***C. prostratus***	***H. rhamnoides***
Cea1.3 (cell suspension)	3 / 0	4 / 0	12 / 0
Cea5.1 (cell suspension)	3 / 0	4 / 0	12 / 0
C. caeruleus crushed nodules	NA	NA	12 / 2
C. caeruleus rhizospheric soil	NA	NA	12 / 11
Uninoculated	2 / 0	2 / 0	6 / 0

Note: NA, not applicable.

PCR amplifications and cloning

Amplification of the 16S rRNA gene was performed using the universal bacterial primers 7f and 1510r (Lane 1991) in a GeneAmp PCR system 2400 (Perkin-Elmer Corp., Norwalk, CT, USA). Each reaction was done in a total volume of 100 μL, under the following conditions; PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 200 μM of each deoxynucleoside triphosphates, 3 mM $MgCl_2$, 0.15 μM of each primer, 0.025% W-1 detergent (w/v), 2.5 U Taq DNA polymerase (Life Technologies, Gaithersburg, MD, USA) and 1 μL template DNA. An

initial denaturation step of 4 min at 94°C was followed by 35 cycles of 45 s at 94°C, 45 s at 50°C and 2 min at 72°C, plus a final post-elongation step of 7 min at 72°C.

Amplification of *nif*H genes was done with primers $nifH_F$ and $nifH_R$ (Mirza *et al.* 1994b), using the same reaction conditions, except that the concentration of each primer was increased to 0.4 μM. In the PCR cycling program, the initial denaturation and final post-elongation times and temperatures were similar, but thermal cycling was extended to 40 cycles of 45 s at 94°C, 45 s at 52°C, and 1 min at 68°C.

Resulting amplicons were purified and concentrated using the QIAquick kit (Qiagen GmbH, Hilden, Germany), then ligated into pGEM-T vector (Promega, Madison, WI, USA), and cloned in *Escherichia coli* JM109 (Promega). Plasmid purification was done with the Wizard kit (Promega). All procedures were performed following the manufacturers' instructions.

The primers $Cea64_F$ (5'AGC GGA CCT TCG GGT CAG) and $Cea996_R$ (5'CCT AAG GAC CCA CCA TCT CTG) were designed to specifically amplify partial 16S rDNA from the isolates. Numbers in the primer names refer to their position in the standard *E. coli* numbering (Brosius *et al.* 1987). Reaction conditions and thermal cycling program were the same as those for 16S rDNA amplification. Specificity of the primers was tested with DNA templates from all isolates and clones from Table 3.2 (p 38), together with all the frankiae and non-actinorhizal strains depicted in Table 3.3 (p 41). DNA extracted from peeled and unpeeled nodule lobes from both *C. caeruleus* and *H. rhamnoides* was included as well (Table 3.3); these same templates were also used for TGGE.

Restriction Fragment Length Polymorphism analysis (RFLP)

To determine differences among isolates and cloned amplicons, and to avoid excessive sequencing, 16S rDNA-RFLP analysis was performed using 3 different restriction enzymes. Approximately 500 ng of each 16S rDNA-cloned fragment or PCR product from the isolates was restricted with each one of the following four-base cutter restriction enzymes; *Alu*I, *Hha*I and *Mbo*I (Life Technologies). Resulting fragments were separated on a 4% agarose gel (Boehringer Mannheim, Germany), and different RFLP groups were formed by visual comparison.

Sequencing

Clones from each RFLP group, containing almost the complete 16S rRNA gene were sequenced on both strands, using an automatic sequencer ABI 373A (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems), following the manufacturer's instructions. Similarly, direct sequencing of PCR products was performed on the amplified 16S rDNA from isolates and *nif*H from the endophyte.

Phylogenetic analysis

A gene databank similarity search was performed by using the algorithm FASTA (Pearson and Lipman 1988). Initial multiple sequence alignment was done with ClustalW program. After inspection, regions of uncertain alignment were removed to exclude them from the analysis.

Calculation of evolutionary distances was done by using the Jukes and Cantor (1969) model. Phylogenetic trees were constructed by the Neighbor-Joining method (Saitou and Nei 1987) with 100 bootstrap replicates, as implemented in the software package TREECON for Windows (van de Peer and de Wachter 1994). Maximum Parsimony trees were constructed with the program PAUP 3.1 (Swofford 1993).

Temperature Gradient Gel Electrophoresis (TGGE)

DNA extracted from peeled and unpeeled nodule lobes from *C. caeruleus* was used as template to amplify a fragment between co-ordinates 968-1401, comprising the V6-V8 variable regions of the 16S rDNA (Neefs *et al.* 1991). In addition, similarly extracted DNA, but from the *H. rhamnoides* nodules obtained during the nodulation tests, was used to further assess the specific presence of the two *Frankia* sequences in nodules from a different host plant.

PCR reactions and TGGE were done as described by Felske *et al.* (1996), with the following minor modifications: urea concentration of the polyacrylamide gel was increased to 8.5 M; ΔT was brought to 15°C (temperature gradient from 36°C to 51°C), and the electrophoresis running conditions were set at 120 V for 16 h. Fixation and silver staining of the gels was according to Sanguinetti *et al.* (1994).

The 16S rDNA sequences from the uncultured endophyte and cultured isolates Cea1.3 and Cea5.1 are available under the accession numbers U69265, U72717 and U72718, respectively. The *nif*H partial sequence of the endophyte is available under accession number U78306.

RESULTS

Isolation

Thirteen actinomycetous strains were isolated from surface-sterilised *C. caeruleus* root nodules. Microscopic examination revealed extensive hyphal formation, but no vesicles or sporangia typical of *Frankia* were observed. All isolates failed to grow on medium without combined N source, and acetylene reduction assays were also negative. Moreover, *nif*H genes were not detected by PCR amplification (data not shown). However, total DNA/RNA hybridisation with the 16S rRNA-targeted FP gave a clear positive signal with 4 out of 13 isolates, whereas three other isolates yielded weaker signals. Four 16S rDNA-PCR products of the endophytes also gave very strong signals (Table 3.2, p 38). Due to the very low growth rate exhibited by 4 of the original 13 strains, they were not included in any further analysis.

FAME analysis

In order to assess their relatedness, five of the FP-positive isolates and two negative ones were subjected to FAME analysis (Table 3.2). Profiles of the seven isolates were compared to approximately 40 FAME profiles from a created database, which included several *Frankia* strains and related actinomycetes. Principal component analysis of the profiles of isolates Cea1.3, Cea1.16, and Cea5.1, showed highest similarity to several *Frankia* strains, whereas the profiles of other four (Cea1.2, Cea1.12, Cea1.15 and Cea5.3) did not match any other FAME profile within the constructed data base. Moreover, when compared to larger commercial databases like ACTIN1 and TSBA (REV 3.80)(Microbial ID), the four *Ceanothus* isolates remained unidentified.

Table 3.2. Characterisation of 9 isolates and 5 cloned 16S rDNA amplicons from *C. caeruleus* root nodules using different approaches: Hybridisation with a genus-specific *Frankia* probe (FP), total cell fatty acid methyl ester analysis (FAME), determination of diaminopimelic acid (DAP) isomer present in the cell wall, and grouping by restriction fragment length polymorphism (RFLP) analysis. An (*) after the group number is used to point the member of each RFLP group that was sequenced.

Isolates and clones	*Frankia* probe	FAME analysis	DAP isomer	RFLP analysis			
				*Alu*I	*Hha*I	*Mbo*I	Group
			Isolates				
Cea1.2	+	no match	meso	C	B	ND	3
Cea1.3	++	*Frankia*	meso	D	A	A	4*
Cea1.11	+	ND	meso	A	A	A	1
Cea1.12	-	no match	meso	C	B	B	3
Cea1.15	-	no match	meso	C	B	B	3*
Cea1.16	++	*Frankia*	ND	A	A	A	1
Cea5.1	++	*Frankia*	meso	A	A	A	1*
Cea5.2	++	ND	meso	A	A	A	1
Cea5.3	+	no match	meso	A	A	ND	1
			Clones				
CloneA	++	NA	NA	B	A	A	2*
CloneB	++	NA	NA	B	A	A	2
CloneC	++	NA	NA	B	A	A	2
CloneD	-	NA	NA	E	C	C	5*
CloneE	++	NA	NA	B	A	A	2

Note: NA, not applicable; ND, not determined.

DAP analysis

Crude cell wall lysates of the isolates showed the presence of meso-DAP as a major distinguishing component of the peptidoglycan, which corresponds to a type III cell wall present in the Frankiaceae (Table 3.2).

Nodulation tests

Due to the limited germination rate of the *Ceanothus* seeds (< 2%), very few plants could be used for the nodulation tests. Sixteen weeks after inoculation none of the three plant species inoculated with the isolates developed nodules; uninoculated control plants did not form nodules either (Table 3.1, p 35). *H. rhamnoides* plants inoculated with *C. caeruleus* crushed nodules or rhizospheric soil suspensions elicited nodule formation after six weeks. The latter inoculum produced nodules in more than 90% of the plants (Table 3.1).

RFLP analysis

RFLP analysis of the 16S rDNA allowed the separation and grouping of the five endophyte-clones from the seven isolate-amplicons. Five RFLP groups were formed, two for the endophytes and three for the isolates. Selected members of each RFLP group were sequenced (Table 3.2). From the three restriction enzymes used, *Alu*I enabled to differentiate five distinct RFLP groups, whereas *Hha*I and *Mbo*I could resolve the same three groups.

Phylogenetic analysis

A preliminary FASTA search using partial sequences, showed that the cloned amplicon in cloneD (RFLP group 5) was a chloroplast-like sequence with 99% similarity over 565 nucleotides to *Ricinis communis* and to *Nicotiana plumbaginifolia* 16S rDNA from chloroplasts. Comparison of the 16S rDNA sequence of isolate Cea1.15 (RFLP group 3) gave highest score of 89.2% similarity to *Streptomyces mutabilis* and 88.6% to *S. ambofaciens* 16S rRNA genes over 1006 nucleotides. The closest *Frankia* 16S rDNA sequence showed only 85.4% similarity, suggesting a significant divergence.

More than 90% of the 16S rRNA gene was sequenced on both strands, for representatives of the remaining RFLP groups (Cea1.3, Cea5.1 and cloneA), these being the first nearly full length 16S rDNA sequences available for isolates and for an uncultured endophyte from *Ceanothus* actinorhiza. Sequence analysis of the two isolates showed a very close relatedness (99.8% similarity), with only three mismatches, in more than 1450 nucleotides. As signature positions, the isolate sequences have two deletions at co-ordinates 68-69 and 83-84 based on the *E. coli* numbering (Brosius *et al.* 1987). FASTA comparison gave several *Frankia* 16S rDNA sequences as highest scores, ranging from 95.5 to 96.8%. Because the available partial sequences (930 nucleotides) of isolates Cn7 and Dc2 (Mirza *et al.* 1992 and 1994c) were within this range, their complete 16S rDNA sequence was determined, to make them comparable.

The cloned 16S rDNA sequence of the endophyte, was 99.7% identical to an uncultured *Frankia* symbiont in *Dryas drummondii* root nodules, with only 4 mismatches in 1432 nucleotides. It appeared also to be very similar to other uncultured endophytes.

Phylogenetic dendrograms constructed by both distance and parsimony methods showed essentially the same topology and the two main clusters formed were conserved in nearly all trees. The phylogenetic trees were constructed using in most cases 1410 nucleotides from several sequences within the Frankiaceae and related genera *Dermatophilus congoliensis* was included as outgroup (Figure 3.1).

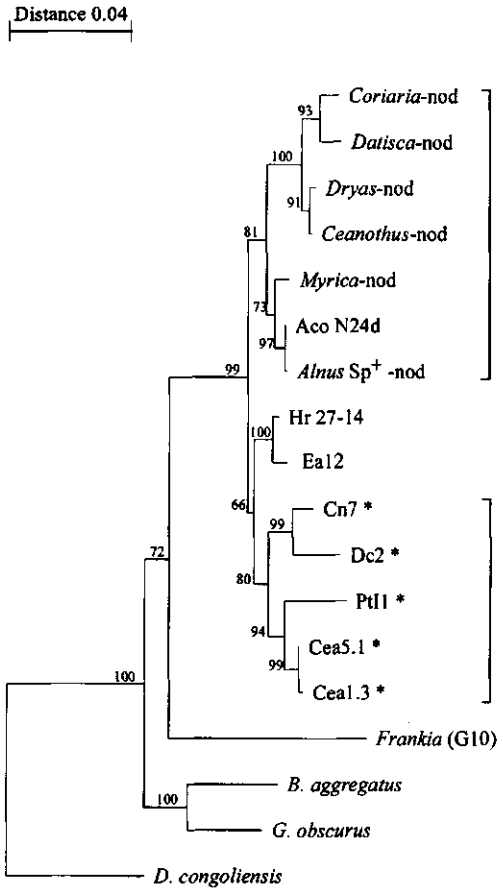

Figure 3.1. Neighbor-Joining phylogenetic tree showing the two main clusters formed; uncultured endophytes and Nod$^-$/Fix$^-$ isolates separated by *Frankia* sequences belonging to Nod$^+$/Fix$^+$ strains. Numbers to the left of the branches correspond to bootstrap values out of 100 replicates. All names followed by the suffix "-nod" refer to endophytes that were sequenced without cultivation, and those with an " * " correspond to Nod$^-$/Fix$^-$ isolates.

Accession numbers and description of the used sequences are: *Coriaria*-nod (*C. nepalensis*, L18981); *Datisca*-nod (*D. cannabina*, L18979); *Dryas*-nod (*D. drummondii*, L40616); *Myrica*-nod (*M. nagi*, L40622); *Alnus Sp*$^+$-nod (*A. rugosa*, L40956); Aco N24d is an *A. cordata* Nod$^+$ isolate (L40610); Hr 27-14 is an *Hippophae rhamnoides* Nod$^+$ isolate (L40617); FE-Ea12 is an *Elaeagnus* sp. Nod$^+$ isolate (L40618); Cn7* (*Coriaria nepalensis*, L18982); Dc2* (*Datisca cannabina*, L19978); Ptl1* (*Purshia tridentata*, L41048); *Frankia* (G10) (non-actinorhizal isolate, X92365); *Blastococcus aggregatus* (L40614); *Geodermatophilus obscurus* subsp. *dictyosporus* (L40621); and *Dermatophilus congoliensis* (L40615).

Nif*H genes*

FASTA comparison of 555 nucleotides from sequenced PCR products of the endophyte *nif*H gene, yielded similarity values of 97.1 and 94.3% to homologous genes of other uncultured symbionts in *C. nepalensis* and *D. cannabina* root nodules (Mirza *et al.* 1994b). Similarity with the *nif*H of *Frankia* strains ArI3 and HRN18a (Normand *et al.* 1988, Normand and Bousquet 1989) was 86.3 and 86.5%, respectively. Translation of the partial gene sequences gave corresponding result. At the amino acid level, similarity values were 97% for both uncultured symbionts, and 89% with strain ArI3.

TGGE

The TGGE banding pattern profiles (Fig. 3.2, p 42) from peeled and unpeeled *C. caeruleus* nodule lobes showed significant differences. Unpeeled nodules gave more complex profiles (lanes 3 - 6) showing two to five extra bands, the most prominent ones being those migrating to the same position as the corresponding bands of the endophyte (lanes 1, 10), and the isolates; Cea1.3 (lanes 2, 9) and Cea5.1 (lane 11). When the nodule periderm was removed (lanes 7, 8), the bands regarded as belonging to the isolates and other unidentified bacteria were not present, indicating that the microorganisms bearing those 16S rDNA sequences were localised either in the outer surface of the nodule or just below the periderm. Peeling of the lobes could have removed some cortical tissue as well.

In Fig. 3.3 (p 42), similar TGGE profiles, but for *H. rhamnoides* nodule lobes obtained after inoculation with suspensions of *C. caeruleus* rhizospheric soil (lanes 5 - 8) or crushed nodules (lane 4) resulted in a slightly different picture. Only the unpeeled sample in lane 4 showed the same specific band positions as those corresponding to the isolates (lanes 1, 2, 10) and the endophyte (lanes 3, 9), plus some additional unidentified bands. The profiles for unpeeled (lanes 5, 6) and peeled (lanes 7, 8) nodule lobes did not show strong differences among them, because in all four cases, the two main bands were conserved, suggesting that peeling did not remove the microorganisms responsible for those bands. This could be attributed to the relatively easy-to-peel *H. rhamnoides* nodules; for these only periderm was removed.

Specific PCR amplification

Design of primer $Cea64_F$ took advantage of the deletions found in the V1 region of the 16S rDNA from the isolates. This primer later appeared to be specific for the Nod$^+$/Fix$^+$ cluster, since a positive PCR product of the expected size was obtained with all the *Ceanothus* isolates from Table 3.2 (p 37), but the result was negative when any of the clones (Table 3.2) was used as target. Interestingly also the non-infective isolates Cn3, Cn7 and Dc2 from Table 3.3 gave a similar product, while the other tested microorganisms yielded negative results. As expected, the DNAs extracted from *C. caeruleus* and *H. rhamnoides* nodule lobes, which have shown on TGGE the corresponding isolate band or a very close one, also gave a positive PCR reaction (see Table 3.3).

Table 3.3. Specificity of PCR primers $Cea64_F$ - $Cea996_R$ was checked with DNA from pure cultures of *Frankia* (*Fr*) strains derived from different actinorhizal plants, and two actinomycetes, *Streptomyces coelicolor* (*S. coeli.*) and *Saccharopolyspora erythraea* (*S. eryth.*). Nodule-extracted DNA refers to the DNA templates used for TGGE which were also tested with the primers.

Host	Source of DNA	Nod type	PCR product	Reference
	Pure culture-extracted DNA			
Alnus sp	*Frankia* ArI3 (HFP 013003)	Nod^+	-	Berry & Torrey 1979
Alnus sp	*Frankia* AgKg84/4	Nod^+	-	Hahn 1990
Alnus sp	*Frankia* Ag45/Mut15	Nod^+	-	Hahn *et al.* 1988
Casuarina sp	*Frankia* BR (ORS 020608)	Nod^+	-	Müller *et al.* 1991
Casuarina sp	*Frankia* D11 (ORS 020602)	Nod^-	-	Gauthier *et al.* 1981
Elaeagnus sp	*Frankia* EAN1pec (ULQ 130100144)	Nod^+	-	Lalonde *et al.* 1981
Hippophaë sp	*Frankia* HrI1 (DDB 140110)	Nod^+	-	Baker unpublished
Coriaria sp	*Frankia* Cn3	Nod^-	+	Mirza *et al.* 1992
Coriaria sp	*Frankia* Cn7	Nod^-	+	Mirza *et al.* 1992
Datisca sp	*Frankia* Dc2	Nod^-	+	Hafeez 1983
Non actinorhizal	*Streptomyces coelicolor* A3(2)	NA	-	Hopwood 1959
Non actinorhizal	*Saccharopolyspora erythraea* (NRRL 2338)	NA	-	Labeda 1987
	Nodule-extracted DNA			
Ceanothus caeruleus	Unpeeled, same as Fig 2, lane 4	NA	+	This study
Ceanothus caeruleus	Unpeeled, same as Fig 2, lane 6	NA	+	This study
Ceanothus caeruleus	Peeled, same as Fig 2, lane 7	NA	-	This study
Ceanothus caeruleus	Peeled, same as Fig 2, lane 8	NA	-	This study
H. rhamnoides	Unpeeled, same as Fig 3, lane 4	NA	+	This study
H. rhamnoides	Unpeeled, same as Fig 3, lane 5	NA	+	This study
H. rhamnoides	Peeled, same as Fig 3, lane 7	NA	+	This study
H. rhamnoides	Peeled, same as Fig 3, lane 8	NA	+	This study

Note: NA, not applicable.

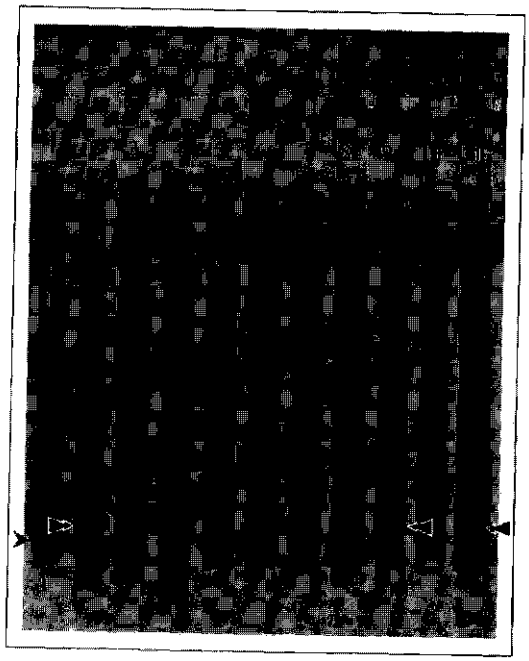

Figure 3.2. TGGE profiles from periderm-peeled and unpeeled Ceanothus caeruleus nodule lobes bands comparable to those of the effective endophyte (marked with single-arrowheads in lanes 1, 10) are present in all nodular samples (lanes 3-8), while the specific bands corresponding to the Nod$^-$ /Fix$^-$ isolates (lanes 2, 9, 11, marked with double arrowheads), are present in most of the unpeeled nodule lobe samples (lanes 3-6), but not in the peeled ones (lanes 7-8).

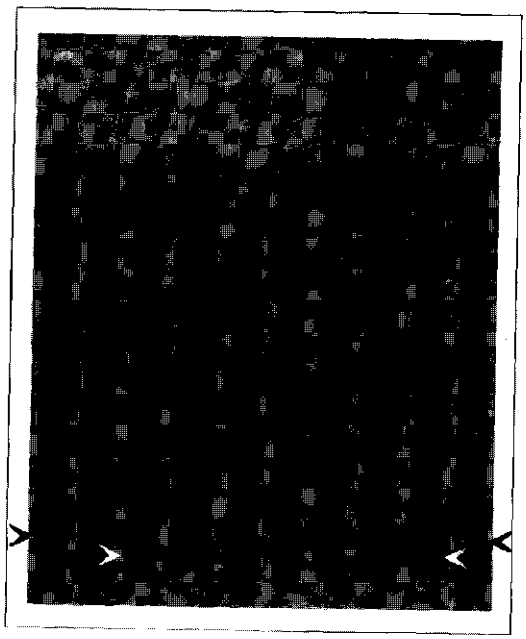

Figure 3.3. TGGE profiles from periderm-peeled (lanes 7, 8) and unpeeled (lanes 4-6) *Hippophae rhamnoides* nodule lobes. Only the sample in lane 4 corresponding to a nodule obtained after inoculation with *C. caeruleus* nodular suspension, has band positions comparable to the reference bands of the *C. caeruleus* endophyte (lanes 3, 9, white arrow heads) and the isolates (black arrow heads) Cea1.3 (lanes 2, 10) and Cea5.1 (lane 1).

DISCUSSION

Three separate attempts to isolate the endophyte from *C. caeruleus* root nodules were performed using the sucrose-gradient method (Baker and O'Keefe 1984). The resulting isolates were *Frankia*-like organisms, which grew from almost all plated material. Care was taken to avoid contaminant soil microorganisms present on the nodule surface.

The isolated actinomycetous strains lacked morphological features of the genus *Frankia,* such as multilocular sporangia and vesicles. A similar observation has been reported previously by Lechevalier and Ruan (1984) for *Frankia* strains LLR03011 and LLR03014, isolated from *C. americanus*.

A preliminary molecular screening of the isolates with a *Frankia* genus-specific probe (Hahn *et al* 1990b) pointed out that some of the isolates bear *Frankia*-16S rDNA sequences, stressing the need for further characterisation.

FAME analysis has been successfully applied in the identification of several microorganisms, among others *Pseudomonas* (Janse 1991a, b), and also *Frankia* strains lacking characteristic features (Mirza *et al.* 1991). Application of FAME and principal component analysis resulted in grouping of isolates Cea1.3, Cea1.16 and Cea5.1, together with other members of the genus *Frankia*. The fact that all three isolates have meso-DAP in their cell wall (Table 3.2) supported further their close relatedness to that genus.

The use of RFLP-based analysis to assess the genetic diversity of *Frankia* strains has proven effective (Simonet *et al.* 1989, Nazaret *et al.* 1989, Jamann *et al.* 1993). Restriction pattern differences between the isolated strains and the clones (Table 3.2, p 38), supported the idea that we did not isolate the real endophyte, but a co-existing organism, as was already supposed by the inability of the isolates to fix nitrogen. Even more, PCR amplification of *nif*H gene was only possible using nodule-extracted DNA as template but always gave a negative result with DNA extracted from the isolated strains.

Solidity of the Neighbor-Joining phylogenetic tree depicted in Fig. 3.1 (p 40) is well supported by high bootstrap values in most branches, although the sequences of *Datisca*-nod and *Coriaria*-nod (endophytes from *D. cannabina* and *C. nepalensis*) are only 925 bases instead of the 1410 used in all other cases. The two main clusters formed, uncultured endophytes and Nod$^-$/Fix$^-$ isolates, are in agreement with a previous description by Normand *et al.* (1996), on their comprehensive work on *Frankia* phylogeny.

Within the endophyte cluster, the strongest lineage with 100% bootstraps, is formed by uncultured symbionts from the AP families Coriariaceae, Datiscaceae, Rhamnaceae (*Ceanothus*) and Rosaceae (*Dryas*); this lineage is also present in the four most parsimonious trees (data not shown). This close relatedness is also reflected by the high similarity values, above 94%, of the partial *nif*H sequences from the uncultured endophytes in *C. caeruleus, C. nepalensis* and *D. cannabina.* Accordingly, the Nod$^-$/Fix$^-$ cluster has also isolates representing the same above-mentioned AP families, i.e. the strains Cn7 (*Coriaria*), Cea1.3 and Cea5.1 (*Ceanothus*) which were isolated from the same nodules as the corresponding uncultured endophytes. These facts support further the idea that the actinorhizal nodules in these plants are

commonly inhabited by two members of the family Frankiaceae belonging to two different and well-defined lineages.

FAME, DAP, and 16S rDNA sequence analyses showed the close relatedness and putative inclusion of the *Ceanothus*-isolates in the genus *Frankia*. However, the taxonomic position of our isolates and the whole Nod^-/Fix^- cluster has to be reconsidered under a polyphasic approach, in order to establish its actual position within the Frankiaceae.

Benson *et al.* (1996), have suggested that the endophyte diversity within members of the Rhamnaceae (*C. griseus*) and Rosaceae (*Purshia tridentata* and *Dryas drummondii*) was low, as they have found among these endophytes identical 16S rDNA partial sequences. This is confirmed by our findings using almost complete 16S sequences; the *C. caeruleus* endophyte sequence is nearly identical (99.7%) to that of *Dryas* uncultured endophyte. Alignment of these last two sequences with those of Benson *et al.* (1996) resulted in identical stretches of 390 bases. It is remarkable that both *Ceanothus* endophytes have a very distant geographic origin, coming from Mexico and New Zealand, the latter being an introduced species grown in greenhouse.

TGGE allowed to identify specific band positions related to the presence in the nodule of the Fix^+ uncultured endophyte and the Fix^- isolate. Because the band characteristic of the isolate was not observed after peeling of the *C. caeruleus* nodule lobes (Fig. 3.2, lanes 7-8, and Table 3.3), and because no microbial growth was observed in P+N liquid medium inoculated with similarly surface-sterilised nodule lobes, we assume that these types of Nod^-/Fix^- isolates inhabit the surface layers of nodule epidermis as co-symbionts or contaminants, where they can escape the disinfecting procedures (Mirza *et al.* 1994b).

In the TGGE profiles of the *H. rhamnoides* nodules (Fig. 3.3) produced with rhizospheric soil inoculum (lanes 5 - 8), the two main bands appreciated did not match exactly with the position of the reference bands for endophyte (lanes 3, 9) and isolates (lanes 1, 2, 10). However, both bands migrated to very close positions, which might indicate high sequence homology and therefore phylogenetic relatedness. Although the identity of the two microorganisms bearing those sequences has not been fully established, it is likely they are related to both inhabitants in *C. caeruleus* root nodules.

Like other *Ceanothus* isolates, the strains Cea1.3 and Cea5.1 were unable to nodulate plants within the same genus, nor were they able to nodulate the promiscuous host *H. rhamnoides*, even though the latter plant was reported as being nodulated by strain R2 isolated from *C. americanus* (Baker 1987). However, the presence of the Nod^-/Fix^- isolates could be demonstrated by PCR (Table 3.3) and TGGE (Fig. 3.3, lane 4), in *H. rhamnoides* nodules induced by *C. caeruleus* nodular suspensions. As mentioned above, closely related signals for endophyte and co-symbiont were similarly detected in nodules produced with rhizospheric soil, indicating that this group of endophytes can also infect *Hippophae* plants, with the presence in all cases of co-symbiotic microorganisms like the isolates or other organisms closely related to them. This group of phylogenetically related Nod^- and/or Fix^- *Frankia*-like strains may play a role separate from nodule contaminants, as they appear to be commonly present in the *Ceanothus* (this study) and in other actinorhizal symbiosis, i.e. *Coriaria* (Mirza *et al.* 1992) and *Datisca* (Hafeez 1983, Mirza *et al.* 1994c).

TGGE is a valuable tool to investigate *Frankia* diversity in actinorhizal root nodules, avoiding the sometimes non-attainable step of isolation. The high resolution achieved by this technique is sequence-specific (Rosenbaum and Riesner 1987). As an example, the electrophoretic separation of the endophyte and isolates bands is due to 17 unevenly distributed substitutions in similar 478 bp amplicons. Bands corresponding to both isolates could not be resolved under the conditions used, as they have only one base difference in the amplified region.

The presence in nodules and soil of Fix^-/Nod^- strains like those described in this paper, may have been largely underestimated, as many of these inconspicuous strains do not show the morphological or physiological features corresponding to those of the Fix^+/Nod^+ *Frankia*. Descriptions of nodule and soil borne actinomycetes, harbouring *Frankia*-related 16S rDNA sequences (Niner *et al.* 1996, Eppard *et al.* 1996), have already pointed out that diversity within the Frankiaceae might be larger than suspected. Application of powerful detection techniques as TGGE, in combination with cloning and sequencing, may help to unveil this hidden diversity.

Acknowledgements

Appreciation is expressed to Dr. M.E. Trujillo for assistance with DAP analysis. This work was in part supported by a fellowship of CONACyT to H.R.S. and financial support through the EC projects STD2.TS2-CT90-0322 and High Resolution Automated Microbial Identification (EC-HRAMI project BIO2-CT94-3098).

CHAPTER 4

*BACTERIAL COMMUNITY CHANGES AND ENRICHMENT OF UNCULTURED *BURKHOLDERIA*-LIKE BACTERIA INDUCED BY CHLORINATED BENZOATES IN A PEAT-FOREST SOIL

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* submitted for publication.

SUMMARY

Bacterial community shifts in a peat-forest soil spiked with 3-chlorobenzoate (3CBA) or 2,5-dichlorobenzoate (2,5DCB) were monitored by PCR-amplification of the V6 to V8 regions of the 16S rRNA and rDNA, followed by separation of the amplicons by temperature gradient gel electrophoresis. 3CBA disappeared to non-detectable levels within 15 days by a biologically mediated process, while 2,5DCB remained at the initial concentration values. Addition of the chlorinated benzoates to the soil resulted in a rapid decrease of the microbial diversity, as judged by a time-dependent reduction in the number of resolved amplicons after temperature gradient gel electrophoresis. In addition, a specific soil-enrichment of several amplicons was detected, that were cloned and characterised by sequence analysis. The identity of the cloned DNA and the corresponding soil amplicons was confirmed by hybridisation with a radioactively labelled V6-probe. Analysis of the 16S rDNA sequences indicated that *Burkholderia*-related bacteria composed the enriched soil populations under 3CBA stress. Enrichment cultures growing on 3CBA as sole C-source were obtained from the respective spiked soil, which were found to contain bacteria with identical 16S rDNA sequences as those induced by 3CBA stress in soil.

INTRODUCTION

The chlorinated benzoates 2,5-dichlorobenzoate (2,5DCB) and 3-chlorobenzoate (3CBA), can be found in soil, sediments and water mainly as by-products of the partial degradation of the man-made polychlorinated biphenyls, which are widely used in industrial and agricultural applications (Focht 1993). Currently, there is a wealth of bacteria that are able to degrade chlorobenzoates under different physiological conditions (Brunsbach and Reineke 1993, 1994, Chaudhry and Chapalamandugu 1991, Krooneman *et al.* 1996, van der Woude 1996). Most of these bacteria have been isolated by selecting for their degrading abilities *in vitro*, but it is uncertain whether these bacteria were predominant or even active in their original habitats. Moreover, little attention has been paid to the ecological impact of chlorinated benzoates on the soil microbial community of natural environments.

It has been widely accepted that with the current microbiological techniques only a minor fraction of all the bacteria present in the environment can be cultured. Therefore, considerable attention has been given to assess bacterial diversity by using molecular methods mainly based on PCR-amplification and analysis of the 16S rRNA (Ward *et al.* 1992). This approach was recently complemented by separation of 16S rDNA- or rRNA-based amplicons with the temperature gradient gel electrophoresis (TGGE), or the equivalent technique denaturing gradient gel electrophoresis (DGGE). The combination of these approaches was found to be effective for analysing complex microbial communities (Felske *et al.* 1998c, Heuer and Smalla 1997, Muyzer *et al.* 1993, Zoetendal *et al.* 1998), detecting uncultured bacteria in natural environments (Felske and Akkermans 1998, Felske *et al.* 1997, Kowalchuk *et al.* 1997), and monitoring the isolation of pure cultures (Teske *et al.* 1996).

The objective of this work was to assess the changes in the bacterial community of a peat-forest soil upon addition of two chlorinated benzoates, 2,5DCB or 3CBA. This was realised by using the 16S rRNA and its encoding gene as molecular markers in combination with the TGGE profiling technique, in order to monitor shifts in the composition of the soil bacterial community, direct the isolation attempts to cultivate the predominant bacteria involved in those changes, and specifically address the phylogenetic relationships of these bacteria.

MATERIALS AND METHODS

Soil sampling

Soil samples were collected during the summer of 1997 from a natural black alder (*Alnus glutinosa*) wet stand at the nature reserve "de Hel", Veenendaal, the Netherlands. A description of the site and physico-chemical properties of the peat soil were reported previously (Wolters *et al.* 1997). Four soil samples (ca. 1 kg each) free of stones and large plant material were taken from different sites, the samples were pooled and stored at 4°C until their use, within the following week.

Microcosms experiments

A fraction of the collected soil was partially air dried to lose 15% (w/v) of the total water content, then three equal samples were taken and reconstituted to the original 45% humidity by adding: a) sterile water to the control treatment, b) 2,5DCB sterile solution to a final concentration of 0.4 μmoles 2,5DCB g^{-1} soil, and c) 3CBA sterile solution resulting in a final concentration of 0.4 μmoles 3CBA g^{-1} soil. The water and the solutions of chlorinated compounds were added to the soil in small aliquots and mixed thoroughly. Two replicate sterile petri dishes filled with 120 g of each treated soil were used as microcosm systems. Soil samples were taken after 0, 5, 10 and 15 days for the HPLC analysis, DNA and rRNA extraction. The petri dishes were incubated in the dark at a cycling temperature of 24/16°C for 16/8 h, respectively, and opened daily under sterile conditions to allow air exchange. Water content was checked every second day and adjusted when necessary.

HPLC analysis for quantification of 2,5DCB and 3CBA

Soils samples were extracted with methanol:water (1:1 v/v) by mixing equal weights of soil and extraction mix in an orbital shaker at 300 rpm for 14 h. The resulting slurry was centrifuged for 10 min at 10,000 X *g*. This extraction procedure resulted in an efficiency around 85%, which was the highest chlorobenzoate recovery reached with this soil type. Aliquots of the supernatants were analysed for 2,5DCB and 3CBA in a HPLC Spectra System (ThermoQuest, Breda, the Netherlands) equipped with a ChromoSphere C_8 reversed-phase column (Chrompack, Middelburg, the Netherlands, Cat No. 28362). The mobile phase was acetonitrile: 0.01 N H_2SO_4 at a volume ratio of 35:65, and a flow rate of 0.6 ml min^{-1}. Under these conditions the retention times for 3CBA and 2,5DCB were 5.1 min and 5.7 min, respectively. UV-absorbance of both compounds was measured at 206 nm.

The cultures used for most probable number (MPN) estimations and the enrichment cultures obtained from them (see below) were centrifuged as described above, and aliquots of the supernatant were similarly analysed to determine residual concentrations of the chlorinated compounds.

DNA and rRNA extraction

DNA and rRNA obtained from isolated ribosomes were extracted simultaneously from soil bacteria as described previously (Felske *et al.* 1998b). The protocol yields two fractions, one was treated to obtain DNA, and the other fraction yielding rRNA was DNase digested, both fractions were resuspended in 100 μl of an appropriate buffer. DNA extraction from enrichment cultures growing on 3CBA as only C source was done by bead beating followed by phenol-chloroform extraction (Mirza *et al.* 1994c).

Temperature gradient gel electrophoresis of PCR and reverse transcriptase (RT)-PCR amplicons

Soil-extracted DNA and rRNA were used as template (1 µl) in PCR and RT-PCR amplifications, respectively, in combination with the bacterial specific primers U968/GC and L1401 (Nübel *et al.* 1996), under previously described conditions (Felske *et al.* 1997). The resulting amplicons were approximately 470 bp (including a 40-mer GC-clamp) (Muyzer *et al.* 1993), and comprised the V6 to V8 variable regions of the 16S rRNA (Neefs *et al.* 1990). Separation of these amplicons was performed in a TGGE system (Diagen, Düsseldorf, Germany). Polyacrylamide gel preparation and electrophoresis running conditions were done as described previously (Felske *et al.* 1998c), except that the temperature gradient was set to range from 38°C to 49°C. Fixation and silver staining of the gels was according to Sanguinetti *et al.* (1994).

Cloning and screening of PCR-amplicons

DNA extracted from the enrichment cultures was used as target to amplify the nearly full-length 16S rRNA gene by using primers 7f and 1510r (Lane 1991). PCR amplification, purification and cloning of the amplicons were done as reported elsewhere (Ramírez-Saad *et al.* 1998).

DNA from randomly selected clones was used as template for PCR-amplification of the V6 to V8 regions. TGGE separation of these amplicons was done in parallel with similar amplicons obtained from the 3CBA treated soil and the enrichment cultures. By this means of screening, the PCR products with identical V6 to V8 sequences were related by their comparable TGGE mobility.

The identity of matching bands was confirmed by transferring the DNA of a non-stained TGGE-gel to a filter that was hybridised with [γ-^{32}P]ATP-labelled, clone-specific V6-targeted probes (Felske *et al.* 1997, Heuer and Smalla 1997). The distribution of the radioactive signal was determined using a Phosphor screen (Molecular Dynamics, Sunnyvale, Cal.), and visualised with a STORM 815 (Molecular Dynamics) scanning and imaging system.

Reamplification of a specific sequence from a silver-stained TGGE band

PCR amplification of a specific sequence was performed by cutting the band of interest immediately after the developing and prior to the fixation steps of the silver staining protocol. The polyacrylamide piece was transferred to an Eppendorf tube, completely covered with approximately 100 µl of TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA), and then shaken for 20 min after which the buffer was changed; this step was repeated three times. After removing the supernatant the gel piece was macerated, and 100 µl TE buffer was added. The suspension was incubated for 2 h at 4°C, and briefly centrifuged to pellet the gel fragments. Two µl of the supernatant was used as template in PCR-amplifications, and the resulting V6 to V8 amplicons were cloned and screened on TGGE as described above.

Cultivation and enumeration of chlorobenzoate-degrading bacteria

Soil microorganisms were extracted from the original unspiked soil sample, and also from the soils that have been incubated for 15 days in the presence or absence of chlorobenzoates. The extraction procedure was done by mixing 5 g of soil with 45 ml 0.1% (w/v) sterile sodium pyrophosphate solution and 5 g sterile glass beads (3 mm diameter). The mixture was shaken for 15 min and allowed to settle down for 10 min; the supernatant was used as soil extract. Serial dilutions of the spiked soil extracts were plated in aerobic conditions, in low-chloride mineral salts medium (LMM) (Gerritse and Gottschal 1992), containing 2.5 mM of either 3CBA or 2,5DCB, as only energy and C-source, while the control soil extracts were plated on both 3CBA or 2,5DCB-amended media.

Alternatively, a MPN approach was used to obtain enrichment cultures and to address the numbers of chlorobenzoate-degrading microorganisms present in the treated soils. Triplicate series of LMM-tubes amended with the respective chlorinated compound were inoculated with aliquots of the serial dilutions. After seven days of incubation at 26°C, the tubes with the highest dilution level showing growth were HPLC-analysed to monitor chlorinated compounds degradation, and scored for MPN determinations. A tube was scored positive when the residual concentration of chlorinated compound was equal or less than 0.5 mM. Aliquots of the most-dilute positive tubes were plated on LMM amended with either of the chlorobenzoates, or enriched by successively transferring 10% of a well-grown culture to fresh liquid medium.

Sequencing and phylogenetic analysis

The cloned 16S rDNA amplicons obtained from plasmid minipreps (Wizard kit, Promega, Madison, Wisc.) were sequenced in both directions by using a Sequenase T7 sequencing kit (Amersham, Slough, United Kingdom), with Infra-Red Dye 41 labelled primers (MWG-Biotech, Ebersberg, Germany), following manufacturers' instructions. The sequencing reactions were run in a 4000L automated sequencer (Li-Cor, Lincoln, Neb.).

Gene data banks search was performed with the improved BLAST tool (Altschul *et al.* 1997). Sequence alignment took into consideration both the primary structure and the putative secondary structure of the 16S rRNA, as implemented in the ARB software package (Ludwig and Strunk 1997). Phylogenetic distance calculation was done according to Jukes and Cantor (1969), unrooted phylogenetic trees were constructed by the Neighbor-Joining method (Saitou and Nei 1987), using options available within the same ARB software package. The accesion numbers (in parenthesis) of the sequences used for tree construction are: *Burkholderia* sp. strains N2P5, N2P6, N3P2 (U37342 to U37344), *Burkholderia* sp. strain JB1 (X92188), *B. caryophylli* (X67039), *B. gladioli* (X67038), *B. andropogonis* (X67037), *B. cepacia* group (M22467, X87275, X87284, X87286, X87287), *Pseudomonas* sp. (U86373), *Alcaligenes* sp. (L31650), *Ralstonia eutropha* (M32021).

The determined sequences were deposited in GeneBank under the accession numbers: AF074711 to AF074713.

RESULTS

Fate of chlorinated compounds added to soil

Peat soil samples were amended with either 2,5DCB or 3CBA to approximately 0.4 μmol g^{-1} soil, and the fate of the added chlorinated benzoates was followed in time. The amount of soil-extractable 2,5DCB remained rather constant during the experiment, while the values of 3CBA decreased to almost non-detectable concentrations after 15 days (Figure 4.1). Autoclaved soils similarly spiked with the chlorinated benzoates did not show any significant reduction of the initial values after two weeks (data not shown).

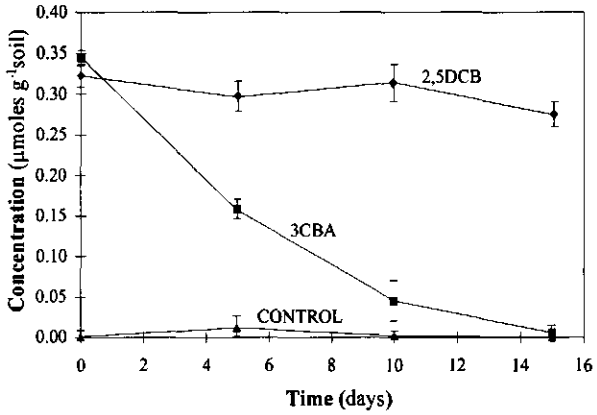

Figure 4.1. Kinetics of 2,5DCB and 3CBA in the soil microcosm experiment at 5-day intervals. Each point represents the mean values of two replicate microcosm systems, and the bars indicate the standard deviation.

Soil-TGGE profiles

To characterise and compare the bacterial communities from the control and the chlorobenzoate-spiked soils, the respective microcosm systems were sampled at different times, and the soil samples processed to obtain V6 to V8 amplicons, which were further separated by TGGE (Figure 4.2). The TGGE-banding profiles of the control (non-spiked) soil remained quite constant in time, while the profiles of the 2,5DCB- and 3CBA-spiked soils showed a strong reduction in time in the number of bands. However, the most noticeable features in the profiles from the chlorobenzoate-spiked soils are the bands appearing with increasing intensity in time, marked U(upper), M(middle), and L(lower) (Figure 4.2). The U band in the 2,5DCB profiles is the most conspicuous after 10 and 15 days, whereas in the 3CBA banding patterns, two other bands marked M and L were also prominent. Comparable prominent bands were also detected in the RT-PCR profiles derived from the spiked soils sampled after 15 days, indicating a high expression of rRNA.

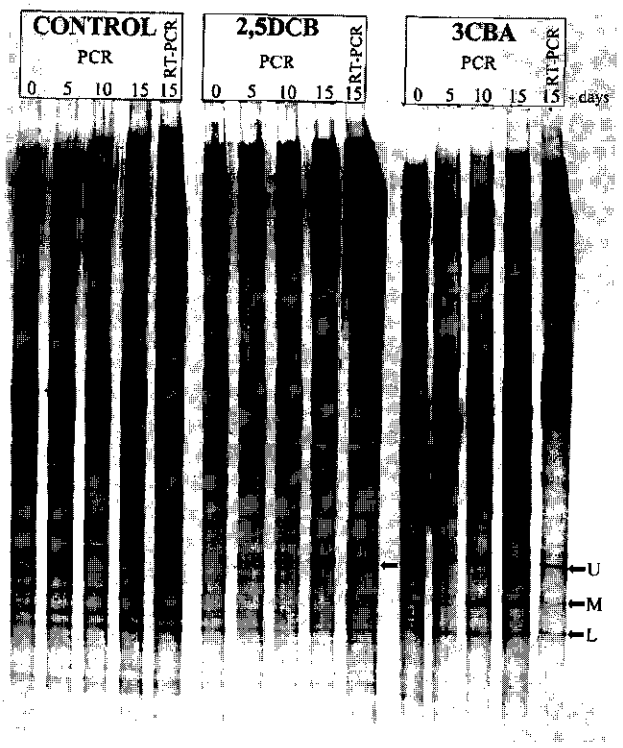

Figure 4.2. TGGE of V6 to V8 rDNA amplicons obtained from the control and the chlorobenzoate-spiked soils. The RNA-based (RT-PCR) profiles included for each treatment showed similar banding patterns as the respective DNA-based (PCR) profiles. The prominent bands U, M, L, are indicated, which were subjected to further analysis.

Cultivation and enumeration of chlorobenzoates-degrading bacteria

Initial trials to isolate either 2,5DCB- or 3CBA-degrading bacteria from the original unspiked soil were not successful. Similar results were obtained with other attempts aimed to isolate such microorganisms from the chlorobenzoate-amended soils. Since 2,5DCB was not degraded in the spiked soil and no enrichment on 2,5DCB-containing media was obtained, further cultivation from this source was not attempted. However, successful enrichments were obtained from the 3CBA-amended soils. The amount of bacteria able to grow in the presence of 3CBA was addressed by MPN counting. Estimation at 95% confidence from two parallel dilution series yielded 8.5 X 10^4 and 1.8 X 10^5 bacteria g^{-1} soil. Additional attempts to cultivate the 3CBA-degraders relied on enrichment of the most diluted positive tubes from both series. After several successive transfers, two enrichment cultures were obtained able to degrade about 80% of the added 3CBA in only 3 days, in contrast to the 7 days needed by the original MPN-cultures to metabolise the same amount of 3CBA.

Sequencing and phylogenetic analysis

Further characterisation of the bacteria present in the 3CBA-degrading enrichment cultures, obtained from the parallel dilution series, was done by analysing their 16S rRNA genes. DNA was extracted from the both enrichment cultures and used as template in two types of PCR amplifications. One reaction was intended to amplify the nearly full length 16S rDNA (primers 7f - 1510r), the obtained products were cloned yielding two clone libraries from the corresponding parallel enrichment cultures. The other reaction was targeting the V6 to V8 regions of the 16S rDNA (primers U968/GC - L1401), and these amplicons were separated by TGGE to address the bacterial composition in the two enrichment cultures (Figure 4.3). Similarly, V6 to V8 amplifications of 15 randomly selected clones from each library allowed TGGE-screening and further characterisation of those clones yielding a band that matched the prominent U, M or L bands in the 3CBA-treated soil profiles.

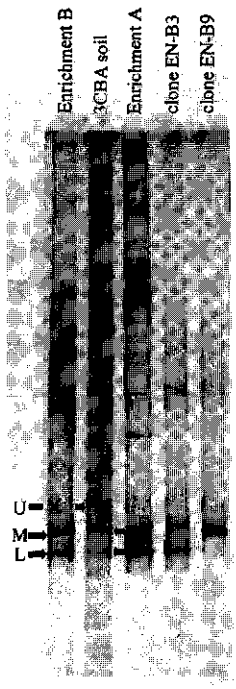

Figure 4.3. TGGE of V6 to V8 rDNA amplicons of the 3CBA-treated soil, the 3CBA-degrading enrichment cultures, and the clones (EN-B3 and EN-B9) obtained from one of the cultures. The bands marked by arrowheads are comparable to those on Fig. 4.2.

The obtained 3CBA-degrading enrichment cultures showed different TGGE profiles. From the prominent arrowhead marked bands only L was common to both of them. In addition, enrichment A had two major bands localised in the upper part of the profile, which were not detected in the 3CBA-spiked soil (Figure 4.3). Hence they were excluded from further analysis.

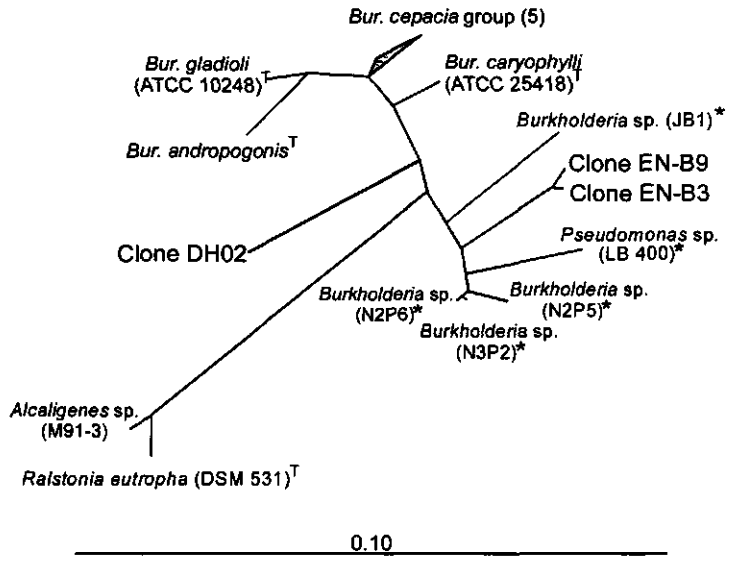

Figure 4.4. Unrooted phylogenetic tree showing the relations of the environmental clone from de Hel soil (DH02), and the clones EN-B3 and EN-B9 from a 3CBA-degrading enrichment culture, with other microorganisms belonging to the β-subclass of Proteobacteria. The dendrogram is based on 429 nucleotides comprising the V6 to V8 variable regions of the 16S rDNA, which is also the analysed sequence length of clone DH02. Those microorganisms marked with an "*" have been reported to degrade aromatic xenobiotic compounds. The bar represents one base substitution per each 10 nucleotides.

Comparison of the V6 to V8 amplicons obtained from the clone libraries showed that 18 of 30 clones yielded an amplicon migrating at the same position as band L (Figures 4.2, 4.3), indicating a predominance of the bacteria harbouring this 16S rDNA sequence. Two clones comparable to the band marked M were present in only one of the libraries (Figure 4.3). The remaining 10 clones migrated to three different positions and did not match with any prominent band within the 3CBA soil profiles.

Three clones were selected for nucleotide sequence analysis. Two of them (clones EN-A5 and EN-B3) that matched the band L, were obtained independently from each of the libraries. The other clone (EN-B9) had a TGGE position comparable to that of band M (Figure 4.3). Nearly full-length 16S rDNA sequences covering more than 1470 bases were determined. As expected by their comparable TGGE mobility, the two clones matching the band L showed identical sequences, and we will only refer to clone EN-B3. Comparison of the rDNA sequence of this clone to that matching the M band (EN-B9) showed a high overall identity of 98.9%, with only 16 nucleotide substitutions in their sequences. Comparison with gene data banks pointed as closest relatives (93 to 94.7%. sequence similarity), different members of the genus

Burkholderia, that includes several strains known to degrade xenobiotic compounds, such as *Burkholderia* sp. strains N2P5, N2P6 and N3P2 (Mueller *et al.* 1997), or strain JB1 (Springael *et al.* 1996) (Figure 4.4).

Remarkably, none of the enrichment cultures presented a band matching the U position on their TGGE-profiles (Figure 4.3), although this band is quite prominent in the profiles of both spiked soils. Therefore, the corresponding band was excised from the TGGE gel, re-amplified and cloned, resulting in clone DH02, whose amplicon showed the same mobility as the band U upon TGGE. Sequence analysis of the V6 to V8 insert of clone DH02 showed it to be homologous to the 16S rRNA from *B. caryophylli*, with 95% identity in the 426 bp sequenced. The relations of the clones with their closest neighbours in the genus *Burkholderia*, and with other members of the β-subclass of Proteobacteria are depicted in Figure 4.4.

V6-probe hybridisation

In order to confirm the identity between the sequenced clones EN-B3, EN-B9 and DH02, and their respective matching bands L, M and U (Figure 4.3), their amplicons were separated by TGGE, together with the amplicons obtained from the chlorobenzoate-treated soils, and electro-blotted on a nylon membrane. Subsequently, radioactively labelled probes of the V6 region from each clone were applied in separate hybridisations. The probes derived from clones EN-B3 and EN-B9 cross-hybridised with each other and with bands L and M, even under stringent conditions (data not shown). Sequence comparison of the V6-region from both clones showed only one mismatch in the 97 nucleotides comprising the probe. In contrast, the radioactively labelled probe based on clone DH02 hybridised with the amplicons derived from this clone. Moreover, significant hybridisation was found with the band U, present in the TGGE-profiles from the soils treated with 2,5DCB or 3CBA (Figure 4.5). This confirms not

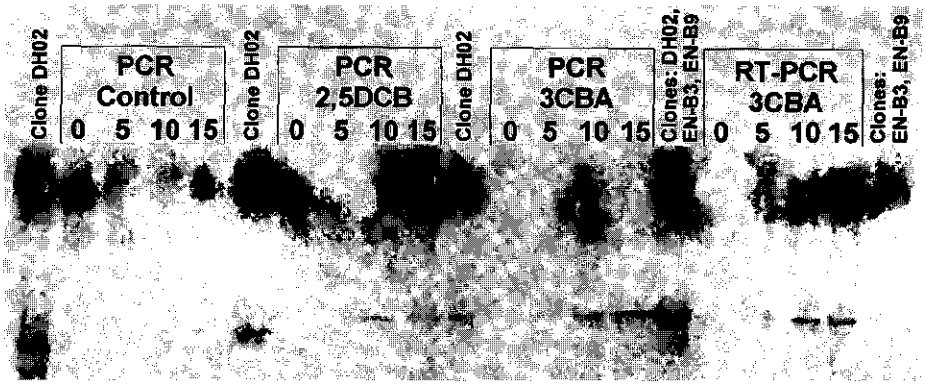

Figure 4.5. Electroblotted TGGE-gel hybridised with a radioactively labeled V6-probe derived from the cloneDH02. The gel was loaded with DNA-based amplicons (PCR) from untreated soil (control, and from both spiked soil (2,5DCB and 3CBA), together with RNA-targeted amplicons (RT-PCR) from 3CBA treated soil. Control lanes with amplicons derived from clones DH02, EN-B3 and EN-B9 are indicated.

only the sequence identity between band U and clone DH02, but also indicates that in the soils amended with either of the chlorobenzoates, phylogenetically identical bacteria are enriched.

DISCUSSION

The here described molecular analysis based on TGGE profiling allowed to detect in a broad scale and without the need of cultivation, the bacterial community changes occurring in a peat-forest soil under chlorobenzoate stress. The specific population changes were assessed, and the phylogenetic position of the predominant bacterial populations determined. Assessment of community shifts at both DNA and rRNA level showed similar results, indicating the metabolic activity of the induced bacterial populations.

From the two chlorobenzoates used, only 3CBA was readily disappearing from the treated soil (Figure 4.1). This disappearance was biologically mediated since rather constant 3CBA concentration values were obtained from soils autoclaved that have been similarly spiked.

The selective pressure induced by the chlorinated benzoates selected some of the bacterial populations originally present in the soil, as reflected in less complex community TGGE-banding profiles. With time, few of the selected bacterial species became dominant populations, clearly visible as the predominant arrowhead marked bands in Figure 4.2.

The bacteria present in the peat-forest soil from De Hel could not degrade the xenobiotic 2,5DCB. However, a *B. caryophylli*-related organism was able to adapt to the stress conditions opposed by 2,5DCB as indicated by the TGGE and sequencing results. Bacteria with similar phylogenetic position could also multiply in the presence of 3CBA, the question arises whether these bacteria contribute to the degradation process in soil. We speculate that the *B. caryophylli*-like bacteria are tolerant to both chlorobenzoates and well adapted to the soil environment. This also may explain why in culture medium they are apparently overgrown by other fast-growing members of the community, which are readily present in the enrichment cultures.

The 3CBA-degrading enrichment cultures A and B (Figure 4.3) developed very different bacterial populations sharing only one predominant band, although both cultures come from the same original soil extract. This could be due to a bias caused by the enrichment procedure itself, as it has been recently reported (Dunbar *et al.* 1997). The cultivation attempts were specifically directed to obtain the bacteria detected as predominant soil populations under 3CBA stress. Nevertheless, those bacteria have resisted the isolation trials.

The *Burkholderia*-related organisms represented by the clones EN-B3 and EN-B9 proved to be predominant in the 3CBA-degrading enrichment cultures and in the spiked soil, as revealed by the molecular analysis. It is tempting to assume that these bacteria are responsible for the 3CBA disappearance in soil. This is in agreement with recent reports describing the capacity of *Burkholderia* spp. to degrade polyaromatic compounds (Mueller *et al.* 1997), mono- and dichlorinated biphenyls (Springael *et al.* 1996), and dichlorinated phenoxyacetic acid

(Cattaneo *et al.* 1997). The use of the enriched organisms in soil bioremediation is tempting, as they have exerted the degrading activity efficiently under microcosm conditions.

We have demonstrated that the chosen approach is successful to identify bacteria involved in the degradation of toxic compounds and may be a useful strategy for future bioremediation studies. Furthermore, TGGE-profiling proved to be an efficient means of monitoring the ecological impact of xenobiotic compounds such as chlorobenzoates, in soil bacterial communities. This approach could be easily applied to other complex environments and to different members of the microbial community.

CHAPTER 5

*MOLECULAR DIVERSITY IN THE BACTERIAL COMMUNITY AND THE FLUORESCENT PSEUDOMONADS GROUP IN NATURAL AND CHLOROBENZOATE-STRESSED PEAT-FOREST SOIL

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SUMMARY

The bacterial community shifts in a peat forest soil spiked with 3-chlorobenzoate (3CBA) or 2,5-dichlorobenzoate (2,5DCB) were monitored in time. The 16S rRNA was used as a molecular marker in combination with the temperature gradient gel electrophoresis (TGGE) profiling technique. The relative band intensities of the TGGE profiles were used to estimate Shannon's diversity (H) and equitability (J) indices, which were taken as parameters of community changes, for the uncultured total bacterial community. The changes occurring in the selected culturable fraction of fluorescent pseudomonads were also evaluated with the H and J indices. Addition of the chlorinated benzoates to soil reduced between 1 and 2 orders of magnitude the overall growth of culturable aerobic bacteria, as evaluated by plate counting in a glucose-containing medium. However, this addition had no effect on the diversity within the group of fluorescent pseudomonads as assessed by differential isolation and a fast TGGE-based ribotyping technique. In contrast, the uncultured bacterial community showed a decrease in the number of bands in the TGGE profiles of the chlorobenzoate-spiked treatments. Accordingly, the diversity and equitability indices of these treatments reflected a decreasing trend in time. The results indicate that chlorobenzoates have a marked negative effect on the soil bacterial community, and the used approach allowed direct analysis of complex bacterial communities. Therefore, it can be proposed as a rapid method to assess community shifts upon contamination of the environment with toxic compounds.

INTRODUCTION

Chlorinated benzoates are a group of compounds with several different agricultural and industrial applications, like insect repellents, industrial solvents, herbicides, fungicides, odorisers and dielectric fluids. As a result of their abundant production and use, both disperse and concentrated contamination has occurred in soils, ground water, sediments and biota (Oliver and Nicol 1982, Schwarzenbach *et al.* 1979, van Zoest and van Eck 1993). Although some of these compounds are recalcitrant in the environment, many pure and enrichment bacterial cultures belonging mainly to the genera *Pseudomonas, Burkholderia* and *Alcaligenes* are able to use chlorobenzoates as sole carbon and energy sources under different physiological conditions (Chaudry and Chapalamandugu 1991, Mohon and Tiedje 1992, Haggblom 1992). However, little is known about the ecological impact of these xenobiotic compounds on the soil bacterial community present in natural environments.

Several concepts of species diversity have been applied to evaluate an ecosystem. Some are simply based on the number of species or Operational Taxonomic Units (OTU) found in a given ecosystem. Other more complex approaches, generally expressed as diversity indices, take into account the relationship between species and individuals (Lloyd *et al.* 1968). In the past, diversity indices have occasionally been applied in microbial ecology studies to get insight into the species richness of the culturable bacteria from different habitats (Atlas 1980, Mills and Wassel 1980). However, these indices have frequently been used in plant and animal ecology, where the species concept has been better determined (Begon *et al.* 1990). More recently, cultivation methods have been used in combination with a molecular typing technique to assess microbial diversity changes under distinct environmental conditions (Bej *et al.* 1991, Dunbar *et al.* 1997, Smit *et al.* 1997). Using this approach, the diversity of plant-associated fluorescent pseudomonads has been assessed by the classical biochemical tests, combined with molecular typing techniques like fatty acid methyl ester analysis, pyrolysis-mass spectrometry and pulsed-field gel electrophoresis (Rainey *et al.* 1994), or in combination with REP-PCR (Lemanceau *et al.* 1995). However, these approaches are constrained to the small fraction of cultivable bacteria.

Estimation of bacterial diversity by culture-independent methods has been addressed by two main groups of approaches. One group seeks to generate information on whole microbial communities. The techniques used are based on the melting behaviour (Torsvik *et al.* 1994) or hybridisation kinetics (Griffiths *et al.* 1996) of community-extracted DNA. These broad-scale approaches have provided crude estimates of the diversity and the relative structure of soil microbial communities, but they have failed in addressing information on specific taxa. The other culture-independent approaches rely on the PCR-amplification of 16S rRNA or rDNA extracted from environmental samples. The subsequent separation or detection of specific amplicons has been achieved by different means, like cloning and sequence analysis (Barns *et al.* 1994), or quantitative hybridisation (Stahl and Amann 1991), using oligonucleotide probes designed with different levels of specificity (Franks *et al.* 1998, Raskin *et al.* 1994). Terminal

restriction fragment length polymorphism (Liu *et al.* 1997) or denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.* 1993) have been proposed as alternatives to resolve the amplicons and analyse microbial community diversity.

The use of DGGE or temperature gradient gel electrophoresis (TGGE) combines the possibilities of a rather broad profiling and assessment of specific bacteria within complex communities (Felske *et al.* 1997, Muyzer *et al.* 1993). These electrophoretic techniques allow separation of amplicons of equal size, by means of their differences in primary structure. The amplicons are generated from a complex mixture of 16S rDNA or rRNA target molecules obtained from an environmental microbial community. Ideally, the final mixture of PCR-products will be reflecting the relative abundance of target molecules (Felske *et al.* 1997, Muyzer *et al.* 1993).

Recently, we reported on the culture-independent detection of bacterial community shifts, and assessment of specific enriched populations in a peat forest soil under induced chlorobenzoate stress. It was shown that 3-chlorobenzoate (3CBA) was completely degraded within 15 days, while 2,5-dichlorobenzoate (2,5DCB) remained unaltered (Chapter 4). In the present work we have monitored and evaluated the bacterial diversity changes at a community level. The capabilities of TGGE as a community profiling technique were extended with a relative quantification of the resulting bands, these values were used to estimate Shannon's diversity and equitability indices (Shannon and Weaver 1963). We aimed to describe the changes in the structure of the soil bacterial community and in the group of fluorescent pseudomonads after addition of 2,5DCB, or 3CBA to the soil. The changes in the fluorescent pseudomonads group were also evaluated with the above mentioned indices. These bacteria are normally present in rhizosphere and soil, they are easy to culture, and so far any strain has been reported as able to grow on chlorobenzoates.

MATERIALS AND METHODS

Soil sampling

Soil samples were collected during the summer of 1997 from a natural black alder (*Alnus glutinosa*) wet stand at the nature reserve "De Hel", Veenendaal, The Netherlands. Details about the site and properties of the peat soil were reported previously (Wolters *et al.* 1997a). Soil samples free of stones and large plant material were taken in the neighbourhood of 4 black alder trees. The samples were pooled and stored at 4°C until their use, within the following week.

Microcosm experiments

The microcosm soil systems were set up as previously described (Chapter 4). Three treatments were established, a) control treatment added with water at 45% (w/v), b) 2,5DCB-spiked treatment to a final concentration of 0.4 μmoles g^{-1} wet soil, and c) 3CBA-spiked treatment with the same final concentration of 0.4 μmoles g^{-1} wet soil. The chlorinated

compounds were added to the soil in small aliquots and mixed thoroughly. Each soil-microcosm system was sampled at 0, 5, and 10 and 15 days, the samples used to extract DNA and rRNA, and to isolate the fluorescent pseudomonads. For the samples taken at time 0 days, the nucleic acids extraction and the bacterial isolation procedures started 3 h after the chlorobenzoates were added to the soil.

Plate counting and isolation of fluorescent pseudomonads

Microorganisms from the original unspiked soil were extracted as previously described (Chapter 4). Serial dilutions of the soil extracts were plated on both a rich medium as tryptone-soya-broth added with 1.5% (w/v) agar (TSA), and a basal medium (BM), made as 1.8 mM $CaCl_2 \cdot 2H_2O$, 4.1 mM $MgSO_4 \cdot H_2O$, 5 mM NH_4Cl, 1.4 mM $K_2(NaH_2)PO_4$ buffer (pH 6.9), and trace elements solutions as Stams *et al.* (1993). The BM medium was supplemented with 5 mM glucose, or 2.5 mM of either 3CBA or 2,5DCB as sole energy and C sources. Additional BM plates containing a combination of glucose and 3CBA, or glucose and 2,5DCB were also inoculated. All the plates were incubated at 24°C, the TSA plates during one day and the others for three days, then the CFU counts were scored.

Isolation of fluorescent pseudomonads was done following the same soil extraction procedure but using samples from the microcosm systems taken at the 4 different sampling times. The diluted soil extracts were first plated in TSA medium containing 50 $\mu g\ ml^{-1}$ cycloheximide. After one day incubation single colonies from the most diluted plates were selected based on their *Pseudomonas*-like morphology and transferred to the differential King's medium B (KB) (King *et al.* 1954). Screening for production of fluorescent pigments was done under UV light after 2 days of incubation on KB medium.

DNA and rRNA extraction

DNA and rRNA from soil microorganisms were simultaneously extracted as described in detail elsewhere (Felske *et al.* 1998b). Briefly, 2 g (fresh weight) of soil were subjected to bead-beating to achieve cell disruption in a buffered environment, followed by differential centrifugation steps allowing separation of nucleic acids and ribosomes from the larger soil particles and cell debris. The resulting fraction was resuspended and divided in two. One fraction was phenol extracted and ethanol precipitated to obtain DNA. The other fraction, yielding rRNA was acid-phenol extracted, followed by ethanol precipitation and DNase digestion. The final pellets of both fractions were dissolved in 100 µl of an appropriate buffer. The amount of 16S and 23S rRNA obtained from each soil sample was estimated by agarose gel electrophoresis in combination with ethidium bromide staining, computer-assisted image analysis and rRNA standards (Felske *et al.* 1997).

Single colonies of the fluorescent pseudomonads were aseptically picked and resuspended in 30 µl sterile distilled water, boiled for 15 min and centrifuged briefly to pellet the cell debris. Aliquots of the crude lysates were used as template for PCR reactions.

PCR, reverse transcription (RT)-PCR and Temperature Gradient Gel Electrophoresis

The PCR and RT-PCR reactions targeting the V6 to V8 variable regions (Neefs *et al.* 1990, 1991) of respectively the 16S rDNA or rRNA were set up according to Felske *et al.* (1996). In all cases 1 μl of soil-extracted DNA or rRNA was utilised as template. Similar PCR-amplifications were performed using 2 - 3 μl of the fluorescent pseudomonads cell lysates.

The amplicons obtained from the soil-extracted DNA and rRNA were separated on a TGGE system (Diagen, Düsseldorf, Germany) (Felske *et al.* 1998c, see also Chapter 4). The high resolution achieved by TGGE was also used as an alternative and fast way to ribotype the fluorescent pseudomonad isolates based on the different mobility of their respective V6 toV8 amplicons. In order to obtain a better separation of these amplicons during TGGE, the ΔT was reduced to 8°C (ranging from 36°C to 44°C), while all other conditions remained the same.

Estimation of Shannon's Diversity (H) and Equitability (J) indices

Species richness and evenness of the bacterial communities in the control and the spiked soils, and for the fluorescent pseudomonads isolates were approached by the ecological indices of Diversity (H) and Equitability (J) proposed by Shannon and Weaver (1963), as implemented by Begon *et al.* (1990). The indices are defined by the equations: $H = -\Sigma Pi \ln Pi$, and $J = H/\ln S$; where *Pi* represents the number of individuals from one species or one OTU, divided by the total number of individuals in the community sample, and S represents the total number of OTU in the sample.

Estimation of both indices for the bacterial community was based on the TGGE profiles, due to the high background the upper part of the gels (indicated with a rectangle on Figure 5.1) was not considered. Each band within a profile was considered as a different OTU, while bands with similar migration position in different profiles were regarded as the same OTU. The silver stained gels were scanned in a flat bed scanner Sharp JX 330, equipped with a second film scanning unit JX-3F6 (Sharp Corp, Japan) for improved resolution. After image digitalisation, the Molecular Analyst (BioRad, Hercules, Cal.) software package was used to localise the bands within each profile, and to calculate band surface and intensity. The relation between band surface and the mean pixel intensity of that area was expressed as band volume values, which were taken as a surrogate measure of OTU's abundance. Band detection within a profile and the respective surface delimitation was performed by automated functions in the program. The number of OTU in a sample (S) was determined by considering only the quantifiable bands, which were those bands accounting for >2.5% of the total band's surface in a profile. This approach was taken since it was not possible to verify without doubt the actual presence and limits of very faint bands in the TGGE gels.

Typing of all the fluorescent isolates obtained through the experiment was based on the high resolution achieved by TGGE. DNA from single colonies treated as described above was used to amplify the V6 to V8 regions of the 16S rDNA. The amplicons were generally producing a single band migrating to a specific position upon TGGE separation, which was called ribotype. Each ribotype was considered as a different OTU.

RESULTS

Effect of 3CBA and 2,5DCB on the culturability of soil bacteria

Although chlorobenzoates can be degraded by a limited number of bacteria, the effect of these compounds on other members of the bacterial community is poorly understood. This question was approached by assessing the differences in the culturability of soil extracted bacteria plated on different media in the presence of either of the chlorobenzoates. The results indicate that de peat soil from De Hel has a very high number of bacteria able to grow on a rich medium or on easily degradable C source such as glucose (Table 5.1). However, when 3CBA or 2,5DCB were used as the sole C and energy sources, only very few bacterial colonies developed (less than 100 CFU g^{-1} soil). None of these isolates were able to grow if transferred to the same culture medium but liquid, and they did not appear when agarose or Noble agar was used instead of agar. Addition of 3CBA or 2,5DCB to the BM medium containing also glucose, resulted in a 10 – 100 fold decrease in the amount of CFU compared to that obtained in the same the medium but containing only glucose. These data clearly indicated that both chlorobenzoates are toxic or inhibitory to a fraction of the cultivable bacterial community.

Table 5.1. Effect of addition of chlorobenzoates to the culture medium, on the number of colony forming units (CFU) obtained from unspiked peat-forest soil. The rich medium tryptone-soya-agar (TSA) was used without supplements. The basal medium (BM) was supplemented with the indicated C sources as described in the Materials and Methods section.

Media and supplements	C.F.U. g^{-1} soil
TSA	$>10^{10}$
BM + glucose	4.9×10^{9}
BM + 3CBA	<100 *
BM + 2,5DCB	<100 *
BM + 3CBA + glucose	1.2×10^{8}
BM + 2,5DCB + glucose	5.1×10^{7}

* regarded as non-chlorobenzoate degraders

Isolation, ribotyping and diversity of fluorescent pseudomonads isolates

Isolation of the fluorescent pseudomonads was done by a two-step approach. The first step plating in rich medium (TSA) was followed by transfer and screening in a differential medium (KB). This approach was intended in order to obtain a better UV-screening and typing of single isolated colonies. The results of isolation, fluorescent screening and ribotyping of the isolates are summarised in Table 5.2. The relation between the number of fluorescent pseudomonads and the number of *Pseudomonas*-like isolates showed differences among the treatments. Multiple χ^2 tests were performed to evaluate the differences in the respective proportions. The comparison between the control and the 3CBA treatments showed no

significant difference in their proportions at 95% confidence, while the fraction of fluorescent pseudomonads in the 2,5DCB treatment was the lower and significantly different to the other treatments. In spite of this, the estimated Shannon's diversity (H) and equitability (J) indices of all treatments showed to be roughly similar, pointing to a minor or no effect of the chlorobenzoates in the structure of the fluorescent pseudomonads group.

The fast TGGE-based ribotyping allowed grouping of the fluorescent pseudomonads in 9 different ribotype groups (Table 5.2). The first four (ribotypes A - D) were common to all treatments and comprised 86% of the total fluorescent isolates. The other 5 ribotypes (V - Z) were less abundant and only found in one or at most two of the treatments. Comparison of the ribotype bands from the fluorescent isolates did not show matching-band positions with prominent bands from the soil TGGE profiles (data not shown). However, it was not possible to completely ascertain this, as several ribotype bands were migrating in the same area as the single stranded DNA (marked with a rectangle in Figure 5.1) which produced a very high background in the stained gel.

Table 5.2. Ribotyping and ribotype abundance of the fluorescent pseudomonads isolated from the unstressed (control) and stressed (2,5DCB and 3CBA) peat soil. Shannon's diversity (H) and equitability (J) indices for each treatment were estimated by using the number of different ribotypes and the ribotype abundance.

	TREATMENTS		
RIBOTYPES	**CONTROL**	**2,5DCB**	**3CBA**
A	16	6	17
B	3	4	6
C	23	13	15
D	11	4	9
V	4	3	
W		4	2
X			1
Y			1
Z	5		
Total number of fluorescent Pseudomonads	62*	34	51*
Total number of *Pseudomonas*-like isolates	327	326	325
Shannon's diversity index (H)	1.55	1.64	1.57
Shannon's equitability index (J)	0.87	0.92	0.81

* the proportion of fluorescent pseudomonads in the two marked treatments were not significantly different at 95% confidence.

rRNA and DNA extraction

The amount of rRNA obtained from all samples ranged between 0.4 to 1.3 μg g^{-1} soil, with an average of 1.0 μg g^{-1} soil, a similar yield has been reported from a different peat-soil, using the same extraction procedure (Felske *et al.* 1997). However, no significant relation could be found between rRNA yield and soil treatments.

The V6 to V8 amplicons obtained from the soil-extracted DNA's and rRNA's were resolved by TGGE. Comparison of the PCR-based profiles with the respective RT-PCR profiles of the control soil did not show marked differences among them (Figure 5.1). The numbers of prominent bands (S) were quite constant in both types of profiles (Table 5.2). Accordingly, the values for the Shannon's Diversity (H) and Equitability (J) indices estimated from the relative band volumes within each profile showed only minor variations (Table 5.2), indicating a stable structure and composition of the prominent bacterial community in the control treatment.

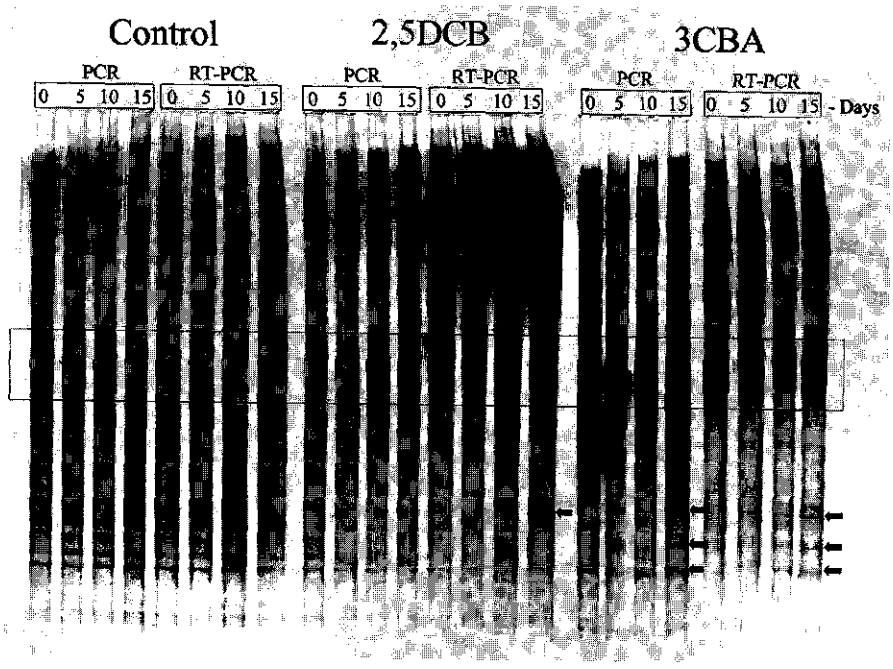

Figure 5.1. DNA- and RNA- based TGGE profiles from the bacterial community in the unspiked (control) and spiked (2,5DCB and 3CBA) peat-forest soil. The area within the rectangle corresponds to single-stranded DNA. Bands with increasing intensity in time are marked with arrowheads. The star points to a band disappearing in time.

In contrast to the control soil, the TGGE profiles of both chlorobenzoate-spiked treatments registered some variations, being the most notorious the bands appearing with increasing intensity in time (marked with arrowheads in Figure 5.1). Other minor bands became fainter or even disappeared (Figure 5.1). The relative quantification and analysis of the DNA-based profiles of both spiked treatments showed a trend in time, towards a diminishing number of quantifiable bands (Table 5.2). The trend was more pronounced in the 2,5DCB-treated soil, where the S values decreased from 17 to 10 bands after 15 days, while the reduction for the 3CBA soil was only from 12 to 10 bands. The initial numbers of quantifiable bands in the spiked treatments were in general lower than the control. This could be indicating a rapid inhibitory effect on the bacterial community occurring only few hours after addition of the chlorobenzoates to the soil. The respective TGGE profiles reflected this fact with an increasing number of very faint bands, which is not reflected so markedly in the H and J values, as most of the bands were non-quantifiable.

Analysis of the rRNA-based profiles of both spiked treatments showed a different trend in the amount of quantifiable bands, as in both cases the smallest S values were obtained on day 5, with according reductions on H and J indices (Table 5.3). The decreases of H values are mostly related to a time-reduction in the number and volume of prominent bands in the respective profiles. Decreasing equitability values (J) are reflecting unequal abundance of target molecules within the original complex mixture.

Table 5.3. Shannon's diversity (H) and equitability (J) indices for each treatment, sampling time, and target molecule (DNA or rRNA). The estimation of H and J was based on the TGGE profiles from Figure 5.1. S represents the number of quantifiable bands (OTU) in each profile.

		TREATMENTS								
	Time	CONTROL			2,5DCB			3CBA		
	(days)	H	J	S	H	J	S	H	J	S
DNA (PCR)	0	2.63	0.88	20	2.55	0.90	17	2.28	0.92	12
	5	2.45	0.85	18	2.45	0.90	15	1.86	0.75	12
	10	2.55	0.87	19	1.48	0.64	10	1.63	0.68	11
	15	2.46	0.85	18	1.55	0.67	10	1.48	0.65	10
rRNA (RT-PCR)	0	2.61	0.89	19	2.39	0.86	16	1.43	0.73	7
	5	2.58	0.88	19	1.78	0.86	8	0.86	0.54	5
	10	2.69	0.88	21	2.05	0.78	14	1.85	0.84	9
	15	2.68	0.89	20	1.54	0.60	13	1.60	0.77	8

DISCUSSION

The effect of chlorinated benzoates on the bacterial community of a peat-forest soil has been assessed by culture-dependent and culture-independent approaches. With the first approach, bacteria extracted from the unspiked soil-sample were cultured in the presence and

absence of 3CBA or 2,5DCB and glucose. In addition, soil-extracted bacteria from the treated microcosm systems were isolated and the fraction of fluorescent pseudomonads was selected for further evaluation. The presence of chlorobenzoates in the culture medium showed a marked negative impact on the viability of the soil-extracted bacteria. However, this effect was not apparent on the fluorescent pseudomonads. This group of bacteria has been isolated in chlorobenzoate-free media from the stressed and unstressed microcosm soil systems, under those conditions some fluorescent strains were cultured that may have been inhibited in the spiked soils. However, this approach was used as the addition of chlorobenzoates to the KB medium inhibited the growth of the fluorescent strains.

TGGE-based ribotyping of the fluorescent pseudomonads has proven a fast and easy way to type a large number of isolates, resulting in single or two-band patterns determining OTU's or groups of bacteria. Furthermore, the isolates typed in this way could be addressed in the context of the community TGGE profiles. However, the ribotype bands of the different fluorescent isolates did not have a corresponding prominent band in the profiles, enabling the idea that this group of bacteria is a minor component of the bacterial community in our model soil.

The toxic effect of both chlorobenzoates was also evident when culture-independent techniques were used to address changes induced by those compounds in the soil bacterial community. TGGE and DGGE profiling have already proven to be powerful tools to analyse complex microbial communities (Felske *et al.* 1997, 1998c, Muyzer *et al.* 1993, see also Chapter 4). The additional relative quantification of the banding patterns enabled to estimate Shannon's diversity and equitability indices providing a better insight on the changes of the community structure. The general picture of the profiling and subsequent analysis of all treatments, pointed to the detection of major changes in the structure and composition of the predominant bacterial populations of the chlorobenzoate spiked soils. We are aware that the actual bacterial soil diversity must be higher than our estimates based on TGGE profiling. This is mainly due to the constraints imposed for the relative quantification of only prominent bands. However, the used approach enabled us to address shifts in the presumably most abundant, active and important fraction of the bacterial community.

The relative band quantification of TGGE profiles was restricted to prominent bands. Considering also minor bands in the analysis (<2.5% of total bands' area) would result in larger differences in the estimated indices, which may not be only reflecting actual variances in diversity due to an effect of the chlorobenzoate, but also biases from the PCR, TGGE or band detection by imaging. Certainly, the whole procedure may suffer the known biases inherent to the use of PCR-based approaches when estimating microbial diversity from environmental samples (reviewed by Head *et al.* 1998, and Wintzigerode *et al.* 1997). However, the possibly introduced biases must be affecting our samples and estimations in an equal manner, as the samples have a common origin and were similarly treated, except for the addition of the chlorobenzoates. It was recently demonstrated that the used primer pair (U968/GC and L1401; Nübel *et al.* 1996) amplified with similar efficiency target molecules from different source (Felske *et al.* 1998a). Suggesting that the biasses may be small.

The application of diversity and equitability indices in combination with a community profiling technique like TGGE is particularly useful when examining time series and population dynamics within the same community. Estimation of changes as numeric values rather than banding patterns may facilitate comparisons and prediction of trends. This approach may therefore be valuable in making decisions when addressing short and long-term responses of a bacterial community to transient environmental perturbations.

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CHAPTER 6

EFFECT OF CHLORINATED BENZOATES IN NATIVE *FRANKIA* POPULATIONS IN A PEAT-FOREST SOIL

SUMMARY

The effect of two halogenated aromatics, 3-chlorobenzoate (3CBA) and 2,5-dichlorobenzoate (2,5DCB) on the *in vitro* growth of *Frankia* strains isolated from *Alnus* spp. was assessed. The ability of four *Frankia* strains to utilise these compounds, or grow in the presence of low concentrations of these xenobiotics was evaluated by comparing the *Frankia* biomass yield in culture. Any of the tested strains could utilise either 3CBA or 2,5DCB as sole carbon and energy source. The presence of 1 mM 3CBA in the culture medium, supplemented with propionate as carbon source, resulted in a significant reduction of biomass yield pointing to a toxic effect of this chlorinated compound. In addition, the impact of 3CBA and 2,5DCB on the native *Frankia* populations from a peat forest soil was evaluated. The uncultured *Frankia* populations were analysed in microcosms systems containing chlorobenzoate-spiked and unspiked soil. Two quantification approaches were used, both of them based on the most probable number concept. The first one was focused on the total number of *Frankia* genomic units (GU) detected by specific PCR reactions, using dilution series of soil-extracted DNA as target. The second approach was based in a plant-traping method to address the amount of *Alnus*-compatible *Frankia* nodulating units (NU) present in dilution series of the treated soils used as plant inoculum. Both chlorinated benzoates had a slight negative effect on the amount of *Frankia* GU, already few hours after their addition to the soil. The reduction on GU was much more pronounced 15 days later, when the differences between the control and the spiked systems were more than one order of magnitude. A similar ten-fold reduction was observed after 15 days in the NU values obtained in both spiked systemss These results point to a marked toxic effect of chlorobenzoates to *Frankia*.

INTRODUCTION

Alder trees (*Alnus* spp.) are widely distributed mainly in the northern hemisphere, where they are found in geographic areas ranging from temperate to subtropical climates. As a general rule, these plants are nodulated by their N_2-fixing symbiont *Frankia*, in almost every field location where they grow (Bond 1977). The actinomycete *Frankia* has also been reported in areas devoid of alder or other 'actinorhizae' plant (Smolander and Sundman 1987, Smolander and Sarsa 1990). In spite of this cosmopolitan distribution *Frankia* strains are difficult to isolate from soil. The main problems encountered are due to their low growth rate, the reduced size of the soil populations, and the lack of knowledge about the most common morphological type present in this habitat (i.e. spores or hyphae). Since 1978 a large number of *Frankia* strains have been isolated mostly from root nodules that are regarded as a natural enrichment source. So far, there is only one report of a *Frankia* strain isolated directly from soil (Baker and O'Keefe 1984). Since several *Alnus*-compatible *Frankia* strains (e.g. ineffective, spore$^+$ type) have resisted isolation, our knowledge on their diversity and abundance in soil is quite limited.

Accurate quantification of *Frankia* populations in soil has been approached in two ways. The first one considers the symbiotic capacity of *Frankia* and is based in a plant-trapping bioassay. Van Dijk (1984) proposed to calculate the number the nodulating units (NU) from a known amount of inoculum source (i. e. soil extract), by counting the number of nodules formed in an inoculated plant. A variation to this approach was provided by Hilger *et al.* (1991) based on the combination of the plant-nodulation bioassay with the well-known statistical concept of most probable number (MPN). Huss-Danell and Myrold (1994) pointed out that the estimations given by both variations of the plant bioassay are usually in agreement. The numbers of *Frankia* NU are heavily influenced by different environmental factors like the soil type and predominant flora (Smolander and Sundman 1987). Typical detection range varied from 10^1 up to 10^3 *Frankia* NU g^{-1} soil (Myrold *et al.* 1994).

The second quantification approach relies on PCR amplifications using *Frankia*-specific oligonucleotide primers and again the MPN concept (Myrold *et al.* 1994). The preferred target for this approach has generally been the 16S rRNA gene, in combination with genus- or group-specific *Frankia* primers. The use of an "optimised booster PCR" (Myrold *et al.* 1990, Picard *et al.* 1992) enhanced the sensitivity of the quantification to about 10 *Frankia* genomic units (GU) g^{-1} soil. A similar sensitivity was achieved by applying a nested PCR procedure (Myrold and Huss-Danell 1994). These procedures were intended as an alternative to increase the sensitivity of the former detection and semi-quantitative approach based on direct hybridisation of soil-extracted rRNA with *Frankia*-specific probes (Hahn *et al.* 1989a, 1990a).

Although both quantification approaches are quite sensitive, they are assessing different aspects of the *Frankia* populations. The first one is quantifying only the fraction of the population able to nodulate a given host plant, the specificity of the plant bioassay is dependent on the host plant used. Recent reports (Wolters *et al.* 1997a,b) have pointed out to the existence of large populations of *Alnus*-compatible ineffective *Frankia*, present mainly in water logged

sites. The second approach is providing an overall estimation of the *Frankia* populations in soil. As it is based on PCR, the resulting data may include *Frankia* GU from spores, non-viable or dead cells. Although unlikely, even free or fossilised DNA could be amplified as well.

Frankia strains have been isolated from most 'actinorhizae' plant genera, though some of the isolates are non-infective in the original host plant (Benson and Silvester 1993). To allow cultivation of the different isolates a number of culture media have been formulated (Lechevalier and Lechevalier 1990). In addition to the metabolism of common C- and N-sources in culture (Benson and Schultz 1990), some plant-derived compounds have been tried as alternative C-sources. Blom (1981) demonstrated that *Frankia* strain AvcI1 was unable to utilise many different compounds, among others vanillic acid, cholesterol and tannin. The effect on the growth of six *Frankia* strains of plant-phenolics added to a rich culture medium was investigated by Perradin *et al* (1983). They found that cinnamic acid-derived compounds were strong inhibitors, while benzoic acid-related phenolics produced no or slight inhibition. Moiroud *et al.* (1985) have reported that low concentrations of some common herbicides on the culture medium did not affect greatly the growth of several *Alnus*-compatible strains, while the fungicide cryptonol (8-hydroxyquinoleine) was a strong inhibitor. Other contaminants like chlorinated benzoates have received no attention, although they can be found in soil and water. One objective of this work was to assess the effect of 3 chlorobenzoate (3CBA) or 2,5 dichlorobenzoate (2,5DCB) on the development of *Alnus*-compatible *Frankia* cultures. A second objective was to evaluate the changes in the total *Frankia* population and in the fraction of *A. glutinosa*-nodulating *Frankia* occurring in a peat-forest soil, upon addition of 3CBA or 2,5DCB.

MATERIALS AND METHODS

Frankia *strains and culture conditions*

The *Frankia* strains ArI3 (Berry and Torrey 1979), Ag45/Mut15 (Hahn *et al.* 1988), Ag29.11 and Ag39.15 were used in the chlorobenzoate-degradation assay. The last two strtains were isolated from *Alnus glutinosa* root nodules collected in Oostvoorne and Drentse A, respectively, in the Netherlands. All strains were cultured for 2 – 3 weeks at 28°C in serum bottles containing 50 ml P+N medium (Meesters *et al.* 1985), the biomass was collected by centrifugation and homogenised by repeated passage trough a syringe and needle. The resulting suspensions were adjusted to 10 ml with sterile SSC [0.15M NaCl, 0.015M sodium citrate pH 7.0], and used as inoculum for the further experiments.

A basal medium for *Frankia* (-P+N) was prepared by omitting the propionate (C-source) from the P+N medium. Sterile solutions of either propionate, 3CBA or 2,5DBC were added as sole C-source at a final concentration of 5mM, resulting in P+N, 3CBA+N and 2,5DCB+N medium respectively, care was taken to keep the final pH between 6.8 – 7.0. Sterile 10 cm glass tubes with screw-cap were filled with 5 ml of each medium. Replicate tubes of each of the 4 media were inoculated with 0.5 ml of the respective inoculum suspension. The growth of each

strain was scored by comparing the original inoculum biomass in the –P+N basal medium, with the biomass obtained in the other media after 2 weeks incubation.

Toxicity of 3CBA was tested in P+N medium amended with 1mM of 3CBA sterile solution (medium P+N+3CBA). The inoculations, culturing conditions, and scoring were the same as above.

Soil samples

The peat forest soil from De Hel, Veenendaal, the Netherlands was also used in this experiment. Details on soil collection were given in Chapter 4, for physico-chemical characteristics of the soil see Wolters *et al.* (1997a)

Soil treatments

The soil samples for this experiment were taken from the different soil microcosm systems described in Chapter 4. Briefly, the soils were treated as: a) control, reconstituted to 45% water content with sterile distilled water, b) 2,5-dichlorobenzoate spiked with sterile 2,5DCB solution to a final concentration of 0.4 μmoles g^{-1} soil, c) 3-chlorobenzoate spiked with a sterile 3CBA solution to a final concentration of 0.4 μmoles g^{-1} soil. The treated soils were placed in replicate sterile petri dishes and incubated for 15 days under described conditions.

Determination of Frankia Genomic Units (GU) by MPN-PCR

DNA was extracted from the microcosm systems as described previously (Chapter 4). The DNA fractions corresponding to sampling times 0 and 15 days from each treatment were selected for the MPN-PCR determinations. Aliquots of the DNA fractions were initially diluted 1/10, and then 8 serial 3-fold dilutions were made (ranging from 1/30 to 1/196 830). Triplicate series of PCR reactions were set up using1 μl of each of these dilutions as target, in combination with the *Frankia*-specific primers 1009_R (Hahn *et al.* 1989), and PCRII (Mirza *et al.* 1992) which is a forward primer complementary to the *Frankia* probe (Hahn *et al.* 1990a), but shortened at the 3' end (*E. coli* positions 183-198). The 25 μl amplification reactions contained 1X PCR buffer [20mM Tris-HCl (pH 8.3), 50mM KCl], 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM each primer, and 1 U of *Taq* DNA polymerase (GibcoBRL-Life Technologies). A thermal cycler Biometra UNO II was set up to perform an initial denaturation of 95°C during 4 min; followed by 35 cycles of 15 s denaturation at 94°C, 15 s annealing at 52°C and 1 min elongation at 68°C, with a final post-elongation step of 4 min at 72°C. The resulting amplicons were visualised in 1.2% agarose gels stained with ethidium bromide, and the number of positive reactions per dilution was scored for the estimation of MPN values.

Plant material

Alnus glutinosa seeds were collected from one tree at the nature reserve of Boezem van Brakel, the Netherlands, and kindly provided by D.J. Wolters. The seeds were surface-sterilised

for 20 min in 15% H_2O_2 and rinsed four times for 5 - 7 min each in sterile demineralized water. Seeds were germinated under sterile conditions, in Petri dishes containing three layers of filter paper moistened with water. The Petri dishes were placed in the dark at 24°C, the first seedlings appeared after 5 days.

Plantlets bearing 2 or 3 true leaves were transferred to modified Leonard's jars filled with a 1:1 mixture (v/v) of perlite:gravel, each jar contained 2 plantlets. Hoagland's nutrient solution (Hoagland and Arnon, 1950) amended with only 1/4 concentration of the N source was used at this initial stage. The jars were placed in a growing chamber set up to16 h light at 24°C, and 8 h dark at 16°C. After 10 days all the nutrient solution was replaced for Hoagland's with N at 1/10 strength, and 10 days later the plantlets were inoculated.

Most Probable Number (MPN)-plant nodulation bioassay

The soil samples used as inoculum were taken from the respective microcosm systems as follow; control soil at time 0; control soil at time 15 days; 2,5DCB soil at time 15 days; and 3CBA soil at time 15 days. The four soil samples were resuspended in one volume of sterile N-free Hoagland's solution at 1/10 strength. These suspensions were considered as the 10^0 dilutions, then ten-fold dilutions corresponding to 10^{-1} to 10^{-4} were prepared.

All the dilutions (10^0 to 10^{-4}) were used as inocula for the MPN assay, which was set up using triplicate series of Leonard's jars containing 2 plantlets per jar. Two ml of each prepared soil suspension were applied to the roots of each plantlet, for this purpose the perlite:gravel substrate was removed as much as possible to uncover the root system without damaging it, once inoculated the roots were covered again. An extra series of plantlets (15 jars) were similarly treated, but using as inoculum sterile N-free Hoagland's solution at 1/10 strength. This last series was taken as negative control.

The plantlets were kept in a growing chamber under the above mentioned conditions. The lower vessels of the Leonard's jars were replenished with sterile demineralised water every third day and the whole solution (Hoagland's with 1/10 strength N) was changed every second week. At week four, the negative control jars were supplemented with Hoagland's solution plus 1/4 strength N.

Seven weeks after inoculation all plants were removed from the jars and individually scored for the presence of nodules. In this way the MPN estimations were based on 6 replicates, considering that each jar had 2 plants.

RESULTS AND DISCUSSION

The possibility that different *Alnus*-compatible *Frankia* strains were able to utilise 3CBA or 2,5DCB as the sole C-source was assayed in small batch cultures. In addition, the toxicity of a low concentration of 3CBA in P+N culture medium was assessed. The results (Table 6.1) after two weeks of incubation demonstrate that none of the tested strains were able to metabolise the chlorobenzoates, as observed by the lack of growth in the xenobiotic-containing

tubes. The presence of 1 mM 3CBA in the P+N culture medium (P+N+3C) was inhibiting the *Frankia* growth to some extent.

Table 6.1. Growth of *Frankia* strains in the presence or absence of chlorinated benzoates. One plus (+) means that the amount of biomass was roughly similar to that found in the basal medium (–P+N) after two weeks, a minus (–) was used when the biomass was less than the control.

	Medium				
Frankia **strain**	**-P+N**	**P+N**	**3CBA+N**	**2,5DCB+N**	**P+N+3CBA**
ArI3	+	+++	+	-	+
Ag45/Mut15	+	+++	+	+	++
Ag29.11	+	++++	+	+	++
Ag39.15	+	+++	-	-	++

To assess the impact of 3CBA and 2,5DCB in the native *Frankia* populations of the peat soil from the natural *Alnus glutinosa* stand in de Hel, soil samples were spiked with 0.4 μmoles of either 3CBA or 2,5DCB, while an unspiked control soil was only treated with water. The effect of these additions at the beginning and end of the 15-day experiment was addressed in two ways. The first approach was focused on the total soil *Frankia* population, as evaluated by MPN-PCR using *Frankia*-specific primers. The second approach addressed the changes in the fraction of the *Frankia* population able to induce nodules in *A. glutinosa* plants. The results of the MPN-PCR assay are summarised in Table 6.2. A slight decrease in the GU values of the spiked treatments was already observed at time 0, although the differences were not statistically significant. Nevertheless, the differences in the GU values between the spiked and the control treatments at time 15 days were more than 10-fold lower, indicating a negative impact of the chlorobenzoates on the total *Frankia* population in the peat-forest soil from de Hel.

Table 6.2. Effect of 3CBA and 2,5DCB on the number of genomic units (GU) in the soil *Frankia* population.

	Frankia **(GU g^{-1} soil)***	
TREATMENT	**Time 0 days**	**Time 15 days**
Control	21 730 a	40 170 c
3CBA	7 252 a	3 510 b
2,5DCB	10 250 a	2 990 b

*) The values followed by the same letter were not significantly different at 95% confidence level.

As already pointed out, PCR-based evaluations may be reflecting the relative abundance of different cell types or target DNA sources, some of which are not readily affected by the presence of the xenobiotic compound. It is not possible to differentiate with this kind of estimation, whether the chlorobenzoates are affecting preferentially a certain fraction of the

population. Evaluation of the *Alnus*-compatible *Frankia* by means of MPN-nodulation bioassay provided a good insight into a selected and more dynamic fraction of the native population. It was found that the nodulation unit (NU) values of the control soil at times 0 and 15 days are 10 to 20 times larger than those values for the spiked treatments (Table 6.3). These differences were pointing to a marked negative impact on the nodulation ability of *Frankia*, 15 days after adding the chlorobenzoates to the soil. Unlike the results obtained by Perradin *et al.* (1983) who found out that 30 days incubation on the presence of benzoic acid-like phenolics did not affect the *Alnus*-nodulation capacity of *Frankia* sp. ACN1^{AG}. Although 3CBA and 2,5DCB are benzoic acid-derived, the presence of the halogen moiety may increase the toxicity of these latter compounds. In addition, the differences on the experimental set up of both experiments make difficult to compare them.

The culture experiments and those based on MPN estimations indicated a general negative effect of the chlorobenzoates on *Frankia*. The suppression of growth or viability in culture and in the environment after addition of chlorobenzoates has been also demonstrated for other members of the same bacterial community (Chapter 5). The extent of this negative effect might vary depending on factors such as the type and concentration of the xenobiotic, culturing or environmental conditions. In addition, a strain-dependant resistance to chlorobenzoates has been noticed in both the *Frankia* strains used here (Table 6.1), and in fluorescent pseudomonads group (Chapter 5).

Table 6.3. Effect of 3CBA and 2,5DCB on the nodulation capacity of native *Alnus*-compatible *Frankia*, as evaluated by a plant nodulation-MPN assay.

TREATMENT	*Frankia* (NU g^{-1} soil)*
Control $T_{0\ days}$	1050 a
Control $T_{15\ days}$	876 a
3CBA $T_{15\ days}$	87 b
2,5DCB $T_{15\ days}$	38 b

*) MPN values followed by the same letter were not significantly different at a 95% confidence level.

Both MPN-based approaches are evaluating different fractions of the *Frankia* population in soil, and the obtained values are given in different units. Therefore, we can not make a direct comparison of them. Both groups of results, those evaluating the total frankiae and the ones assessing the *Alnus*-nodulating units, indicated a marked negative effect of the chlorobenzoates on the evaluated *Frankia* populations. Previous results (Chapter 5) have demonstrated that this inhibition is rather general on members of the bacterial community, and only some specific bacteria within the genus *Burkholderia* were enriched under chlorobenzoate stress. It still has to be determined whether the negative impact could be reduced under natural conditions, where the amount and distribution of the contaminants may be different.

CHAPTER 7

BACTERIAL DECHLORINATION OF 3-CHLOROBENZOATE IN LIQUID CULTURES UNDER THE INFLUENCE OF *ALNUS GLUTINOSA* ROOTS.

SUMMARY

A liquid culture model system was tried to assess the influence of black alder (*Alnus glutinosa*) on the dechlorination of 3-chlorobenzoate (3CBA) by *Pseudomonas* sp. strain B13. Preliminary results demonstrated that 3CBA was highly toxic to young black alder plantlets, and that the culture medium used to grow the bacteria could not completely sustain the growth of the plants. A modified culture medium was developed able to support the growth of bacteria and plants, and in which 3CBA-dechlorination could be monitored. The working hypothesis was focussed on demonstrating that enhanced dechlorination should occur under the influence of *A. glutinosa* roots. Five treatments covering the possible combinations of presence/absence of *Pseudomonas* sp. B13 and xenobiotic 3CBA were established, and plant survival and dechlorination activity were followed in time during 6 weeks. Although the expected enhanced dechlorination could only be partially demonstrated, the results suggest that the presence of *Pseudomonas* B13 provides a protective effect to black alders against 3CBA toxicity.

INTRODUCTION

Black alder trees (*Alnus glutinosa* (L.) Gaertn.) can be found throughout western Europe generally growing on wet or swampy ground, and along rivers and ditches, as they are well adapted to wet sites. They have also been planted for forestry and land reclamation purposes, on coal-mine spoils and eroded lands. Black alders are able to establish two types of symbiotic root-interactions, actinorhizal nodules with the nitrogen-fixing actinomycete *Frankia* as symbiont, and mycorrhizae with a number of fungi. *A. glutinosa* trees are resistant to high concentrations of some heavy metals (i.e. Bo, Cd, Zn, Pb) and few gases, like SO_2 (Wheeler and Miller 1990). Additionally, black alders have also been used in combination with other marsh plants in filter beds to purify water from microbial and chemical contaminants (patent Max Planck Institute 1974).

Pseudomonads are well-known for their abilities to colonise different environments and utilise a wide variety of compounds, including several chlorobenzenes. These compounds have been extensively used as solvents, fumigants, deodorants, pesticides, herbicides and synthetic intermediates. Because of their patterns of use, a large fraction of the chlorobenzenes produced are released into the environment (Spain 1990). Due to its solubility in water, 3-chlorobenzoate (3CBA) can be found as contaminant in soil, ground and surface water. *Pseudomonas* sp. B13, originally isolated from a sewage-plant, is well-known for its ability to degrade 3CBA (Dorn *et al.* 1974). Such capacity has proved effective under a large concentration range of 3CBA (Tros 1996), and even in a simulated marine environment, different than the one from which it was isolated (Leser *et al.* 1995). This adaptability makes *Pseudomonas* B13 a good candidate to test its degrading performance when inoculated in the rhizosphere of black alder, in a phytoremediaton assay. Phytoremediation encompasses the use of plants for the remediation of contaminated environments. Removal of hazardous wastes can be accomplished by the plant through bioaccumulation, adsorption or stabilisation. Biodegradation of toxic organic compounds is mainly performed by microorganisms, and several of them can take advantage of the rhizosphere effect improving their performance and survival in soil (DOE 1994).

The objectives of this study were: a) to select a nutrient solution that allows growth and dechlorination activity of *Pseudomonas* sp. B13, and growth of *A. glutinosa* plantlets, b) to demonstrate that enhanced dechlorination of 3CBA by *Pseudomonas* sp. B13 occurs under the influence of *A. glutinosa* roots, in a water culture system.

MATERIALS AND METHODS

Nutrient solutions

Five nutrient solutions were tested for their capacity to support plant and *Pseudomonas* sp B13 growth: 1) Low-chloride mineral salts medium [LMM] which was modified from the original formulation (Gerritse and Gottschal, 1992) by increasing the amount of $Ca(NO_3)_2$ to

0.3 g/l. 2) Hoagland's solution [Hoagland] prepared as described by Hoagland and Arnon (1950). 3) Arnon's solution [Arnon] prepared according to Arnon and Hoagland (1940). 4) Medium Z3 modified [Z3mod] prepared as [0.46 g Na_2HPO_4, 0.92 g KH_2PO_4, 0.5 g $MgSO_4$, 0.06 g $Ca(NO_3)_2$, 0.4 g NH_4NO_3, in 1 l demineralised water]. 5) Tap water, 1 l of tap water was amended with 0.01 g KNO_3 and 0.03 g $Ca(NO_3)_2$. The last two nutrient solutions were added with 1 ml of each; the iron and the micronutrients Hoagland's solutions. All the solutions were sterilised according to the respective recipes, or by autoclaving. The final pH of the solutions was adjusted to 6.4 – 6.6 when necessary. The Arnon, Z3mod and Tap water solutions did not include chloride in their formulations, while LMM contained only traces.

Plant material

A. glutinosa seeds (Pelgrum-Vink materialen, Westervoort, the Netherlands) were surface sterilised by soaking for 10 min in a commercial solution of sodium hypochlorite (1% active chlorine) and rinsing several times with sterile demineralised water. The seeds were germinated in the dark under sterile conditions, on a bed of glass beads (3 mm ϕ) covered with demineralised water. Plant-tube growth systems were made up by transferring under aseptic conditions 2-week old plantlets to 25 X 200 mm glass tubes, containing 25 g of glass beads covered to the top with the respective nutrient solution. The plant-tube systems were kept in a growth chamber set up to a photoperiod of 16 h light and 8 h dark; and a thermoperiod of 24°C and 16°C, respectively. The light intensity was 3 500 lux provided by Phillips 33 fluorescent tubes, and the relative humidity was kept at 60%.

Cultivation of Pseudomonas sp. B13

The 3CBA-degrader *Pseudomonas* sp. B13 (Dorn, *et al.* 1974) was routinely cultured in Z3 medium amended with 5 mM 3CBA as sole C- and energy source. Incubation was done on static conditions at 28°C during 2 – 3 days. Inoculum preparation for growth experiments was done by harvesting a 50 ml culture, and rinsing with sterile Z3 medium without C-source. The final suspension was adjusted to an $O.D._{660} = 0.2$.

Nutrient solution selection

The plant-tube growing systems were filled to the top of the glass beads-support with each of the solutions tested (Arnon, Hoagland, LMMmod, Z3mod and Tap water), the liquid level was kept constant by addition of water. The total nutrient solution was changed every two weeks. Five plantlets were used for each solution, and raised as above described during 8 weeks. Plant development was scored visually based on colour and general appearance of the plants.

The same 5 nutrient solutions but amended with 10 mM 3CBA were used as culture medium for *Pseudomonas* sp. B13. Duplicate serum bottles containing 40 ml of each amended medium were inoculated with 0.5 ml of a *Pseudomonas* B13 inoculum. After 5 days incubation the cell density was measured in a spectrophotometer at a wavelength of 660 nm.

3CBA dechlorination under the influence of A. glutinosa roots

Plant-tube growing systems were set up and the plantlets were nourished with Z3mod nutrient solution during 4 weeks, then four treatments were applied consisting of 5 plant-tube systems each. The complete nutrient solution was replaced as follows: a) Z3mod [-X-B13], b) Z3mod inoculated with *Pseudomonas* sp. B13, [-X+B13], c) Z3mod amended with 3CBA [+X-B13], d) Z3mod amended with 3CBA and inoculated with *Pseudomonas* sp. B13 [+X+B13], an extra set of tubes with glass beads but without plant was also added with the latter suspension [NP+X+B13]. The last treatment was used as control to see the possible effect of plants on the bacterial dechlorination activity. All growing systems were kept for six more weeks, and 0.5 ml samples of the nutrient solutions were taken every 3 – 4 days to monitor dechlorination. Release of Cl^- into the medium was measured with a MicroChlor-O-counter (Maurius, Utrecht, the Netherlands), using an external standard of NaCl.

RESULTS AND DISCUSSION

The first objective of this work was dealing with the selection of a nutrient solution that allowed the growth and 3CBA-dechlorination activity of *Pseudomonas* sp. B13, and at the same time could support *A. glutinosa* growth. The solution must be Cl-free to allow monitoring the release of Cl^- into the medium.

Five different solutions were assayed on separate cultures for *Pseudomonas* and *Alnus*. The original LMM and Z3 formulations were developed as bacterial culture media, but modified by increasing the concentration of the N-source and lowering the pH to allow plant development. The Hoagland's and Arnon's solutions were formulated as plant nutrient solutions, addition of 3CBA as C-source might enable them as culture medium for *Pseudomonas* B13. The results are summarised in Table 7.1.

Table 7.1. Growth of *Pseudomonas* sp. B13 (after 5 days) and development of *Alnus glutinosa* plantlets (after 8 weeks) in different culture media. Their appearance was scored from (++++) for a large plant with many leaves, to (++) for small plants with few leaves.

Solution	***Pseudomonas* sp. B13**	***Alnus glutinosa* plantlets**	
	OD_{660}*	**Colour**	**Appearance**
Arnon	0.095 ± 0.009	PALE GREEN	++++
Hoagland	0.168 ± 0.011	GREEN	+++++
LMMmod	0.212 ± 0.006	PALE GREEN	+++
Z3mod	0.265 ± 0.005	PALE GREEN	+++
Tap water	0.012 ± 0.002	YELLOWISH	++

* values are the mean of 2 replicate cultures.

As expected, the best growth of *Pseudomonas* was obtained with the bacterial culture media, while the best-developed *Alnus* plantlets were obtained in the plant nutrient solutions. Therefore, a compromise between bacterial and plant development had to be reached. Although the Hoagland's solution produced the best results in the general appearance of the plant, the presence of Cl^- in their formulation hampered its use. Unfortunately it was found that Arnon's solution, which is a Cl^--free alternative, did not yield a good bacterial growth. Based on plant-bacterial growth balance, the Z3mod medium was chosen for further experiments.

The second objective was to determine the influence of *A. glutinosa* roots on the dechlorination of 3CBA by *Pseudomonas* sp. B13. A plant-liquid culture with support of glass beads was chosen as growth system, which allowed constant monitoring of dechlorination activity and bacterial growth. It was expected that the presence of root exudates might enhance the degradation performance of *Pseudomonas* B13.

In order to have stronger plants able to resist the presence of 3CBA, the black alder plantlets were grown for 4 extra weeks after being transferred to the tube growth systems. In previous assays the 3CBA proved to be highly toxic for the plantlets (data not shown). After the 4 weeks period, there were already differences in the size of the alder plants that may be attributed to differences in the genetic background of the seeds. The groups of 5 plant-tube systems used for each treatment included plants of every size range, in order to make the treatments as comparable as possible. During the next 2 weeks after addition of the chlorobenzoate to the growth systems, few of the smaller plants have died. In the treatment added with 3CBA and non-inoculated with *Pseudomonas* B13 [+X-B13], 3 out of the 5 plants died while in the treatment suplemented with 3CBA and inoculated with the bacteria [+X+B13] two plants were dead. In the former treatment only one plant survived at the end of the experiment. On Figure 7.1 are shown the plant-tube growth systems with some of the surviving plants. Six weeks after the treatments started there were strong differences in the development and general appearance of the plants growing in the absence (-X) or presence (+X) of 3CBA. In the latter treatments the plants inoculated with *Pseudomonas* sp. B13 [+X+B13] not only survived better, but also had a better general appearance compared to those without the bacteria [+X-B13]. These findings suggest a protective effect of *Pseudomonas* B13 to the *Alnus* plants.

Dechlorination of 3CBA was monitored by release of Cl^- into the nutrient solution. Since 3CBA was the only external source of Cl^-, the concentration of this ion in the medium is reflecting an equimolar dechlorination of 3CBA. Comparison of the dechlorination activity (Figure 7.2) occurring in the [+X+B13] and [NP+X+B13] treatments pointed to an increased dechlorination rate in the former treatment that was under the influence of *A. glutinosa* root-system. Although the differences were significant at the beginning of the experiment (i.e. days 6 to 9), later the behaviour of both treatments was more alike, hampering the interpretation as a real enhancement of the dechlorination activity.

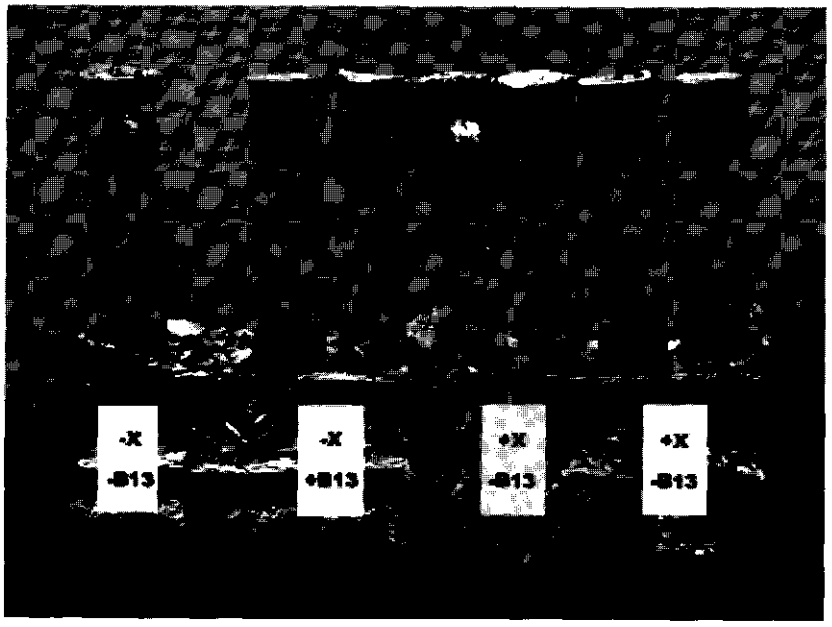

Figure 7.1. Tube growing systems with *A. glutinosa* plants (12 weeks old) at the end of the experiment. At this time only one plant (4th tube from the right) remained alive in the treatment [+X-B13], the small plant next to it was already dead as appreciated by its very dark (brown) colour.

As shown in Figure 7.2, dechlorination occurred under the influence of *A. glutinosa* roots in the inoculated [+X+B13] and even in the non-inoculated [+X-B13] treatment. The rate of Cl^- released is quite similar in both treatments, though most of the values for the latter treatment were based upon one plant that survived the experiment. Alternative explanations for the high dechlorination activity registered early in time may point to a cross-contamination with *Pseudomonas* B13. However, since any 3CBA-degrader could be isolated from the nutrient solutions of that treatment, we consider this option unlikely. Another possibility is to relate the dechlorination activity to this particular *Alnus* plant. Several plants are known to adsorb xenobiotic compounds into their roots (Schwab *et al.* 1998), while others like poplars and alfalfa are able to uptake and transform them (Thompson *et al.* 1998, Gordon, *et al.* 1997, Ferro *et al.* 1997). However, no further evidence of dechlorination activity by this *Alnus glutinosa* plant was obtained. Finally, we may consider that part of the Cl^- is not derived from 3CBA but released from the plant after the prolonged stress conditions, and therefore we should not focus too much on the period after 9 days of incubation.

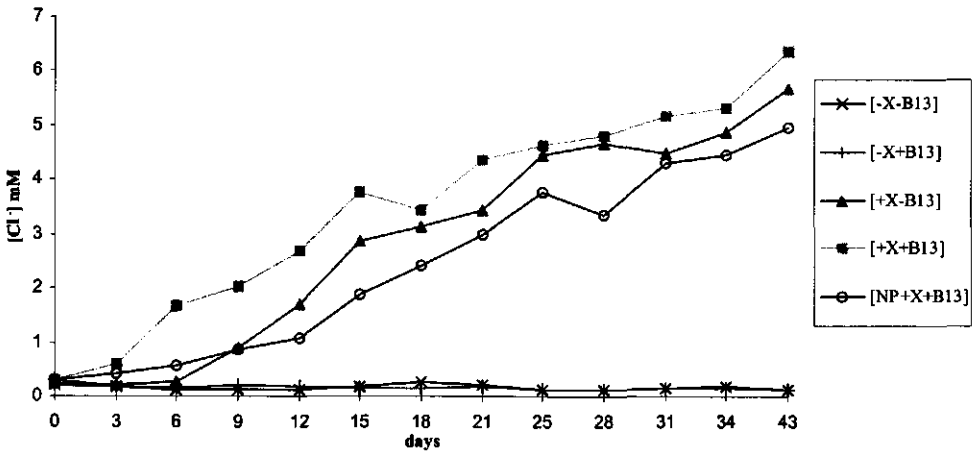

Figure 7.2. Dechlorination of 3CBA under the influence of *A. glutinosa* roots. The Cl^- ions present in the nutrient solution were measured by a titration procedure.

The fact that Cl^- release was measured in all 3 surviving plants on the [+X+B13] treatment, together with a more healthy appearance suggests that the beneficial effect of *Pseudomonas* B13 to the plants goes beyond the detoxification of the chlorobenzoate. Several *Pseudomonas* spp. are known to act as plant-growth promoting bacteria, and one of the proposed growth promoting mechanisms is the production of fytohormones and growth regulators (Lynch 1982). This may also explain the presence of a more developed root system i.e. increased number of secondary and tertiary roots in all inoculated (+B13) plants (data not shown). The nature of the plant-growth promoting activity of *Pseudomonas* sp B13 on *Alnus* remains unknown.

The results obtained with this experimental model point to interesting possibilities in the field of phytoremediation. The combination of xenobiotic-resistant or tolerant plants, with bacteria able to degrade the compound has been postulated as a low-cost and aesthetic means of soil remediation (DOE 1994). This combination would be enhanced if the bacteria were able not only to colonise the roots, but also to produce plant-growth-promoting substances resulting in vigorously growing plants, with highly developed root systems covering larger soil volumes.

CHAPTER 8

SUMMARY AND CONCLUDING REMARKS

Microbial Ecology studies aim to describe and assess the behavior and activity of microorganisms in their natural environments (Brock 1966). Nowadays it is clear that the large number of existing microorganisms has surpassed our capabilities to rapidly characterise them by traditional culturing methods. This has resulted in a poor understanding of the structure and composition of microbial communities. As an alternative, microbial communities can be described on the basis of 16S rRNA sequence diversity, without the bias-introducing step of cultivation.

In the present thesis a molecular analysis is given of two ecosystems that harbour several uncultured bacteria. The first part of the thesis is focused on the detection and characterisation of *Frankia* in actinorhizal nodules and soil. Elucidation of the actual diversity within the family Frankiaceae was hampered by the inability to obtain isolates from all known actinorhizal plants. So far, the Nod^+/Fix^+ *Frankia* symbionts in root nodules of plants from the families Coriariaceae, Datiscaceae, Rosaceae and Rhamnaceae (with exceptions reported by Carú 1993, Carú *et al.* 1990, and Carrasco *et al.* 1995) have resisted isolation. Best opportunities to characterise those uncultured endophytes require molecular methods that relay heavily on an easy and efficient technique to extract DNA from the respective actinorhizal nodules. Chapter 2 describes the techniques to isolate DNA from root nodules of different actinorhizal plants such as *Casuarina* sp, *Alnus* sp and *Ceanothus* sp. The procedure has also been successfully applied by Wolters *et al.* (1997b) in the minuscule ineffective nodules on *Alnus glutinosa*.

Several attempts to characterise the uncultured endophytes from *Coriaria* sp. and *Datisca* sp. plants pointed on the one hand, to the presence in those actinorhizae of *Frankia*-related actinomycetes. This assumption was based mainly on the repeated isolation from those nodules of Nod^-/Fix^- *Frankia*-like strains (Hafeez 1983, Mirza *et. al.* 1994b, c). On the other hand, the effective (Fix^+), non-isolated symbionts showed to be phylogenetically closely related (Mirza *et al.* 1993a), forming a separate lineage within the genus, in spite of the distant geographical distribution of the plants (Nick *et al.* 1992).

The work described in Chapter 3 is focussed on the localisation and phylogenetic position of the nitrogen-fixing *Frankia* and Nod^-/Fix^- actinomycetes, both present in root nodules of the Mexican actinorhizal plant *Ceanothus caeruleus*. Application of the TGGE technique allowed localising the Nod^-/Fix^- actinomycete in the outer layers of the *C. caeruleus* nodules. Similar bacteria were also detected in *Hippophaë rhamnoides* nodules induced with soil inoculum that was collected in the vicinity of the former plant. The fact that a second nodule inhabitant was commonly present in these nodules containing recalcitrant endophytes may allow some speculations about their possible role in the symbiosis. However, it seems worthwhile to apply the same TGGE methodology to other actinorhizal nodules, even to those containing *Frankia* strains that are easy-to-isolate (i.e. *Elaeagnus, Casuarina, Alnus* spp.), since the detection of *Frankia*-related actinomycetes, in addition to the Fix^+ endophyte, would provide further evidence about the need for their presence. Coincidentally, the Nod^-/Fix^- isolates from *Coriaria, Datisca* and *Ceanothus* are phylogenetically related, pointing again to a certain specificity for

their presence in the nodules. This relatedness has also been supported by analysis of low molecular weight RNA (i.e. 5S rRNA and tRNA's) using staircase electrophoresis (Velázquez *et al.* 1998).

The 16S rDNA sequence from the non-isolated Fix^+ endophyte in *C. caeruleus* root nodules (Chapter 3), was the first full sequence obtained from a field-collected *Ceanothus* symbiont. Parsimony and phylogenetic distance analyses grouped it within the *Dryas* cluster that originally contained only the uncultured endophytes from *Dryas*, *Coriaria* and *Datisca* as proposed by Normand *et al.* (1996). Benson *et al.* (1996) redefined this cluster by adding other uncultured endophytes present in *Ceanothus griseus* (Rhamnaceae), *Purshia tridentata* and *Dryas drummondii* (Rosaceae) root nodules. Since the determined partial 16S rDNA sequences were almost identical, they suggested that the *Frankia* diversity from these actinorhizal plant families might be low. However, Clawson *et al.* (1998) demonstrated that *Frankia* isolates obtained from several genera within the Rhamnaceae (i.e. *Talguenea*, *Colletia*, *Discaria*, *Retanilla* and *Trevoa*) were phylogenetically different than those in *Ceanothus*, grouping in the *Elaeagnus* cluster. These findings were consistent with morphological differences of the endophytes *in planta*, since the vesicles found in the *Ceanothus* symbionts resemble more to those in the Rosaceae, while all the latter host plants in the Rhamnaceae family have endophytes like those in *Elaeagnus*.

The results reported in the first part of the thesis have demonstrated that TGGE and sequence analysis of 16S rDNA provide an accurate picture for the identification of recalcitrant endophytes in root nodules of actinorhizal plants. It has also been demonstrated that besides the N_2-fixing endophyte, root nodules of *C. caeruleus* also harbour *Frankia*-related actinomycetes. Since these have also been observed in other actinorhizae, a further study is needed to understand the possible function of these co-symbionts.

The work described in the second part of the thesis was addressing the changes occurring under chlorobenzoate stress in the soil bacterial community and other selected groups of bacteria present in peat soil collected from a natural *Alnus glutinosa* stand. A combination of culturing and non-culture based approaches was used for the assessment. Among the latter approaches, the possibilities offered by TGGE were exploited in several ways. Profiling of complex communities and subsequent analysis of specific bacterial groups has been one of the major applications of TGGE (Felske *et al.* 1996). With this approach, major population shifts induced by either 3CBA or 2,5DCB were detected in the uncultured bacterial community (Chapter 4). Although only the former compound was readily metabolised in soil, both xenobiotics promoted similar changes. Several bacterial populations were reduced or suppressed, while few others were enriched in time, as assessed by shifts in the TGGE banding patterns of the total bacterial community. To characterise the soil-enriched bacterial populations, 3CBA-degrading enrichment cultures were obtained and their composition was addressed by TGGE. Further isolation attempts were directed by this means to prove that the isolated strains were indeed the same enriched organisms as detected in soil. One of the enrichment cultures contained two of the soil-enriched bacteria as predominant components.

Although isolation was not achieved, both bacteria were identified as belonging to the genus *Burkholderia*. The bacterial group detected as predominantly enriched in both spiked soils was not present in any of the enrichment cultures, suggesting that the microorganisms belonging to this groups are either unable to degrade 3CBA or not growing under the used culturing conditions. In any case their fitness to the soil conditions imposed by the addition of chlorobenzoates was high, but the mechanisms involved were not elucidated. These bacteria were also identified as *Burkholderia* by partial 16S rDNA sequence analysis (Chapter 4).

The diversity (H) and the equitability (J) indices are important parameters used by ecologists to assess the species richness and the species evenness, respectively, within a community. As the estimation of such indices relies heavily on species definition and individuals enumeration, their application in microbial ecology studies is seldomly possible. Furthermore, assessment of H and J in uncultured bacterial communities must rely on the interpretation of community fingerprints, which should provide means to distinguish between species or operational taxonomic units (OTU), and to estimate their abundance. TGGE community profiling offers both possibilities, and the community changes occurring in the model soil system were evaluated with this original approach (Chapter 5). In addition, H and J indices were also estimated for the fluorescent pseudomonads group, a selected culturable fraction of the bacterial community. OTU recognition was addressed by using TGGE as a ribotype-fingerprinting technique for the isolated fluorescent pseudomonads. Estimation of H and J at the community level without culturing by TGGE profiling, and at the group level by a combination of culturing and TGGE ribotyping should allow to address and compare the population changes occurring, since the target molecule used in both TGGE was the same. Such comparison was only partially possible since most of the bands corresponding to the fluorescent pseudomonads could not be assessed in the community profiles. However, estimation of H and J indices indicated a clear reduction of species richness and individuals abundance in the uncultured community, which was related to the presence of chlorobenzoates in soil. Evaluation of population shifts by indexed values as H and J proved to be a useful means for analysing the community structure in time, and may be used to assess short and long-term responses of a bacterial community to environmental perturbations.

Chapter 6 describes the changes in the total frankiae in soil and in the fraction of the population that is able to produce root nodules in *Alnus glutinosa* seedlings. Culture-independent approaches based on the most probable number concept were used, one in combination with a *Frankia*-specific PCR detection and another in combination with a plant-nodulation bioassay. After 15 days of incubation in the presence of chlorobenzoates both fractions of the soil *Frankia* populations were reduced in more than one order of magnitude, while the populations in the unspiked control soil were not affected. The results indicated that 3CBA and 2,5DCB both had a negative effect on the size of the native *Frankia* population from the used peat forest soil. This negative effect was also evident during *in vitro* experiments using *Frankia* strains isolated from *Alnus* sp. The presence of 1 mM 3CBA in the culture medium, in addition to the normal carbon source, resulted in reduction or suppression of biomass yield.

The influence of *Alnus glutinosa* on the dechlorination of 3CBA by *Pseudomonas* sp. strain B13 was assessed in hydroponic cultures. It was expected that root exudates could enhance the dechlorination activity of *Pseudomonas* B13. When the bacteria were incubated in the presence of an alder plant, only a slight increase in the dechlorination rate of 3CBA was registered in comparison to the control without plant. The main observed effect in the alder plants appears to be a protection against 3CBA toxicity, as the alders inoculated with *Pseudomonas* B13 showed a better survival rate and grew more vigorously than the non-inoculated plants.

The toolbox for microbial ecology studies is increasing constantly by means of developing new techniques or by adapting foreign tools into the field, such as indices to evaluate species diversity and eveness. Although the information provided by these community parameters facilitates comparisons and assessment of changes, their suitability to evaluate bacterial communities is still uncomplete. Estimation of diversity indices requires the recognition of bacterial species as discrete units, and this condition is far from real in natural environments. Species-independent approaches to evaluate diversity must be developed, that consider the bacterial diversity as a continuous range of phylogenetically related taxonomic units.

In conclusion, the work described in the first part of the thesis strengthen the current phylogenetic division of the Frankiaceae, by adding new evidence supporting two of the already described clusters (*Dryas* cluster, Nod^-/Fix^- cluster). Although the current taxonomic status of the latter cluster must be better evaluated in order to assess its pertinence to the genus *Frankia*. In addition, the common occurrence of Nod^-/Fix^- *Frankia*-like actinomycetes in nodules containing recalcitrant endophytes, mainly from the *Dryas* cluster, was also demonstrated for *Ceanothus* actinorhiza. In the second part, the suitability and versatility of molecular tools such as TGGE were demonstrated by their application in community profiling and estimation of bacterial diversity. Community changes occurring in stressed and unstressed soil systems were easily detected and assessed by this means. In addition, specific populations such as the *Burkholderia*-like bacteria that strongly reacted to the addition of chlorobenzoates to soil were further characterised. Moreover, TGGE was shown to be a fast ribotyping technique that may enable its use in combination with the community profiles to address shifts of specific groups within bacterial communities. It is tempting to suggest that this general approach will be of importance to direct the isolation of hitherto uncultured bacteria from soil.

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SAMENVATTING*

Microbieel ecologische studies hebben tot doel het gedrag en de activiteit te beschrijven en te voorspellen van micro-organismen in hun natuurlijke omgeving. Vanwege het grote aantal verschillende en grotendeels nog onbekende micro-organismen in het milieu was het vrijwel onmogelijk de samenstelling van de microbiële gemeenschap te beschrijven met behulp van conventionele microbiologische technieken. Hierdoor bleef de kennis van de structuur en samenstelling van microbiologische leefgemeenschappen beperkt. Recentelijk is het mogelijk geworden de samenstelling van microbiële gemeenschappen ook te beschrijven op basis van 16S rDNA basenvolgorde (=DNA sequentie) diversiteit, zonder de langdurige (om)weg van kweken. In dit proefschrift wordt een moleculaire analyse gegeven van twee ecosystemen waarin niet te cultiveren (=recalcitrante) bacteriën voorkomen. Het eerste deel van het proefschrift richt zich op de detectie en karakterisering van *Frankia* in wortelknolletjes en in grond. De opheldering van de diversiteit binnen de familie Frankiaceae werd bemoeilijkt door de onmogelijkheid isolaten te verkrijgen van alle bekende 'actinorhizae' planten (= planten die wortelknollen vormen met stikstofbindende actinomyceten uit de *Frankia* groep). Tot dus verre was het niet mogelijk wortelknolvormende, stikstofbindende (Nod^+/Fix^{+}) endofieten als reincultuur te kweken uit wortelknollen van de plantenfamilies Coriariaceae, Datiscaceae, Rosaceae en Rhamnaceae (met uitzonderingen beschreven door Carú 1993, Carú *et al.* 1990, en Carrasco *et al.* 1995). Karakterisering van deze niet-cultiveerbare endofieten vereiste daarom methoden waarmee op een eenvoudige en efficiënte manier DNA uit de door deze micro-organismen gevormde wortelknollen kan worden geëxtraheerd. Hoofdstuk 2 beschrijft de technieken om DNA te isoleren uit wortelknollen van de verschillende 'actinorhizae' planten, zoals *Casuarina* sp, *Alnus* sp, *Ceanothus* sp. Deze procedure werd ook met succes toegepast op de minuscule ineffectieve knolletjes van *Alnus glutinosa* (Wolters *et al.* 1997b).

Verscheidene pogingen om de recalcitrante endofieten uit wortelknollen van *Coriaria* spp. en *Datisca* spp. te karakteriseren leidden tot de isolatie van *Frankia*-achtige Nod^-/Fix^- actinomyceten (Hafeez 1983, Mirza *et al.* 1994 b,c). De effectieve (Fix^+), niet te isoleren microsymbionten bleken fylogenetisch nauw verwant (Mirza *et al.* 1993a) en vormden een afzonderlijke groep binnen het genus ondanks de verschillende geografische verspreiding van de planten (Nick *et al.*1992).

In hoofdstuk 3 wordt de lokalisatie en de fylogenie beschreven van de stikstofbindende endofiet *Frankia* en hieraan verwante Nod^-/Fix^- actinomyceten in wortelknollen van de Mexicaanse plant *Ceanothus caeruleus*. Met behulp van de TGGE techniek werd aangetoond

* Voetnoot van de auteur: met speciale dank aan Ans Geerling voor haar nauwkeurige en snelle vertaling.

dat de Nod⁻/Fix⁻ actinomyceet zich in de buitenste laag van de *C. caeruleus* knollen bevond. Verwante bacteriën werden ook aangetoond in *Hippophaë rhamnoides* knollen die waren geïnduceerd met grondent-materiaal dat was verzameld in de omgeving van *Ceanothus caeruleus*. Het feit dat een tweede 'knolbewoner' algemeen aanwezig was in de wortelknollen doet vermoeden dat deze een nog onbekende rol speelt in de symbiose met moeilijk te isoleren *Frankia* stammen. In verband hiermee lijkt het zinvol dezelfde TGGE techniek toe te passen op de wortelknollen die makkelijk te isoleren *Frankia* stammen (zoals *Elaeagnus, Casuarina, Alnus* spp.) bevatten. Toevalligerwijs, zijn de Nod⁻/Fix⁻ isolaten van *Coriaria, Datisca* en *Ceanothus* fylogenetisch verwant, wat weer duidt op een zekere specificiteit voor hun aanwezigheid in de knollen. Deze relatie wordt ook gesteund door analyse van 5SrRNA en tRNA's met behulp van 'staircase' electroforese (Velázquez *et al.* 1998).

De in hoofdstuk 3 beschreven 16S rDNA sequentie van de recalcitrante Fix⁺ endofiet in *C. caeruleus* wortelknollen, was de eerste volledige sequentie verkregen van een uit het veld verzamelde *Ceanothus* symbiont. Met de parsimonie en fylogenetische afstand analysemethoden groepeerde de sequentie in het door Normand *et al.* (1996) voorgestelde *Dryas* kluster. Deze cluster bevatte aanvankelijk alleen de niet kweekbare endofieten van *Dryas, Coriaria* en *Datisca* spp. Benson *et al.* (1996) herdefinieerde dit cluster door andere niet cultiveerbare endofyten uit *Ceanothus griseus* (Rhamnaceae), *Purshia tridentata* en *Dryas drummondii* (Rosaceae) wortelknollen toe te voegen. Daar de partiele sequenties van het 16S rDNA binnen de cluster vrijwel identiek waren, mag voorlopig aangenomen worden dat de diversiteit van *Frankia* in wortelknollen van de genoemde groep planten gering is. Weliswaar demonstreerde Clawson *et al.* (1998) dat *Frankia* isolaten verkregen uit verschillende genera binnen de Rhamnaceae (namelijk *Talguenea, Colletia, Discaria, Retanilla* en *Trevoa*) fylogenetisch verschilden van die uit *Ceanothus* wortelknollen en gegroepeerd bleken in het *Elaeagnus* cluster. Deze ontdekkingen kwamen overeen met de morfologische verschillen tussen de endofieten *in planta*: de blaasjes (vesicles) van de *Ceanothus* symbionten lijken meer op die bij Rosaceae, terwijl al de laatstgenoemde gastheerplanten binnen de Rhamnaceae familie endofieten hebben met blaasjes zoals die in *Elaeagnus* knollen voorkomen.

De resultaten die in het eerste deel van dit proefschrift zijn beschreven hebben aangetoond dat de identificatie van de endofieten in wortelknollen van 'actinorhizae' planten snel en nauwkeurig kan worden uitgevoerd door middel van een TGGE fingerprint en sequentie analyse van het 16S rDNA. Bovendien werd aangetoond dat wortelknollen van *Ceanothus caeruleus* behalve de stikstofbindende endofiet ook nog een aan *Frankia* verwante actinomyceet bevatte. Daar deze bacteriën ook in wortelknollen van andere 'actinorhizae' planten aangetroffen werden, is verder onderzoek naar de mogelijke functie van deze co-symbionten gewenst.

In het tweede deel van dit proefschrift wordt het microbiologisch onderzoek beschreven naar de effecten van chlorobenzoaat stress op de samenstelling van de bacteriële gemeenschap in de grond. Het onderzoek werd uitgevoerd met veengrond afkomstig van een natuurlijke *Alnus glutinosa* locatie waarin *Frankia* algemeen aanwezig was. Het effect van de milieuvreemde verbindingen werd bepaald met conventionele kweekmethoden en met

moleculaire analyse van het 16S rDNA. De mogelijkheden die TGGE analyse bieden werden op verschillende manieren toegepast. Een van de belangrijkste toepassingen van TGGE is het beschrijven van de complexe samenleving en de vervolganalyse van de specifieke groepen van bacteriën (Felske *et al.* 1996). Met deze methode konden de belangrijkste populatie verschuivingen, geïnduceerd door de chloorbenzoaten 3CBA of 2,5DCB, worden beschreven aan de hand van verschuivingen in het TGGE bandenpatroon. Ondanks dat alleen 3CBA makkelijk werd afgebroken, bevorderde beide xenobiotica vergelijkbare veranderingen in de bacteriegemeenschap. Een aantal bacteriepopulaties werden gereduceerd of onderdrukt, terwijl enkele anderen zich in de loop van de tijd uitbreidden. Om de in de grond verrijkte bacteriepopulaties te karakteriseren, werden 3CBA afbrekende ophopings-cultures gemaakt en werd de bacteriesamenstelling door middel van TGGE bekeken. Verdere isolatie pogingen werden hierdoor gestuurd door te bewijzen dat de geïsoleerde stammen dezelfde waren als de bacteriestammen die in de grond groeiden. Een van de ophopings-cultures bevatte twee bacteriën die ook in de behandelde grond voorkwamen. Ondanks dat de bacteriën niet in reinculturen werden verkregen, werden beiden geïdentificeerd als *Burkholderia*. De bacteriën die in beide behandelde gronden ophoopten, waren niet aanwezig in de ophopings-cultures. Hieruit kan worden geconcludeerd dat deze organismen of niet in staat zijn 3CBA af te breken of dat de kweekcondities niet geschikt waren voor de groei van deze bacterie. In ieder geval blijkt de aanpassing aan de grondcondities, opgewekt door de toevoeging van chlorobenzoaten, groot, hoewel het mechanisme dat dit veroorzaakt onopgehelderd blijft. Door middel van partiële 16S rDNA sequentie bepaling werd aangetoond dat deze bacteriën ook tot het geslacht *Burkholderia* behoren (hoofdstuk 4).

De diversiteit (H) en de 'equitability' (J) indices zijn belangrijke parameters die worden gebruikt door ecologen om de soortenrijkdom en het voorkomen van soorten te beschrijven. Daar de schatting van deze indices sterk afhangt van de definitie van het soortbegrip en van het bepalen van aantallen cellen is de toepassing in microbieel ecologische studies veelal niet mogelijk. Verder is de bepaling van H en J van niet opgekweekte bacteriepopulaties afhankelijk van de interpretatie van de DNA fingerprints van de bacteriegemeenschap. In de fingerprints moet onderscheid gemaakt kunnen worden tussen soorten of, meer neutraal, operationele taxonomische eenheden (OTU), en moet hun voorkomen gekwantificeerd kunnen worden. De TGGE fingerprints van het 16S rDNA boden beide mogelijkheden en de optredende populatieveranderingen in de grond konden worden geëvalueerd (hoofdstuk 5). Tevens werden de H en J indices bepaald van fluorescente pseudomonaden, welke als relatief gemakkelijk te kweken bacteriegroep was gekozen. De isolaten van de fluorescerende pseudomonaden werden ingedeeld als 'ribotype' op grond van de verschillende bandposities van 16S rDNA fragmenten in de TGGE. Schattingen van H en J indices op het niveau van de gemeenschap zonder te kweken door TGGE profielen, en op groepsniveau door een combinatie van kweken en TGGE ribotypering, maakten het mogelijk populatieveranderingen te beschrijven. Dit kan omdat in beide TGGE's vergelijkbare delen van het 16S rDNA werden geanalyseerd. Deze vergelijking was slechts gedeeltelijk mogelijk omdat de meeste banden die corresponderen aan de fluorescente pseudomonaden niet zichtbaar waren in de populatie profielen. De schatting van H

en J toonden echter duidelijk aan dat het toevoegen van chloorbenzoaten aan de grond resulteerde in een sterke afname van de soortenrijkdom en individuele aantallen bacteriën. Hiermee werd aangetoond dat het bepalen van deze indices een goed bruikbare manier is om een populatiestructuur in de tijd te volgen en effecten van milieuverstoringen op de bacteriegemeenschap te beschrijven.

In hoofdstuk 6 werd in het hierboven beschreven grondmodel het effect bestudeerd van chloorbenzoaten op de *Frankia* populaties in de grond en het deel hiervan dat bij *Alnus glutinosa* wortelknollen vormde. In beide gevallen was de detectiemethode van *Frankia* gebaseerd op het meest waarschijnlijke aantal ('most probable number'). Bij de eerste bepaling werd een verdunningsreeks van bodem-DNA als target gebruikt voor de amplificatie van 16S rDNA met *Frankia*-specifieke primers. Bij de tweede bepaling werd een verdunningsreeks van grond als entstof voor elzenplanten gebruikt in een biotest. Na een incubatie van 15 dagen in de aanwezigheid van chloorbenzoaten bleek in beide bepalingen de *Frankia* populatie sterk gereduceerd, terwijl de populaties in de onbehandelde grond gelijk gebleven waren. De resultaten tonen aan dat zowel 3CBA als 2,5DCB een negatief effect op de grootte van de oorspronkelijke *Frankia* populatie hebben in de onderzochte veengrond. Dit negatieve effect werd ook duidelijk tijdens *in vitro* experimenten met *Frankia* stammen geïsoleerd uit *Alnus* sp. De aanwezigheid van 1 mM 3CBA in het kweekmedium met propionaat als koolstofbron, resulteerde in een sterke reductie van opbrengst.

Naast de analysen van de effecten van chloorbenzoaten op de bacteriegemeenschap in de grond werd de mogelijke invloed van *Alnus glutinosa* op de dechlorering van 3CBA bestudeerd. In hoofdstuk 8 werd beschreven in welke mate *Alnus glutinosa* planten in vloeibare cultures beschermd kunnen worden tegen chloorbenzoaten door toevoegen van een dechlorerende *Pseudomonas* sp. stam B13. Uit vooronderzoek was gebleken dat 3CBA reeds bij lage concentratie toxisch is voor de kiemplanten van de els. Verwacht werd dat wortelexudaten de dechloreringsactiviteit van *Pseudomonas* B13 zouden kunnen versterken. Wanneer de bacteriën samen met elzenplaten geïncubeerd werden in 3CBA bevattend medium, werd slechts een kleine toename van de dechlorering van 3CBA gevonden, in vergelijking met de controle zonder plant. Uit de groeiproeven bleek dat elzen geënt met de *Pseudomonas* B13 een betere overlevingskans bezaten en beter groeiden dan de niet geënte planten. Hiermee werd aangetoond dat dechlorerende bacteriën door afbraak van 3CBA de plant een bescherming kunnen bieden.

De mogelijkheden voor microbieel ecologisch onderzoek nemen steeds verder toe door de ontwikkeling van nieuwe technieken en door de aanpassing van methoden uit andere onderzoeksgebieden, zoals het gebruik van diversiteitindices. Hoewel hiermee populatieverschuivingen als gevolg van milieuveranderingen kunnen worden bepaald is de bruikbaarheid nog verre van compleet. Voor de schatting van de diversiteit moeten de bacteriesoorten als afzonderlijke eenheden herkend kunnen worden, en deze conditie is verre van reëel in een natuurlijk milieu. Soort-onafhankelijke methoden om diversiteit te vast te stellen moeten ontwikkeld worden, waarbij de bacteriële soortenrijkdom beschouwd wordt als een continue reeks van fylogenetisch verwante taxonomische eenheden.

Samenvattend kan worden gesteld dat het werk dat in het eerste deel beschreven staat de huidige fylogenetische indeling van de Frankiaceae ondersteunt, door nieuw bewijsmateriaal toe te voegen aan twee al eerder beschreven clusters (*Dryas* cluster, Nod$^-$/Fix$^-$ cluster). Verder onderzoek zal uit moeten maken of de Nod$^-$/Fix$^-$ cluster binnen het genus *Frankia* gehandhaafd kan worden. Tevens werd aangetoond dat de Nod$^-$/Fix$^-$ *Frankia*-achtige actinomyceten niet alleen voorkomen in wortelknollen met recalcitrante endofieten uit de *Dryas* cluster, maar ook wortelknollen van *Ceanothus caeruleus*. In het tweede deel werd de geschiktheid en de veelzijdigheid van moleculaire technieken zoals TGGE aangetoond als methoden om diversiteit van een bacteriegemeenschap te beschrijven. De methoden werden toegepast om populatieveranderingen te bepalen als gevolg van milieustress. Aangetoond werd ook dat de TGGE methode als een snelle ribotyperingstechniek geldt voor *Burkholderia* stammen die sterk reageren op de toevoeging van chloorbenzoaten aan grond. In combinatie met TGGE profielen van de gehele bacteriegemeenschap kunnen hiermee snel verschuivingen van bepaalde groepen organismen zichtbaar gemaakt worden. Het is te verwachten dat deze analyse van bacteriegemeenschappen van grote waarde zal zijn bij het gericht ophopen en isoleren van bacteriën.

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Wageningen, NL. May, 1999.

CURRICULUM VITAE

Hugo César Ramírez Saad was born in Mexico city on the 23th March, 1957, and after few years of illiteracy he started to attend public schools in his home town according to a tradition in the family. He studied Biology with an orientation to the management of aquatic natural resources, at the Universidad Autónoma Metropolitana-Xochimilco. After a short and unrewarding professional experience, he decided to move into Microbiology under the guidance of Horacio Sandoval. It was under this scientific influence that he learned about actinomycetes, and French food and wine. Latter he made a M.Sc. and started to work with *Frankia* under the supervision of María Valdés.

Already at a very early age he felt very much inclined to know, probably influenced by his initial readings of The Bible (Gen 4:1, 4:17, 4:25; Jdg 19:25; Mat 1:25). This idea was ripened for a long time and at a mature age he decided to pursue a PhD, as one of the most politically correct expressions of knowing. The results of this last venture are summarised in the present thesis.

After July 1999, he will resume his position as lecturer at the UAM-X in Mexico City. His address will be the following:

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