16S rRNA as molecular marker in ecology of *Frankia*

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STELLINGEN

1. De introductie van stikstof-bindende planten biedt geen oplossing voor stikstoftekorten in beheerde bossen.
   Wheeler et al. (1986) Plant and Soil 90: 393-406

2. Door de complexiteit van natuurlijke ecosystemen is vertaling van laboratorium gegevens naar het veld amper mogelijk.
   Dit proefschrift

3. Kwantificeren van bacteriën is een subjectieve maat die afhankelijk is van correlatie factoren.
   Dit proefschrift

4. Er is onvoldoende bewijs voor de conclusies die Simon et al. uit de competitie experimenten trekken.
   Simon et al. (1988) FEMS Microbiol. Lett. 51: 13-18

5. Fylogenetische studies met slechts enkele bacterie stammen leveren geen blijvende informatie.
   Hahn et al. (1989) Syst. Appl. Microbiol. 11: 236-242

6. Er is onvoldoende experimentele evidentie voor de door Kuczek en Mordarski opgestelde screenings procedure voor Streptomyceten.
   Kuczek and Mordarski (1989) FEMS Microbiol. Lett. 61: 257-260

7. De frustraties van een hele natie kunnen zich over de jaren heen in de beslissing van een onpartijdige manifesteren.

8. Het is te betreuren dat de wetenschappelijke belangstelling in sommige onderzoeksgebieden gedreven wordt door economische motieven.

9. De stelling dat alleen mensen in staat zijn hun milieu aan zich aan te passen is overdreven.
   Bronowski (1988) The ascent of man. Futura Publ. London

10. Het karakteriseren van een bevolking met trefwoorden heeft met de werkelijkheid niets te maken.

11. Het bedenken of laten bedenken van stellingen net voor de vereiste datum schiet zijn doel voorbij.

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Chapter 1

Introduction

Introduction

Nitrogen fixation in forest ecosystems

Biological nitrogen-fixation is one of the dominant transformation processes in the nitrogen cycle. Only procaryotes are able to reduce molecular nitrogen and to grow in the absence of combined nitrogen sources. The ability to reduce N_2 to NH_4^+ requires the presence of a special enzyme system, the nitrogenase, and certain conditions such as limited availability of combined nitrogen. Nitrogen fixation is distributed among many species of oxygenic and anoxygenic phototrophic bacteria and of aerobic and anaerobic chemoautotrophic and chemoheterotrophic bacteria. N_2-fixing procaryotes comprise both free-living and symbiotic microorganisms. Free-living N_2-fixers belong to many genera including C-autotrophic, phototrophic and C-heterotrophic species (Postgate, 1981; Kennedy et al., 1981; Bothe et al., 1984; Siebold et al., 1985), whereas symbiotic N_2-fixers mainly belong to three taxa, *Rhizobium*, *Frankia* and Cyanobacteria (mainly *Anabaena* and *Nostoc*) (Akkermans and Houwers, 1983).

Investigations on symbiotic nitrogen fixation have mainly been concentrated on the interaction between *Rhizobium* and annual leguminous plants in agricultural systems (Bergersen, 1971). Agricultural systems often obtain in addition to natural nitrogen inputs from leguminous crops nitrogen from fertilization. In contrast managed forest systems depend primarily on natural nitrogen inputs (Bormann et al., 1977). Nitrogen inputs from biological nitrogen fixation and other natural sources, however, are seldom sufficient for optimal tree growth (Dawson, 1983). The total amount of nitrogen in most forest soils, however, is quite high. The limiting factor usually is not the amount of nitrogen but its availability to the trees (Mikola et al., 1983). In managed, i.e. coniferous forests the unbalanced ratio between the production and decomposition of organic matter results in the accumulation of raw humus where nitrogen is blocked from circulation in non-available form in the humus layer. Two principal ways are available to improve the nitrogen nutrition of trees, a) the activation of the decomposition of soil organic matter and the acceleration of nitrogen mineralization, and b) the addition of nitrogen to the soil in easily available form, i.e. the application of nitrogen fertilizers (Mikola et al., 1983). Nitrogen fertilization in forests involves some drawbacks. It is expensive and, to be efficient, the treatment must be repeated quite frequently every 2-3 years (Baule and Fricker, 1967). To avoid these drawbacks, the application of biological nitrogen fixation has repeatedly suggested. The use of actinorhizal and leguminous trees and shrubs to increase productivity of managed forest systems becomes interesting. In tropical regions leguminous trees, e.g. *Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora* (Trinick, 1980) are often used for reforestation, as shade trees and as nitrogen-fixing nurse crops (Doebereiner and Campelo, 1977). In temperate and subtropical regions nonleguminous, nitrogen-fixing plants are more important for these purposes (Dawson, 1983).

The presence of nodular structures on the roots of non-leguminous plants has been first reported for alder in 1829 (see Bond, 1983). At the end of the 19th century a total of 12 species belonging to the genera *Alnus*, *Hippophae*, *Myrica*, *Ceanothus* and *Casuarina* have been known to form nodules with the nitrogen-fixing actinomycete

Frankia (Bond, 1983). Today, nodule formation on woody plants has been demonstrated on more than 160 species distibuted over 21 genera of angiosperms (Akkermans and van Dijk, 1981; Bond, 1983; Lechevalier, 1984). Economically, actinorhizal plants are interesting for reforestation and reclamation of depauperate, nitrogen-limiting soils, especially in developing countries. Only *Alnus rubra* and *Casuarina* spp. attein heights which lend their wood for commercial use as lumber (Anonymous, 1980; Lechevalier, 1988). In subtropical regions the importance of *Casuarina* for the agricultural economies of developing countries is increasingly recognised. Besides its use for reforestation and reclamation of poor soils this tree is also important as a source of firewood (Anonymous, 1980; Wheeler et al., 1986).

In temperate regions *Alnus* species in general and *Alnus cordata*, *A. incana*, *A. rubra* and *A. glutinosa* in particular, have the highest potential for use in forestry (Gordon and Dawson, 1979; Tremblay et al., 1986). They are used as nurse trees in mixed plantations with valuable tree species, i.e. by interplanting actinorhizal plants with suitable tree crops such as walnut, for production of fuelwood and as source of timber in monocultures (Fessenden, 1979; Zavitkovsky et al., 1979; Teissier du Cros et al., 1984; Gordon, 1983). Studies in the USA and Canada on mixed stands of red alder (*A. rubra*) with poplar or douglas fir have demonstrated large yield increments in wood production due to the additional nitrogen input of the *Frankia*-alder system (Miller and Murray, 1979; Binkley, 1980; Hanson and Dawson, 1982; Heilman, 1982; Heilman and Ekuan, 1982; Heilman and Stettler, 1983; Binkley et al., 1984; Cote and Camire, 1985).

The results of the American studies could in part be confirmed in European forestry studies on mixed plantations of *Alnus glutinosa*, the dominating alder species in Northwest Europe, with *Pinus sylvestris*, *Picea abies* and *Populus* spp. (Virtanen, 1957; Mikola, 1966; Mikola et al., 1983; Teissier du Cros et al., 1984; Wheeler et al., 1986). Large variations in yield improvement, however, have discouraged many European foresters from making such plantings, except on the most difficult sites where the nitrogen fixing species can be of value as a protective nurse and as a source of nitrogen (Wheeler et al., 1986). Reasons for the variable test results can be found in the high competitive ability of *Alnus* depending on suitable planting sites, in allelopathic effects of polyphenolic compounds of poplar on the *Frankia-Alnus* symbiosis decreasing the performance of the symbiosis, in the influence of large contents of soil-nitrogen and even in high nitrogen inputs by air pollution. Questions for the usefulness of the symbiosis between *Frankia* and *Alnus glutinosa* as system to increase the availability of nitrogen in managed forests are also concerned with economical purposes. Net worth analyses of theoretical systems for managing actinorhizal plants instead of fertilization have indicated that from the economic point of view fertilization is still less expensive as long as energy costs are low (Tarrant et al., 1983). Interest in biological nitrogen fixation was therefore directly correlated to the price of nitrogen fertilizers. Because of the above reasons, biological nitrogen fixation in forestry practice has mainly remained at the experimental stage and even conclusive results of experiments are scanty. Studies on the interaction between plant and microbiont are needed in order to make biological nitrogen fixation competitive to fertilization. The efficiency of nitrogen fixation of the *Frankia-Alnus glutinosa* system and the commercial value of black alder must be improved in order to reduce costs for plants and manpower and to make biological nitrogen fixation more attractive for practical application in forestry.

Frankia-Alnus glutinosa symbiosis

An improvement of the nitrogen-fixing symbiosis may be achieved both by selection of superior host plant genotypes and of the microsymbiont *Frankia*. Clonal propagation of superior improved cultivars of mature trees is possible (Huss-Danell, 1981; Lundquist and Torrey, 1984) but time consuming and of limited success (Barghchi, 1988). To establish a uniform and high quality plantation, procedures of micropropagation are good alternatives to conventional clonal propagation (Garton et al., 1981; Perinet and Lalonde, 1983a; Perinet and Lalonde, 1983b; Tremblay and Lalonde, 1984; Tremblay et al., 1984; Perinet and Tremblay, 1987; Barghchi, 1988; Montpetit and Lalonde, 1988).

Recently, different ecotypes of black alder (*Alnus glutinosa* (L.) Gaertn.) have been shown to differ significantly in their optimal cultivation system and their root nodulation with the actinomycete *Frankia* (Verweij, 1983). The well-known pioneer ecotypes (e.g. "Weerribben") are characterized by a relatively small size, rapid youth growth and early extensive fruiting (Verweij, 1983). Plants of economical interest can be found within the forest ecotype "Bentheim" of *Alnus glutinosa*. This ecotype is characterized by a relatively slow youth growth during the first 8 years and a delayed generative phase. It forms large and erect trunks and is an excellent source of timber. The economical importance of the forest ecotype can be improved by selecting the right combination of plants (i.e. clones) and their microsymbiont *Frankia* (Hall and Maynard, 1979; Dawson and Sun, 1981; Simon et al., 1985). As a result, higher timber yields can be obtained without nitrogen fertilization (Hall et al., 1979).

The establishment of the symbiosis between actinorhizal plants and the nitrogen-fixing actinomycete *Frankia* can be influenced by several factors (Hall and Maynard, 1979). There are genetically determined factors of the plant-actinomycete interaction such as host-plant compatibility (Baker, 1987; Hooker and Wheeler, 1987; van Dijk et al., 1988) and nodule forming capacity (Dawson and Sun, 1981; Lechevalier et al., 1983; Simon et al., 1985; MacKay et al., 1987). In addition, environmental circumstances may play an important role in the performance of this symbiosis. Abiotic factors like nitrogen-nutrition and pH are known to influence the optimal establishment of an effective symbiosis by introduced *Frankia* strains (MacConnell and Bond, 1957; Stewart and Bond, 1961; Knowlton and Dawson, 1983; Griffiths and McCormick, 1984; Smolander et al., 1988; Smolander and Sundman, 1987). The presence of microorganisms others than *Frankia* can also have an influence (Knowlton et al., 1980). Roots of *Alnus glutinosa* can interact with soil-borne organisms like *Streptomyces* (Suetin et al., 1988), *Nocardia* (Dobritsa and Sharaya, 1986), *Penicillium* (Van Dijk, 1984; Capellano et al., 1987; Valla et al., 1989) and several mycorrhizal fungi (Gardner et al., 1984;). Nitrogen fixation and plant growth can be improved by the presence of other microorganisms, e.g. *Pseudomonas cepacia* (Knowlton and Dawson, 1983), or by inoculating with mixed cultures of effective *Frankia* strains instead of a pure culture (Prat, 1989). An overview of the different interactions between microbes and *Alnus* has been described elsewhere (Akkermans et al., 1989).

Investigations on the ecology of *Frankia* are often hampered by problems encountered with isolation and identification of this microorganism. The application of markers that can be used to identify *Frankia* in complex systems, depends on the isolation and cultivation of the strains to be studied. However, isolation techniques are

always selective and in case of *Frankia* hardly reproducible because only a few isolation attempts succeed in general (St-Laurent and Lalonde, 1987). Information on the distribution and occurrence of *Frankia* in soil is therefore rather fragmentary (Houwers and Akkermans, 1981; Smolander and Sundman, 1987; Smolander et al., 1988). No information is available about the genetic variability and the competitive abilities of *Frankia* in natural environments. Recently, ineffective *Frankia* strains have been shown to occur quite commonly in natural habitats (van Dijk and Sluimer, submitted; Hahn et al., 1988; van Dijk and Sluimer-Stolk, 1984). These atypical strains are not able to fix nitrogen, do not form vesicles and are sometimes not able to infect their original host plants (Baker et al., 1980; van Dijk and Sluimer-Stolk, 1984; Hahn et al., 1988). They lack all morphological and physiological characteristics of *Frankia* and are difficult to isolate.

Characteristics of *Frankiaceae*

Although the knowledge about the interaction of woody plants and the actinomycete *Frankia* has a long history, pure cultures of this actinomycete have been obtained only recently. First attempts to taxonomically characterize the microsymbiont *Frankia* have been made by using its ability to enter a symbiotic association with higher plants and its morphological and structural differences from other actinomycetes (Becking, 1970). The combination of these criteria with cytochemical characteristics has led to the emendation of the family *Frankiaceae* with the genus *Frankia* (Becking, 1970; Becking, 1974). First successful isolations were reported just ten years ago (Callaham et al., 1978). Since that time hundreds of isolates have been obtained using different isolation techniques (Baker et al., 1979a; Baker et al., 1979b; Berry and Torrey, 1979; Baker and O'Keefe, 1984). However, detailed information on the phylogenetic position of pure cultures of *Frankia* and the inter- and intrageneric relationships of strains is still fragmentary.

Frankia strains resemble *Geodermatophilus obscurus* and *Dermatophilus congolensis* in certain morphological features, e.g. hyphae dividing in more than one plane, but differ from them in the possession of sporangia. *Geodermatophilus* was designated to be member of the family *Dermatophilaceae* because both type species *Dermatophilus* and *Geodermatophilus* divide in both transverse and longitudinal planes to form clusters of coccoid cells (Luedemann, 1968). However, both type species show significant differences in physiological characteristics (Goodfellow and Pirouz, 1982), the menaquinone composition (Collins et al., 1984) and 16S rRNA catalogs (Stackebrandt et al., 1983). These arguments are in favour to reinvestigate the taxonomic position of the two genera. Another organism, "*Blastococcus*" ssp., an invalidly described isolate from the baltic sea (Ahrends and Moll, 1970) is reported to show a *Geodermatophilus*-like reproduction. Studies on the ultrastructure of this organism also indicate a close relationship to *Geodermatophilus* (Ishiguro and Wolfe, 1970).

Morphological features and cell wall type of both *Frankia* and *Geodermatophilus* were the basis for previous classification in a taxon "Multilocular sporangia" (Goodfellow, 1986). Even though frankiae show many more phenotypic similarities to dermatophili than to geodermatophili, their dissimilar whole cell sugars, host specificities and serology argue for caution in making conclusions about their close relationship.

Investigations on DNA base composition showed that the G+C% of frankiae and dermatophili were very different and in addition, there was a lack of DNA homology between representatives of the two genera (Samsonoff et al., 1977; An et al., 1985, 1987).

Because of their ubiquity, their large size and their genetic stability, ribosomal RNA sequences have intensively been used to investigate quantitative evolutionary relationships amoung numerous bacteria (Olsen et al., 1986; Woese, 1987). Oligonucleotide catalogs obtained by partial sequence analysis using oligonucleotide fragments produced by digestion with ribonuclease T_1 have until recently been proven to be the most useful for establishing distant phylogenetic relationships (Stackebrandt, 1986; Stackebrandt et al., 1985). Today, new sequencing techniques allow rapid determination of total or almost complete 16S rRNA sequences (Lane et al., 1985; Smida, 1988; Embley et al., 1988). The analyses of conserved sequences of 16S rRNA of *Frankia* and the comparison of these sequences with that of reference actinomycetes has led to the clustering of *Frankia*, "*Blastococcus*" and *Geodermatophilus* into one family *Frankiaceae* (Hahn et al., 1989; Akkermans et al., in press; Chapter 6). Further information is needed to specify the phylogenetic composition of the family *Frankiaceae*.

Characteristics of *Frankia*

The first classification within the genus *Frankia* was based on host plant relationships using crushed nodules as inocula sources (Becking, 1970). However, further investigations with pure cultures demonstrated that the host specificity groups were quite different (Normand and Lalonde, 1986; Baker, 1987). All isolates obtained from nodules were assigned to the genus *Frankia* on the basis of (i) morphological features, such as sporangia and vesicle formation in submerged liquid culture, (ii) chemical composition of certain cell constituents such as cell wall type III, phospholipid type PI and the presence of the diagnostic sugar 2-O-methyl-mannose and (iii) the ability to fix nitrogen and to nodulate plants (Lechevalier, 1984; Lechevalier and Lechevalier, 1984).

Cytochemical criteria have been shown to be helpful to study close phylogenetic relationships. All *Frankia* strains so far studied contained 2-O-methyl-mannose, a cell wall sugar which is specific for the genus *Frankia* (Mort et al., 1983). Quantitative analyses of sugars of whole-cell hydrolysates allowed the separation of the tested *Frankia* strains into their host specificity groups, the *Alnus* and the *Elaeagnus* group, respectively (St-Laurent et al., 1987). Quantitative analyses of total fatty acid composition have also been shown to be useful for taxonomical investigations allowing the discrimination between type species *alni* and *elaeagni* and the subspecies *pommeri* and *vandijkii* of the proposed species *Frankia alni* (Lalonde et al., 1988). Strains clustered in the subspecies *vandijkii* have been derived from nodules in which *Frankia* produces many spores (sp(+) nodules). The genetic stability of this feature is still disputable and not well documented in literature.

Methods to measure small phylogenetic distances among closely related species and to distinguish between strains include serological studies (Baker et al., 1981; Lechevalier et al., 1983) as well as electrophoretic patterns of proteins and isoenzymes (Benson and Hanna, 1983; Benson et al., 1984; Gardes and Lalonde, 1987; Gardes

et al., 1987). Both serological and cytochemical methods show that the genetic diversity among *Frankia* strains is very large. Based on immunodiffusion assays and 1-D-SDS-Page two groups of *Frankia* strains could be distinguished, mainly comprising the *Alnus*-compatibility group and the *Elaeagnus*-compatibility group. However, immunofluorescence studies on the same strains failed to show reliable patterns. Furthermore, the electrophoretic characteristics of some strains did not correlate to their original compatibility group indicating the insufficiency of nodulation tests for taxonomic purposes. Further subgrouping of the strains on the basis of SDS-Page within the compatibility groups is also possible. Related strains could also be distinguished on the basis of electrophoretic separation of isoenzymes and could be delineated in groups of strains.

Molecular methods like restriction enzyme analysis of total DNA (Dobritsa, 1985) or determination of DNA base composition and DNA/DNA homology (An et al., 1985; An et al., 1987; Fernandez et al., 1989) also demonstrate the genetic diversity among *Frankia* strains because only low levels of homology are obtained. Ratios of homology in genomic DNA/DNA hybridizations between 67 and 94% within one compatibility group or levels lower than 50% between different compatibility groups are obtained. However, DNA/DNA homology studies can characterize the compatibility groups and have led to the emendation of *Frankia alnii* (Fernandez et al., 1989). It has been demonstrated that Nif genes have a conserved character (Ruvkun and Ausubel, 1980) and can therefore be used to investigate large phylogenetic relationships (Henneke et al., 1985). Different restriction sites within these genes can supply specific hybridization patterns of digested total DNA with Nif gene probes indicating a possible usefulness of these patterns in taxonomical investigations and as specific markers in ecology oriented research (Normand and Lalonde, 1986; Simonet et al., 1988; Normand et al., 1988; Meesters, 1988; Simonet et al., 1989a, b).

Molecular probes in *Frankia* ecology

Investigations on the behaviour and distribution of typical and atypical *Frankia* and related organisms are always hampered by problems encountered with isolation and identification of this recalcitrant organism. The development of reliable isolation and/or sensitive detection and identification methods is the only way to obtain more information on the development of an optimal actinorhizal plant-*Frankia* symbiosis. Marker molecules that can be used to identify *Frankia* strains introduced to natural environments are necessary to follow the establishment of these strains in nodules and in soil. They can further be used to investigate the competitive abilities of *Frankia* with natural bacterial populations. Identification problems can be solved by applying specific probes that can be used to identify *Frankia* strains without isolation and cultivation (Simonet et al., 1988). Nif-genes have been shown to be possible candidates to act as specific markers in nitrogen-fixing strains. The development of quantitative DNA extraction methods from natural environments, i.e. soil or aquatic environments, makes DNA markers an attractive tool for ecological research (Ogram et al., 1988; Holben et al., 1988; Steffan et al., 1989; Sommerville et al., 1989; Fuhrman et al., 1988). Methods that can be used to amplify minute quantities of target sequences (Polymerase Chain Reaction) are currently developed (Saiki et al., 1988; Steffan and Atlas, 1988) and could be used in combination with specific probes to detect bacteria that are present

in small amounts.

Stable, universal markers can also be found within sequences of 16S rRNA. Differences in the rRNA molecule are not distributed randomly across the entire molecule, but are rather clustered into specific regions. It is therefore not always necessary to determine the entire nucleic acid sequence in order to obtain a suitable probe sequence. Analyses of variable regions of 16S rRNA of closely related organisms indicate sufficient variation to design the probe of interest, despite the fact that DNA/DNA homology studies suggested these two species might actually be one and the same. This makes rRNA sequences an attractive target for diagnostic research (Kohne et al., 1986; Viscidi and Yolken, 1987). Ribosomal RNA is preferable to DNA as a probe target because of its relative abundance and stability in the cell.

Synthetic complementary oligonucleotides serve as specific probes in the DNA/rRNA hybridization reaction. Group- or species-specific sequences that can be used as specific targets for probes in hybridization experiments are found within various microorganisms (Embley et al., 1988; Giovannoni et al., 1988; Goebel et al., 1987; Stahl et al., 1988). Because of the high specificity of the probe-target system, the detection limit becomes very low. Under certain circumstances, e.g. pure cultures, as little as a single bacterium can be detected (Giovannoni et al., 1988; DeLong et al., 1989). Even in mixed cultures where target organisms may occur in low numbers these hybridization technique forms a powerful tool for detection of microorganisms, esp. for pathogens, symbionts and parasites, where isolation and cultivation are difficult (Lane et al., 1988; Tenover, 1988). Although the potentials for molecular probes in ecology look highly promising, our current extraction methods of nucleic acids are far from optimal and do not allow us to measure microbial biomass quantitatively. This is particularly the case with filamentous organisms, e.g. actinomycetes.

Outline of the thesis

The aim of this study is to investigate the factors affecting the establishment of the nitrogen-fixing symbiosis between *Alnus glutinosa* and *Frankia*. Investigations on the role of introduced *Frankia* strains on the development of an optimal symbiosis depend on the standardization of the environmental and host-plant depended factors. Nodulation experiments on defined plant material of physiologically different origin under artificial conditions should give some information about the genetically determined nodulation capacity and nitrogen fixing activity of selected *Frankia* strains. The first part is focussed on the selection and propagation of proper plant clones (Chapter 2) and the development of a standardized nodulation test system (Chapter 3). Results of a screening procedure under axenic conditions indicate the existence of qualitatvely different *Frankia* types, i.e. Nif^+ and Nif^- types (Chapter 4). Studies on competitive abilities of these *Frankia* types indicate some influence of dual inoculation of both types of *Frankia* on plant growth (Chapter 5). Further investigations on the reasons for the observed effects are hampered by problems in identification of the strain composition in nodules. Due to the recalcitrant nature of *Frankia* an identification system is needed that can be used without reisolation and cultivation of the introduced strains. Therefore reliable markers and sensitive detection systems are required. Ribosomal RNA sequences commonly used to investigate phylogenetic relationships (Chapter 6) represent such stable genetic markers. Variable regions on 16S rRNA can

act as targets for specific probes in hybridization experiments (Chapter 7). An approach to use rRNA sequences for identification of the recalcitrant microorganism *Frankia* in nodules is presented in Chapter 8. The usefulness of this detection method for direct identification of *Frankia* specific sequences in soil is also shown (Chapter 9). The relevance of the molecular probes will be further discussed in the final Chapter 10.

Chapter 2 Micropropagation and selection of *Alnus glutinosa* ecotype clones reports studies on multiplication of *Alnus glutinosa* ecotype clones, that can be used as physiologically different test plants in inoculation experiments with *Frankia* strains.

Chapter 3 Selection of *Frankia* strains under standardized nodulation conditions gives a description of a test system used to investigate the genetically determined nodulation capacity and nitrogen-fixing activity of *Frankia* strains on two plant clones under axenic conditions.

Chapter 4 Variable compatibility of cloned *Alnus glutinosa* ecotypes against ineffective *Frankia* strains shows some results of the screening procedure for superior *Frankia* strains indicating the existence of atypical *Frankia* strains.

Chapter 5 Growth increment of *Alnus glutinosa* upon dual inoculation with effective and ineffective *Frankia* strains deals with some ecological aspects of introduced typical *Frankia* strains to large populations of atypical strains.

Chapter 6 Evidence for a close phylogenetic relationship between members of the genera *Frankia*, *Geodermatophilus*, and "*Blastococcus*" and emendation of the family *Frankiaceae* gives some information on the phylogenetic position of *Frankia* by sequence comparison of conserved regions of 16S rRNA of a typical *Frankia* strain and several other actinomycetes.

Chapter 7 Synthetic oligonucleotide probes for identification of *Frankia* strains shows some data of sequence differences between typical and atypical *Frankia* strains that can be used as target sequences for oligonucleotide probes in hybridization experiments.

Chapter 8 Oligonucleotide probes against rRNA as a tool to study *Frankia* strains in root nodules shows the development of an rRNA isolation method from nodules and gives some information on the application of oligonucleotide probes in ecological research on *Frankia*.

Chapter 9 Extraction of ribosomal RNA from soil for detection of *Frankia* with oligonucleotide probes reports about the development of a *Frankia*-specific probe against 16S rRNA and the application against target sequences directly isolated from soil.

Chapter 10 gives a short summary of the presented results.

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Chapter 2

Micropropagation and selection of *Alnus glutinosa* ecotype clones

Micropropagation and selection of *Alnus glutinosa* ecotype clones

Abstract

Clones of two ecotypes of *Alnus glutinosa* (L.)Gaertn. (pioneer ecotype, forest ecotype) were propagated by *in vitro* techniques. Multiple shoots were formed from seedling cultures on modified Heller medium supplemented with 1-5 µM of benzyladenine. Formation of both adventitious and axilliary shoots ensured a high rate of multiplication. Rooting of the shoots occurred after transfer of the individual shoots to media containing indoleacetic acid (1 µM). After root initiation plantlets were transferred to tubes in order to set up a standardized system for nodulation experiments under axenic conditions. Clones derived from both ecotypes were characterized on the basis of isoenzyme patterns, growth rate and nodulation capacity. Isoenzyme patterns of esterases, acid phosphatases and leucylaminopeptidase were identical in all clones of both ecotypes, while growth rate and nodulation capacity showed large differences between clones independent of the original ecotype. Based on these experiments, clones B II and W I were selected as non representative, but easy to propagate, fast growing and physiologically different model clones of the forest and the pioneer ecotype, respectively.

Introduction

Nitrogen-fixation of the symbiotic system actinorhizal host-plant-*Frankia* ensures high yields in biomass production on nitrogen-deficient sites (Zavitkovski et al., 1979). Among the actinorhizal plants, *Alnus* species, i.e. *Alnus glutinosa*, *Alnus cordata*, *Alnus incana* and *Alnus rubra* have the highest potential for use in forestry under temperate climates (Dawson and Gordon, 1979). They are commonly used in land reclamation and soil improvement, as nurse plants in mixed cultures or as pure cultures for fibre production (DeBell, 1979; Fessenden, 1979; Resch, 1979). Recently, ecotypes of black alder (*Alnus glutinosa* (L.) Gaertn.) have been shown to differ significantly in their optimal cultivation system and their root nodulation with the actinomycete *Frankia* (Verweij, 1983). Plants of economical interest can be found within the forest ecotype "Bentheim" of *Alnus glutinosa*. This ecotype is characterized by a relatively slow youth growth during the first 8 years and a delayed generative phase. It forms large and erect trunks and is an excellent source of timber. It is aberrant of the better-known pioneer ecotypes (e.g. "Weerribben") which are characterized by rapid youth growth, early extensive fruiting and relatively small size (Verweij, 1983). The economical importance of the forest ecotype can be improved by selection of the right combination of plants (i.e. clones)(Hall and Maynard, 1979; Dawson and Sun, 1981; Simon et al., 1985) and their nitrogen-fixing microsymbiont *Frankia* in order to produce higher timber yields without nitrogen fertilization in monoculture and mixed plantations (Hall et al., 1979).

The research reported in this paper focussed on "Bentheim", the forest ecotype of

Alnus glutinosa. Using tissue culture techniques (Garton et al., 1981; Perinet and Lalonde, 1983; Tremblay and Lalonde, 1984; Tremblay et al., 1984) plant material from the forest ecotype "Bentheim" and the pioneer ecotype "Weerribben" was micropropagated in order to obtain cloned plant material for nodulation studies under axenic conditions. Nodulation with the effective *Frankia* strain Ag45/Mut15, plant growth and cytochemical criteria (isoenzyme patterns) of clones were compared with results obtained from seedling batches of both ecotypes in order to obtain representative plant material for both ecotypes.

Material and methods

Micropropagation

After one week of vernalization at 4°C, seeds of *Alnus glutinosa* (L.) Gaertn. from different provenances (Bentheim, Weerribben), supplied by the Research Institute for Forestry and Landscape Planning "De Dorschkamp" (Wageningen, The Netherlands) were disinfected in a 1% solution of NaOCl with a drop of Tween 20 for 2 hours and washed three times with sterile distilled water. The treated seeds were placed in Petri dishes containing water agar (0.5%) and glucose (0.5%) so that microbial contaminants could be detected. After germination, the axenic shoots were excised and transferred to tubes of Heller medium (Heller, 1957) containing either 1, 5 or 10 µM of BAP (initiation medium). The shoots of non-axenic plantlets bearing 2-3 leaves after two weeks were surface sterilized in a 1% solution of NaOCl with a drop of Tween 20 for 10-15 minutes, washed three times and placed directly in tubes of initiation medium.

After 6-8 weeks on initiation medium shoots were excised into 2-3 pieces, each with 1-2 buds, and transferred to Heller medium containing 1 µM of BAP (multiplication medium).

After 4-6 weeks on multiplication medium, well-developed shoots were excised and transferred to rooting medium (Heller medium at half strenght) containing 1 µM of IAA. Callus and small shoots were replaced on multiplication medium.

Transfer to artificial medium

After one to two weeks on rooting medium, shoots showing root primordia were transferred to glass tubes (15 x 150 mm) containing an artificial growth substrate (perlite, 9 ml per tube) and a modified Heller salt solution (6 ml per tube) containing $NaNO_3$ as nitrogen source.

Nodulation experiments on different clones

Micropropagated axenic plants of two clones from the pioneer ecotype "Weerribben" (W I, W II) and plants of five clones from the forest ecotype "Bentheim" (B II, B VI, B VII, B VIII, B X) were grown in tubes containing perlite as artificial medium supplemented with a modified Heller salt solution containing 20 ppm NO_3-N as nitrogen-source at pH 5.4 for 4-5 weeks. Well-developed plants were inoculated with

the infective *Frankia* strain Ag45/Mut15. Strain Ag45/Mut15 had been grown on P+N medium (Meesters et al., 1985) for one week, washed twice with Heller salt solution without combined nitrogen and homogenated in the same solution through repeated passages into a needle (0.6 mm in diameter) with a sterile syringe. Plantlets were inoculated with one milliliter of this suspension containing an amount of *Frankia* equivalent to 20 µg of total protein (Moss and Bard, 1957). Plant height, nodulation and acetylene reduction activity were measured after 7 weeks and compared with results obtained with axenic seedling batches of both ecotypes grown under the same conditions in to select representative clones for both ecotypes.

In addition, two clones D II and Weg I from local *A. glutinosa* plants were used as reference clones in the study of isoenzyme patterns.

Uninoculated plants of all clones were also transferred to plastic containers (25 x 25 cm) containing perlite and the modified Heller salt solution. After an adaption period of 4 weeks in the greenhouse under high humidity the plants were potted in 1.5 l pots with perlite as substrate and finally, after a short growth period of two weeks, inoculated with defined amounts of *Frankia* strain Ag45/Mut15. Plant height, dry weight and nodulation of the plants were determined after 4 months of growth and compared with measurments obtained from nodulation experiments with strain Ag45/Mut15 on seedling batches from both ecotypes.

Cultural conditions

Cultures for the initiation and multiplication steps were grown in a growth chamber with a thermoperiod of 25/21 °C and a photoperiod of 16/8 day/night under 2500 lux provided by Philips 33 fluorescent tubes. The conditions for rooting, inoculation and nodulation experiments under axenic conditions were the same, except that the lighting was 7500 lux.

Acetylene reduction assay

The tubes were sealed with rubber caps seven weeks after inoculation and used for the acetylene reduction assay. The plants were incubated in a 10% (v/v) acetylene in air for two hours. Samples of 100 µl of the gaseous phase were analysed for C_2H_4 with a Packard gas chromatograph (model 417) equipped with FID and a 0.325 mm x 1 m colomn packed with Porapak R (80- to 100-mesh). Effectivity is expressed as nmol C_2H_4/h/nodulated plant.

Isoenzyme patterns of *Alnus glutinosa* clones

Isoenzyme patterns of acid phosphatases, esterases and leucylaminopeptidase (Pain, 1986) were determined according to Bousquet et al. (1986) with small variations in composition of the extraction buffer. Samples of 300 mg fresh tissue (green parts of 7-week-old nodulated plants) were grounded in 0.5 ml extraction buffer containing 8% PVP(7 g PVP-K 25 (Fluka) and 1 g PVP 360 (Sigma) in 100 ml) at pH 7.5. After centrifugation (3 min., 12000 rpm) 200 µl of the supernatant and 2 µl of 1% bromphenol blue were mixed. Samples of 5-10 µl were run on native polyacrylamide

gels (stacking gel 3%, running gel 11%) for 3 hours at 200 V after prerunning without samples for one hour at 250 V. Enzyme essays were done for acid phosphatases according to Pain (1986), for esterases according to Wetter and Dyck (1983) and for leucylaminopeptidase according to Scandalios and Sorensen (1977).

Results

Initiation

After 5 weeks on the basic medium containing 5 or 10 µM of BAP, the epicotyl of the initiated shoots had not elongated and formed many small, sturdy shoots from lateral buds. The shoots on basic medium containing 10 µM of BAP also formed much callus on those parts of the explants that were in contact with the medium, and the development was slower. No axilliary or adventitious buds were formed on basic medium containing 1 µM of BAP. Normal shoot elongation was observed and after 3-4 weeks roots developed. Shoots initiated on basic medium containing 5 µM of BAP produced small and sturdy shoots from lateral buds (Fig. 1a). These shoots were excised and transferred to multiplication medium.

Multiplication

Two concentrations of BAP were tested for shoot development. Multiple shoot development was only sufficient in subculture on medium containing 1 µM of BAP (Fig. 1b). Lateral buds were formed and callus was produced but there was no shoot elongation after subculture on medium containing 5 µM of BAP. Shoots growing on media containing 1 µM of BAP elongated and developed basal axilliary buds. Clusters of shoots and buds were formed rapidly and could be separated for subculure after 4-6 weeks. The multiplication rate ranged from 2-10 elongating shoots per explant. On average, 5 shoots per explant (ranging in length from 0.5 cm to 1.5 cm) were excised and transferred to rooting medium. The remaining mass of shoot-producing tissue was divided into 4-5 pieces and used for subculture on multiplication medium. The size and development of the shoots depended on the origin of the explant. Shoots originating from seeds of the pioneer ecotype (Weerribben) could be used for rooting and subculture after 3 weeks, whereas explants of the forest ecotype (Bentheim) needed about five weeks to reach this stage.

Root initiation

Individual shoots were excised from 4-week-old cultures on multiplication medium and placed in tubes or Erlenmeyer flasks containing the rooting medium. The first root primordia appeared after 6-7 days and rooting was 100% after 14-20 days (Fig. 1c).

Transfer to artificial substrate

At different times *Alnus glutinosa* plantlets were transferred to tubes containing an

artificial substrate. Shoots directly transferred from multiplication medium to the growth substrate supplemented with 1 μM of IAA showed a very slow root initiation. The first root primordia were observed after 3-4 weeks. After 8 weeks, about 80% of the transferred shoots were well-developed and could be used for nodulation experiments (Fig. 1d). The same percentage of well-developed plantlets was obtained after roots had been initiated on rooting medium for one week and these plantlets that showed root primordia had been transferred to growth substrate for another 3 weeks.

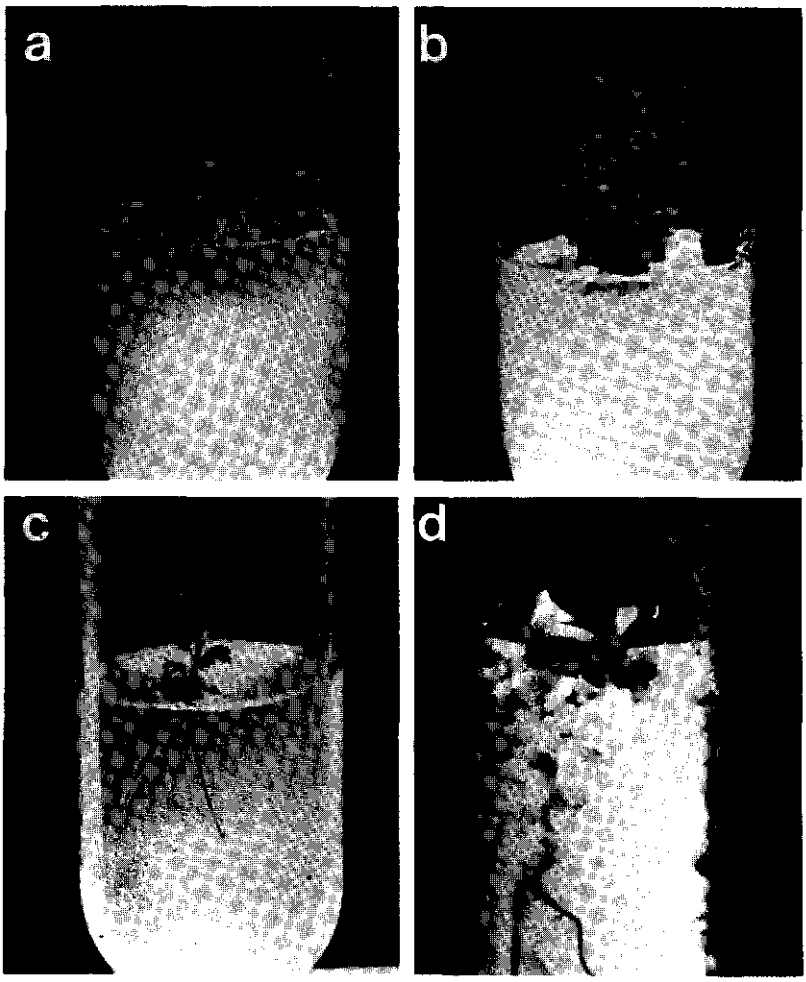

Fig. 1 Initiation (a), multiplication (b), rooting (c) and inoculation (d) steps in micropropagation of black alder

For the pioneer ecotype clone WI the average survival was 80% for developed plants. Only 65% of the forest ecotype clone BII developed to plants. Plantlets transferred to the growth substrate at a later stage (2-3 weeks) often showed a decreased growth and a low survival rate, because most of the roots were damaged by the transfer to the artificial substrate.

Characterization and selection of clones

Nodulation experiments with the nitrogen-fixing *Frankia* strain Ag45/Mut15 showed significant variation among the different clones 7 weeks after inoculation of the axenic plants (Hahn et al., 1988). Large differences between five clones of the forest ecotype "Bentheim" were obtained in the percentage of nodulated plants (42% of clone B X and 100% of clones B II and B VII), nodule number per plant (2.2 nodules of clone B VI and 6.8 of clone B VIII) and effectivity (5.4 nmol C_2H_4/plant/h of clone B VIII and 26.1 nmol C_2H_4/plant/h of clone B II). The diversity between these clones became also apparent by the average of plant length was compared 7 weeks after inoculation. The values were between 9.4 mm (clone B VI) and 38.3 mm (clone B II). Similar variations were shown within the two "Weerribben" clones.

These results obtained under axenic conditions were confirmed in greenhouse experiments under semi-axenic conditions (Table 1). Similar nodulation experiments with seedlings of both alder ecotypes did not show specific nodulation or growth pattern characteristic for the ecotype. None of both criteria could therefore be used as selection criterium (Table 2, Table 3).

Table 1 Growth characteristics of several *Alnus glutinosa* ecotype clones inoculated with the equivalent of 100 µg protein of the effective *Frankia* strain Ag45/Mut15. Plants were grown in the greenhouse under semi-axenic conditions for six months (n= 20; X ± SE)

Clone	Number of nodules	Plant height (cm)	Total dry weight (g)	Shoot/root ratio
B II	442 ± 166	85.0 ± 16.7	27.8	2.2
B VII	230 ± 78	84.0 ± 8.3	37.6	2.4
B VIII	155 ± 66	66.4 ± 18.6	16.1	1.9
B X	249 ± 53	85.6 ± 13.6	42.1	2.1
W I	853 ± 319	65.0 ± 16.9	18.8	1.9
W II	318 ± 136	73.2 ± 9.5	31.6	1.8
Weg I	547 ± 179	68.7 ± 7.9	40.7	1.9

A characterization of all clones by isoenzym patterns using esterases, acid phosphatases and leucylaminopeptidase did not show differences, neither between clones of the same ecotype nor between the two ecotypes (Fig. 2). However, the acid phosphatase patterns of clones B II and W I differed significantly of the two reference

clones, D II and Weg I.

Table 2 Growth characteristics of *Alnus glutinosa* ecotypes "Bentheim" and "Weerribben" inoculated with the equivalent of 20 µg protein of the effective *Frankia* strain Ag45/Mut15. Plants were grown in the greenhouse under semi-axenic conditions for three months (n= 156 "B" and 124 "W"; X ± SE)

Ecotype	Number of nodules	Plant height (cm)	Total dry weight (g)	Shoot/root ratio
"Bentheim"	31 ± 18	34 ± 15	2.4	1.7
"Weerribben"	31 ± 26	31 ± 13	3.1	2.0

Table 3 Growth characteristics of *Alnus glutinosa* ecotype "Bentheim" and "Weerribben" inoculated with the equivalent of 20 µg protein of *Frankia* strain Ag45/Mut15. Plants were grown in tubes under axenic conditions for 7 weeks (n= 70 "B" and 65 "W"; X ± SE)

Ecotype	Nodulation (%)	Effectivity (nmol/h/plant)	Number of nodules	Plant height (mm)
Bentheim	100	19.9 ± 8.3	9.7 ± 4.4	39.4 ± 12.2
Weerribben	89	27.6 ± 9.4	9.1 ± 3.8	44.7 ± 11.6

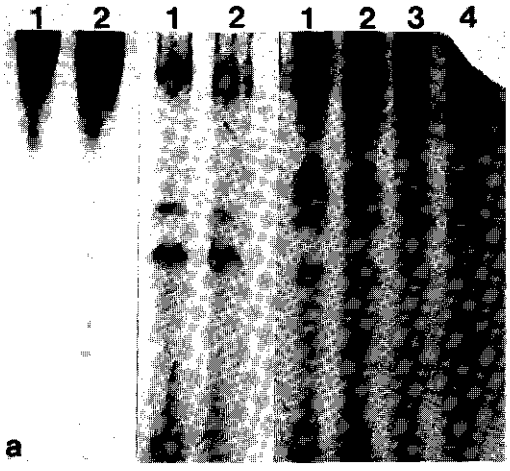

Fig. 2 Isoenzyme patterns of alder clones W I (1), B II (2), D II (3) and Weg I (4) (acid phosphatases (a), leucylaminopeptidase (b), esterases (c)) doe not show specific patterns characteristic for one of the ecotype clones

Discussion

Alnus glutinosa was easily propagated from seedling material by *in vitro* techniques. This opens up the the possibility of large-scale production of interesting *Alnus* clones (Perinet and Tremblay, 1987); not only of *Alnus glutinosa* ecotypes (e.g. Bentheim) but also of other *Alnus* species (Tremblay and Lalonde, 1984). When refined, these technique could be a powerful tool, especially if mature trees of superior genotypes could be propagated and used in mass production (Tremblay et al., 1986). Because it is still impossible to use shoots of mature trees for micropropagation, seedlings or shoots of young trees must be used for tissue culture. This hinders the determination of morphological features being an important criterium to select economically interesting clones of *Alnus glutinosa*. Considerable differences in physiological characteristics of two interesting ecotypes of *Alnus glutinosa*, the forest ecotype "Bentheim" and the pioneer ecotype "Weerribben", have been found in short-term experiments in shade tolerance, germination, juvenile growth and number and size of the root nodules (Verweij, pers.comm.). These characteristics were therefore important criteria for the selection of representative clones for both ecotypes. Numerous clones of both ecotypes of *Alnus glutinosa* were obtained by micropropagation. Nevertheless, criteria like plant growth rate, nodulation and nitrogen-fixing activity in combination with effective *Frankia* strains used in our short-therm experiments to determine ecotype specific patterns and to select representative clones from seedling cultures did not show specific figures for the ecotypes but large variation within clones of one ecotype. Additional experiments with isoenzyme patterns did not show specific patterns of clones and also no ecotype-specific patterns, at least for the three enzyme groups tested. Higher shade tolerance as additional criterium for the forest ecotype (Grime, 1981; Southwood, 1981) indicated that two selected clones (B II and W I) belong to the expected ecotypes (Verweij, pers. comm.). Membership to the expected ecotypes is also supported by the earlier dormancy of the pioneer ecotype clone W I. The selection of one clone of both ecotypes (B II and W I) was therefore based on practical aspects, i.e. rapid growth in tissue culture and large differences in nodulation and growth features between these clones.

The *in vitro* propagated clones can be used to compare the effects of selected superior *Frankia* isolates on the growth of genetically defined host plants. The latter has been done under non-axenic conditions with clones obtained from cuttings (Dawson and Sun, 1981). Nevertheless, culture conditions and natural populations of microorganisms can influence the development of actinorhizal plants (Huss-Danell, 1980; Knowlton et al., 1983; Simon et al., 1985; MacKay et al., 1987). The axenic conditions of our growth system open up the possibility to investigate the performance of the *Alnus-Frankia* symbiosis without the interference of other microorganisms (Perinet and Lalonde, 1983). A standardized nodulation system supplying optimized nodulation conditions must be developed in order to minimize the influence of nutrition and inoculation conditions on the performance of the symbiosis.

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Chapter 3

Selection of *Frankia* strains under standardized nodulation conditions

Selection of *Frankia* strains under standardized nodulation conditions

Abstract

Clones of two *Alnus glutinosa* ecotypes, the forest ecotype clone B II and the pioneer ecotype clone W I, have been micropropagated and used to set up a standardized infection system under axenic conditions. Perlite as artificial substrate ensured a constant pH of about 6.5 during the growth period of 4-5 weeks and, after inoculation with *Frankia*, also during the nodulation period of 7 weeks. The single addition of a modified Heller salt solution supplemented with 20 ppm NO_3-N provided sufficient nutrition for optimal nodulation in our closed system. Inoculation of rooted plants with amounts of *Frankia* strain Ag45/Mut15 equivalent to 2 µg of protein showed maximum nodulation on the forest ecotype clone, whereas the pioneer ecotype clone showed increasing nodule formation with increasing inoculation amounts.

Due to the restricted growth conditions and the plant clone dependent nodulation conditions, this axenic test system was only reliable in qualitative studies. It was less useful for quantitative investigations on nodulation capacity and nitrogen-fixing ability of *Frankia*. Pot experiments in the greenhouse with perlite as artificial substrate and three selected *Frankia* strains confirmed the obtained results of the axenic test system in part. Two of the three strains, however, showed different patterns of nodulation and plant growth.

Inoculation of strain Ag45/Mut15 to plants of both ecotype clones growing on sterilized and non-sterilized soil ("Weerribben" and "Bentheim") did not show any influence on plant growth during 6 months. Nodule formation was only enhanced on "Weerribben" soil.

Introduction

The establishment of an efficient symbiosis between the nitrogen-fixing actinomycete *Frankia* and woody plants of the genus *Alnus* can be influenced by the source of genotypes of both partners (Hall and Maynard, 1979). Plants of economical interest could be found within ecotypes of *Alnus glutinosa*, i.e. the forest ecotype "Bentheim", that forms an excellent source of timber (Verweij, 1983). Clones of this ecotype and of the pioneer ecotype "Weerribben" have been micropropagated using tissue culture techniques in order to obtain homogenous plant material for nodulation studies (Hahn et al., 1988). It has been shown that significant differences in growth and nitrogen-fixation can occur between clones of actinorhizal plants (Dawson and Sun, 1981; Huss-Danell, 1980; MacKay et al., 1987). These differences can largely be explained by the plant genotype (Simon et al., 1985). Another significant factor is the genotype of the *Frankia* strain (Normand and Lalonde, 1982; Vandenbosch and Torrey, 1984). The optimal use of selected clones of the forest ecotype of *Alnus glutinosa* therefore involves the improvement of the symbiosis through selection of superior genotypes of *Frankia*. Criteria like nitrogen-fixing capacity and compatibility of *Frankia* in combination with competitiveness of introduced strains must be checked in order to find suitable

inoculum strains.

Absolute performance of the *Alnus-Frankia* system can best be evaluated under optimized conditions (MacKay et al., 1987). In order to investigate and to compare the genetically determined nodulation capacity of *Frankia* strains a standardized infection system under axenic conditions that supplies optimal nodulation conditions must be developed. Stable growth and infection conditions are supported by a pH in the range of pH 6.5-7.0 (Knowlton et al., 1983). In closed systems single N- fertilization with NO_3 as sole nitrogen source supports the establishment of a pH in the neutral range (Troelstra et al., 1985; Troelstra et al., 1987). Since it is known that high concentrations of combined nitrogen inhibit nodulation and depress nitrogen-fixation in alder (Ingestad, 1980) and low concentrations can support nodulation (Stewart and Bond, 1961) optimal N-concentrations for nodulation under axenic conditions must be determined when performance of the *Alnus-Frankia* symbiosis is investigated (Sellstedt and Huss-Danell, 1984). Optimal performance also requires optimal concentrations of introduced *Frankia* strains.

In this study a standard infection system is set up in order to supply optimal conditions for nodulation experiments on the forest ecotype clone B II and the pioneer ecotype clone W I. Nodulation experiments on both clones are performed under axenic standardized conditions in order to investigate the genetically determined nodulation capacity of different *Frankia* strains.

The reliablility of the test results was checked under semi-axenic growth conditions with perlite as artificial substrate and three selected *Frankia* strains as inoculum. One of these strains was further tested for its influence on plant growth and nodule formation under non-axenic conditions with soil as natural substrate.

Material and methods

Plant material

Clones of two ecotypes of *Alnus glutinosa*, the pioneer ecotype "Weerribben" (clone W I) and the forest ecotype "Bentheim" (clone B II) were micropropagated using tissue culture techniques (Perinet and Lalonde, 1983; Tremblay and Lalonde, 1984; Tremblay et al., 1984) as described in Chapter 2. Rooted plantlets were transferred axenically to tubes containing perlite as artificial substrate supplemented with a modified Heller salt solution (Heller, 1953) and $NaNO_3$ as nitrogen-source at pH 5.4. After 4-5 weeks well-developed plants were used in nodulation tests.

For the development of a standard nodulation system plants of both clones still growing in tubes were inoculated with suspensions containing defined amounts of *Frankia* and grown axenically for another 7 weeks.

For greenhouse experiments uninoculated plants of both clones were transferred to plastic containers (25 x 25 cm) containing perlite and the modified Heller salt solution. After an adaptation period of 4 weeks in the greenhouse under high humidity the plants were potted in 1.5 l pots with perlite as artificial substrate or soil as natural substrate and finally, after a short growth period of two weeks, inoculated with defined amounts of *Frankia* strains.

Standard axenic test system

Four to five weeks after their transfer to the artificial medium, the in vitro propagated plantlets were inoculated with homogenous suspensions of Frankia strain Ag45/Mut15. Strain Ag45/Mut15 had been grown on P+N medium (Meesters et al., 1985) for one week, washed twice with Heller salt solution without combined nitrogen and homogenated in the same solution by repeated passages through a needle (0.6 mm in diameter) fixed to a sterile syringe.

Inoculation experiments were performed with constant amounts of this *Frankia* strain equivalent to 20 µg of total protein (Moss and Bard, 1957) and single N-nutrition (20 ppm N). Different pH of the inoculum solution (pH 5.4; pH 5.8; pH 6.2; pH 6.6) were used in order to determine the buffer capacity of the growth substrate perlite.

Comparative experiments were done with varying amounts of *Frankia* inoculum equivalent to 200 µg, 20 µg, 2 µg, 0.2 µg and 0.02 µg of total protein and single N-nutrition (20 ppm NO_3-N) at pH 5.4. Finally, varying N-nutrition (10 ppm, 20 ppm, 30 ppm, 40 ppm and 50 ppm NO_3-N) was tested at pH 5.4 and constant *Frankia* inoculum (20 µg protein) in order to find optimal nodulation conditions.

Plant height, nodulation and acetylene reduction activity were measured after 7 weeks as described previously (Chapter 2).

Cultural conditions

Nodulation experiments under axenic conditions were performed in a growth chamber with a thermoperiod of 25/21 °C and a photoperiod of 16/8 day/night witha quantum flux density of about 125 µmoles/m^2/s provided by Philips 33 fluorescent tubes for 7 weeks.

Acetylene reduction assay

Seven weeks after inoculation tubes with test plants were sealed with rubber caps and used for the acetylene reduction assay. The plants were incubated for two hours in a 10% (v/v) acetylene in air. Samples of 100 µl of the gaseous phase were analysed for C_2H_4 with a Packard gas chromatograph (model 417) equipped with FID connected to a 0.325 mm x 1 m colomn filled with Porapak R (80- 100-mesh).

Selection of *Frankia* strains under axenic conditions

Seventeen *Frankia* strains were grown in pure culture for inoculum production as described above. Amounts equivalent to 20 µg protein were used as inoculum for plants grown in tubes containing perlite and a Heller salt solution supplemented with 20 ppm NO_3-N.

Nodulation, acetylene reduction activity and plant growth were determined 7 weeks after inoculation. Nodulation was expressed as % of nodulated plants and the number of nodules per nodulated plant (nodule forming capacity).

Pot experiments in the greenhouse

Nodulation tests on perlite in the greenhouse were performed as a model for a nitrogen-limited environment. Three *Frankia* strains (Ag45/Mut15, AgB32, AgKG'84/4) were tested. Ag45/Mut15 and AgKG'84/4 were isolated from spore(-) nodules originating from *A. glutinosa* plants of the pioneer ecotype (Grossensee and Krems, West-Germany) whereas AgB32 was an isolate from spore(+) nodules obtained from *Alnus glutinosa* plants of the forest ecotype (Bentheim, West-Germany)(Hahn et al., 1989). Inoculum preparations of *Frankia* were done as described above. A volume of 5 ml of the homogenized suspension containing the equivalent of 20 µg, 2 µg, 0.2 µg or 0.02 µg of protein (Moss and Bard, 1957) was inoculated to each plantlet grown on perlite in 1.5 l pots. Inoculated plants were grown in the greenhouse under natural light conditions (April-July). Plant height and diameter were measured monthly, nodule formation and dry weight were determined after 4 months.

Inoculation studies in soil

Surface samples (10 cm depth) were collected from two soils, a sandy loam from Bentheim (B), West-Germany, and a peaty soil from Weerribben (W), The Netherlands. Soil characteristics were: (B) soil organic matter was 8.1% of dry weight with a C/N of 13.3 at a $pH_{(H2O)}$ of 3.9. Water content was 57% of soil dry weight. (W) soil organic matter was 71.5% of dry weight with a C/N of 17.7 at a $pH_{(H2O)}$ of 3.6. Water content was 329% of soil dry weight.

Samples of each soil were filled in 1.5 l pots, and half of the pots were sterilized by gamma irradiation (2.5 Mrad) in order to obtain *Frankia*-free soil and allowed to recolonize with air-borne microorganisms in the greenhouse. After two months cloned alder plants were transferred to pots containing sterilized and non-sterilized soil samples. After an adaptation period of two weeks, the equivalent of 100 µg of protein was inoculated to plants growing in the greenhouse. Plant height and diameter were measured monthly, nodule formation and dry weight were determined after 6 months.

Results

Axenic test system

Perlite as artificial substrate was able to buffer the infection medium efficiently. Independent of the pH of the inoculation solution (pH 5.4, pH 5.8, pH 6.2, pH 6.6) the resulting pH was stabilized at pH 6.3-6.5 during the whole growth period. A slight increase of pH during the nodulation period from pH 6.3 up to 6.9 was still within the range for optimal nodulation. Due to the comparable growth conditions in these experiments no differences in plant growth and in nodule formation with *Frankia* strain Ag45/Mut15 could be detected (data not shown).

The single addition of combined nitrogen at different concentrations (10 ppm, 20 ppm, 30 ppm, 40 ppm and 50 ppm NO_3-N) to the artificial medium was sufficient for plant growth when concentrations higher than 10 ppm NO_3-N were used. For both

Table 1 Nitrogen-dependent nodule induction of *Frankia* strain Ag45/Mut15 on *Alnus glutinosa* clone W I (n=30) after 7 weeks of growth ($^*NO_3$-N; X ± SE)

N-nutrition*	Nodulation (%)	Nodule number per plant	Effectivity (nmol/plant/h)	Plant height (mm)
10 ppm	96	8.0 ± 4.0	5.9 ± 5.5	9.8 ± 3.4
20 ppm	96	9.8 ± 6.3	10.6 ± 6.2	12.5 ± 5.4
30 ppm	100	11.3 ± 3.2	15.0 ± 6.1	16.1 ± 5.9
40 ppm	100	10.2 ± 4.1	13.5 ± 7.6	13.1 ± 4.1
50 ppm	100	8.7 ± 4.6	14.9 ± 8.5	12.2 ± 4.6

Table 2 Nitrogen-dependent nodule induction of *Frankia* strain Ag45/Mut15 on *Alnus glutinosa* clone B II (n=35) after 7 weeks of growth ($^*NO_3$-N; X ± SE)

N-nutrition*	Nodulation (%)	Nodule number per plant	Effectivity (nmol/plant/h)	Plant height (mm)
10 ppm	89	3.7 ± 2.5	11.3 ± 11.6	23.5 ± 10.5
20 ppm	83	4.6 ± 3.7	16.9 ± 12.4	25.3 ± 11.8
30 ppm	95	5.2 ± 3.0	21.9 ± 15.5	35.6 ± 13.6
40 ppm	97	5.6 ± 3.3	25.1 ± 19.0	37.2 ± 13.4
50 ppm	100	6.3 ± 2.7	35.6 ± 15.0	40.5 ± 13.0

Table 3 Nodule induction of *Frankia* strain Ag45/Mut15 on *Alnus glutinosa* clone B II (n=20) after 7 weeks of growth, dependent on inoculum concentration (*µg protein)

Inoculum*	Nodulation (%)	Nodule number per plant	Effectivity (nmol/plant/h)	Plant height (mm)
0	0	0	0	16.4 ± 1.3
0.002	50	1.4 ± 1.4	11.8 ± 16.5	18.3 ± 6.1
0.02	40	1.2 ± 1.6	19.3 ± 18.7	17.0 ± 3.2
0.2	74	2.1 ± 2.0	22.5 ± 10.2	21.1 ± 8.3
2	80	4.8 ± 2.1	22.6 ± 10.2	25.8 ± 8.5
20	100	4.4 ± 1.6	22.8 ± 11.3	30.9 ± 9.9
200	100	4.1 ± 2.1	23.7 ± 20.5	27.1 ± 8.9

Table 4 Nodule induction of *Frankia* strain Ag45/Mut15 on *Alnus glutinosa* clone W I (n=20) after 7 weeks of growth, dependent on inoculum concentration (*µg protein)

Inoculum*	Nodulation (%)	Nodule number per plant	Effectivity (nmol/plant/h)	Plant height (mm)
0	0	0	0	11.4 ± 2.0
0.002	33	1.1 ± 1.7	29.3 ± 6.4	11.6 ± 3.1
0.02	69	1.6 ± 1.6	15.7 ± 13.7	12.4 ± 3.1
0.2	78	3.7 ± 2.5	12.3 ± 12.5	11.4 ± 2.3
2	100	5.9 ± 2.6	22.3 ± 10.3	14.1 ± 4.0
20	100	7.6 ± 2.5	19.9 ± 11.0	16.2 ± 5.8
200	100	11.1 ± 2.6	46.3 ± 12.3	15.5 ± 3.4

clones optimal nodulation was obtained at concentrations higher than 10 ppm N. Higher nitrogen concentrations did not show significant changes in nodule formation (Table 1, 2).

Inoculation of clone B II with different concentrations of *Frankia* showed optimal nodulation (nodule number per plant), effectivity and plant growth at concentrations equivalent to 2 µg of protein (Table 3). Similar results were obtained with clone W I. With the exception that the number of formed nodules increased with the inoculum concentration till the highest *Frankia* inoculum concentration (Table 4).

In all inoculation experiments with *Frankia* strain Ag45/Mut15 *Alnus glutinosa* clone W I always showed about twice as much nodules as the forest ecotype clone B II while the initial growth rate of W I (expressed in plant height) remained significantly lower than of B II.

Selection of *Frankia* strains under axenic conditions

Nodulation tests with different effective (Nif$^+$) *Frankia* strains that fix N_2 in pure culture have shown that most strains were infective on both clones W I and B II of *Alnus* glutinosa (Table 5). Nodulation percentage of most strains was high both on clones W I and B II. Significant differences in nodule number per plant between the two ecotype clones were obtained with four strains (AgP, AgP_1R_3C, AgGS'84/45, Ag45/Mut15). Generally, these strains induced about twice as much nodules on the pioneer ecotype clone W I than on the forest ecotype clone B II. Other strains (AgN_2Cl_2, AgN_3Cl_2, AgP_1R_1, AgP_1R_2C, AgGS'84/44, AgKG'84/4, An2.24, Arl3, AgB20, AgB32) gave similar nodulation on both ecotype clones with different averages in nodule number per plant (3.5 of strain AgP_1R_1 and 9.5 of strain AgKG'84/4). Two strains, AgN_1Cl_2 and Cpl.2, formed only low numbers of nodules or were non-infective under the given conditions. In addition, strain AgB16 seemed to be ineffective 7 weeks after inoculation in spite of its ability to fix N_2 in pure culture. All strains except AgB16 and Cpl.2 were effective on both clones, but there were large variations in acetylene

Table 5 Nodulation tests with *Frankia* pure cultures under axenic conditions on *Alnus glutinosa* ecotype clones W I and B II

Strains	Origin*	Clone	Nodulation (%)	Nodule number per plant	Effectivity (nmol/plant/h)	Height (mm)
AgN_1CL_2	sp(-)	W I	36	1.8 ± 1.0	35.5 ± 2.1	12.7 ± 3.3
		B II	33	1.0 ± 0.0	2.8 ± 4.7	22.4 ± 2.8
AgN_2Cl_2	sp(-)	W I	65	5.2 ± 2.0	22.5 ± 19.4	10.9 ± 3.8
		B II	80	4.9 ± 2.3	36.8 ± 23.0	26.1 ± 9.4
AgN_3Cl_2	sp(-)	W I	85	5.9 ± 1.6	10.7 ± 8.1	15.0 ± 5.6
		B II	90	3.9 ± 1.4	22.4 ± 11.4	38.1 ± 9.5
AgP	sp(+)	W I	85	8.1 ± 3.3	30.3 ± 17.3	19.4 ± 7.7
		B II	89	3.7 ± 1.9	30.7 ± 16.8	25.1 ± 8.8
AgP_1R_1	sp(+)	W I	85	4.0 ± 2.2	28.2 ± 29.7	16.5 ± 7.6
		B II	50	3.1 ± 1.7	42.0 ± 35.3	24.3 ± 9.7
AgP_1R_2C	sp(+)	W I	100	6.9 ± 2.8	41.0 ± 24.8	25.4 ± 9.4
		B II	85	4.2 ± 1.5	62.7 ± 21.6	33.5 ± 9.4
AgP_1R_3C	sp(+)	W I	100	8.9 ± 2.7	47.3 ± 14.6	23.7 ± 7.2
		B II	100	3.9 ± 1.4	56.8 ± 21.7	33.2 ± 7.7
AgGS84/44	sp(-)	W I	85	9.0 ± 2.9	72.3 ± 23.1	22.6 ± 9.0
		B II	85	6.2 ± 2.0	96.2 ± 38.6	51.0 ± 9.9
AgGS84/45	sp(-)	W I	100	5.0 ± 1.6	35.6 ± 19.6	21.6 ± 7.0
		B II	80	2.4 ± 0.7	25.5 ± 15.2	31.4 ± 9.9
Ag45/Mut15	sp(-)	W I	100	9.4 ± 3.0	20.3 ± 7.2	15.1 ± 4.2
		B II	100	5.5 ± 1.6	20.0 ± 13.8	31.1 ± 4.3
AgKG84/4	sp(-)	W I	95	9.9 ± 2.7	18.2 ± 9.7	17.7 ± 5.1
		B II	95	7.8 ± 2.8	37.9 ± 14.2	30.0 ± 8.6
An2.24	sp(-)	W I	90	7.3 ± 2.9	44.3 ± 19.8	19.5 ± 5.7
		B II	89	4.5 ± 2.5	36.1 ± 24.3	21.5 ± 4.6
Arl3	sp(-)	W I	85	6.3 ± 2.8	77.0 ± 26.7	16.9 ± 7.5
		B II	90	7.2 ± 1.9	88.4 ± 34.3	42.9 ± 9.9
Cpl.2	sp(-)	W I	0	0	0	8.6 ± 2.4
		B II	0	0	0	19.9 ± 3.3
AgB16	sp(+)	W I	100	9.8 ± 5.2	0	14.4 ± 2.6
		B II	95	9.8 ± 3.5	0	17.3 ± 2.4
AgB20	sp(+)	W I	100	9.0 ± 3.0	49.5 ± 24.1	28.1 ± 9.9
		B II	100	7.8 ± 2.3	43.1 ± 20.9	35.3 ± 9.9
AgB32	sp(+)	W I	80	4.5 ± 2.7	23.1 ± 17.7	17.4 ± 5.0
		B II	84	4.5 ± 2.2	51.2 ± 29.4	31.5 ± 9.9
control		W I	0	0	0	10.4 ± 1.2
		B II	0	0	0	14.9 ± 3.0

* nodule type from which strains have been isolated

reduction activity per plant. Large differences were also obtained in plant height. On the average W I plants remained smaller than B II plants. Depending on the *Frankia* strain, clone B II showed significant variation in plant height, ranging from 21.5 mm (An2.24) to 51.0 mm (AgGS'84/44).

Table 6 Plant height (cm) of *Alnus glutinosa* clones BII and W I, inoculated with different *Frankia* strains. Plants were grown on perlite in the green house for 4 months (n=15)

		Amount of inoculum (µg protein)			
	Strain	20	2	0.2	0.02
B II	Ag45/Mut15	55 ± 5	55 ± 2	45 ± 4	16 ± 4
	AgB32	48 ± 7	40 ± 12	42 ± 11	23 ± 13
	AgKG'84/4	49 ± 5	50 ± 4	34 ± 9	30 ± 9
W I	Ag45/Mut15	38 ± 4	26 ± 7	31 ± 8	19 ± 3
	AgB32	51 ± 7	50 ± 4	53 ± 8	45 ± 8
	AgKG84/4	48 ± 9	48 ± 7	40 ± 5	19 ± 7

Table 7 Nodule formation (nodule number per plant) on *Alnus glutinosa* clones BII and W I, inoculated with different *Frankia* strains. Plants were grown on perlite in the greenhouse for 4 months (n=15)

		Amount of inoculum (µg protein)			
	Strain	20	2	0.2	0.02
B II	Ag45/Mut15	159 ± 23	62 ± 18	9 ± 3	0
	AgB32	61 ± 21	34 ± 19	23 ± 15	2 ± 3
	AgKG'84/4	21 ± 9	12 ± 4	4 ± 3	2 ± 1
W I	Ag45/Mut15	65 ± 21	28 ± 13	15 ± 8	0
	AgB32	67 ± 18	64 ± 29	37 ± 13	5 ± 4
	AgKG'84/4	55 ± 21	27 ± 9	11 ± 4	2 ± 1

Pot experiments in the greenhouse

Inoculation with different amounts of three effective *Frankia* strains (Ag45/Mut15, AgKG'84/4 and AgB32) to the artificial substrate perlite as model for a nitrogen-limited environment showed that plant growth was not only dependent on the plant clone but was also influenced by the *Frankia* strain and the amount of inoculum (Table 6).

Inoculation with strain Ag45/Mut15 resulted in significantly larger plant height of the forest ecotype clone B II compared with clone W I. These differences in growth between the alder clones were not obtained after inoculation with strains AgB32 and AgKG'84/4. Clone W I responded differently to the *Frankia* strains showing growth values ranging from 38 cm (Ag45/Mut15) to 51 cm (AgB32). The variation of growth of clone B II was less significant (Table 6). An inoculum amount of *Frankia* equivalent to about 2 µg protein was required for optimal plant growth. Larger inoculum amounts did not show increasing effects. Lower amounts could be sufficient for optimal plant growth depending on the *Frankia* strain (AgB32) and the plant clone (W I). These results were not only expressed in plant height but also in plant dry weight (data not shown).

Table 8 Growth of two ecotype clones (BII, WI) of *Alnus glutinosa* on two soil types, τ-radiated and natural, after introduction of the effective *Frankia* strain Ag45/Mut15 (plant heigth in cm)

Clone	Treatment	"Bentheim" soil τ-radiated	"Bentheim" soil natural	"Weerribben" soil τ-radiated	"Weerribben" soil natural
BII	control	37 ± 6	43 ± 7	36 ± 6	20 ± 5
	Ag45/Mut15	36 ± 6	44 ± 6	40 ± 4	18 ± 2
WI	control	27 ± 6	36 ± 4	29 ± 4	11 ± 2
	Ag45/Mut15	21 ± 6	35 ± 5	28 ± 4	11 ± 2

Table 9 Nodule formation of two ecotype clones (BII, WI) of *Alnus glutinosa* on two soil types, τ-radiated and natural, after introduction of the effective *Frankia* strain Ag45/Mut15)(nodule number per plant)

Clone	Treatment	"Bentheim" soil τ-radiated	"Bentheim" soil natural	"Weerribben" soil τ-radiated	"Weerribben" soil natural
BII	control	0	40 ± 31	0	50 ± 21
	Ag45/Mut15	0	26 ± 16	107 ± 35	83 ± 18
WI	control	0	46 ± 23	0	51 ± 23
	Ag45/Mut15	0	64 ± 26	55 ± 21	105 ± 30

Nodule formation was positively correlated with the amount of inoculum, i.e. the higher the inoculum amount the more nodules were formed (Table 7). The number of nodules was not correlated with plant growth but dependent on the plant clone and the *Frankia* strain (Table 7). *Frankia* strain Ag45/Mut15 formed about twice as much nodules on the forest ecotype clone B II than on the pioneer ecotype clone W I. Strain

AgB32 did not show significant differences in nodule formation between both clones, whereas strain AgKG'84/4 showed twice as much nodules on clone W I than on clone B II (Table 2).

Table 10 Spore type in nodules of two ecotype clones (BII, WI) of *Alnus glutinosa* on two soil types, τ-radiated and natural, after introduction of the effective, spore (-) *Frankia* strain Ag45/Mut15

Clone	Treatment	"Bentheim" soil τ-radiated	"Bentheim" soil natural	"Weerribben" soil τ-radiated	"Weerribben" soil natural
BII	control	0	77% sp+	0	83% sp+
	Ag45/Mut15	11% sp+	95% sp+	3% sp+	5% sp+
W I	control	0	87% sp+	0	99% sp+
	Ag45/Mut15	8% sp+	76% sp+	3% sp+	4% sp+

Inoculation studies in soil

Introduction of *Frankia* strain Ag45/Mut15 to natural environments, i.e. soils containing natural populations of *Frankia*, did not show any significant influence of the introduced strain on plant growth (Tab. 8) and plant dry weight (data not shown).

Nodule formation on plant clone B II was also not significantly influenced by inoculation independent of the soil type. Nodule formation on clone W I showed significant increment after inoculation of the effective *Frankia* strain Ag45/Mut15 to non-sterilized "Weerribben" soil (Tab. 9).

Microscopical determination of spore types in the nodules showed that the spore type was only significantly changed when the spore (-) strain Ag45/Mut15 was inoculated to the non-sterilized "Weerribben" soil (Table 10).

Discussion

The nitrogen status of the host plant plays an important role in the early development of the symbiosis (Ingestad, 1980; Granhall et al., 1983). Our selected clones W I and B II respond similarly to the N-treatments. Inoculation with different amounts of *Frankia*, however, shows different nodulation figures for the clones. At a concentration of 2 µg of total *Frankia* protein maximum nodule formation on clone B II is obtained. Contrary, clone W I reacts with increasing nodule number with increasing inoculum concentrations. These results indicate that the amount of inoculum is a critical paramenter in optimizing the the nodulation.

Due to the restricted growth conditions of our axenic test system most of the differences in nodulation and plant height are statistically not significant. Even when large amounts of cloned plants are used for nodulation studies, the restricted growth conditions for plants and roots result in large variation in nodulation and plant growth.

The low numbers of nodules and the very small size of the axenic plants obtained in small tubes do not permit to draw general conclusions. Quantitative studies will only be reliable when large differences in nodulation of *Frankia* strains on one clone or on different clones are expected. The data obtained with the nodulation studies show clearly the limitation of our axenic test system. Sufficient information can be obtained in qualitative studies, i.e. on infectivity or effectivity of *Frankia* strains. Quantitative studies, i.e. comparing nitrogen-fixing abilities between different effective *Frankia* strains are hampered by the large variation in growth and nodulation of the clones due to the restricted growth conditions. A selection of superior *Frankia* strains based on our axenic test system must therefore be seen with certain precautions. The establishment of the symbiosis between the plant clones and the *Frankia* strains can be subject of the test conditions, i.e. dependent on different optimal inoculum concentrations for nodulation of the clones. Consequently, our test system should only be used for qualitative nodulation studies.

Nodulation studies in the greenhouse on three selected *Frankia* strains show large variation in plant development dependent on the plant clone, the *Frankia* strain and the inoculum amount. Many of the obtained results are similar to the results obtained under axenic conditions in tubes, but some, e.g. the influence of the *Frankia* strain on the plant clone, are variable. Only strain Ag45/Mut15 shows identical results under axenic and greenhouse conditions. These results demonstrate that inoculation is highly effected by environmental conditions and still unpredictable. Pronounced differences in nodulation which can be obtained under contained, axenic conditions may disappear when plants are grown longer under other conditions.

The latter conclusion becomes even more important when we look to the attempts to inoculate plants in natural (non-sterile) soil. Introduction of *Frankia* to soils containing natural populations of *Frankia* does not show any significant influence on plant growth. This may in part be due to the high nitrogen content of these soils, especially that of the "Bentheim" soil. The failture to obtain any nodules when plants were inoculated in τ-radiated "Bentheim" soil can in part be explained by the high nitrogen content. The nitrogen content of the "Weerribben" soil is much lower. Therefore, a large nodule formation on both alder clones can be obtained on τ-radiated "Weerribben" soil. The lower nitrogen content of this soil can also be responsable for the increased nodule formation when the effective *Frankia* strain is introduced to the untreated soil. This effect can not be found on natural "Bentheim" soil. Differences in plant growth between τ-radiated and natural soils depend on factors that can support plant growth, i.e. mycorrhizae forming fungi in "Bentheim" soil, or show suppressing effects, i.e. plant pathogenic oomycetes in "Weerribben" soil.

The origin of the formed nodules can not reliably be determined because no strain-specific markers are available. Presence or absence of spores in nodules can give some indication about the performance of the symbiosis with the introduced strain. However, spore formation of *Frankia* in nodules is still an unexplored field and surely not a strong marker. It is therefore not clear whether the increment of nodule formation is due to infection by the introduced *Frankia* strain or the natural *Frankia* population. The significant suppression of the percentage of spore(+) nodules formed on plants in "Werribben" soil suggests that the success of inoculation with strains may depend on the soil type and interactions with other microorganisms including *Frankia*. Competition experiments under nitrogen-limited conditions show a large influence of

a combined inoculation of different *Frankia* strains (Hahn et al., 1990). In case of low nitrogen contents in soil an increase of nodule formation after introduction of *Frankia* strains to natural populations has also been shown. Nevertheless, a positive influence of introduced *Frankia* strains to soil on plant growth has not been shown because plant growth was always influenced by the soil-born nitrogen level during the relatively short growth period of 6 months.

The inoculation experiments descibed so far are not in favour to put major efforts in inoculation programmes on *Alnus glutinosa*, unless more basic knowledge is available about the population dynamics of introduced *Frankia* strains in soil. To improve inoculum strains for application in forestry, additional criteria including the ability to form nodules promptly, to persist in soil and to compete with natural *Frankia* strains must be proposed. Given the fact that many soils, particularly eroded soils or steamed garden soils in nurseries, are low in nitrogen content and/or contain too low numbers of *Frankia*, inoculation with one or a mixture of effective *Frankia* remains advisable for practical purposes (Prat, 1989). The use of selected pure cultures of *Frankia* as inoculum instead of soil or crushed nodules (Houwers and Akkermans, 1981) is more a controlled way to supress spread of root pathogens than to obtain an optimal symbiosis, unless more basic knowledge is available about the population dynamics of introduced *Frankia* in soil.

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Chapter 4

Variable compatibility of cloned *Alnus glutinosa* ecotypes against ineffective *Frankia* strains

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Variable compatibility of cloned *Alnus glutinosa* ecotypes against ineffective *Frankia* strains

Key words: Alder, *Alnus glutinosa*, Clones, Compatibility, Ecotypes, *Frankia*, Ineffectivity, N_2-fixation, Nodulation

Abstract

Nodulation tests on *in-vitro* propagated clones of *Alnus glutinosa* ecotypes (forest ecotype, pioneer ecotype) with *Frankia* strains originating from both ecotypes indicated differences in host-plant compatibility. Inoculated plants of the pioneer ecotype clone were not infected by strains, that were unable to fix nitrogen in pure culture. Nodulation could only be induced on the clone of the forest ecotype, but no nitrogen-fixing activity could be detected. Ultrastructural observations of the nodules by SEM and TEM indicated that ineffectivity of these strains was correlated with the lack of vesicles in the infected cells. Cells were only filled with hyphae; neither sporangia nor vesicles could be detected. In contrast, effective nodules could be obtained on both alder clones after inoculation with an effective strain, showing normal development of vesicle clusters in infected cells. In pure culture the ineffective strains produced no vesicles; sporangia were found only during early stage of growth. The results demonstrate the existence of *Frankia* strains which were either non-infective or inefective on different clones of *Alnus glutinosa*.

Introduction

Recent research has revealed that ecotypes or clones of black alder (*Alnus glutinosa* (L.)Gaertn.) differ significantly in their optimal cultivation system and their root nodulation with the actinomycete *Frankia* (Dawson and Gordon, 1979; Simon et al., 1985; I.A. Verweij, pers. comm.). The host-plant specificity and nitrogen-fixing activity of different *Frankia* strains are also genetically determined (Dillon and Baker, 1982). Thus, the right combination of plants (ecotypes, clones) and *Frankia* strains must be found in order to obtain an optimal *A. glutinosa-Frankia* symbiosis (Dawson and Gordon, 1979; Dillon and Baker, 1982; Hall et al., 1979). Therefore, phenomena like incompatibility and ineffectivity that do not only occur in inter- and intrageneric inoculation studies with several actinorhizal plants (Mackintosh and Bond, 1970; Rodriguez-Barrueco and Miguel, 1979; VandenBosch and Torrey, 1983; van Dijk et al., 1988) but also in intraspecific nodulation tests (van Dijk and Sluimer-Stolk, 1984) must be excluded.

Plants of economical interest could be found within the forest ecotype "Bentheim" of *Alnus glutinosa*. This ecotype is characterized by a relatively slow youth growth during the first 8 years and a delayed generative phase. It forms large and erect trunks and is an excellent course of timber. It is aberrant of the better-known pioneer ecotypes (e.g. "Weerribben") which are characterized by rapid youth growth, early extensive fruiting and relatively small size (Verweij, 1983).

During the last years many *Frankia*-like organisms have been isolated from root nodules of both ecotypes of *A. glutinosa*. These strains were unable to fix N_2 and did not form vesicles. Nodulation tests with seedling cultures showed large variation in infectivity and effectivity of these strains and remained irreproducable (I. Sastre, R.T. Dickhoff, pers. comm.). Because *Alnus* spp. are anemophilus and genetically highly variable, the use of cloned plant material instead of seedling cultures is essential in nodulation studies.

The aim of this study is to investigate the infectivity and effectivity of these atypical *Frankia* strains, that so far have had little attention and usually were discarded during the early selection procedure, under standardized infection conditions by using clones of both ecotypes.

Material and methods

Plant material

Plant material from the forest ecotype "Bentheim" and the pioneer ecotype "Weerribben" of *Alnus glutinosa* was cloned by tissue culture techniques (Garton et al. 1981; Perinet and Lalonde, 1983; Tremblay and Lalonde, 1984) in order to obtain homogenous plant material for nodulation studies under axenic conditions. Multiple shoots were formed from seedling cultures on Heller medium (Heller, 1953) containing 1-5 µM of benzyladenine (Sigma). Rooting of the individual shoots was induced on half strength Heller medium containing 1 µM of indoleacetic acid (Sigma). After root initiation plantlets showing root primordia were transferred to glass tubes (15 x 150 mm) containing 9 ml perlite as an inert artificial substrate supplemented with 6 ml Heller salt solution and 20 ppm NO_3-N at pH 5.4. Twenty-one days after their transfer to the artificial medium, the *in-vitro* propagated plantlets were inoculated with a homogenous suspension of *Frankia*.

Frankia strains

Inoculation tests were performed with *Frankia* strains isolated from nodules originating from both *Alnus glutinosa* ecotypes. *Frankia* strains AgB1.5, AgB1.7, AgB1.9, AgB1.10, AgB2.6, AgB2.9 and AgB2.10 were isolated by D. Baker and T.M. Meesters from nodules of alders belonging to the forest ecotype (Bentheim, West-Germany), whereas AgW1.1 was isolated from nodules of alders of the pioneer ecotype (Weerribben, The Netherlands). In both cases the major nodule population consisted of the spore(+) (i.e. spore containing) type. Ag45/Mut15 was isolated from spore(-) nodules originating from *A. glutinosa* plants of the pioneer ecotype (Grossensee, West-Germany). All *Frankia* strains were maintained in pure culture in P+N medium (Meesters et al., 1985) containing propionic acid and ammonium chloride as C- and N-source, respectivily, at pH 6.8.

Inoculation

One-week-old *Frankia* cultures were harvested, washed twice in a Heller salt solution without combined nitrogen and homogenized in the same solution through repeated passages into a needle (0.6 mm in diameter) with a sterile syringe. A volume of 1 ml of the homogenized suspension containing 20 µg of protein determined by a modified Lowry method (Moss and Bard, 1957) was inoculated to each plantlet.

Thirty plantlets of *Alnus glutinosa* clone "Bentheim II" (B II) and 25 plantlets of clone "Weerribben I" (W I) were used per *Frankia* strain and incubated in a growth chamber with a thermoperiod of 24/21 °C and a photoperiod of 16/8 day/night with a quantum flux density of about 125 µmoles/m^2/s provided by Philips 33 fluorescent tubes. After 7 weeks half of the plants were harvested and the acetylene reduction activity, nodulation and plant length were measured. The remaining plants were fertilized with 1 ml of Heller salt solution containing 20 ppm NO_3-N and grown for another 7 weeks. Uninoculated control plants were grown under the same conditions.

Cloned plants from other seedling cultures (clones W I, W II, B II, B VI, B VII, B VIII, B X) were used in additional nodulation tests.

Acetylene reduction assay

Seven or forteen weeks after inoculation the tubes were sealed with rubber caps and used for the acetylene reduction assay. The plants were incubated for 2 hours (linear accumulation of C_2H_4) in a 10% (v/v) acetylene in air. Samples of 100 µl of the gaseous phase were analysed in a Becker Packard gas chromatograph (mode 417) equipped with FID and a 3.25 mm x 1 m Porapak R, 80-100-mesh, filled colomn.

Transmission electron microscopy (TEM)

Nodules harvested 7 and 14 weeks after inoculation were fixed intact in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 overnight at 4 °C. The nodules were rinsed twice with buffer, postfixed in 1% osmium tetroxide in buffer for two hours, rinsed again in distilled water and slowly dehydrated in ethanol (10-100%) followed by propylene oxide. The nodules were embedded in an epon mixture (40:1 mixture of propylene oxide : epon for two hours followed by 20:1, 10:1, 5:1 and 1:1 mixtures each for another two hours; subsequently for 12 hours in a 1:3 mixture and finally in 100% epon for 12 hours). The epon resin capsules were polymerized for 48 hours at 60 °C. The blocks were sectioned on an ultramicrotome and the sections were stained with uranyl acetate and lead citrate. The stained sections were examined with an electron microscope, model Philips TEM 301.

Scanning electron microscopy (SEM)

Nodules and attached root pieces were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 overnight at 4 °C. They were rinsed twice in distilled water and transferred to 25 % (v/v) Dimethyl sulfoxide (DMSO, Merck) in water for one hour, finally to 50% (v/v) DMSO in water. These nodules were fixed on blocks with glue and

transferred to liquid propane and finally liquid nitrogen. Frozen nodules were sectioned in an ultramicrotome (Reichert FC 4D). The remaining pieces of nodules still fixed on the blocks were rinsed twice in 25 % DMSO in water and finally distilled water, each for half an hour, slowly dehydrated in ethanol (10-100%) and critical point dried (Baltzers Union)(A. van Aelst, pers. comm.). The objects were sputtered with gold and examined with a Jeol JSM-35 C Scanning Electron Microscope.

Pure cultures of *Frankia* were filtered through Nuclepore membrane filters (0.4 µm), fixed and dehydrated as described above and used after critical point drying and sputtering with gold for scanning electron microscopy.

Results

Frankia pure cultures

Growth of the *Frankia* strains AgW1.1, AgB1.5, AgB1.7, AgB1.9, AgB1.10, AgB2.6, AgB2.9 and AgB2.10 could only be obtained in media containing combined nitrogen; nitrogen-fixing activity was never obtained. The strains were different with respect to the degree of spore formation, colony shape, pigmentation and growth rate. All strains formed a very fine mycelium with a diameter less than 0.3 µm. Sperical sporangia of different sizes could be observed in an early growth stage (3-4 days) of all strains (Fig. 1a). Material from older cultures of these slow growing strains (generation time about 3 days) could not be used because hyphae lysed very soon and formed a

Table 1 Inoculation test on two alder ecotype clones (B II and W I), 7 weeks

Strain	Clone	Nodulation[b] %	Nodule number[c] per plant	Effectivity[c] (nmol/plant/h)	Plant height[c] (mm)
Ag45/Mut15	B II	100	3.9 ± 2.0	12.5 ± 5.6	30.1 ± 9.1
	W I	100	6.1 ± 2.9	10.4 ± 6.4	14.0 ± 4.5
AgW1.1	B II	40	1.9 ± 0.8	0	18.6 ± 2.9
	W I	0	0	0	9.6 ± 2.5
AgB1.5[a]	B II	0	0	0	18.8 ± 3.6
	W I	0	0	0	10.9 ± 3.3
AgB1.9	B II	57	3.5 ± 2.2	0	17.7 ± 3.0
	W I	0	0	0	11.7 ± 3.6
AgB1.10	B II	10	0.1 ± 0.3	0	19.0 ± 3.5
	W I	0	0	0	12.5 ± 3.0
control	B II	0	0	0	18.2 ± 3.4
	W I	0	0	0	10.0 ± 2.3

[a] similar results were obtained with the strains AgB1.7, AgB2.6, AgB2.9 and AgB2.10
[b] number of plants n= 15 [c] X ± SE

nontransparant mixture of growing and lysed cells, so that cell differentiation was not seen. Vesicles were never detected, neither in media containing combined nitrogen nor in media with N_2 as sole N-source.

Contrary, cell differentiation into vesicles was commonly detected in pure cultures of Ag45/Mut15, both in P-N and in P+N medium (Fig. 1b). Under this growth conditions sporangia were only formed occasionally. Acetylene reduction activity was detected under nitrogen limiting conditions (up to 175 nmol C_2H_4/mg protein/h). Hyphae were much thicker in diameter (1 µm) than those of the other strains.

Nodulation

Differences in infectivity between *Frankia* strains in relation to *Alnus glutinosa* ecotype clones were found between strains that were able to fix nitrogen and those that were unable (Tab. 1). Nodules on both clones of *A. glutinosa* were obtained two weeks after

Fig. 1 a) Pure culture of AgW1.1, grown with combined nitrogen for 4 days, showing hyphae (h), young (ys) and mature (ms) sporangia. Bar represents 1 µm. b) Pure culture of Ag45/Mut15, grown with combined nitrogen for 4 days (h=hyphae, v=vesicles). Bar represents 1 µm. c) Sectioned ineffective nodule of *Alnus glutinosa* clone B II induced by AgW1.1, showing loose-packed hyphae (h) within infected cells (14 weeks after inoculation). Bar represents 10 µm. d) Sectioned effective nodule of clone B II induced by Ag45/Mut15, showing *Frankia* vesicles (v) embedded in the cytoplasm of cortical cells (7 weeks after inoculation). Bar represents 10 µm.

Table 2 Inoculation test on different alder ecotype clones with *Frankia* Ag45/Mut15 (7 weeks)

Clone	Nodulation[a] (%)	Number of nodules[b]	Effectivity[b] (nmol/plant/h)	Plant height[b] (mm)
Weerribben I	100	10.1 ± 4.0	18.6 ± 7.9	14.2 ± 3.7
Weerribben II	100	5.8 ± 3.6	34.1 ± 17.3	30.1 ± 10.7
Bentheim II	100	6.4 ± 1.9	26.1 ± 8.4	38.3 ± 10.3
Bentheim VI	55	2.2 ± 0.8	5.6 ± 3.7	9.4 ± 2.4
Bentheim VII	100	6.0 ± 2.3	25.2 ± 7.2	25.3 ± 7.2
Bentheim VIII	50	6.8 ± 6.2	5.4 ± 7.2	13.2 ± 8.5
Bentheim X	42	4.9 ± 2.3	18.7 ± 17.5	20.5 ± 13.9

[a] number of plants n= 15 [b] X ± SE

Table 3 Inoculation test on two alder ecotype clones (B II and W I), 14 weeks

Strain	Clone	Nodulation[b] %	Nodule number[c] per plant	Effectivity[c] (nmol/plant/h)	Plant height[c] (mm)
Ag45/Mut15	B II	100	4.4 ± 2.1	40.8 ± 6.9	69.8 ± 8.6
	W I	100	10.6 ± 3.6	19.6 ± 8.2	18.8 ± 2.0
AgW1.1	B II	100	8.4 ± 7.9	0	18.8 ± 2.4
	W I	0	0	0	11.8 ± 1.3
AgB1.5[a]	B II	0	0	0	17.4 ± 4.7
	W I	0	0	0	11.4 ± 4.8
AgB1.9	B II	100	10.8 ± 5.2	0	21.2 ± 3.8
	W I	0	0	0	11.0 ± 1.7
AgB1.10	B II	0	0	0	19.0 ± 1.4
	W I	0	0	0	12.0 ± 0.7
control	B II	0	0	0	20.0 ± 1.7
	W I	0	0	0	12.3 ± 2.5

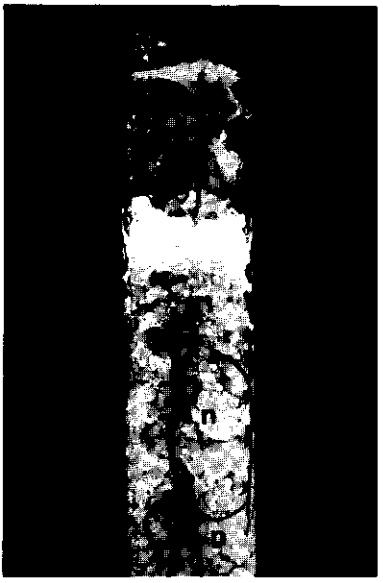
[a] similar results were obtained with the strains AgB1.7, AgB2.6, AgB2.9 and AgB2.10
[b] number of plants n= 15 [c] X ± SE

inoculation with the nitrogen-fixing *Frankia* strain Ag45/Mut15. The pioneer ecotype clone W I formed much more nodules with this strain than the forest ecotype clone B II (significant difference >99% after 14 weeks following F-test). Additional experiments with different clones of both ecotypes showed remarkable variation within the ecotypes with respect to nodule formation, acetylene reduction activity and plant height (Tab. 2). The higher nodule formation on clone W I is therefore not necessarily a general

feature of pioneer ecotypes.

Controvers results were obtained after inoculation with strains that were not able to fix nitrogen in pure culture. Only strains AgW1.1, AgB1.9 and AgB1.10 could infect roots of the forest ecotype clone B II, producing very small nodules that were first visible six weeks after inoculation (Fig. 2). Plants of the pioneer ecotype clone W I were totally resistant against infection of these strains under the applied conditions (Tab. 1). Total
resistance was obtained on both clones B II and W I after inoculation with the strains AgB1.5, AgB1.7, AgB2.6, AgB2.9 and AgB2.10.

The percentages of nodulated plants were very low, i.e. 40% for AgW1.1 and 57% for AgB1.9, respectivily. Strain AgB1.10 also formed some nodules, but the percentage was even lower (2 nodules per 15 plants). One-hundred-percent nodulation was obtained with the strains Ag45/Mut15, AgW1.1 and AgB1.9 after 14 weeks, while the strain AgB1.10 did not show additional nodule formation (Tab. 3). Effective nodules showed multiple root lobe development after 7 weeks, while ineffective nodules remained small.



In all cases of nodulation of *Frankia* strains originating from "Bentheim" or "Weerribben" nodules, nitrogenase activity (acetylene reduction) could not be detected. The leaves of these plants were chlorotic because of the lack of nitrogen. In contrast, plants producing effective nodules with strain Ag45/Mut15 grew normally and foormed green leaves. The leaves of the control plants were less but still green after 7 weeks owing to previous nitrogen uptake. Inoculation with ineffective and non-infective strains had no influence on plant length, whereas inoculation with the effective strain gave significant growth increment on both clones. Differences in growth rate between both clones were a feature of their genotype, independent of inoculation (Tab. 1, Tab. 3).

Fig. 2 *Alnus glutinosa* clone B II plantlet, producing ineffective, hardly visible nodules (n) after inoculation with AgB1.9 (14 weeks)

SEM actinorhizae

The effective nodules formed by Ag45/Mut15 showed normal external structure and typical internal organization with the endophyte developing vesicles within the cortical cells of the host tissue (Fig. 1d). Ineffective nodules showed a similar organization but failed in developing vesicles. Only loose-packed mycelium developed, filling most of the host cell (Fig. 1c). No morphological differentiation of the endophyte occurred, neither vesicle development nor sporangia formation could be observed. Mycelium development was not restricted to cortical cells but could also be observed in

deformed root hairs (Fig. 3), supporting the assumption that root hairs were the sites of infection.

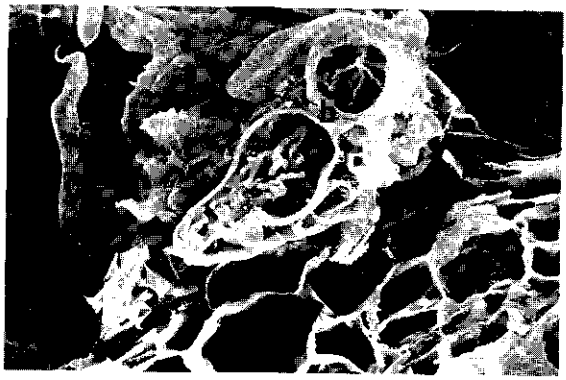


Fig. 3 Section through a deformed root hair containing hyphae (h) of AgW1.1 embedded in cytoplasm. Hyphae are also visible at the surface of the root hair (7 weeks after inoculation). Bar represents 10 µm.

TEM actinorhizae

Endophytic cells were frequently detected in the host cytoplasm, commonly surrounded by a capsule. The characteristic organization of hyphae and vesicles was expressed in effective nodules of strain Ag45/Mut15 showing numerous hyphae occupying the center of the cells, while vesicles were orientated around the periphery of the host cells (Fig. 4a). In contrast to the densely filled cells of the effective nodules, ineffective nodules of the strains AgW1.1, AgB1.9 and AgB1.10 showed only loose-packed hyphae within the infected cells (Fig. 4b). Vesicles were not expressed by these strains. No sporangia, neither in infected cells nor in the intercellular space could be observed.

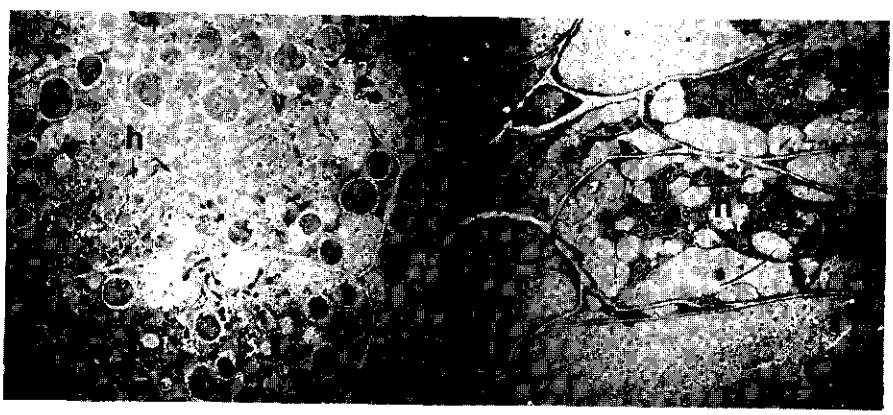


Fig. 4 a) Electronmicrograph of an effective nodule, showing numerous hyphae (h) in the center of an infected cell and vesicles (v) at the periphery of the cell. Bar represents 5 µm. b) Electronmicrograph of an ineffective nodule (AgW1.1) with loose-packed hyphae (h) in infected cells. Bar represents 5 µm.

Discussion

Using genetically homogenous plant material and defined bacterial strains we have demonstrated the existence of different types of interactions between *Frankia* strains and *Alnus glutinosa* clones. The effective, i.e. N_2-fixing strain Ag45/Mut15 is infective on all *A. glutinosa* clones tested, however, the number of formed nodules is host-dependent. Host-dependent interaction of *Frankia* has been reported for several *Alnus* spp. clones (Dawson and Sun, 1981; Simon et al., 1985, Tremblay et al., 1984), however, without information about the original ecotypes. The additional experiments with different clones of both ecotypes cannot indicate that the higher number of nodules of clone W I is a general feature of pioneer ecotypes.

No infection appears on plants of the pioneer ecotype clone when ineffective, i.e. non N_2-fixing *Frankia* strains are used as an inoculum. Plants of the forest ecotype clone show resistance against some strains but can be nodulated by other strains. The total resistance of the pioneer ecotype clone is not correlated with morphological abnormalities in root hair formation as was found for a hardly nodulating *A. crispa* clone (Tremblay et al., 1984).

The ability to induce nodules by the ineffective *Frankia* strains can be due to a) genetically determined infectivity of these strains indicated by a delayed infection of the strains AgW1.1 and AgB1.9 and the non-infectivity of the strains AgB1.5, AgB1.7, AgB2.6, AgB2.9 and AgB2.10 b) suboptimal infection conditions indicated by the very low percentage of nodulation of strain AgB1.10 and c) host plant controlled compatibility indicated by the observed resistance of the pioneer ecotype clone. The latter is further supported by the variable degree of nodulation in seedling batches of different origin (van Dijk and Sluimer-Stolk, 1984). The existence of fully resistance against ineffective *Frankia* strains might be of ecological significance, particularly for plants growing at low nitrogen levels in soil. The non-infectivity of strains AgB1.5, AgB1.7, AgB2.6, AgB2.9 and AgB2.10 is only based on two *Alnus glutinosa* clones. A more extensive screening of additional clones is needed to determine the possible range of infectivity.

Recent research on pure cultures of *Frankia* has revealed that nitrogenase is localized in vesicles (Meesters, 1987; Noridge and Benson, 1986). Nitrogenase activity in the absence of vesicles has recently been found in one *Casuarina* strain (Ccl.3) at very low oxygen tension (Murray et al., 1985) and is commonly observed in *Casuarina* nodules. Contrary, vesicle formation is characteristic for nitrogen-fixing *Frankia* in alder nodules. Ultrastructural observations of the ineffective nodules of *A. glutinosa* clone B II indicate that the ineffectivity is related to the lack of vesicles in the infected cells. Sporangia, commonly observed in other ineffective actinorhizae (Baker et al., 1980; Lechevalier et al., 1983; VandenBosch and Torrey, 1983) could not be observed in nodules, whereas sporangia formation is commonly detected in pure culture. The ineffective strains are morphologically identical, with respect to the form of hyphae and sporangia. Nevertheless, these morphological features and the results of nodulation studies cannot really indicate that all isolates tested are *Frankia*. Other criteria are needed to discriminate between these *Frankia* strains and related actinomycetes.

So far ineffective strains have only been isolated from root nodules of the Weerribben and Bentheim types of *Alnus glutinosa*, which are predominantly of the spore(+) type. Spore(+) strains are difficult to obtain in pure culture (Quispel, 1979) and so far no

confirmative strains are available, which are isolated from spore(+) nodules. The isolation of ineffective strains from these nodules indicates the occurrence of either gene instability of *Frankia* strains or the multiple presence of *Frankia* strains in or on a nodule.

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Chapter 5

Growth increment of *Alnus glutinosa* upon dual inoculation with effective and ineffective *Frankia* strains

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Plant and Soil, in press

Growth increment of *Alnus glutinosa* upon dual inoculation with effective and ineffective *Frankia* strains

Key words: *Alnus glutinosa*, competition, *Frankia*, ineffectivity, N_2-fixation

Abstract

Investigations on the ecological function of ineffective *Frankia* strains and their behaviour in competition with effective *Frankia* strains indicated an enhanced plant growth upon dual inoculation with increasing amounts of effective (i.e. N_2-fixing) *Frankia* strains and simultaneous inoculation with a constant amount of an ineffective *Frankia* strain. Enhanced plant growth was measured as increase in plant height and total dry weight at constant shoot/root ratio. The stimulating effect of the ineffective strain was independent of the plant clone and was obtained with both *Alnus glutinosa* clones W I and B II, which were resistant and susceptable, respectively, to the ineffective strain. Stimulation was also independent of the nodulation conditions. Short-term studies (7 weeks) under axenic conditions and greenhouse experiments during 3 months showed comparable results, not only in plant growth but also in nodule formation. Increment in plant growth was not necessarily correlated to higher nodule formation with the effective *Frankia* strains.

Introduction

Recent investigations have revealed that ineffective *Frankia* strains occur quite commonly in natural habitats (Van Dijk and Sluimer-Stolk, submitted; Hahn et al., 1988; van Dijk and Sluimer-Stolk, 1984; Lechevalier et al., 1983). Sequence analysis of 16S rRNA and hybridization against 16S rRNA with different oligonucleotide probes (Hahn et al., 1989; Hahn, Starrenburg, unpubl. results) indicate that the occurrence of this type of *Frankia* is not due to gene instability. The demonstration of genetically distinct and morphologically and physiologically variable strains of ineffective *Frankia* (Hahn et al., 1988) in natural environments indicates that these strains are separate from effective strains. Investigations on ecological niches established by ineffective *Frankia* are in progress (van Dijk and Sluimer-Stolk, submitted). However, less is known of the ecological function of these actinomycetes and their behaviour in competition with other soil organisms and effective *Frankia* strains. It is speculated that at sites with high populations of these ineffective strains competition with effective *Frankia* strains might reduce the number of formed effective nodules and might have a negative influence on the establishment of an optimal symbiosis.

The selection of improved strains of *Frankia* able to compete with other *Frankia* strains or natural populations is hampered by the lack of convenient methology to detect introduced *Frankia* strains. In order to determine the origin of formed nodules, *Frankia* strains with specific markers have to be used in competition experiments. The use of ineffective *Frankia* strains (Hahn et al., 1988) or natural enrichments (Van Dijk

and Sluimer-Stolk, 1984) forming nodules that are easily distinguishable from effective nodules opens up the possibility of investigating the influence of large ineffective *Frankia* populations on introduced nitrogen-fixing *Frankia* strains.

The aim of our investigations was to determine the influence of ineffective *Frankia* strains on the establishment of an effective symbiosis with introduced effective *Frankia* strains. In order to eliminate any host plant variation and to investigate the influence of physiologically different host plants, i.e. plants resistant and susceptible against the ineffective strain, cloned plant material was used (Hahn et al., 1988), originating from two *Alnus glutinosa* ecotypes (Verweij, 1983). Furthermore, two test systems, a standardized nodulation system in tubes under axenic conditions (Hahn et al., 1988) and pot experiments under semi-axenic conditions in the greenhouse were used in order to determine the performance of the plant-*Frankia* interaction under different conditions.

Material and methods

Plant material

Clones of the pioneer ecotype "Weerribben" (W I) and the forest ecotype "Bentheim" (B II) of *Alnus glutinosa* (L.) Gaertn. were micropropagated using established procedures (Garton et al., 1981; Perinet and Lalonde, 1983; Tremblay and Lalonde, 1984). Rooted plantlets were grown axenically in tubes containing perlite as artificial medium supplemented with a modified Heller salt solution (Heller, 1953) containing 20 ppm NO_3-N as nitrogen-source at pH 5.4 (Hahn et al., 1988). After 4-5 weeks well-developed plants were used as source for both test systems.

For the standard nodulation system plants still growing in tubes were inoculated with suspensions containing defined amounts of *Frankia* and grown axenically for another 7 weeks.

For greenhouse experiments uninoculated plants of both clones were transferred to plastic containers (25 x 25 cm) containing perlite and the modified Heller salt solution. After an adaptation period of 4 weeks in the greenhouse under high humidity the plants were potted in 1.5 l pots with perlite as substrate and finally, after a short growth period of two weeks, inoculated with defined amounts of *Frankia* strains.

Frankia strains

Nodulation tests were performed with three effective *Frankia* strains (Ag45/Mut15, AgB32, AgKG'84/4) and the ineffective *Frankia* strain AgB1.9. Ag45/Mut15 and AgKG'84/4 were isolated from spore(-) nodules originating from *A. glutinosa* plants of the pioneer ecotype (Grossensee and Krems, West-Germany) whereas AgB32 and AgB1.9 were isolates from spore(+) nodules obtained from *Alnus glutinosa* plants of the forest ecotype (Bentheim, West-Germany)(Hahn et al., 1989). All *Frankia* strains were maintained in pure culture at pH 6.8 in P+N medium (Meesters et al., 1985) containing propionic acid and ammonium chloride as C- and N-source, respectivily.

Inoculation

One-week-old *Frankia* cultures were harvested, washed twice in a Heller salt solution without combined nitrogen and homogenized in the same solution by repeated passages through a needle (0.6 mm in diameter) with a sterile syringe.

For nodulation experiments under axenic conditions a volume of 1 ml of the homogenized suspension containing pure cultures of *Frankia* equivalent to 20 µg, 2 µg, 0.2 µg or 0.02 µg of protein were inoculated. Mixtures of the effective *Frankia* strains at the same concentrations with 20 µg of the ineffective strain were also inoculated to each plantlet.

Greenhouse experiments were done with the same amounts of inoculum supplied in 5 ml Heller salt solution. One µg of protein was equivalent to 9.5×10^3 CFU (colony forming units) of strain AgB1.9, to 3.8×10^2 CFU of Ag45/Mut15, to 9.5×10^2 CFU of strain AgB32 and to 5.0×10^3 CFU of AgKG'84/4.

In axenic experiments twenty plantlets of *Alnus glutinosa* clone "Bentheim II" (B II) and twenty plantlets of clone "Weerribben I" (W I) were used with pure cultures or combinations of *Frankia* strains and incubated in a growth chamber with a thermoperiod of 24/21 °C and a photoperiod of 16/8 day/night with a quantum flux density of about 125 µmol m^{-2} s^{-1} provided by Philips 33 fluorescent tubes. After 7 weeks the plants were harvested and the acetylene reduction activity, nodulation and plant height were measured (Hahn et al., 1988).

In greenhouse experiments fifteen plants were used per alder clone and treatment. Inoculated plants were grown under natural light conditions for three months (April 6th to July 6th). Plant height, dry weight, shoot/root ratio and nodulation of the plants were determined after this growth period.

Analytical methods

Determination of inoculum concentrations were done using a modified Lowry protein determination method (Moss and Bard, 1957). Statistical analyses were done using standard LSD tests.

Results

Competition experiments under axenic conditions

Nodulation experiments under axenic conditions on two clones of *Alnus glutinosa* (clone W I and B II) with combinations of variable amounts of the effective *Frankia* strain Ag45/Mut15 and constant amounts of the ineffective *Frankia* strain AgB1.9 showed better plant growth when compared to treatments in which corresponding inoculum amounts of pure cultures of the *Frankia* strains were used (Table 1). Similar increment of plant growth occurs in competition experiments with two additionally used effective strains (AgKG'84/4 and AgB32) and the ineffective strain AgB1.9 (data not shown). Growth increment was obtained on both plant clones, clone W I, that is resistant against ineffective *Frankia* strains and the susceptible clone B II (Table 1).

Table 1 Plant height (mm) of *Alnus glutinosa* clones WI and B II grown in tubes after combined inoculation of the effective *Frankia* strain Ag45/Mut15 and the ineffective strain AgB1.9 (n=20; t= 7 weeks; X ± SE)

Clone		Inoculum (µg protein) 20	2	0.2	0.02
W I	Ag45/Mut15	17 ± 3	16 ± 4	14 ± 2	11 ± 2
	AgB1.9	12 ± 4	nd	nd	nd
	Ag45/Mut15/ AgB1.9 (20µg)	20 ± 6^{ns}	19 ± 8^{ns}	17 ± 5*	12 ± 3^{ns}
B II	Ag45/Mut15	31 ± 12	26 ± 9	21 ± 8	17 ± 3
	AgB1.9	15 ± 2	nd	nd	nd
	Ag45/Mut15/ AgB1.9 (20µg)	40 ± 8*	42 ± 5*	36 ± 6*	25 ± 10^{ns}

*significance P ≤ 0.01; **significance P ≤ 0.05; ^{ns} non-significant; nd not determined

Table 2 Nodule formation (number of nodules) on *Alnus glutinosa* clones WI And B II grown in tubes after combined inoculation of the effective *Frankia* strain Ag45/Mut15 and the ineffective strain AgB1.9 (n=20; t= 7 weeks; X ± SE)

Clone		Inoculum (µg protein) 20	2	0.2	0.02
W I	Ag45/Mut15	8.7 ± 2.8	9.7 ± 2.6	6.4 ± 1.8	3.5 ± 1.3
	AgB1.9	0	nd	nd	nd
	Ag45/Mut15/ AgB1.9 (20µg)	7.6 ± 1.5^{ns}	8.8 ± 2.1^{ns}	7.3 ± 2.3^{ns}	2.3 ± 1.7^{ns}
B II	Ag45/Mut15	4.4 ± 1.6	4.8 ± 2.1	2.1 ± 2.0	1.2 ± 1.6
	AgB1.9	0.1 ± 0.5	nd	nd	nd
	Ag45/Mut15/ AgB1.9 (20µg)	5.8 ± 1.7**	6.9 ± 1.8*	5.1 ± 1.6*	4.5 ± 2.5*

*significance P ≤ 0.01; **significance P ≤ 0.05; ^{ns} non-significant; nd not determined

Nevertheless, growth increment on both clones was not always significant under the restricted growth conditions used. Increment in plant growth was not necessarily correlated to higher nodule formation with the effective *Frankia* strains, i.e with strain Ag45/Mut15 on clone W I (Table 2). Significantly higher nodule formation was only

obtained with strain Ag45/Mut15 on clone B II (Table 2).

Table 3 Total dry weight (g per plant) of *Alnus glutinosa* clones W I and BII grown on perlite under greenhouse conditions after combined inoculation of the effective *Frankia* strain Ag45/Mut15 and the ineffective strain AgB1.9 (n=15, t= 3 months; X ± SE)

Clone		Inoculum (µg protein) 20	2	0.2	0.02
W I	Ag45/Mut15	5.0 ± 0.4	3.6 ± 1.4	4.9 ± 0.8	2.9 ± 1.8
	AgB1.9	2.7 ± 1.2	2.9 ± 1.5	3.6 ± 1.5	2.9 ± 1.1
	Ag45/Mut15/ AgB1.9 (20µg)	7.5 ± 1.2*	6.0 ± 2.0*	7.4 ± 2.2*	5.0 ± 2.5*
B II	Ag45/Mut15	7.8 ± 2.5	6.8 ± 2.1	5.3 ± 1.7	3.0 ± 1.3
	AgB1.9	3.5 ± 1.4	5.8 ± 1.3	4.3 ± 0.5	4.7 ± 0.9
	Ag45/Mut15/ AgB1.9 (20µg)	11.7 ± 2.2*	9.8 ± 2.3*	7.8 ± 2.4*	4.5 ± 1.4*

*significance $P \leq 0.01$; **significance $P \leq 0.05$; ns non-significant

Table 4 Shoot/root ratio (dry weight) of *Alnus glutinosa* clones W I and BII grown on perlite under greenhouse conditions after combined inoculation of the effective *Frankia* strain Ag45/Mut15 and the ineffective strain AgB1.9 (n=15, t= 3 months; X)

Clone		Inoculum (µg protein) 20	2	0.2	0.02
W I	Ag45/Mut15	2.6	1.8	1.7	0.9
	AgB1.9	0.3	0.4	0.3	0.5
	Ag45/Mut15/ AgB1.9 (20µg)	2.6	2.3	2.0	1.2
B II	Ag45/Mut15	2.6	2.7	1.6	0 . 5
	AgB1.9	0.4	0.2	0.5	0.3
	Ag45/Mut15/ AgB1.9 (20µg)	3.0	2.6	1.3	0.4

Table 5 Nodule formation on *Alnus glutinosa* clones W I and BII grown on perlite under greenhouse conditions after combined inoculation of the effective *Frankia* strain Ag45/Mut15 and the ineffective strain AgB1.9 (n=15; t= 3 months)

Clone		Inoculum (µg protein) 20	2	0.2	0.02
W I	Ag45/Mut15	65 ± 21	28 ± 13	17 ± 14	2 ± 2
	AgB1.9	0	0	0	0
	Ag45/Mut15/ AgB1.9 (20µg)	81 ± 24**	39 ± 23^{ns}	15 ± 8^{ns}	4 ± 3^{ns}
B II	Ag45/Mut15	159 ± 23	62 ± 18	9 ± 3	0.1 ± 0.3
	AgB1.9	2.4 ± 2.4	0.3 ± 0.9	0.4 ± 0.5	0
	Ag45/Mut15/ AgB1.9 (20µg)	242 ± 49*	62 ± 30^{ns}	6 ± 4^{ns}	0.5 ± 0.5^{ns}

*significance P≤0.01; **significance P≤0.05; ^{ns} non-significant

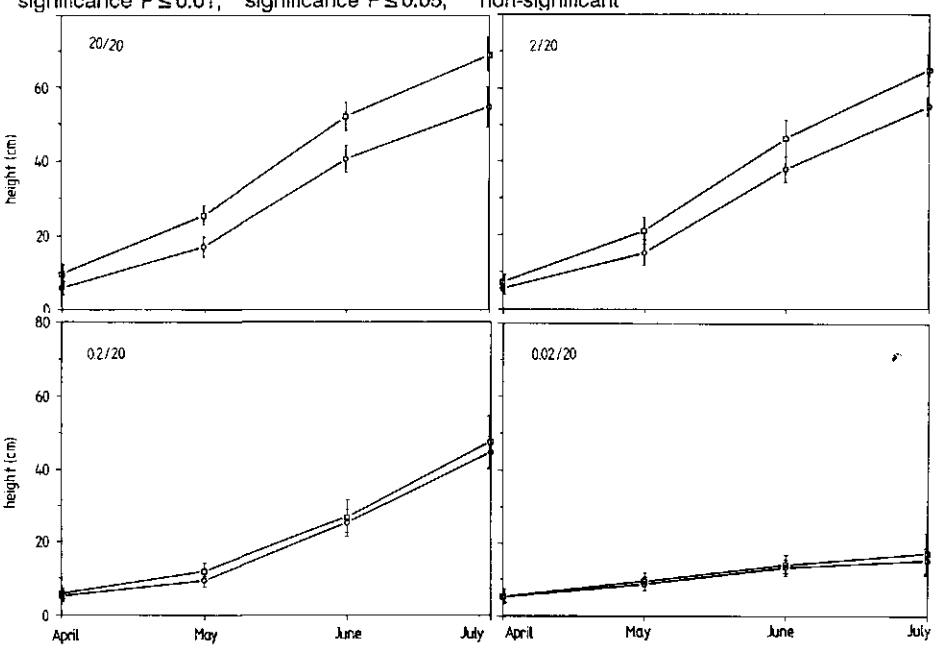

Fig. 1 Growth of *Alnus glutinosa* clone B II (n=15; X ± SE) under greenhouse conditions during three months (April 6th - July 6th), inoculated with variable amounts of the effective *Frankia* strain Ag45/Mut15 (0.02 µg, 0.2 µg, 2 µg and 20 µg) and combinations of this strain with a constant amount of the ineffective strain AgB1.9 (20 µg).

Competition experiments under greenhouse conditions

Nodulation experiments under greenhouse conditions on both clones of *Alnus glutinosa* with combinations of the same effective and ineffective *Frankia* strains showed similar phenomena as under axenic conditions. Increment of plant growth was observed in competition experiments with all three effective *Frankia* strains. Inoculation of *Frankia* strain Ag45/Mut15 at different concentrations mixed with large amounts of the ineffective strain AgB1.9 resulted significant growth increment on both alder clones, measured either as plant height (Fig. 1) or total dry weight (Table 3). Increment of growth depended on the inoculum concentration of the effective *Frankia* strain and seemed to be most pronounced during the first weeks (Fig. 1). The stimulating effect was reflected in the total dry weight and not restricted to the roots as indicated by comparable shoot/root ratios (Table 4).

Significantly higher nodule formation was only obtained with the highest concentrations of strain Ag45/Mut15 on clone B II and not on clone W I ($P \leq 0.05$) (Table 5). The increase in nodule formation on clone B II was due to higher nodule formation of the effective strain. In competition with effective strains the ineffective strain AgB1.9 does not form nodules, except when low concentrations of strain AgKG'84/4 (0.02 µg) are used as inoculum (data not shown). Experiments with strains AgKG'84/4 and AgB32 as effective strains and simultaneous addition of the ineffective strain AgB1.9 on clones W I and B II had no effect on the total number of formed nodules (data not shown).

Discussion

The optimal establishment of an effective symbiosis between *Frankia* and actinorhizal plants can be influenced by environmental circumstances like pH and soil-nitrogen (MacConnell and Bond, 1957; Steward and Bond, 1961; Knowlton and Dawson, 1983; Griffiths and McCormick, 1984; Smolander et al., 1988; Smolander and Sundman, 1987). In our inoculation experiments on *Alnus glutinosa* clones external influence of nutrition on plant development can be excluded because all plants are grown under the same nitrogen limited conditions. Recent research has revealed that growth increment of alder plants can be obtained when mixed cultures of effective *Frankia* strains are used as inoculum instead of a pure culture (Prat, 1989). The results obtained in our nodulation experiments indicate that ineffective, i.e. Nif^- *Frankia* strains can also stimulate plant growth when effective *Frankia* strains are present. Stimulation of plant growth is not correlated with higher nodule formation but depends on a sufficient level of effective *Frankia*. Stimulation is expressed in comparable increment of root and shoot growth as indicated by similar shoot/root ratios.

Both physiologically different alder clones W I and B II show a large increase of plant growth but growth increment is not necessarily correlated to higher nodule formation. When higher nodule formation is obtained, the nodules are always induced by the effective and not the ineffective strains. In competition with very small amounts (0.02 µg) of the effective *Frankia* strains the ineffective strain AgB1.9 does not form nodules indicating a very low competitive ability. Similar results have been obtained in competition experiments with *Elaeagnus*-compatible effective and ineffective strains

(Simon et al., 1988). Competition experiments with *Alnus glutinosa* seedling batches inoculated with nodule homogenates or enrichment cultures of ineffective *Frankia* also support these results (van Dijk and Sluimer-Stolk, submitted). The latter experiments, however, do not exclude the possible involvement of other microorganisms present in the nodule homogenates.

The presence of microorganisms other than *Frankia* can have an influence on the establishment of the symbiosis (Knowlton et al., 1980). Roots of *Alnus glutinosa* can interact with soil-borne organisms like *Streptomyces* (Suetin et al., 1988), *Nocardia* (Dobritsa and Sharaya, 1986), *Penicillium* (Van Dijk, 1984; Capellano et al., 1987; Valla et al., 1989) and several mycorrhizal fungi (Gardner et al., 1984;). Nitrogen fixation and nodule formation with *Frankia* and plant development can be improved by these biotic factors, i.e. in the presence of *Pseudomonas cepacia* (Knowlton and Dawson, 1983). Growth stimulation could possibly be induced by the larger population of microorganisms, independent of the type of organism. This population effect is supported by comparable results obtained with *Pseudomonas* (Knowlton et al., 1983). Large populations of microorganisms possibly compete for soil borne nitrogen and induce growth increment of the roots in the early non-nitrogen fixing phase. After induction of active nodules the larger root network could supply a better uptake of other nutrients.

Root extension can also be induced directly by bacteria, i.e. by the excretion of vitamins or phytohormones like auxins or cytokinins that can have stimulating effects on root development (Strzelczyk and Pokojska-Burdziej, 1984; Strzelczyk and Leniarska, 1985). *Frankia* is shown to produce low amounts of auxins (Wheeler et al., 1984). However, the present strains were unable to produce detectable amounts of these auxins (C. Weniger, Ital, Wageningen, unpubl. results). Furthermore, cytokinins applied to young plants of *Alnus glutinosa* are shown to have a negative effect on root and plant growth (Bermudez de Castro et al., 1977).

Larger bacteria populations can also have a more direct effect on growth increment of the plants, i.e. by inducing more efficient root colonization of the effective *Frankia* strains. Plate counts of several combinations of *Frankia* strains used in nodulation experiments under axenic conditions do not indicate any influence of the ineffective strain on the development of the effective strains, neither in CFU contents in the medium nor in CFUs on the rhizosphere. Antagonistic effects on growth on plates or in liquid cultures between the strains could also not be detected (unpublished results).

Our investigations cannot show the reason for the growth stimulation by the ineffective *Frankia* strain. This is due to the lack of convenient methology for the isolation and identification of *Frankia* strains in natural systems, i.e. in nodules and in soil. Recently, molecular genetic markers that could possibly be used to identify *Frankia* without reisolation are found in 16S rRNA sequences (Hahn et al., 1989). The use of synthetic oligonucleotide probes in the detection of specific *Frankia* strains however depends on the development of reliable isolation methods for rRNA. The application of these probes in hybridization experiments could give much more insight in ecological problems of the ineffective *Frankia* strains, i.e. in the distribution of these strains in natural environments, in soil and in nodules.

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Chapter 6

Evidence for a close phylogenetic relationship between members of the genera *Frankia*, *Geodermatophilus*, and "*Blastococcus*" and emendation of the family *Frankiaceae*

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Evidence for a close phylogenetic relationship between members of the genera *Frankia*, *Geodermatophilus*, and "*Blastococcus*" and emendation of the family *Frankiaceae*

Key words: *Frankia*, *Geodermatophilus*, *Dermatophilus*, Phylogeny, 16S rRNA cataloguing, Reverse transcriptase sequencing

Abstract

The phylogenetic position of two *Frankia* strains, isolated from two different host plants, was analysed by reverse transcriptase sequencing and/or oligonucleotide cataloguing of their 16S ribosomal RNA. The two strains are highly related, showing a distinct relationship to *Geodermatophilus obscurus* and a strain of "*Blastococcus*". These organisms constitute a main subline of descent within the phylogenetic radiation of the order *Actinomycetales*. In contrast to the present classification the genera *Geodermatophilus* and *Dermatophilus* cannot be considered members of the same family. *Geodermatophilus* is transferred into the family *Frankiaceae* for which an emended description is given.

Introduction

In a recent survey on the phylogenetic relationships of members of the phenotypically defined families of the order *Actinomycetales* (Gottlieb, 1974) the heterogeneity of the family *Dermatophilaceae* (Austwick, 1958, 42, emend. mut. char. Gordon, 1964, 521) was demonstrated (Stackebrandt et al., 1983) by significant differences in the primary structures of the 16S rRNAs of the type species of *Dermatophilus* and *Geodermatophilus*. While *D. congolensis* DSM 43037 clustered with members of the *Arthrobacter-Cellulomonas-Brevibacterium* subline of descent, *G. obscurus* DSM 43160 represented an individual subline, showing no close relationship to any of the actinomycete taxa investigated up to that time by 16S rRNA cataloguing. The only other organisms reported to show a *Geodermatophilus*-like reproduction are *Crenothrix polyspora* (Cohn, 1870) for which no culture is available, and "*Blastococcus*" ssp., an invalidly described isolate from the baltic sea (Ahrends and Moll, 1970). *Frankia* strains, on the other hand, resemble *G. obscurus* and *D. congolensis* in certain morphological features, e.g. hyphae dividing in more than one plane, but differ from them in the possession of sporangia. A comparison of some of their other phenotypic characteristics is given in Table 1. In 1984 it was pointed out (Lechevalier and Lechevalier, 1984) that even though frankiae show many more phenotypic similarities to dermatophili than to geodermatophili, their dissimilar whole cell sugars, host specificities and serology argued for caution in making conclusions about their close relationship. Later work showed that the GC%'s of frankiae and dermatophili were very different and that furthermore, there was a lack of DNA homology between representatives of the two genera (Samsonoff et al., 1977; An et al., 1985, 1987).

Table 1 Phenotypic characteristics of members of the genera *Frankia*, *Geodermatophilus* and *Dermatophilus*

	Frankia	*Geodermatophilus*	*Dermatophilus*
Hyphae, well-developed	+	-	-
Multilocular sporangia[1]	+	+	+
Vesicles	+	-	-
Outer membraneous spore layer	+	-	-
Capsule	-	-	+
Motile spores	-	+	+
Relation to O_2	A/MA[2]	A	MA
Cell wall type	III	III	III
Whole cell sugars[3]	B, C, D and fucose	C	B
Major Menachinone	MK 9(H_4)[4]	MK 9(H_4)[5]	MK 8(H_4)[5]
GC%[6]	67-72	70-75	56-59
Habitat	angiospermous nodules, soil	soil, sea	mammalian epidermis

[1] The entire thallus is a sporangium in the case of *Geodermatophilus* and *Dermatophilus*
[2] A = aerobic; MA = microaerophilic
[3] Lechevalier and Lechevalier (1970)
[4] Lechevalier et al. (1987)
[5] Collins et al. (1984)
[6] Samsonoff et al. (1977); An et al. (1985; 1987)

Chemotaxonomic properties that could indicate a possible membership of "*Blastococcus*" to frankiae, dermatophili or geodermatophili are not available for the former taxon.

In order to determine their relationships, 16S rRNA catalogues of "*Blastococcus*" ssp. IFAM 1494 and *Frankia* strain AirI1 were generated and compared to the existing data base. In addition, a long stretch of the 16S rRNA from *Frankia* strain Ag45/Mut15 was sequenced by reverse transcriptase which allows placement of this organism into the phylogenetic tree of the actinomycetes.

Materials and Methods

Frankia strain AirI1 (LLR 01321) was isolated from spore(-) nodules of *Alnus incana* ssp. *rugosa* (Lechavalier et al., 1983). For 16S rRNA analysis, it was grown statically for six weeks at 28°C in S+TW medium (Lechevalier et al., 1983) and the cells stored in Tris-EDTA buffer. Strain Ag45/Mut15 was isolated from spore(-) nodules of *Alnus glutinosa* (Grossensee, F.R.G.) and maintained in a medium containing propionic acid and ammonium chloride as C- and N-source, respectively, at pH 6.8 (Meesters et al., 1985). The cultivation of "*Blastococcus*" ssp. IFAM 1494 was done as described by

Ahrends and Moll (1970).

16S rRNA cataloguing, calculation of similarity coefficients and generation of a dendrogram were carried out as described by Stackebrandt et al. (1985). The procedure of the reverse transcriptase sequencing of 16S rRNA followed the description of Lane et al. (1985) with the modifications indicated by Embley et al. (1988a). The sequence generated was aligned to those of about 40 reference actinomycetes (Smida 1988; Embley et al., 1988b; Collins et al., 1988a,b,c; Charfreitag et al., 1988). Due to sequence gaps and alignment problems only the regions between positions 200-470 and 477-1450 were used for the calculation of homology values. Prior to the determination of these values with the aid of the Beckman Microgenie programme (Queen and Korn, 1984) invariable positions were removed, reducing the number of nucleotides compared to 490. Calculation of evolutionary distances (Knuc) values (Hori and Osawa, 1979) and generation of a phylogenetic tree (Fitch and Margoliash, 1967) by the distance matrix method contained in a programme written by Felsenstein (Phylip version 2) for the IBM PC followed described methods.

Results and Discussion

The partial sequence of the 16S rRNA of *Frankia* strain Ag45/Mut15, covering regions 1-79 and 98-1462 (according to the *E.coli* numbering system, Brosius et al., 1978) is shown in scheme 1. These two stretches, comprising 1403 nucleotides, were aligned

Fig. 1 1410 nucleotides long fragment of the 16S rRNA from *Frankia* strain Ag45/Mut15.
* -invariable nucleotide within the 16S rRNA of actinomyces. ...non-sequenced regions. N - undetermined nucleotide. small letters - unproven composition. double line - conserved oligonucleotides for the *Frankia-Geodermatophilus* subline of descent. single line - additional oligonucleotides of *Frankia* strain Ag45/Mut15.

Table 2 Phylogenetic parameters of the comparison of 16S rRNAs. Lower left section: homology values; upper right section: Knuc values (evolutionary distances).

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 *Frankia* Ag45/Mut15	-	0.300	0.304	0.285	0.352	0.325	0.276	0.256	0.424	0.297	0.400	0.372	0.593
2 *Dactylosporangium aurantiacum* DSM 43157	75.3	-	0.336	0.309	0.336	0.330	0.253	0.242	0.325	0.280	0.304	0.363	0.506
3 *Streptoverticillium baldaccii* DSM 40845	75.0	72.9	-	0.391	0.360	0.360	0.303	0.286	0.388	0.304	0.388	0.383	0.510
4 *Saccharopolyspora hirsuta* ATCC 27875	76.3	74.7	69.5	-	0.239	0.239	0.248	0.252	0.316	0.335	0.363	0.371	0.500
5 *Pseudonocardia thermophila* ATCC 19285	71.9	72.9	71.4	79.5	-	0.289	0.273	0.251	0.322	0.368	0.385	0.370	0.572
6 *Mycobacterium tuberculosis* H37rv	73.6	73.3	71.4	79.5	77.4	-	0.211	0.202	0.266	0.294	0.344	0.292	0.512
7 *Nocardia asteroides* DSM 43005	76.9	78.5	75.1	78.9	77.1	81.6	-	0.131	0.292	0.273	0.381	0.327	0.524
8 *Rhodococcus erythropolis* DSM 43188	78.3	79.3	76.2	78.6	78.7	82.3	88.0	-	0.260	0.235	0.346	0.285	0.492
9 *Corynebacterium glutamicum* DSM 20300	67.6	73.6	69.7	74.2	73.8	77.6	75.8	78.0	-	0.339	0.391	0.376	0.502
10 *Nocardioides albus* DSM 43109	75.5	76.6	75.0	73.0	70.9	75.7	77.1	79.8	72.7	-	0.380	0.285	0.502
11 *Actinomyces bovis* DSM 43014	69.0	75.0	69.7	71.2	69.9	72.4	70.1	72.3	69.5	70.2	-	0.400	0.422
12 *Propionibacterium freudenreichii* DSM 20271	70.7	71.2	70.0	70.7	70.8	75.8	73.5	76.3	70.4	76.3	69.0	-	0.508
13 *Bifidobacterium bifidum* DSM 20456	59.0	63.2	63.0	63.5	60.0	62.9	62.3	63.9	63.4	63.4	67.7	63.1	-

to the 16S rRNAs of members of various actinomycete genera whose sequences have been published previously, i.e. *Nocardia*, *Rhodococcus* and *Gordona* (Stackebrandt et al., 1988), *Corynebacterium* (Collins et al., 1988a), *Pimelobacter* (Collins et al., 1988b), *Tsukamurella* (Collins et al., 1988c), *Pseudonocardia* and allied taxa (Embley et al., 1988b), *Mycobacterium* and *Actinoplanes* (Smida 1988) and *Streptoverticillium* (Witt and Stackebrandt, unpublished). The region used for determination of homology values is indicated by arrows. Nucleotides found to be highly invariable in this region of the 16S rRNA of members of the order *Actinomycetales* sensu Stackebrandt (1982) were omitted from the calculation of homology values (Table 2, lower left triangle). These nucleotides are marked with asteriks. Homology values for *Frankia* strain Ag45/Mut15 and representatives from other major actinomycetes sublines of descent range between 69.0 and 78.3%. These values are as high as those obtained for members of the individual sublines (69.5-79.3%). A phylogenetic tree of representative actinomycetes, based on evolutionary distance values (Knuc, upper right triangle in Tab. 2) shows the position of the *Frankia* strain Ag45/Mut15 (Fig. 2).

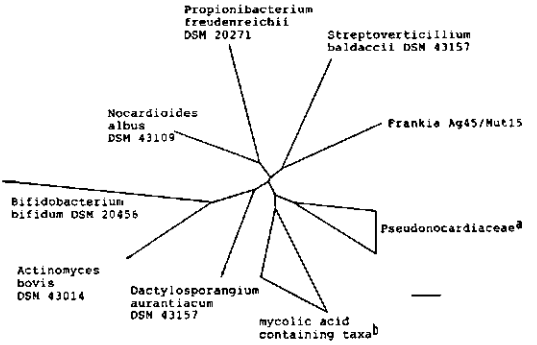


Fig 2 Phylogenetic position of Frankia strain Ag45/Mut15 within the family tree of actinomycetes as derived from the analysis of large 16S rRNA fragments. Bar represents 0.04 Knuc. [a] according to Embley et al. (1988b) [b] this taxon includes the genera *Corynebacterium* (Collins et al., 1989a), *Mycobacterium* (Smida, 1988), *Tsukamurella* (Collins et al., 1988) and *Nocardia*, *Rhodococcus* and *Gordona* (Stackebrandt et al., 1988).

Table 3 Binary similarity coefficients (S_{AB} values) between the 16S rRNA catalogues of strains of *Frankia*, *Geodermatophilus* and "*Blastococcus*" and average S_{AB} values for these strains and various actinomycete taxa, whose catalogues have been published (Stackebrandt and Woese, 1981; Stackebrandt et al., 1983; Fowler et al., 1985). Number of strains investigated in brackets.

	1	2	3	4
1 *Frankia* ssp AirI1	1.00			
2 *Frankia* ssp Ag45/Mut15	0.84	1.00		
3 *Geodermatophilus obscurus*	0.66	0.62	1.00	
4 "*Blastococcus*"	0.69	0.68	0.80	1.00
Actinoplanes (2)		0.51 - 0.55		
Dactylosporangium aurantiacum		0.53 - 0.56		
Micromonospora chalcea		0.60 - 0.66		
Corynebacterium (2)		0.38 - 0.40		
Mycobacterium phlei		0.46 - 0.52		
Nocardia asteroides		0.53 - 0.59		
Rhodococcus (2)		0.48 - 0.57		
Saccharotrix australiensis		0.56 - 0.59		
Pimelobacter simplex		0.50 - 0.51		
Streptomyces (3)		0.52 - 0.58		
Streptoverticillum baldaccii		0.53 - 0.58		
Streptosporangium roseum		0.58 - 0.60		
Actinomadura (4)		0.47 - 0.60		
Nocardiopsis dassonvillei		0.48 - 0.54		
Thermomonospora curvata		0.47 - 0.54		
amycolate wall type IV organisms (3)		0.47 - 0.54		
Arthrobacter/Micrococcus group (8)		0.44 - 0.58		
Dermatophilus congolensis		0.54 - 0.56		
Actinomyces (2)		0.42 - 0.48		

In contrast to the rather few 16S rRNA sequences available from actinomycetes the number of 16S rRNA catalogues from these organisms is high which in addition cover a much broarder spectrum of the many sublines of this order (Stackebrandt and Woese, 1981; Stackebrandt, 1985; Fowler et al., 1985; Stackebrandt and Schleifer, 1985; Fox and Stackebrandt, 1987). For comparison of the sequence information of strain Ag45/Mut15 with that of catalogues the putative RNase T_1 resistant oligonucleotides of hexamer size and larger were extracted and compared with the existing data base of actinomycetes. Two highly conserved oligonucleotides (UAACAAG and AUCACCUCCUUUCU) were added to the catalogue of strain Ag45/Mut15. These occur at position 1498 and 1531, respectively, at the 3' terminal region of any actinomycets 16S rRNA, a part that has not been sequenced in strain

Table 4 RNase T_1 resistant oligonucleotides (hexamers) of the 16S rRNAs from *Frankia* strains AirI1 (1) and Ag45/Mut15 (2), *Geodermatophilus obscurus* DSM 43060 (3) and *"Blastococcus"* subsp. IFAM 1494 (4), arranged according to their distribution in these strains.

Occurrence in all strains (1–4)

CACAAG	AACACCG	CCACACUG	UACACACCG	CUUAACACAUG
UCCACG	CAACCUG	CCUAUCAG	CCUACCAAG	AAUCCCAAAAAG
CUAACG	CAACUCG	CUACAAUG	CAACCCUCG	CUAAAACUCAAAG
UAAACG	UAACACG	ACUCAUAG	ACUCCUACG	UAAACCUCUUUCAG
AAUACG	UAACAAG	UCAACUCG	CUUAAUUCG	AUCACCUCCUUUCU
CCUUCG	UACAAAG	AUACCCUG	CCAACUACG	
UUCCCG	AUAUCAG	AAUUCCUG		
UCUCAG	UAAUACG	AUUUAUCG		
AUCCUG	CAUUAAG	AAUUAUUG		
UAAUCG	AAUAUUG			
UCUCUG				

Occurrence in *Frankia* strains only (1 and 2)

ACCCCG	UCCUAUG	UCAUCAUG	UAACACCCG	ACCUUCCACG
CUAAUG			CCCCUUACG	CCUAACCCUUG
				AACCUUACCAG

Occurrence in *G. obscurus* and *"Blastococcus"* ssp. only (3 and 4)

ACACUG	CCCCUUG	ACCCCAUG	CAACAACUG	AUAACUCCAAG
UCAAUG		CAAACAUG	CCCCUUAUG	AACCUUACCUAG
				UCAAAUCAUCAUG

Others

CCCCCG (3)	ACUCAUG (3)	ACAUCACUG (1)
AAACCG (1)	UUCCAUG (1)	CUCAACCCCG (2)
AACCUG (3)	UCAUCUS (2)	CUCAACUCCG (3)
AUACCG (1,3,4)	UUCUAUG (4)	CCAUUCCACG (3)
CUUCCG (3)	AAAACCCG (3)	AAUAACCUCG (2)
UACCUG (1–3)	CACACAUG (1)	AAUAACUUCG (1)
CAUCUG (1)	AAAACCUG (3)	CUCAUUCCAG (4)
AAAUUG (1,3,4)	CAUCUACG (3)	CUCAACCUCAG (4)
CCCAAAG (1)	AAAUCUCG (3)	CCCAACCCUUG (4)
UAAACCG (2)	AAAUAUCG (2)	AAAUCCUCCAG (2,4)
ACACAUG (2)	CUAACUACG (2)	AUAUCACAUUG (2)
UCAACUG (1)	CUAAUACCG (4)	CCCAACCCCUUG (1)

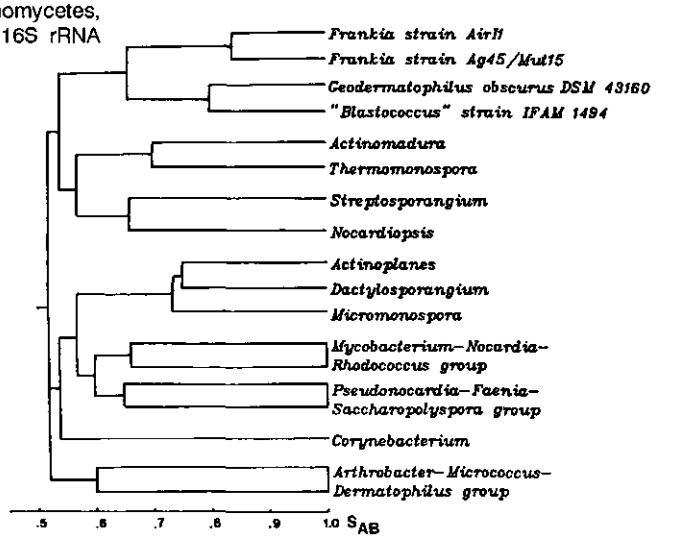
Ag45/Mut15. Table 3 compiles the similarity coefficients (S_{AB} values) obtained for this strain and its closest relatives, i.e. *Frankia* strain AirI1, *"Blastococcus"* sp. IFAM 1494 and *Geodermatophilus obscurus* (Stackebrandt eta l., 1983). The two *Frankia* strains and the pair *G. obscurus* and *"Blastococcus"* show a high degree of relationship (S_{AB} values of 0.84 and 0.80, respectively). Values obtained between the two pairs range between 0.62 and 0.69 which on the average are significantly higher than those found for these strains and members of the various phylogenetically defined groupings of

actinomycetes (0.42-0.60). A dendrogram of relationships, derived by average linkage clustering (amoung the merged groups) is shown in Fig. 2. Oligonucleotides present in the catalogues of all four organisms investigated in this study are indicated in Scheme 1 by a double line. Plain lines mark the additional oligonucleotides making up the catalogue of strain Airl1. Arranged according to their degree of conservatism, Table 4 compiles the oligonucleotides of the two *Frankia* strains, "*Blastococcus*" IFAM 1494 and *G. obscurus*. The catalogue of the latter species has only been partially determined by Stackebrandt et al. (1983).

Because of the high degree of phylogenetic relationship between *Frankia* ssp. Airl1 and *G. obscurus* 16S rRNA, the two genera have recently been considered to constitute the family *Frankiaceae* (Fox and Stackebrandt, 1987). The validity of this informal proposal is now supported by the results of this study. The high correlation between the branching patterns obtained by almost complete 16S rRNAs and oligonucleotide cataloguing is encouraging in that it verifies the phylogenetic conclusions based on the restricted information of the cataloguing method. Both strains of *Frankia* used in this study are of type B, which means that, among other characteristics, they are infective for their original host (see Lechevalier and Lechevalier, 1984). No A-types (not infective for original host) have yet been successfully analysed for their 16S rRNAs, thus the description of the family *Frankiaceae* is made with the full awareness that future work may modify the present proposed definition. "*Blastococcus*" has to be transferred into *Frankiaceae* once its generic status has been validated.

With the transfer of *Geodermatophilus* into the emended family *Frankiaceae*, *Dermatophilus* is the only remaining genus of the family *Dermatophilaceae*.

Fig. 3 Phylogenetic tree of the genera *Frankia*, *Geodermatophilus* and "*Blastococcus*" within the dendrogramm of actinomycetes, based on the analysis of 16S rRNA oligonucleotide catalogues.



Emendation of *Frankiaceae* Becking, 1970, 201.

Hyphae may be rudimentary (*Geodermatophilus* (G)) to extensive (*Frankia* (F)). Aerial mycelium is absent. Hyphal diameter varies from 0.5-3.0 μm, but averages 1.0 μm. Branching may be rare. A part (F) or all (G) of the thallus is composed of irregularly-shaped cuboid to oval cells dividing in more than one plane. These cells serve as propagules and may be motile (G) or non-motile (G,F) and may germinate to give filaments (F,G) or bud to give motile cells (G). Motile cells bear a tuft of terminal flagella. Gram-positive to gram-variable. Aerobic to microaerophilic. Members utilize sources of nitrogen such as amino acids and ammonia; atmospheric nitrogen is fixed by *Frankia* strains *in vivo* and *in vitro*. Carbon sources include carbohydrates, organic and fatty acids. Found in soil (F,G), the sea (G) and as symbionts of higher plants (F). Cell wall of type III (*meso* diaminopimelic acid, glutamic acid, alanine, glucosamine and muramic acid). Whole cell sugar patterns include B (3-O-methyl-D-galactose, madurose), C (no characteristic sugar), D (xylose) and fucose (no sugar group assigned). Phospholipid patterns PI (F) and PII (G). Major menaquinones of MK 9(H). Phylogenetically it constitutes one of the several major sublines of descent of the order *Actinomycetales*. Type genus is *Frankia*.

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Chapter 7

Synthetic oligonucleotide probes for identification of *Frankia* strains

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Synthetic oligonucleotide probes for identification of *Frankia* strains

Key words: *Frankia*, Oligonucleotide probes, rRNA sequences, Filter hybridization, *In-situ* hybridization

Abstract

Reverse transcriptase sequence analyses of variable regions of 16S rRNA of the nitrogen-fixing (Nif$^+$) *Frankia* strain Ag45/Mut15 and the Nif$^-$ strains AgB1.9 and AgW1.1 showed large differences in two of three variable regions between both *Frankia* groups. Synthetic oligonucleotides complementary to sequences in one of these different regions were used in hybridization experiments against isolated rRNA of several *Frankia* strains belonging to three compatibility groups. Ribosomal RNA of eleven effective *Frankia* strains obtained from different *Alnus* species strongly hybridized with the probe against the effective strain Ag45/Mut15, whereas ineffective strains and effective strains obtained from other hosts (*Elaeagnus*, *Comptonia*, *Coriaria*, *Hippophaë*, *Colletia* spp.) did not hybridize. Strong hybridization was also obtained with the effective *Casuarina* strain Ccl3. In the group of effective alder strains one strain showed weaker hybridization indicating small sequence differences. Different sequences were also found after hybridization with the probe against the ineffective *Frankia* strains AgB1.9 and AgW1.1. Only these two strains showed hybridization. The same results were obtained by *in-situ* hybridizations. Tests of these probes against rRNA of several microorganisms indicated a high specificity.

Introduction

Because of their ubiquity, their large size and their genetic stability, ribosomal RNA sequences have intensively been used to investigate quantitative evolutionary relationships amoung numerous bacteria (Olsen et al., 1986; Woese, 1987). Oligonucleotide catalogs obtained by partial sequence analysis using oligonucleotide fragments produced by digestion with ribonuclease T_1 have until recently been proven to be the most useful for establishing distant phylogenetic relationships (Stackebrandt, 1986; Stackebrandt et al., 1985). Today, new sequencing techniques allow rapid determination of total or almost complete 16S rRNA sequences (Lane et al., 1985; Smida, 1988; Embley et al., 1988). Total 16S rRNA sequences indicated the presence of variable regions that could be used to unravel close phylogenetic relationships. Furthermore, the large amounts of rRNA per cell together with the presence of short variable regions made them an attractive target for diagnostic research (Kohne et al., 1986; Viscidi and Yolken, 1987). Group- or species-specific sequences that could be used as specific targets for probes in hybridization experiments were found within various microorganisms (Embley et al., 1988; Giovannoni et al., 1988; Goebel et al., 1987; Stackebrandt and Smida, 1988). Because of the high specificity of the probe-target system, the detection limit became very low. Under certain circumstances, i.e. pure cultures, as little as a single bacterium could be detected. Even in mixed cultures

where target organisms may occur in low numbers these hybridization technique formed a powerful tool for detection of microorganisms, esp. for pathogens, symbionts and parasites, where isolation and cultivation were limiting factors (Lane et al., 1985; Tenover, 1988).

The application of this molecular biological tool in ecology of the nitrogen-fixing microsymbiont *Frankia* could solve many problems in identification of this recalcitrant organism. Successful attempts to isolate *Frankia* from soil were reported only once (Baker and O'Keefe, 1985). Usually *Frankia* were isolated from nodules that were natural enrichments of this microorganism. Nevertheless, isolation techniques were selective and unreproducible, because only small percentages of isolation attempts succeeded (St-Laurent and Lalonde, 1987). Available markers of *Frankia* strains like protein and isoenzyme patterns, sugar analysis, antibiotic resistance, fatty acid composition or morphological criteria, i.e. colour of strains could not be used to distinguish reliably between strains without reisolation (Benson and Hanna, 1983; Gardes and Lalonde, 1987; Normand and Lalonde, 1986; St-Laurent et al., 1987; Wheeler et al., 1986; Hafeez et al., 1984). However, investigations on strain composition in nodules or competition experiments were always influenced by reisolation problems because pure cultures have to be used in all investigations using markers described above (Simon et al., 1988).

The aim of this research was to search for variable regions in 16S rRNA of two types of *Frankia* strains. Using the reverse transcriptase sequencing method (Lane et al., 1985: Embley et al., 1988) we analyzed variable regions of 16S rRNA of the effective (i.e. Nif$^+$) *Frankia* strain Ag45/Mut15 isolated from spore(-) nodules of *Alnus glutinosa*. Variable regions of this strain were compared with homologous regions of two ineffective (i.e. Nif$^-$) strains AgB1.9 and AgW1.1, that were found to be selectively infective on *Alnus glutinosa* clones (Hahn et al., 1988). The observed differences were used to design complementary synthetic oligonucleotides that could be used as specific molecular markers in hybridization experiments in order to discriminate between Nif$^+$ and Nif$^-$ strains.

Material and methods

Frankia strains

Pure cultures of *Frankia* strains described in Table 1 were grown in P+N-medium (Meesters et al., 1985) for 2 weeks at 30°C. Cells were harvested by centrifugation (3000 xg), washed twice with PBS buffer (145 mM NaCl, 100 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) and stored at -70°C.

Extraction of RNA

About 2 g wet weight of cells of *Frankia* strains were resuspended in 10 ml cold (4°C) standard saline citrate (SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Lysis of strains Ag45/Mut15, AgB1.9 and AgW1.1 used for sequencing was performed by shaking with 45 g glass beads (0.10-0.11 mm, Braun) for 35 s in a cell homogenizer

(Braun)(Embley et al., 1988). Lysis of strains used in dot blot experiments was performed by French pressure. Extraction of RNA was done according to the method of Embley et al. (1988).

Tab. 1 *Frankia* strains

Pos.	Strain	N_2-fixation	Host species	Origin	Reference
Ia	Ag45/Mut15	+	*Alnus glutinosa*	F*, Grossensee (FRG)	Hahn et al., 1988
IIa	AgGS'84/45	+	*Alnus glutinosa*	F, Grossensee (FRG)	Hahn, unpublished
IIIa	AgN_2Cl_2	+	*Alnus glutinosa*	R AgN_2, Rijngeest (NL)	Burggraaf, 1984
IVa	AgN_3Cl_2	+	*Alnus glutinosa*	R** AgN_3,	Burggraaf, 1984
Va	AgP_1R_2C	+	*Alnus glutinosa*	R AgP, Hoogmade (NL)	Burggraaf, 1984
VIa	AgKG'84/4	+	*Alnus glutinosa*	F, Krems-Goels (FRG)	Hahn, unpublished
Ib	AgB16	+	*Alnus glutinosa*	F, Bad Bentheim (FRG)	Hahn,Starrenburg,
IIb	AgB32	+	*Alnus glutinosa*	F, Bad Bentheim (FRG)	Hahn, Starrenburg,
IIIb	Arl3	+	*Alnus rubra*	F, Oregon (USA)	Berry, Torrey,1979
IVb	An2.24	+	*Alnus nitida*	R An2,	Hafeez et al., 1985
Vb	Avcl1	+	*Alnus viridis*	F, Ontario (Can)	Baker, Torrey, 1980
Ic	AgW1.1	-	*Alnus glutinosa*	F, Weerribben (NL)	Hahn et al., 1988
IIc	AgB1.5	-	*Alnus glutinosa*	F, Bad Bentheim (FRG)	Hahn et al., 1988
IIIc	AgB1.7	-	*Alnus glutinosa*	F, Bad Bentheim (FRG)	Hahn et al., 1988
IVc	AgB1.9	-	*Alnus glutinosa*	F, Bad Bentheim (FRG)	Hahn et al., 1988
Vc	AgB1.10	-	*Alnus glutinosa*	F, Bad Bentheim (FRG)	Hahn et al., 1988
VIc	AgI5	-	*Alnus glutinosa*	F, Oostvoorne (NL)	Akkermans, vanDijk,
Id	Eul1	-	*Elaeagnus um.*	F, Petersham (USA)	Baker et al., 1980
IId	Hrl1	+	*Hippophaë rh.*	F, Petersham (USA)	Baker, unpublish.
IIId	Cc1.17	+	*Colletia cruc.*	F,	Meesters et al., 1985
IVd	Cpl.2	+	*Comptonia per.*	R Cpl.1,	Meesters et al., 1985
Vd	Ccl3	+	*Casuarina cun.*	F, Tampa (USA)	Zhang et al., 1984
VId	CN_3	-	*Coriaria nepal.*	F,	Akkermans, unpubl.

* field isolate, ** reisolate

Reverse transcriptase sequencing

Variable regions of 16S rRNA of *Frankia* strains Ag45/Mut15, AgB1.9 and AgW1.1 were sequenced using a modified method of Lane et al.(1985) (Embley et al., 1988). Primers used in sequencing reactions (*E.coli* 3' positions 357, 536 and 1115) were described in Embley et al. (1988).

Probe synthesis and labelling

Primers as well as oligonucleotide probes were synthesized by standard phosphoamidize methods using an Applied Biosystems 3801A or a New Brunswick Scientific Cyclone DNA synthesizer. 200 ng of oligonucleotides complementary to variable sequences of two different *Frankia* strains (Tab. 4) were 5'-labelled using

phage T_4 polynucleotide kinase (BRL) and 50 µCi of [τ-^{32}P]adenosine-5'-triphosphate (3000 Ci/mmol; Amersham) (Maniatis et al., 1982). The kinase mixture was used directly in hybridization experiments without separation of labelled oligonucleotides from unincorporated [τ-^{32}P]ATP.

Filter hybridization

RNA solutions were applied to nitrocellulose filters (Schleicher & Schüll) either by pipette or with a HYBRI.DOT manifold (BRL) and dried at 80 °C for two hours; each spot contained 10 ng (*Frankia* strains) or 100 ng (other microorganisms) of rRNA. Filters were prehybridized in 10 ml of hybridization solution in plastic bags without labelled probe for 2 hours at 40 °C. After the addition of the probe, hybridization was performed at the same temperature for 16 hours. The hybridization solution contained 1 x Denhardt solution [0.2 g Ficoll (Pharmacia; average M_w,400000), 0.2 g of polyvinylpyrrolidone (PVP-360; Sigma) and 0.2 g of bovine serum albumine (Sigma, fraction V) per liter], 100 µg/ml denatured salmon sperm DNA (Sigma), 0.1% sodium dodecylsulfate (SDS; BRL) and 6 x SSC buffer.

After hybridization the filters were washed twice in 6 x SSC, 0.1% SDS and 1 x Denhardt's for 30 minutes at 30 °C, twice in 2 x SSC and 0.1% SDS and three times in 0.2 x SSC and 0.1% SDS for 30 minutes at 30 °C. Additional washing steps at higher temperatures were always performed in 0.2 x SSC and 0.1% SDS.

Washed filters were sealed in plastic bags and exposed to X-ray film (Kodak XAR 5) for 6 to 50 hours.

In-situ hybridization

For cell blot hybridizations (Yu and Gorovsky, 1986) glutaraldehyde-fixed cells were used. Two weeks old cultures were harvested by centrifugation, washed once in PBS buffer and fixed in 0.5% glutaraldehyde in PBS buffer (v/v). Fixed cultures were homogenized by repeated passages through a needle (0.5 mm in diameter) and cells (about 2 µg of protein (Moss and Bard, 1957)) spotted onto dry nylon filters (GeneScreen Plus, Du Pont). Filters were carefully air dried, immersed in an aequeous solution containing 0.1 M triethanolamine, pH 8 (Merck) for 10 minutes at room temperature. Acetic anhydride was added to 0.25% (v/v) and filters were incubated for additional 10 minutes (Giovannoni et al., 1988). Filters were then rinsed in 2 x SSC and air dried. Dried filters were prehybridized in 2 x SSC, 1 x Denhardt's solution, 100 µg/ml ssDNA and 0.1% SDS for 2 hours at 40 °C. Hybridization occurred at 40 °C for 16 hours. Washing conditions were as indicated for filter hybridization, except that 2 x SSC replaced 6 x SSC.

Results

Reverse transcriptase sequencing

Sequence analysis of three variable regions of 16S rRNA of the effective (Nif$^+$) *Frankia*

strain Ag45/Mut15 and the two ineffective (Nif$^-$) and selectively infective strains AgB1.9 and AgW1.1 showed large differences in two regions between the effective and the ineffective strains. Differences in nucleotide sequence between the effective *Frankia* strain and the ineffective strains are illustrated in Figures 1 and 2 showing secondary structures of variable regions at positions 1000-1040 and 140-210 (*E.coli* pos.). The third region was found to be identical. This region was found to be between nucleotides 430-500 (*E.coli* pos.)(Fig. 3). The sequences of both ineffective strains were completely identical.

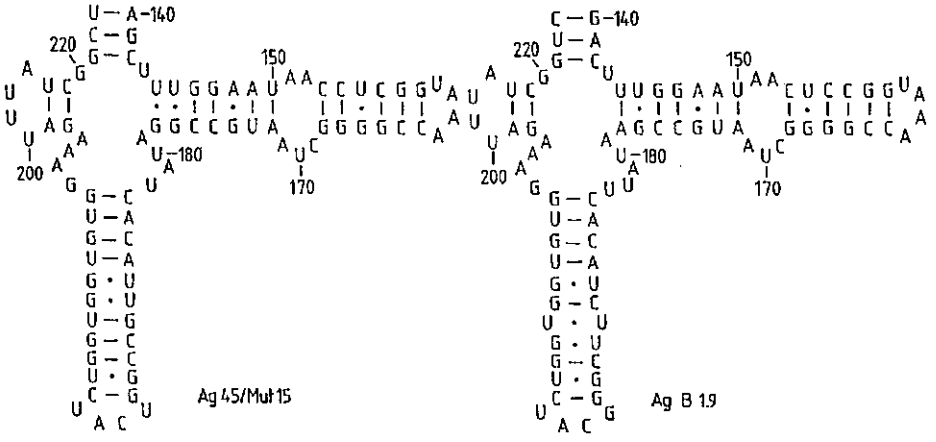

Fig. 1 Secondary structure of 16S rRNA region 140-220 (*E.coli* pos.) showing numerous differences between the effective *Frankia* strain Ag45/Mut15 and the ineffective strain AgB1.9.

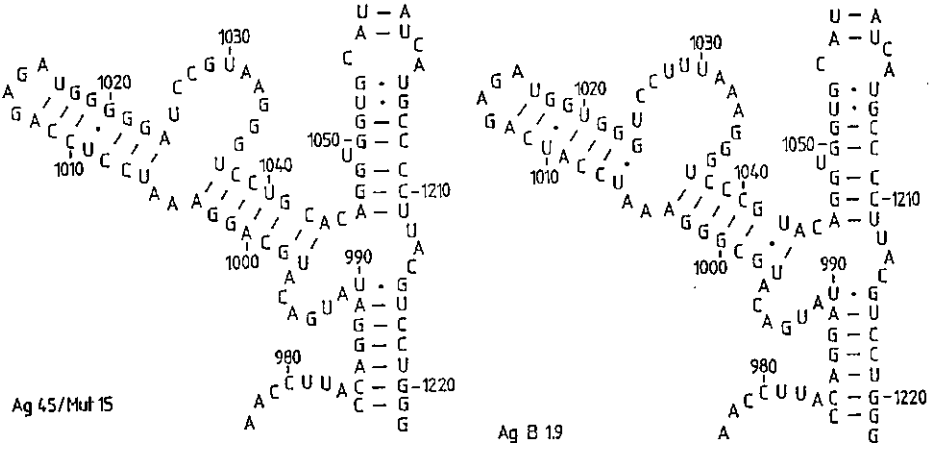

Fig. 2 Secondary structure of 16S rRNA region 980-1060 (*E.coli* pos.) showing numerous differences between positions 1000-1040 of the effective *Frankia* strain Ag45/Mut15 and the ineffective strain AgB1.9.

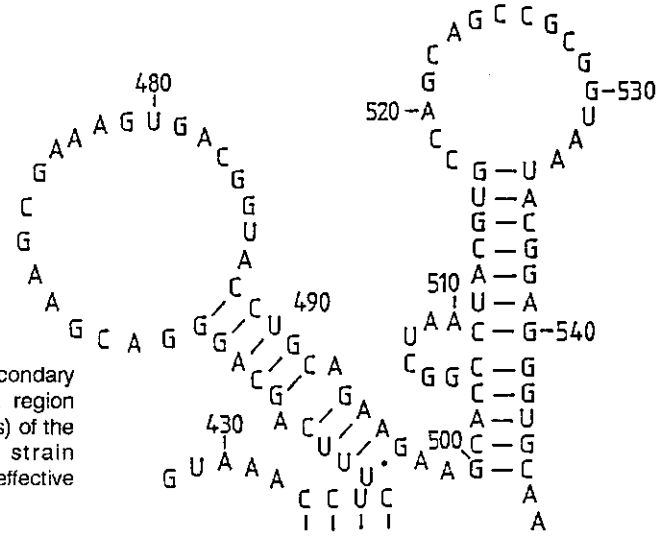

Fig. 3 Identical secondary structure of 16S rRNA region 430-540 (*E.coli* positions) of the effective *Frankia* strain Ag45/Mut15 and the ineffective strain AgB1.9.

Probe design

The variable sequences of the effective *Frankia* strain Ag45/Mut15 and the ineffective strains AgB1.9 and AgW1.1 were compared and showed regions with large differences. Region 1020-1042 (*E.coli* pos.) showed five differences in nucleotide composition spread over the whole sequence and additionally two missing bases (Fig. 4). It was therefore choosen as target (Fig. 4). A universal probe, commonly used as primer in 16S rRNA sequencing reactions (primer 1115, Embley et al., 1988), was used as positive control.

3'	C C C T A G G - C A - T T C C C A G G A C G	5' probe EFP
5'	G G G *A* U C C - *G* U - A A G G G U C C *U* G *C*	3' Ag45/Mut15
5'	*U* G G G U C C U *U* U A A A G G G U C C C G *U*	3' AgB1.9
3'	A C C C A G G A A A T T T C C C A G G G C A	5' probe iFP

Fig. 4 Sequences of synthetic oligonucleotide probes aligned with corresponding sequences of 16S rRNAs (*E.coli* pos. 1020-1042 (Brosius et al., 1981)) of an effective (Ag45/Mut15) and an ineffective (AgB1.9) *Frankia* strain. Differences are in italic type.

Filter hybridization

Temperature dependence and sequence specificity of probe hybridization are illustrated in Figure 5. Hybridizations were performed under low stringency conditions followed by washes at higher temperatures. Hybridization of the universal probe to various rRNAs immobilized on nitrocellulose filters indicated that rRNA was present in all RNA preps. Twenty-three *Frankia* strains of different origin were used in these experiments (Tab. 1). Ribosomal RNA of eleven effective *Frankia* strains obtained from

different *Alnus* species strongly hybridized with the probe against the effective strain (probe EFP) whereas ineffective strains and effective strains obtained from other host plants (*Elaeagnus*, *Comptonia*, *Coriaria*, *Hippophaë*, *Colletia* spp.) did not hybridize, even not under low stringency conditions. Strong hybridization signals with probe EFP

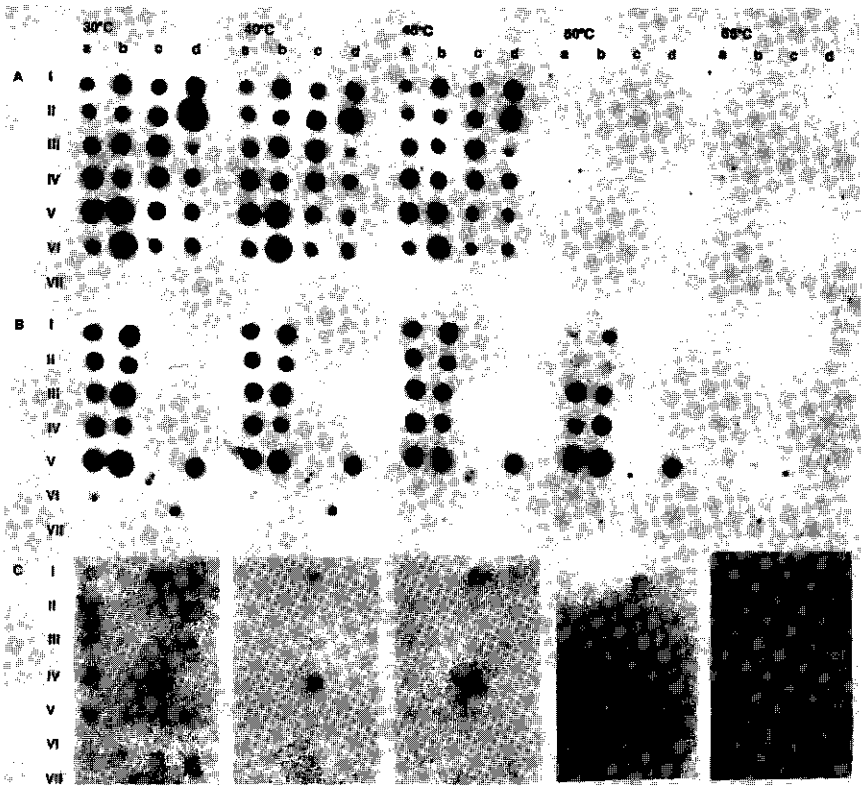

Fig. 5 Temperature dependence and sequence specificity of probe hybridization to nitrocellulose-bound rRNAs. Each spot contains 10 ng of RNA. Spot positions are described in table 1. Additional tests are carried out using RNA of *Streptomyes lividans* (pos. VIb) and DNA of *Alnus glutinosa* (pos. VIId). Abbreviations: A, probe primer 1115 (Embley et al., 1988), B, probe EFP, C, probe IFP

were also obtained with the effective *Casuarina* strain Ccl3. Within the effective alder strains one strain AgKG'84/4 showed temperature dependent hybridisation.

Specific hybridization was also found with the probe against the ineffective strains AgB1.9 and AgW1.1 (probe IFP). Only these two strains, but none of the other strains, neither the ineffective nor the effective strains showed hybridization.

Additional tests of probe specificity to immobilized rRNA of different micoorganisms did not show any significant hybridization, neither with probe EFP nor with probe IFP (Fig. 6).

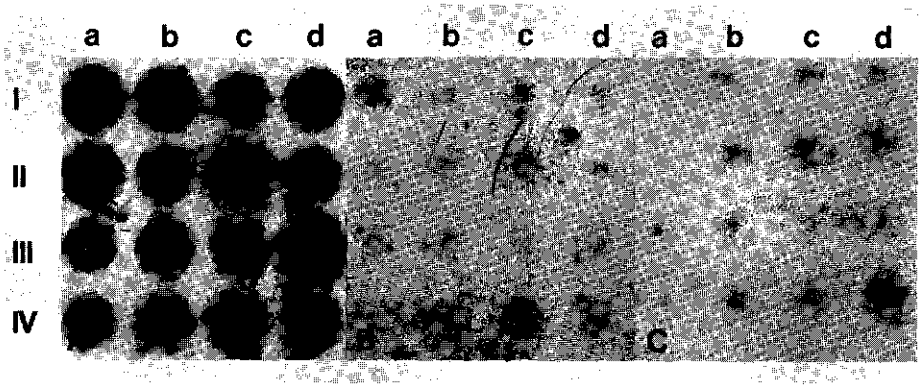

Fig. 6 Sequence specificity of probe hybridization to nitrocellulose-bound rRNAs of different microorganisms. Each spot contains 100 ng of RNA. Spot positions: Ia *Streptomyces albus* (DSM 40313), Ib *Streptomyces cyaneus* (DSM 40108), Ic *Streptomyces kauaiensis* (DSM 43360), Id *Streptoverticillium baldaccii* (DSM 40845), IIa *Streptoverticillium luteoreticuli* (DSM 40509), IIb *Actinomyces bovis* (DSM 43014), IIc *Propionibacterium thoenii* (DSM 20276), IId *Nocardioides albus* (DSM 43109), IIIa *Pimelobacter simplex* (NCIB 8929), IIIb *Terrabacter tumescens* (NCIB 8914), IIIc *Tsukamurella* (NCTC 10741), IIId *Nocardiopsis dassonvillei* (DSM 43235), IVa *Casiobacter polymorphus* (NCDO 2097), IVb *Dermatophilus congolensis* (DSM 43037), IVc *Geodermatophilus obscurus* (DSM 43160), IVd *Frankia*
Abbreviations: A, probe primer 1115 (Embley et al., 1988), B, probe EFP, C, probe IFP

In-situ hybridization

Binding of the probes to fixed, intact cells immobilized on nylon filters is shown in Figure 7. All strains bound the universal probe, showing large variation in hybridization intensity though all of the cell spots contained the same cell mass (2 µg protein). Hybridization with probe EFP showed the same specific binding as obtained for rRNA bound on nitrocellulose filters. Hybridization occurred only with the effective *Alnus* strains and the *Casuarina* strain. The temperature dependent hybridization effect of strain AgKG'45/4 could also be detected. In contrast, probe IFP against the ineffective strains AgB1.9 and AgW1.1 also bound to cell spots of several effective strains.

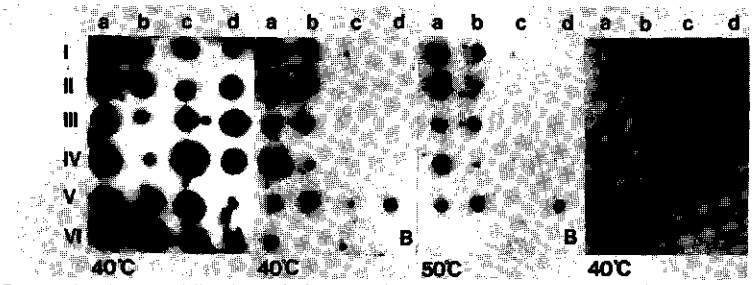

Fig. 7 *In-situ* hybridization of a) the universal probe primer 1115 (Embley et al., 1988), b) the probe EFP and c) the probe IFP. Each spot contains identical cell masses (2 µg protein). Spot positions are described in Table 1.

Discussion

Reverse transcriptase sequencing of 16S rRNA of two types of *Frankia* strains, the N_2-fixing strain Ag45/Mut15 and the ineffective strains AgB1.9 and AgW1.1 indicate the existence of two highly variable regions at positions 140-210 and 1000-1040 (*E.coli* pos.). A third region between nucleotides 430-540, observed to be commonly variable in 16S rRNA of Actinomycetes (Smida, 1988), is found to be identical. The variable character of these regions and the large differences in nucleotide sequences between both *Frankia* types make them an attractive tool for DNA-RNA hybridizations. Large differences, i.e. 5 different nucleotides and two missing nucleotides spread over a short sequence of 20 nucleotides, favours region 1020-1042 as target for synthetic oligonucleotides specific for the effective *Frankia* strain Ag45/Mut15. The same region is choosen for synthesis of an oligonucleotide, complementary to the sequence of the ineffective strains. The position of this variable region adjacent to a primer binding site makes sequencing analysis of other strains attractive because future designation of putative target sites will be greatly facilitated.

Specificity of both probes to other *Frankia* isolates is shown in hybridization experiments against rRNA of *Frankia* strains belonging to different compatibility groups (Baker, 1987). Hybridization of probe EFP only occurs with RNA of effective strains also obtained from alders and, surprisingly, to RNA of the *Casuarina* compatible strain Ccl3 belonging to a separate compatibility group. Ineffective strains and strains belonging to the *Elaeagnus* compatibility group do not hybridize with probe EFP. The effective *Comptonia* strain Cpl.2 belonging to the *Alnus* compatibility group also indicates sequence differences. Temperature dependent hybridization to probe EFP is obtained with RNA of the effective *Alnus glutinosa* strain AgKG'84/4, indicating small differences in nucleotide composition of that region. Specificity of the probe against the ineffective strains AgB1.9 and AgW1.1 (probe IFP) is also demonstrated. Only these two but not heterologous strains can be detected by hybridization.

The probes described here are of relatively small size (20-22 nucleotides). This minimizes problems of cellular permeability and access to binding sides. *In-situ* hybridizations of fixed *Frankia* cells with probe EFP give the same results as hybridizations with immobilized RNA. In contrast, hybridization with probe IFP does not show the same specificity. Theoretically, this technique of *in-situ* DNA-RNA hybridizations can be used for taxonomical research because large amounts of strains can be tested in a short period of time. However, the results obtained from hybridization experiments with 23 *Frankia* strains from different compatibility groups indicate more strain specific sequences than group specific sequences so that the use of both probes in taxonomical investigations might be of limited value. Screening of much more *Frankia* strains should give more information about the real application of these probes in taxonomical research within the family *Frankiaceae*.

Large nucleotide differences within the variable regions also minimize crossreaction with sequences of other soil microorganisms. Specificity of the synthetic oligonucleotides to *Frankia* strains is demonstrated by the lack of hybridization with large amounts of immobilized rRNA obtained from several other actinomycetes. No significant crossreaction can be seen, even not with *Geodermatophilus obscurus*, the actinomycete most closely related to *Frankia* (Stackebrandt, 1986).

The use of specific nucleic acid hybridization probes in detection and identification

of *Frankia* strains can be a powerful tool in ecological investigations. Specificity of probes, the low detection limit of target sequences and the simple application in *in-situ* hybridization experiments can avoid problems of reisolation and identification in pure culture. Natural enrichments of one organism, i.e. like *Frankia* in nodules are preferred for investigations. Detection of double-infections with two different strains or competition experiments with defined strains will be feasible. Combinations of strains differing in only one or two nucleotides, i.e. AgKG'84/4 and the other *Alnus* strains, simplify the identification process and are therefore especially useful for competition experiments and ecological investigations.

A *Frankia* specific probe can possibly be found within region 430-540 that is identical in both types of strains. The sequence of this region should be aligned and compared to sequences of other microorganisms in order to detect a *Frankia* specific target for probes.

Acknowledgements

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Chapter 8

Oligonucleotide probes against rRNA as a tool to study *Frankia* strains in root nodules

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Oligonucleotide probes against rRNA as a tool to study *Frankia* strains in root nodules

Abstract

Oligonucleotide probes which hybridized with specific sequences in variable regions of the 16S rRNA of the nitrogen-fixing actinomycete *Frankia* were used for the identification of *Frankia* strains in nodules. *Frankia* cells were released from plant tissue by grinding glutaraldehyde fixed root nodules in guanidine-hydrochloride solution. Ribosomal RNA was obtained after sonification, precipitation with ethanol and purification by phenol/chloroform extraction. Northern blots indicated physical destruction of the isolated rRNA. Hybridization with synthetic oligonucleotides was not influenced by this destruction. Nodules of about 1 mg fresh weight provided sufficient rRNA for reliable detection of the *Frankia* strain. The utility of this rRNA extraction method was tested in a competition experiment with two effective *Frankia* strains on cloned *Alnus glutinosa* plants.

Introduction

The actinomycete *Frankia* is a nitrogen-fixing organism that forms root nodules in symbiosis with woody plants, such as black alder (*Alnus glutinosa*) and sea buckthorn (*Hippophaë rhamnoides*)(Bond, 1983). In root nodules typical *Frankia* strains form specialized cell clusters that can fix molecular nitrogen. The formation of these so-called "vesicles" is also obtained in pure cultures of *Frankia* grown under nitrogen-limited conditions (Meesters et al., 1985; Meesters, 1987). Beside the typical strains, atypical *Frankia* strains exist that do not fix nitrogen, do not form vesicles and are sometimes not able to infect their original host plants (vanDijk and Sluimer-Stolk, 1984; Hahn et al., 1988). These atypical strains lack all morphological and physiological characteristics of *Frankia* and usually resist isolation. Investigations on the ecology of *Frankia* are very often hampered by problems encountered with isolation and identification. In the presence of other bacteria it is often impossible to obtain this organism in pure culture. In general, *Frankia* is isolated from nodules which naturally enrich for this organism. Successful attempts to isolate *Frankia* from soil have been reported only once (Baker and O'Keefe, 1984). Up to now, no selective isolation techniques have been developed with the result that only small percentages of isolation attempts succeeded (St-Laurent and Lalonde, 1987). The difficulties with isolation and identification of *Frankia* could be ameliorated by specific markers for identifying these bacteria without first isolating them.

In a first attempt to identify *Frankia* strains in nodules, plasmids were used as molecular probes (Simonet et al., 1988). Alternatively, identification of strains in nodules can also be done using restriction enzyme patters of total DNA and subsequent hybridization with Nif-genes (Normand and Lalonde, 1986; Normand et al., 1988; Simonet et al., 1988). The application of plasmid and DNA probes is restricted to endophyte enrichments, i.e. to large nodules or pure cultures, supplying sufficient DNA

for direct hybridization experiments.

Another possibility to use molecular markers is the application of synthetic oligonucleotides in hybridization experiments against rRNA (Giovannoni et al., 1988; Goebel et al., 1987; Olsen et al., 1986; Stahl et al., 1988). By using the reverse transcriptase sequencing method (Lane et al., 1985; Embley et al., 1988; Smida, 1988) the 16S rRNA nucleotide sequence of the effective (i.e. Nif$^+$) *Frankia* strain Ag45/Mut15 isolated from spore(-) nodules of *Alnus glutinosa* was analysed in order to investigate the phylogenetic position of *Frankia* (Hahn et al., 1989). Variable regions of this typical *Frankia* strain showed several differences with homologous regions of two atypical, ineffective (i.e. Nif$^-$) strains. These regions were used to design complementary synthetic oligonucleotides. The resulting probes were suitable for use in hybridization experiments to discriminate between Nif$^+$ and Nif$^-$ strains and to discriminate strains within both physiologically different groups (Hahn et al., 1989). Nodules, that are metabolically highly active enrichments of *Frankia* containing large amounts of rRNA, are predestinated for the application of these probes for identification.

The application of oligonucleotide probes in the detection of specific *Frankia* strains in nodules depends on the development of a reliable isolation method for rRNA. Pure rRNA preparations must be obtained to ensure specific hybridization signals of enriched target sequences. Aspecific binding to DNA or polyphenolic compounds must be avoided. Polyphenols, rather common in actinorhizal plant tissue are quite difficult to remove from nucleic acid preparations and always disturb the quantification of hybridization signals (Simonet et al., 1988). The presence of large amounts of polyphenols in *Alnus glutinosa* hinders the application of RNA extraction procedures commonly used with other plants (Govers et al., 1985; Verwoerd et al., 1989). As often large numbers of nodules per plant must be investigated, a rapid rRNA extraction would be very handy. Additionally, the detection limit must be very low to allow the identification of *Frankia* strains in very small nodules.

The aim of our investigations was to develop a rapid RNA isolation method from nodules ensuring high yields of pure rRNA of the endophyte that could be used in hybridization experiments to detect *Frankia* strains without reisolation and cultivation. In the following a method is described which is rapid and sensitive enough to detect specific target sequences in small nodules.

Material and methods

RNA extraction

Root nodules of actively growing *Alnus glutinosa* plants were harvested and fixed in 1% glutaraldehyde. Nodules up to 1 mg fresh weight were ground in a mortar in 1 ml of 7.5 M guanidine-hydrochloride/ 1 M Tris, pH 7.0 and centifuged at 3000 xg to precipitate plant cells and released *Frankia* cells. The pellet was resuspended in 0.4 ml guanidine-hydrochloride solution and sonified 3-6 times for 30 sec. at 60 W (Ultra sonics, Danbury, CT, USA) to disrupt *Frankia* cells. To check the efficiency of the extraction, also pure cultures of *Pseudomonas putida* and the typical, nitrogen fixing *Frankia* strain Ag45/Mut15 (1 g fresh weight) were sonified under the same conditions. After centrifugation at 1000 xg for 10 minutes soluble substances of the supernatant

were precipitated with 2.5 vol ethanol. The resulting pellet was resuspended in sterile distilled water and extracted twice with phenol/chloroform. Phenol was buffered with 10 mM Tris, pH 7.4. After two additional chloroform extractions RNA was precipitated with 2.5 vol ethanol, washed with 70% ethanol, dried and dissolved in 10 µl distilled water.

RNA isolations were checked on northern blots, hybridized with a universal eubacterial probe commonly used as primer in 16S rRNA sequencing reactions (primer 1115, (5)). Additionally, two probes against 16S rRNA sequences specific for effective *Frankia* strains belonging to the *Alnus* compatibility group (3' C C C T A G G C A T T C C C A G G A C G; probe EFP (Hahn et al., 1989)) and generally for *Frankia* (3' G A C C A C C A C A C C T T T C T A A A T A (Hahn, unpublished) were used to detect 16S rRNA sequences. The specificity of both probes has been demonstrated in dot blot hybridization experiments with immobilized rRNA of 23 *Frankia* strains belonging to different compatibility groups.

Detection of *Frankia*

RNA from different amounts of nodules (1 to 10 mg fresh weight) of *Frankia* strain Ag45/Mut15 was used in dot blot experiments with probe EFP to estimate the detection limit. In order to show the purity of the RNA preparation, i.e. the absence of polyphenols, temperature dependent hybridization of this probe with 16S rRNAs of the effective *Frankia* strains Ag45/Mut15 and AgKG'84/4 was also tested in dot blot experiments. Probe EFP has been designed on the basis of 16S rRNA sequences of *Frankia* strain Ag45/Mut15. Even under stringent conditions this probe hybridized with 16S rRNA sequences of nitrogen-fixing strains obtained from alder plants (Hahn et al., 1989).

Competition experiments

The usefulness of the RNA extraction method was tested in competition experiments with the effective *Frankia* strains Ag45/Mut15 and strain AgKG'84/4. Plants of *Alnus glutinosa* clone B II (Hahn et al., 1988) were grown in 1.5 l pots in the greenhouse with perlite as substrate and inoculated with defined amounts of both *Frankia* strains. After 3 months of growth under natural light conditions plant height, plant dry weight and number of nodules per plant were determined. Nodules were harvested, fixed in 1% glutaraldehyde solution and stored in this solution at 4°C until used for RNA isolation. About 20 nodules per plant were selected at random and used to determine strain compositions.

Filter hybridization

Northern blots and dot blot hybridization experiments were performed on GeneScreen filters (DuPont). Ribosomal RNA was applied with a Hybri.Dot manifold (BRL), immobilized by UV light and hybridized according to Church and Gilbert (1984). Northern blots (Maniatis et al., 1982) were hybridized under the same conditions, except that filters were incubated at 80 °C for 30 minutes to remove formaldehyde

prior to UV light immobilization. Oligonucleotide probes were 5'-labelled using phage T_4 polynucleotide kinase (BRL) and 20-50 μCi of [τ-^{32}P]adenosine-5'-triphosphate (3000 Ci/mmol; Amersham) (Maniatis et al., 1982).

Northern blots and filters containing rRNA from nodule preparations were always prewashed after rRNA immobilization in 0.1 x standard saline citrate (SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1% SDS for 15 minutes at 80°C in order to remove possible contaminations with polyphenolic compounds.

The same washing procedure was also used to remove probes bound to immobilized target rRNA on the filters.

Results

RNA extraction

The RNA extraction method results in an rRNA preparation without detectable contamination by DNA. Northern blots of RNA from pure cultures of *P. putida* and *Frankia* strain Ag45/Mut15 show distinct hybridization bands of 16S rRNA with the universal probe. Specific hybridization of 16S rRNA of *Frankia* strain Ag45/Mut15 is obtained with probe EFP, that should crosshybridize with DNA of *P. putida* (Fig. 1). In the rRNA preparations isolated by this method from pure cultures some degradation is observed. Degradation is even more pronounced when the rRNAs are isolated from nodules, probably as a result of mechanical disruption during the sonification step (Fig. 2).

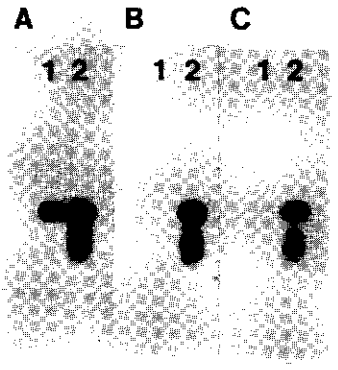

Fig. 1 Northern blot of RNA preparations (50 ng per slot) from pure cultures of *Pseudomonas putida* (1) and *Frankia* strain Ag45/Mut15 (2) hybridized with a universal probe (a), probe EFP, specific for effective *Frankia* strains obtained from *Alnus* plants (b) and a *Frankia*-specific probe (c).

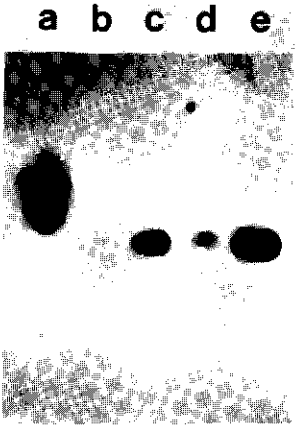

Fig. 2 Northern blot of RNA isolated from nodules induced by *Frankia* strains Ag45/Mut15 (b, d) and AgKG'84/4 (c, e). Preparations are done on the same sample after three (b, c) and six times (d, e) of sonification for 30 sec. at 60 W and compared with rRNA isolation from a pure culture of strain Ag45/Mut15 (a) after three times of sonification. Hybridization is performed with probe EFP.

A more intensive sonification yields more rRNA without further degradation of the RNA. Small nodules of about 1 mg fresh weight (about 1 mm in diameter) contain sufficient target sequences for clear hybridization signals (Fig. 3). The hybridization signals of isolated rRNA from nodules of 1 mg, 5 mg and 10 mg fresh weight with probe EFP correlate more or less with that of 1 ng, 5 ng and 10 ng of rRNA of *Frankia* strain Ag45/Mut15.

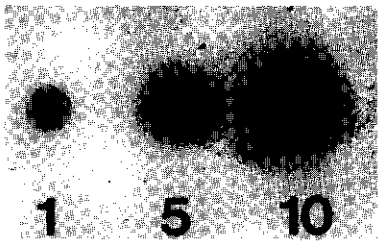

Fig. 3 Dot blot hybridization of immobilized RNA isolated from nodules formed by *Frankia* strain Ag45/Mut15. Nodules of 1 mg, 5 mg and 10 mg fresh weight are sonified six times for 30 sec. at 60 W.

The isolated rRNA is pure enough to give specific hybridization signals (Fig. 4). Crossreaction with contaminating polyphenols would result in aspecific binding disturbing clear signals also at high stringency conditions. With rRNA of *Frankia* strain AgKG'84/4 no aspecific binding was found. This rRNA contains one additional base within the target sequence (Hahn, unpublished). At high stringency pure RNA preparations of this strain did not show any hybridization signal, whether specific or aspecific. The same results, indicating the isolation of pure RNA, are obtained when two probes, probe IFP, specific for an ineffective *Frankia* strain and probe EFP, are used one by one (data not shown).

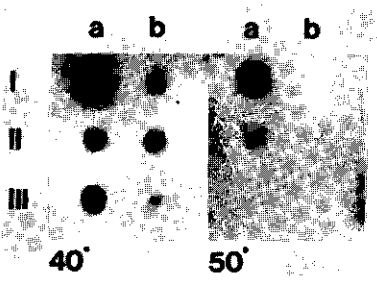

Fig. 4 Dot blot hybridization of isolated RNA from nodules (about 10 mg fresh weight) formed by *Frankia* strains Ag45/Mut15 (IIa) and AgKG'84/4 (IIb) compared with RNA preparations from pure cultures (Ag45/Mut15: I with 10 ng (a) and 1 ng (b); AgKG'84/4: III with 10 ng (a) and 1 ng (b)). Hybridization with probe EFP shows temperature dependent signal patterns indicating the purity of the rRNA preparation.

Competition experiments

Competition experiments with both effective *Frankia* strains that can be distinguished by temperature dependent hybridization (Hahn et al., 1989) were set up to investigate the applicability of the isolation and hybridization procedure in "natural systems". Due to the restricted usefulness of this temperature dependent hybridization that cannot distinguish between strain Ag45/Mut15 and double infections with strains Ag45/Mut15 and AgKG'84/4, these experiments were only used to demonstrate how this technique works. Inoculations with each strain alone or with both strains together showed small influence on plant growth but significant influence on nodule formation. In all cases

dual inoculation decreased the amount of nodules when compared with identical inoculation amounts of the dominating *Frankia* strain (Table 1).

Table 1. Competition experiment between the effective *Frankia* strains AgKG'84/4 and Ag45/Mut15 on *Alnus glutinosa* clone B II (n=15;t=3 months)

AgKG'84/4 (µg total protein)	Ag45/Mut15 (µg total protein)	height (cm)	shoot/root ratio	nodules (no)	AgKG'84/4 % nodules	Ag45/Mut15[a] % nodules
20	-	38 ± 9	4.4	55 ± 21	100	0
2	-	48 ± 7	4.2	27 ± 9	100	0
20	2	40 ± 8	1.9	14 ± 6	75	25
20	20	43 ± 10	4.3	95 ± 41	5	95
2	20	49 ± 7	2.8	110 ± 32	0	100
-	2	52 ± 3	2.3	134 ± 14	0	100
-	20	55 ± 7	2.0	282 ± 83	0	100

[a] % nodules formed by strain Ag45/Mut15 or both strains

Temperature dependent hybridizations used to unravel strain composition in nodules per plant showed that about 75% of the nodules, obtained after inoculation with a mixture of AgKG'84/4 cells equivalent to 20 µg total protein and Ag45/Mut15 cells (2µg total protein), were formed by strain AgKG'84/4 (Table 1). This result is calculated from the disappearence of hybridization signals with probe EFP under high stringency conditions. Nodule formation of this strain in any other inoculation combination was suppressed even when large amounts of cells were inoculated. Combined with identical amounts of strain Ag45/Mut15 (2 µg total protein) only about 5% of the nodules are formed by strain AgKG'84/4. In the presence of higher amounts of strain Ag45/Mut15 no nodules containing strain AgKG'84/4 could be detected. The strain composition of nodules showing hybridization signals also at high stringency conditions was not investigated due to the lack of a second probe specific for strain AgKG'84/4.

Discussion

The simple and rapid isolation method for rRNA from nodules described here and the subsequent use of oligonucleotides for the detection of specific target sequences on rRNA is attractive for ecological investigations. Specificity of probes, the low detection limit of target sequences and the simple application in hybridization experiments can avoid problems of reisolation and identification in pure culture. Natural enrichments of *Frankia*, i.e. nodules, are a good starting material for these investigations. The very low detection limit of the probe-target system allows in this case a reliable identification of the *Frankia* strain in nodules that are about one millimeter in diameter. The procedure enables the investigation of the strain

composition in larger nodules consisting of several lobes. Even the strain composition of each lobe can be studied separately. Using different specific probes detection of double-infections with two strains or competition experiments with defined strains will be feasible.

Combinations of strains differing in only one or two nucleotides, e.g. *Frankia* strains AgKG'84/4 and Ag45/Mut15, theoretically simplify the identification process because single hybridization and washing at two stringencies can distinguish between both strains. However, temperature dependent hybridization cannot be used to study possible double infections in one nodule. Here, two specific probes are necessary to identify the infecting strains. In our experiment the identification of *Frankia* strains in nodules obtained after dual inoculation of both *Frankia* strains at different concentrations indicates that strain Ag45/Mut15 is a superior competitor than strain AgKG'84/4. However, this assumption includes some speculation because nothing is known about possible double infections. Even the application of two specific probes could be of limited value when the concentration of one of the infecting strains is below the detection limit.

The phenomenon of reduced nodule formation after dual inoculation of *Frankia* in *Alnus glutinosa* plants is similar to results found in competition experiments with *Rhizobium* strains (Lie et al., 1988; Winarno and Lie, 1979). More information on possible interference between both *Frankia* strains in nodule formation on *Alnus glutinosa* plants might therefore be obtained after independent inoculation of both *Frankia* strains at different times in combination with strain identification.

Group- or species specific sequences of 16S rRNA have been found within various microorganisms (Kohne et al., 1986; Goebel et al., 1987; Giovannoni et al., 1988). Analyses of variable regions of 16S rRNA of closely related organisms indicate sufficient variation to design probes of interest, despite the fact that DNA/DNA homology studies suggest these species might actually be one and the same. This makes rRNA sequences an attractive target for diagnostic research (Kohne et al., 1986; Tenover, 1988; Viscidi and Yolken, 1987). The application of the RNA extraction method in combination with specific probes can supply rapid information on the occurrence and the establishment of recalcitrant organisms in natural populations, e.g. populations in natural environments like soil. Problems encountered with the isolation of RNA from soil samples are quite similar to that encountered with the isolation from nodules, i.e. the presence of large amounts of humic acids instead of polyphenols. During our isolation procedure all polyphenols are removed from the rRNA sample. The usefulness of 16S rRNA sequences directly isolated from soil samples as targets for oligonucleotide probes should therefore only depend on the quality of the probe ensuring the detection of small amounts of target sequences within large amounts of background rRNA.

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Chapter 9

Extraction of ribosomal RNA from soil for detection of *Frankia* with oligonucleotide probes

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Extraction of ribosomal RNA from soil for detection of *Frankia* with oligonucleotide probes

Abstract

Sequences of 16S rRNA of the nitrogen-fixing *Frankia* strain Ag45/Mut15 and the ineffective *Frankia* strain AgB1.9 were used to design a genus-specific oligonucleotide probe. Hybridization experiments of this *Frankia* probe and a second probe, specific for Nif$^+$-*Frankia* strains only, were used to detect *Frankia* specific target sequences in RNA isolations from soil. A method is described for direct isolation of RNA from a loamy soil and a peat. Yields of about 10 ng RNA/g wet soil are obtained without detectable contamination with humic acids. Isolation of RNA after initial extraction of bacteria from soil resulted in significantly lower RNA yields, compared to the direct isolation procedure. Hybridization with both probes against rRNA isolations from *Frankia*- containing soil could detect target sequences within RNA isolations from 1 g wet soil with an estimated detection limit of 10^4 cells.

Introduction

Recent developments in molecular biological techniques offer chances to study recalcitrant bacterial populations in natural systems without their isolation and cultivation (Olsen et al., 1986; Holben et al., 1988; Steffan and Atlas, 1988; Fuhrman et al., 1988; Sommerville et al., 1989). Investigations on the nitrogen-fixing actinomycete *Frankia* are often hampered by problems encountered with its isolation and identification (St-Laurent and Lalonde, 1987). First attempts to study the strain composition of this recalcitrant organism in nodules were made using plasmid DNA as probe-target system in hybridization experiments (Simonet et al., 1988). Identification of strains in nodules was also done using restriction enzyme patterns of total DNA and subsequent hybridization with Nif-genes (Meesters, 1988; Simonet et al., 1988; Normand et al., 1988).

An alternative to DNA as target for identification of bacteria is the use of rRNA as target in hybridization experiments (Kohne et al., 1986; Goebel et al., 1987; Giovannoni et al., 1988; Stahl et al., 1988). Actinorhizal nodules are metabolically highly active enrichments of *Frankia*, and consequently contain large amounts of *Frankia* rRNA. Synthetic oligonucleotides were used as specific probes in hybridization experiments against 16S rRNA sequences in order to discriminate between different *Frankia* groups and strains (Hahn et al., 1989). For the application of these probes in identification of *Frankia* strains in nodules, a rapid rRNA extraction method has been developed which ensures high yields of RNA without contamination with polyphenols that cause aspecific binding of the probes (Hahn et al., submitted).

So far, hybridizations against specific DNA sequences or against rRNA sequences were developed for detection of *Frankia* in enrichments such as nodules and pure cultures from which sufficient DNA or rRNA can be obtained. Detection of this recalcitrant organism in soil is normally performed in a biotest by detecting nodule

formation on their host plants (van Dijk, 1984; van Dijk et al., 1988; Smolander and Sundman, 1987; Smolander et al., 1988). This detection method is rather selective, excluding e.g. non-infective *Frankia* strains (Hahn et al., 1988) and *Frankia* strains belonging to other compatibility groups (Baker, 1987).

Our research focussed on the development of an oligonucleotide probe that could be used to detect different types of *Frankia* strains directly in the soil. Sequences within or close to variable regions in 16S rRNA of effective and ineffective *Frankia* strains were compared with sequences of other actinomycetes (Smida, 1988) and used to design this *Frankia* specific probe. The application of the probe in detection of *Frankia* in soil included the development of a reliable RNA extraction method from different soils that generally contain low populations of *Frankia*. The usefulness of rRNA directly isolated from soil as target for *Frankia* specific synthetic oligonucleotides in hybridization experiments is demonstrated.

Material and methods

Probe design

Frankia strains Ag45/Mut15, AgB1.9 and AgW1.1 were grown and harvested as described elsewhere (Hahn et al., 1989). Ribosomal RNA extraction and reverse transcriptase sequencing was done according to Embley et al., 1988. Sequences of variable regions of these strains were compared with published sequences of other actinomycetes (Smida, 1988). Oligonucleotide synthesis and labelling were described in Hahn et al., 1989. The probe was tested in dot blot hybridization experiments with immobilized rRNA of twenty-three *Frankia* strains (Hahn et al., 1989) belonging to different compatibility groups (Baker, 1987). Specificity of the probe for *Frankia* was tested in dot blot hybridizations with rRNA of several other actinomycetes which were kindly provided by Prof. E. Stackebrandt (Dept. of Microbiology, Univ. Kiel, FRG).

Filter hybridization

Northern blots and dot blot hybridization experiments were performed on GeneScrene filters (DuPont). About 100 ng rRNA per dot applied with a Hybri. Dot manifold (BRL) was immobilized by UV light and hybridized according to Church and Gilbert (1984) for 2-4 hours at 52°C. Similar amounts of RNA were applied in northern blots (Maniatis et al., 1982) hybridized under the same conditions, except that filters were incubated at 80°C for 30 minutes prior to UV light immobilization to remove formaldehyde and prewashed in 0.1 x SSC/1% SDS for 15 minutes at 80°C prior to prehybridization. Oligonucleotide probes were 5'-labelled using phage T_4 polynucleotide kinase (BRL) and 20-50 µCi of [τ-^{32}P]adenosine-5'-triphosphate (3000 Ci/mmol; Amersham) (Maniatis et al., 1982).

Soil characteristics and treatment

Surface samples (10 cm depth) were collected from two soils, a sandy loam from

Bentheim (B), West-Germany, and a peaty soil from Weerribben (W), The Netherlands. Soil characteristics were: (B) soil organic matter was 8.1% of dry weight with a C/N of 13.3 at a $pH_{(H20)}$ of 3.9. Water content was 57% of soil dry weight. (W) soil organic matter was 71.5% of dry weight with a C/N of 17.7 at a $pH_{(H20)}$ of 3.6. Water content was 329% of soil dry weight.

Samples of each soil were filled in 1.5 l pots, sterilized by gamma irradiation (2.5 Mrad) in order to obtain *Frankia*-free soil and allowed to recolonize with air-borne microorganisms in the greenhouse for two months. Cloned plants of two *Alnus glutinosa* ecotypes, originating from Bentheim (forest ecotype) and Weerribben (pioneer ecotype) were grown in these pots for two weeks. Half of the plants were inoculated with homogenous suspensions of the nitrogen-fixing *Frankia* strain Ag45/Mut15 (Hahn et al., 1988). The amount of inoculum was equivalent to 20 μg total protein (Moss and Bard, 1957). After six months of growth plants were harvested and controlled for nodule formation. *Frankia*-free and *Frankia*-containing soils were maintained in plastic vessels per pot at 4°C.

Enumeration of viable heterotrophic bacteria was performed by standard plate count procedures on 5% PYTA agar (Balkwill and Ghiorse, 1985). Plates were incubated at 20°C and CFU were determined after one week.

Extraction of bacterial cells

The bacterial fraction of both soils was isolated according to Faegri et al. (1977) and Torsvik (1980). Ten gram of soil was suspended in 40 ml 0.1 M $Na_4P_2O_7$, shaken for 15 minutes and centrifuged at 1000 xg to remove fungi and inorganic substances. The pellet was extracted once more with 20 ml TE-buffer (10 mM Tris/ 1 mM EDTA, pH 8). The pooled supernatants were centrifuged at 15000 xg and the resulting pellet washed twice with TE-buffer to remove soluble amounts of humic acids. This bacterial fraction, still contaminated with humic acids, was used for rRNA isolation.

Ribosomal RNA extraction

Pure cultures of *Pseudomonas putida* and *Frankia* strain Ag45/Mut15 (1 g fresh weight), the extracted bacterial fractions of 10 g soil, and soil (2 g wet weight) were each sonified (3-6 x 30 sec., 60 W, Ultra Sonics, Danbury, CT, USA) in 5 ml of 7.5 M guanidine-hydrochloride/ 1 M Tris, pH 7.0 and centrifuged at 3000 xg to separate unsoluble residues. Soluble substances of the supernatant were precipitated with ethanol. The ethanol insoluble pellet was resuspended in sterile distilled water and extracted twice with phenol, pH 7.4/chloroform. After subsequent chloroform extraction (2 x) the colourless rRNA was precipitated with ethanol, dried and resuspended in distilled water (Hahn et al., submitted). If pellets were brown they were resuspended in 0.4 ml guanidine-hydrochloride solution, precipitated with ethanol and the resulting pellet again resuspended in distilled water and extracted with phenol/chloroform. This procedure was repeated until the pellet was colourless.

RNA extraction was controlled on northern blots hybridized with a universal eubacterial probe commonly used as primer in 16S rRNA sequencing reactions (primer 1115, Embley et al., 1988). This control was also performed on all dot blots with RNA

extractions from soil.

The amount of RNA was determined from the intensity of the hybridization signals with the universal probe and comparative ethidium bromide signals on gels.

Filters containing rRNA from soil preparations were always prewashed after rRNA immobilization in 10% standard saline citrate (SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1% SDS for 15 minutes at 80°C in order to remove rests of contaminating humic acids.

Detection of *Frankia*

Dot blot hybridizations of RNA directly isolated from *Frankia*-free or *Frankia*-containing soil (1 g wet weight of sandy loam) or from extracted bacteria of the same soil samples were performed with the following probes: a) probe EFP, an oligonucleotide probe, specific for nitrogen-fixing *Frankia* strains obtained from *Alnus* nodules (Hahn et al., 1989) and b) the *Frankia* probe to detect *Frankia* specific target sequences.

The possible interference of background on the hybridization signals was tested by mixing low amounts of rRNA of *Frankia* strain Ag45/Mut15 with large amounts of RNA obtained from *Pseudomonas putida*. One picogramm to 1 ng of *Frankia* rRNA was mixed with 10 ng to 1 µg rRNA of *P. putida*. The mixtures were used in dot blot hybridization experiments with probe EFP and the *Frankia* probe.

Additionally, *Frankia*-free soil was inoculated with homogenous suspensions of *Frankia* strain Ag45/Mut15 (late stationary phase) at different concentrations (0.001 µg protein/g soil to 10 µg/g soil). The samples were thoroughly mixed and incubated at room temperature for one hour to allow some binding to the soil. Two g of the inoculated soil and a *Frankia*-free control-soil were used to isolate rRNA directly from soil. Extracted rRNA was hybridized with probes described above and with the universal probe.

Results

Design of *Frankia* probe

Sequences of variable regions of the effective *Frankia* strain Ag45/Mut15 and the ineffective strains AgB1.9 and AgW1.1 were compared. They showed regions with large differences and one identical region (region 430-540, *E.coli* pos.)(Hahn et al., 1989). However, a comparison of this region with the homologous region of thirty actinomycetes (Smida, 1988) indicated several similar or identical sequences. Another sequence close to the variable region 180-240 (*E.coli* pos., Brosius et al., 1981) was found to be unique for *Frankia* and was therefore chosen as target for complementary synthetic oligonucleotides with the sequence: 3' G A C C A C C A C A C C T T T C T A A A T A

Probe specificity

Temperature dependence and sequence specificity of probe hybridization are

illustrated in Figure 1. Hybridizations were performed under low stringency conditions followed by washes at higher stringency, i.e. at higher temperatures. Hybridization of the *Frankia* probe to immobilized rRNAs of *Frankia* strains belonging to different compatibility groups showed signals of all twenty-three *Frankia* strains at washing temperatures up to 40°C. Lack of hybridization at higher washing temperatures indicated small sequence differences of several *Frankia* strains.

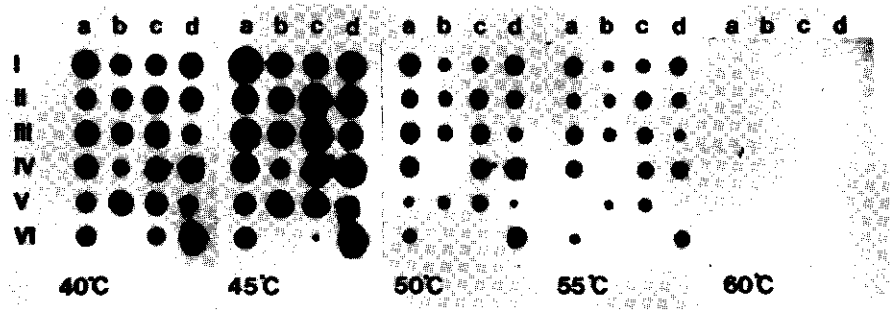

Fig. 1 Temperature dependence and sequence specificity of probe hybridization to nylonfilter-bound rRNAs of several physiologically different *Frankia* strains belonging to distinct compatibility groups (Hahn et al., 1989). Lanes a and b contain rRNA from nitrogen-fixing *Frankia* strains obtained from alder nodules, lane c contains rRNA from non-nitrogen-fixing strains from alder nodules and lane d rRNA from strains obtained from different hosts (*Colletia*, *Elaeagnus*, *Casuarina*, *Hippophaë*, *Comptonia* and *Coriaria*). RNA of *Streptomyces lividans* (VIb) is used as negative control. The position of the strains is identical to those described previously (Hahn et al., 1989) Each spot contains 10ng of RNA.

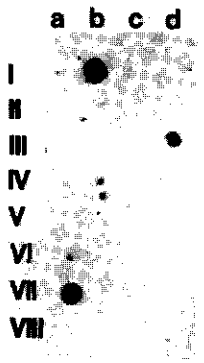

Fig. 2 Dot blot hybridization against immobilized rRNA (10 ng) of several actinomycets. Positive hybridization signals are obtained with RNA of *Frankia* strain Ag45/Mut15, *Actinomadura* sp. and *Microbispora rosea*, also under stringend washing conditions (0.04 M Phosphate buffer/ 1% SDS at 55°C (Church and Gilbert, 1984)). Ia *Streptoverticillium hachijoense* (2011), IIa *Streptoverticillium olivoreticuli* (0278), IIIa *Streptospor angium amethystogenes* (DSM 43179), IVa Streptosporangium corrugatum (DSM 43316), Va *Streptosporangium indianense* (DSM 43803), VIa *Thermomonospora mesophila* (DSM 43048), VIIa *Microbispora rosea* (DSM 43025), VIIIa *Saccharothrix australiensis* (DSM 43800), Ib *Actinomadura* (DSM 43768), IIb *Actinomyces naeslundii* (DSM 43013), IIIb *Actinomyces viscosus* (DSM 43027), IVb *Actinomyces odontolyticus* (DSM 43331), Vb *Actinomyces bovis* (DSM 43014), VIb *Propionibacterium thoenii* (DSM 20276), VIIb *Propionibacterium acnes* (1897), VIIIb *Propionibacterium jensenii* (DSM 20535), Ic *Nocardioides albus* (DSM 43109), IIc *Nocardioides luteus* (DSM 11455), IIIc *Pimelobacter simplex* (8929), IVc *Pimelobacter jensenii* (20641), Vc *Terrabacter tumescens* (8914), VIc *Tsukamurella aurantiaca* (10741), VIIc *Nocardiopsis dassonvillei* (DSM 43235), VIIIc *Corynebacterium polymorphus* (2097), Id *Dermatophilus congolensis* (DSM 43037), IId *Geodermatophilus obscurus* (DSM 43160), IIId *Frankia* Ag45/Mut15, IVd *Arachnia propionica* (DSM 43307), Vd *Rhodococcus bronchialis* (DSM 43247), VId *Amycolatopsis* sp., VIId *Kibdellosporangium*, VIIId *Clostridium* sp., + *Streptomyces cyaneus* (DSM 40108)

The specificity of the probe to *Frankia* is shown in hybridization experiments against rRNA of several other bacteria including a number of soil actinomycetes. Aspecific hybridization was only obtained with two other bacteria, *Actinomadura* and *Microbispora rosea* (Fig. 2).

Ribosomal RNA isolation

RNA was isolated in small amounts directly from both soils, the sandy loam (B) and the peat soil (W). Their yield was about 10 ng rRNA per g soil depending on the soil (Fig. 3). The difference reflected the different amounts of bacteria in each soil. CFU of soil B were about 1.2×10^7 and of soil W about 1.4×10^6. The total yield of RNA was in general much lower when bacteria were first separated from soil (Fig. 3). The difference in yield was due to the amount of washing steps used to remove contaminating humic acids from bacteria suspensions.

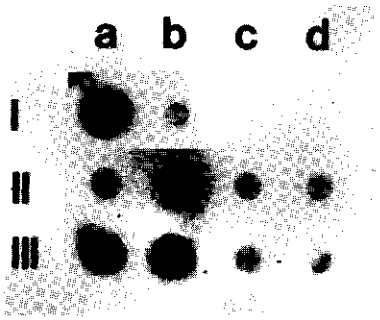

Fig. 3 Dot blot hybridization of RNA isolated directly (a, b) or after bacterial isolation (c, d) from the sandy loam (II) and the peat (III). Lanes a and c contain RNA of *Frankia*-free soil whereas lanes b and d contain RNA from *Frankia*-containing soil. Lane I consists of rRNA isolated from *P.putida* (a: 10 ng; b: 1 ng). Hybridization is performed with a universal probe (primer 1115 (Embley et al., 1988)).

Detection of *Frankia*

Detection of *Frankia*-specific sequences was feasable with rRNA isolations directly from soil (Fig. 4, 5). When rRNA was obtained from the bacterial fraction of the same soil, no *Frankia* specific sequences could be detected. Concomitant hybridization with both probes, the *Frankia*-probe and probe EFP, could only be seen with RNA isolated directly from *Frankia*-containing soil (Fig. 4).

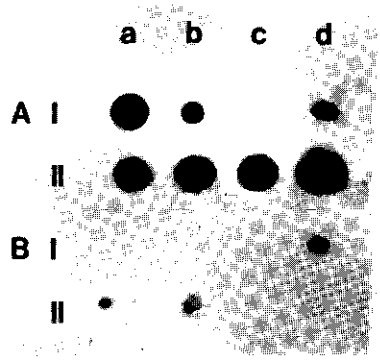

Fig. 4 Dot blot hybridization of RNA isolated directly (IIa, IIb) or after bacterial isolation (IIc, IId) from the sandy loam. Lanes a and c contain RNA of *Frankia*-free soil whereas lanes b and d contain RNA from *Frankia*-containing soil. Lane I consists of rRNA isolated from *P.putida* (a: 10 ng; b: 1 ng) and from *Frankia* Ag45/Mut15 (d: 1 ng). Hybridization is performed with a universal probe (A) and the *Frankia* probe (B).

Compared with hybridization signals obtained from RNA of *Frankia*-free soil, signals from RNA of *Frankia*-containing soil showed a much higher intensity after hybridization with probe EFP under low stringency conditions, i.e. at washing temperatures of 30°C (Fig. 5). Under high stringency conditions the background signal of the RNA of *Frankia*-free soil disappeared whereas the RNA of the *Frankia*-containing soil still showed hybridization signals (Fig. 5).

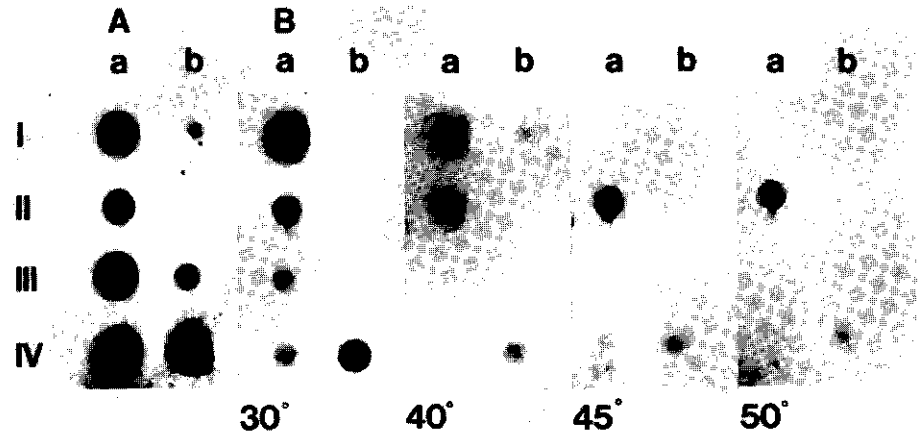

Fig. 5 Dot blot hybridization of RNA isolated directly from soil (1 g wet weight)(IVa: *Frankia*-free sandy loam; IVb: *Frankia*-containing sandy loam) with a universal probe (A) and probe EFP (B) at different stringencies. Lane I consists of rRNA of *Frankia* strain AgKG'84/4 (a: 10 ng; b: 1 ng), lane II of rRNA of strain Ag45/Mut15 and lane III of rRNA of *P. putida*.

The detection of target sequences in the presence of large amounts of background rRNA was determined in dot blot hybridization experiments with both probes using mixtures of target sequences (up to 1 pg *Frankia* rRNA) with large amounts of background rRNA (1 µg *Pseudomonas putida* rRNA). Similar to the hybridization experiments with RNA from *Frankia*-free and *Frankia*-containing soil enhanced signals with target sequences were obtained under low stringency conditions. Positive

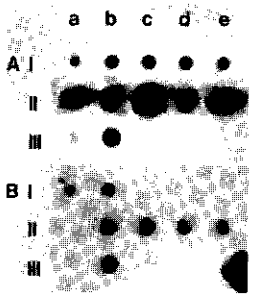

Fig. 6 Dot blot hybridization of RNA isolations directly from soil with the *Frankia* probe at different stringencies (A: 30°C; B: 50°C). Lane I contains control preparations of different amounts of *Frankia* rRNA (1 ng (a, b), 100 pg (c), 10 pg (d) and 1 pg (e)) mixed with 10 ng of *P.putida* rRNA (b-e). Lane II consists of rRNA isolations from *Frankia*-free soil (B) inoculated with *Frankia* strain Ag45/Mut15 (1 µg - 0.001 µg total protein in spots b-e). Lane III contains 10 ng *P.putida* rRNA (IIIa) and rRNA isolated from 1 g *Frankia*-containing soil type B obtained in the inoculation experiment after 6 months (IIIb).

hybridization reactions were obtained with 1 pg *Frankia* rRNA in 1 µg *P. putida* rRNA (data not shown). The same amount of pure rRNA of *Frankia* could not be detected, however. The detection limit depended on the amount of background rRNA. Lower amounts (100 ng) showed a decreased detection limit of the target sequence (100 pg) with both probes. Under high stringency conditions the enhanced signals were less significant.

Inoculation experiments with different amounts of *Frankia* to *Frankia*-free soil (B) showed positive detection with inoculum amounts of *Frankia* equivalent to 0.001 µg protein. Smaller inoculum sizes could not be detected (Fig. 6).

Discussion

Analyses of variable regions of 16S rRNA of closely related organisms indicate sufficient variation to design specific probes. This makes rRNA sequences an attractive target for diagnostic research (Kohne et al., 1986; Viscidi and Yolken, 1987). Ribosomal RNA is preferable to DNA as a probe target because of its relative abundance and stability in the cell. For the design of a probe specific for the genus *Frankia* and therefore specific for strains belonging to different compatibility groups, sequences within and closed to a commonly variable region on 16S rRNA were used. The design of the *Frankia* probe was a compromise, however. Small sequence differences between several *Frankia* strains and homologuous sequences with two actinomycetes other than *Frankia* could not be avoided.

The RNA extraction method originally developed for the isolation of RNA from nodules (Hahn et al., submitted), could also be used for direct isolation of RNA from soil containing large amounts of humic acids. In contrast to rRNA isolations from nodules, the removal of humic acids from soil extractions represents still a problem. Most of the humic compounds could be removed in the first extraction step with phenol/chloroform. Depending on the soil the precipitated RNA was still contaminated with some humic acids after this first step. By repeating the extraction procedure or by washing the nylon filters at high temperatures after rRNA had been fixed with UV light the humic acid contaminations could be removed entirely. In contrast to usual DNA isolation techniques from soil (Ogram et al., 1987; Steffan et al., 1988) our method provides a rapid extraction of target sequences, i.e. rRNA, directly from the natural environment within several hours. It is a simple method which can be used without complex equipment. Sonification of the bacterial cells for the release of rRNA resulted in a physical disruption of the RNA. However, hybridization with short oligonucleotide probes was not influenced by this disruption.

The isolation of ribosomal RNA directly from soil yields about 10 ng rRNA/g soil. In our inoculation experiment this amount is sufficient to detect *Frankia* specific target sequences, either at low stringency conditions when the presence of large amounts of background RNA enhances signal expression of the hybridizing probes, or also under high stringency conditions. Because *Frankia* is a hyphae forming organism, it is quite difficult to determine the exact amount of *Frankia* cells inoculated to the soils. Theoretical calculation of the introduced amount of *Frankia* cells using 10^{-12}g fresh weight for one cell results in about 10^5 cells/g soil. Quantitative analyses of the hybridization signals, however, are unreliable because nothing is known about the

actual amount of *Frankia* cells and their metabolic activity after six months of incubation. Inoculation experiments where metabolically inactive *Frankia* (late stationary phase) were added to *Frankia*-free soil indicated that the detection limit could be quite low (10^4 cells/g soil). Because the amount of target sequences in the total RNA preparation is very low the expression of homologuous hybridization signals becomes more and more difficult. This problem could be circumvented by using larger soil samples.

The combination of ribosomal RNA sequences and the rapid RNA extraction method from soil offers possibilities in detection of natural populations of organisms which are difficult to isolate. Ribosomal RNA sequences could be used as stable and reliable markers of physiologically different types of one genus such as effective and ineffective strains of *Frankia* that lack stable DNA markers, i.e. Nif-genes. Suitable oligonucleotide probes together with the rRNA extraction method allow the detection of low numbers of soil-borne microorganisms even when the advantage of large amounts of target sequences per cell in metabolically active cells might be lost in starved cells. For an increase in sensitivity it should be possible to combine the advantages of rRNA as stable target and the rapid extraction of RNA from soil with amplification methods commonly used with DNA or mRNA (Saiki et al., 1988; Steffan and Atlas, 1988).

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Chapter 10

Summary

Summary

The research described in this thesis focusses on the role of biotic factors encountered with the establishment of the symbiosis between black alder plants (*Alnus glutinosa*) and introduced *Frankia* strains. A selection of plant clones and *Frankia* strains that gave optimal nodulation and nitrogen fixation in forestry was made. For this reason nodulation tests with increasing complexity were set up. An attempt was made to investigate whether introduced strains behaved differently on plants grown under axenic and non-axenic conditions. Since *Frankia* strains were difficult to identify by conventional techniques, special attention was given to the development of new molecular techniques for identification of the strains at the nucleic acid level.

Inoculation tests

Initially, plant material of two physiologically different ecotypes of *Alnus glutinosa*, the forest ecotype "Bentheim" and the pioneer ecotype "Weerribben", respectively, was selected. Using tissue culture techniques plant material of both ecotypes was cloned in order to obtain genetically identical plants (Chapter 2). These micropropagated plants were used to set up a standardized inoculation system under axenic conditions in order to study the genetically determined nodulation ability and nitrogen-fixing capacity of *Frankia* strains and to select superior *Frankia* strains as source of inoculum (Chapter 3). The usefulness of selected *Frankia* strains as inoculum was further tested under more practical conditions, in perlite as model environment for nitrogen-limited conditions and in two soils, representing natural environments with different nutritional factors and different microbial populations. The results of the inoculation tests under axenic conditions were confirmed by studies under greenhouse conditions.

The performance of the symbiosis was effected by many variables, e.g. the plant genotype, the *Frankia* strain and environmental conditions. The influence of the environmental conditions became more pronounced when plants were grown on either a sandy loam ("Bentheim") or a peat ("Weerribben") soil and inoculated with *Frankia* strains. Plant growth was positively influenced, e.g. by mycorrhizal fungi in "Bentheim" soil, or negatively influenced, e.g. by oomycetes in "Weerribben" soil. The effects of inoculation with *Frankia* on plant growth remained minimal. The establishment of the introduced *Frankia* strain was also dependent on the soil conditions. The introduced spore(-) *Frankia* strain was only able to compete with the natural spore(+) population of the "Weerribben" soil. Introduction of this strain to "Bentheim" soil did not show any establishment of the introduced strain. In contrast to the sandy loam of "Bentheim" which was rich in nutrients, the peat of "Weerribben" was a representative of poor soils. It could therefore be used for feasability studies in inoculation programmes. The use of pure cultures of *Frankia* as inoculum instead of soil or crushed nodules, has the advantage to prevent the contamination of the plant with root pathogens. Pure cultures did not result in a better symbiosis.

Atypical *Frankia* strains

Screening of several isolates obtained from nodules of both alder ecotypes indicated the existence of atypical, ineffective *Frankia* strains. The alder clones used showed variable resistance against infection of the ineffective strains (Chapter 4). When compared with growth after the addition of a single strain dual inoculation of typical, effective *Frankia* strains and an ineffective *Frankia* strain to both alder clones showed growth increment of the plants (Chapter 5). The growth enhancing effect of the ineffective *Frankia* strain was not paralleled by increased number of nodules. Nothing is known yet about the growth stimulation by atypical *Frankia* strains. The results indicate that simultaneous inoculation of different *Frankia* strains to *Alnus* plants can be profitable for the host plant.

Ribosomal RNA

Because the ineffective *Frankia* strains lacked morphological and physiological characteristics of typical *Frankia* strains and because nodule formation on actinorhizal plants might be reduced or even absent, detection of the ineffective strains and studies on their competitive abilities were quite difficult. Reliable markers which could be used to detect both types of *Frankia* in nodules and in soil without reisolation had not been available at that moment. An attempt was made to find specific markers in a molecule which was commonly used to unravel evolutionary relationships: the 16S ribosomal RNA. New sequencing techniques allowed the rapid determination of total or almost total 16S rRNA sequences. Total 16S rRNA sequences indicated the presence of conserved and variable regions. Conserved regions had been used to investigate quantitative evolutionary relationships amoung bacteria. The conserved regions of the total 16S rRNA sequence of the effective *Frankia* strain Ag45/Mut15 were compared with aligned sequences of other actinomycetes and used to determine the position of the family *Frankiaceae* in the phylogenetic tree of the actinomycetes (Chapter 6). Analyses of variable regions of 16S rRNA of closely related organisms indicated sufficient variation, despite the fact that DNA/DNA homology studies suggested these two species might actually be one and the same. Large differences in DNA/DNA homology studies of *Frankia* which were also obtained between strains of one compatibility group suggested chances on large variation within the variable regions of different strains. Sequence analyses of variable regions of 16S rRNA of two ineffective *Frankia* strains (i.e. AgB1.9 and AgW1.1) and the effective strain Ag45/Mut15, all belonging to the *Alnus*-compatibility group, showed large differences in base composition. These sequences were used to design complementary synthetic oligonucleotides that could act as specific probes in hybridization experiments. The specificity of these probes was shown in hybridization experiments against immobilized rRNA from 23 *Frankia* strains belonging to different compatibiliy groups and of several related soil actinomycetes. The probes were able to distinguish between Nif^+ and Nif^- strains, between several Nif^- strains and between several *Alnus* compatible Nif^+ strains and strain AgKG'84/4 also belonging to the *Alnus*-compatibility group (Chapter 7). Strong strain specific sequences, however, were not obtained. The design of oligonucleotide probes opens up the possibility to investigate competitive abilities of

selected strains under defined conditions, e.g. in model systems with perlite and defined *Frankia* strains. The question whether competition studies under these controlled conditions are ecologically relevant needs further investigations because little basic knowledge on *Frankia* population dynamics is yet available. The application of probes to identify introduced strains in soil remains restricted, due to the low specificity for strains. Up to now we are not able to design reliable strain specific probes that can be used to follow the establishment of introduced *Frankia* strains in natural environments. A much more promising application of probes towards rRNA is concerned with the development of a genus-specific oligonucleotide probe against *Frankia* (Chapter 9) that theoretically enables quantitative detection of total *Frankia* populations.

RNA extraction

The application of oligonucleotide probes in the detection of specific *Frankia* strains does not only depend on specificity of the probes but also on the development of a reliable isolation method for target sequences. Ribosomal RNA is preferable to DNA as target because of its relative abundance in large amounts in metabolically active cells. Actinorhizal nodules represent enrichments of *Frankia*, which are metabolically highly active and consequently contain large amounts of *Frankia* RNA. Our investigations resulted in the development of a rapid RNA extraction method that was sensitive enough to investigate strain composition also from very small nodules or lobes (Chapter 8). The detection of target sequences, however, remained limited by the design of specific probes and the ratios of different target sequences in one sample. For reliable signal expression in hybridization experiments quite similar amounts of target sequences per sample were needed.

So far, the usefulness of rRNA sequences as targets for oligonucleotide probes was only shown in combination with pure cultures of *Frankia* (Chapter 7) or in metabolically highly active enrichments, e.g. nodules (Chapter 8). Terrestrial environments like soil contain populations of many different microorganisms. These populations normally grow under suboptimal nutrition conditions. Bacteria adapt to these conditions by forming special starvation cells, which are metabolically inactive and contain only low amounts of rRNA. The starvation respons often results in viable, but non-culturable populations. The recalcitrant character of *Frankia*, which are difficult to isolate, makes it a useful model microorganism of soil bacteria. The application of oligonucleotide probes for detection of *Frankia* in soil depends on the development of an extraction method for RNA. RNA directly isolated from soil as target for *Frankia* specific oligonucleotide probes was useful in detection of *Frankia* (Chapter 9). Quantification of the obtained signals, however, is still unreliable because *Frankia* is a hyphae forming organism. It is also quite difficult to correlate cell numbers (theoretical estimation) to the amount of RNA. The concentration of these molecules in an organism is a function of the activity of the individual cell. Quantification of hybridization signals therefore depends on the availability of basic information of the metabolic activity of *Frankia* cells in soil. This information, however, is very difficult to obtain for recalcitrant microorganisms like *Frankia*. It is much easier for other microorganisms, e.g. for *Streptomyces*. *Streptomyces* spores are quite easy to isolate from soil and the establishment of *Streptomyces* cells, i.e. as spores or as mycelium

in soil, is well studied. Quantification based on hybridization signals must be possible when this basic knowledge is available. In case of *Frankia* methods that enable quantification must still be developed. Similar to *Streptomyces* these quantification methods for *Frankia* can be of direct character, e.g. quantitative extraction of spores, or of indirect character, e.g. determination of mycelium by phage counts.

The development of rapid and sensitive methods to detect *Frankia* on the basis of rRNA sequences opens up new ways to study other recalcitrant microorganisms in the environment. This molecular approach in microbial ecology can definitely further be explored when the advantages of rRNA as stable target and the rapid extraction of RNA from soil can be combined with *in vitro* amplification methods commonly used with DNA or mRNA. Promising approaches can also be expected in *in situ* studies using hybridization signal intensity of fluorescent dye labelled oligonucleotides and the amount of rRNA as criterium for bacterial activity.

Samenvatting

Het in dit proefschrift beschreven onderzoek richt zich op de rol van biotische faktoren betrokken bij het tot stand komen van de symbiose tussen zwarte els (*Alnus glutinosa*) en geïntroduceerde *Frankia* stammen. Er werd een selectie gemaakt van planten klonen en *Frankia* stammen, die een optimale knolvorming (nodulatie) en stikstof binding geven. Hiervoor werden nodulatie testen met toenemende complexiteit opgezet. Daarnaast werd een poging ondernomen om te bekijken of geïntroduceerde stammen zich anders gedragen op planten onder axenische dan onder niet axenische omstandigheden. Omdat *Frankia* stammen met conventionele microbiologische technieken moeilijk te volgen zijn, werd speciale aandacht besteed aan de ontwikkeling van nieuwe technieken voor de identificatie van stammen op moleculair niveau.

Inoculatie testen

In hoofdstuk 2 wordt de initiële selectie van twee fysiologisch verschillende ecotypen van *Alnus glutinosa* beschreven, nl. het bos ecotype "Bentheim" en het pioniers ecotype "Weerribben". Plant materiaal van beide ecotypen werd door middel van weefselkweek technieken gekloneerd om genetisch identieke planten te verkrijgen. Deze planten werden gebruikt om een gestandardiseerd inoculatie system onder axenische ("steriele") condities op te zetten. Dit systeem werd gebruikt om de nodulatie capaciteit en het stikstofbindend vermogen van *Frankia* stammen te bepalen. Aldus verkregen superieure *Frankia* stammen werden als inoculum gebruikt (hoofdstuk 3). De bruikbaarheid van geselecteerde *Frankia* stammen als inoculum is verder getest onder praktijk omstandigheden, nl. in perlite als model grond voor stikstof gelimiteerde condities. Tevens werden twee soorten grond gebruikt, nl. een zandige leemgrond (Bentheim) en een veengrond (Weerribben). De gronden verschillen in nutriëntgehalte en microbiele populatie. Inoculatie met *Frankia* stammen aan potten met perlite als kunstmatige matrix toonde de beperking van ons axenisch test systeem aan. Dit was te wijten aan de slechte groei omstandigheden in buizen. De resultaten van de inoculatie test onder axenische condities worden gedeeltelijk bevestigd in studies onder kas condities. Het optreden van symbiose wordt door vele factoren beïnvloed, zoals het genotype van de plant, de *Frankia* stam en omgevingsfactoren. Het belang van de omgevingsfactoren wordt meer benadrukt als de planten in bovengenoemde twee grondsoorten werden gekweekt en met *Frankia* stammen beënt werden. Bodemparameters beïnvloeden de planten groei positef, b.v. het voorkomen van mycorrhizae schimmels in Bentheim grond, of negatief, zoals de aanwezigheid van wortelpathogene oomyceten in Weerribben grond. De invloed van inoculatie met *Frankia* blijft daardoor minimaal. De vestiging van de geïntroduceerde *Frankia* stam was ook afhankelijk van de bodem omstandigheden. De geïntroduceerde sporen negatieve *Frankia* stam was alleen in staat om met de natuurlijke sporen vormende *Frankia* populatie op Weerribben grond te concureren. In tegenstelling tot de zandige leem grond uit Bentheim die rijk is aan voedingsstoffen is de veen grond uit Weerribben meer representatief voor arme gronden die geschikt zijn voor inoculatie programmas.

Gegeven het feit dat vele bodems, vooral geërodeerde gronden of gestoomde tuinaarde een laag stikstofgehalte en/of te lage hoeveelheden aan *Frankia* bevatten, blijft beënten met *Frankia* adviseerbaar voor de praktijk. Gebruik van reincultures van *Frankia* in plaats van grond of gemalen knollen is eerder een meer gecontroleerde manier om verspreiding van wortelpathogenen te onderdrukken dan om een optimale symbiose te verkrijgen. Voor dit laatste is meer basiskennis nodig over de groei en overleving van geïntroduceerde *Frankia* stammen in de bodem.

Atypische *Frankia* stammen

Tijdens de screening van verschillende isolaten uit knollen van beide elzen ecotypen werden naast effectieve, N_2 bindende stammen ook atypische, d.w.z. ineffectieve *Frankia* stammen gevonden. De gebruikte elzen klonen bezaten een variable resistentie tegen infectie met de ineffectieve *Frankia* stammen (hoofdstuk 4). Inoculatie van beide elzen klonen met typische, effectieve *Frankia* stammen en atypische ineffectieve stammen tegelijkertijd wijzen op groeitoename van de planten, vergeleken met de groei van planten die slechts met een enkele stam beënt waren (hoofdstuk 5). Het versterkte groei effect van de ineffectieve *Frankia* stam wordt echter niet uitgedrukt in extra knolvorming. Het mechanisme van dit effect is nog onbekend omdat men nog niets weet van de populatie groei en de ontwikkeling van deze nieuwe *Frankia* groep. De resultaten geven echter aan dat gelijktijdige inoculatie van elzen planten met verschillende *Frankia* stammen gunstig kan zijn voor de gastheer plant.

Ribosomaal RNA

Omdat de ineffectieve stammen geen morfologische en fysiologische kenmerken van typische *Frankia* stammen bezitten en knolvorming aan planten verminderd of zelfs afwezig kan zijn, is de detectie van de ineffectieve stammen en studies van hun concurentie mogelijkheden behoorlijk moeilijk. Betrouwbare markers die gebruikt kunnen worden om zowel effectieve als ineffectieve *Frankia* stammen zonder reïsolatie in knollen en grond aan te tonen waren op dat moment niet aanwezig. Wij hebben een poging ondernomen om specifieke markers te vinden in een molecuul dat algemeen gebruikt wordt om evolutionaire verwantschap te ontrafelen, nl. het 16S ribosomaal RNA. Met de moderne sequentie technieken is het mogelijk snel de komplete of nagenoeg komplete basenvolgorde (= sequentie) van het 16S rRNA te bepalen. Totale 16S rRNA sequenties wijzen op de aanwezigheid van geconserveerde en variabele regio's. Geconserveerde sequenties zijn veel gebruikt om kwantitatieve evolutionaire verwantschappen tussen bacteriën te bepalen. Dit proefschrift bevat de beschrijving van geconserveerde regio's uit de totale 16S rRNA van de effectieve *Frankia* stam Ag45/Mut15. De resultaten werden vergeleken met vergelijkbare sequenties van andere actinomyceten en gebruikt om de positie van de familie *Frankiaceae* in de stamboom van de actinomyceten te bepalen (hoofdstuk 6).
Analyses van de variable regios van het 16S rRNA van nauw verwante organismen tonen behoorlijke variatie ondanks het feit dat DNA/DNA homologie studies suggereerden dat deze twee soorten wellicht het zelfde zouden zijn. Grote verschillen in DNA/DNA homologie studies aan *Frankia* stammen die bij een infectie group horen veronderstelde een grote kans op variaties in de variabele

regio's. Sequentie analyses van variabele regio's van 16S rRNA van twee ineffectieve *Frankia* stammen (nl. AgB1.9 en AgW1.1) en de effectieve stam Ag45/Mut15, allen behorend tot de op *Alnus*-infectieve groep, tonen grote verschillen in de basenvolgorde tussen beide typen *Frankia*. De verschillen in de sequencies worden gebruikt om komplementaire synthetische oligonucleotiden te maken die als specifieke probes kunnen fungeren in hybridizatie experimenten. De specificiteit van de probes wordt aangetoond in hybridizatie experimenten tegen geïmobiliseerd rRNA uit 23 *Frankia* stammen behorend tot verschillende kruisinoculatie groepen en tegen rRNA uit verschillende verwante bodemactinomyceten. Met de probes is men in staat onderscheid te maken tussen Nif^+ en Nif^- stammen, d.w.z. stammen die al (Nif^+) of niet (Nif^-) N_2 kunnen binden. Er kan zelfs onderscheid gemaakt worden tussen verschillende Nif^- stammen onderling en tussen verschillende Nif^+ stammen behorend bij de elzen infectie group en de bij dezelfde group behorende stam AgKG84/4 (hoofdstuk 7). Sterke stam specifieke probes werden echter niet verkregen. De ontwikkeling van deze probes opent de mogelijkheid om het concurentie vermogen van geselecteerde stammen onder gedefineerde condities te onderzoeken, zoals in model systemen als perlite met gedefineerde *Frankia* stammen. Om de vraag te beantwoorden of competitie studies onder deze omstandigheden ecologisch relevant zijn, is meer onderzoek aan de groei en ontwikkeling van *Frankia* populaties nodig. De toepassing van probes om geïntroduceerde stammen in grond te herkennen blijft beperkt. Dit is te wijten aan de lage specificiteit van de probes voor stammen. Tot nu toe zijn wij niet in staat betrouwbare stamspecifieke probes te ontwerpen, die gebruikt kunnen worden om de vestiging van geïntroduceerde *Frankia* stammen te volgen in natuurlijke milieus. Een meer belovende toepassing van probes tegen rRNAs heeft te maken met de ontwikkeling van een genus specifieke oligonucleotide probe tegen *Frankia*, die theoretisch een kwantitatieve detectie van de totale *Frankia* populatie mogelijk maakt.

RNA extractie

De toepassing van oligonucleotide probes bij de detectie van specifieke *Frankia* stammen is niet alleen afhankelijk van de specificiteit van de probes maar ook van de ontwikkeling van een betrouwbare isolatie methode van de doel sequentie. Vanwege zijn voorkomen in grote hoeveelheden in metabolisch actieve cellen wordt ribosomaal RNA verkozen boven DNA als doel. Knollen bevatten grote hoeveelheden aan *Frankia* die metabolisch heel actief zijn en bevatten dus ook grote hoeveelheden *Frankia* rRNA. Ons onderzoek resulteerde in de ontwikkeling van een snelle RNA extractie methode uit wortelknollen. Deze is gevoelig genoeg om stammen in zelfs zeer kleine knollen te identificeren (hoofdstuk 8). De detectie van doel- (= target) sequenties blijft echter beperkt door de specificiteit van de probes en door de hoeveelheid van verschillende doelsequenties in een monster. Voor een betrouwbaar signaal in hybridizatie experimenten zijn ongeveer vergelijkbare hoeveelheden doelsequenties per monster nodig.

De bruikbaarheid van rRNA sequenties als doel voor oligonucleotide probes werd tot dan toe alleen getoond in combinatie met reinculturen van *Frankia* (hoofdstuk 7) of in metabolisch zeer actieve milieus zoals knollen (hoofdstuk 8). Andere natuurlijke milieus zoals b.v. grond bevatten veel verschillende soorten bacteriën. Deze groeien meestal onder suboptimale omstandigheden. Ze adapteren aan deze omstandigheden als metabolisch inactieve cellen die

vermoedelijk weinig rRNA bevatten. Ze leven onder deze omstandigheden nog wel, zijn alleen niet meer in staat om te groeien en kunnen moeilijk geisoleerd worden. Omdat veel bodembacteriën evenals *Frankia* moeilijk of niet te isoleren (=recalcitrant) blijken, kan *Frankia* op dit punt als een model organisme voor bodemmicroben beschouwd worden. De toepassing van oligonucleotide probes voor de detectie van *Frankia* in grond is weer afhankelijk van een betrouwbare rRNA isolatie methode. Direct uit de grond geïsoleerd RNA als doel voor *Frankia* specifieke oligonucleotide probes is bruikbaar voor de detectie van *Frankia* (hoofdstuk 9). Het kwantificeren van de verkregen signalen is echter moeilijk omdat *Frankia* hyfen vormt. Het is ook vrij moeilijk om cel aantallen (theoretisch bepaald) en hoeveelheden RNA te coreleren, omdat RNA hoeveelheden afhankelijk zijn van de cel activiteit. Het kwantificeren van hybridizatie signalen is daarom afhankelijk van de basis informatie met betrekking op groei en activiteit van *Frankia* cellen in de bodem. Deze informatie is moeilijk te verkrijgen voor recalcitrante microoganismen zoals *Frankia*. Het is veel eenvoudiger in het geval van andere oranismen zoals *Streptomyces*. *Streptomyces* sporen zijn vrij makkelijk te isoleren uit de grond en de schatting van *Streptomyes* cellen als sporen of als mycelium in de grond is goed bestudeerd. Het kwantificeren van de verkrijgen hybridizatie signalen moet mogelijk zijn als deze basiskennis beschikbaar is. In het geval van *Frankia* moeten methoden die het kwantificeren mogelijk maken nof ontwikkeld worden. Vergelijkbaar met *Streptomyces* kunnen deze kwantitatieve methoden voor *Frankia* direct zijn zoals kwantitatieve extractie van sporen of indirect zoals voor de bepaling van mycelium door faag tellingen.

De ontwikkeling van een snelle en gevoelige methode om *Frankia* op basis van hun rRNA sequencies aan te tonen geeft nieuwe mogelijkheden om recalcitrante microorganismen in grond te bestuderen. Deze moleculaire benadering in de microbiële ecologie moet zeker nader onderzocht worden. Vooral als de voordelen van rRNA als stabiele marker molecuul en de snelle extractie methode van RNA uit grond gecombineerd kunnen worden met *in-vitro* amplificatie methoden die algemeen gebruikt worden voor DNA en mRNA. Of in combinatie met *in-situ* studies die gebruik maken van signaal intensiteit van een oligonucleotide, gemerkt met een fluorescerende stof en het aantal rRNA moleculen per cel om activiteiten per cel te bepalen.

Nawoord

Op deze plaats wil ik graag iedereen bedanken, die heeft meegewerkt aan het tot stand komen van dit proefschrift: iedereen, die zich heeft ingezet voor mijn onderzoek, het goed laten functioneren van het laboratorium, het correctiewerk van het proefschrift, de tekeningen en de fotografische verzorging. Ook al die mensen die het mij mogelijk gemaakt hebben om mij als Duitser in Wageningen een beetje thuis te laten voelen wil ik bedanken. Slechts één naam wil graag vermelden in dit nawoord, die van Marjo Starrenburg. Marjo wil ik niet alleen bedanken voor de prettige werksfeer en het uitvoeren van een groot aantal experimenten, maar ook voor het geduld en de manier waarop zij mijn beperkingen in het gebruik van de Nederlandse taal accepteerde.

Curriculum vitae

The author of this thesis was born in July, 1958, in Neumünster, West-Germany. In 1979 he started the study of biology as a Diploma candidate at the University of Hamburg, West-Germany. After termination of the basic studies, he specialized in the disciplines of pedology (Prof. Scharpenseel), general botany (Prof. Doerffling) and applied botany (Prof. von Weihe). From December 1985 to November 1989 he was working as a PhD student at the Department of Microbiology in Wageningen, The Netherlands. The results of this PhD study are presented in this thesis. Since November 1989 he is working on a postdoctoral position at the Department of Microbiology in Wageningen.