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Cover figure: *In situ* hybridisation on a nodule homogenate of *Alnus incana* with fluorescently (CY3)-labeled oligonucleotide probe Eub338 targeting Bacteria and corresponding epifluorescence micrograph after DAPI staining (top). Bar represents 5 μm.

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To Ykä and Saku

In all cases it will be evident
that the more we know,
the more we realize what
we still have to understand.

-Quispel-

Contents

List of original publications

ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which in the text are referred to by their Roman numerals.

I **Elo, S., Maunuksela, L., Salkinoja-Salonen, M., Smolander, A. and Haahtela, K.** 2000. Humus bacteria of Norway spruce stands: plant growth promoting properties and birch, red fescue and alder colonizing capacity. FEMS Microbiol. Ecol. 31:143-152.

II **Maunuksela, L., Zepp, K., Koivula, T., Zeyer, J., Haahtela, K. and Hahn, D.** 1999. Analysis of *Frankia* populations in three soils devoid of actinorhizal plants. FEMS Microbiol. Ecol. 28: 11-21.

III **Maunuksela, L., Hahn, D and Haahtela, K**. 2000. Effect of freezing of soils on nodulation capacities of total and specific *Frankia* populations. Symbiosis 29: 107-119.

IV **Maunuksela, L., Hahn, D and Haahtela, K.** Comparative analysis of a 23S rRNA insertion of cultured and uncultured frankiae of the *Alnus* host infection group. (Manuscript).

ABBREVIATIONS

bp	base pair
CAS	chrome azurol S
DAPI	4´6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
ERIC	enterobacterial repetitive intergenic consensus
FAME	fatty acid methyl esther
gln	glutamine synthetase
gu	genomic units
HCN	hydrogen cyanide
HIG	host infection group
IAA	indole-3-acetic acid
IGS	intergenic spacer
MPN	most probable number
nif	nitrogen fixation
nu	nodulation units
PC	propionate-casaminoacid
PCR	polymerase chain reaction
Rep	repetitive extragenic palindromic
RFLP	restriction fragment length polymorphism
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
RNA	ribonucleic acid
Sp+/Sp-	spore-positive/spore-negative
UPGMA	unweighted pair group method with arithmetic means

1. Introduction

1.1 Soil and rhizosphere microbes

Soil is a complex habitat where a large number of different microorganisms including bacteria, fungi, protozoa and algae interact. Only 1% to 10% of the soil microbiota can be cultured so there is still much to learn about soil as an environment for microbial life. Bacteria are by far the most numerically abundant soil microorganisms. They can be found free-living or attached to the surface of soil particles in bulk soil, but a large number of soil bacteria also interact with the roots of plants, in what is termed the rhizosphere.

The rhizosphere is frequently divided into the endorhizosphere, the rhizoplane and the ectorhizosphere (Lynch 1990). These respective compartments encompass the root tissues, the root surface and associated soil. Soil further away from the rhizosphere is often termed as bulk soil. Root symbiotic mycorrhizal fungi are also important since they provide a link between bulk soil and plant roots in the mycorrhizosphere (Linderman 1988). Observations have shown that the concentration of bacteria found around the roots of plants is generally much greater than in the surrounding soil and that the rhizosphere supports higher microbial growth rates and activities as compared to the bulk soil (Söderberg and Bååth 1998). One of the main reasons for these higher growth rates is the increased availability of soluble organic compounds that results from plant root exudation. These are typically carbohydrate monomers, amino acids and sugars, but the composition and quantity of root exudates varies depending on plant species (Smith 1976) and abiotic conditions such as water content and temperature (Martin and Kemp 1980). In turn, rhizosphere microorganisms increase root exudation through production of plant hormones or more directly by physically damaging the roots (Grayston et al. 1996). In general, the nutrient-rich rhizosphere is naturally colonized by many beneficial or pathogenic bacteria and fungi which may have a considerable impact on plant growth, development and productivity. The numerous interactions between bacteria, fungi and roots may have beneficial, harmful or neutral effects on the plant, the outcome being dependent on the type of symbiont interaction and the soil conditions (Fig. 1) (e.g. Lynch 1990; Smith and Read 1997).

1.1.1 Microbes under trees in low temperature boreal forest soils

Coniferous trees are estimated to transfer 60-70% of their assimilated carbon below-ground as rhizodeposition supporting growth of root associated bacteria (reviewed in Grayston et al. 1996). Apart from root associated bacteria, mycorrhizal fungi are also present and play an important role in plant nutrient and water uptake and in protecting roots from plant pathogens (Smith and Read 1997). Boreal forest trees are nearly exclusively ectomycorrhizal and infection specificity varies from host specific to broad host-range fungi (Smith and Read 1997). Furthermore, infection may be affected by “mycorrhization” helper bacteria (Garbaye 1994). Shishido et al. (1996) suggested that a metabolic interaction between mycorrhizae and PGPR *Pseudomonas* strains resulted in enhanced growth promotion of pine. Studies on the interactions between Scots pine (*Pinus sylvestris*) and rhizosphere bacteria showed that forest - and nursery -soil derived bacterial communities in defined *Suillus bovinus* and *Paxillus*

involutus mycorrhizospheres clearly differed in their species composition and physiological traits (Timonen et al. 1998).

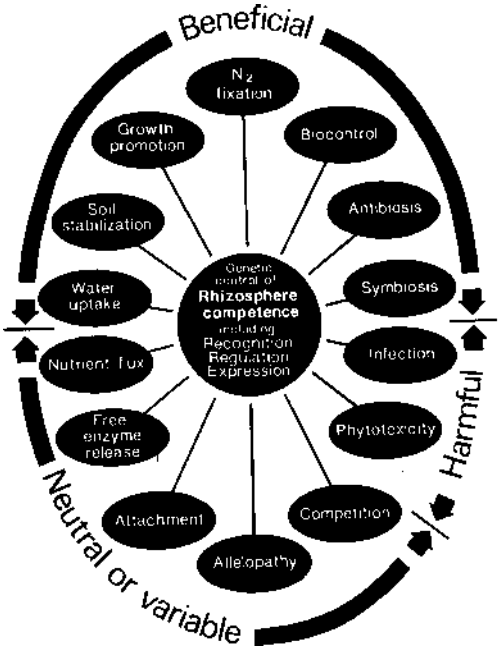

Figure 1. The associations between rhizosphere organisms and plant roots (Lynch 1990).

Boreal forest soils are classified as podzols consisting of an upper organic rich humus (mor) layer (Lundström et al. 2000). Compared to mineral soils, these forest soils contain considerable amounts of organic matter. However, this organic matter is relatively unavailable as a carbon source since it is humified to humus by a slow, chemical process (Paul and Clark 1996). Boreal forest soils are also naturally acidic, the pH of the humus layer in Finnish forest soils ranges from 3.4 to 5.8. In boreal forest soils with high organic matter content and low pH, nitrogen is the main limiting nutrient (Tamm 1991). Conifers, especially spruce, may reduce soil pH, whereas birch may raise pH and improve soil nutrient status (Priha and Smolander 1999). Furthermore, soils under birch (*B. pendula, B. pubescens*) have been shown to possess a more active and richer microflora than those under Scots pine (*Pinus sylvestris*) and especially Norway spruce (*Picea abies*) (Priha and Smolander 1999). This is probably due to more favourable temperature and light conditions in birch stands and the fact that birch litter is more easily decomposed than spruce litter. Birch roots usually penetrate deeper than those of pine, and especially spruce (Laitakari 1929; Laitakari 1935) which may be another factor. Tree species composition also influence the microbial community structure which may reflect differences in carbon rhizodeposition. Birch stands favoured the growth of gram-negative

bacteria, whereas in pine and spruce stands gram-positive species were more common (Priha et al. 2001).

There has been an increased pressure for reforestration of agricultural fields due to agricultural overproduction in central Europe. For successful reforestration, knowledge on soil and root microbial interactions is an essential prerequisite. In a study by Priha and Smolander (1997) no tree-specific changes in soil microbial biomass and activities could be found in birch, spruce and pine soils from afforestration sites in former agricultural fields where Scots pine, Norway spruce or Silver birch had been planted 24 years earlier. This contrasted with clear host species-specific differences in microbial activities found in birch, spruce and pine forest soils. Understorey vegetation can influence soil microbes and their activity, but very little is known about the rhizosphere bacteria of herbaceous plants growing on the forest floor. These bacteria may have an important role as a microbe pool in the early phases of tree development and the plant growth promoting properties of boreal coniferous forest bacteria may be of importance when replanting for forest regeneration. In the same study on spruce (Priha and Smolander (1997), comparison of understorey vegetation at these originally similar field afforestration sites showed differences under the different tree species. Ground vegetation consisted mainly of grasses and herbs under birch and pine whereas mosses or no vegetation with a thick layer of needles were found under spruce.

Unlike in most studied sites, the climate in Finland is characterized by long cold winters which results in soil freezing for a period of several months every year. Kubin (1983) showed that in Finnish boreal forest soils, a considerable portion of the annual decomposition of spruce litter takes place during the summer months, but that decomposition also occurs during winter. Furthermore, a thick snow cover often prevents the soil from freezing throughout the winter (Kurka 2000). Nevertheless, little is known about the presence and function of rhizosphere microbes in these low-temperature boreal forest soils. Previous studies on the nitrogen-fixing actinomycete *Frankia* in Finnish forest soils (Smolander 1990; Smolander *et al.* 1990; Smolander and Sarsa 1990) and the bacterial populations in pine mycorrhizospheres in forest humus (Timonen et al. 1998) have mainly applied basic physiological tests and electron microscopy techniques. Due to the difficult nature of boreal forest soils with a high organic matter content containing humic acids, the application of molecular techniques to study these soils remains difficult.

Other studies on rhizosphere bacteria of forest trees include investigations on actinomycetes isolated from roots of Douglas-fir seedlings as agents for biological control of soil-borne fungal pathogens (Axelrood et al. 1996). Interactions between *Pinus radiata* and rhizosphere bacteria has been studied by Garbaye and Bowen (1989) and with a hybrid spruce host (*Picea glauca* x *P. engelmannii*) by Shishido et al. (1999). Shishido et al. (1995; 1999) described plant growth promoting *Bacillus* strains capable of stimulating conifer seedling growth and the capability of two plant growth promoting strains belonging to the genera *Bacillus* and *Pseudomonas* to enter spruce root tissue. The effect of rhizosphere *Bacillus* and *Pseudomonas* strains on plant growth was also studied by Probanza et al. (1996) using alder (*Alnus glutinosa*).

1.1.2 Plant growth promotion

Rhizosphere microbes may improve the uptake of nutrients by plants and/or produce plant growthpromoting compounds. They also protect plant root surfaces from colonization by pathogenic microbes through direct competitive effects and production of antimicrobial agents. These plant growth promoting bacteria can enter into a symbiotic relationship with plants (i.e *Rhizobium*-legume and *Frankia*-actinorhizal plant symbiosis), but also non-symbiotic, free-living soil bacteria can promote plant growth (reviewed in Glick 1995). Free-living bacteria are often referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper et al.1989) These PGPR can indirectly or directly affect plant growth. Indirect plant growth promotion includes the prevention of the deleterious effects of phytopathogenic organisms. This can be achieved by the production of siderophores, i.e small iron-binding molecules. In soils, iron is found predominately as ferric ions, a form that cannot be directly assimilated by microorganisms. Siderophore production enables bacteria to compete with pathogens by removing iron from the environment (O'Sullivan and O'Gara 1992). Siderophore production is common among pseudomonads (O'Sullivan and O'Gara 1992), but also *Frankia* (Boyer et al. 1998) and *Streptomyces*-species (Loper and Buyer 1991) have been shown to produce iron-chelating compounds. The synthesis of antibiotics has also been reported in several bacterial species (O'Sullivan and O'Gara 1992; Haansuu et al. 1999). Another mechanism by which PGPR can inhibit phytopathogens is the production of hydrogen cyanide (HCN) and/or fungal cell wall degrading enzymes e.g chitinase and β-1,3-glucanase (Friedlender et al.1993). Although pectinolytic capability is usually associated with phytopathogenic bacteria, non-phytopathogenic species such as *Rhizobium* (Angle 1986), *Azospirillum* (Umali-Garcia 1980; Tien et al. 1981), some strains of *Klebsiella pneumoniae* and *Yersinia* (Chatterjee et al. 1978) and *Frankia* (Seguin and Lalonde 1989) are also able to degrade pectin. In general, pectinolytic enzymes play an important role in root invasion by bacteria.

Symbiotic and non-symbiotic PGPR may also promote plant growth directly through production of plant hormones such as auxins (Tien et al. 1979), gibberellins (Gutiérrez-Mañero et al. 2001) and ethylene (Lynch 1990). Production of indole-3-ethanol or indole-3-acetic acid (IAA), compounds belonging to the auxins, have been reported for several bacterial genera, such as *Frankia* (Berry et al. 1989; Smolander et al. 1990; Mansour and ElMelegy 1997), *Klebsiella* and *Enterobacter* (Haahtela et al. 1988), *Pseudomonas* (Yrjönen et al. 2001) and *Bacillus* (Gutiérrez-Mañero et al. 1996). Most importantly, for rhizobacteria to act beneficially, they must be able to efficiently colonize and multiply in the plant rhizosphere. Different abiotic and biotic factors influencing bacterial colonization and viability were reviewed in Benizri et al. (2001).

1.1.3 Nitrogen fixation

Providing the plant with essential nutrients, eg. NH_4 - N through atmospheric nitrogen fixation or aiding the plant in nutrient uptake are also considered as direct plant growth promotion (Glick 1995). Nitrogen is essential to all living organisms. Although 78% of the atmosphere consists of dinitrogen, nitrogen in this form cannot be used by most organisms and consequently the availability of nitrogen in a form suitable for assimilation is often a major

limiting factor for growth. Biological nitrogen fixation is a process of reduction of atmospheric nitrogen to ammonia by free-living, or symbiotic bacteria possessing the enzyme nitrogenase. Symbiotic nitrogen-fixing bacteria include the cyanobacteria, the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Frankia* (e.g. Paul and Clark 1996; Brock 2000). The symbiosis between *Rhizobium* and legumes has been studied intensively and lately the symbiosis between *Frankia* and non-leguminous plants, the so-called actinorhizal plants, has also been receiving increased attention.

Free-living nitrogen-fixing bacteria maintain a looser interaction with host plants which results in root associated, as opposed to symbiotic, nitrogen-fixing activity. These associative nitrogen fixers, for example bacteria belonging to the species *Azospirillum*, *Enterobacter*, *Klebsiella* and *Pseudomonas*, have been shown to attach to the root and efficiently colonize root surfaces (Haahtela et al. 1988). It is still unclear how loose this plant-bacterium interaction is, but it is likely that, apart from atmospheric nitrogen fixation, other bacterial processes of importance to the plant also take place in the rhizosphere. For instance, in the case of *Azospirillum* spp., auxin production, rather than nitrogen fixation, is mostly responsible for the stimulation of rooting and enhanced plant growth (Bloemberg and Lugtenberg 2001). Although associative nitrogen-fixing bacteria in the rhizosphere are considered to play a minor role in nitrogen fixation, this is not always the case. For example, *Acetotobacter diazotrophicus* may provide up to 80% of the nitrogen required by sugarcane through biological nitrogen fixation (Boddey et al. 1991). Since nitrogen-fixing plants can grow independently of soil nitrogen levels, there have also been attempts to engineer new nitrogen-fixing symbiotic associations to economically important non-nodulated plants (Swensen and Mullin 1997).

Most of the studies concerning nitrogen fixation have been carried out in gnotobiotic systems or agricultural soils. In boreal forests, nitrogen-fixing bacteria play a major role since nitrogen is the most important nutrient limiting the growth of trees. However, not much is known concerning bacterial nitrogen fixation in natural forest soil systems. The role of legumes as hosts for nitrogen-fixers is not very significant in northern boreal forests. Here, actinorhizal plants provide the main input of biologically fixed nitrogen.

1.2 *Frankia* - actinorhizal plant symbiosis

Another important plant-microbe interaction is the symbiosis between members of the nitrogen-fixing actinomycetous genus *Frankia* and non-leguminous woody plants, the actinorhizal plants. The term actinorhizal plant describes the symbiotic interaction between frankiae (actino) and plant roots (rhiza). Unlike the *Rhizobium*-legume symbiosis, where most of the host plants belong to a single large family, *Frankia* can form root nodules in symbiosis with actinorhizal plants distributed among eight families consisting of over 200 species of angiosperms (e.g. Baker and Schwintzer 1990; Benson and Silvester 1993).

Actinorhizal plants have a worldwide distribution and represent mostly perennial, dicotyledonous woody shrubs and trees. They are commonly used in forestry as fuelwood, pulpwood or saw timber. Due to their ability to enter into symbiosis with N-fixing *Frankia*, actinorhizal plants are successful pioneer species frequently found in low-nitrogen sites that are

unfavourable for other N-dependent plant species and after disturbances such as fires, volcanic eruptions and floodings. Furthermore, a well-developed actinorhizal plant root system favours soil-binding capacity which improves the quality of impoverished soils and strongly supports the use of these plants in land reclamation. Actinorhizal plants are also commonly used as intercrops for other tree species (Wheeler and Miller 1990; Dommergues 1997).

The most studied *Frankia*-plant symbiosis is that with alder (*Alnus* sp.). From an ecological point of view, the *Frankia*-alder interaction is of great importance because this interaction is found world-wide from the arctic to the tropics. The contribution to nitrogen fixation in the *Frankia*-alder symbiosis can be up to 300 kg/ha/yr (Tarrant and Trappe 1971). Although the nitrogen-fixing potential of alder-*Frankia* symbiosis may be high, the amount of nitrogen actually fixed in the field is often low because of unfavorable environmental conditions. Therefore, proper management practices that optimize efficiency of the nitrogen-fixing system are required (Dommergues 1997). Native actinorhizal plant species found in Finland are: *Alnus incana*, *A. glutinosa*, *Hippophaë rhamnoides*, *Myrica gale* and *Dryas octopetala*. Of these species, alders are the most common and often found under other tree species and in mixed stands. The main input of fixed nitrogen into the soil N pool is through leaf litter (Huss-Danell 1986). Since nitrogen in alder leaf litter is easily mineralized, alders considerably improve the nitrogen status of the soil (Mikola 1958).

1.2.1 *Frankia* as an endophyte

Frankia populations inhabit three distinct ecological niches, the root nodules, the rhizosphere, and the soil, where *Frankia* can be found as a symbiont of actinorhizal plants, an associate of non-host plants or as a saprophyte, respectively. Although the biochemical and molecular basis of the *Frankia*-actinorhizal plant symbiosis are not as well understood as in the case of the counterpart *Rhizobium*-legume symbiosis, there is a regulated series of events leading to this close association between infective *Frankia* and compatible host plant and the subsequent formation of root nodules on host plant roots. Root nodules are perennial, morphologically differentiated clusters of lateral roots consisting of multiple lobes (Fig. 2).

Infection can be either through a deformed root hair (i.e. *Alnus*, members of the *Casuarinceae* and *Myricaceae*) or by direct penetration of the root through the intercellular spaces of the epidermis and cortex (i.e. Elaeagnaceae and *Ceanothus*) (Fig. 3) (reviewed in Huss-Danell 1997). In *Alnus*, infection is initiated via root hairs, which become branched in response to *Frankia* contact (Berry et al. 1986). Following *Frankia* penetration of root hair cells, a series of cell divisions occurs in the hypodermis and cortex of the root. The host cell produces wall-like material containing pectin, hemicellulose and cellulose and encapsulates the *Frankia* hyphae within the invaded host cells (Berg 1990). Division of root cortical cells results in the formation of a prenodule (Callaham et al. 1979). The actual nodule lobe originates in the pericycle and becomes infected by penetrating *Frankia* hyphae.

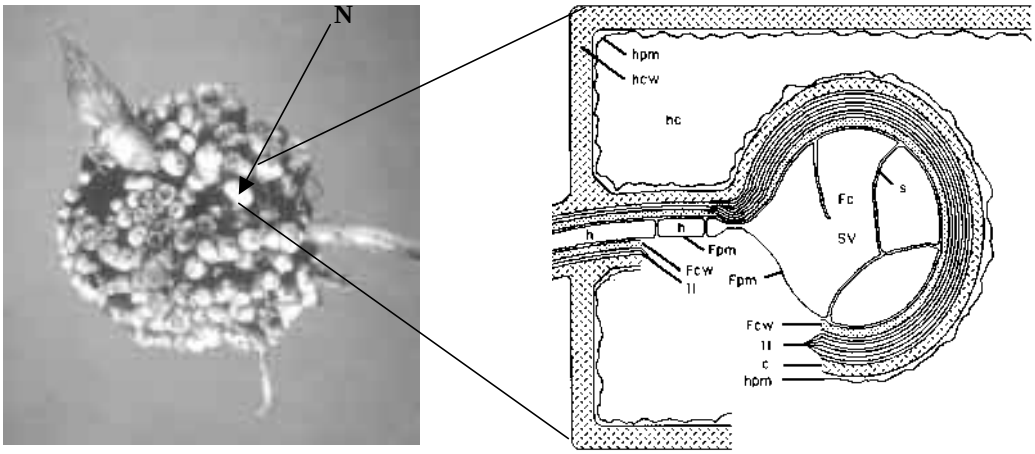

Figure 2. Actinorhizal root morphology. Left panel shows a densely compact root nodule on *Alnus* sp. N; nodule lobe. A schematic magnification of this infected *Alnus* nodule cell (right panel) shows *Frankia* hypha (h), symbiotic vesicle (SV), host cell wall (hcv), host plasma membrane (hpm), host cytoplasm (hc), capsule of host origin (c), lipid laminae (ll), *Frankia* cell wall (Fcw), *Frankia* plasma membrane (Fpm), septum (s) and *Frankia* cytoplasm (Fc). Modified from Huss-Danell (1990).

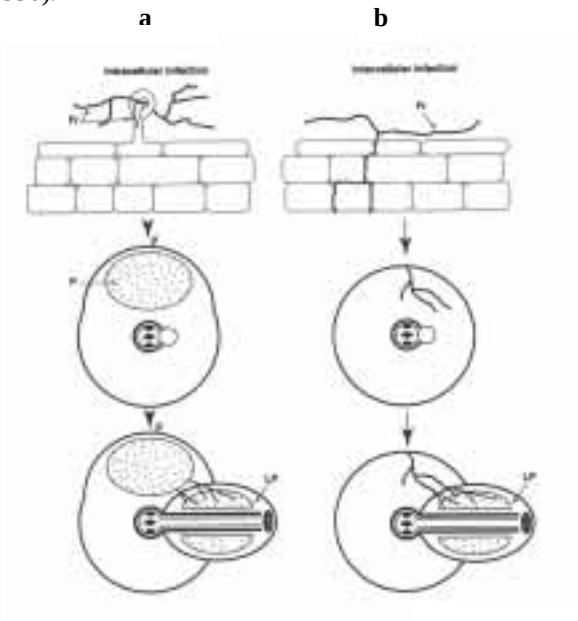

Figure 3. Infection of an actinorhizal plant nodule lobe. *Frankia* hyphae (Fr) penetrate a curled root hair (a, intracellular infection) or the middle lamella between adjacent root epidermal cells (b, intercellular infection). Sustained cell divisions and expansion of infected cells give rise to the prenodule (P) in intracellular infection. The nodule lobe (LP) is initiated in the pericycle and *Frankia* hyphae progress through root cortex to the young nodule lobe. (Franche et al. 1998).

In Elaeagnaceae, *Ceanothus* and *Cercocarpus* sp., the infection pathway bypasses the root hairs and consequently no root hair deformation is observed. In these hosts, *Frankia* hyphae colonize the root surface and penetrate the root through the middle lamellae between epidermal cells (Miller and Baker 1986). No cortical cell divisions leading to prenodule formation take place. However, as a result of infection a nodule primordium is similarly induced in the pericycle, and plant cells are invaded by *Frankia* hyphae in the cortex as the developing nodule lobe develops and penetrates the root surface (Fig. 3). The infection mechanism of *Frankia* is assumed to be determined by the host plant, since strains with the ability to infect via both routes have been observed (Miller and Baker 1986; Racette and Torrey 1989). In the fully developed symbiotic tissue, *Frankia* produce vesicles in the host cells in which nitrogenase is expressed that supports nitrogen fixation (Huss-Danell and Bergman 1990).

Two types of root nodules can be distinguished: those with and those without spores of which van Dijk (1978) introduced the terms spore-positive (Sp+) and spore-negative (Sp-). In pure cultures, all *Frankia* strains produce sporangia, even if they originate from Sp (-) nodules. Attempts to isolate *Frankia* from Sp (+) nodules have failed to give isolates that retain the Sp (+) phenotype upon reinoculation of the compatible host (Torrey 1987). Since Sp (+) type nodules contain 100-1000 times more infective *Frankia* propagules (van Dijk et al. 1988), it has been speculated that the ability to grow saprophytically could be the advantage of Sp (-) type *Frankia* over Sp (+) type *Frankia* in competing and persisting in soil. This is supported by observations of mainly Sp (-) type *Frankia* in soil sites devoid of host plants (Weber 1986; Smolander and Sundman 1987) suggesting saprophytic growth for this type of *Frankia.* In contrast to a study where the sporulation capacity of nodules was considered to be a genetically stable character of the endophyte and not influenced by the host plant (Vandenbosch and Torrey 1985), the findings of Zepp et al. (1997a) do support the genetic identity of vesicle-and spore-forming *Frankia*. The authors discovered *Frankia* populations belonging to host infection groups IIIa or IV (Hönerlage et al. 1994) from both Sp (+) and Sp (-) nodules and suggest that spore-formation is induced by environmental factors.

1.2.3 Classification of *Frankia*

Typical *Frankia* strains can be assigned to the genus *Frankia* by morphological, cytochemical and physiological criteria. Morphological criteria include the formation of septate, branching hyphae, the production of multilocular sporangia, the presence of non-motile spores in multilocular sporangia and the production of thick-walled, lipid-encapsulated structures called vesicles, which are the site for nitrogen fixation (Fontaine et al. 1984; Lechevalier and Lechevalier 1984; Meesters et al. 1985; Meesters et al. 1987). Most *Frankia* isolates can be grouped into four major host specificity groups: 1) *Alnus* and *Myrica*, 2) *Casuarina* and *Myrica*, 3) *Myrica* and *Eleagnus* and 4) members of the Elaeagnaceae (*Elaeagnus*, *Hippophaë*, *Shepherdia*) (Baker 1987). However, this classification is not complete since it has not been possible to isolate *Frankia* from all actinorhizal plant species. Several *Frankia* strains have also been isolated which lack the morphological, cytochemical and physiological criteria assigned to *Frankia.* These atypical *Frankia* include non-infective strains, which are unable to form root nodules on plants from which they were initially isolated. Inefficient strains, unable

to fix nitrogen in symbiosis or pure culture have also been characterized (Baker et al. 1980; Hahn et al. 1988). In addition, Bosco et al. (1992) isolated a group of broad-host range *Frankia* strains that infected several host plant species. In addition to host specificity, morphological, physiological, and nucleic acid based criteria have been proposed for the classification of members within the genus *Frankia*, but so far no standardized criteria for *Frankia* species definition have been established (reviewed by Lechevalier 1994).

Based on comparative sequence analysis of 16S rDNA sequences of *Frankia* pure cultures and uncultured endophytes in nodules, *Frankia* was considered to be the only genus within the family *Frankiaceae* (Normand et al. 1996). Furthermore, four main subdivisions within the genus *Frankia* were proposed: 1) a group consisting of *Frankia alni* and other typical nitrogen-fixing strains belonging to the *Alnus* and *Casuarina* host infection groups, 2) uncultured symbionts of *Dryas*, *Coriaria* and *Datisca* species, 3) *Elaeagnus* infective strains and 4) atypical non-nitrogen-fixing strains (Fig. 4) (Normand et al. 1996). Another analysis based on an actinomycetes-specific 23S rRNA insertion mostly agreed with the former differentiation and grouped 35 *Frankia* strains into roughly seven different host infection groups (Fig. 6). Strains belonging to the *Casuarina* and *Elaeagnus* host infection groups (HIG II and VI, respectively) showed only small sequence variation, whereas *Alnus* infecting *Frankia* could be grouped into four different subgroups (HIG I, IIIa, IIIb, and IV) (Hönerlage et al. 1994).

1.2.3 *Frankia* as a soil and rhizosphere organism

Little is known about *Frankia* populations in soil. In the presence of other soil bacteria, it is nearly impossible to isolate *Frankia* pure cultures and only one attempt to isolate *Frankia* from soil has been successful (Baker and O'Keefe 1984). Although the first isolation of a *Frankia* strain was achieved over 20 years ago (Berry and Torrey 1979) and several hundred *Frankia* strains have since been isolated, there is still no universal, standardized isolation method. Of the many isolations typically carried out in a study, only a very few succeed. Isolation and identification of *Frankia* is hampered by long generation times, the requirement for special isolation factors (Quispel et al 1989) and the lack of specific nutritional requirements of pure cultures (Akkermans et al. 1992).

Ecological studies of *Frankia* populations in soil have until recently been based mainly on plant bioassays in which a quantification of the nodulation capacity on a specific host plant (expressed as nodulation units (nu) g^{-1} soil) is used to describe the infective *Frankia* population. The nodulation capacity of a *Frankia* source is defined as the number of *Frankia* particles per unit of *Frankia* source, each of which induces one nodule in a nodulation test. Studies can be done with capture plant seedlings grown directly in the soils studied after which the nodules formed on plant roots are calculated. More quantitative methods include alder species as capture plants which are inoculated with serial dilutions of the studied soils. In the MPN (most probable number) method, nodulation is scored as +/- and the number of nodulation units estimated based on MPN statistics (Toomsan et al. 1984; Huss-Danell and Myrold 1994). In the nodule counting (or capacity) method, nodules of alders in each dilution

are counted. Nodule numbers obtained from suitable dilutions, taking the intrinsic nodulation capacity of the plant into account, are used to estimate nodulation units (comparable with colony counting) (van Dijk 1979). Using these methods, nodulation units between 0 and 4,600 nu g^{-1} soil have been obtained for different soils (Myrold et al. 1994). Myrold and Huss-Danell (1994) detected a good correlation between the MPN plant bioassay and the nodule capacity methods especially between 30 and 300 nu g^{-1} soil.

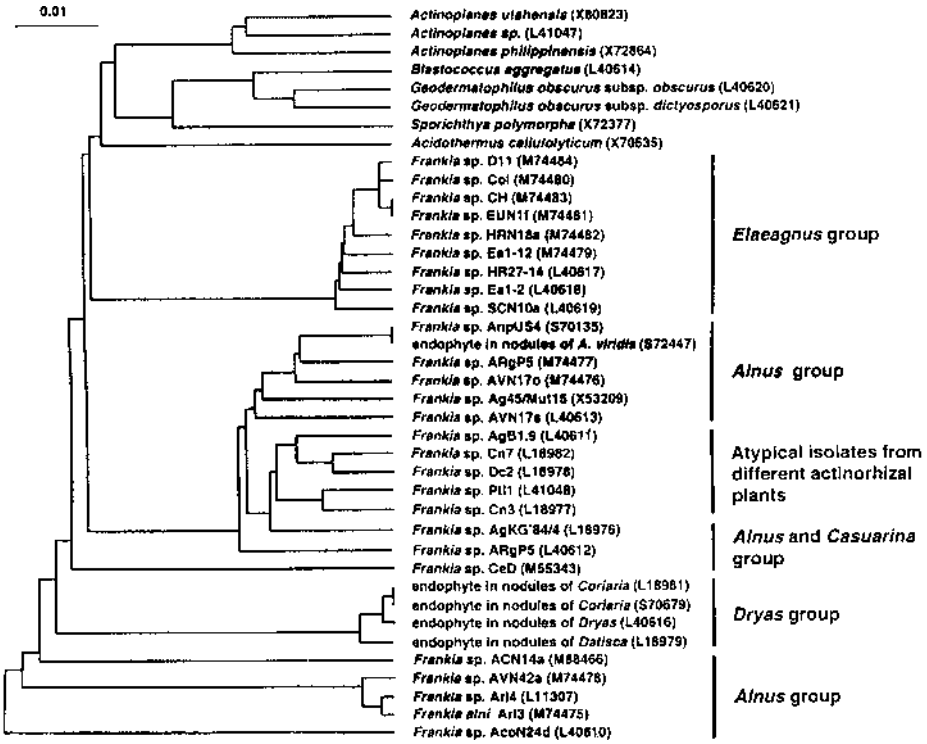

Figure 4. Phylogenetic tree obtained by the neighbor-joining method of selected members of the genus *Frankia* and closely related organisms derived from the EMBL/GenBank databases. The alignment of the sequences was done using CLUSTAL W. Upper clusters represent relevant outgroup actinomycetes. The distance scale indicates the expected number of changes per sequence position. (Hahn et al. 1999).

Detection of *Frankia* via plant bioassays depends on the capture plant species and cultivation conditions used (Huss-Danell and Myrold 1994). Other drawbacks of these plant bioassays include the failure to analyze specific *Frankia* populations, the inability to quantify competition for infection between *Frankia* populations in a sample, and inherent variable compatibilities of host plants to *Frankia* populations (Hahn et al. 1988; Huss-Danell and Myrold 1994). Due to autoregulation in the *Alnus* symbiosis, the efficiency of *Frankia* nodulation decreases with increased nodule numbers (Wall and Huss-Danell 1997). *Frankia*

nodulation units in mineral soil sites have also been shown to increase with increasing pH, at least in the pH range of 3.4 to 4.4 (Smolander and Sundman 1987). Storage conditions, such as the temperature regime and moisture contents, have also been shown to affect soil nodulation capacities although their impact depended on the *Frankia* strain (Sayed et al. 1997). While the nodulation capacity of one strain, for example, decreased in time under a large variety of temperature and moisture conditions, the nodulation capacities of others were negatively effected by a smaller subset of specific conditions (Sayed et al. 1997).

Quantification of *Frankia* populations in soil using rRNA as a target molecule resulted in detection of frankiae with an estimated detection limit of 10^4 cells (Hahn et al. 1990). This is comparable to the detection limit of 10^4 genomic units g^{-1} soil obtained by Myrold and Huss-Danell (1994) using PCR. A genomic unit (gu) is defined as the amount of frankiae containing a single genome. *Frankia* genomes contain two rRNA operons (Normand et al. 1992). Quantification of *Frankia* populations is can be achieved using the PCR-MPN method and nested (Myrold and Huss-Danell 1994) or booster (Picard et al. 1992) PCR to guarantee sufficient sensitivity to detect *Frankia* populations in soils. In these methods, *Frankia* DNA is selectively amplified from serial dilutions of extracted soil DNA using PCR. Comparisons made using the PCR-MPN based method and a plant bioassay revealed that only a small proportion of the total *Frankia* population were able to nodulate. These results suggest that the nu:gu ratio is an indicator of *Frankia* activity in soil (Myrold and Huss-Danell 1994).

A limited number of studies have focused on *Frankia* in the rhizosphere. These studies have been carried out in gnotobiotic systems, where axenic plant cultures were inoculated with known microorganisms. In a study where axenic alder, birch and grass seedlings were inoculated with *Frankia* pure cultures, microscopical analysis revealed vigorous growth of *Frankia*, including the production of sporangia and vesicles in the birch rhizosphere (Smolander et al. 1990; Rönkkö et al. 1993). Filaments, spores and vesicles were also detected in the rhizosphere of the grass species (Rönkkö et al. 1993). The authors also demonstrated in the same study that *Frankia* was able to grow in the rhizosphere of birch seedlings without the addition of an exogenous carbon source. Birch root exudates were likely to provide the attached bacterial population with nutrients and thereby promote growth in the rhizosphere. Clearly more work needs to be done to explain the relationship between *Frankia* and non-host plants and the function of *Frankia* in the rhizosphere of birch. Future work applying molecular methods may allow the detection of *Frankia* in non-host rhizosphere *in situ*.

1.2.4 Molecular methods for the detection of *Frankia*

Over the recent years, DNA based methods have proved to be powerful tools in microbial ecology studies. Since these methods are based on the isolation and detection of molecules instead of organisms, they allow to study microbes unbiased by the limitations of culturability. Molecular markers, such as the rRNA-encoding genes, containing conserved and highly variable areas, provide good targets for the detection, identification and phylogeny of cultured and uncultured microorganisms at different levels of resolution (e.g for bacteria see Amann et

al. 1995). Recently, molecular biological techniques have also enabled the direct detection of *Frankia* populations from soil and nodules and the identification and classification of *Frankia* isolates (reviewed in Hahn et al. 1999). Techniques used for analysis of *Frankia* populations are based on nucleic acid hybridization using 16S rRNA- and 23S rRNA sequences as markers (Hahn et al. 1990; Nazaret et al. 1991; Simonet et al. 1994; Benson et al. 1996; Normand et al. 1996; Zepp et al. 1997a; Zepp et al.1997b; Clawson et al.1999; Ritchie and Myrold 1999). Usually PCR-amplified partial 16S rRNA sequences from isolates or uncultured *Frankia* populations in root nodules are analyzed (e. g. Mirza et al. 1994a; Simonet et al. 1991; Simonet et al. 1994). Furthermore, specific *Frankia* populations can be analyzed by probing PCR products with specific oligonucleotide probes or primers directed against the 16S rRNA gene in dot blots (Mirza et al. 1994b) or in PCR assays (Simonet et al. 1991).

Apart from frankiae from the *Alnus* and *Casuarina* host infection groups, significant sequence differences between *Frankia* strains belonging to other different host infection groups have been obtained when using 16S rRNA as a target (Nazaret et al. 1991). However, differentiation between *Frankia* strains within host infection groups is difficult since many typical *Frankia* strains belonging to the same host infection group have identical 16S rRNA sequences and cannot be differentiated (Hahn et al. 1989; Nazaret et al. 1991). Compared to the 16S rRNA, 23S rRNA exhibits considerable sequence and length variations. In high G+C gram-positive bacteria a large insertion specific for these organisms is found in domain III of the 23S rRNA (Fig. 5) (Roller et al. 1992). Sequence variation within this insertion in three *Frankia* strains belonging to the *Alnus* host infection group suggested that the domain could be used as a target in the differentiation of *Frankia* strains (Roller et al. 1992). Comparative sequence analysis of PCR amplified and cloned 23S rRNA inserts from *Frankia* strains belonging to different host infection groups demonstrated that the *Casuarina* and *Elaeagnus* groups show limited sequence variation whereas the *Alnus* group could be separated into four subgroups, three (IIIa, IIIb and IV) containing typical nitrogen-fixing strains and a fourth (I) containing only non-nitrogen-fixing strains (Hönerlage et al. 1994).

This highly variable insertion has been used as a target when characterizing uncultured *Frankia* populations in root nodules of *Alnus* by *in situ* hybridization using digoxigenin- or fluorochrome-labelled probes (Zepp et al. 1997a, Zepp et al. 1997b). The *in situ*, or whole cell hybridization, technique is based on the microscopical detection of naturally amplified target molecules like ribosomal RNAs. The targets are detected after the formation of stable hybrids with fluorochrome-, digoxigenin- or enzyme-labeled oligonucleotide probes in fixed cells (Amann et al. 1995). Apart from alder root nodules (Hahn et al. 1993; Zepp et al. 1997a, Zepp et al. 1997b), *in situ* hybridization has been used to analyze bacterial cells in aquatic systems (Maszenan et al. 2000; McSweeney et al. 1993; Wagner et al. 1994), sediments (Ramsing et al. 1993) and soils (Hahn et al. 1992; Hönerlage et al. 1995; Chatzinotas et al. 1998). In contrast to digoxigenin- and horseradish peroxidase-labeled probes, small fluorescent oligonucleotide probes can be used without additional permeabilization pretreatments (Chatzinotas et al. 1998; Zepp et al. 1997a). However, autofluorescence from plant material may be a problem when fluorochrome-labelled probes are used, since it may mask true probe-conferred signals. The application of the red-fluorescent cyanine Cy3 reactive dye (Mujumdar et al. 1989) and the use of filter systems optimized for this dye allowed for a reliable differentiation between probe-conferred signals and autofluorescence in root nodules (Zepp et al. 1997a). An optimized detection protocol was used to analyze *Frankia* populations at the subgroup-level resulting in

reliable detection of *Frankia* vesicles, spores and filaments from alder nodule homogenates (Zepp et al. 1997a). *In situ* hybridization analysis has allowed detection of *Frankia* populations in nodules of *A. glutinosa*, *A. incana*, *A.viridis* and *A. nepalensis* at the subgroup level to a detection limit of one cell (Zepp et al. 1997a). In the same study the authors also identified the presence of a single *Frankia* population in each nodule homogenate. This technique was also applied in competition studies of nodule formation in different *Frankia* strains (Zepp et al. 1997b). Unfortunately, detection of *Frankia* in heterogenous environments such as soil has not been possible mostly due to low numbers of target organisms (Hahn et al. 1988) and binding of oligonucleotide probes to contaminating humic acids (Hahn et al. 1990).

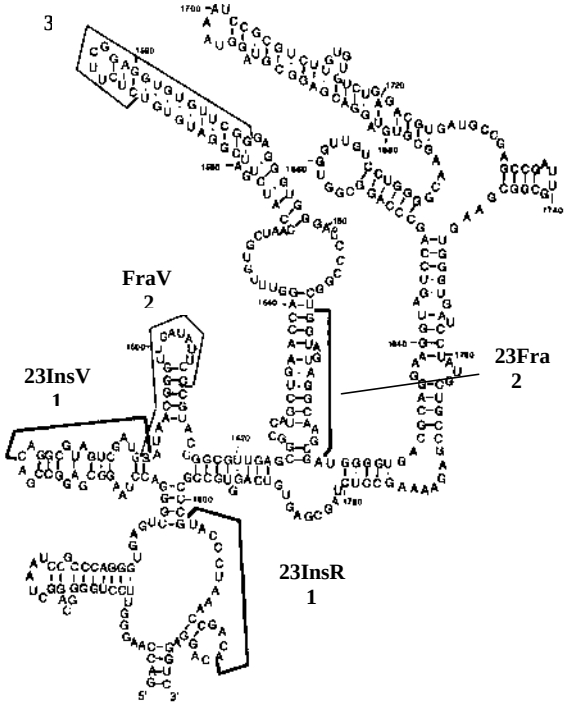

Figure 5. Secondary structure model of Domain III of the 23S rRNA of *Frankia* strain ORS020606 (Normand et al. 1992). A highly variable insertion specific for bacteria with a high DNA G+C content is located at position 1525-1623 (Roller et al. 1992). The target sites of 1) two amplification primers (23InsV and 23InsR) for *Frankiaceae*, 2) two amplification primers (FraV and 23Fra) for the genus *Frankia* and 3) specific probes detecting *Frankia* of the *Alnus* host infection subgroups are marked by solid lines. Modified from Hahn et al. (1997).

Frankia diversity has mostly been studied through the analysis of PCR-amplified nucleic acids of isolates or uncultured endophytes in nodules (reviewed in Hahn et al. 1999). Detection of frankiae at a low taxonomic level has been achieved via restriction fragment length polymorphism (RFLP) analysis. Baker and Mullin (1994) showed considerable diversity

among *Ceanothus* infecting *Frankia* strains following RFLP analysis on DNA extracted from *Ceanothus* nodule lobes and subsequent hybridization with *Frankia* or *nif*D-H gene probes. RFLP-analysis of PCR-amplified 16S rDNA (McEwan et al. 1994; Huguet et al. 2001) and the intergenic spacer (IGS) of the 16S-23S rRNA operon (Maggia et al. 1992; Rouvier et al. 1996; Ritchie and Myrold 1999), IGS between the nitrogenase *nif*H and *nif*D (*nif*H-D) genes (Cournoyer and Normand 1994), *nif*D and *nif*K (*nif*D-K) genes (Jamann et al.1993; Rouvier et al. 1996; Nalin et al. 1997) or the *gln*II gene (Cournoyer and Normand 1994; Cournoyer and Lavire 1999) have been used in classification of *Frankia* isolates and *Frankia* in nodules. Recently, squalene-hopene cyclase gene sequence fragments have also been used in phylogenetic comparisons of *Frankia* (Dobritsa et al. 2001). To distinguish closely related *Frankia* strains, arbitrary primers (Sellstedt et al. 1992) and primers targeting consensus motifs of repetitive elements common to prokaryotic genomes such as REP (repetitive extragenic palindromic), ERIC (enterobacterial repetitive intergenic consensus), DR (direct repeat) or BOX elements (rep-PCR) have been used (Murry et al. 1995; Murry et al. 1997; Jeong and Myrold 1999; Jeong and Myrold 2001). Genetic markers, such as resistance to antibiotics (Tisa et al. 1999) and phenotypic characteristics including pigment production and isoenzyme polymorphism have also been used to characterize *Frankia* strains (Dobritsa 1998; Igual et al. 2001).

When studying uncultured *Frankia* in soil, PCR-based techniques can provide sequence information. However, the use of genus-specific primers that do not bind to groups of uncultured *Frankia* may result in an underestimation of the *Frankia* genus, since PCR is carried out with primers designed on sequence information obtained from only a few cultured or uncultured frankiae (Hahn et al. 1999). Furthermore, technical problems are often encountered when studying forest soil with a high organic matter content using molecular methods.

1.2.5 *Frankia* in soils devoid of host plants

Plant bioassays have demonstrated that members of the genus *Frankia* can survive and remain infective in soils that are devoid of host plants (Arveby and Huss-Danell 1988; Huss-Danell and Frej 1986; Smolander 1990; Smolander et al. 1988; Smolander et al. 1990; Smolander and Sarsa 1990; Smolander and Sundman 1987). The nodulation capacity of soil from a Silver birch-stand was shown to be much higher than that of the soils from Scots pine- or Norway spruce-stands (Smolander 1990; Smolander et al. 1990; Smolander and Sarsa 1990) and even higher than in soil under alder (Smolander 1990). Although birch species are not hosts for *Frankia*, they belong to the same plant family (Betulaceae) as alder. Certain alder and birch species are thought to be more closely related compared to other alder species (Bosquet and Lalonde 1990). Thus, the stimulation of *Frankia* nodulation capacity in alder in soil supporting birch species may be due to rhizosphere similarities with alders or merely to the “leaky” nature of birch root systems. Birch roots have been shown to exude large amounts of organic compounds including carbohydrates, amino acids and organic acids that stimulate the growth of *Frankia* (Smith 1976).

Explanations for the persistence of *Frankia* in soils lacking host plants centre on the presence of long-lived spores that can act as inoculum sources or to the presence of free-living *Frankia* in soil. To date, there is no direct proof that *Frankia* grows saprophytically on fixed nitrogen in soil. Several studies, however, support this assumption. In addition to the occurrence of infective *Frankia* in soils devoid of actinorhizal plants (Burleigh and Dawson 1994; Paschke and Dawson 1992a; Paschke and Dawson 1992b) and the already mentioned ability of *Frankia* to grow in the rhizosphere of birch and grass species (Smolander et al. 1990; Rönkkö et al. 1993), there are reports of *Frankia* siderophore production (Aronson and Boyer 1994; Arahou et al. 1998; Boyer et al. 1998), and the production of antagonistic compounds (Gerber and Lechevalier 1984; Medentsev et al. 1989; Haansuu et al. 1999). There are other studies demonstrating a correlation between nodulation capacities and soil properties (moisture, organic matter and pH) (Righetti et al. 1986; Dawson et al. 1989; Smolander et al. 1988; Young et al. 1992). Furthermore, cellulolytic (Safo-Sampah and Torrey 1988) and pectinolytic (Séguin and Lalonde 1989) activities may also play a role in maintaining a saprophytic growth phase in soil. It is clear that more studies are needed to confirm *Frankia* presence and function in non-host soils.

2 Aims of the study

The beneficial effects of symbiotic and non-symbiotic PGPR on plant growth and production in agriculture and forestry makes them an interesting field of study. However, very little is known about the rhizosphere bacteria and *Frankia* of forest trees and their potential plant growth promoting activities. The main aim of this work was to investigate the diversity and function of plant growth promoting bacteria under forest trees with special emphasis on nitrogen-fixing *Frankia* in soils devoid of host plants. The specific aims of the study were:

- to isolate and identify forest soil bacteria from a Norway spruce stand and characterize their potential to supply beneficial rhizobacteria to birch, alder and fescue grass (Paper I).
- to detect and characterize *Frankia* populations from three soils devoid of actinorhizal plants using molecular rDNA markers (Papers II and IV)
- to study the effect of freezing and subsequent low-temperature storage of soils and the effect of different host plants on the nodulation capacities of total and specific *Frankia* populations (Paper III).
- to characterize unidentified *Frankia* populations from *Alnus* root nodules using *in situ* rDNA probing methodology (Papers III and IV).

3 Materials and methods

Soil characteristics
Norway spruce (*Picea abies* (L.) Karst.) stands at Heinola and Kerimäki, aged 42 and 60 years, in southern Finland, were developed on mineral soils of the podzol type. Plots had been limed, limed and fertilized, fertilized or were untreated. The fertilization histories of the plots are described in Smolander et al (1995). Soil organic matter content varied from 16 to 67% and pH between 3.6 and 4.7. Twenty-eight soil cores collected from the upper humus layer were combined to give one composite sample per plot (Paper I).

The soils used in papers II-IV were collected from an afforestation site at Karttula, Finland, established in a former agricultural field. The stand was 24 years old and plots contained monocultures of birch (*Betula pendula* Roth), pine (*Pinus sylvestris* L.) and spruce (*Picea abies* L.) The physical and chemical characteristics of these three stands were similar, consisting of a mineral soil with 12-14 % organic material and a pH between 5.3 and 5.5 (Priha and Smolander 1997). Twenty soil cores from 1-10 cm depth were taken and combined. Green plant material was removed from all the soil samples studied, samples were sieved and stored at 6^{o}C.

Plant material and bacterial isolation
Grey alder (*A. incana* (L.) Moench) (I, II, III) and black alder (*A. glutinosa* (L.) Gaertner) (III) were used as capture plants in bioassays to analyze the nodulation capacities of soils. Red fescue (*Festuca rubra*) and silver birch (*Betula pendula* Roth) seedlings were used to isolate rhizosphere bacteria from humus (Paper I). Bacteria from humus and plant roots were isolated by plating humus suspension or homogenized roots (in PBS) on different media as described in Paper I. The origin and identification of nitrogen-fixing isolates from spruce humus, birch and fescue roots are described in Table 2 of Paper I. *Frankia* strains isolated from nodules induced after inoculation of capture plants with birch, spruce or pine soil suspensions were cultivated in PC-medium (Weber et al. 1988). 23S rRNA insertion nucleotide sequences from frankiae are described in Paper II (Fig. 1) and Paper IV (Fig. 1). *Frankia* isolates from root nodules, uncultured *Frankia* in nodules and *Frankia* reference strains used in this thesis are listed in Table 1. The methods used in this thesis are described in the original publications and are summarized in Table 2.

Table 1. *Frankia* isolates, reference strains, sequence accession numbers and uncultured *Frankia* in nodules (Papers II-IV).

***Frankia* isolate**	**Origin**	**Genbank accession No.**	**Reference**
AiBp3, AiBp4, AiBp5, AiBp6	*Betula pendula* stand, Finland[a] (HIG IVa)	AJ003036	This study
AiPa1, AiPs2, AiPs4	*Picea abies* stand, Finland [a] (HIG IVb)	AJ003031	This study
AiPs1	*Pinus sylvestris* stand, Finland [a] (HIG IIIa)	AJ003032	This study
Ag4b, Ag4d	*Alnus glutinosa*, Finland (HIG IVa)		Weber et al. 1988
Ag5b	*A. glutinosa*, Finland (HIG IIIc)		Weber et al. 1988
Ai1a	*A. incana*, Finland (HIG IVb)		Weber et al. 1988
Ai7a	*A. incana*, Finland (HIG IVc)		Weber et al. 1988
Ai17	*B. pendula* stand, Finland [a] (HIG IVa)		Smolander and Sarsa 1990
AgB1.7	*A. glutinosa* (HIG I)		Hahn et al. 1988
AgB1.9	*A. glutinosa* (HIG I)	M85113	Hahn et al. 1988
ORS020609	*Casuarina cunninghamiana* (HIG II)		Zhang et al. 1984
ArI3	*A.rubra* (HIG IIIa)	M85112	Berry and Torrey 1979
AgB32	*A. glutinosa* (HIG IIIb)		Hahn et al. 1989
ARgP5	*A.rugosa* (HIG IIIb)		Normand and Lalonde 1982
AcoN24d	*A.cordata* (HIG IIIb)		Simonet et al. 1984
AgKG'84/4	*A.glutinosa* (HIG IIIb)		Hahn et al. 1989
Ag45/Mut15	*A. glutinosa* (HIG IVa)	M85114	Hahn et al. 1988
AVN17s	*A.viridis* (HIG IVb)		Fernandez et al. 1989
CNNod	*Coriaria nepalensis* (HIG V)		Mirza et al. 1992
Cc1.17	*Colletia cruciata* (HIG VI)		Meesters et al. 1985
SCN10a	*Sheperdia canadensis* (HIG VI)		Mort et al. 1983
WgCc1.17	*Colletia cruciata*		Nittayajarn et al. 1990
ACNs	*A.crispa*		Normand and Lalonde 1982
EAN	*Elaeagnus angustifolia*		This study (YMBO)
Ea1.12	*Elaeagnus angustifolia* (HIG VI)		Fernandez et al. 1989
S. ambofaciens	*Streptomyces ambofaciens*	M27245	Roller et al. 1992
$AiBp2_{Nod}$, , $AiPs23_{Nod}$,	*A. incana* nodule (Paper III)		This study
$AgPa11_{Nod}$, $AgPs7_{Nod}$	*A.glutinosa* nodule (Paper III)		This study
$AgBp24_{Nod}$, $AgBp21_{Nod}$	*A.glutinosa* nodule (HIG IVa) (Paper III)		This study
$AiPs3_{Nod}$, $AiPs5_{Nod}$	*A. incana* nodule (HIG IVa) (Paper III)		This study
$AgPs5_{Nod}$, $AgPs4_{Nod}$	*A.glutinosa* nodule (HIG IVb) (Paper III)		This study
$AiPa11_{Nod}$, $AiPa12_{Nod}$	*A. incana* nodule (HIG IVb) (Paper III)		This study
AiPs621-623Nod, AiPs634Nod	*A. incana* (HIG IIIa) (Paper IV)		This study
$AiBp20_{Nod}$, $Bp19_{Nod}$, $Pa12_{Nod}$	*A. incana* nodule (HIG IIIc) (Paper IV)		This study

Table 1. Continued.

***Frankia* isolate**	**Origin**	**Genbank accession No.**	**Reference**
AiBp320$_{Nod}$, AiPa8$_{Nod}$, AiPa63$_{Nod}$, AiPa66$_{Nod}$, AiPa68$_{Nod}$, AiBp36$_{Nod}$	*A. incana* (HIG IVb) (Paper IV)		This study
AiPa4$_{Nod}$, AiPa6$_{Nod}$, AiPa53$_{Nod}$, AiPa59$_{Nod}$, AiBp4$_{Nod}$, AiBp23$_{Nod}$, AiBp34$_{Nod}$, AiBp37$_{Nod}$, AiBp19$_{Nod}$, AiPs20-22$_{Nod}$, AiPs24$_{Nod}$	*A. incana* (HIG IVa) (Paper IV)		This study

[a] Isolated from *A incana* root nodule, induced by soil from a *B. pendula* (Bp), *Picea abies* (Pa) or *Pinus sylvestris* (Ps) stand.
HIG; *Alnus* host infection group according to Hönerlage et al. (1994).

Table 2. Methods used in this study.

Analysis	Method	Description	Reference
Nodulation capacity of soil	Plant bioassay	Papers I-III	Smolander and Sundman 1987
Nitrogen-fixing activity	Acetylene reduction assay	Paper I	Haahtela et al. 1981
Isolation of *Frankia*	OsO_4 surface-sterilization of nodules	Paper II	Normand and Lalonde 1982
Identification of bacterial isolates			
Physiological profiling	API strip test	Paper I	BioMerieux SA, France
Whole cell fatty acid composition	Gas chromatography of methyl esters	Paper I	Pirttijärvi et al.1996
Rep-PCR	PCR using REP -, BOX – and ERIC-primers	Paper I, II, III	Versalovic et al. 1991, 1994
Fingerprint analysis	UPGMA	Paper II,III	Sneath and Sokal 1984
Detection of uncultured *Frankia*			
In nodules	*In situ* hybridization	Paper II, III	Zepp et al. 1997a
In soil	PCR amplification of partial 23S rRNA gene and southern hybridization	Paper II	Zepp et al. 1997b, this thesis, Church and Gilbert 1984
Antagonistic activity of bacteria isolates	Agar assay	Paper I	This thesis
Production of plant growth beneficial substances			
Hydrogen cyanide production	Picric acid filter paper method	Paper I	Lorck 1948
Chitinolytic activity	Plating on medium containing chitin	Paper I	Modified from Chernin et al. 1995
Hydrolysis of pectin	Plating on pectin agar	Paper I	Chatterjee and Starr 1972
Carboxymethylcellulase (CMC) activity	Plating on CMC agar	Paper I	Andro et al. 1984
Siderophore production	Chrome azurol S (CAS) agar and microtiter assay	Paper I	Alexander and Zuberer 1991
Indoleacetic acid (IAA) production	Rapid *in situ* assay	Paper I	Bric et al. 1991
Nucleic acid extraction and purification			
Frankia isolates and nodules	Enzymatic lysis	Paper II, IV	Modified from Hönerlage et al. 1994
Soil	Bead beating protocol	Paper II, IV	Modified from Hönerlage et al.1995
Cloning of PCR products			
Ligation and transformation	SureClone^{TM} Ligation Kit	Paper IV	Pharmacia Biotech, Invitrogen, Sambrook et al. 1989
Determination of ligation	Restriction	Paper IV	Sambrook et al. 1989
Plasmid DNA isolation	Wizard Plus Minipreps	Paper IV	Promega
Sequencing			
16S rRNA	Solid phase method	Paper I	Hultman et al. 1991
Partial 23S rRNA of PCR products and cloned PCR products	Cyclic dideoxy method	Paper II, IV	Applied Biosystems
Sequence analysis and tree constuction	ABI Prism Genetic Analyzer	Paper IV	Applied Biosystems
	FASTA, PileUp	Paper II, IV	Devereux et al. 1984
	ClustalW	Paper IV	Thompson et al. 1994
	Genedoc	Paper IV	Nicholas and Nicholas 1997
	Treeview	Paper IV	Page 1996

4 Results and Discussion

4.1. Identification of bacterial isolates from humus and humus exposed seedlings

The capability of coniferous forest humus bacteria to colonize birch or grass roots was studied by exposing axenic silver birch and fescue grass seedlings to humus extracts obtained from four differently fertilized plots from two spruce stands (Paper I). Altogether 667 isolates, 241 from the humus, 209 from birch seedlings and 217 from fescue grass seedlings were obtained. Of these isolates, 131 (humus), 90 (birch) and 104 (fescue grass) were tentatively identified (Fig. 1 of Paper 1). The same species were obtained from the humus, regardless of the fertilization history of the plot. Identities of the heterotrophic aerobic isolates from the humus samples and from the exposed seedlings are compiled in Fig. 1 of Paper I. Aerobic spore-forming bacilli as well as *Nocardia* sp. actinomycetes were prevalent in the humus layer of the Norway spruce stands and the number and variety of gram-negative isolates were low. This is in accordance with the results of Timonen et al. (1998) who found low numbers of fluorescent gram-negatives but diverse spore-forming *Bacillus* spp. in Scots pine mycorrhizospheres developed in Finnish Scots pine forest humus. In contrast, Sørheim et al. (1989), who investigated the acid floor of Norwegian deciduous forest (beech) found that the colony-forming isolates were dominantly gram-negative, *Pseudomonas* and *Alcaligenes*, whereas the frequency of spore-forming bacteria was low. Taken together these studies suggest that the compositions of bacterial communities in forest soils are not determined merely on the basis of pH. Timonen et al. (1998) found a more enriched gram-negative community in Scots pine mycorrhizospheres in sphagnum peat with similar acidic pH to humus (pH 4.3 and 3.9, respectively).

Results from this study showed that the capture seedling species exposed to the test forest humus positively selected certain genera from the humus inoculum, notably the gram-negatives *Pseudomonas*, *Comamonas* and *Alcaligenes* and the actinobacteria *Nocardia* and *Rhodococcus*. The roots of fescue grass, a monocotyledon, also attracted spore-forming bacilli. A similar situation was shown in agricultural soil where the roots of rice, a major monocotyledon crop species, were mainly colonized by members of the alpha and beta subdivisons of proteobacteria while gram-positive bacteria dominated in the surrounding bulk soil (Rosencrantz et al. 1998). Host plant specific differences in rhizosphere bacterial communities may relate to composition of root exudates as they are known to differ with plant species (Smith 1976). There have been reports indicating that plant species are selective in their interaction with root bacteria from soil (Wilkinson et al. 1994; Lemanceau et al. 1995; Latour et al. 1996; Latour et al. 1999). In our study, the rhizospheres of the bait plants enriched gram-negatives, even though red fescue also attracted spore-forming bacilli. Latour et al. (1996) suggested, from the results of plant/soil factorial exposure experiment, that soil was responsible for the diversity of the bacterial populations associated with the roots. In contrast, our results rather indicate that plant species select bacteria associated with the roots from the bacterial pool in the soil.

4.2 Antagonistic activity and production of siderophores or IAA by spruce humus bacteria

Antifungal activities of rhizobacteria have been described by many authors (e.g. Hagedorn et al. 1989; Crawford et al. 1993; Axelrood et al. 1996). In this study, the plant-growth promoting potential of spruce humus bacteria was analysed by testing their capability to inhibit plant-pathogenic fungi *in vitro*. Pseudomonads, bacilli and streptomycetes were selected for testing as they are known to be potential antagonists. Two target strains of a uninucleate *Rhizoctonia* sp. (*Ceratobasidium bicorne*), 263 and 264 (Hietala et al. 2001), originating from a conifer tree nursery, represented conifer root pathogens. In addition, three species of common plant-pathogenic fungi were used as target organisms. The inhibition of fungal growth by *Streptomyces*, bacilli and fluorescent gram-neagative isolates from spruce humus and birch and grass roots is illustrated in Figures 2 and 3 of Paper I. Most of the strong (> 4 mm) antagonists were *Streptomyces* isolates from spruce humus, but also other gram-positives (*B. mycoides* and *B. sphaericus*) and *Ps. fluorescens* suppressed several of the tested fungi.

Of the *Streptomyces* isolates from spruce humus, 46% suppressed one or more of the indicator fungi, similar to the frequency reported previously for rhizosphere and non-rhizosphere isolates (Crawford et al. 1993; Axelrood et al. 1996). It was interesting that the fungal root pathogens originating from the conifer nursery were highly susceptible to several of the *Streptomyces* isolates from spruce humus. The humus bacteria may thus have a natural potential for attenuating autochthonous fungi as biological control agents in spruce nurseries. Several reports indicate a correlation between *in vitro* growth inhibition and biocontrol (Fravel 1988), however many other studies have found no advantage in screening the strains *in vitro* (Fravel 1988; Hagedorn et al. 1989; Crawford et al. 1993). Broadbent et al. (1971) noted that non-antagonistic strains in plate assays were generally also inactive in soil. Although there are limitations in the use of *in vitro* assays, they might aid in the preliminary screening of strains that should be further studied under relevant plant cultivation conditions.

It has been suggested that synthesis of hydrogen cyanide (HCN) by certain pseudomonads could be linked to the ability of these strains to inhibit growth of pathogenic fungi (Glick 1995). Therefore, to further elucidate the plant-growth promotion potential of the spruce humus bacterial isolates, HCN production by mostly fluorescent pseudomonads was determined. Ten fluorescent isolates appeared positive of which six also inhibited fungal growth. Thus the growth inhibition *in vitro* was not explainable by HCN production alone. In addition, sixty-six presumptive plant-growth promoting isolates with antagonistic, nitrogen-fixing or HCN producing capacity were tested for their ability to excrete cell wall degrading enzymes (chitinase, pectinase, cellulase). Antagonistic spore-forming bacilli and *Streptomyces*, and nitrogen-fixing isolates appeared to commonly produce the target enzymes and many of them hydrolyzed all the tested substrates (Fig. 4 of Paper I). The siderophore and IAA assays on antagonistic or nitrogen-fixing bacterial isolates highlighted active production of both classes of bioactives in *Pseudomonas* and *Comamonas* isolates whilst most *Streptomyces* isolates produced siderophores. Eighty-one and 34% of the tested isolates possessed siderophore and IAA producing capability, respectively (Table 3 of Paper I).

In summary, about one-third of the *Streptomyces*, the spore-forming bacilli and the fluorescent gram-negative isolates from the humus layer of the Norway spruce stands and from the roots of bait plants possessed antifungal properties based on the assays on pathogenic fungi of conifer and crop plants. In addition, many of the antagonistic bacteria were able to produce fungal cell wall hydrolyzing enzymes, siderophores or IAA *in vitro*.

4.3 Nitrogen-fixing taxa in the humus of spruce stands and in humus exposed seedlings

Of the 813 isolates from the Norway spruce humus of the non-fertilized control plots, birch and fescue grass seedling rhizospheres developed in the humus, five percent gave a positive in acetylene reduction assay after enrichment. To determine the diversity of N-fixing isolates, all isolates were analysed by rep-PCR. Nine unique fingerprint profiles were found and a further four patterns were shared by 32 isolates resulting in a total of 13 rep-PCR groups. In all cases, isolates within the same group also shared the same origin (humus, birch or fescue grass).

The 16S rRNA genes of representatives of each of the 13 different rep-PCR groups were amplified and sequenced and resulting sequences compared to those in the EMBL database. The identities based on 16S rDNA homology are presented in Table 2 of Paper I. The nitrogen-fixing isolates were identified to the genera *Rhodococcus*, *Paenibacillus*, *Pseudomonas* and *Phyllobacterium*. In addition, isolates of two unknown γ-proteobacterial genera were recognized on the basis of their 16S rDNA sequences. Two species of nitrogen-fixing *Rhodococcus*, *R. fascians* and *R. erythropolis*, were found; one in humus (KM6) and the other (HN41) from the roots of fescue grass. To our knowledge, nitrogen-fixing isolates of *Rhodococcus* have not earlier been reported. Additionally, two isolates (HM29 and HM35) from humus and one isolate (HK40) from birch roots were identified as species of *Pseudomonas*, *Phyllobacterium* and *Stenotrophomonas*, respectively, with 98-99% sequence homologies. Our findings of nitrogen-fixing *Pseudomonas* in spruce humus soil are in accordance with results from Haahtela et al. (1981) for red fescue roots collected from boreal organic rich meadows. Birch seedlings have been shown to positively respond to the presence of nitrogen-fixing *Enterobacter*, *Klebsiella* and *Pseudomonas* through observed changes in root morphology and increased plant biomass (Rönkkö et al. 1993).

It is notable that out of the 13 independent genotypes tested, seven were identified as *Paenibacillus* sp. This taxon was found in the humus, and in the roots of both the fescue and birch, which strongly supports rhizosphere enrichment of nitrogen-fixing *Paenibacillus* species from the spruce humus inoculum. Nitrogen-fixing *Paenibacillus* strains have been found to be amply present in the rhizospheres of different grasses and soils (e.g. Seldin et al. 1998) but the present report is the first description of nitrogen-fixing *Paenibacillus* in silver birch roots that originated from conifer forest humus inoculum. 16S rDNA sequences of the seven independent (judged by rep-PCR) nitrogen-fixing paenibacilli and the two gram-negative isolates were sufficiently divergent (less than 98%) to other known sequences in the databases, suggesting that these belonged to novel unidentified species. Accordingly, based on analysis of phylogenetic, phenotypic and chemotaxonomic characteristics, these nitrogen-fixing isolates were proposed as a new species, *Paenibacillus borealis* sp. nov. (Elo et al. 2001). Further

studies on the identification and function of these bacteria *in planta* are needed in order to elucidate their role in nitrogen-limiting forest soil.

4.4 Nodulation capacity of soils

Alnus incana (Papers I-III) and *A. glutinosa* (Paper III) seedlings were used as capture plants in bioassays for enumerating nodulation positive *Frankia* propagules in the humus (Paper I) and fresh and stored soils from birch, spruce and pine stands (Papers II and III). All soils tested contained nodule-forming *Frankia* populations. The nodulated test plants grew faster, were larger, and developed greener leaves in comparison with non-inoculated control seedlings that showed no nodule development indicating that they were not fixing nitrogen. The nodulation capacities of these soils are summarized in Table 3.

Table 3. Nodulation capacity[a] (nodulation units g^{-1} soil) on seedlings of *A. incana* and *A. glutinosa* inoculated with soil collected from sites under birch, spruce or pine.

	A. incana[b]	*A. incana*[c]	*A. glutinosa*[c]
"Pine" soil	2267	40	560
"Spruce" soil	2747	180	180
"Birch" soil	3160	140	1300
Spruce humus at Kerimäki (Ca)[d]	2800		
Heinola	2400		
Spruce humus at Kerimäki (N)[d]	< 10		
Heinola	< 10		
Spruce humus at Kerimäki (CaN)[d]	560		
Heinola	560		
Spruce humus at Kerimäki (0)[d]	1400		
Heinola	240		

[a] calculated from bioassays using 0.025 g of soil as inoculum (nodule counting method).

[b] nodulation units of fresh soils (Papers I and II)

[c] nodulation units of soils stored at -20°C for several years (Paper III)

[d] limed (Ca), nitrogen fertilized (N), limed and nitrogen fertilized (CaN) or untreated (0) plots (Paper I)

4.4.1 Nodulation capacity of spruce humus soil

The alder nodulating capacity of the spruce humus soils taken from differently fertilized plots varied greatly, whereas other bacterial species were similarly distributed among the eight plots. For better comparison of nodulation capacities between the different soils in Papers I-III, nodulation capacity values expressed per g^{-1} soil organic matter calculated in Paper I were converted to nodulation units g^{-1} soil and the mean nodulation capacities were calculated from nodule numbers obtained after inoculation with 0.025 g of soil in all the experiments. The limed plots of both stands contained high numbers of nodule forming *Frankia*: 2800 and 2400 nu g^{-1} soil, respectively, whereas there was almost no nodulation capacity (< 10 nu g^{-1}) in the humus of the nitrogen-fertilized plots of the same stands. The limed and nitrogen-treated plots from both stands had nodulation capacities of 560 nu g^{-1}, whereas nodulation capacities of untreated plots varied from 240 nu g^{-1} (Heinola stand) to 1400 nu g^{-1} (Kerimäki stand). The higher soil organic matter content in the Kerimäki stand as compared to the Heinola stand (51 vs. 20 %) suggests that organic matter is important for saprophytic growth of frankiae. Nitrogen fixation by pure cultures of *Frankia* is known to be inhibited by combined nitrogen (e.g. Fontaine et al. 1984). Our results indicate that long-term nitrogen fertilization debilitated the alder nodulation capacity of the acid conifer humus although there was no observable effect on the aerobic heterotrophic flora measurable by plate counting. Sensitivity towards N-fertilization has also been reported for the endophytic nitrogen-fixers of sugarcane (Fuentes-Ramírez et al. 1999). *Frankia* nodulation capacity in the limed humus soils (pH 4.7 and 5.3) was higher than in the unlimed plots with lower pH (pH 3.7 and 4.2). This is in accordance with a study by Smolander and Sundman (1987) where a positive correlation between soil pH and the nodulation capacity was noted.

4.4.2 Nodulation capacity of soils from birch, pine, or spruce stands of an afforestration site

The mean nodulation capacity of the soil from the birch stand was 3160 nu g^{-1} soil, from the pine and spruce stand 2267 nu g^{-1} soil and 2747 nu g^{-1} soil, respectively (Table 3). Compared to these results, nodulation capacities on the same soils frozen (-20°C) and stored for four years were much lower on both the *Alnus* species (Table 3). Nodulation capacities on *A. incana* decreased by more than one order of magnitude for all three soils equivalent to approximately 2, 7, and 4% of the nodulation capacities of fresh soils of pine, spruce, and birch stands, respectively. Using *A. glutinosa* as the capture plant, nodulation capacities of the frozen soils were still lower than those in fresh soils obtained with *A. incana* as the capture plant (Table 3). Differences, however, were less pronounced than with *A. incana* as the capture plant. However, since no information on *A. glutinosa* nodulation with fresh soil inocula were available, we can only speculate the effect of soil freezing with *A. glutinosa* as a host plant. In conclusion, these results demonstrate that even after several years of storage at -20°C, *Frankia* populations retained their infectivity in these three soils. However, the lower nodulation capacities in the frozen soil treatments compared to those in fresh soils indicate a negative impact of freezing and low temperature-storage on physiologically active *Frankia* populations

in soil, which most likely accounted for the decreased numbers of infective frankiae in the soils.

In contrast to the published data (Smolander and Sundman 1987; Smolander et al. 1988; Smolander 1990) that has identified much lower nodulation capacities in soils from both the pine (0 to 63 nu g^{-1}) and the spruce (0 to 50 nu g^{-1}) stands compared to that in soil from a birch stand (490 to 6500 nu g^{-1}) (assuming a bulk density of 1.0 g cm^{-3}), differences in nodulation capacities of soils from birch, pine and spruce stands in this study were only small. The high similarity of physico-chemical factors in the soils used in our study tied to the identical seasonal conditions at all the forest sites are both supposed to significantly influence the nodulation capacities of soils (Smolander and Sundman 1987; Myrold and Huss-Danell 1994). Nodulation capacities of soils are also assumed to be influenced by the composition of local vegetation (Smolander and Sundman 1987, Smolander 1990; Myrold and Huss-Danell 1994) and by variable host infection compatibilities of different *Frankia* populations (Weber 1986; Hahn et al. 1988; Van Dijk et al. 1988; Van Dijk and Sluimer 1994). At present, it is unclear whether other non-host plant species than birch can also form associations with *Frankia* and thereby enrich the forest soil *Frankia* population. This study does not support earlier data on obvious impacts of vegetation on the overall nodulation capacity. In contrast to the birch, spruce and pine soils used in the studies by Smolander and Sundman (1987) and Smolander (1990), the soils used in this study were from young afforestration sites in former agricultural fields. These soils had a earlier cropping history that included liming and fertilization treatments which has resulted in a higher pH compared to natural Finnish forest soils. In accordance with our results, indicating similar numbers of infective *Frankia* in birch, spruce and pine soils, Priha and Smolander (1997) were also unable to find tree host specific changes in soil microbial biomass and activities from these soils. These results suggest that the impact of tree-planting on soil microbial communities in former agricultural soils is difficult to detect in the early phase of forest stand development.

Previous studies have demonstrated that bioassay results are dependent on cultivation conditions as well as on the capture plant used, with nodule numbers consistently being greatest on *A. rubra*, intermediate on *A. incana*, and lowest on *A. glutinosa* (Huss-Danell and Myrold 1994). In contrast, *A. glutinosa* nodulation capacities in soils from pine- and birch-stands in our study were, respectively, about 14- and 9-fold higher than with *A. incana*. Values for soil from the spruce-stand, however, were comparably low with 7% of the nodulation capacity of fresh soil (Table 3). These results suggest that studies on nodulation capacities of soils from birch, pine or spruce stands using *A. incana* and *A. glutinosa* are impacted by variable compatibilities of the host plants to specific *Frankia* populations.Variable compatibilities of *A. incana* and *A. glutinosa* to different *Frankia* populations have been demonstrated (Van Dijk et al. 1988). In Finland, for example, nodules on *A. glutinosa* have been mostly of the Sp (-) type and nodules on *A. incana* of the Sp (+) type (Weber 1986). Neither *in situ* hybridization nor DAPI staining (section 4.5.2) could detect spores in any of the nodules in this study, which indicates that all nodules were of the Sp (-) type, which is in accordance with previous results that soil from sites devoid of host plants induced only Sp (-) type nodules on capture plants (Smolander 1990). Based on these observations, one might speculate that the higher *A. glutinosa* nodulation capacities of the test soils were a result of strong associations of Sp (-) type *Frankia* populations to *A. glutinosa*. In order to detect

possible differences in specific *Frankia* populations in the Sp (-) nodules, group specific probes and the *in situ* hybridisation technique were used.

It must also be remembered that *A. glutinosa* and *A. incana* seedlings were grown in artificial nutrient solutions, which likely induced stress on the plant and reflected on the quantity and composition of plant root exudates. It can be hypothesised that *A. glutinosa*, which usually grows on lake and sea shores, was better adapted to these *in vitro* growth conditions than *A. incana*, which prefers drier mineral soil sites. Furthermore, differences in size of the plant seedlings at the time of inoculation and a decrease in pH in the solution, the rate of acidification being greater in the presence of larger plants, have been suggested to cause differences in nodulation numbers between host plant species in *in vitro* bioassays (Huss-Danell and Myrold 1994).

4.5 Detection and characterization of *Frankia*

4.5.1 Isolates obtained from root nodules

A total of eight isolates, three from *A. incana* nodules inoculated with soil from the birch-(AiBp3-AiBp6) and pine (AiPs1-2 and AiPs4) stands, and one obtained from nodules of capture plants inoculated with soil from the spruce-stand (AiPa1), were selected for further characterization by sequence analysis of a highly variable actinomycetes-specific insertion in Domain III of the 23S rRNA and by rep-PCR. Finnish *Frankia* strains Ag4b, Ag4d, Ag5b, Ai1a, and Ai7a, isolated from *A. incana* or *A. glutinosa* root nodules from different ecotypes in Finland and *Frankia* strain Ai17, isolated from *A. incana* nodules induced by soil from a *B. pendula* stand were included in the sequence analysis (Paper IV).

Based on a comparative sequence analysis of PCR amplified 135 bp fragments containing the 23S rRNA insertion in domain III, the examined isolates could be assigned to two groups of *Frankia* within the *Alnus* host infection group (Hönerlage et al. 1994), namely group IIIa and group IV (Fig. 1 of Paper II). The assignment was partially correlated to the origin of the soil used as inoculum. Sequences of isolates obtained from root nodules induced with soil from the birch-stand (AiBp3 to AiBp6) were identical and showed only a single nucleotide substitution to that of the reference *Frankia* strain Ag45/Mut15 for the *Alnus* host infection group IV (Hönerlage et al. 1994). Sequences of these isolates revealed 21 differences to isolate AiPs1 obtained from nodules formed through inoculation with soil from the pine-stand. The sequence from isolate AiPs1 again showed a single nucleotide substitution compared to the sequence of *Frankia* strain AvcI1 belonging to the *Alnus* host infection group IIIa (Fig. 1 of Paper II) (Hönerlage et al. 1994). Sequences of isolates AiPs2 and AiPs4 obtained from nodules after inoculation with soil of the same pine stand as well as that of isolate AiPa1 obtained from nodules formed after inoculation with soil from the spruce-stand were identical, but showed 27 differences compared to the sequence of isolate AiPs1 and 18 differences compared to those of isolates AiBp3 to AiBp6. Isolates AiPs2, AiPs4 and AiPa1 showed two differences to the sequence of *Frankia* strain AvN17s that was assigned to the *Alnus* host infection group IV (Hönerlage et al. 1994). Due to the previously observed sequence divergence between strain AvN17s and the reference strain of group IV, strain Ag45/Mut15 (Hönerlage et al. 1994), a reorganization of the *Alnus* host infection group IV into two groups IVa and IVb incorporating

Frankia strains Ag45/Mut15 and AvN17s as reference strains for the respective groups is justified. This reorganization results in the assignment of all isolates to one of three distinct groups within the *Alnus* host infection group classification system, namely groups IIIa, IVa and IVb. Accordingly, sequences obtained from *Frankia* isolates Ag4b, Ag4d, Ag5b, Ai1a, Ai7a and Ai17 as well as sequences from uncultured frankiae from root nodules could be assigned to these existing subgroups (IIIa, IVa and IVb) (Fig. 1 of Paper IV). A part of the aligned sequences showed identical sequences to those of reference *Frankia* strains, whilst others displayed very high similarities. No sequences showing homology to reference strains from subgroups I and IIIb were found. However, a comparison of target sites for existing probes designed against the references strains of the subgroups, *Frankia* strain Ai7a assigned to subgroup IVa allowed identification of two mismatches to probe 23Mut(II) targeting members of this subgroup (Fig.1 of Paper IV). These mismatches could explain failures to identify certain *Frankia* populations in root nodules.

Even though small differences in sequence variation can be attributed to artefacts caused by PCR cycling or represent microheterogeneities of the ribosomal operon in the *Frankia* genome, sequence differences in both the entire insertion as well as the target sites for existing probes may justify the design of probes targeting additional subgroups. *Frankia* strain Ai7a that exhibited 9 mismatches in the whole insertion and 2 mismatches in the probe target site to strain Ag45/Mut15 would represent a new subgroup IVc, separated from subgroup IVa and IVb (Fig. 6). The identified differences in probe target sites allowed the design of a new probe targeting the same site. Sequences from isolate Ag5b and uncultured nodule populations with identical sequences induced by frankiae from soil under birch ($AiBp19_{nod}$, $AiBp20_{nod}$) or under spruce ($AiPa12_{nod}$) clustered within host infection group IIIa, showing 5 differences to reference strain ArI3 which are all outside the target site for probe 23ArI3 (Fig. 1 of Paper IV). Again, the sequence divergences would justify the establishment of a new subgroup (IIIc) within the *Alnus* host infection groups, which would, however, require a shift in the probe target site for future detection (Fig. 6).

Using sequence variation in both the entire insertion as well as the target sites for existing probes as criteria for the establishment of new subgroups, a further differentiation could focus on subgroup IIIb. Based on sequence divergence between *Frankia* strains of this group (up to 13 mismatches), three subgroups represented by the original references strain AgB32 (IIIb), as well as the strains ARgP5 (IIId) and AgKG'84/4 (IIIe), respectively, could be included within this *Alnus* host infection subgroup. Similar considerations of subgroup I comprising non-nitrogen fixing *Frankia* strains may also be justified (Fig. 6). In order to confirm the new groupings, however, analysis of larger *Frankia* populations are needed.

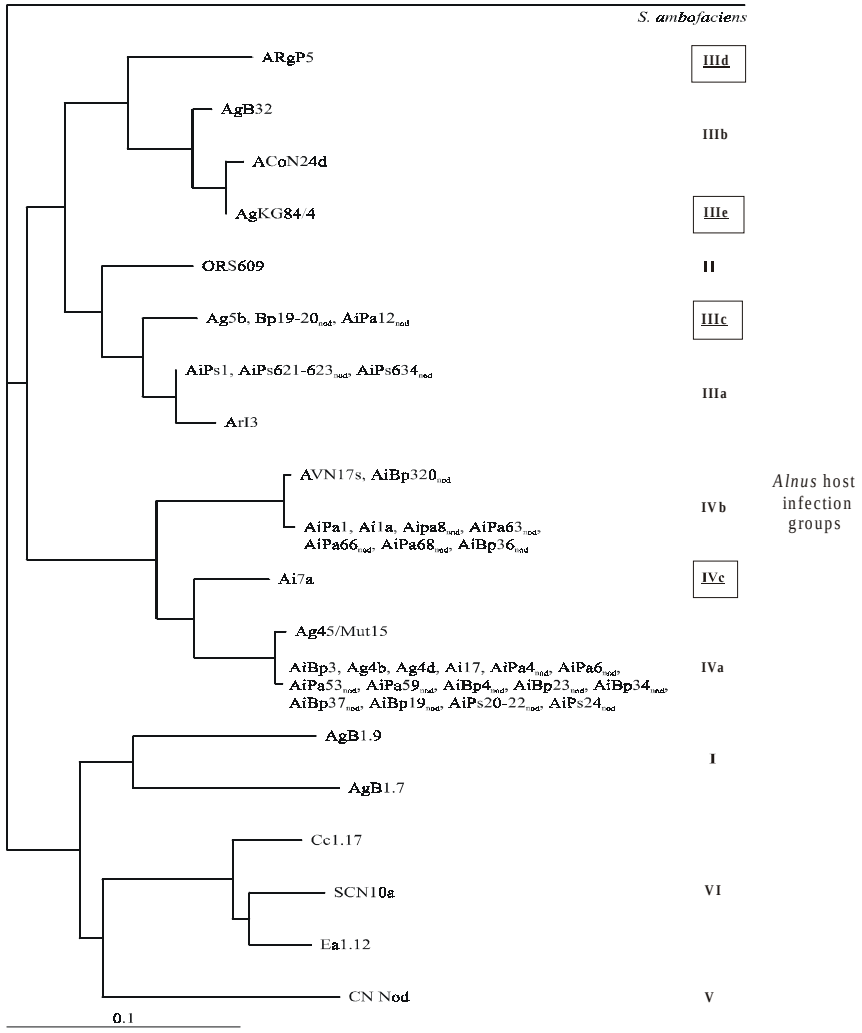

Figure 6. A neighbor-joining dendrogram based on aligned sequences of an insertion in 23S rRNA from pine (Ps), birch (Bp) and spruce (Pa) nodule clones, *Frankia* strains representing host infection groups I-VI and *S. ambofaciens* (Genbank AC#M27245). Proposed new *Alnus* host infection groups are underlined and boxed. The scale bar represents 0.1 substitutions per site.

The assignment of the *Alnus* host infection groups IIIa, IVa and IVb was confirmed by the analysis of genomic fingerprints of the eight birch, spruce and pine soil isolates generated by rep-PCR. On average, 14 bands representing a unique fingerprint for each strain were generated (Fig. 2 of Paper II). An UPGMA analysis of fingerprint patterns depicted as a dendrogram showed that the isolates could be clustered into five groups (Fig. 3 in Paper II):

group A includes isolates AiPa1, AiPs2 and AiPs4, group B isolates AiBp3 to AiBp6 and reference strain EAN, group C isolate AiPs1, group D reference strain WgCc1.17 and group E reference strain ACN. Considering that rep-PCR fingerprints may reflect strain-specific genomic structure (Versalovic et al. 1994), these results strongly support each isolate being a unique strain. Isolates with similar rep-PCR fingerprints could be assigned to the same group within the *Alnus* host infection group. A comparably good correlation between degrees of relatedness determined by comparative RNA sequence analysis and fingerprinting has been shown for 16S rRNA analysis and rep-PCR fingerprinting of *Frankia* strains (Murry et al. 1995). Our results involving co-analysis of 23S rRNA sequence analysis and rep-PCR fingerprints patterns show that different *Frankia* strains, belonging to the same group within the *Alnus* host infection group, inhabit the same soil and are infective on *A. incana*. The data also suggests that, depending on the soil source, different *Frankia* populations can nodulate the *Alnus* capture plants. This assumption, however, is only based on the analysis of a limited number of isolates obtained from nodules. Because isolation procedures can be extremely selective, a final proof of whether the isolates represent the total *Frankia* populations in nodules must include a comparison of isolates with uncultured *Frankia* populations within root nodules.

4.5.2 Uncultured *Frankia* populations in root nodules

Uncultured *Frankia* populations in root nodules were analyzed by *in situ* hybridization (Papers II and III). Altogether 20 (Paper II) and 52 (Paper III) nodule lobes chosen randomly from each soil treatment and both host plant species (Paper III) were analyzed. The results are summarized in Table 4. *In situ* hybridization with the bacterial probe EUB338 on lobe homogenates resulted in intensive hybridization signals in vesicles present in all samples. This demonstrated that the vesicles were sufficiently permeable to allow probe penetration and contained sufficient amounts of target sequences per cell. For details of nodule structure see Fig. 2.

Frankia populations in lobes induced after inoculation with fresh soil from the birch stand exclusively hybridized with probe 23Mut(II) thereby confirming the presence of frankiae of the *Alnus* host infection group IVa (Fig. 7a). No hybridization signals were obtained with probe 23AvN targeting *Frankia* of group IVb or with probe 23AvC targeting *Frankia* of group III (Fig.7b). The analysis of *Frankia* populations in lobes induced after inoculation with fresh soil from the pine-stand confirmed the presence of frankiae of the *Alnus* host infection groups III and IVb. In lobes induced after inoculation with fresh soil from the spruce-stand, *Frankia* populations hybridized with probe 23Mut(II) and probe 23AvN targeting *Frankia* of groups IVa and IVb. Here, no hybridization signals were obtained with probe 23AvC. A comparative analysis of the latter samples with control isolates, however, was greatly hampered by the availability of only one isolate, AiPa1. The results on fresh soils were based on the analysis of only 20 nodule lobes from each soil. For more quantitative results, increased nodule numbers should have been analyzed. However, we can conclude that freezing and storage at -20ºC for several years induced only small differences in the structure of nodulating *Frankia* populations (Table 4). The large reduction in nodulation capacity of all three soils after freezing and storage on *A. incana* is therefore not correlated to the reduction or the increment of specific *Frankia* populations.

Figure. 7. *In situ* hybridization on a nodule homogenate of *A. incana* induced after inoculation with a birch soil with CY3-labeled oligonucleotide probe 23Mut(II) targeting *Frankia* of the *Alnus* host infection group IVa (a, upper panel) and 23AvC targeting *Frankia* of the *Alnus* host infection group IIIa (b, lower panel). Hybridization with prober 23Mut(II) shows intensive signals on vesicles (v), whereas probe 23AvC reveals only low signal intensities due to autofluorescence. The left panels show the corresponding epifluorescence micrographs after DAPI staining. Bar represents 5 μm.

In order to evaluate possible variable compatibilities of *A. incana* and *A. glutinosa* we investigated the structure of the *Frankia* populations forming root nodules on both capture plant species inoculated with soils from pine, spruce and birch stands, respectively, using the *in situ* hybridization technique. Comparison of *Frankia* populations in nodules formed on *A. incana* or *A. glutinosa* inoculated with frozen and stored "pine", "spruce" or "birch" soil showed only small differences. In nodules obtained on both *A. incana* and *A. glutinosa* that were induced by frankiae from "spruce" and "birch" soil only *Frankia* groups IVa and IVb, but not IIIa and IIIb were detected (Table 4). *Frankia* group IVa was dominant inhabiting three to four times as many lobes as group IVb frankiae. In nodules induced by frankiae from "pine" soil, *Frankia* populations belonging to group IVa were also detected in comparable numbers of nodules on both plant species, however, nodules on *A. glutinosa* were much more often inhabited by group IVb frankiae than those on *A. incana* (48 *versus* 29%) (Table 4). On *A. glutinosa*, none of the nodules contained *Frankia* groups IIIa or IIIb. Group IIIb was also not detected in nodules on *A. incana*, however, frankiae of group IIIa were detected in 25% of the lobes. Although there were small differences in *Frankia* populations between both capture plant species that support the theory of variable compatibilities of the host plants to specific *Frankia* populations, the differences cannot be correlated to the large differences in the nodulation capacities of the different soils obtained between both plant species.

Table 4. *In situ*-based detection of uncultured *Frankia* populations in nodule lobe homogenates of *A. incana* and *A. glutinosa* bioassay seedlings inoculated with pine, spruce or birch test soils.

	Domain Bacteria[a]	*Frankia* populations[b]				Unidentified[c]
		IIIa	IIIb	IVa	IVb	
***A. incana*[d]**						
"Pine" soil	100	25	0	0	40	35
"Spruce" soil	100	0	0	35	25	40
"Birch" soil	100	0	0	100	0	0
***A. incana*[e]**						
"Pine" soil	100	25	0	42	29	4
"Spruce" soil	100	0	0	63	27	10
"Birch" soil	100	0	0	71	12	17
***A. glutinosa*[e]**						
"Pine" soil	100	0	0	40	48	12
"Spruce" soil	100	0	0	73	13	13
"Birch" soil	100	0	0	77	15	8

[a] % lobes in which *Frankia* vesicles strongly hybridized to probe EUB338 targeting all members of the Domain Bacteria

[b] % lobes in which *Frankia* vesicles hybridized to probes 23AvC targeting *Frankia* of the *Alnus* host infection subgroup IIIa), 23B32(II) (*Frankia* subgroup IIIb), 23Mut(II) (*Frankia* subgroup IVa), and 23AvN (*Frankia* subgroup IVb)

[c] % lobes in which *Frankia* vesicles did not hybridize to probes 23AvC, 23B32(II), 23Mut(II), or 23AvN

[d] bioassay with fresh soils (Paper II)

[e] bioassay with soils stored at -20^{o}C for several years (Paper III)

This study showed the applicability of the *in situ* hybridization technique for the analysis of specific *Frankia* populations in alder root nodules. Further analysis of even larger numbers of nodules would result in more accurate and quantitative results. However, based on the results from this study we can conclude that nodulating *Frankia* isolates represent a significant portion of the *Frankia* population in soils from the birch, pine or spruce stands. Nevertheless, nodules induced after inoculation with all three soils (fresh or frozen) also contained *Frankia* populations which could only be identified with specific probes, the amount varying slightly depending on the soil and the host plant. All lobes contained vesicles that gave positive

hybridization signals with the bacterial probe EUB338. Therefore it is evident that nodules developed in soils from all stands were also inhabited by *Frankia* populations which had not been isolated and which could not be identified. It is not surprising that certain nodulating *Frankia* populations were not identified, since several *Frankia* strains have already been isolated that require probe modification for detection (Hahn et al. 1999; Zepp et al. 1997a) and only a limited number of oligonucleotide probes were used which restricted the detection of *Frankia* populations to four groups within the *Alnus* host infection group in this study.

Since it had been demonstrated that comparative 16S or 23S rRNA sequence analysis of *Frankia* strains showed a good correlation between degrees of relatedness with fingerprinting techniques such as rep-PCR fingerprinting (Murry et al. 1995), we tried to investigate the relatedness of unidentified *Frankia* populations in these nodules to identified *Frankia* populations and previously isolated strains by rep-PCR.

4.5.3 Unidentified *Frankia* populations in root nodules

Analysis of rep-PCR fingerprint patterns of 16 nodule lobe homogenates showed, on average, 13 bands (Fig. 1 of Paper III). The complexity of the fingerprint patterns obtained were similar to the patterns obtained using DNA from *Frankia* isolates. They were also comparable to those obtained from nodules in other studies (Jeong and Myrold 1999). The UPGMA dendrogram of the fingerprint patterns allowed us to cluster the *Frankia* populations in the root nodules into five groups (Fig. 2 of Paper III): All the patterns from the identified nodules clustered with the pure cultures, whereas the unidentified *Frankia* populations ($AiBp2_{Nod}$, $AgPa11_{Nod}$, $AiPs23_{Nod}$ and $AgPs7_{Nod}$) in nodules clustered into two groups outside our analyzed subgroups within the *Alnus* host infection group. Furthermore, the rep-PCR results correlated with the *in situ* hybridization results and allowed us to assign *Frankia* populations in root nodules with similar fingerprints to the same group within the *Alnus* host infection group. To further characterize these populations, comparative sequence analysis of PCR-amplified and cloned 23S rRNA gene fragments from nodule and soil was performed.

Sequence alignments from cloned nodule DNA and comparison with corresponding sequences of *Frankia* isolates representing five subgroups (I, IIIa, IIIb, IVa and IVb) of the *Alnus* host infection group, *Casuarina* host infection group (II) and *Elaeagnus* host infection group (VI) is shown in Figure 1 of Paper IV. Based on sequence similarities of the *Frankia* strains and nodule clones a dendrogram showing the existing subgroups was generated (Fig. 6). All clones from nodule DNA grouped within the five alder host infection groups and nearly all had identical sequences with alder host infection groups IIIa, IVa and IVb (Fig. 1 of Paper IV). New sequences within the *Alnus* host infection groups were found from two “birch” soil induced nodule clones and one “spruce” soil induced nodule clone. The birch nodule clone (AiBp19 and AiBp20) sequences and spruce nodule clone (AiPa12) sequence were identical with each other and showed four differences to the sequence from *Frankia* isolate AiPs1 (Fig. 1 of Paper IV).

Because the design of the group-specific oligonucleotide probes was based on a limited number of pure cultures (Hönerlage et al. 1994), further studies on the diversity of *Frankia* populations in nodules may result in the discovery of new *Frankia* populations. Nevertheless, the data presented here demonstrate that different *Frankia* populations formed nodules on the capture plants after inoculation with soil from the birch, pine or spruce stands. However, whether these populations in nodules represent the total *Frankia* population in these soils or only a fraction of physiologically active, infective frankiae still needed further investigation.

4.5.4 Uncultured *Frankia* populations in soils

To determine whether *Frankia* populations in nodules represent a major portion of the total *Frankia* population in soils, uncultured *Frankia* populations in soil were analyzed by PCR on DNA extracted from soil of the birch, pine and spruce stand, respectively. A 135 bp product was obtained from all soil samples and reference strain DNA subjected to genus-specific amplification (Fig. 4A of Paper II). To increase the sensitivity of detection, a nested PCR was carried out using primer combinations FraV and 23B1.9, AFAr and 23AvC, AFMr and 23AvN or AFMr and 23Mut(II) on a portion of PCR products obtained after genus-specific amplification with primers FraV and 23Fra. Amplification products of approx. 65 bp on DNA extracted from soil of the birch, pine and spruce stand, as well as on DNA obtained from pure cultures of the respective target groups (Fig. 4B-E of Paper II) were obtained. The presence of homologous sequences was confirmed by southern hybridization with probes targeting representative strains of the respective target groups. These PCR-based results indicated that the total *Frankia* population was similar in soils of the birch, pine and spruce stands.

In conclusion, we detected different *Frankia* populations in the nodules of the capture plant *A. incana*, depending on whether the soil originated from the birch, pine or spruce stands (Table 4). Based on results from PCR experiments, these differences were not reflected in the *Frankia* populations present in the respective soils because all *Frankia* populations analyzed were found to be present in all three soils. Comparative analysis of nodulation units with genomic units of soils determined by the PCR-MPN technique using nested PCR indicated that only a small portion of the total population of *Frankia* was able to nodulate (Myrold and Huss-Danell 1994; Myrold et al. 1994). Based on our study, it was suggested that the nodulation capacity of a soil was controlled largely by the physiological status of *Frankia*, as indicated by infectivity, rather than by the total population size. The physiological status of a specific *Frankia* population in soil might be triggered by environmental factors such as e.g. the vegetation favouring saprophytic growth of this population thus increasing its competitive abilities with respect to root nodule formation. This assumption was supported by studies in which only one population of *Frankia* was detected in nodules of the host plant at the respective site by *in situ* hybridization, though different *Frankia* populations were detected in soil by PCR (Zepp et al. 1997b). Based on the analysis of *Frankia* populations in nodules and soils in this study, it must also be assumed that *Frankia* populations in nodules of the capture plants represent a fraction of physiologically active, infecting frankiae in the test soils rather than the total *Frankia* population. However, since PCR products do not necessarily reflect the abundance of the target sequences in the original sample (Suzuki and Giovannoni 1996) the size of the different *Frankia* populations detected might have differed significantly. Furthermore, the analysis only focused on a small number of target groups and may therefore be incomplete. This assumption

is supported by the discovery of *Frankia* populations in nodules of the capture plants after inoculation with soil from the pine-, spruce- and birch-stands which could not be identified. Nevertheless, the results showed that the nodulation capacities of these soils were determined by a limited number of specific populations rather than a function of the size of all *Frankia* populations present.

Many questions on *Frankia* under non-host plants still remain unanswered. Although indirect results support the theory of *Frankia* nitrogen fixation and growth in soils devoid of actinorhizal plants, more studies on the function, i.e. nitrogen-fixation, protection of plant roots and production of plant growth promoting substances, of *Frankia* in soil are needed. Interactive relationships with mycorrhizas, also major symbionts of these non-host plants in boreal forests, are an interesting and untouched field of research.

5 Summary and future prospects

In this study, isolation and identification of bacteria from the humus horizon of a Norway spruce stand and from birch and fescue grass seedlings exposed to this soil inoculum showed that seedling roots selectively attracted bacteria from the surrounding soil. Gram-negative genera including *Pseudomonas*, *Alcaligenes* and *Comamonas* predominated on seedling roots, whereas gram-positive genera, *Bacillus*, *Paenibacillus*, *Arthrobacter*, *Nocardia*, *Rhodococcus* were most frequently found in the humus. In addition, new classes of nitrogen-fixing bacteria, *Rhodococcus erythropolis*, *R. facians* and a novel *Paenibacillus* sp. were discovered in the rhizosphere and humus. Studies on the antagonistic potential of the isolated bacteria showed that the rhizoflora and humus contained high proportions of bacteria with antagonistic activity towards plant pathogenic fungi. These isolates also commonly produced siderophores and/or cell wall degrading enzymes. Further studies, however, are required to assess the antagonistic and plant growth promoting activities of these strains in more natural conditions.

Studies on the presence of *Frankia* in soils lacking host plants showed high nodulation capacities in long-term limed plots of a Norway spruce stand and in soils from pine, spruce and birch stands taken from an afforestration site on former agricultural soils. In contrast to previous studies, where especially high nodulation capacities were found in soils under birch as compared to those under spruce and pine, there were no differences beween the nodulation capacities of the pine, spruce and birch stands in this study. This was probably due to the young age of the trees and also related to the agricultural history of the sites, which had impacted on soil properties e.g. higher pH than in natural Finnish forest soils. Root infection by *Frankia* was also plant-species dependent: soils from pine- and birch-stands displayed approximately ten times higher nodulation capacities with *A. glutinosa* than with *A. incana* when used as capture plants. This suggests variable compatibilities of the host plants to *Frankia* populations. Future studies should also include other non-host plants in forest communities which might interact and enrich for *Frankia*. It is clear that *Frankia* will interact with other rhizosphere organisms such as those identified here and important fungal groups such as mycorrhiza and pathogenic fungi. Recent work in our group has shown that one of the *Frankia* strains used in our study is also able to produce antimicrobial compounds (Haansuu et

al. 2001). We now have the methods to study such interactions under more natural forest soil conditions.

Based on comparative sequence analysis of an insertion in Domain III of the 23S rRNA of selected *Frankia* isolates from birch, spruce and pine soils, isolates could be assigned to two groups within the *Alnus* host infection group, namely group IIIa and group IV. The assignment was partially correlated to the origin of the soil used as inoculum. Subsequent PCR-based analysis of specific *Frankia* populations from the birch, spruce and pine soils revealed the presence of *Frankia* belonging to the *Alnus* host infection groups I, IIIa, IVa and IVb. However, only *Frankia* belonging to groups IIIa, IVa and IVb were infective on *A. incana* as shown by *in situ* hybridization. In addition, different *Frankia* populations induced nodules on the host plant depending on the soil inoculum source: frankiae from group IIIa were infective in pine soils compared to IVa group frankiae in birch soils. These results suggest that *Frankia* populations in nodules of the capture plants represent only a fraction of physiologically active, infecting frankiae rather than the total *Frankia* population in soil. Future studies are needed on the detection and spatial localization of these saprophytic *Frankia* populations in soil.

In situ hybridization and sequence analysis of uncultured *Frankia* populations in nodules revealed several *Frankia* populations that could not be detected using presently available probes. Consequently, new subgroups within the *Alnus* host infection group were suggested. The design of new probes will allow the detection of unidentified frankiae in nodules and soil and aid in further studies on infection biology and the ecology of *Frankia*. Functional genes, i.e. *nif* gene probes and monitoring of mRNA as an indicator for gene expression will open up studies on the function and metabolic activity of *Frankia* and other nitrogen-fixing bacteria in the rhizosphere of forest trees. In addition, development of the *in situ* hybridization technique, for instance by using probes containing multiple fluorescent labels, will allow *Frankia* detection in more complex environments like soil. All this will in turn help in gaining a better understanding on the distribution, function and the various interactions of these beneficial micro-organisms in boreal forest ecosystems.

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