Characterization of uncultured *Frankia* strains by 16S rRNA sequence analysis

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Stellingen

1. The results of probing PCR amplificates from environmental samples suggest that any conclusions should be verified by sequence analysis.

   "This thesis"

2. The observation by Nick and co-workers that the endophytes of *Coriaria* nodules from different parts of the world have similar 16S rRNA sequence should be extended to other variable regions of 16S rRNA.

   Nick et al., 1992. Mol. Ecol. 1:175-181

3. The conclusion that *Frankia* strains can be divided into three groups on the basis of fatty acid composition may change when more strains from different hosts are analysed.

   Mirza et al., 1991. FEMS Microbiol. 83:91-98

4. The pressure to publish can often lead to insufficient confirmation of data.

   Nature, 343:665 (1993)

5. Research funding uncertainties can lead to the researcher being more concerned with financial considerations than scientific excellence.

   Nature, 364:571 (1993)

6. It is more important for an idea to be fruitful than true.

   A.N. Whitehead

7. The majority of the people who migrate fail to realize that social values are changing rapidly in their original country in which they may not find easy to re-adjust.
8. Student exchanges should be made more frequent to increase knowledge about racism/fundamentalism and wine/hot food (the latter being more important, of course).
9. For a person working at the Department of Microbiology, it is hard to believe that The Netherlands is a "vlak land".
10. Women have more freedom in the West, but enjoy more respect in the East.

Wageningen, 12 November 1993. **M. Sajjad Mirza**

For aunt Mrs. Sultana Mirza & Uncle M. Sharif

Contents

Chapter 1

Introduction

Introduction

The ability to fix molecular nitrogen occurs in a diverse groups of microoganisms that exist, either as free-living diazotrophs or in symbiotic associations with plants (Young, 1992). The root nodule is the most important symbiotic association between nitrogen-fixing microorganisms and higher plants (Akkermans and Houwers, 1979; Dixon and Wheeler, 1986). Actinorhizal root nodule formation involves the symbiotic association of an actinomycete *Frankia* and the roots of dicotyledonous plants belonging to eight plant families and 24 genera (Benson and Silvester, 1993). Actinorhizal plants have current and potential applications in reforestation and soil improvement, timber and pulp production, and acting as nurse and fuelwood plants (Dawson, 1990; Diem and Dommergues, 1990; Gordon and Wheeler, 1983)

In symbiotic associations, *Frankia* inhabits the cortical region of the nodules. In most actinorhizal root nodules, the infected cells are uniformly distributed within the cortical region around a central stele (Becking, 1968; Hafeez *et al.*, 1984c; Lalonde and Knowles, 1975; Newcomb *et al.*, 1978). In nodules of *Coriaria* and *Datisca*, infected cells are present on one side of the stele, resulting in asymmetric nodule structure in transverse sections (Canizo and Rodriguez-Barrueco, 1978; Hafeez *et al.*, 1984a; Newcomb and Pankhurst, 1982). Three cell types *i.e.* hyphae, vesicles and sporangia have been identified in *Frankia*. Within the infected cells, depending on the host species, the vesicles may be globose, pear shaped or elongate (Torrey, 1985). Spores and sporangia of the endophyte have also been detected in actinorhizal nodules (Schwintzer, 1990; van Dijk, 1978; van Dijk and Merkus, 1976).

The isolation of nitrogen-fixing endophytes from actinorhizal nodules proved difficult for many years, until the first reproducible isolation of *Frankia* from *Comptonia peregrina* was reported (Callaham *et al.*, 1978). To date, *Frankia* strains from a number of other actinorhizal species are available in pure culture (Lechevalier, 1986; Benson and Silvester, 1993) and their growth requirements have been optimized (Akkermans *et al.*, 1983: Blom, 1982; Meesters *et al.*, 1985; Tjepkema *et al.*, 1980). *Frankia* spp. in pure culture are slow growing actinomycetes with poorly branched hyphae that range in diameter from 0.5 to 2.0 μm. Contrary to other actinomycetes, no aerial mycelia are formed on solid media. The formation of sporangia in submerged liquid culture is

considered a characteristic of the genus *Frankia* (Lechevalier and Lechevalier, 1990). The spores are thick-walled, somewhat polygonal in shape and non-motile. In nitrogen-free media, spherical "vesicles" are formed. With few exceptions e.g. in strains ACN1ag, EAN1pec, Cc1.17 (Meesters *et al.*, 1985; Tisa *et al.*, 1983), vesicle formation is suppressed in the presence of combined nitrogen. Vesicles are the principal locus of nitrogenase, the enzyme responsible for nitrogen fixation (Meesters *et al.*, 1987).

1. Molecular Characterization of *Frankia* strains.

Members of the genus *Frankia* can be distinguished from other actinomycetes on the basis of their characteristic morphology, ability to nodulate plants and to fix nitrogen. However, differentiation of *Frankia* strains has proved difficult due to their relatively similar morphology, non-availability of pure cultures from several actinorhizal plants and some controversial cross-inoculation data (Becking, 1970; Baker, 1987). This means that there is a requirement for biochemical and molecular techniques for the characterization and detection of genetic diversity among members of the genus *Frankia* (Mullin and An, 1990; Simonet *et al.*, 1990). Application of molecular techniques like PCR has made it possible to study phylogenetic relationships of uncultured *Frankia* strains and to characterize non-infective strains that lack some morphological and physiological characteristics of this genus.

1.1. Chemical analyses.

Chemical analyses of whole organisms or cell fractions is increasingly being used to describe and discriminate between bacteria, notably the actinomycetes (Williams *et al.*, 1989). The important compounds include cell polymers e.g peptidoglycan, polysaccharides, cellular and membrane fatty acids. Members of the genus *Frankia* have a cell-wall composition of type III, containing meso-diaminopimelic acid, alanine, glutamic acid, muramic acid, and glucosamine (Lechevalier, 1984; Lechevalier *et al.*, 1983). Among the variety of sugars present in *Frankia* polysaccharides, xylose and fucose are the most common differentiating compounds (Lechevalier and Lechevalier, 1990). Another commonly occurring sugar, 2-*O*-Methyl-D-mannose is also considered to be the genomic marker (Mort *et al.*, 1983; St. Laurent *et al.*, 1987). However, some *Frankia* strains lack this sugar in amounts detectable on paper or thin-layer chromatography

(Lechevalier and Lechevalier, 1990). This compound does not occur uniquely in *Frankia*. Recently, 2-*O*-Methyl-mannose has also been identified as a constituent of the polysaccharides from mould genera belonging to the order of Mucorales (*Zygomycetes*) (De Ruiter, 1993).

Total cellular fatty acid composition has been frequently used in taxonomic investigations of actinomycetes (Lechevalier and Lechevalier, 1980). Qualitative and quantitative analysis of total fatty acid composition of several *Frankia* strains have been investigated (Lalonde *et al.*, 1988; Lechevalier *et al.*, 1983; Simon *et al.*, 1989; Wheeler *et al.*, 1986). The analysis of fatty acid profiles of *Frankia* strains has allowed discrimination between the type species *F. alni* and *F. elaeagni* and also identification of the subspecies *pommeri* and *vandijkii* within the proposed species *Frankia alni* (Lalonde *et al.*, 1988; Simon *et al.*, 1989). Although *Frankia* strains showed fatty acid pattern distinct from those of other actinomycetes including *Actinomyces, Geodermatophilus, Nocardia, Mycobacterium* and *Streptomyces* (Mirza *et al.*, 1991), no clear taxonomic marker that would place an unknown strain within the genus has as yet been identified.

1.2 DNA-DNA hybridization studies on *Frankia*.

The measurement of the extent to which single-stranded DNA fragments from one bacterial strain reassociate or hybridize with single-stranded DNA from another strain, has been used to determine nucleic acid sequence similarty or DNA homology. DNA-DNA reassociation data are mainly relevant when investigating relationships within and between bacterial species. Generally, bacteria within the same genomic species have DNA homology values above 70% (Wayne *et al.*, 1987). The existence of a large diversity among *Frankia* strains is clearly reflected by the DNA relatedness that ranged from 3 to 100% among the strains tested (Akimov and Dobritsa, 1992; An *et al.*, 1985a; Bloom *et al.*, 1989; Fernandez *et al.*, 1989). Fernandez *et al.* (1989) identified at least nine genomic species among 43 *Frankia* strains on the basis of DNA relatedness. Akimov and Dobritsa (1992) estimated DNA relatedness among 27 *Frankia* strains that allowed differentiation into nine genospecies. Five genospecies were delineated among 18 strains compatible with plants of the genus *Alnus* (isolates from *Alnus* and *comptonia*) and four genospecies were delineated among 9 strains compatible with plants of the family *Elaeagnaceae* (isolates from *Elaeagnus, Hippophae, Shepherdia* and *Colletia*).

1.3 Restriction fragment patterns.

Restriction fragment patterns of total genomic DNA provide a sensitive measure of diversity within populations of *Frankia*. Using restriction fragment patterns of total genomic DNA, a number of distinct groups were identified among the *Frankia* strains compared (An *et al.*, 1985b; Bloom *et al.*, 1989; Dobritsa, 1985). Nine distinct restriction patterns were found among 16 *Frankia* strains isolated from *Myrica pensylvanica* nodules from five different locations (Bloom *et al.*, 1989).

1.4 Protein patterns.

Total cellular protein and isozyme patterns have also been successfully used to study heterogenity among *Frankia* strains (Benson and Hanna, 1983; Benson *et al.*, 1984; Gardes and Lalonde 1987; Gardes *et al.*, 1987). In a study of *Frankia* isolates from an *Alnus incana* stand, Benson and Hanna (1983) placed 43 nodule isolates into five groups based on one-dimentional SDS-polyacrylamide gel electrophoresis of total cell proteins.

1.5. 16S rRNA sequence analysis.

Ribosomal RNA sequence analysis has been extensively used to study phylogenetic relationships between microorganisms (Woese, 1987), as well as for taxon identification (Woese *et al.*, 1985). A significant achievment of molecular evolution studies has been the recognition of three domains, the Bacteria, the Archaea and the Eucarya, each containing two or more kingdoms (Woese *et al.*, 1990). The primary structure of 16S rRNA contains stretches of sequence conserved to varying degrees and their positions are mostly known. Sequence information from the conserved regions is useful for studying phylogenetic relationships (Woese *et al.*, 1985) as well as for the design of universal oligonucleotide probes and primers used for identification and amplification, respectively (Givannoni, 1991). Variable regions provide sequence data to develop specific probes and primers for detection of microorganisms by hybridizations or with polymerase chain reaction (Stahl and Amann 1991; Ward *et al.*, 1992). Using conserved primers, the 16S rRNA gene can be easily amplified by PCR not only from pure cultures but also directly from the environmental samples (Giovannoni *et al.*, 1990; Olsen *et al.*, 1986; Pace *et al.*, 1986; Ward *et al.*, 1992).

Sequence information of 16S rRNA has not only been obtained from *Frankia* pure cultures, but also directly from the root nodules and is being used to study phylogenetic relationships and for ecological research (Hahn *et al.*, 1989a,b; Hahn *et al.*, 1990a; Mirza

et al., 1992; Nazerat *et al.*, 1991; Nick *et al.*, 1992; Normand *et al.*, 1992). Based on 16S rRNA sequence information, *Frankia*, *Geodermatophilus* and "*Blastococcus*" have been classified in the family *Frankiaceae* (Hahn *et al.*, 1989b). Several atypical, non-infective isolates were confirmed as *Frankia* strains by hybridization with a 16S rRNA-targeted oligonucleotide probe and highly related 16S rRNA sequences (Mirza *et al.*, 1992). Partial 16S rRNA sequence analysis allowed Nazaret *et al.*, (1991) to determine close relatedness of the *Frankia* strains belonging to the *Alnus* infectivity group to those of the *Casuarina* infectivity group. The same study concluded that strains of both groups are distantly related to those included in the *Elaeagnus* infectivity group. Using sequence data of the 16S rRNA gene it has been reported that endophytes of *Coriaria* nodules form a distinct lineage within the genus *Frankia* (Nick *et al.*, 1992).

Based on 16S rRNA sequence analysis, a *Frankia* genus-specific probe and two probes specific for effective and ineffective *Frankia* strains from *Alnus* have been designed (Hahn *et al.*, 1989a; Hahn *et al.*, 1990a). The *Frankia* genus probe reacts with RNA from all *Frankia* strains tested but, also with the RNA of *Actinomadura* and *Microbispora* (Hahn *et al.*, 1990a). Ribosomal RNA of effective *Frankia* strains from different *Alnus* species was found to hybridize strongly with the probe developed against the effective strain, except one strain (Hahn *et al.*, 1989a). This probe does not cross-react with rRNA of ineffective and effective strains from other hosts (*Elaeagnus, Comptonia, Coriaria, Hippophae, Colletia* spp.), the only exception being an isolate from *Casuarina* (Hahn *et al.*, 1989a).

1.6 *Frankia nif* genes.

Despite the widespread occurrence of nitrogen fixation among phylogenetically diverse bacteria, it was found that structural genes from many bacterial sources are very similar (Normand and Bousquet, 1989; Normand *et al.*, 1988; Ruvkun and Ausbel, 1980). Nucleotide sequence comparisons of conserved genes, like structural genes of nitrogenase, provides valuable information to study the evolution of various groups of diazotrophs and their relationships with other nitogen-fixing microorganisms (Hennecke *et al.*, 1985).

In all nitrogen-fixing microorganisms, the nitrogenase enzyme complex consists of two proteins. The structural genes of the nitrogenase complex are referred to as *nifD* and *nifK* for the alpha and beta subunits of the MoFe-protein and *nifH* for the Fe-protein. Ruvkun and Ausubel (1980) reported the hybridization of a cloned fragment of *Klebsiella* DNA

carrying the nitrogenase structural genes to DNA from *Frankia* strain CpI1. In five *Frankia* strains tested, Normand *et al.*, (1988) found that *nifH* was contiguous with *nifK* and *nifD*. However hybridization data from Ligon and Nakas (1987) suggests that the genes for Fe-protein and MoFe-protein are not contiguous in the *Frankia* strain FaCl. Restriction patterns of the *nif* genes have been used to study genetic diversity among *Frankia* strains and also as specific markers in ecological research (Jamann *et al.*, 1992; Nazaret *et al.*, 1989; Simonet *et al.*, 1989).

The amino acid sequence derived from nucleotide sequence analysis indicates that *nifH* is highly conserved. The complete nucleotide sequence of the *nifH* open reading frame from a *Frankia* strain (ArI3) was found to be 861 bp in length (Normand *et al.*, 1988). The *nifH* from a *Frankia* strain isolated from *Hippophae rhamnoides* showed 93% nucleotide sequence similarty and 96% derived amino acid similarty with *nifH* from ArI3 (Normand and Bousquet, 1989). Comparison of *nifH* gene sequences from *Frankia* and other nitrogen-fixing bacteria shows that the *Frankia* sequences are more similar to those from *Anabaena* than to other gram-positive nitrogen-fixing species, which supports the hypothesis that *nif* genes were transmitted horizontally among bacteria rather than vertically from a common ancestor (Normand and Bousquet, 1989; Normand *et al.*, 1988).

2. Atypical *Frankia* strains.

Besides the nitrogen-fixing root nodules, formation of ineffective nodules has also been reported on many actinorhizal plants. In cross-inoculation experiments, formation of ineffective nodules was initially reported by Bond (1967). The occurrence of host-induced ineffectiveness in soil populations of *Frankia* has been observed for strain type AiSp$^+$ Finland, which is effective on *Alnus incana*, but ineffective on *Alnus glutinosa* (van Dijk *et al.*, 1988). A similar phenomenon has also been observed at the intergeneric level for a *Myrica gale* endophyte (Vandenbosch and Torrey, 1983). In a survey on nodulation potential of Scottish soils, occasional development of very small, ineffective nodules was detected (Wheeler *et al.*, 1981). Van Dijk and Sluimer-Stolk (1990) have reported that ineffective strains can occur as major components of soil populations of *Frankia* in natural stands of *Alnus glutinosa*.

Several atypical *Frankia* strains have been isolated from root nodules of different actinorhizal plants (Baker *et al.*, 1980; Hafeez, 1983; Hahn *et al.*, 1988; Lechevalier *et al.*, 1983; Mirza *et al.*, 1991; Mirza *et al.*, 1992). These strains have been classified as members of the genus *Frankia* on the basis of their similar morphology, induction of atypical nodules, identical fatty acid pattern and high sequence homology of 16S rRNA (Baker, 1980; Hahn *et al.*, 1988; Hahn *et al.*, 1990b; Mirza *et al.*, 1991; Mirza *et al.*, 1992). Atypical strains are either non-infective on the host plants, or induce only ineffective nodules. Atypical strains obtained from *Alnus* were found to have beneficial effect on the growth of the host plants when used as inoculum in combination with typical effective *Frankia* strains (Hahn *et al.*, 1990b).

3. Ecological significance of *Coriaria* and *Datisca*.

Coriaria nepalensis and *Datisca cannabina* are the two most abundant and widely distributed actinorhizal plants in the northern hilly areas of Pakistan. It has been suggested that these plants play an important role in erosion control (Akkermans *et al.*, 1985: Chaudhary *et al.*, 1985). The genus *Coriaria* shows a relatively ancient origin, as is apparent from its wide, disjunct distribution through the northern and southern temperate zones (Bond, 1983; Silvester, 1977). The presence of root nodules and their significant contribution to the nitrogen nutrition of various species of the genus *Coriaria* was recognized long ago (Bond, 1962; Bond, 1967; Harris and Morrison, 1958; Kataoka 1930; Shibata and Tahara, 1917; Stevenson, 1958).

Datisca cannabina is a small dioecious herb. Although the occurrence of root nodules and their anatomy have been described during first quarter of this century (Montmartini, 1905; Severini, 1922; Trotter, 1902), *Datisca cannabina* was included in the list of actinorhizal plants relatively recently (Chaudhary 1979), shortly after which the nitrogen-fixing ability and actinomycetous nature of the nodule endophyte was reported (Hafeez *et al.*, 1984 a, b). *D. glomerata,* the only other species of the genus, has also been found to bear actinorhizal nodules in California (Calvert *et al.*, 1979; Chaudhary, 1979; Winship and Chaudhary, 1979).

4. Outline of the thesis.

The main aim of the present research was to characterize uncultured *Frankia* strains from the root nodules of *Coriaria nepalensis* and *Datisca cannabina* by using molecular biological techniques. Research on the actinorhizal plants *Coriaria nepalensis* and *Datisca cannabina* was initiated due to their important role in erosion control in the northern hilly areas of Pakistan. Nitrogen fixing ability of the nodules was confirmed by the acetylene reduction assay (chapter 3). Besides their potential practical applications in soil improvement, both actinorhizal plants offer interesting features for basic microbial ecological research. Nodule structure and morphology of the endophyte within the nodules of these plants is unique among the actinorhizal plants (chapter 2). Moreover, repeated efforts to obtain the endophytes of both the plants in pure culture have failed, indicating that the endohytes are physiologically different from most other *Frankia* strains which were isolated on the commonly used isolation media. Only atypical strains could be isolated that were non-infective and failed to fix nitrogen. These strains were described previously as *Frankia*-like organisms on the basis of similar morphology. To characterize these isolates further, fatty acid profiles were compared with those of confirmed *Frankia* strains and other actinomycetes (chapter 4). The major objectives of the study were to investigate phylogenetic relationships of atypical *Frankia* strains and the endophytes within the root nodules of *Coriaria* and *Datisca*, as well as to develop oligonucleotide probes for detection of the endophytes (chapter 5 & 6). The relationship between the endophytes of *Coriaria* and *Datisca* nodules was also investigated by comparing *nifH* sequences obtained from the nodules by PCR (chapter 6). Nodulation of *Datisca* seedlings by the endophyte of *Coriaria* nodules was confirmed by 16S rRNA sequence analysis (chapter 7). Presence of *Coriaria* and *Datisca* compatible *Frankia* strains in the soils collected from different areas of Pakistan was investigated by nodulation tests. The diversity among *Frankia* strains nodulating *Datisca* was determined by using 16S rRNA targeted oligonucleotides and sequence analysis (chapter 8). The results of the thesis are summarized in chapter 9.

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Chapter 2

Ultrastructure of the endophyte and localization of *nifH* transcripts in root nodules of *Coriaria nepalensis* Wall. by *in situ* hybridization.

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Ultrastructure of the endophyte and localization of *nifH* transcripts in root nodules of *Coriaria nepalensis* Wall. by *in situ* hybridization.

Summary

Studies on the root nodules of *Coriaria nepalensis* Wall. using light and electron microscope revealed that the infected cortical cells are enlarged and form a compact kidney-shaped region with an acentric stele. Within a single cell, the actinomycetous endophyte has branched, septate hyphae and elongate vesicles. Hyphae are penetrating the host cell wall in different directions. Inside the host cell, the hyphae are located in the peripheral region of the cytoplasm near the host cell wall whilst the vesicles are positioned towards the centre of the cell around the central vacuole. No septa formation was observed in the microsymbiont vesicles. Host cell mitochondria were found aggregated near the hyphal/ vesicular junction of the endophyte. *In situ* hybridization studies demonstrated that the *nifH* transcripts are unequally distributed over the infected cortical cells. In infected cells, *nifH* mRNA was located in the region occupied by the elongate vesicles of the endophyte near the host cell central vacuole. The results indicate that the elongate vesicles of the *C. nepalensis* nodule endophyte are functionally identical to the spherical vesicles of the *Alnus* nodule endophyte.
Key words: Nitrogen fixation, *Coriaria nepalensis*, *Frankia*, *In situ* hybridization, *nifH* transcripts.

Introduction

Members of the actinorhizal genus *Coriaria* are found distributed in widely separated parts of the world. Actinorhizal nodules occur in 16 species belonging to this genus (Bond, 1983). Nitrogen fixing ability of the *Coriaria* nodules was demonstrated by growth of plants in nitrogen-free medium, acetylene reduction assay and isotopic tests with N^{15} (Harris & Morrison, 1958; Stevenson, 1958; Bond, 1967; Canizo & Rodriguez-Barrueco, 1978). *C. nepalensis* is a common pioneer plant in the northern hilly region of Pakistan. Nodulation in *C. nepalensis* in its natural habitat was first reported from Pakistan by Chaudhary *et al.*, (1981) while nodules in a specimen of the species growing in a Portuguese botanical garden

were observed by Rodriguez-Barrueco (Bond, 1976).

In pure culture *Frankia* proliferates as filamentous mat consisting of branched septate hyphae and forms spherical terminal vesicles in the medium lacking fixed nitrogen compound. In host plants, shape of *Frankia* vesicles may be spherical, pear-shaped, club-shaped or elongate (Torrey, 1985). The spherical terminal vesicles of *Frankia* formed in N-free medium are specialized structures known to contain the nitrogen fixing enzyme nitrogenase (Meesters, Van Vliet & Akkermans, 1987). In symbiotic stage, a similar localization of the enzyme has been reported in vesicles in root nodules of *Elaeagnus pungens* (Sasakawa, Hiyoshi & Sugiyama, 1988) and *Alnus incana* (Huss-Danell & Bergman, 1990). So far no reports are available on localization of nitrogenase in other types of vesicles. This research has been further complicated by the difficulties in growing the microsymbiont in pure culture (Mirza, Hahn & Akkermans, 1992).

The enzyme responsible for conversion of atmospheric nitrogen to ammonia is present in a number of free-living or symbiotic procaryotes. In all nitrogen fixing bacteria, the nitrogenase enzyme complex consists of two proteins. The nitrogenase Fe protein is encoded by *nifH* which is highly conserved among *nif* genes (Hennecke *et al.*, 1985; Ruvkun and Ausubel, 1980). The complete nucleotide sequence of the *nifH* open reading frame from a *Frankia* strain (ArI3) was found to be 861 bp in length (Normand, Simonet & Bardin, 1988).

This paper details the anatomy of the *C. nepalensis* root nodules with particular attention given to the fine structure of the infected cells and the endophyte using light, scanning- and transmission electron microscopy. Furthermore, the localization of *nifH* transcripts in the elongate vesicles of nodule endophyte is discussed.

Materials and Methods

Light microscopy: Root nodules were collected from the plants growing in a natural habitat at Murree, Pakistan. The nodules, fixed in FAA (90 ml of 70 % ethanol, 5 ml glacial acetic acid and 5 ml formalin), were dehydrated in series of ethanol (80 %, 90 %, 100 %) and embedded in paraffin wax. Longitudinal and transverse sections, 6-10 μm thick were cut with a microtome (Cambridge, Shandon). The Saffranin-Fast Green staining procedure (Sass, 1958) was used to stain the cortical and xylem cells differentially in order to study their relative arrangement. The endophyte in the nodules was stained, using the Brown & Brenn

(1931) staining procedure.

Transmission electron microscopy (TEM): Root nodules were collected from greenhouse grown *C. nepalensis* plants and washed with water to remove adhering soil particals. Excised nodules were cut into small pieces and fixed in glutaraldehyde (2 %) in 0.05 M potassium phosphate buffer (pH 6.8) at 27°C and 4°C for four hours each. These fixed specimens were then washed with phosphate buffer overnight and further fixed in buffered OsO_4 solution (1 %) for one hour. After washing for twenty minutes in phosphate buffer, the fixed samples were then passed through a dehydration series of ethanol in distilled water (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 % ethanol, respectively) twice for 10 minutes each, followed by propylene oxide for 25 minutes. The samples embedded in epon mixture (1:1 mixture of propylene oxide: epon, for one hour: subsequently for 24 hours in a 1:4 mixture and finally in 100 % epon for four days). Following this the resin capsules were polymerized for 24 hours at 35°C, 48°C and 60°C.

The blocks were sectioned at 500 Å on an ultramicrotome and stained with uranyl acetate and lead citrate. The stained sections were examined with an electron microscope (Philips EM 200)

Scanning electron microscopy (SEM): Washed nodules were cut into pieces and fixed for four hours in glutaraldehyde (2 %) in cacodylate buffer (0.25 M, pH 6.8). After three washings with cacodylate buffer, the material was fixed for two hours in OsO_4 (1 %) in the same buffer and subsequently dehydrated in different mixtures of acetone and water (10, 20, 35, 50, 65, 80, 90, 100 %, respectively), 15 minutes per step. The acetone saturated nodule pieces were cut into small pieces (2-4 mm) in liquid nitrogen. After critical point drying (Baltzers Union Critical Point Dryer) the nodule pieces were fixed on a holder and sputtered with gold. Nodule sections were observed under scanning electron microscope (Jeol JSM 35C).

***In situ* hybridization:** The ~380 bp *XhoI*/*BglII* fragment of pFQ148 (Simonet, Normand & Bardin, 1988) containing part of the coding region of *Frankia* ArI3 *nifH* was subcloned in pBluescript KS^+, and antisense RNA was transcribed using T7 polymerase (BRL) and ^{35}S-UTP (Amersham, UK). The probes were partially degraded to an average length of 150 nucleotides by heating for 90 min. at 60°C in a buffer containing 0.2 M Na_2CO_3 and 0.2 M $NaHCO_3$.

Washed *C. nepalensis* nodules were fixed for four hours at room temperature in 4 %

paraformaldehyde and 0.25 % glutaraldehyde in 100 mM phosphate buffer pH 7.2. Dehydration was performed in graded ethanol and xylene series, and samples were embedded in para clean (Klinipath, The Netherlands). Seven μm thick sections were attached to poly-L-lysine-coated slides, deparaffinized with xylene and rehydrated through a graded ethanol series.

Hybridization pretreatment, hybridization and washing conditions were essentially as described by Cox and Goldberg (1988) and adapted by van de Wiel *et al.* (1990). Slides were coated with microautoradiography emulsion LM-1 (Amersham, UK) and exposed for 4 weeks at 4°C. They were developed in Kodak D19 developer for 5 min. and fixed in Kodak fix. Sections were stained with 0.025 % toluidine blue O for 5 min. and mounted with DPX.

Results

Light microscopy: Light microscopic studies of *C. nepalensis* root nodule sections demonstrated an organization similar to that of other actinomycete-induced root nodules. Each nodule lobe consisted of an outer multilayered epidermis, a thick cylinder of cortex within which a single layer of endodermis surrounded the vascular cylinder or stele. In transverse sections the vasular cylinder was present on one side of the compact kidney-shaped region formed by the infected cortical cells, resulting in an asymmetric structure (Fig. 1). Non-infected cells were absent in the infected zone of the cortex. A 2-3 cell layers thick region of cortex separated the infected region from the endodermis. These infection free cells in the cortex contained large starch grains. The infected cells had a larger diameter than non-infected cells. In the infected cells, the endophyte occupied the outer region of the cytoplasm, leaving the centre of the host cell infection free.

Transmission- and scanning electron microscopy: Transmission and scanning electron microscopic observations of *C. nepalensis* root nodule sections showed that a large portion of the infected cells was occupied by the hyphae and vesicles of the endophyte (Fig. 2, 3). Only the central part of the host cell remained infection free. No endophyte spores or sporangia were observed in the nodules. The branched hyphae of the endophyte, ranging in diameter from 0.5-0.8 μm, were present near the host cell wall (Fig. 3, 4). The outer boundary of the endophyte cells consisted of a plasma membrane and cell wall. Adjacent to these structures were the capsule and host plasma membrane; thus the endophyte cytoplasm

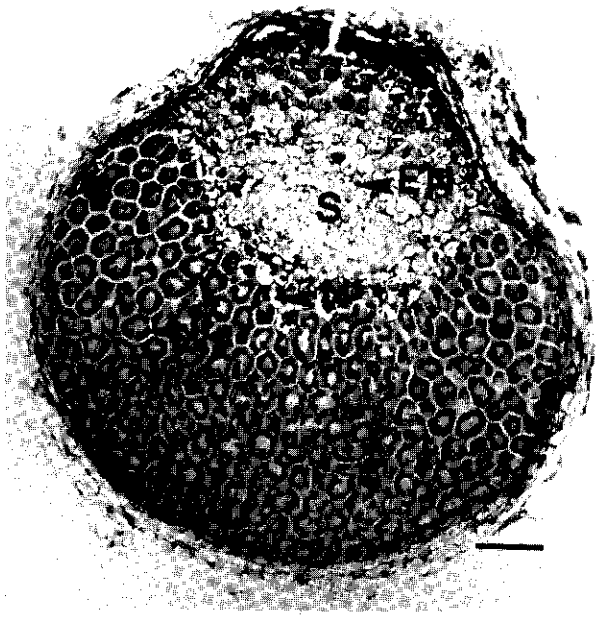

Figure 1. Light micrograph of cross section of *Coriaria nepalensis* root nodules showing kidney-shaped region formed by the infected cells (IC). Acentric stele (S); non-infected cortical cells (N); epidermis (P); endodermis (EN). Bar= 60μm

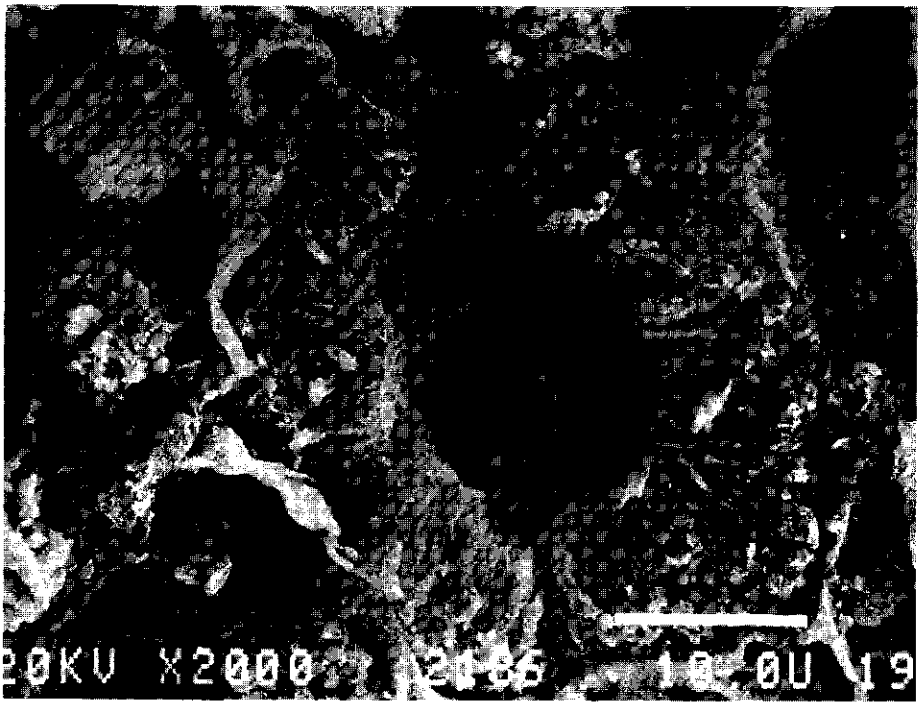

Figure 2. SEM of an infected cortical cell. A large part of the host cell is occupied by hyphae (H) and vesicles (V) of the endophyte. Host cell wall (W).

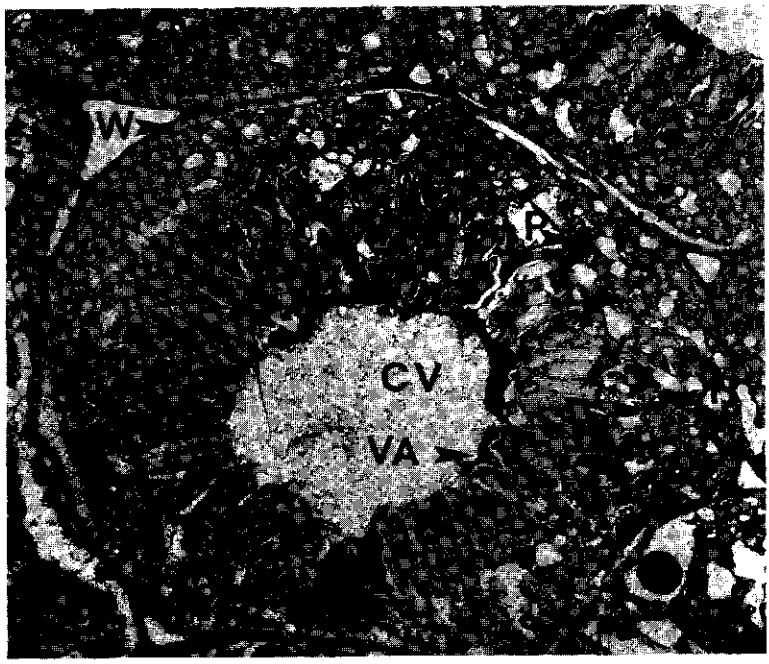

Figure 3. TEM of an infected cortical cell with an outer most region of ramified hyphae (H) and filamentous vesicles (V) oriented towards the large central vacuole (CV). Host cell wall (W); mitochondria (M); small vacuoles (VA); plastids (P). Bar= 5μm

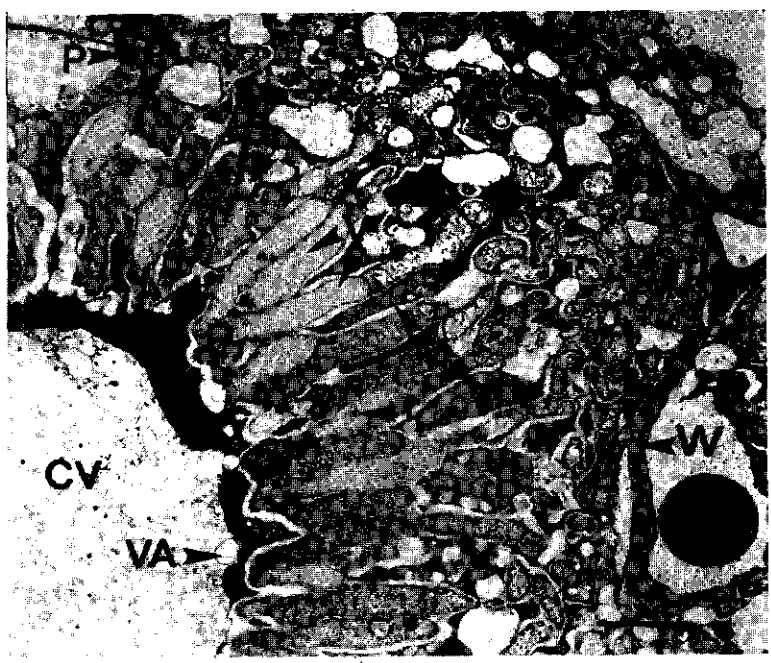

Figure 4. TEM of an infected cortical cell with an outer most region of ramified hyphae (H) and non-septate filamentous vesicles (V) oriented towards the large central vacuole (CV). Host cell wall (W); mitochondria (M); plastids (P); small vacuoles (VA); electron dense inclusions (I). Bar= 2μm

was always separated from the host cytoplasm by four layers: the endophyte plasma membrane, cell wall, capsule as well as the host plasma membrane (Fig. 5,6).

Elongate vesicles were formed at the end of the hyphae near the host cell central vacuole. The vesicles were arranged parallel to each other and attained more compact and organised arrangement (Fig. 3, 4). The elongate vesicles ranged in diameter from 0.6-0.8 μm, while vesicle length varied between 4-8 μm. The vesicles were separated from the hyphae by septa (Fig. 5). The septa were connected with the endophyte cell walls and the entire plasmalemma was visible on each side of the cross wall. Neither compartmentalization nor septa formation was observed within the vesicles. Cytoplasm of the vesicles was more homogenous as compared to that of the hyphae. However, sometimes unidentified inclusions were observed in the vesicles (Fig. 4). The vesicles, like the hyphae, were surrounded by a capsule and a plasma membrane envelope. The capsule of vesicles was continuous with those of the parental hyphae. No void area or space between the capsule and the endophyte cell wall was observed.

The endophyte infection from one cell to another was by penetration of hyphae through host cell walls. Usually many penetrating hyphae were found in a single section of the host cell wall. Some of the penetrating hyphae were empty and contained only a few globules near the endophyte cell wall, others were living and completely filled with the cytoplasm (Fig. 6). The penetrating hyphae of the endophyte were surrounded by the capsule which was continuous with the host cell wall. Near the basal end of the vesicles, the space was completely packed by the host mitochondria containing numerous cristae (Fig. 3, 4, 5). Mitochondria were only rarely found elsewhere between the peripheral hyphae and the vesicles. In addition to uninfected cortex cells completely packed with starch grains found near the endodermis, large plastids were also observed in the infected cells (Fig. 3,4).

Localization of *nifH*: Sections of the *C. nepalensis* nodules were hybridized to antisense RNA transcribed from partial *nifH* insert cloned from *Frankia* (ArI3). Transverse nodule sections showed a hybridization pattern similar to that of infected cells in the cortex. Thus *nifH* transcripts were restricted only to one part of the cortex in transverse sections where *Frankia* cells are localized (Fig. 7). In the infected cells, *nifH* was expressed in the region of the host cell occupied by the endophyte vesicles (Fig. 8). No background signals were observed in host cell central vacuole and peripheral parts of the host cell where hyphae of the endophyte are present.

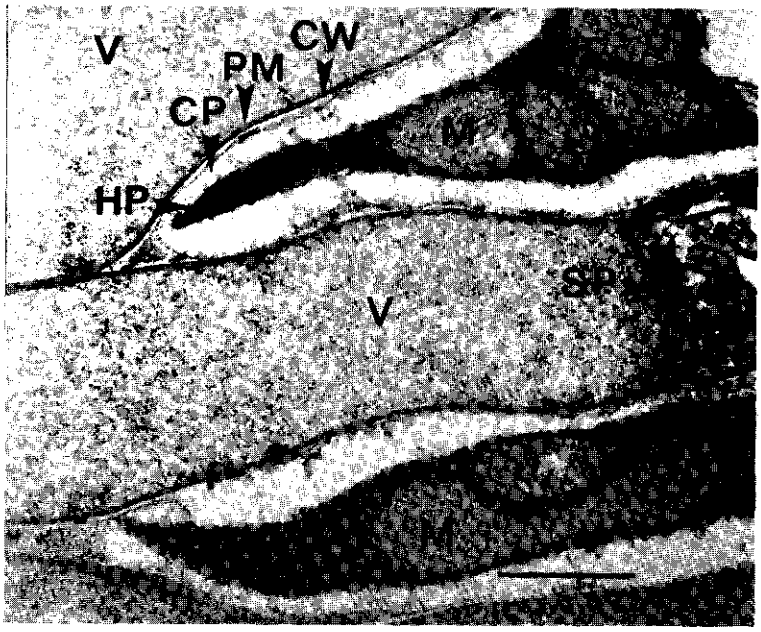

Figure 5. TEM of an infected cell. The cytoplasm of the endophyte hyphae (H) and vesicles (V) is separated from the host cytoplasm containing mitochondria (M) by four layers; the endophyte plasma membrane (PM) and cell wall (CW), the capsule (CP) and the host plasma membrane (HP). Septa (SP) are formed between the parental hyphae and the vesicles. Bar= 0.34μm

Figure 6. TEM of an infected cortical cell showing a detail of the penetrating site of the host cell wall (W) by endophyte hyphae (H). The penetrating hyphae are surrounded by the capsule (CP) which is continuous with the host cell wall. Bar= 0.36μm

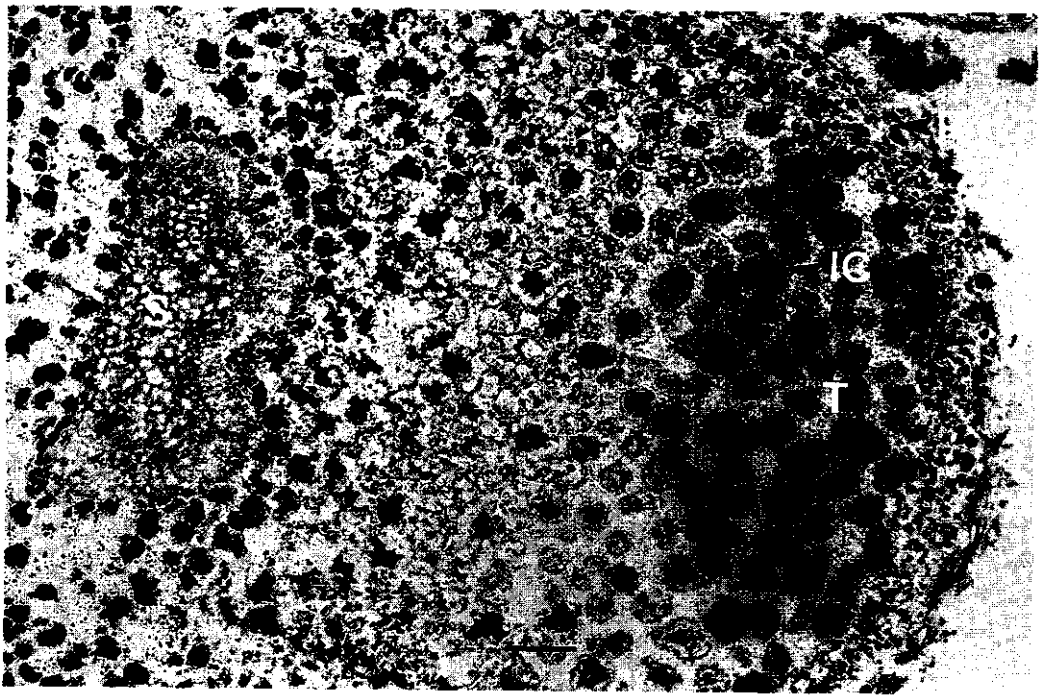

Figure 7. Transverse section of the nodule lobe showing the *nifH* transcripts (black silver grains) in the infected region of the cortex. Infected cortical cells (IC); *nifH* transcripts (T); stele (S); epidermis (P). Bar= 50μm

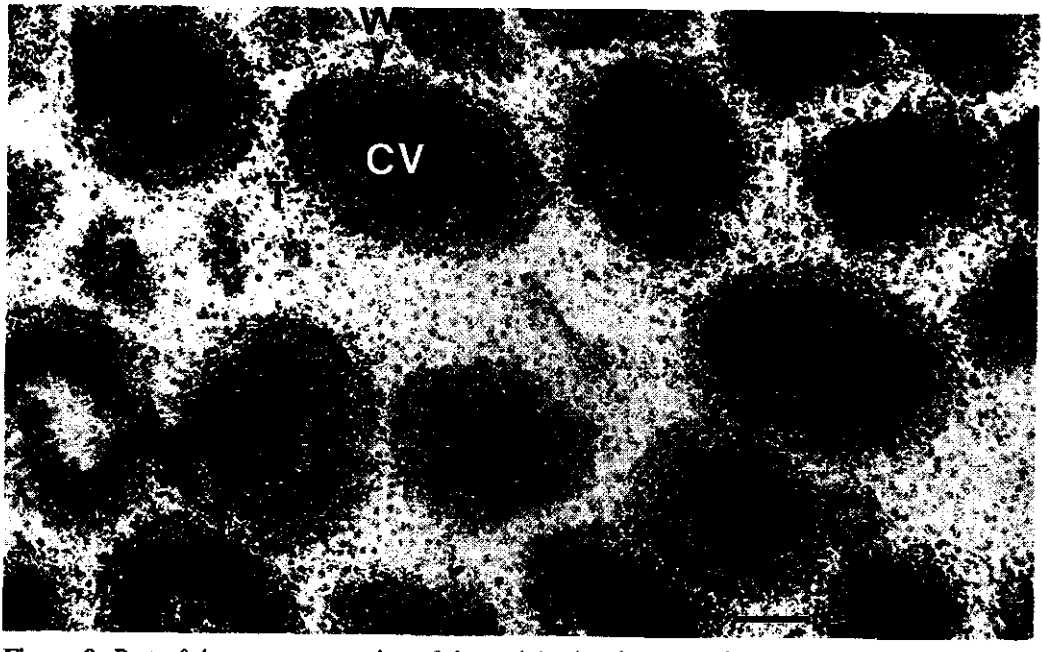

Figure 8. Part of the tranverse section of the nodule showing the *nifH* transcripts (T; black silver grains) near the central vacuole (CV) of the host cells where endophyte vesicles are located. Host cell wall (W). Bar= 10μm

Discussion

In transverse sections, the nodules of *C. nepalensis* show a kidney-shaped infected cortical region around an acentric stele. Such an asymmetric nodule structure is found only in *Datisca* (Hafeez, Akkermans & Chaudhary, 1984a) and *Coriaria* (Canizo & Rodriguez-Barrueco, 1978; Newcomb & Pankhurst, 1982a; Silvester & Harris, 1989). In the nodules of most other actinorhizal plants, the infected cells are uniformally distributed in the enlarged cortex, interspaced by non-infected cells and the stele is central (Becking, 1968; Lalonde & Knowles, 1975; Newcomb *et al.*, 1978; Hafeez, Chaudhary & Akkermans, 1984b).

The endophyte of *C. nepalensis*, like that of *Datisca cannabina* (Hafeez *et al.*, 1984a) and *Coriaria arborea* (Newcomb & Pankhurst, 1982a) does not occupy the centre of the host cell which remains free of endophyte tissue. In actinorhizal genera other than *Datisca* and *Coriaria*, the endophyte forms a compact tangled mass that occupies the centre of the infected cells (Gatner & Gardner, 1970; Lalonde & Knowles, 1975; Lalonde & Quispel, 1977; Hafeez *et al.*, 1984b).

The present study has added to the earlier evidence (Newcomb & Pankhurst, 1982a) that the endophyte of *C. nepalensis* nodules is an actinomycete. The actinomycetous nature of the endophyte is reflected by the size of the hyphae and the lack of a nuclear envelope. Thus this organism is similar to the actinomycete *Frankia* which induces root nodules of several non-legumes (Lalonde & Quispel, 1977; Newcomb, 1981; Newcomb & Pankhurst, 1982a, 1982b; Newcomb & Heisey, 1984; Hafeez *et al.*, 1984a, 1984b; Miller & Baker, 1985). Endophyte spores or sporangia were not detected in *C. nepalensis* nodules. These structures have been found in some other actinorhizal plants e.g. *Alnus* nodules (van Dijk & Merkus, 1976).

No rupturing of the host cell wall was observed at the point of entry of penetrating hyphae of the endophyte into the host cell. This indicates that the entry of the endophyte into the host cell is by enzymatic degradation rather than the physical breakdown of the host cell wall. Continuity of the endophytic capsule with the host cell wall also reflects a similar origin and chemical composition of both structures (Lalonde & Knowles, 1975; Lalonde, 1979). Encapsulation of the endophyte in infected cells is assumed to be a host cell defence reaction (Lalonde & Knowles, 1975). A capsule was not detected in *Frankia* in pure culture (Lalonde, 1979).

Three characteristics of the endophyte vesicles in *Coriaria* and *Datisca* i.e. filamentous

shape, absence of septa and the orientation of the vesicles are not found together in the nodules of any other actinorhizal species. The elongate to club-shaped nature of the vesicles in *Coriaria* and *Datisca* is also found in *Comptonia* (Newcomb *et al.*, 1978) and *Myrica* (Henry, 1977). However, in this case the vesicles are oriented towards the periphery like the spherical vesicles of *Alnus* (Lalonde & Knowles, 1975; Hafeez *et al.*, 1984b) *Dryas* (Newcomb, 1981), *Discaria* (Newcomb & Pankhurst, 1982b), *Hippophae* (Gatner & Gardner, 1970) and *Elaeagnus* (Baker, Newcomb & Torrey, 1980; Miller & Baker, 1985). Unlike compartmentalized endophyte vesicles of several other actinorhizal genera (Gatner & Gardner, 1970; Lalonde & Knowles, 1975; Newcomb & Pankhurst, 1982b; Hafeez *et al.*, 1984b; Miller & Baker, 1985), no septa formation occurs in vesicles of *Coriaria* and *Datisca*. The orientation of the vesicles towards cell centre in *Coriaria* and *Datisca* may be an adaptation to oxygen concentration or to some other requirement from the central vacuole for optimum activity of the nitrogenase enzyme.

In the infected cells, host mitochondria are embedded in between the closely packed hyphae near the base of the vesicles. The host cell mitochondria may be involved in keeping oxygen tension low near the O_2 sensitive nitrogenase known to be present in vesicles (Meesters, 1987; Meesters *et al.*, 1987; Huss-Danell & Bergman, 1990). Aggregation of mitochondria near the vesicles also indicates their involvement in providing energy rich compounds (Akkermans *et al.*,1983)

Unlike spherical vesicles found in *Alnus* nodules (Hafeez *et al.*, 1984b), the vesicles in *Coriaria* nodules are morphologically least differentiated. In *Alnus* nodules, the endophyte vesicles are compartmentalized and the cell walls are thicker than those of hyphae (Silvester, Silvester & Torrey, 1988). The so-called "vesicles" in *C. nepalensis* nodules are non-compartmentalized and the cell walls of both hyphae and vesicles are of the same thickness. Only the peculiar arrangement of the endophyte cells near the central vacuole of the host cell suggests that these cells might be the endophyte vesicles. Presence of the nitrogen fixing enzyme (nitrogenase) in the symbiotic stage in the nodules has been confirmed in spherical vesicles of *Alnus* (Huss-Danell & Bergman, 1990) and *Elaeagnus* (Sasakawa *et al.*, 1988) by immunological techniques. It is evident from the results of *in situ* hybridization studies that *nifH* is strongly expressed in the region of the host cell where "vesicles" are located. These results indicate that filamentous vesicles are functionally identical to spherical vesicles. The opposite orientation of the vesicles in the cell also suggest that the transport system of fixed

nitrogen is probably different than that of *Alnus* nodules. This aspect needs further attention.

The present investigations revealed that *C. nepalensis* nodules show striking resemblance to that of *Datisca* nodules indicating that the endophytes of both (taxonomically unrelated) plant species are related. Recent studies on the phylogeny of microsymbionts of *C. nepalensis* (Mirza *et al.*, 1992) and *Datisca cannabina* (Mirza *et al.*, submitted) showed that both microbes have *Frankia* specific sequences in 16S rRNA suggesting that the symbionts can be classified as *Frankia*.

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Chapter 3

Seasonal fluctuations in nitrogen-fixing ability (C_2H_2 reduction) and hydrogen uptake by root nodules of *Coriaria nepalensis* and *Datisca cannabina*.

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Seasonal fluctuations in nitrogen-fixing ability (C_2H_2 reduction) and hydrogen uptake by root nodules of *Coriaria nepalensis* and *Datisca cannabina*.

Summary

A study was made on the seasonal fluctuations in N_2-fixing (C_2H_2 reduction) activity and uptake hydrogenase activity of the root nodules of *Coriaria nepalensis* and *Datisca cannabina*. Nitrogenase activity of the nodules of both plants showed biphasic curves with peaks in spring and late summer. Acetylene reduction by the root nodules of *Coriaria nepalensis* was highest (16.4 μmol. $C_2H_4.g^{-1}$ fresh nodule wt. h^{-1}) during July while the peak acetylene reduction activity (14.8 μmol. C_2H_4. g^{-1} fresh nodule wt. h^{-1}) of *Datisca cannabina* nodules was determined in April. Less than 15% of the enzyme activity was retained by the nodules in winter. The nodules of both plant species consumed hydrogen on incubation with a gas mixture containing H_2, indicating an uptake hydrogenase activity. The H_2 uptake by the excised nodules of both *Coriaria* and *Datisca* was highest (2.16 and 1.95 μmol. H_2 consumed g^{-1} fresh nodule wt. h^{-1}, respectively) in May. The presence of an uptake hydrogenase was confirmed in nodule homogenates with phenazine metasulphate as artificial electron acceptor. Purified vesicle cluster suspensions (20 μm residue) showed highest hydrogenase activity, indicating that the enzyme is associated with the endophyte.

Introduction

Vegetation cover from the hills in the northern part of Pakistan is disappearing rapidly due to extensive cuttings, overgrazing and soil erosion. Nitrogen fixing plants nodulated by an actinomycete (*Frankia*) may be used effectively for erosion control and reforestation of such sites (Akkermans *et al.*, 1985; Chaudhary *et al.*, 1985). Out of six genera of actinorhizal plants reported from Pakistan (Chaudhary *et al.*, 1981), species of five genera (*Alnus, Coriaria, Datisca, Elaeagnus, Hippophae*) grow naturally in the northern hilly area. Actinorhizal shrubs *Coriaria nepalensis* and *Datisca cannabina* have great potential for utilization as fast growing pioneers in the areas effected by landslides and erosion.

Like other N_2-fixing microorganisms, members of the actinorhizal genus *Frankia* can reduce atmospheric nitrogen with the help of the nitrogenase system. During reduction of N_2 by microorganisms, considerable amount of energy is lost in the simultaneous process of reduction of protons to H_2 by the nitrogenase (Arp, 1990; Evans *et al.*, 1987). Several nitrogen fixers posses an uptake hydrogenase which recycles this H_2, and regenerates energy and reducing power for reutilization by nitrogenase (Emerich *et al.*, 1979; Evans *et al.*, 1987; Schubert *et al.*, 1978). It has been suggested that this reaction also provides a means of protection of nitrogenase from oxygen (Dixon, 1972: Emerich *et al.*, 1979).

Uptake hydrogenase activity has been reported in the *Frankia* induced nitrogen fixing root nodules of non-legumes (Benson *et al.*, 1980; Hafeez *et al.*, 1984b; Huss-Danell, 1990; Mirza *et al.*, 1987; Roelofsen and Akkermans, 1979; Sellstedt, 1989; Sellstedt *et al.*, 1986). In pure culture, the actinomycete *Frankia* proliferates as a filamentous mat consisting of branched septate hyphae and forms spherical terminal vesicles in the medium lacking combined nitrogen. In symbiotic state, the endophyte *Frankia* inhabits cortical cells of the actinorhizal nodules in the form of "vesicle clusters". The symbiotic vesicle clusters are composed of multicellular hyphae and filamentous or spherical vesicles. The endophyte vesicle clusters of *Coriaria* and *Datisca* are morphologically different from those found in other actinorhizal nodules (Hafeez *et al.*, 1984a; Mirza *et al.*, subm.). The clusters can be easily isolated from the host cells by filtration techniques as described originally by Akkermans and coworkers (Akkermans, 1971; Akkermans *et al.*, 1981,1983; Akkermans and Roelofsen, 1979) and further improved by Vikman and Huss-Danell (1987). Vesicle cluster suspensions have been successfully used to study various physiological processes such as nitrogenase activity, uptake hydrogenase activity and other metabolic processes of the endophyte (Akkermans *et al.*, 1979; Benson *et al.*, 1979; Benson *et al.*, 1980; Hafeez *et al.*, 1984b; Roelofsen and Akkermans, 1979; Vikman, 1992). Uptake hydrogenase has been localized by immunogold-labelling in both the symbiotic and the free living *Frankia* in hyphae as well as in vesicles (Sellstedt and Lindblad, 1990)

In this chapter, seasonal variations in nitrogen fixing ability (C_2H_2 reduction) and hydrogen uptake by the root nodules of *Coriaria nepalensis* and *Datisca cannabina* is discussed. Furthermore, hydrogen uptake by different fractions of the nodule homogenates using artificial electron acceptors is described.

Materials and methods

Plant growth: To avoid extensive travelling for collection of fresh nodules from the plants growing in their natural habitats, seedlings of *Coriaria nepalensis* and *Datisca cannabina* were raised in the nursery of Quaid-e-Azam University Islamabad, Pakistan. Temperatures at Islamabad are considerably higher as compared to the natural habitats of *Coriaria* and *Datisca*. This is especially true for Swat where *Datisca* plants remain covered with snow for a considerable period of time during winter. *Coriaria* nodules used for inoculum were collected from Murree while *Datisca* nodules were collected from Swat. The seedlings were grown in sterile sand and inoculated at the age of 8 weeks with crushed nodule suspension. N-free Hoagland nutrient solution (half strength) was added once a week. Nodules were collected from the plants for one year (March 1986-Feb. 1987) and used for measuring acetylene reduction activity and uptake hydrogenase activity.

Acetylene reduction: Nitrogenase activity was measured by incubating nodulated roots in air-tight plastic bottles (500 ml) with air and acetylene (10 %) at ambient air temperature. One ml gas samples from these plastic bottles were transferred after every 15 minutes to vacutainer tubes (5.6 ml). The gas samples were stored in these tubes and analyzed for C_2H_4 production with FID system of gas chromatgraph (Hitachi Model 163). Carrier gas (N_2) was 40 ml. min^{-1}. Porapak R, packed loop stele column, 1.5 m long and 3 mm in ID was used for separation.

H_2-uptake by excised root nodules: The nodules were washed with water, dried on a filter paper and one gram nodules incubated at ambient temperature in air tight bottles (7.5 ml) with a gas mixture containing 1 % H_2. Control (a) contained only 1 % H_2 in air, while (b) contained only nodules and air for the detection of any H_2 evolution from the nodules. After preincubation for 10 minutes, the gas samples were taken at different time intervals to determine hydrogen uptake by the nodules using gas chromatograph (Hitachi Model 163), equipped with one m long molecular sieve column and a thermal conductivity detector.

Preparation of nodule homogenate for H_2 uptake: The nodule homogenates, from green house collected material, were prepared as described by Roelofsen and Akkermans (1979). Six g washed nodule lobes were homogenized anaerobically in N_2 atmosphere in 50 ml buffer with virtis mixer (45-HI speed) for 3 minutes at 3000 rpm. The homogenization buffer (50

mM, P-buffer, pH 7) contained sucrose (0.3 M), soluble polyvinyl pyrolidone (4 %), sodium dithionite (10 mM) and dithioerythritol (10 mM). Under continuous flow of nitrogen the homogenate was passed through 100 μm and 20 μm filters respectively to separate the vesicle clusters of the endophyte. The 20 μm residue was washed with phosphate buffer (50 mM, pH 7; dithioerythritol and sodium dithionite free) and suspended in six ml of the buffer (vesicle cluster suspension). The 20 μm filtrate was centrifuged for 10 minutes at 12,000 xg. The pellet was resuspended in six ml of phosphate buffer (20 μm filtrate suspension) and the supernatant was also stored for the assay.

Fractions of vesicle cluster suspension in 1.8 ml quantities were injected in 13.5 ml vials with 0.2 ml solution of 10 mM phenazine metasulfate (PMS) used as electron acceptor. The gas phase was nitrogen with 1 % H_2. Hydrogen uptake was measured on TCD system of gas chromatgraph (Hitachi model 163) after preincubation for 15 minutes.

Results

Root nodules of *Coriaria* and *Datisca* collected from the plants growing under similar conditions showed nitrogenase activities of the same order of magnitude. Acetylene reduction activity recorded during different seasons varied considerably and showed peak enzyme activity twice a year (Fig. 1). The activity was low during winter and increased progressively during spring. The acetylene reduction activity decreased during hottest months of May and June and recovered again during July after moonsoon rains. The highest acetylene reduction activity (16.4 μmol. C_2H_4. g^{-1} fresh nodule wt. h^{-1}) by *Coriaria* nodules was determined in July. In *Datisca* root

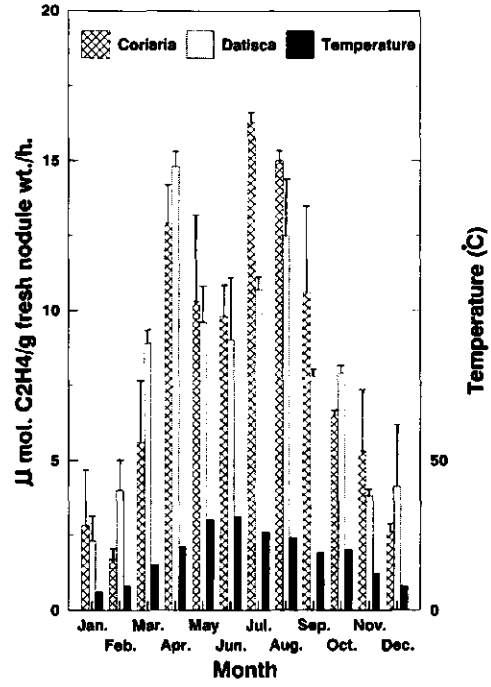

Figure 1: Seasonal fluctuations in nitrogen-fixing ability (C_2H_2 reduction) by root nodules of *Coriaria nepalensis* and *Datisca cannabina*.

nodules, peak acetylene reduction activity (14.8 μmol. C_2H_4. g^{-1} fresh nodule wt. h^{-1}) was recorded in April.

Root nodules of *Coriaria* and *Datisca* did not evolve any detectable amount of hydrogen throughout the year. However, the nodules consumed hydrogen on incubation with a gas mixture (Fig. 2). For both *Coriaria* and *Datisca* nodules, the highest H_2 uptake activity (2.16 and 1.95 μmol. H_2 consumed. g^{-1} fresh nodule wt. h^{-1}, respectively) was determined in May. The uptake hydrogenase activity was detected in both fractions of the nodule homogenates. The activity was higher in 20 μm residual fraction than 20 μm filtrate (table 1). The activity was absent in the supernatant obtained by centrifugation of

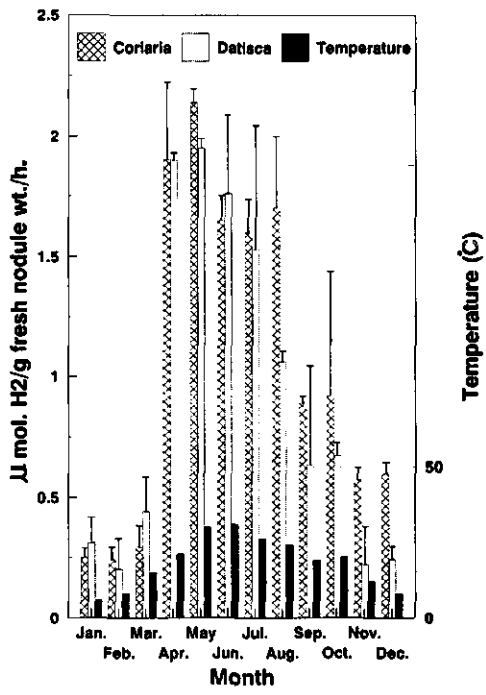

Figure 2: Seasonal fluctuations in uptake hydrogenase activity by root nodules of *Coriaria nepalensis* and *Datisca cannabina*.

Table 1: H_2 uptake by intact nodules and nodule homogenates of *Coriaria nepalensis* and *Datisca cannabina* .

Nodule material used	*Coriaria nepalensis* *μmol. H_2 consumed. g^{-1} fresh nodule wt.h^{-1}.	*Datisca cannabina* *μmol. H_2 consumed. g^{-1} fresh nodule wt.h^{-1}.
Intact nodules	1.9	1.6
Nodule homogenate	1.3	0.7
20 μm residue (vesicle cluster suspension)	1.0	0.5
20 μm filtrate pellet	0.3	0.2
20 μm filtrate supernatant	0	0

* Each value represents average of three determinations

20μm filtrate fraction. Intact nodules of *Coriaria* and *Datisca* used for preparation of homogenates consumed H_2 at the rate of 1.9 and 1.6 μmol. H_2 consumed. g^{-1} fresh nodule wt. h^{-1}, respectively. The activity of uptake hydrogenase measured in case of nodule homogenate (20 μm residue + filtrate) of *Coriaria* and *Datisca* in the presence of phenazine metasulfate were 1.3 and 0.7 μ mol. H_2. g^{-1} fresh nodule wt. h^{-1}., respectively.

Under the microscope, the 20 μm residue mainly consisted of vesicle clusters of the endophyte. The 20 μm filtrate contained only broken parts of host cells and of the endophyte.

Discussion

The rate of acetylene reduction by nodules of *Coriaria nepalensis* and *Datisca cannabina* are comparable to those already reported for actinorhizal plants of Pakistan (Chaudhary *et al.*, 1985; Hafeez *et al.*, 1984b). During winter the plants survive in leafless dormant state and the enzyme activity is low due to low temperatures and limited supply of photosynthates required for the energy intensive process of nitrogen fixation. With the emergence of leaves in early spring, the activity increased and reached its peak in April. After relatively low enzyme activity in hottest months of summer when temperatures are considerably higher than those of the natural habitats, the activity recovered again during rainy season (July-August). This again is correlated with the vigorous vegetative growth of plants under the given growing conditions. In an early study using nodules of *Alnus glutinosa* and *Alnus rubra*, it was observed that the period of maximum nitrogenase activity coincides with that of maximum growth (Wheeler *et al.*, 1981).

Like several other actinorhizal nodules (Benson *et al.*,1980; Hafeez *et al.*, 1984; Roelofsen and Akkermans, 1979) an uptake hydrogenase is also present in nodules of *Coriaria nepalensis* and *Datisca cannabina*. This indicates presence of a more efficient nitrogen fixing system as has been reported for *Rhizobium*-nodulated plants (Arp, 1990; Evans *et al*,1981). The nodules always consumed H_2 from the gas phase while no net H_2 evolution was detected during any season. Using nodules collected from natural habitat, H_2 consumption by *Coriaria* nodules was detected throughout the year (Mirza *et al.*, 1987). It has been reported previously (Hafeez *et al.*,1984b) that *Datisca* nodules evolve H_2 in summer. However, this H_2 evolution was suggested to be due to the deterioration of the nodule material used for the

assay during transportation from a long distance. The results of the present study using fresh nodules of *Coriaria* and *Datisca* nodules differ from that of *Alnus* where H_2 evolution have been detected during autumn (Roelofsen and Akkermans, 1979). High uptake hydrogenase activity of both *Coriaria* and *Datisca* nodules was detected during early summer when conditions for vegetative growth of the host plant are favourable or possibly due to stimulation by the increase in H_2 production by nitrogenase.

Hydrogenase activity is present in vesicle cluster suspension as well as filtrate fractions of the nodule homogenates. High enzyme activity in 20 μm residue is obviously due to the presence of intact vesicle clusters of the endophyte. The activity in the filtrate is also particle bound as exhibited in the fraction while activity was absent in the supernatant. The fact that uptake hydrogenase activity of the nodule homogenate (20μm residue + filtrate) is less than that of the intact nodules may be due to partial inactivation of the enzyme or alternately all of the vesicle clusters may not be released from the host cells during homogenization. The results obtained with *Datisca* nodule homogenates in the present study are comparable to those reported previously (Hafeez *et al.*, 1984b).

The results with nodule homogenates of *Coriaria* and *Datisca* indicate that the uptake hydrogenase is associated with the endophyte. Further localization of the enzyme into hyphae or vesicles of the endophyte in the nodules of *Coriaria* and *Datisca* will be difficult. In *Coriaria* and *Datisca* nodules, the filamentous vesicles are morphologically least differentiated (Hafeez *et al.*, 1984; Mirza *et al.*, submitted). In most other nodules e.g. *Alnus*, the spherical nodules are attached to the parental hyphae by delicate stalks and separation of vesicles from symbiotic vesicle clusters is easier. Recently symbiotic vesicles have been isolated from *Alnus* nodules by isopycnic centrifugation of disrupted vesicle clusters and successfully used to study activities of various respiratory enzymes (Vikman, 1992). Use of modern techniques e.g. *in situ* hybridization and isolation of the endophyte will greatly facilitate localization as well as molecular characterization of the uptake hydrogenase.

Present studies with *Coriaria* and *Datisca* indicate that maximum nitrogenase activity of the nodules coincides with the period of vigorous plant growth. This suggests that during fast growth period increased nitrogenous demands of the host plant are met with the elevated nitrogen fixation rates by the endophyte.

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Chapter 4

Identification of atypical *Frankia* strains by fatty acid analysis.

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Identification of atypical *Frankia* strains by fatty acid analysis.

Key words: Actinorhizal plants, *Coriaria*, Actinomycetes, *Frankia*, *Geodermatophilus*, Fatty acid pattern.

Summary

Ineffective, non-infective actinomycetous isolates obtained from actinorhizal nodules of *Coriaria nepalensis* and *Datisca cannabina* were identified as *Frankia* using whole cell fatty acid analysis. The isolates exhibited fatty acid patterns very similar to those of confirmed *Frankia* strains from other host plants (*Alnus*, *Casuarina*, *Colletia*, *Comptonia*, *Elaeagnus* and *Hippophae)*. All *Frankia* strains, including *Coriaria* and *Datisca* isolates, showed fatty acid profiles very distinct from those of other actinomycetes used as controls (*Actinomyces*, *Geodermatophilus*, *Nocardia*, *Mycobacterium* and *Streptomyces*). For the genus *Frankia*, a characteristic pattern of five fatty acids (15:0; 15:1; 16:0 iso; 17:0 and 17:1) was found. These fatty acids comprised 75% or more of the total content. All *Frankia* strains could be placed into three subgroups. *Coriaria* isolates were found in the largest subgroup which contained most *Frankia* strains from other hosts while ineffective strains from *Alnus*, *Elaeagnus* and *Datisca* were distributed in all 3 subgroups of *Frankia*.

Introduction

During the last decade hundreds of *Frankia* isolates have been obtained from actinorhizal nodules using different isolation techniques [1-4]. All isolates obtained from nodules were assigned to the genus *Frankia* on the basis of (i) morphological features, such as sporangium 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 [5-7].

Recently, many isolates lacking some of these morphological and physiological characteristics of typical *Frankia* have been obtained from actinorhizal nodules. These isolates were not able to fix nitrogen, did not form vesicles and were sometimes not able to

infect the host plants from which they had been isolated [8-11]. During past few years, several *Frankia*-like actinomycetes have been purified from *Coriaria* (Mirza, unpubl.) and *Datisca* [12] root nodules. All isolates formed hyphae and large multilocular sporangia typical of *Frankia* but failed to fix N_2 in pure culture and to form root nodules on their host plants. The application of the above mentioned criteria in the characterization of these atypical isolates is therefore not sufficient to identify these isolates as members of the genus *Frankia*.

Qualitative and quantitative analyses of total fatty acid composition have been shown to be useful for taxonomic investigations [13-15]. First attempts to use total fatty acids of *Frankia* to characterize strains focused on typical isolates obtained from *Alnus* and other hosts [16,17]. The analysis of fatty acid profiles of *Frankia* strains belonging to different compatibility groups has led to discrimination between the type species *F. alni* and *F. elaeagni* and also of the subspecies *pommeri* and *vandijkii* of the proposed species *Frankia alni* [18,19]. All these investigations showed a characteristic fatty acid profile for all strains tested.

The aim of our investigations was to show the applicability of total cell fatty acid analysis in the identification of atypical *Frankia* strains. The comparison of fatty acid patterns of the isolates from *Coriaria* and *Datisca* as well as those of other atypical *Frankia* strains with the patterns of confirmed *Frankia* strains and several other actinomycetes was primarily carried out to establish the relationships of the isolates with the genus *Frankia*.

Materials and Methods

Confirmed *Frankia* strains and the isolates from *Coriaria* and *Datisca* (Table 1) were grown in P+N medium [20] at 30°C for 2 weeks without agitation. *Actinomyces sp.* (A5, Dept. of Microbiol., Wageningen), *Arthrobacter globiformis* (ATCC 8010), *Mycobacterium phlei* (M9, Dept. of Microbiol., Wageningen), *Nocardia convoluta* (ATCC 4275), *Nocardia vaccinii* (ATCC 11092), *Rhodococcus chlorophenolicus* (DSM 43826), *Streptomyces albus* (ATCC 3004) and *Streptomyces griseoruber* (ATCC 23919) were grown in tryptone soya-broth [21] with 10% sucrose at 30°C, shaken at 120 rpm. *Geodermatophilus obscurus* (ATCC 25078) was grown on malt-yeast agar medium [22].

Table 1: List of the *Frankia* strains used for fatty acid analysis.

Strain	*Subgp.	Host Plant	**N2-Fix.	+Inf.	Reference
Typical					[10]
Ag45/Mut15	(I)	*Alnus glutinosa*	+	+	
AgKG84/4	(I)	*A. glutinosa*	+	+	[23]
AgB32	(I)	*A. glutinosa*	+	+	[23]
AgPM2	(I)	*A. glutinosa*	+	+	Akkermans unpubl.
An1	(I)	*A. nitida*	+	+	[24]
Ar13	(I)	*A. rubra*	+	+	[3]
Avcl1	(I)	*A. viridis*	+	+	[25]
Cpl1	(II)	*Comptonia peregrina*	+	+	[26]
WgCpl.2	(I)	*C. peregrina*	+	+	[20]
Cc1.17	(II)	*Colletia cruciata*	+	+	[20]
Cj	(I)	*Casuarina junghuhniana*	+	+	[27]
EAN	(I)	*Elaeagnus angustifolia*	+	+	[28]
Hrl1	(II)	*Hippophae rhamnoides*	+	+	Baker, unpubl.
Atypical					
Eul1	(II)	*Elaeagnus umbellata*	-	+	[8]
AgB1.5	(III)	*Alnus glutinosa*	-	-	[10]
AgB1.9	(I)	*A. glutinosa*	-	+	[10]
AgW1.1	(III)	*A. glutinosa*	-	+	[10]
Agl5	(III)	*A. glutinosa*	-	+	Akkermans unpubl.
Dc2	(III)	*Datisca cannabina*	-	-	[12]
Cn1,Cn3,Cn4,Cn6, Cn7,CnM,CnM1,CnM 2,CnM3,CnM4,CnM7	(I)	*Coriaria nepalensis*	-	-	Mirza, unpubl.

* *Frankia* subgroups are explained in Results & Discussion

** N2-Fix. = Nitrogenase activity ; + Inf. = Infectivity (nodulation ability)

Bacterial cells were harvested by centrifugation and washed twice with sterile distilled water. Whole-cell fatty acids were saponified, methylated and extracted as described by Miller and Berger [14]. The samples were analysed using MIDI Microbial Identification System (Microbial ID, Newark, DE, USA) which consists of a Hewlett Packard HP 5890A gas chromatograph with a 25m x 0.2mm 5% methylphenyl silicone fused silica capillary column, H_2 as carrier gas and a flame-ionization detector, an automatic sampler, an integrator and a computer. The latter identifies the fatty acids, using data of a fatty acid library and a calibration mix of known fatty acids (Microbial ID) as a reference. Library generating software and a statistical program CLUS developed by Microbial ID, were used for principle component and cluster analysis of strains and also for creating a reference library for *Frankia*.

Results and Discussion

Total cell fatty acid profiles of *Frankia*-like actinomycetes isolated from nodules of *Coriaria nepalensis* and *Datisca cannabina* clearly resemble those of confirmed *Frankia* strains isolated from *Alnus*, *Casuarina*, *Colletia*, *Comptonia*, *Elaeagnus* and *Hippophae* (Table 2). The fatty acid composition is qualitatively similar to the composition reported previously for several *Frankia* strains [16,17,19]. However, comparatively lower amounts of 16:0 and 18 carbon fatty acids (18:0 cis9 & 18:2 cis9,12 or 18:0 anteiso) and higher amounts of 15:0 and 17:1 were detected in the strains tested in the present study. A profile of five major fatty acids (15:0; 15:1; 16:0 iso; 17:0 and 17:1) appears to be characteristic for the genus *Frankia*, as such high amounts of the same acids were not detected in any other actinomycete included in this study [Table 3]. These fatty acids constituted 75% or more of the total fatty acids detected in the majority of *Frankia* strains including the *Coriaria* and *Datisca* isolates as well as the ineffective strains isolated from *Alnus glutinosa*. In *Actinomyces*, *Geodermatophilus*, *Mycobacterium*, *Nocardia* and *Streptomyces*, these fatty acids were either absent or detected in comparatively minor amounts (Table 3). The overall fatty acid profile of these organisms differs significantly from that of *Frankia*.

Compared with overall fatty acid profiles of confirmed *Frankia* strains, atypical (ineffective/noninfective) strains from *Alnus, Coriaria, Datisca* and *Elaeagnus* did not show any significant differences in fatty acid composition. However, most atypical strains contained 16 carbon hydroxy fatty acids (16:0 3OH & 16:1 2OH). These fatty acids were absent in typical *Frankia* strains.

Fatty acid analysis data when subjected to cluster analysis very clearly separated all *Frankia*, including *Coriaria* isolates and other atypical strains, from bacteria (*Arthrobacter*, *Actinomyces*, *Geodermatophilus*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces*) used as a reference (Figure 1). Because of the high degree of phylogenetic relationship between *Frankia* and *Geodermatophilus*, the two genera have recently been considered to constitute the family *Frankiaceae* [29,30]. The fatty acid profile of *Geodermatophilus*, however, is quite distinct from that of *Frankia* (Table 3).

Within the *Frankia* cluster three subgroups were found to be present (Figure 1&2. See table 1 for the strains included in the three subgroups). The subdivision of *Frankia* strains used in this study into three subgroups is mainly determined by the relative amounts of five key

Table 2: Fatty acid composition[+] of *Frankia* subgroups* identified on the basis of fatty acid analysis.

Fatty acids	*Frankia* I				*Frankia* II		*Frankia* III	
	Frankia strains (11)		*Coriaria* isolates (11)		(4 strains)		(4 strains)	
	C	M%(SD)	C	M%(SD)	C	M%(SD)	C	M%(SD)
Saturated								
12:0**	9	0.3(0.2)	10	0.3(0.1)	3	0.3(0.2)	1	0.05(0.08)
13:0	11	2.3(1.3)	11	0.7(0.1)	4	1.2(0.5)	3	0.2(0.1)
14:0	10	0.9(0.6)	11	0.6(0.1)	4	1.6(0.7)	4	0.3(0.08)
15:0	10	12.7(5.2)	11	19.7(3.2)	4	19.4(4.5)	4	10.7(4.0)
16:0	11	4.0(2.7)	11	2.0(0.4)	4	10.8(5.4)	4	1.5(0.1)
17:0	11	6.9(3.0)	11	4.7(1.5)	4	8.8(3.5)	4	5.2(2.6)
Unsaturated								
15:1	11	6.9(2.5)	11	6.3(1.9)	4	2.7(1.1)	4	2.2(0.8)
16:1 cis9	11	3.1(1.8)	11	2.6(0.4)	4	5.1(2.8)	4	1.3(0.4)
16:1 iso	9	1.1(1.0)	11	1.0(0.5)	2	2.4(4.5)	3	1.7(1.3)
17:1	11	26.0(4.7)	11	26.1(1.2)	4	20.0(4.5)	4	19.7(5.9)
18:1 cis9	10	1.2(1.2)	10	0.5(0.2)	4	3.1(1.5)	2	0.4(0.4)
18:1 iso	7	0.2(0.2)	10	0.3(0.2)	3	0.3(0.2)	4	0.9(0.2)
18:1 cis11/t9/t6	2	0.3(0.7)	2	0.1(0.3)	1	0.1(0.2)	2	0.1(0.1)
Branched								
12:0 iso	10	0.4(0.3)	3	0.02(0.03)	1	0.05(0.08)	0	-(-)
14:0 iso	11	2.0(1.1)	10	0.5(0.2)	4	0.7(0.1)	4	0.7(0.2)
15:0 anteiso	3	0.1(0.2)	9	0.2(0.1)	2	0.1(0.1)	0	-(-)
16:0 iso	11	26.8(7.3)	11	31.7(5.1)	4	19.8(14.0)	4	43.5(3.8)
17:0 anteiso	5	0.3(0.5)	11	0.7(0.4)	1	1.2(2.1)	4	3.1(1.9)
Hydroxy								
12:0 3OH	2	0.03(0.08)	8	0.2(0.1)	1	0.07(0.1)	0	-(-)
16:0 iso 3OH	5	0.4(1.1)	2	0.01(0.03)	0	-(-)	0	-(-)
16:1 2OH	1	0.08(0.2)	1	0.2(0.8)	1	2.0(3.5)	3	5.9(5.1)

[+] Fatty acids (14:1 cis9/t9;15:1 anteiso;16:1;17:1 anteiso;18:3 cis6,12,14;15:0 iso;12:0 2OH;13:0 iso 3OH;15:0 3OH;16:0 3OH;17:0 3OH;18:0 3OH) detected in minor amounts and/or only in a few strains were not included in the table. All strains were tested in duplicate.* See table 1 for the strains included in three subgroups of *Frankia*.** Number of carbon atoms in fatty acid : Number of double bonds per molecule. C = Counts; M = Mean (%) of total fatty acids; SD = Standard Deviation.

Table 3: Key Fatty Acids [+] in *Frankia*, *Actinomyces*, *Geodermatophilus*, *Mycobacterium*, *Nocardia* and *Streptomyces*.

Fatty Acids	*Frankia* M%(SD)**	*Actinomyces* sp.	*Geodermatophilus obscurus*	*Mycobacterium phlei*	*Nocardia convoluta*	*Streptomyces albus*
14:0 iso*	1.1(0.9)	12.7	1.8	-	-	6.1
15:0	15.9(5.7)	1.0	2.0	2.8	6.0	0.7
15:0 iso	0.04(0.1)	5.8	19.0	1.1	-	16.1
15:0 anteiso	0.1(0.1)	30.0	5.7	6.1	-	24.9
15:0 iso 2OH/ 16:1 t9	-(-)	-	-	20.3	16.0	-
15:1	5.5(2.7)	0.2	2.0	-	0.4	-
16:0	3.8(3.8)	4.3	3.8	26.7	31.0	1.9
16:0 iso	29.9(10.0)	26.2	16.2	-	-	17.8
16:1 cis 9	3.1.(1.4)	1.1	13.0	2.1	2.3	0.7
17:0	6.1(2.9)	0.2	1.9	1.5	2.8	-
17:0 iso	-(-)	1.1	1.7	0.6	-	7.5
17:0 anteiso	1.0(1.4)	6.1	3.6	1.2	-	11.5
17:1	24.4(4.9)	-	10.4	1.5	4.9	-
18:1 cis9	1.3(1.5)	-	6.6	9.4	8.7	-
TSBA 10ME 18:0	-(-)	-	-	11.6	11.5	-

[+] Five key fatty acids for each genus are underlined. All strains tested in duplicate.
* Number of carbon atoms in fatty acid : Number of double bonds per molecule.
** M= Mean(%) of fatty acids in 30 *Frankia* strains; SD= Standard deviation.

fatty acids and three 16-carbon fatty acids(16:0; 16:1 cis9; 16:1 2OH). These subgroups do not correspond to the compatibility groups reported by Baker [31]. All isolates from *Coriaria* and most *Frankia* strains from other hosts cluster in one main group. In addition to 11 isolates from *Coriaria*, *Frankia* subgroup I includes 8 strains from *Alnus* and one strain each from *Casuarina*, *Comptonia* and *Elaeagnus*. The remaining 8 *Frankia* strains were distributed equally into two subgroups. The subgroup II contains one strain each from *Colletia* , *Comptonia* , *Elaeagnus* and *Hippophae* . Wheeler [17] described a *Colletia* strain which deviated from other strains in having a low content of 16:0 iso and a high content of 16:0. Similar amounts of the above mentioned fatty acids were found in subgroup II, also including a *Colletia* strain. Two *Comptonia* strains i.e. CpI1 and an isolate (WgCpI.2) obtained from a single cell colony of CpI1, were found in separate subgroups. This shows presence of more than one isolates in the original CpI1 culture. The third subgroup consists of four atypical strains from *Alnus* and *Datisca*. However, this group may not be considered as representative for all atypical strains as ineffective strains from *Alnus*, *Elaeagnus* and *Coriaria* were also found to be randomly distributed among the other two subgroups of *Frankia*.

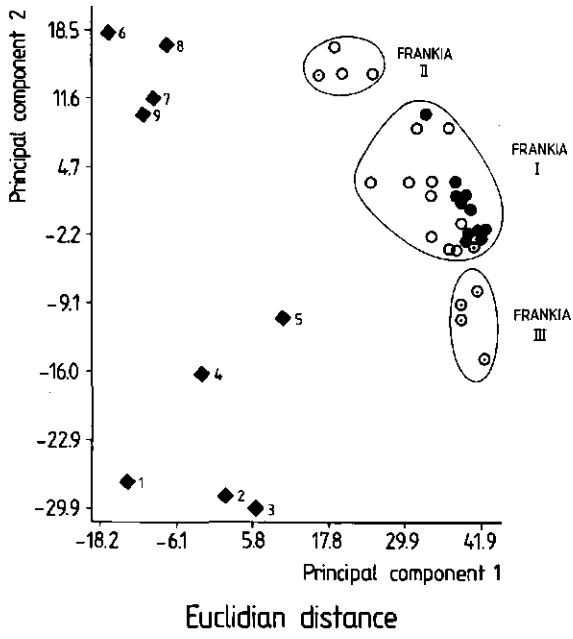
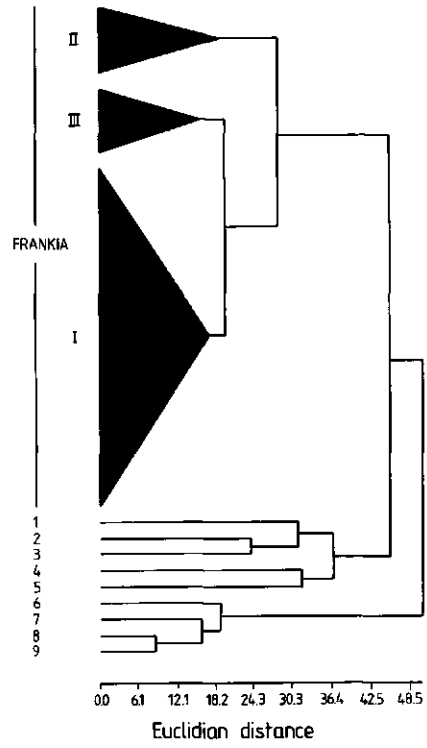


Figure 1: Two-dimensional plot of principal component analysis differentiating 30 *Frankia* strains from other bacteria,based on fatty acid profiles. *Coriaria* isolates = O ; typical *Frankia* = O ; atypical *Frankia* = O; other bacteria = O (1= *Streptomyces griseoruber*; 2=*Streptomyces albus*; 3= *Actinomyces* sp.; 4= *Arthrobacter globiformis*; 5=*Geodermatophilus obscurus*; 6= *Nocardia vaccinii*; 7= *Rhodococcus chlorophenolicus*; 8= *Nocardia convoluta*; 9= *Mycobacterium phlei*). See table 1 for the strains included in the three subgroups of *Frankia*.

Fatty acid analysis proved a powerful taxonomic tool for the characterization and identification of *Frankia* strains, especially for atypical strains difficult to identify on the

Figure 2: Dendrogram showing relationships between *Frankia* fatty acid subgroups (I,II,III) and other bacteria (numbers refer to the same bacterial species as in figure 1). See table 1 for the strains included in the three subgroups of *Frankia*.



basis of standard tests, e.g. acetylene reduction assays and nodulation tests. Because fatty acid profiles appear to be quite conserved at the genus-level, *Frankia*-like isolates from *Coriaria*, *Datisca* and other atypical *Frankia* strains could easily be grouped within the genus *Frankia*. The presented results show that the analysis of total cellular fatty acids can be a good and rapid addition to molecular techniques like DNA/DNA hybridization [32] or rRNA sequencing [30] in the taxonomy of the genus *Frankia*.

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Chapter 5

Isolation and characterization of *Frankia* strains from *Coriaria nepalensis*

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Summary

Nine *Frankia*-like actinomycetes were isolated from root nodules of *Coriaria nepalensis*. All isolates formed hyphae and sporangia typical of *Frankia* but failed to induce nodules on *Coriaria* seedlings or to reduce acetylene in pure culture. Hybridization with a 16S rRNA targeted oligonucleotide probe indicated that the isolates did belong to the genus *Frankia*. This was confirmed for one of the isolates (Cn7) by comparison of partial 16S rRNA sequences of the cloned amplification products that showed 94.7 and 96.5% homology with those of the confirmed *Frankia* strains from *Alnus* (Ag45/Mut15) and *Casuarina* (ORS 020606), respectively. Similar values were obtained when 16S rRNA sequences of Cn7 were compared with those of the amplification products obtained directly from nodules of *Coriaria nepalensis*. Analysis of two variable regions of 15 cloned amplification products from nodules showed two types of 16S rRNA sequences, slightly different from each other. In both variable regions, none of the isolates showed identical sequences to those obtained from nodules. The results argue strongly that the isolates are members of the genus *Frankia*. However, their identity as endophytes of *Coriaria* nodules could not be proved.

Key words: *Coriaria*, *Frankia*, oligonucleotide probes, rRNA,

Introduction

Coriaria nepalensis is a widespread actinorhizal shrub in the northern hilly areas of Pakistan. The importance of *Coriaria* is reflected by its ability to colonize and stabilize eroded soils and to add nitrogen to forest ecosystems (*Chaudhary* et al., 1984). The ability of root nodules of various species of the genus *Coriaria* to fix atmospheric nitrogen has been confirmed by isotopic tests with ^{15}N and by acetylene reduction assay (*Harris* and *Morison*, 1958; *Stevenson*, 1958; *Bond*, 1967; *Canizo* and *Rodriguez-Barrueco*, 1978; *Chaudhary* et al., 1984; *Rodriguez-Barrueco* et al., 1988; *Silvester* and *Harris*, 1989).

A comparison of the internal structure of *Coriaria* nodules and the morphology of the

infecting endophyte shows a number of differences to other actinorhizal plants (*Newcomb* and *Pankhurst*, 1982). Cross-sections of the nodules show a kidney-shaped region formed by infected cortical cells and a stele pushed to one side of the nodule lobe. In infected cells, the vesicles of the endophyte are filamentous and oriented towards the infection-free cell centre. The only other known genus of actinorhizal plants bearing nodules of similar internal structure is *Datisca* (*Datiscaceae*), with one species also commonly growing in the same area of Pakistan (*Hafeez* et al., 1984a).

In contrast to other actinorhizal plants, from which hundreds of *Frankia* isolates have been obtained during the last two decades (*Callaham* et al., 1978; *Berry* and *Torrey*, 1979; *Baker* and *Torrey*, 1980; *Hafeez* et al., 1984b), the endophyte from *Coriaria* has not yet been isolated and characterized. This failure may be more a result of difficulties in the identification of isolates obtained rather than in the isolation of the endophyte itself. *Frankia* isolates are mostly identified on the basis of morphological features like sporangia and vesicle formation and the ability to form nitrogen fixing nodules (*Lechevalier*, 1984). During the last few years, many isolates lacking some of these characteristics have been obtained from actinorhizal nodules. These isolates showed the basic characteristic morphology of *Frankia* like hyphae and sporangia formation but were not able to fix nitrogen, did not form vesicles and were sometimes not able to infect the host plants from which they had been isolated (*Baker* et al., 1980; *Lechevalier* et al., 1983; *Hahn* et al., 1988). Confirmation of some of these isolates as *Frankia* strains was done cytochemically using fatty acid patterns (*Mirza* et al., 1991) or genetically using DNA or rRNA sequence analysis and probing (*Hahn* et al., 1989a; *Hahn* et al., 1990a; *Harry* et al., 1991; *Nazaret* et al., 1991).

The aim of this study was to isolate the endophyte from root nodules of *Coriaria nepalensis* and to characterize the isolates obtained on the basis of morphological characteristics generally present in typical and atypical *Frankia* strains, and on genetic characteristics based on 16S rRNA sequence analysis.

Material and Methods

Isolation of the endophyte: Root nodules of several *Coriaria nepalensis* plants collected from Murree (Pakistan) were surface-sterilized with 1% sodium hypochlorite solution for 15 minutes, washed several times with sterile distilled water and homogenized in a mortar to

release vesicle clusters of the endophyte from host cells. The vesicle clusters were separated from cell debris and contaminating bacteria by filtration through a 100 μm nylon filter onto a 20 μm nylon filter followed by several washings with sterile water (*Benson*, 1982). Washed vesicle cluster samples were resuspended in distilled water and used to inoculate agar plates containing P+N medium (*Meesters* et al., 1985). The plates were incubated at 30°C for several weeks and *Frankia*-like colonies were transferred to new P+N plates and finally grown in liquid P+N medium. The *Frankia*-like isolates thus obtained were encoded with acronyms (Cn1, Cn3, Cn6, Cn7, Cnm, Cnm1, Cnm2, Cnm3, Cnm4).

Bacterial strains: The isolates and the confirmed *Frankia* strains Ag45/Mut15, AgB1.7 (*Hahn* et al., 1988), AgI5, An2.24 and AgKG'84/4 (*Hahn* et al., 1989a) were grown in liquid P+N medium at 30°C for 7 to 14 days, whereas *Streptomyces lividans* TK24 (P. A. Hopwood, John Innes Institute, Norwich, UK) was grown in R5 liquid medium (*Hopwood* et al., 1985) supplemented with 10% sucrose at 30°C for 2 days.

Scanning Electron Microscopy: Cells of the isolates as well as of *Frankia* strain AgKG'84/4 were harvested by centrifugation after 7 to 14 days, washed with PBS (8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4.2H_2O$ and 0.23 g $NaHPO_4.H_2O$ / litre of water) and fixed for 16 hours in 2% glutaraldehyde in 25 mM cacodylate buffer, pH 6.8 (*Hafeez* et al., 1984b). Fixed cells were rinsed three times with distilled water, slowly dehydrated with alcohol (20-100%) and critical point dried (Balzers Union CPD 11 120). The samples were sputtered with gold and examined with a Philips SEM 535 scanning electron microscope.

Extraction of Nucleic Acids: Glutaraldehyde-fixed nodules of *Coriaria nepalensis* (200 mg fresh weight) were ground in a mortar in 1 ml of homogenization buffer (200 mM Tris/HCl, pH 8.5; 1.5% lauryl sulfate; 300 mM LiCl; 10 mM Na_3EDTA; 1% sodium deoxycholate (w/v); 1% octylphenol ethylene oxide condensate (Nonidet P-40, w/v); 5mM thiourea; 1 mM aurintricarboxylic acid; 10 mM dithiotreithol) to release cells of the endophyte and then sonicated for 30 sec. at 60 W (Ultra sonics, Danbury, CT, USA) to disrupt the cells of the endophyte. Pure cultures of the isolates, *Streptomyces lividans* TK24 and confirmed *Frankia* strains (200 mg fresh weight) were suspended in 3 ml of homogenization buffer and sonicated for 30 sec. at 60 W. The crude homogenates of both nodules and pure cultures were mixed with 0.3 vol. of 8.5 M potassium acetate, pH 6.5. After incubation on ice for 15 minutes and subsequent centrifugation at 5000 x g for 15 minutes the supernatant was mixed with 1/9 vol. 3.3 M sodium acetate and 0.5 vol. isopropanol and incubated at -20°C for one hour. This

was then centrifuged at 5000x g for 15 min. and the pellet obtained was dissolved in 800 µl TE-buffer. 1/2 vol. of 10 M LiCl was added and the preparation was incubated at 4°C for 5 -12 hours. It was then centrifuged at 5000x g for 30 min., the pellet was dissolved in 200 µl TE-buffer, prior to addition of 1.5 vol. 5 M potassium acetate, incubation on ice for 3-5 hours and centrifugation at 5000x g for 30 minutes (*Hughes* and *Galau*, 1988). The nucleic acid pellets were dissolved in 100 µl of TE-buffer and used for filter hybridization and PCR amplification.

Filter hybridization: Dot blot hybridization experiments were performed on GeneScreen filters (DuPont). Ribosomal RNA (100 ng/ dot) was applied with a Hybri.Dot manifold (BRL), immobilized by UV light and hybridized according to *Church* and *Gilbert* (1984). Oligonucleotide probes were 5'-labeled using phage T_4 polynucleotide kinase (BRL) and 10-20 µCi of [γ-^{32}P]adenosine-5'-triphosphate (3000 Ci/mmol; Amersham) (*Maniatis* et al., 1982).

Oligonucleotides: Ribosomal RNA targeted oligonucleotides were used as probes in hybridization experiments or as primers for PCR. The eubacterial primer 1115 (*Embley* et al., 1988, $^{5'}$AGGGTTGCGCTCGTTG) was used as universal probe while probe EFP, specific for nitrogen-fixing *Frankia* from *Alnus* and *Casuarina* (*Hahn* et al., 1989a, $^{5'}$GCAGGACCCTTACGGATCCC), and probe FP, designed as *Frankia* genus probe (*Hahn* et al., 1990a, $^{5'}$ATAAATCTTTCCACACCACCAG), were used as specific probes. Specific PCR amplification was done with primer 1115 as universal primer and PCR II, which was complementary to FP but shortened at the 3'end ($^{5'}$CTGGTGGTGTGGAAAG). For sequence analysis after PCR amplification, probe 1115 was synthesized with an additional restriction site (*Hind*III, $^{5'}$GTG*AAGCTT*AGGGTTGCGCTCGTTG). The second primer was complementary to primer 124 (*Embley* et al., 1988) and equipped with an additional *Bam*HI site ($^{5'}$CAC*GGATCC*GGACGGGTGAGTAACACG). Both primers were used in sequencing reactions as well as in PCR. The 16S rRNA sequence of a *Frankia* strain from *Casuarina* (ORS 020606; GenBank 58598; *Normand* et al. 1992) was kindly provided by Dr. Normand and Dr. Simonet (Lyon, France).

PCR amplification, cloning and sequencing: Standard PCR was performed in a total volume of 100 µl containing 10 µl 10 x PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris/HCl pH 8.4, 1 mg/ml bovine serum albumin), 1 µl dNTPs (each 10 mM in 10 mM Tris/HCl pH 7,5), 0.2 µl Taq polymerase (BRL, 5 U/ µl), 0.5 µl of both primers (100 ng/

μl) and 1 μl nucleic acid preparation. Thirty-five rounds of temperature cycling (94°C for 1 minute, 50°C for 2 minutes and 72°C for 3 minutes) were followed by a final seven minute incubation at 72°C.

Prior to cleavage with restriction enzymes (*Bam*HI and *Hind*III, BRL) and cloning, the amplification products were treated with proteinase K (BRL, final concentration 60 ng/100 μl) at 37°C for 15 minutes (*Crowe* et al., 1991), phenol/chloroform extracted and precipitated with ethanol. The DNA-fragments were obtained by TAE-agarose gel electrophoresis followed by excision of the fragments and subsequent purification by the Gene-Clean procedure (Bio 101, La Jolla, California). Cloning of the amplification products into pUC19 was done by standard methods (*Maniatis* et al., 1982). Plasmid preparation was performed by using the alkaline lysis method (*Maniatis* et al., 1982).

Cloned amplification products were sequenced using the T7 DNA polymerase kit (Sequenase, U.S.Biochemicals) following the manufacturer's instructions.

Results and Discussion

The attempts to isolate the endophyte from root nodules of *Coriaria* resulted in the purification of nine *Frankia*-like actinomycetes. Both light microscopy and scanning electron microscopy of the isolates revealed branched, septated hyphae and sporangia of different sizes and shapes (Fig.1). Young sporangia were visible after one week of growth in P+N liquid medium. Non-motile spores released from sporangia could be detected in the second week of growth and intact sporangia were visible only rarely after 2 weeks. Both terminal and intercalary sporangia were formed by isolates from *Coriaria* and the *Frankia* strain AgKG'84/4. Terminal sporangia were pear-shaped in the early stages of differentiation. Sporangia formation by divisions of the cells in three planes is considered a morphological characteristic of the genus *Frankia* (*Lechevalier*, 1984) and the related genus *Geodermatophilus* of the family *Frankiaceae* (*Hahn* et al., 1989b).

Vesicles were never detected, either in media containing combined nitrogen or in media with N_2 as sole N-source. In our experiments the isolates also failed to nodulate seedlings of *C. nepalensis*. This, however, could be due to limitation inherent in the host plants because nodule formation on *C. nepalensis* is only found occasionally even when crushed nodules are used as inoculum. In natural habitats also, profuse nodulation of *C. nepalensis* is observed

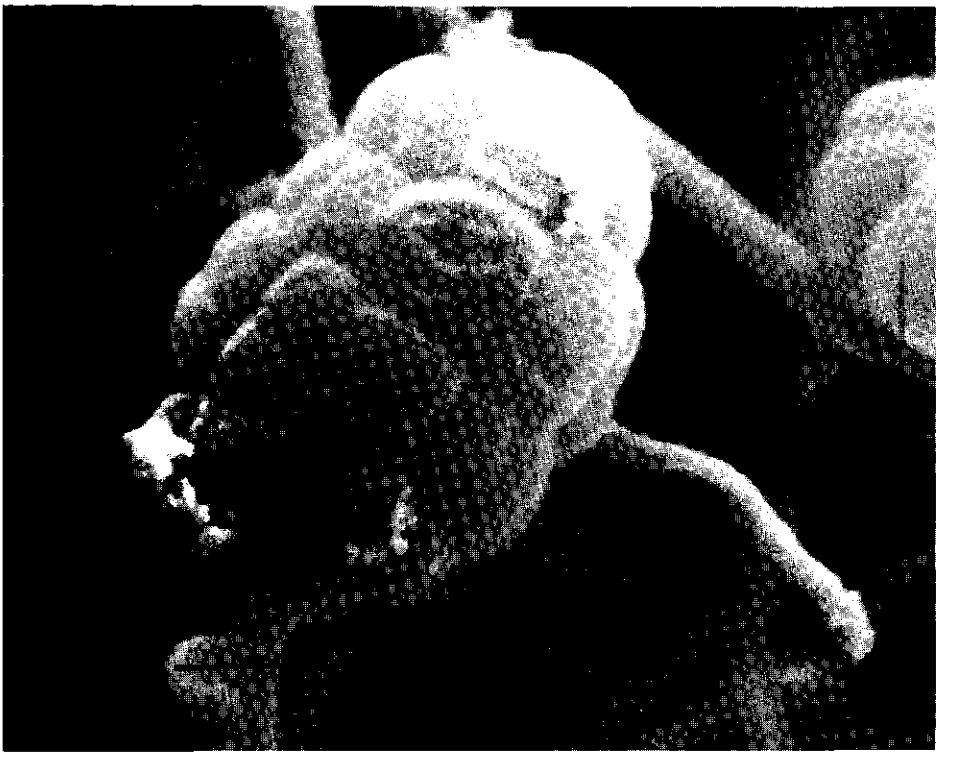

Fig. 1. Pure culture of isolate Cn7 from *Coriaria* nodules, showing hyphae and terminal sporangia formed by divisions of the cells in three planes. Bar represents 1 μm.

only rarely. The lack of these characteristics in our isolates required alternative methods to prove their identity as *Frankia* strains. Hybridization of rRNA isolated from pure cultures of the isolates and from two confirmed *Frankia* strains, the nitrogen-fixing strain Ag45/Mut15 and the atypical, ineffective strain AgB1.7 with the probe FP showed strong hybridization signals under stringent washing conditions, giving evidence of a strong relationship of the isolates to the genus *Frankia* (Fig. 2). The probe used, however, is not absolutely specific for *Frankia*, as it showed cross-hybridization to two members of other genera tested and also differences in intrageneric hybridization patterns at high stringency washing conditions (*Hahn* et al., 1990b).

Due to the small number of nodules available and the small amount of target RNA obtained from these nodules, the presence of the *Frankia* genus sequence on the 16S rRNA of the

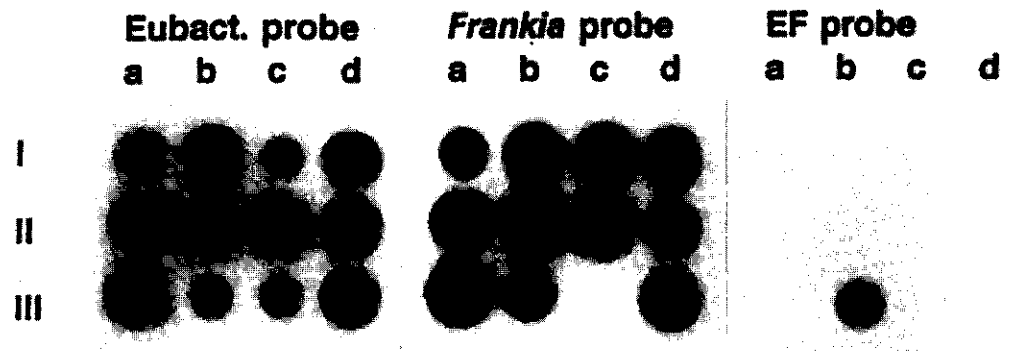

Fig. 2. Dot blot hybridization of rRNA of nine isolates from *Coriaria* (Ia-d, IIa-d, IIIa) and the control organisms *Streptomyces lividans* TK21 (IIIc) and two confirmed *Frankia* strains (Ag45/Mut15 [IIIb] and AgB1.7 [IIId] with probe FP (B). Hybridization signals were obtained with all isolates from *Coriaria* and both *Frankia* strains from *Alnus*. Hybridization with a eubacterial probe (primer 1115) was used to confirm the presence of rRNA in all dots (A) whereas hybridization with probe EFP (C) was used as negative control to exclude contamination of *Frankia* from the *Alnus* or *Casuarina* compatibility group.

endophyte in nodules of *Coriaria* could not be confirmed by direct hybridization. *Frankia*-specific sequences in nodules as well as in the isolates were detected by specific amplification of partial 16S rRNA sequences by PCR. An oligonucleotide (PCR II) complementary to the probe FP but shortened at the 3'-end was used as specific primer together with the universal primer 1115 (*Embley* et al., 1988). The amplification products of the isolates and nodules as well as that of the confirmed *Frankia* strain showed the expected size of about 0.9 kb (Fig. 3). Hybridization of the PCR products with probe EFP, specific for effective *Frankia* obtained from *Alnus* and *Casuarina* (*Hahn* et al., 1989a) showed hybridization signals only with the amplification product of strain Ag45/Mut15 which indicated that the amplification products of isolates from *Coriaria* and nodules were not the result of amplification of contaminating *Frankia* commonly used in our laboratory.

PCR on nucleic acids isolated from both nodules and the isolates with two universal primers (primer 1115 and 124) equipped with restriction sites resulted in amplification products of about 1 kb which were cloned and sequenced. Sequences corresponding to *E.coli* positions 129-1073 from PCR products of *Coriaria* nodules and the isolate Cn7 were aligned and compared (Fig. 4) with those of confirmed *Frankia* strains (*Hahn* et al., 1989b; *Normand* et al., 1992). One amplification product obtained from nodules, the isolate Cn7 and sequences of confirmed *Frankia* strains showed high homology (Table 1). Homology values of 94.7 and

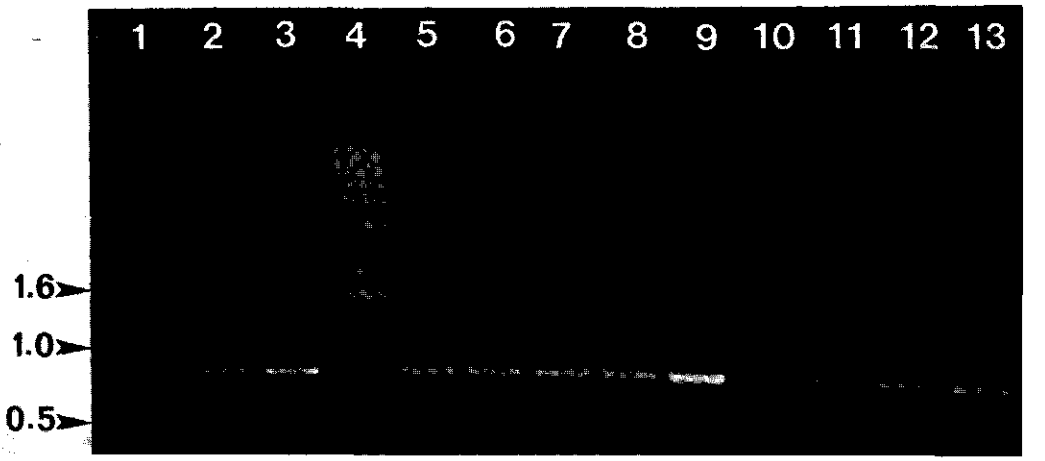

Fig. 3. Amplification of partial 16S rRNA sequences with primer 1115 (*E. coli* position 1100-1115) as universal primer and PCR II (*E. coli* position 190-199) as specific primer. PCR products were obtained from confirmed *Frankia* strain Ag45/Mut15 (Lane 2), *Coriaria* nodules (Lane 3) as well as from all isolates from *Coriaria* (Lanes 5-13). No PCR product was obtained from *Streptomyces lividans* TK21 (Lane 1). Lane 4= 1 kb ladder (BRL). Sizes are in kilobases.

Table 1. Homology values among partial 16S rRNA sequences of *Frankia* based on the analysis of 925 nucleotides.

	1	2	3	4	5
1. Ag45/Mut15	-				
2. AgB1.9	96.6	-			
3. ORS 020606	95.1	94.1	-		
4. Cn7	94.7	95.5	96.5	-	
5. *Coriaria* nodule	93.8	93.1	97.2	95.8	-

96.5% were obtained between the isolate Cn7 and the *Frankia* strains from *Alnus* (Ag45/Mut15) and *Casuarina* (ORS 020606), respectively. Similar values were obtained when sequences of Cn7 were compared with those of amplification products obtained from nodules of *C. nepalensis*.

Recently, the analysis of variable regions of several *Frankia* strains has shown that the majority of the strains with identical sequences belong to a single genomic species identified on the basis of DNA-DNA homology (*Fernandez* et al., 1989; *Nazaret* et al., 1991). To investigate the variability of the isolates and to confirm them as microsymbionts of *Coriaria*,

```
          0
Mut15     AACCTGCCTC  GAGCACTGGA  ATAACCTCGG  TAAACCGGGG  CTAATGCCGG
Cas       ........C.  ....T.....  ..........  G.........  ..........
Nod       ........C.  ....T....G  .....T....  G.........  .....A....
Cn7       ........C.  AG..T.....  .....TC...  G.........  ..........
          50
Mut15     ATAT-CACATT GCCGGTCATC  TGGTGGTGTG  GAAAGATTTA  TCGGCTCGGG
Cas       .....G.....  .....G....  ..........  ..........  ..........
Nod       .....G...C.  A.T..A....  ..........  ..........  ......T...
Cn7       ....T....CC  TT...G....  ..........  ..........  .T....T...
          100
Mut15     ATGG-CCCGC  GGCCTATCAG  CTAGTTGGTG  GGGTAATGGC  CTACCAAGGC
Cas       ....G.....  ..........  ..T.......  ....G.....  ..........
Nod       ....G.....  ..........  ..T.......  ......C...  ..........
Cn7       ....G.....  ..........  ..T.......  ....G.C...  ..........
          150
Mut15     GACGACGGGT  AGCCGGCCTG  AGAGGGCGAC  CGGCCACACT  GGGACTGAGA
Cas       ..........  ..........  ..........  ..........  ..........
Nod       ..........  ..........  .........T  ..........  ..........
Cn7       .....T....  ..........  ..........  ..........  ..........
          200
Mut15     CACGGCCCAG  ACTCCTACGG  GAGGCAGCAG  TGGGGAATAT  TGCGCAATGG
Cas       ..........  ..........  ..........  ..........  ..........
Nod       ..........  ..........  ..........  ..........  ..........
Cn7       ..........  ..........  ..........  ..........  ..........
          250
Mut15     GCGAAAGCCT  GACGCAGCGA  CGCCGCGTGA  GGGATGACGG  CCTTCGGGTT
Cas       ...G......  ..........  .........G  ..........  ..........
Nod       ...G......  ..........  .........G  .......A..  ..........
Cn7       ..........  ..........  ..........  ..........  ..........
          300
Mut15     GTAAACCTCT  TTCAGCAGGG  ACGAAGCGAA  AGTGACGGTA  CCTGCAGAAG
Cas       ..........  ..........  .........G  ..........  ..........
Nod       ..........  ..........  ........C.  ..........  ..........
Cn7       ..........  ..........  ........C.  ..........  ..........
          350
Mut15     AAGCACCGGC  TAACTACGTG  CCAGCAGCCG  CGGTAATACG  GAGGGTGCGA
Cas       ..........  C.........  ..........  ..........  T.......A.
Nod       ..........  ..........  ..........  ..........  T.......T.
Cn7       ..........  ..........  ..........  ..........  T.......A.
          400
Mut15     GCGTGGTCCG  GAATTATTGG  CGGTAAAGAG  CTTGTAGGCG  GCCTGTCACG
Cas       ....T.....  ..........  GC........  ..C.......  ......TG..
Nod       ....T.....  ..........  GC........  ..C.......  ..T.......
Cn7       ....T.....  ..........  GC........  ..C.......  .......G..
          450
Mut15     TCGGCTGTGA  AATATCGGGG  CTCAACCCCG  GGCCTGCAGT  CGATACGGGC
Cas       ..........  ..ACC.....  ......T...  ..........  ..........
Nod       ..........  ...CC.....  ......T...  ...G...G..  ..........
Cn7       ..........  ..AC.T....  ..........  .......G..  ..........
          500
Mut15     AGACCAGAGT  CCGGCAGGGG  AGACTGGAAT  TCCTGGTGTA  GCGGTGAAAT
Cas       ..G.T.....  ..........  ..........  ..........  ..........
Nod       ..G.T.....  ....T.....  ..........  ..........  ..........
Cn7       ..G.T.....  ....T.....  ..........  ..........  ..........
          550
Mut15     GCGC-AGATA  TCAGGAGGAA  CACCGGTGGC  GAAGGCGGGT  CTCTGGGCCG
Cas       ..........  ..........  ..........  ..........  ..........
Nod       ....T.....  ..........  ..........  ..........  ........T.
Cn7       ....T.....  ..........  ..........  ..........  ........T.
          600
Mut15     GAACTGACGC  TAAGGAGCGA  AAGCGTGGGG  GTCGAACAGG  ATTAGATACC
Cas       ..........  ..........  ..........  AG........  ..........
Nod       .T........  ..........  ..........  AG........  ..........
Cn7       ..........  ..........  ..........  AG........  ..........
          650
Mut15     CTGGTnGTCC  ACGCCGTAAA  CGTTGGGCGC  TAGGTGTGGG  GGACCTTCCA
Cas       .....A....  ..........  ..........  ..........  ..........
Nod       .....A....  ..........  ..........  ..........  ..........
Cn7       .....A....  ..........  ..........  ..........  ..........
          700
Mut15     CGGCCTCCGT  GCCGC-GCTA  ACGCATTAAG  CGCCCCGCCT  TGGGAGTACG
Cas       ..........  .....A....  ..........  ..........  G.........
Nod       ..........  .....A....  ..........  ..........  G.........
Cn7       ..........  .....A....  ..........  ..........  G.........
```

```
           750
Mut15      GCCGCAAgGC  TAAAACTCAA  AGGAATTGac  gggggcccGC  ACAAGcggcg
Cas        .......G..  ..........  ........AC  GGGGGCCC..  .....CGGCG
Nod        .......G..  ..........  ........AC  GGGGGCCC..  .....CGGCG
Cn7        .......G..  ..........  ......C.AC  GGGGGCCC..  .....CGGCG
           800
Mut15      gagcatgNgG  CTTAATTCGa  tgcaacGCGA  AGAACCTTAC  CAGGATATGA
Cas        GAGCATGTG.  .........A  TGCAAC....  ..........  ....GCT...
Nod        GAGCATGTG.  .........A  TGCAAC....  ..........  ..A.GCT...
Cn7        GAGCATGTG.  .........A  TGCAAC....  ..........  ....GCT...
           850
Mut15      CATGCA-GGA  AATTCTCCAG  AGATGGGGGA  TCCGTAAGGG  TCCTGTACAG
Cas        ......G...  ...CTCGT..  ....AC...G  ..........  .....C....
Nod        ......G...  ...CTCGT..  ....AC...G  ..........  CT...C....
Cn7        .....TG...  ...C......  .........G  ...T..G...  ...A.C....
           900
Mut15      GTGGTGCATG  GCTGTCGTCA  GCTCGT
Cas        ..........  ..........  ......
Nod        ..........  ..........  ......
Cn7        ..........  ..........  ......
```

Fig. 4. Alignment of a part of 16S rRNA nucleotide sequences from *Frankia* strains corresponding to *E. coli* positions 129-1073. Nucleotide sequences from *Coriaria* nodules (Nod) and Cn7 are aligned with those of the *Frankia* strains from *Alnus* (Ag45/Mut15; *Hahn* et al., 1989b) and *Casuarina* (ORS 020606; *Normand* et al., 1992). Base positions in the sequence are numbered consecutively and nucleotide 0 corresponds to nucleotide 129 in the standard *E. coli* 16S rRNA numbering (*Woese* et al., 1983). Gaps (-) are introduced to facilitate the alignment. Only nucleotides that differ from Ag45/Mut15 are shown, while identities are denoted by dots.

```
A.
                                                  3'GACCACCACACCTTTCTAAATA    Frankia probe
                                                  5'CTGGTGGTGTGGAAAG          PCR II
CCUCGGUAAACCGGGGCUAAUGCCGGAUAU-CACAUUGCCGGUCAUCUGGUGGUGUGGAAAGAUUUAU  Ag45/Mut15#
.UC.....................A....U.....CUU...G.....................  AgB1.9##, AgW1.1##
......G.....................G...C......G.....................  An2.24,
......G.....................G....CA.U..G.....................  AgKG'84/4
.UC...G.....................U.....CUU...G.............U.......  AgI5
.U....G.....................G...C...U..G.................-GGG..  AcnI1*
.UC...G.....................U...GC.A....G.................-GGG..  PtI1*
......G.....................G.........G.....................  ORS 020606**
.....................A.........G...C.A.U..A.....................  Cn nod1-7
.U....G.....................A.........G...C.A.U..A.....................  Cn nod8-15
.UC...G.....................U....CCUU...G.....................  Cn1/7,Cnm3/4
.UC...G.....................C....CCUU...G.....................  Cnm
.UC...G.....................CCUU...G.....................  Cn4/8
.UC...G.....................A.........G.....G.....................  Cn3,Cnm2

B.
                    CCCTAGGCA--TTCCCAGGACG          EF probe
AUGACAUGCA-GGAAAUUCUCCAGAGAUGGGGGAUCCGU--AAGGGUCCUGUACAGGUGG  Ag45/Mut15#
.........-G......C.AU.........U..G...U.UA.........C.........  AgB1.9##,AgW1.1##,AgI5
U.........G......CUCGU......AC...G.................C.......  An2.24,AcnI*
U.........G......C.AU.........U..G....C.............C.......  AgKG'84/4
u.........GG.....C.AU.........U..G...U....G......C..G......  PtI1*
U.........G......CUCGU......AC...G.................C.......  ORS020606**
U.........G......CUCGU......A-...G......A.....CU...C.......  Cn nod1-7
U.........G......CUCGU......AC...G.............CU...C.......  Cn nod8-15
U.........UG.....C.................G...U....G......A.C.......  Cn1/7,Cn4/8
U.........UG.....C.................G...U..A-G......A.C.......  Cnm,Cnm2/3/4
U.........GG.....C.AU.........U..G...U....G......C.........  Cn3
```

Fig. 5. Alignment of two variable regions of 16S rRNA nucleotide sequences from *Frankia* strains. Nucleotide sequences from nodules (Cn nod_{1-7} and Cn nod_{8-15}), the isolates from *Coriaria* as well as from *Alnus* strains are aligned with those published for *Frankia* (# *Hahn* et al., 1989b;## *Hahn* et al., 1989a; * *Harry* et al., 1991; ** *Normand* et al., 1992). Gaps (-) were introduced to facilitate the alignment. Only the nucleotides that differ from Ag45/Mut 15 are shown, while identities are denoted by dots. (A: *E. coli* positions 153-204; B: *E. coli* positions 991-1051).

two variable regions of 16S rRNA of all isolates and of clones of amplification products from nodules were sequenced using the amplification primers as sequencing primers. The amplification products from nodules were checked by hybridization with the probe FP and sequence comparison of twenty clones which were selected at random. Twenty-five percent of the clones obtained did not react with the probe FP. When compared with sequences of confirmed *Frankia* strains (*Hahn* et al., 1989b; *Harry* et al., 1991; *Nazaret* et al., 1991) the primary and secondary structures of the sequences of these clones showed large differences indicating their origin from other bacteria contaminating the nodule material.

Strong hybridization with the probe FP was observed with all the remaining clones originating from nodules. The presence of the target sequence in these strains was confirmed by sequence analysis. Sequence analysis of both variable regions revealed two types of sequences in PCR products obtained from nodules with only small differences between them (Fig. 5). Both sequences were different from any other sequence of confirmed *Frankia* strains used as a reference in our study or published (*Hahn* et al., 1989a,b; *Harry* et al., 1991; *Nazaret* et al., 1991). Among the isolates obtained in culture, four types of sequences were found in one region (*E.coli* positions 153-204) and three different sequences in the second variable region (*E. coli* positions 991-1051). The observation that these sequences are different between most of the isolates indicates that distinct strains have been obtained. Only isolates Cn1 and Cn7, Cn4 and Cn6 and isolates Cnm_3 and Cnm_4 showed identical sequences and thus could be duplicate isolates of the same strain. With the exception of isolates Cn3 and Cnm_2 differences in sequences between the isolates were only small. Two effective *Frankia* strains from *Alnus* (An2.24 and AgKG,84/4) included in the present study have different sequences in both variable regions while three atypical strains (AgB1.9, AgW1.1, AgI5) showed identical sequences in the variable region corresponding to *E. coli* positions 991-1051. Strain An2.24 shared the same sequence with another *Frankia* strain (AcnI) from *Alnus* (*Harry* et al., 1991) in the region corresponding to *E. coli* positions 991-1051. The fact that some strains shared the same sequence in one variable region but showed a different sequence in the other region reflects the need to sequence longer stretches of 16S rRNA for comparison of bacterial strains. The sequence complimentary to the probe FP was detected in all isolates from *Coriaria* as well as in *Frankia* strains from other hosts except for AgI5 and the published strains AcnI1 and PtI1 (*Harry* et al., 1991).

In both variable regions, none of the strains obtained in pure culture showed identical

sequences to those found in *Coriaria* nodules nor to those used as references (Fig. 5). In our study nodules used for isolation and PCR-amplification consisted of different nodule lobes formed by natural populations of *Frankia* strains of unknown genetic variation. Isolation of different strains was therefore not surprising. Probing with 16S rRNA targeted oligonucleotides as well as sequence comparison between the isolate from *Coriaria* (Cn7) and confirmed *Frankia* strains adds weight to our conclusion based on SEM and fatty acid analysis (*Mirza* et al., 1991) that the isolates belong to the genus *Frankia*. Their symbiotic association with *Coriaria* or any other host remains unknown until successful nodulation of the host by improved inoculation methods or detection of identical 16S rRNA sequences using different nodule samples can be achieved.

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Chapter 6

Phylogenetic studies on uncultured *Frankia* populations in nodules of *Datisca cannabina*.

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Phylogenetic studies on uncultured *Frankia* populations in nodules of *Datisca cannabina*.

Abstract

Part of the 16S rRNA gene was amplified directly from uncultured endophyte populations within the root nodules of *Datisca cannabina* and three strains isolated from nodules of *Alnus glutinosa* (AgKG'84/4), *Coriaria nepalensis* (Cn3) and *Datisca cannabina* (Dc2). Sequence comparison based on 930 nucleotides indicated that the endophyte of *Datisca* nodules belongs to the genus *Frankia* and is highly related to the endophyte of *Coriaria nepalensis* root nodules. The relatedness of the endophytes of *Coriaria* and *Datisca* nodules was also reflected by closely related *nifH* sequences amplified from the nodules. 16S rRNA sequence analysis of the non-infective strains obtained from both *Datisca* (Dc2) and *Coriaria* (Cn3) nodules also revealed their close relationship to the genus *Frankia*.

Key words: Nitrogen fixation, *Frankia*, 16S rRNA, *nifH*

Introduction

Datisca cannabina is an actinorhizal plant forming root nodules in symbiosis with a nitrogen-fixing actinomycete. The actinomycetous nature of the root nodule endophyte of *Datisca cannabina* as well as the nitrogen-fixing ability of the endophyte has been shown (Hafeez *et al.* 1984a,b). The nodule structure of *Datisca* is quite different from those of other actinorhizal plants like *Alnus*, *Casuarina* or *Elaeagnus* spp., but very similar to that of *Coriaria* nodules (Berg and McDowell 1987; Canizo and Rodriguez-Barrueco 1978; Hafeez *et al.* 1984c; Lalonde and Knowles 1975; Miller and Baker 1985; Mirza *et al.* submitted; Newcomb and Pankhurst 1982). In contrast to the availability of a number of *Frankia* strains from plants belonging to the *Alnus*, *Casuarina* or *Elaeagnus* compatibility groups, strains capable of inducing nodulation in *Datisca* and *Coriaria* are not yet available in pure culture. Isolation of non-infective strains from *Coriaria* and *Datisca* nodules, however, has been reported (Hafeez 1983; Mirza *et al.* 1991; Mirza *et al.* 1992).

A possibility to study phylogenetic relationships of the uncultured endophytes within the

nodules of *Coriaria* and *Datisca* is offered by PCR assisted, selective sequence retrieval and comparative sequence analysis of phylogenetically important macromolecules. Sequence analysis of the PCR-amplified 16S rRNA gene has greatly facilitated the systematic, evolutionary and ecological studies of various microorganisms (Olsen *et al.* 1986; Pace *et al.* 1986; Ward *et al.* 1992; Woese 1987). This technique is particularly valuable for non-culturable microorganisms and has been extensively used for the elucidation of the phylogenetic relationships of various microorganisms (Giovannoni *et al.* 1990; Ward *et al.* 1990), including *Frankia* populations within the root nodules (Mirza *et al.* 1992; Nazaret *et al.* 1991; Nick *et al.* 1992). Based on 16S rRNA sequence analysis, a number of atypical *Frankia* strains from alder nodules that lacked some of the morphological and physiological characteristics of typical *Frankia* strains were identified as members of the genus *Frankia* (Hahn *et al.* 1989a). Comparison of partial 16S rRNA sequences of the non-infective strain Cn7, isolated from *Coriaria* and the *Frankia* population within nodules of *Coriaria* indicated that this strain belonged to the genus *Frankia* (Mirza *et al.* 1992). Due to the differences in in the 16S rRNA sequences of the strain Cn7 and those obtained from the nodules by PCR, its identity with the *Coriaria* nodule endophyte could not be proved.

Additional information on uncultured *Frankia* populations in nodules can be gained by sequence comparison of other conserved genes, e.g. the *nifH* gene that encodes the nitrogenase Fe-protein and is highly conserved among the *nif* genes (Hennecke *et al.* 1985; Ruvkun and Ausubel 1980). The complete nucleotide sequence of the *nifH* open reading frame of *Frankia* strain ArI3 was found to be 861 bp in length, encoding a polypeptide of 287 amino acids (Normand *et al.* 1988). Compared to the sequence of *nifH* from *Frankia* strain HRN18a, 96% similarity at the amino acid level and 93% similarity at the nucleotide level was observed (Normand and Bousquet 1989). Comparison with *nifH* gene sequences of other nitrogen-fixing organisms showed that the sequence of ArI3 was more similar to *nifH* sequences of *Anabaena* and *Azotobacter* spp. than to those of *Clostridium pasteurianum* and *Methanococcus voltae*. The combination of comparative sequence analysis of *nifH* and 16S rRNA has previously been used to study natural relationships of *Rhizobium* and other nitrogen-fixing bacteria (Eardly *et al.* 1992; Hennecke *et al.* 1985).

The present study deals with the characterization of the uncultured endophyte in nodules of *Datisca cannabina* by PCR assisted retrieval of partial 16S rRNA sequences from the nodules. For comparisons, partial 16S rRNA sequences were obtained from the *Frankia*

strain AgKG'84/4, as well as from non-infective strains Cn3 and Dc2 isolated from *Coriaria* and *Datisca* nodules, respectively. The endophyte in *Datisca* and *Coriaria* nodules was further characterized on the basis of *nifH* sequence comparison with those of the *Frankia* strains and other nitrogen-fixing organisms.

Materials and Methods

Nucleic acids extraction: *Frankia* strain AgKG'84/4 from *Alnus glutinosa* (Hahn *et al.* 1990b) and strains Cn3 and Cn7 from *Coriaria nepalensis* (Mirza *et al.* 1991) and Dc2 from *Datisca cannabina* (Hafeez 1983) were grown in liquid P+N medium (Meesters *et al.* 1985) at 30°C for two weeks. Bacteria were harvested by centrifugation at 3000 x g for 10 minutes and washed with TE buffer. Cells (approx. 100 mg fresh weight) were disrupted in 500 µl TE along with 200 mg of glass beads (0.1-0.11 mm dia., Braun, Melsungen, Germany) by vigourous shaking in a bead beater (Braun, Melsungen) for 10 minutes.

Seeds of *Coriaria nepalensis* and *Datisca cannabina* were germinated and grown in a greenhouse. Root nodules used to inoculate *Coriaria* and *Datisca* plants were collected from Murree and Swat areas of Pakistan, respectively. Nodules were washed with liquid detergent (Decondi 118, Otares, Enschede, The Netherlands) for 5 minutes, followed by 3 washings with sterile water. Two hundred mg of nodules were homogenized in a mortar with 500 µl of TE buffer. The homogenate was transferred to an Eppendorf tube containing 200 mg of glass beads and cells were disrupted in a bead beater.

Purification of nucleic acids was performed after pelleting of cell debris and glass beads by centrifugation at 13000 x g for 10 minutes. The supernatant was extracted twice with phenol/chloroform, followed by two extractions with chloroform/isoamyl alcohol (24:1). After adding 1/10 vol. of sodium acetate (3 M, pH 5.2) and 0.5 vol. of isopropanol, the supernatant was incubated at -70°C for 20 minutes. The nucleic acids were then precipitated by centrifugation at 13000 x g for 20 minutes and the pellet was washed with 70% ethanol before drying under vacuum. The nucleic acids pellet was then dissolved in 50-100 µl of TE buffer.

PCR amplification, cloning and sequencing: Oligonucleotides used as probes or primers in PCR reaction are listed in table 1. PCR was performed in a total volume of 100 µl

Table 1. List of the oligonucleotides used as probes or primers in PCR.

Primer	Sequence*	Restriction site	Reference
P 124	5'CAC*GGATCC*GGACGGGTGAGTAACACG	*Bam*HI	Embley *et al.* 1988
P 1115	5'GTG*AAGCTT*AGGGTTGCGCTCGTTG	*Hind*III	Embley *et al.* 1988
Probe FP	5'ATAAATCTTTCCACACCACCAG		Hahn *et al.* 1990a
*nifH*F	5'CAC*GGATCC*GCAAGGGTGGTATTGG	*Bam*HI	Normand *et al.* 1988
*nifH*R	5'GTG*AAGCTT*CTCGATGACCGTCATCCGGC	*Hind*III	Normand *et al.* 1988

* Restriction sites are given in italics.

containing 10 μl 10x PCR buffer (KCl, 500 mM; $MgCl_2$, 25 mM; Tris/HCl, 200 mM, pH 8.4; bovine serum albumin, 1 mg/ml); dNTPs, 1 μl (each 10 mM in 10 mM Tris/HCl, pH 7.5); Taq polymerase, 0.2 μl (BRL, 5 U/μl); primers, 0.5 μl each (100 ng/μl); and nucleic acid preparation, 1 μl. Thirty five rounds of temperature cycling (94°C for 1 minute, 50°C for 2 minutes and 72°C for 3 minutes) were followed by a final incubation at 72°C for 7 minutes.

The amplification products were treated with proteinase K (BRL, final concentration 60 ng/100 μl) at 37°C for 15 minutes (Crowe *et al.* 1991), phenol/chloroform extracted and precipitated with ethanol. The DNA pellet was dissolved in 15 μl of TE and digested with *Bam*H1 and *Hind*III (BRL). The DNA-fragments were obtained by TAE-agarose gel electrophoresis followed by excision of the fragments and subsequent purification by the Gene-Clean procedure (Bio 101, La Jolla, California). Cloning of the amplification products into pUC19 was done by standard methods (Maniatis *et al.* 1982). Plasmid preparation was performed by using the alkaline lysis method (Maniatis *et al.* 1982).

For dot blot hybridizations, plasmid DNA was denatured with 0.5 N NaOH (1:1, v/v) and applied to Hybond-N^+ nucleic acid transfer membranes (Amersham) with a Hybri.Dot. manifold (BRL). The target DNA was immobilized by UV light and hybridized according to Church and Gilbert (1984). Oligonucleotide probe FP (Hahn *et al.* 1990a) was 5'-labeled

using phage T_4 polynucleotide kinase (BRL) and 10-20 μCi of [γ-^{32}P]adenosine-5'-triphosphate (3000 Ci/mmol; Amersham) according to Maniatis *et al.* (1982).

Cloned amplification products were sequenced using the T7 DNA polymerase kit (Pharmacia) following the manufacturer's instructions. Based on the partial 16S rRNA sequence information, a phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei 1987).

Results and Discussion

The amplification of partial 16S rDNA sequences (124-1115 *E.coli* positions) from *Datisca cannabina* root nodules as well as from pure cultures of the strains AgKG'84/4, Cn3 and Dc2 was achieved by PCR. Screening for the presence of *Frankia* genus-specific 16S rRNA sequences in clones originating from *Datisca* nodules was done with the oligonucleotide probe FP (Hahn *et al.* 1990a). Twenty clones containing inserts strongly hybridizing with the FP probe were sequenced with the forward PCR primer 124. This allowed confirmation of the *Frankia* sequence in the inserts. All 20 clones from the *Datisca* nodules contained inserts with an identical sequence in the variable V2 region (Neefs *et al.* 1991). The *Frankia* genus-specific 16S rRNA sequence complementary to the FP probe was also present in inserts of the clones originating from pure *Frankia* strain AgKG'84/4 and strain Cn3.

The partial 16S rRNA sequence (930 nucleotides) amplified from *Datisca* nodules and the pure cultures of AgKG'84/4, Cn3 and Dc2 is given in Fig. 1. Homology values from 93% to 98% were observed between *Frankia* 16S rRNA sequences generated in the present study or published previously (Table 2). The sequence obtained from the *Datisca* nodules was closely related to sequences from *Frankia* strains isolated from *Alnus* (AgKG'84/4), *Casuarina* (ORS 020606; Normand *et al.* 1992), *Coriaria* (Cn3) and to the sequence which was amplified from *Coriaria nepalensis* nodules (Mirza *et al.* 1992), with homology values higher than 96%. These results indicate that the endophyte of *Datisca* nodules belongs to the genus *Frankia*. The close relatedness of the *Coriaria* and *Datisca* nodule endophytes within the genus *Frankia* is clearly shown in the phylogenetic tree (Fig. 2). Among the other *Frankia* strains compared, the endophytes of both *Coriaria* and *Datisca* nodules showed quite close relatedness to a strain (ORS 020606) from *Casuarina* nodules. Based on partial 16S rRNA sequence analysis of 250 nucleotides, close relatedness was previously observed

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            1                                                        50
AgKG'84/4   AACCTGCCCC GAGCTCTGGA ATAACCTCGG GAAACCGGGG CTAATGCCGG
Cn3         AACCTGCCCC GAGCTCTGGG ATAACTTCGG GAAACCGGGG CTAATACCGG
Dat.        AACCTGCCCC GAGCTCTGGG ATAACTTCGG GAAACCGGGG CTAATACCGG
Dc2         AACCTGCCCC AGGCTCTGGA ATAACTCCGG GAAACCGGGG CTAATGCCGG
           51                                                       100
AgKG'84/4   ATAT-GACAT CACTGGGCAT CTGGTGGTGT GGAAAGATTT ATCGGCTTGG
Cn3         ATAT-GACAT TGCCGGGCAT CTGGTGGTGT GGAAAGATTT ATTGGCTTGG
Dat.        ATAT-GACAT TGCCGGGCAT CTGGTGGTGT GGAAAGATTT ATCGGCTCGG
Dc2         ATATTCACAT GCTCGGGCAT CTGGGTGTGT GGAAAGATTT ATTGGTCTGG
           101                                                      150
AgKG'84/4   GATGGGCCCG CGGCCTATCA GCTTGTTGGT GGGGTGATGG CCTACCAAGG
Cn3         GATGGGCCCG CGGCCTATCA GCTTGTTGGT GGGGTAATGG CCTACCAAGG
Dat.        GATGGGCCCG CGGCCTATCA GCTTGTTGGT GGGGTGATGG CCTACCAAGG
Dc2         GATGGGCCCG CGGCCTATCA GCTTGTTGGT GGGGTGACGG CCTACCAAGG
           151                                                      200
AgKG'84/4   CGACGACGGG TAGCCGGCCT GAGAGGGCGA CCGGCCACAC TGGGACTGAG
Cn3         CGACGACGGG TAGCCGGCCT GAGAGGGCGA TCGGCCACAC TGGGACTGAG
Dat.        CGACGACGGG TAGCCGGCCT GAGAGGGCGA TCGGCCACAC TGGGACTGAG
Dc2         CGACGACGGG TAGCCGGCCT GAGAGGGCGA CCGGCCACAC TGGGACTGAG
           201                                                      250
AgKG'84/4   ACACGGCCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA TTGCGCAATG
Cn3         ACACGGCCCA GACTCCTTCG GGAGGCAGCA GTGGGGAATA TTGCGCAATG
Dat.        ACACGGCCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA TTGCGCAATG
Dc2         ACACGGCCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA TTGCGCAATG
           251                                                      300
AgKG'84/4   GGCGAAAGCC TGACGCAGCG ACGCCGCGTG AGGGATGACG GCCTTCGGGT
Cn3         GGCGAAGGCC TGACGCAGCG ACGCCGCGTG AGGGATGACG GCCTTCGGGT
Dat.        GGCGGAAGCC TGACACAGCG ACGCCGCGTG GGGGATGACG GCCTTCGGGT
Dc2         GGCGAAAGCC TGACGCAGCG ACGCCGCGTG AGGGATGACG GCCTTCGGGT
           301                                                      350
AgKG'84/4   TGTAAACCTC TTTCAGCAGG GACGAAGCGA AAGTGACGGT ACCTGCAGAA
Cn3         TGTAAACCTC TTTCAGCAGG GACGAAGCGC AAGTGACGGT ACCTGCAGAA
Dat.        TGTAAACCTC TTTCAGCAGG GACGAAGCGA AAGTGACGGT ACCTGCAGAA
Dc2         TGTAAACCTC TTTCAGCAGG GACGAAGCGA GAGTGACGGT ACCTGCAGAA
           351                                                      400
AgKG'84/4   GAAGCACCGG CCAACTACGT GCCAGCAGCC GCGGTAATAC GTAGGGTGCA
Cn3         GAAGCACCGG CCAACTACGT GCCAGCAGCC GCGGTAATAC GTAGGGTGCA
Dat.        GAAGCACCGG CCAACTACGT GCCAGCAGCC GCGGTAATAC GTAGGGTGCA
Dc2         GAAGCACCGG CCAACTACGT GCCAGCAGCC GCGGTAATAC GTAGGGTGCA
           401                                                      450
AgKG'84/4   AGCGTTGTCC GGAATTATTG GGCGTAAAGA GCTCGTAGGC GGCCTGTCGC
Cn3         AGCGTTGTCC GGAATTATTG GGCGTAAAGA GCTCGTAGGC GGCCTGTCGC
Dat.        AGCGTTGTCC GGAATTATTG GGCGTAAAGA GCTCGTAGGC GGCTTGTTAC
Dc2         AGCGTTGTCC GGAATTATTG GCGGTAAAGA GCTCGTAGGC GGCTTGTCGC
           451                                                      500
AgKG'84/4   GTCGGCTGTG AAATATCGGG GCTCAACCCC GGGCCTGCAG TCGATACGGG
Cn3         GTCGGCTGTG AAATATCGGG GCTCAACCCC GGGCCTGCAG TCGATACGGG
Dat.        GTCGGCTGTG AAATCCCGGG GCTCAACTCC GGGCGTGCAG TCGATACGGG
Dc2         GTCGGCTGTG AAAACTCGGG GCTCAACCCC GGGCCTGCAG TCGATACGGG
           501                                                      550
AgKG'84/4   CAGGCTAGAG TCCGGCAGGG GAGACTGGAA TTCCTGGTGT AGCGGTGAAA
Cn3         CAGGCTAGAG TCCGGTAGGG GAGACTGGAA TTCCTGGTGT AGCGGTGAAA
Dat.        CAGGCTAGAG TCCGGCAGGG GAGACTGGAA TTCCTGGTGT AGCGGTGAAA
Dc2         CAGGCTAGAG TCCGGTAGGG GAGACTGGAA TTCCTGGTGT AGCGGTGAAA
           551                                                      600
AgKG'84/4   TGCGC-AGAT ATCAGGAGGA ACACCGGTGG CGAAGGCGGG TCTCTGGGCC
Cn3         TGCGC-AGAT ATCAGGAGGA ACACCGGTGG CGAAGGCGGG TCTCTGGGCC
Dat.        TGCGC-AGAT ATCAGGAGGA ACACCGGTGG CGAAGGCGGG TCTCTGGGCC
Dc2         TGCGC-AGAT ATCAGGAGGA ACACCGGTGG CGAAGGCGGG TCTCTGGGCC
           601                                                      650
AgKG'84/4   GGAACTGACG CTAAGGAGCG AAAGCGTGGG GAGCGAACAG GATTAGATAC
Cn3         GGAACTGACG CTAAGGAGCG AAAGCGTGGG GAGCGAACAG GATTAGATAC
Dat.        GGTACTGACG CTAAGGAGCG AAAGCGTGGG GAGCGAACAG GATTAGATAC
Dc2         GGAACTGAAG CTAAGGAGCG AAAGCGTGGG GAGCGAACAG GATTAGATAC
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          651                                                        700
AgKG'84/4 CCTGGTAGTC CACGCCGTAA ACGTTGGGCG CTAGGTGTGG GGGACCTTCC
Cn3       CCTGGTAGTC CACGCCGTAA ACGTTGGGCG CTAGGTGTGG GGGACCTTCC
Dat.      CCTGGTAGTC CACGCCGTAA ACGTTGGGCG CTAGGTGTGG GGGACCTTCC
Dc2       CCTGGTAGTC CACGCCGTAA ACGTTGGGCG CTAGGTGTGG GGGACCTTCC
          701                                                        750
AgKG'84/4 ACGGCCTCCG TGCCGC-GCT AACGCATTAA GCGCCCCGCC TGGGGAGTAC
Cn3       ACGGCCTCCG TGCCGCAGCT AACGCATTAA GCGCCCCGCC TGGGGAGTAC
Dat.      ACGGCCTCCG TGCCGCAGCT AACGCATTAA GCGCCCCGCC TGGGGAGTAC
Dc2       ACGGCCTCCG TGCCGCAGCT AACGCATTAA GCGCCCCGCC TGGGGAGTAC
          751                                                        800
AgKG'84/4 GGCCGCAAGG CTAAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGC
Cn3       GGCCGCAAGG CTAAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGC
Dat.      GGCCGCAAGG CTAAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGC
Dc2       GGCCGCAAGG CTAAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGC
          801                                                        850
AgKG'84/4 GGAGCATGTG GCTTAATTCG ATGCAACGCG AAGAACCTTA CCAGGGTTTG
Cn3       GGAGCATGTG GCTTAATTCG ATGCAACGCG AAGAACCTTA CCAGGGCTTG
Dat.      GGAGCATGTG GCTTAATTCG ATGCAACGCG AAGAACCTTA CCAAGGCTTG
Dc2       GGAGCATGTG GCTTAATTCG ATGCAACGCG AAGAACCTTA CCAGGGCTTG
          851                                                        900
AgKG'84/4 ACATGCAGGG AAATCCATCA GAGATGGTGG GTCCGC-AAG GGTCCTGCAC
Cn3       ACATGCAGGG AAATCCATCA GAGATGGTGG GTCCTT-AGG GGTCCCGTAC
Dat.      ACATGCAGGG AAATCTCGTA GAGATACGGG GTCCGT-AAG GGCTCTGCAC
Dc2       ACATGCGGGG AAATCCTCCA GAGATGGGGG GTCCTT-AGG GGTCCCGCAC
          901                                  930
AgKG'84/4 AGGTGGTGCA TGGCTGTCGT CAGCTCGTGT
Cn3       AGGTGGTGCA TGGCTGTCGT CAGCTCGTGT
Dat.      AGGTGGTGCA TGGCTGTCGT CAGCTCGTGT
Dc2       AGGTGGTGCA TGGCTGTCGT CAGCTCGTGT
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Figure 1. Alignment of a part of 16S rRNA nucleotide sequences (129-1075 *E. coli* positions) from the endophyte of *Datisca* nodules (Dat) and *Frankia* strains AgKG'84/4, Cn3 and Dc2. Base positions in the sequence are numbered consecutively and nucleotide 1 corresponds to nucleotide 129 in the standard *E. coli* 16S rRNA numbering (Woese *et al.* 1983). Gaps (-) are introduced to facilitate the alignment. The target region of probe FP is underlined and two mismatches in this region in the Dc2 sequence are given in bold letters.

between the *Casuarina*-infective strains and endophytes of *Coriaria* spp. (Nick *et al.* 1992).

Relatively low homology values of about 95% were obtained between the sequences of Dc2 and the nodule endophyte of *Datisca cannabina* (Table 2). The phylogenetic tree (Fig. 2) shows a quite close relatedness of strain Dc2 to strain Cn7 obtained from *Coriaria nepalensis* (Mirza *et al.* 1991), but a distant relatedness to the nodule endophyte from *Datisca cannabina*. Analysis of the target sequence of probe FP of strain Dc2 showed two substitutions (Fig. 1). Even though the presence of the *Frankia* genus sequence has been shown for most *Frankia* strains, some exceptions have been reported (Hahn *et al.* 1990a; Harry *et al.* 1991; Mirza *et al.* 1992). Moreover, relatedness of Dc2 to a number of *Frankia* strains has also been shown on the basis of identical fatty acid patterns (Mirza *et al.* 1991). Therefore, it can not be ruled out that the selection of clones harbouring 16S rRNA

Table 2. Homology values (%) among partial 16S rRNA sequences of *Frankia* spp. based on the analysis of 930 nucleotides.

	1	2	3	4	5	6	7	8
1. Ag45/Mut15[1]	-							
2. AgKG'84/4	95.9	-						
3. ORS 020606[2]	95.1	97.7	-					
4. AgB1.9[3]	96.6	95.8	94.1	-				
5. Cn3	95.1	97.9	96.8	95.4	-			
6. Cn7[4]	94.7	97.2	96.5	95.5	97.3	-		
7. *Coriaria* nodule[4] endophyte	93.8	96.4	97.2	93.1	96.5	95.8	-	
8. *Datisca* nodule endophyte	94.4	96.7	98.3	93.4	96.5	95.5	98.7	-
9. Dc2	94.4	96.5	96.2	95.2	96.7	98.2	95.0	95.1

[1] Hahn *et al.* 1989b; [2] Normand *et al.* 1992; [3] Hahn unpublished results; [4] Mirza *et al.* 1992.

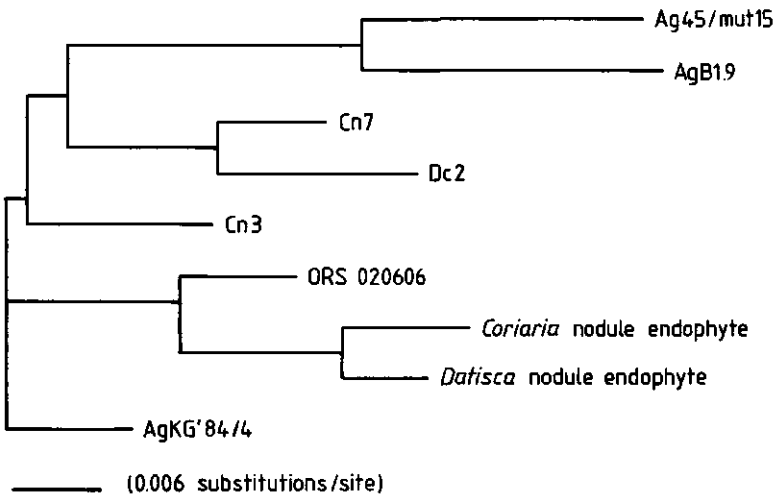

Figure 2. Phylogenetic tree showing relationships of the endophyte of *Datisca cannabina* and strains Dc2, Cn3 and AgKG'84/4 with *Frankia* strains obtained from *Alnus* (Ag45/Mut15; Hahn *et al.* 1989b, AgB1.9; Hahn unpublished), *Casuarina* (ORS 020606; Normand *et al.* 1992), *Coriaria* (Cn7; Mirza *et al.* 1992) and the endophyte within the nodules of *Coriaria nepalensis* (Mirza *et al.* 1992). The neighbor-joining tree was obtained by using the aligned 930 nucleotides sequence information from 129-1075 (*E. coli* 16S rRNA positions).

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          1                                                           60
ArI3      CAG CAG AAC ACC ATG GCG GCC ATG GCC GAG ATG GGC CAG CGG GTC ATG ATC GTC GGG TGT
Cor       CAG CAG AAC ACC ATG GCC GCA ATG GCT GAG CGT GGC AAC CGG GTC ATG ATC GTC GGT TGC
Dat       CAG CAG AAC ACC ATG GCC GCA CTG GCT GAG CGG GGC AAC CGG GTC ATG ATC GTC GGT TGT
          61                                                          120
ArI3      GAC CCG AAG GCT GAC TCG ACC CGC CTG ATC CTG CAC TCG AAG GCG CAG ACC TCC GTC ATC
Cor       GAC CCG AAG GCG GAC TCG ACC CGT CTC ATC CTG CAC TCC AAG GCC CAG CAC ACC GTC ATC
Dat       GAC CCG AAG GCT GAC TCG ACC CGT CTG ATC CTG CAT TCC AAG GCC CAG CAC ACC GTC ATC
          121                                                         180
ArI3      CAG CTC GCG GCC GAG AAG GGT TCG GTC GAG GAC CTG GAG CTC GAC GAG GTG CTC GTC GAG
Cor       GAG AAG GCG GCC GAG AAG GGC TCG GTG GAG GAC CTC GAG CTG GAA GAG GTT CTG CTC GAG
Dat       GAG AAG GCC GCC GAG AAG GGC TCG GTG GAG GAC CTC GAG CTC GAG GAG GTC CTG CTC GAG
          181                                                         240
ArI3      GGC CAG TGG GGC ATC AAG TGC GTC GAG TCC GGT GGC CCG GAG CCG GGC GTC GGC TGC GCC
Cor       GGC GCA TGG AAC ATC CGG TGT GTC GAG TCG GGT GGT CCG GAG CCG GGT GTC GGC TGC GCC
Dat       GGC GCG TGG AAC ATT CGG TGT GTC GAG TCC GGT GGC CCG GAG CCG GGT GTC GGC TGC GCC
          241                                                         300
ArI3      GGC CGT GGC GTC ATC ACC TCC ATC ACC TAC CTG GAG GAG GCC GGC GCG TAC GAG AAC CTC
Cor       GGC CGA GGT GTC ATC ACC GCC ATC ACC TTC CTG GAG GAG AAC CGC GCC TAC GAG AAC CTC
Dat       GGC CGA GGT GTC ATC ACC GCC ATC ACC TTC CTG GGG GAG AAC GGC GCC TAC GAG GAC CTC
          301                                                         360
ArI3      GAC TTC GTG ACC TAC GAC GTC CTC GGT GAC GTT GTG TGT GGT GGC TTC GCG ATG CCG ATC
Cor       GAC TTC GTC ACC TAC GAC GTC CTC GGT GAC GTC GTG TGC GGT GGT TTC GCC ATG CCG ATC
Dat       GAC TTC GTC ACC TAC GAC GTC CTC GGT GAC CTC GTC TGT GGC GGG TTC GCC ATG CCG ATC
          361                                                         420
ArI3      CGC CAG GGC AAG GCG CAG GAG ATC TAC ATC GTC ACC TCC GGC GAG ATG ATG GCG ATG TAC
Cor       CGC CAG GGC AAG GCC CAG GAG ATC TAC ATC GTC ACC TCC GGT GAG ATG ATG GCC ATG TAC
Dat       CGC CAG GGC AAG GCC CAG GAG ATC TAC ATT GTC ACC TCC GGT GAG ATG ATG GCC ATG TAC
          421                                                         480
ArI3      GCG GCG AAC AAC ATC GCC CGA GGC ATC CTG AAG TAC GCG CAC TCC GGC GGC GTC CGG CTC
Cor       GCG GCG AAC AAC ATT GCC CGA GGC ATC CTC AAG TAC GCC CAC AGC GGT GGT GTC CGC CTC
Dat       GCG GCG AAC AAC ATT GCC CGA GGC ATC CTC AAG TAC GCC CAC AGC GGT GGT GTC CGC CTC
          481                                                         540
ArI3      GGC GGG CTC ATC TGC AAC AGC CGC AAG ACC GAC CGC GAG GAC GAG CTG ATC ATG GAG CTC
Cor       GGT GGG CTG ATC TGC AAC AGC CGT AAC ACC GAC CGT GAG GAC GAG CTC ATC ATC GAG CTC
Dat       GGT GGG CTG ATC TGC AAC AGC CGT AAC ACC GAC CGT GAG GAC GAG CTC ATC ATC GAG CTC
          541                                                         600
ArI3      GCC CGC CGC CTC AAC ACC CAG ATG ATC CAC TTC ATC CCG CGT AAC AAC GTC GTG CAG CAC
Cor       GCC CGC CGC CTG AAC ACC CAG ATG ATC CAC TTC GTT CCG CGT GAC AAC ATC GTC CAG CAC
Dat       GCC CGC CGC CTG AAC ACC CAG ATG ATC CAC TTC GTT CCG CGT GAC AAC ATC GTC CAG CAC
          606
ArI3      GCC GAG
Cor       GCC GAG
Dat       GCC GAG
```

Figure 3. Alignment of partial *nifH* sequences from the endophytes of *Coriaria nepalensis* (Cor) and *Datisca cannabina* (Dat) nodules with that of *Frankia* ArI3 sequence (*nifH* positions 51-657; Normand *et al.* 1988).

amplification products of DNA from nodules of *Datisca cannabina* by hybridization with probe FP excluded clones containing amplification products identical or similar to that obtained from strain Dc2.

Comparison of *nifH* sequences (Fig. 3) based on 606 nucleotides (51-657 *nifH* positions; Normand *et al.* 1988) shows that the *nifH* sequences amplified from *Coriaria* and *Datisca* nodules are highly related and differ in only 23 nucleotides. Both sequences showed 87% nucleotide homology to the *nifH* of *Frankia* strains ArI3 and HRN18a (Normand *et al.* 1988; Normand and Bousquet 1989), whereas the homology value between *Coriaria* and *Datisca* nodule endophytes was over 96%. Amino acid sequences translated from the nucleotide sequences of *nifH* from *Coriaria* and *Datisca* nodules showed even higher homology (98%).

At the amino acid level, the sequences from *Coriaria* and *Datisca* nodules showed 90% and 89% identity, respectively, to that of ArI3 (Normand *et al.* 1988). Among the nitrogen-fixing microorganisms other than *Frankia*, the *nifH* sequences of *Anabaena* (Mevarech *et al.* 1980) and *Azotobacter* (Brigle *et al.* 1985) were found highly related to those amplified from both *Coriaria* and *Datisca* (84% and 80% homology, respectively). Similar results were previously reported for *Frankia* strains ArI3 and HRN18a (Normand *et al.* 1988; Normand and Bousquet 1989).

Attempts to amplify *nifH* from strains obtained from *Datisca* (Dc2) and *Coriaria* (Cn3 and Cn7) were not successful. These atypical strains failed to grow in N-free media and did not reduce acetylene. It has been suggested that atypical strains have lost the ability to fix nitrogen but retained the ability to associate with the host plants as co-symbionts or as contaminants in the surface layers of the nodule epidermis where they escape disinfection treatments (Hahn *et al.* 1990b).

The results of both *nifH* and 16S rRNA sequence comparison of the endophytes of *Coriaria* and *Datisca* suggest that the endophytes in nodules of these plants are closely related *Frankia* strains. This relatedness of the endophytes was also suggested by the strikingly similar nodule structures and morphology of the endophytes in the nodules (Hafeez *et al.* 1984a; Mirza *et al.* submitted). Isolation of nitrogen-fixing endophytes from both *Coriaria* and *Datisca* nodules will help in further confirmation of the relatedness of the endophytes on the basis of morphology, physiology and/or genetics.

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Chapter 7

PCR-amplified 16S rRNA sequence analysis to confirm nodulation of *Datisca cannabina* L. by the endophyte of *Coriaria nepalensis* Wall.

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Plant and Soil (submitted)

PCR-amplified 16S rRNA sequence analysis to confirm nodulation of *Datisca cannabina* L. by the endophyte of *Coriaria nepalensis* Wall.

Key words: Nitrogen fixation, Actinorhizal plants, *Frankia*, Cross-inoculation, PCR, 16S rRNA.

Abstract

Different *Frankia* strains and crushed nodule suspensions were tested for their ability to nodulate *Coriaria nepalensis* and *Datisca cannabina*. *Datisca cannabina* seedlings were nodulated effectively by both crushed nodule suspension from *Coriaria nepalensis* and *Datisca cannabina*. The origin of the endophyte in *Datisca* nodules induced by crushed nodules of *Coriaria* was confirmed by comparing partial PCR-amplified 16S rRNA sequences with those of the endophytes of both plants. *Coriaria* seedlings could only be nodulated by crushed nodule suspensions of *Coriaria nepalensis*. All pure cultures of *Frankia* used as a single inoculum source or in combinations with a nodule filtrate, failed to induce nodulation on *Coriaria*. Two atypical *Frankia* strains Cn3 and Cn7 isolated from *Coriaria* nodules showed no acetylene reduction activity and did not induce nodulation on the host seedlings.

Introduction

Early attempts to classify the genus *Frankia* were based on results of cross-inoculation trials (Becking, 1970) before the first pure culture became available (Callaham *et al.*, 1978). Crushed nodule suspensions, the most common source of inoculum used in these studies, offer difficulties as the presence of more than one *Frankia* strain as a contaminant or co-symbiont cannot be ruled out (Benson and Hanna, 1983; VandenBosch and Torrey, 1983). On the basis of the host specificity of 50 pure-cultured strains, Baker (1987) divided *Frankia* into four host specificity groups and pointed out the existence of additional groups depending on the availability of more pure cultures.

When available, pure cultures of *Frankia* provide the most convenient and efficient source of inoculum (Torrey, 1990). However, pure-cultured cells of *Frankia* are not

available from several genera out of the 24 currently known to bear *Frankia* induced root nodules (Baker and Schwintzer, 1990). *Frankia* from the actinorhizal families *Coriariaceae* and *Datiscaceae* have proved particularly difficult to bring into pure culture (Allen *et al.*, 1966; Chaudhary and Mirza, 1987; Hafeez, 1983; Nick *et al.*, 1992). Only non-infective strains have been isolated from *Coriaria nepalensis* (Mirza *et al.*, 1991; Mirza *et al.*, 1992) and *Datisca cannabina* (Hafeez, 1983). These strains lack some physiological and morphological characteristics of typical *Frankia* strains obtained from other actinorhizal plants.

Sequence analysis of 16S rRNA is being extensively used to study phylogenetic relationships of the microorganisms (Olsen *et al.*, 1986; Pace *et al.*, 1986; Woese, 1987). Amplification of the 16S rRNA gene by PCR from pure cultures or directly from the uncultured microorganisms in the environmental samples has greatly facilitated evolutionary and ecological studies (Giovannoni *et al.*, 1990; Ward *et al.*, 1990; Ward *et al.*, 1992). Sequence information from a number of *Frankia* strains is now available and has been used to characterize and identify strains and to determine phylogenetic relationships (Hahn *et al.*, 1989; Hahn *et al.*, 1990; Mirza *et al.*, 1992; Nazaret *et al.*, 1991; Nick *et al.*, 1992; Normand *et al.*, 1992)

The present contribution deals with the results of nodulation trials on *Coriaria nepalensis* and *Datisca cannabina* seedlings using *Frankia* pure cultures and crushed nodule suspensions. Nodulation of the *Datisca* seedlings by the endophyte of *Coriaria* nodules was confirmed by analysis of the 16S rRNA sequences amplified from the nodules by PCR.

Materials and methods

***Frankia* strains**: The *Frankia* strains used in the present study included isolates from *Alnus* (Ag45/Mut15, Hahn *et al.*, 1988; LDAgp1r1, LDAgn1, Burggraaf, 1984), *Coriaria* (Cn3, Cn7, Mirza *et al.*, 1991) and *Datisca* (Dc2, Dc12, Hafeez, 1983). All *Frankia* strains were grown in P+N broth (Meesters *et al.*, 1985) at 30°C for 14 days. The cells were collected by centrifugation at 4000 xg for 10 minutes and used as an inoculum. For measuring acetylene reduction activity, strains Cn3 and Cn7 were grown in P-N medium (Meesters *et al.*, 1985).

Preparation of inoculum: Root nodules of *Coriaria nepalensis* and *Datisca cannabina* were collected from the plants grown in the greenhouse. Nodules used to inoculate *Coriaria* and *Datisca* plants were collected from the Murree and Swat areas of Pakistan, respectively. The nodules were washed with liquid detergent (Decondi 118, Otares, Enschede, The Netherlands) for 10 minutes followed by washing with sodium hypochlorite (1%, W/V) for 5 minutes. The nodules were washed three times with sterile water, homogenized with a pestle and mortar and suspended in water (10 g fresh wt./ l).

Frankia pure cultures were washed twice with sterile distilled water and homogenized by passing through a syringe needle (0.8mm dia.). A 10 ml of cell suspension (1 g fresh wt./ l) was used for each plant.

The filtrate from *Coriaria* and *Datisca* nodules was prepared by filtering nodule suspension through a 0.22 μm filter (Rodriguez-Barrueco *et al.*, 1988) and used in combination (1 ml/10 ml of the inoculum) with crushed nodule suspensions or pure cultures of *Frankia*.

Plant inoculations: Seeds of *Coriaria nepalensis* and *Datisca cannabina* were surface sterilized with 1% sodium hypochlorite (w/v) for 10 minutes and washed three times with sterile water. The seedlings were grown in sterile perlite moistened with Hoagland solution (10X diluted) in a growth chamber (thermoperiod, 24/21°C; photoperiod, 16/8 hours day/night). One seedling was grown in each plastic pot of one liter capacity. Six weeks old seedlings were inoculated with crushed nodule suspensions or *Frankia* pure cultures. For each treatment, 6 replicates were used and the same number of seedlings were kept as uninoculated controls. Ten ml of inoculum were added to the partly exposed root system of each seedling. Each pot was given 100ml of N-free nutrient solution (Hoagland solution; 10X diluted) once a week.

Acetylene reduction activity: Root nodules were collected from the plants three months after inoculation and tested for acetylene reduction activity on a gas chromatograph (Packard, Model 417) equipped with FID and a 3.25 mm x 1m porapak R, 80-100-mesh, filled column. Acetylene reduction activity of the strains Cn3 and Cn7 isolated from *Coriaria* nodules was also checked.

Extraction of Nucleic Acids: Root nodules of *Datisca cannabina* were collected from plants inoculated with crushed nodule suspension from *Coriaria nepalensis*. The nodules were washed with liquid detergent (Decondi 118, Otares, Enschede, The Netherlands),

for 5 minutes followed by three washings with sterile water. For extraction of nucleic acids, 200 mg nodules were homogenized with pestle and mortar in 500 μl TE buffer and transferred to an Eppendorf tube. The homogenate was sonicated (Sonics & Materials Sonifier, CT, USA) for two minute and the plant cell debris was pelleted by centrifugation at 13000 xg for 10 minutes. The supernatant was extracted twice with phenol/chloroform, followed by two extractions with chloroform/isoamyl alchol (24:1). After adding 1/10 vol. of sodium acetate (3 M, pH 5.2) and 0.5 vol. of isopropanol, the supernatant was incubated at -70°C for 20 minutes. The nucleic acids were precipitated by centrifugation at 13000 xg for 20 minutes and the pellet was washed with 70% ethanol and dryed under vacuum. The nucleic acid pellet was dissolved in 50-100 μl of TE buffer and used as a template for PCR.

PCR amplifications, cloning and sequencing: Amplification of partial 16S rRNA gene was carried out from template DNA obtained from the nodules. Primer P124 (Embley *et al.*, 1988; complementary primer with an additional *Bam*HI site 5'CA*CGGATCC*GGACGGGTGAGTAACACG) and P1115 (Embley *et al.*, 1988; with an additional *Hind*III site 5'GTG*AAGCTT*AGGGTTGCGCTCGTTG) were used to amplify the partial 16S rRNA gene. Each reaction mixture (100 μl) contained, 0.2 μl Super Tth Taq polymerase (5U/μl; HT biotechnology, UK), 10 μl of 10X Super Tth buffer; dNTPs (final concentration 200 μM each), 1 μl (100 ng/μl) of each primer (P124 & P1115) and 1 μl nucleic acid preparation. Thirty rounds of temperature cycling (94°C for 1 minute, 50°C for 2 minutes and 72°C for 3 minutes) were followed by a final incubation at 72°C for 7 minutes.

The amplification products were treated with proteinase K (BRL, final concentration 60 ng/100 μl) at 37°C for 15 minutes (Crowe *et al.*, 1991), phenol/chloroform extracted and precipitated with ethanol. The DNA pellets were redissolved in 15 μl of TE and digested with *Bam*HI and *Hind*III. The DNA-fragments were obtained by TAE-agarose gel electrophoresis followed by excision of the fragments and subsequent purification by the Gene-Clean procedure (Bio 101, La Jolla, California). Cloning of the amplification products into pUC19 was done by standard methods (Maniatis *et al.*, 1982).

Cloned PCR products were sequenced with the amplification primer 124, using the T7 DNA polymerase kit (Pharmacia) following the manufacturer's instructions.

Results and discussion

Nodulation of *Coriaria nepalensis* seedlings was achieved only with nodule homogenates of the same plant species. *Frankia* strains LDAgp1r1, LDAgn1, Cn3, Cn7 and the nodule suspension from *Datisca cannabina*, when used alone or in combination with filtrate from *Coriaria* nodules, failed to nodulate seedlings of *Coriaria nepalensis*. Nodulation in *Coriaria myrtifolia*, the European representative of the family *Coriariaceae*, has been reported by using crushed nodule suspensions from other *Coriaria* species and several species of *Myrica* (Miguel *et al.*, 1978; Rodriguez-Barrueco and Miguel, 1979; Cervantes *et al.*, 1988). Moreover, it was shown earlier that a filtered nodule extract from *C. myrtifolia* was required to achieve effective nodulation by *Alnus glutinosa* crushed nodule suspensions and *Frankia* pure cultures (LDAgp1r1, LDAgn1) obtained from this actinorhizal species (Cervantes *et al.*, 1988; Rodriguez-Barrueco *et al.*, 1988). In spite of several attempts, similar results could not be obtained with *Coriaria nepalensis* inoculated with *Frankia* strains LDAgp1r1 and LDAgn1 in the presence of nodule filtrates.

The isolates Cn3 and Cn7 from *Coriaria nepalensis* root nodules failed to reduce acetylene and did not induce nodulation on *Coriaria* or *Datisca* seedlings. These atypical strains were found to be related to other *Frankia* strains on the basis of their characteristic sporangia formation, fatty acid pattern and 16S rRNA analysis (Mirza *et al.*, 1991; Mirza *et al.*, 1992). It has been proposed that the atypical strains have lost ability to nodulate and fix nitrogen, but maintained association with the host plant as a contaminant or cosymbiont within the nodules (Hahn *et al.*, 1988).

Nodulation in all seedlings of *Datisca cannabina* was induced not only with nodule suspensions of *Datisca cannabina,* but also with those of *Coriaria nepalensis.* Effectiveness of the *Datisca* nodules induced by nodule homogenates of *Coriaria* was indicated both by the physical appearence of the seedlings, as well as by acetylene reduction activity (4.91 ± 1.44 μmol C_2H_4/ g fresh nodule wt^{-1}. h^{-1}.). Unpublished results indicate that *Datisca cannabina* can also be nodulated easily with nodule homogenates of *Datisca glomerata* and rhizosphere soil of *Ceanothus americanus* (Torrey, 1990). However, nodule suspensions from *Alnus nitida* were not found effective for nodulation of *Datisca* plants (Hafeez *et al.*, 1984).

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          1                                                        50
a)        AACCTGCCCC GAGCTCTGGG ATAACTTCGG GAAACCGGGG CTAATACCGG
b)        AACCTGCCCC GAGCTCTGGG ATAACTTCGG GAAACCGGGG CTAATACCGG
c)        AACCTGCCCC GAGCTCTGGG ATAACTTCGG GAAACCGGGG CTAATACCGG

          51                                                      100
a)        ATATGACACT ACTGGACATC TGGTGGTGTG GAAAGATTTA TCGGCTTGGG
b)        ATATGACACT ACTGGACATC TGGTGGTGTG GAAAGATTTA TCGGCTTGGG
c)        ATATGACATT GCCGGGCATC TGGTGGTGTG GAAAGATTTA TCGGCTCGGG

          101                                                     150
a)        ATGGGCCCGC GGCCTATCAG CTTGTTGGTG GGGTAACGGC CTACCAAGGC
b)        ATGGGCCCGC GGCCTATCAG CTTGTTGGTG GGGTAACGGC CTACCAAGGC
c)        ATGGGCCCGC GGCCTATCAG CTTGTTGGTG GGGTGATGGC CTACCAAGGC
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Figure 1. Alignment of partial 16S rRNA sequences PCR-amplified from nodules of *Coriaria nepalensis* (a; Mirza *et al.*, 1992), *Datisca cannabina* (c; Mirza *et al.*, (a) submitted) and *Datisca* nodules (b) induced by crushed nodule suspension of *Coriaria nepalensis*. First nucleotide in the sequence corresponds to the nucleotide 129 in the standard *E. coli* 16S rRNA numbering (Woese, *et al.*, 1983). Mismatches in the sequence are given in bold letters.

The origin of the endophyte in the nodules of *Datisca* induced by crushed nodule suspensions of *Coriaria* was confirmed by 16S rRNA sequence analysis. Part of the 16S rRNA gene was amplified from the nodules by PCR for sequence comparison with those of the nodule endophytes of *Coriaria* and *Datisca* (Mirza *et al.*, 1992; Mirza *et al.*, (a) submitted). In a stretch of 150 nucleotides, no sequence difference was observed among the five clones originating from two different PCR-products. This sequence, which covered the variable region V2 of 16S rRNA (Neefs *et al.*, 1991), was identical to the sequence of the *Coriaria* nodule endophyte (Fig. 1). Sequence information especially from the V2 region was previously found sufficient to discriminate between the endophytes of *Coriaria* and *Datisca* root nodules (Mirza *et al.*, (b) submitted). These results indicate that the *Datisca* nodules were indeed induced by the endophyte of *Coriaria* and not by the *Datisca* nodule endophyte through contamination.

The present results indicate the usefulness of this molecular technique to identify strains in cross-inoculation experiments with complex inoculum sources like nodules or soil samples.

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Chapter 8

Genetic diversity of *Datisca*-compatible *Frankia* strains, as determined by sequence analysis of the PCR-amplified 16S rRNA gene.

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Appl. Environ. Microbiol (submitted)

Genetic diversity of *Datisca*-compatible *Frankia* strains, as determined by sequence analysis of the PCR-amplified 16S rRNA gene.

Summary

The presence of *Frankia* strains in soil samples collected from northern areas of Pakistan was detected by inoculating *Coriaria nepalensis* and *Datisca cannabina* plants. Abundance of compatible *Frankia* strains in some areas was indicated by profuse nodulation of the host plants, while soil samples from other localities failed to induce nodulation. An oligonucleotide probe (COR/DAT) directed against 16S rRNA of the endophytes of *Coriaria* and *Datisca* was developed that did not cross-react with RNA of pure *Frankia* strains isolated from other hosts. Genetic diversity among *Frankia* strains nodulating *Datisca cannabina* was determined by sequence analysis of partial 16S rRNA gene PCR-amplified from nodules induced by soil samples from different localities. Four types of *Frankia* sequences and one non-*Frankia* sequence were detected by hybridization with a *Frankia* genus probe and the COR/DAT probe as well as by sequence analysis of the cloned PCR-products. The significance of the oligonucleotide probes in studies involving PCR amplified targets from environmental samples is discussed.

Introduction

Nitrogen-fixing root nodules induced by the actinomycete *Frankia* have been reported in more than 200 non-leguminous plant species (4). After the first successful isolation of a *Frankia* strain from *Comptonia peregrina* (10), hundreds of isolates have been purified from different actinorhizal nodules. With the exception of one attempt (3), all *Frankia* strains were obtained from nodules where the endophyte is found in relative abundance and purity as compared to soil. As research on *Frankia* remained focused mainly on its biology in pure culture or in root nodules, relatively little published information is available concerning the asymbiotic stage or extra nodular survival of the endophyte in soil (7,20,27,41,43,44,47,54). These studies confirmed the existence of viable *Frankia* cells in soil by successful nodulation of the host plants. Increasing interest in the practical

application of actinorhizal plant species (14,50) in forestry, soil erosion control and soil improvement projects requires further research on *Frankia* populations in soil.

Successful nodulation of plants inoculated with soil can confirm the existence of compatible *Frankia* cells, but the possibility of the presence of more than one infective strain cannot be ruled out. This can be proved by isolation and characterization of the endophyte in pure culture. Genetic diversity among *Frankia* strains in pure culture obtained from different or the same host plants has been shown previously by DNA-DNA hybridization studies (1,8,18), restriction fragment pattern analysis (2,8,16), total cellular protein patterns (5,6) and 16S rRNA sequence analysis (23,25,33,36).

The isolation procedures are tedious and particularly difficult with some actinorhizal plants like *Coriaria* and *Datisca*, where isolation attempts have repeatedly failed. For studying genetic diversity of the endophyte laborious isolation procedures can be avoided by amplification of 16S rRNA gene using polymerase chain reaction and sequence analysis (40,49). Highly variable regions like V2 and V6 (37) within the 16S rRNA provide an opportunity to synthesize complementary oligonucleotide probes for identification and detection of specific microorganisms in pure cultures or in environmental samples (19,40,42,48,49,51). In the V6 region of 16S rRNA, sequences amplified from *Datisca* and *Coriaria* nodules were found to be identical (33,34), but quite distinct from those of the *Frankia* strains from other hosts (23,36,38,39). 16S rRNA sequence analysis has also been successfully applied to characterize non-infective *Frankia* strains isolated from *Coriaria* and *Datisca* nodules (33,34).

The present study deals with the detection of *Coriaria* and *Datisca* compatible *Frankia* strains with a 16S rRNA targeted oligonucleotide probe. Genetic diversity of *Frankia* strains associated with *Datisca* nodules was determined by sequence analysis of PCR-amplified 16S rRNA gene.

Materials and Methods

Collection of nodule and soil samples: Soil samples were collected from different areas in the northern part of Pakistan. Samples included rhizosphere and non-rhizosphere soil as well as soil from sites effected by landslides in those areas. Soil samples were transported to The Netherlands in polythene bags. Root nodules were also collected from *Coriaria*

nepalensis or *Datisca cannabina* plants growing in the same areas. Both nodules and the soil samples were used to inoculate seedlings of *Coriaria nepalensis* and *Datisca cannabina*.

Seeds of *Coriaria* and *Datisca* were collected from the Murree and Swat areas of Pakistan, respectively. The seeds were surface sterilized with 1% sodium hypochlorite (w/v) and grown in sterile perlite in a growth chamber (thermoperiod, 24/21°C; photoperiod, 16/8 hours day/night). An inoculum of 100 ml (10g soil/l or 1g/l crushed nodule suspension) was added to each pot of 1 litre capacity with *Coriaria* or *Datisca* seedlings. Each pot was given 100 ml of N-free nutrient solution (10x diluted Hoagland solution) once a week. Root nodules were collected from the plants four months after inoculation and tested for acetylene reduction activity on a gas chromatograph (Packard, Model 417) equipped with FID and a 3.25 mm x 1m porapak R (80-100-mesh) filled column.

Oligonucleotides: Sequences of the oligonucleotides used as probes or primers in PCR are given in table 1.

Table 1. List of the oligonucleotides used as probes or primers in PCR.

Primer	Sequence*	Restriction site	Reference
P 124	5'CAC*GGATCC*GGACGGGTGAGTAACACG	*Bam*HI	17
P 1115	5'GTG*AAGCTT*AGGGTTGCGCTCGTTG	*Hind*III	17
probe FP	5'ATAAATCTTTCCACACCACCAG		24
COR/DAT probe	5'GAGCCCTTACGGACCCCGTATC		This paper
Dc2 probe	5'GCGGGACCCCTAAGG		This paper

* restriction sites are given in italics.

Bacterial strains: The *Frankia* strains used in the present study included isolates from *Alnus* (Ag45/Mut15, AgKG'84/4, and atypical strains AgB1.9, AgW1.1; 22), *Casuarina* (CeD ORS02060; 15; CcI3; 53), *Colletia* (Cc1.17; 30), *Comptonia* (CpI2; 30), *Coriaria* (Cn3, Cn7; 32), *Datisca* (Dc2, Dc12; 21), *Elaeagnus* (EAN1; 28) and *Hippophae* (HrI1; Baker, unpublished). All *Frankia* strains were grown in P+N broth (30) at 30°C for 14

days, whereas *Streptomyces lividans* TK24 (D. A. Hopwood, John Innes Institute, Norwich, UK) was grown in R5 liquid medium (26) supplemented with 10% sucrose at 30°C for 2 days.

Cells from pure cultures were collected by centrifugation at 5000 xg for 10 minutes and washed with TE buffer. Nucleic acids from *Frankia* pure cultures and *Coriaria* nodules were extracted by bead beating (see below).

Extraction of Nucleic Acids from nodules: Root nodules of *Datisca cannabina* were collected from plants inoculated with soil from five different localities. The nodules were washed with liquid detergent (Decondi 118, Otares, Enschede, The Netherlands), for five minutes followed by three washings with sterile water. For extraction of nucleic acids, 200 mg nodules were homogenized in a pestle and mortar with 500 μl TE buffer and transferred to an Eppendorf tube. After adding 200 mg of glass beads (0.1-0.11 mm dia.), the homogenate was shaken vigoursly in a bead beater (Braun, Melsungen, Germany) for 10 minutes. The nodule debris and glass beads were pelleted by centrifugation at 13000 xg for 10 minutes. The supernatant was extracted twice with phenol/chloroform, followed by two extractions with chloroform/isoamylalchol (24:1). After adding 1/10 vol. of sodium acetate (3 M, pH 5.2) and 0.5 vol. of isopropanol, the supernatant was incubated at -70°C for 20 minutes. The nucleic acids were precipitated by centrifugation at 13000 xg for 20 minutes and the pellet was washed with 70% ethanol and dried under vacuum. The nucleic acids pellet was dissolved in 50-100 μl of TE buffer and used for filter hybridizations and PCR amplifications.

PCR amplifications and cloning: Amplification of partial 16S rRNA gene was carried out from template DNA in duplicate from nodules induced by each of the five soil inocula. Each reaction mixture (100 μl) contained, 0.2 μl Super Tth Taq polymerase (5U/μl; HT biotechnology, UK), 10 μl of 10X Super Tth buffer, dNTPs (final concentration 200μM each), 1 μl (100 ng/μl) of each primer (124 & 1115) and 1 μl nucleic acid preparation. Thirty-five rounds of temperature cycling (94°C for 1 minute, 50°C for 2 minutes and 72°C for 3 minutes) were followed by incubation at 72°C for seven minutes.

The amplification products were treated with proteinase K (BRL, final concentration 60 ng/100 μl) at 37°C for 15 minutes (13), phenol/chloroform extracted and precipitated with ethanol. The DNA pellets were redissolved in 15 μl of TE and digested with

restriction enzymes *Bam*HI and *Hind*III (BRL). The DNA-fragments were obtained by TAE-agarose gel electrophoresis followed by excision of the fragments and subsequent purification by the Gene-Clean procedure (Bio 101, La Jolla, California). Cloning of the amplification products into pUC19 was done by standard methods (29).

Filter hybridization: Plasmid DNA was isolated by the alkaline lysis method (29). Dot blot hybridizations were performed on Hybond-N^+ nucleic acid transfer membranes (Amersham). Plasmid DNA from 200 clones was used in dot blot hybridizations. Each inoculum sampling area (Athmokam, Chitral, Kaghan, Murree, Puddar) was represented by 40 clones originating from two different PCR products. Plasmid DNA was denatured with equal volumes of 0.5 N NaOH. The denatured plasmid DNA (100 ng/dot) was applied with a Hybri.Dot manifold (BRL). The nucleic acids were immobilized by UV light and hybridized according to Church and Gilbert (12). Oligonucleotide probes were 5'-labeled using phage T_4 polynucleotide kinase (BRL) and 10-20 μCi of [γ-^{32}P]adenosine-5'-triphosphate (3000 Ci/mmol; Amersham) (29). The blots were initially washed with 5x SSC/1% SDS at 30°C for 15 minutes and then with 1% SSC/0.1% SDS at 50°C for 15 minutes (29).

Sequencing: Cloned PCR products were sequenced with amplification primers 124 and 1115, using the T7 DNA polymerase kit (Pharmacia) following the manufacturer's instructions.

Results and Discussion

Nodulation of *Coriaria* and *Datisca*: The nodulation experiments using soil suspensions from different areas as inoculum for *Datisca* plants indicated a wide distribution of compatible *Frankia* strains. Nodulation was achieved with soil inocula from all five localities tested (Table 2). This included one sampling area (Murree), where *Coriaria nepalensis* was recorded as the only representative of actinorhizal plants (11). *Datisca* plants were nodulated with all soil samples used as an inoculum, with the exception of one collected from an eroded area of Puddar. Soil samples from seven different sites were used as an inoculum for *Coriaria* plants. Poor nodulation of *Coriaria*, as compared to *Datisca* plants, was observed. With soil inocula from two sites (Chitral & Kaghan), only one plant was nodulated, while nodulation was absent altogether when soil samples from

Table 2. Nodulation and acetylene reduction of *Coriaria* and *Datisca* plants using inocula* collected from different localities of Pakistan

Locality/ Inoculum source	*Coriaria nepalensis*			*Datisca cannabina*		
	No. of plants nodulated[1]	No. of nod./nodulated plant[2]	μmol.C_2H_4/ g fresh nod.wt./h.[3]	No. of plants nodulated[1]	No. of nod./nodulated plant[2]	μmol.C_2H_4/ g fresh nod.wt./h[3].
Abbotabad						
E	4	25 ± 15	0.30 ±0.1	ND	ND	ND
N	4	44 ± 22	0.14 ±0.1	ND	ND	ND
NR	4	33 ± 10	0.24 ±0.1	ND	ND	ND
R	4	38 ± 18	0.19 ±0.2	ND	ND	ND
Athmokam						
E	0	0	0	2	2 ± 1	6.58 ±3.6
N	ND	ND	ND	3	2 ± 1	2.72 ±3.8
NR	0	0	0	3	1 ± 1	12.5 ±5.0
R	0	0	0	3	1 ± 1	5.97 ±4.0
Chitral						
E	1	4	0.08	1	1	11.61
N	ND	ND	ND	3	3 ± 2	3.64 ±0.0
NR	0	0	0	3	2 ± 1	1.08 ±0.5
R	0	0	0	1	11	0.42
Kaghan						
E	0	0	0	3	18 ± 9	4.61 ±4.9
N	ND	ND	ND	4	20 ± 16	4.24 ±4.0
NR	1	1	0.19	4	17 ± 10	1.71 ±0.6
R	0	0	0	4	20 ± 14	3.03 ±0.4
Murree						
E	1	1	0.74	3	2 ± 1	5.54 ±3.7
N	3	2 ± 1	0.58 ±0.0	ND	ND	ND
NR	2	1 ± 0	0.22 ±0.0	2	5 ± 2	2.12 ±1.0
R	2	1 ± 0	0.19 ±0.0	2	1 ± 0	2.44 ±3.1
Puddar						
E	0	0	0	0	0	0
N	ND	ND	ND	3	2 ± 1	5.83 ±1.2
NR	0	0	0	1	1	1.09
R	0	0	0	3	5 ± 4	0.97 +0.0
Rawalakot						
E	4	5 ± 2	0.11 ±0.0	ND	ND	ND
N	0	0	0	ND	ND	ND
NR	3	5 ± 5	0.16 ±0.0	ND	ND	ND
R	3	3 ± 3	0.17 ±0.1	ND	ND	ND

*(E= eroded soil; N= nodules from the same plant; NR; non-rhizosphere soil; R= rhizosphere soil).
All four plants used as control remained non-nodulated.
[1] Four plants were used for each treatment. [2] Each value represents the average number of nodules/nodulated plant. [3] Each value represents the average of two determinations. ND = not determined.

Athmokam and Puddar were used. Although *Coriaria* plants have been found to be distributed in Kaghan (11), they were absent from those particular sites where soil samples were collected. Poor nodulation of *Coriaria* has been reported previously both under natural as well as laboratory conditions (9,31). Therefore absence or scarcity of nodulation of *Coriaria* may not be due to the absence of compatible *Frankia* strains in the particular areas.

Exceptionally good nodulation of *Coriaria* and *Datisca* by soil samples collected from Abbottabad and Kaghan, respectively, reflects the abundance of compatible *Frankia* strains in these soils. This also indicates that these soils can be exploited for use as inocula for large scale inoculations. Collection of nodules, the alternate source of inoculum, is laborious and difficult to perform throughout the year. Acetylene reduction by all nodule samples of both plant species confirmed that the endophyte is effectively fixing nitrogen (table 2) and this was also apparent from the physical appearence of the nodulated plants.

Probe design: For the specific detections, a 22mer oligonucleotide probe (COR/DAT) was developed against V6 region of 16S rRNA of the endophyte of *Coriaria* and *Datisca* nodules. The probe hybridized specifically with the 16S rRNA extracted from *Coriaria* nodules and the cloned PCR product from *Datisca* nodules, but did not cross-react with rRNA extracted from pure *Frankia* strains isolated from *Alnus*, *Casuarina*, *Colletia*, *Comptonia*, *Elaeagnus*, *Hippophae* spp. and atypical strains from *Alnus*, *Coriaria* and *Datisca* nodules (Fig. 1 & 2). These results also indicate that the sequence amplified from *Coriaria* nodules originates from a predominant *Frankia* strain. As PCR products from *Datisca* nodules were used as a target for the probe because of our inability to obtain pure RNA, no conclusions can be drawn about the abundance of the strain with this particular sequence.

Screening of the clones: To study the usefulness of 16S rRNA-targeted oligonucleotide probes in ecology of *Frankia* strains associated with *Datisca*, the *Frankia* genus probe and COR/DAT probe were used for screening the clones (Fig. 2). Partial 16S rRNA gene (124-1115 *E. coli* positions) was PCR amplified and cloned from two different DNA extracts of nodules originating from each of the five types of soil inocula used. Out of the total 200 clones screened, three clones originating from nodules induced by soil inocula from Athmokam (A2-12, A2-19) and Kaghan (K2-6) hybridized with probe FP but failed

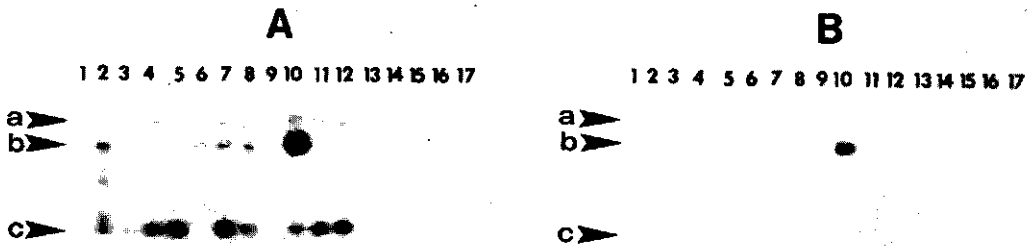

Figure 1. Specific hybridization of the COR/DAT probe with membrane bound rRNA (northern blot) isolated from *Coriaria nepalensis* root nodules (Lane 10). The probe did not hybridize with rRNA of different *Frankia* strains isolated from *Alnus* (Ag45/Mut15, AgKG'84/4, AgB1.9, AgW1.1; Lane 2-5), *Casuarina* (CeD ORS02060 and Ccl3; Lane 6 & 7), *Colletia* (Cc1.17; Lane 8), *Comptonia* (CpI2; Lane 9), *Coriaria* (Cn3, Cn7; Lane 11 & 12), *Datisca* (Dc2, Dc12; Lane 13 & 14), *Elaeagnus* (EAN1; Lane 15) and *Hippophae* (HrI1; Lane 16) as well as with rRNA of *E. coli* (Lane 1) and *Streptomyces lividans* (Lane 17). a = 23S rRNA; b = 16S rRNA; c = sheared nucleic acids. A: northern blot washed at 30°C; B: northern blot washed at 50°C..

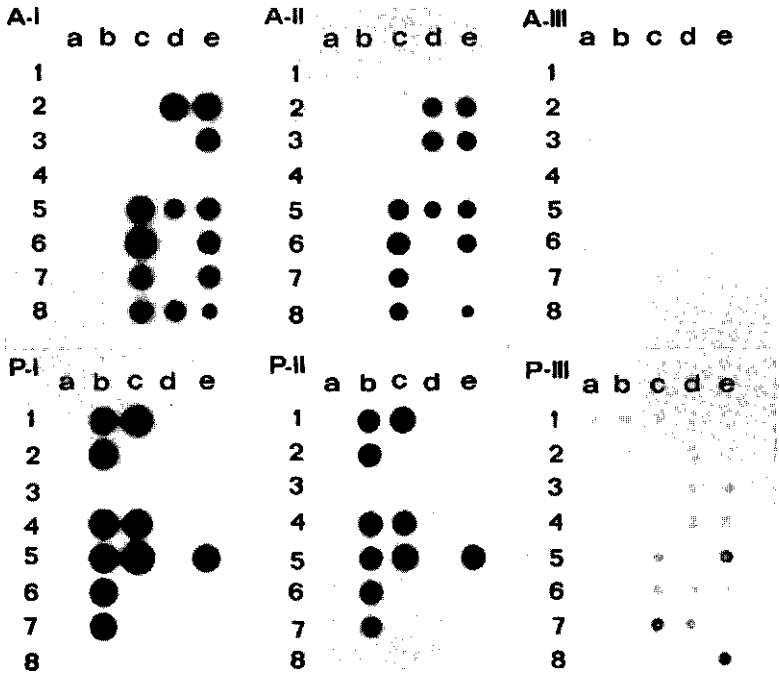

Figure 2. Dot blot hybridization of the oligonucleotide probes with the plasmid DNA from clones with inserts originating from the PCR-amplified 16S rRNA gene from *Datisca cannabina* nodules. Filters representing two areas (Athmokam, Puddar) were prepared with the plasmid DNA from 40 clones and hybridized with the *Frankia* genus probe FP (AI, PI), COR/DAT probe (AII, PII) and the DC2 probe (AIII, PIII). Plasmid DNA from the clones A2-12 (blot A-I, position d8) and A2-19 (blot A-I, position e7) hybridized with the probe FP but did not react with the COR/DAT probe, indicating the presence of a new *Frankia* specific sequence. Presence of a non-*Frankia* sequence was indicated in clone A2-7 (blot A-I, position d3) as it failed to hybridize with the *Frankia* genus probe.

to react with the COR/DAT probe. These results indicate that the three clones represent *Frankia* strains associated with *Datisca* nodules different from those already characterized by 16S rRNA sequence analysis (34). As nodulation of *Datisca cannabina* by the endophytes of other actinorhizal plants has been reported (35,46), detection of more than one *Frankia* sequence in the nodules can be expected. Moreover one clone, originating from nodules induced by a soil inoculum collected from Athmokam (A2-7) hybridized with the COR/DAT probe but did not hybridize with the *Frankia* genus probe FP.

Genetic diversity among *Frankia* strains: To detect any diversity among the clones with inserts reacting with both probes, the variable regions V2 and V6 were sequenced from four clones originating from each of the five soil inocula used in nodulation experiments. All clones showed sequences in the V6 region identical to those reported previously for *Coriaria nepalensis* and *Datisca cannabina* nodule endophytes (33,34). However in the other variable region (V2), three different types of sequences were observed. Sequences A2-1, C2-6 and K2-7 (Fig. 3), detected in all clones corresponding to three areas i.e. Athmokam, Chitral and Kaghan, were identical to one already reported for the *Datisca* nodule endophyte (34). The sequence P1-9, present in clones originating from the nodules induced by the soil collected from Puddar was different from all others, indicating the occurrence of a different *Frankia* strain in this soil. The sequence M2-2 representing four clones corresponding to Murree soil was identical to one published for the *Coriaria* nodule endophyte (33) which is the only actinorhizal plant that grows in this particular area. This confirms the results of our cross-inoculation experiments in which *Datisca* plants were successfully nodulated with crushed nodule inoculum from *Coriaria* from this particular area (35).

Three clones (A2-12, A2-19, K2-6) that only reacted with the *Frankia* genus probe and did not react with the COR/DAT probe, showed a sequence in the V2 region identical to that already reported (34), but had a quite different sequence in the V6 region. Whether this new sequence represents a true endophyte of *Datisca* or a *Frankia* strain present as contaminant or cosymbiont is not yet clear.

One clone (A2-7), that hybridized with the COR/DAT probe but did not react with the *Frankia* genus probe, showed a non-*Frankia* sequence in V2 variable region. Sequence analysis of the V2 variable region indicated very high sequence homology (97%) to the 16S rRNA gene from tobacco chloroplasts (45).

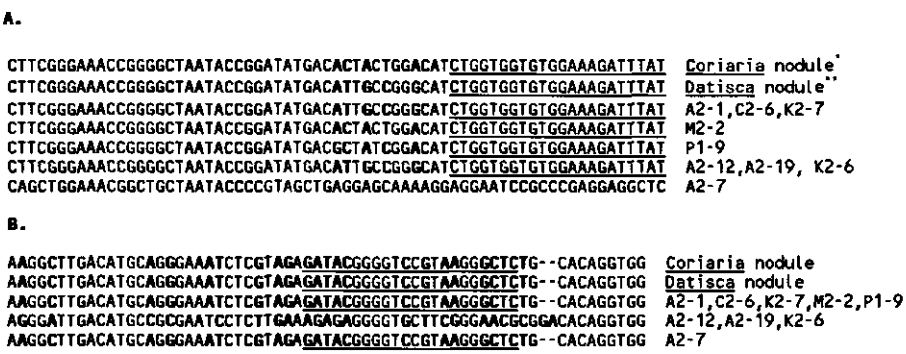

Figure 3. Alignment of the sequences from V2 and V6 variable regions of the 16S rRNA PCR-amplified from *Datisca cannabina* nodules.(A: *E. coli* positions 153-204; B: *E. coli* positions 981-1051; 52). Target sequences of the probe FP (Fig. 3A) and the COR/DAT probe (Fig. 3B) are underlined. Mismatches in the sequences are given in bold letters except for A2-7 (Fig. 3A). (*33; **34)

Dc2 probe: The probe Dc2 developed against the 16S rRNA of an atypical isolate (Dc2) from a *Datisca* nodule did not react with any of the clones originating from nodules induced by five different soil inocula. This indicates that the strain Dc2 may be a representative of a minor population that escaped amplification and detection by PCR.

Conclusions: Sequence analysis enabled the detection of heterogenity existing among *Datisca* nodule 16S rRNA sequences that escaped detection by using only oligonucleotide probes. *Datisca* nodule clones, reacting with both the *Frankia* genus probe and the COR/DAT probe showed three types of sequences. Based only on the probing data with the COR/DAT probe, a sequence (A2-7) would have been easily scored as a *Frankia* sequence. Recently, remarkable homogeneity was reported (38) among 16S rRNA sequences of *Coriaria* nodules originating from geographically widely separated regions (Mexico, France, Pakistan, New Zealand). These observations were based on a continuous stretch of 250 nucleotides that also covered the V6 region. Our data indicate that the V2 region is more suitable than the V6 region for detection of genetic diversity among *Frankia* strains. *Coriaria* and *Datisca* nodule endophyte sequences, that were identical in the V6 region, could be differentiated by sequence analysis of the V2 region. Thus, the sequence information of the V2 variable region may also be useful for the detection of genetic diversity among *Frankia* strains nodulating *Coriaria* spp. and other actinorizal plants.

The nodulation experiments have provided evidence for a wide distribution and abundance of *Coriaria* and *Datisca* nodule endophytes in the soils of northern areas of Pakistan. Genetic diversity of *Frankia* strains inducing nodulation on *Datisca* plants was successfully determined by PCR-amplified 16S rRNA sequence analysis. Moreover, some limitations of using only one probe for screening PCR-amplified targets from the environmental samples were noticed that necessitated the use of more than one probe simultaneously and sequence analysis.

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Chapter 9

Summary

Summary

Studies on the root nodules of *Coriaria nepalensis* using light and electron microscope revealed that the nodule structure and morphology of the endophyte is unique among the actinorhizal plants and similar only to those of *Datisca cannabina*. Plant cells containing the endophyte were relatively enlarged as compared to the non-infected cells. Transverse sections of the nodules revealed that the infected cells form a compact kidney-shaped zone around the stele which is located on one side of the nodule lobe. Inside the host cell, the hyphae of the endophyte were located in the peripheral region of the cytoplasm whilst the elongate vesicles were present near the central vacuole. Host cell mitochondria were abundant and mostly found in close vicinity of the hyphal/vesicular junction of the endophyte. *In situ* hybridization studies showed localization of the *nifH* mRNA within the elongate vesicles of the endophyte. The results indicate that the elongate vesicles of the *Coriaria* nodule endophyte are functionally identical to the spherical vesicles of the *Alnus* endophyte.

Acetylene reduction (C_2H_2 reduction) and uptake hydrogenase activity of the root nodules of *Coriaria nepalensis* and *Datisca cannabina* were measured to study seasonal fluctuations in the enzyme activities. Nitrogenase activity of the nodules of both plants showed biphasic curves with peaks in spring and late summer. Less than 15% of the enzyme activity was retained by the nodules in winter. Results showed that maximum nitrogenase activity coincides with the period of vigorous plant growth in both hosts. Uptake hydrogenase activity was detected in nodules of both plants throughout the year. The peak enzyme activity in both *Coriaria* and *Datisca* nodules was recorded in May. Vesicle cluster fraction of the crushed nodule suspensions showed the highest uptake hydrogenase activity, indicating that the enzyme is associated with the endophyte.

Nine *Frankia*-like actinomycetes were isolated from root nodules of *Coriaria nepalensis*. All isolates formed hyphae and sporangia typical of *Frankia*, but failed to induce nodules on *Coriaria* seedlings, or reduce acetylene in pure culture. The relationship of these strains to atypical and typical *Frankia* strains isolated from other actinorhizal plants and various other actinomycetes was investigated by comparing fatty acid patterns. All *Frankia* strains, including atypical isolates, showed fatty acid profiles distinct from those of *Actinomyces*, *Geodermatophilus*, *Nocardia*, *Mycobacterium* and *Streptomyces*. For

Frankia strains a characteristic pattern of five fatty acids was found that comprised 75% or more of the total content. Three subgroups were identified among 30 *Frankia* strains compared. Atypical strains isolated from *Coriaria* were found in the largest subgroup which contained most *Frankia* strains from other hosts, while ineffective strains from other hosts were distributed in all three subgroups.

Non-infective, atypical strains isolated from *Coriaria* nodules were characterized further by partial 16S rRNA sequence analysis. Ribosomal RNA isolated from all atypical strains hybridized strongly with a *Frankia* genus probe and indicated that the isolates did belong to the genus *Frankia*. This was confirmed for two of the isolates (Cn3 and Cn7) from *Coriaria* nodules by comparison of partial 16S rRNA sequences of the cloned amplification products. Both isolates showed about 95% sequence homology to those of the confirmed *Frankia* strains from other hosts. Similar high homology values were obtained when 16S rRNA sequences of Cn3 and Cn7 were compared with those of the amplification products obtained directly from nodules of *Coriaria nepalensis*. In two variable regions compared, none of the isolates showed identical sequences to those obtained from nodules. These results suggest that the isolates are members of the genus *Frankia*, but different from the strain identified within the root nodules of *Coriaria* by 16S rRNA sequence analysis.

The endophyte of the root nodules of *Datisca cannabina* was identified as *Frankia* by sequence analysis of the partial 16S rRNA gene, amplified directly from the nodules by polymerase chain reaction. Moreover, 16S rRNA sequence analysis indicated that the endophyte of *Datisca* nodules is closely related to that of the *Coriaria* nodule endophyte, to which it also resembles morphologically in the symbiotic state. Relatedness of the endophytes of *Coriaria* and *Datisca* nodules was further proved by the closely related *nif*H sequences obtained from the nodules by PCR. 16S rRNA sequence analysis of the non-infective atypical strain Dc2 obtained from the *Datisca* nodules revealed its close relationship to the genus *Frankia*.

Nodulation in *Datisca* was achieved with crushed nodule suspensions from both *Coriaria* and *Datisca*, whereas various pure *Frankia* strains failed to induce nodulation. The origin of the endophyte in *Datisca* nodules induced by crushed nodules of *Coriaria* collected from Murree (Pakistan) was investigated by comparing partial 16S rRNA sequences with those of the endophytes of both plants. The sequences in the variable region were found

to be identical to those of *Coriaria* nodule endophyte, confirming that the endophyte of *Coriaria* can cross-nodulate *Datisca* plants. Acetylene reduction by the root nodules indicated effectiveness of the nodules. *Coriaria* seedlings could only be nodulated by crushed nodule suspensions of *Coriaria nepalensis*. All pure cultures of *Frankia* including atypical strains used as single inoculum or in combination with the nodule filtrate, failed to induce nodulation on *Coriaria* seedlings.

Sequence information obtained from the root nodules of *Coriaria* and *Datisca* by PCR amplification of partial 16S rRNA gene of the endophyte, was used to develop an oligonucleotide probe. The probe was designed against the V6 variable region of the 16S rRNA that was identical in the 16S rRNA of the endophytes of *Coriaria* and *Datisca*. The probe did not cross-react with RNA of typical and atypical *Frankia* strains isolated from *Alnus, Casuarina, Colletia, Comptonia, Elaeagnus* and *Hippophae*.

To investigate the presence of *Frankia* strains infective on *Coriaria* and *Datisca*, soil samples were collected from different areas of Pakistan and used as inoculum. Three types of soil samples i.e. rhizosphere soil, non-rhizosphere soil and soil from sites effected by erosion were used to inoculate seedlings of *Coriaria* and *Datisca*. Abundance of compatible *Frankia* strains in most areas was indicated by profuse nodulation of the host plants, while soil samples from Athmokam and Puddar failed to induce nodulation on *Coriaria* seedlings. Exceptionally good nodulation of *Coriaria* and *Datisca* with soil samples collected from Abbottabad and Kaghan respectively, reflects abundance of compatible *Frankia* strains in the soils. This also indicates that these soils can be used effectively as an inoculum for large scale inoculation programmes instead of nodules that are difficult to obtain throughout the year.

Successful nodulation of plants inoculated with soil suspensions can confirm the existence of compatible *Frankia* cells, but the possibility of the presence of more than one infective strain cannot be ruled out. Genetic diversity among the *Frankia* strains in *Datisca* nodules originaly induced by soil inoculum collected from different areas of Pakistan, was determined by sequence analysis of 16S rRNA. Part of the 16S rRNA gene was amplified from nodules and the cloned PCR products were screened initially with a *Frankia* genus probe and then with the specific probe. Three categories of the clones were identified i.e. those reacting with both probes and those hybridizing with only the *Frankia* genus probe, or only with the probe specific for the *Datisca* endophyte. Four types of

Frankia sequences and one non-*Frankia* sequence were identified by sequence analysis of the cloned PCR products. The results suggest the presence of more than one *Datisca*-compatible *Frankia* strains in the soils collected from different areas.

Samenvatting

Coriaria nepalensis en *Datisca cannabina* zijn twee pionierplantensoorten uit het Noordelijk deel van Pakistan. De planten vormen wortelknollen met een stikstofbindende actinomyceet als microsymbiont. In het hier beschreven onderzoek werden de morfologie, fysiologie en taxonomie van deze wortelknolsymbionten beschreven. Tevens werd getracht deze microsymbionten in reinkultuur te kweken en te karakteriseren.

Een licht- en electronenmicroscopische studie heeft aangetoond dat de structuur en morfologie van wortelknollen van *Coriaria nepalensis* en *Datisca cannabina* onderling overeenkomen, maar sterk afwijken van die van wortelknollen bij andere "actinorhizal plants", zoals de els en de duindoorn. In de wortelknollen van *Coriaria* en *Datisca* zijn de geïnfecteerde cellen groter dan de niet geïnfecteerde cellen. In dwarsdoorsnede vormt het geïnfecteerde weefsel een niervormige zone aan één zijde van de vaatbundel. De microsymbiont (= endofiet) vormt een netwerk van actinomyceethyfen aan de periferie van de geïnfecteerde cellen. Van hieruit lopen langgerekte palissadenhyfen ("vesicles") die naar de centrale vacuole zijn gericht. In het cytoplasma van de gastheercel, nabij de verbinding van de gewone hyfen en de palissadehyfen, bevinden zich grote aantallen mitochondriën. De palissadehyfen zijn metabolisch zeer actief en spelen een belangrijke rol bij de binding van N_2. *In-situ* hybridisatieonderzoek werd uitgevoerd in coupes van wortelknollen van *Coriaria nepalensis*. Met behulp van DNA probes, die specifiek hybridiseren met mRNA van *nifH*, werd aangetoond dat het *nifH* gen tot expressie komt in de palissadehyfen. Deze resultaten tonen aan dat deze palissadenhyfen dus functioneel identiek zijn aan de bolvormige blaasjes ("vesicles") van de endofiet in wortelknollen van de els (*Alnus*) en de "vesicles" die worden gevormd in reinkultuur van *Frankia* stammen geïsoleerd uit wortelknollen van o.a. *Alnus* spp.

In potproeven in Islamabad in Pakistan werd de seizoensfluctuatie bepaald in de acetyleenreductie (stikstofbindings- activiteit) en de-opname hydrogenaseactiviteit van wortelknollen van beide plantensoorten. De nitrogenaseactiviteit van de knollen van beide planten vertoonde een tweetoppige kurve met pieken in het voorjaar en aan het einde van de zomer. Tijdens de winterperiode was de activiteit tot minder dan 15 % van de maximum-activiteit gedaald. De stikstofbindingsactiviteit was gecorreleerd met de groei van beide plantensoorten. De opname hydrogenaseactiviteit was in beide plantensoorten

gedurende het gehele jaar aantoonbaar, met een piek in mei. Aansluitend op metingen van de activiteit van intacte wortelknollen werd de hydrogenaseactiviteit bepaald van knolhomogenaten en "vesicle cluster" fracties. Deze experimenten toonden aan dat de H_2-opnameactiviteit in de endofiet plaatsvindt.

Uit wortelknolmateriaal van *Coriaria nepalensis* werden 9 actinomyceetstammen geïsoleerd. Alle stammen vormden actinomyceethyfen en sporangia welke karakteristiek zijn voor *Frankia*, afkomstig uit wortelknollen van andere "actinorhizal plants". De stammen uit *Coriaria* wortelknollen bleken echter geen N_2 te binden en vormden geen wortelknollen bij *Coriaria* zaailingen. De taxonomische verwantschap van deze stammen met andere actinomyceten en *Frankia* stammen afkomstig uit wortelknollen van andere plantensoorten werd ondermeer bepaald op grond van vetzuurpatronen. Het vetzuuranalyses van alle onderzochte *Frankia* stammen, inclusief "atypische stammen", d.w.z. stammen die geen N_2 binden en geen of slechts ineffectieve knollen vormen, was sterk afwijkend van het vetzuurpatroon van andere onderzochte actinomyceten (*Actinomyces, Geodermatophylus, Nocardia, Mycobacterium* and *Streptomyces* spp.). Alle *Frankia* stammen vertoonden een karakteristiek patroon van vijf vetzuren. Deze vijf vetzuren vormden in totaal meer dan 75 % van het totale vetzuurgehalte. Op grond van de vetzuurpatronen werden de 30 onderzochte *Frankia* stammen in drie subgroepen ingedeeld. De atypische stammen afkomstig uit *Coriaria* knollen bleken geclusterd in de grootste subgroep. Atypische stammen afkomstig van andere "actinorhizal plants" kwamen voor in alle drie subgroepen.

Teneinde een taxonomische karakterisering te kunnen geven van de niet-cultiveerbare microsymbionten van *Coriaria* en de verkregen isolaten uit deze wortelknollen, werd de nucleotidenvolgorde bepaald van een deel van het 16S rRNA. Dit molecuul wordt thans als meest betrouwbare fylogenetische merker gebruikt in taxonomisch onderzoek. Een door Hahn ontwikkelde *Frankia* "genus-specifieke" probe, welke met ribosomaal RNA uit alle geteste typische en atypische *Frankia* stammen reageerde, hybridiseerde tevens met gekloneerde amplificatieproducten van 16S rRNA sequenties van isolaten Cn3 en Cn7 uit *Coriaria* wortelknollen. Uit sequentieonderzoek bleek dat deze producten ongeveer 95 % homologie vertoonden met die van bekende *Frankia* stammen afkomstig uit andere typen wortelknollen. Een ongeveer gelijke sterke homologie werd gevonden bij het vergelijken van de 16S rRNA sequenties van de stammen Cn3 en Cn7 met die van de

amplificatieproducten welke direct uit wortelknollen van *Coriaria nepalensis* waren verkregen. In twee variabele regio's in het 16S rRNA bleken de stammen Cn3 en Cn7 niet volledig identiek te zijn aan de sequenties van amplificatieproducten uit de wortelknollen. Uit deze resultaten kon worden geconcludeerd dat de verkregen isolaten tot het genus *Frankia* kunnen worden gerekend, maar niet identiek zijn aan de niet-cultiveerbare microsymbiont in de wortelknollen van *Coriaria*.

Naar analogie van het hiervoor beschreven onderzoek met *Coriaria* werd de identiteit bepaald van de endofiet van de wortelknollen van *Datisca cannabina*. Delen van de 16S rRNA genen werden direct uit wortelknollen geamplificeerd en gekloneerd, waarna de nucleotidenvolgorde werd bepaald. Het onderzoek wees uit dat ook deze microsymbiont tot het genus *Frankia* kon worden gerekend. De sequentieanalyse toonde aan dat de endofiet van de onderzochte *Datisca* wortelknollen zeer verwant was met die in *Coriaria* wortelknollen. Deze gegevens bevestigen de conclusies die uit eerder verricht morfologisch onderzoek werden getrokken. De sterke verwantschap tussen de microsymbionten van wortelknollen van *Datisca* en *Coriaria* werd verder gesteund door sequentieonderzoek van amplificatieproducten van het *nifH* gen uit de wortelknollen. Tot op heden is het niet mogelijk gebleken de microsymbiont van *Datisca* wortelknollen in reincultuur te verkrijgen. Een eerder door Hafeez geïsoleerde, niet-infectieuse, stam (Dc2) uit *D. cannabina* wortelknollen, kon op grond van 16S rRNA sequentiegegevens als "atypische" *Frankia* stam worden gekarakteriseerd.

Zaailingen van *D. cannabina* bleken wortelknollen te vormen na enten met een wortelknolhomogenaat van zowel *Datisca cannabina* als *Coriaria nepalensis*. Geen van de onderzochte *Frankia* stammen in reincultuur bleek in staat wortelknollen bij *Datisca* te vormen. De herkomst van de endofiet in de *Datisca* wortelknollen, welke waren gevormd door homogenaten van *Coriaria* wortelknollen afkomstig van Murree (Pakistan), werd bepaald door de nucleotidevolgorde van delen van het 16S rRNA gen van de microsymbionten in beide planten te vergelijken. De sequenties in de variabele regio's bleken identiek aan die van de *Coriaria* endofiet. Hiermee werd aangetoond dat de *Coriaria* endofiet *Datisca* kan infecteren. Aanvullend onderzoek heeft aangetoond dat de gevormde wortelknollen effectief waren, d.w.z. stikstof konden binden.

Zaailingen van *Coriaria* bleken uitsluitend wortelknollen te vormen na enten met wortelknolhomogenaten van *Coriaria nepalensis*. Reinkultures van *Frankia*, inclusief

atypische stammen, bleken niet in staat wortelknollen te vormen bij *Coriaria* zaailingen. Evenmin bleken deze stammen in staat wortelknollen te vormen wanneer aan het entmateriaal een wortelknolfiltraat werd toegevoegd.

Op grond van de sequentieinformatie van amplificatieproducten van de 16S rRNA genen uit de wortelknollen van *Coriaria* en *Datisca* werd een oligonucleotide probe ontwikkeld. De probe werd ontwikkeld tegen de variabele regio (V6) van het 16S rRNA, welke identiek is in het 16S rRNA van de microsymbionten van *Coriaria* en *Datisca*. De probe bleek specifiek te hybridiseren met rRNA afkomstig uit wortelknollen van *Coriaria nepalensis* en *Datisca cannabina*. Er werd geen kruisreaktie verkregen met RNA van *Frankia* stammen afkomstig uit wortelknollen van *Alnus, Casuarina, Colletia, Comptonia, Elaeagnus* en *Hippophae* spp.

De verspreiding van de *Frankia* stammen in Pakistan die *Coriaria* en *Datisca* planten kunnen infecteren werd bepaald door zaailingen van beide plantensoorten te enten met grond afkomstig uit verschillende delen van Pakistan. Er werden drie typen grondmonsters getest, nl. rhizosfeergrond, niet-rhizosfeergrond en grond afkomstig van plaatsen die door erosie waren aangetast. Compatibele *Frankia* stammen werden in de meeste gebieden aangetoond door vorming van veel wortelknollen aan de zaailingen, terwijl grondmonsters afkomstig uit Athmokam en Puddar geen wortelknollen vormden bij *Coriaria* zaailingen. De beste nodulatie van de zaailingen van *Coriaria* en *Datisca* werd verkregen na enten met grondmonsters afkomstig uit resp. Abbottabad en Kaghan. Hieruit werd geconcludeerd dat deze gronden de geschikte *Frankia* stammen in voldoende mate bevatten en dus een eenvoudig inoculum vormen voor grootschalige enten van planten.

Hoewel de nodulatie van de planten na enten met grondmonsters de aanwezigheid van compatibele *Frankia* stammen bevestigt, was niet uitgesloten dat er meer dan één infectieve stam aanwezig was. Een aanwijzing voor genetische diversiteit van *Frankia* stammen binnen een verzameling *Datisca* wortelknollen welke met grond waren beënt, werd gevonden door 16S rRNA sequenties uit verschillende wortelknollen te vergelijken. Een deel van het 16S rRNA gen werd uit verschillende wortelknollen geamplificeerd en de gecloneerde PCR-producten werden gescreened met een *Frankia* genus-specifieke probe en vervolgens met een *Datisca*-specifieke probe. Er werden drie katagoriën van klonen geïdentificeerd, nl. klonen die met beide probes reageerden, klonen die uitsluitend met de *Frankia* specifieke probe reageerden, en klonen die alleen door de *Datisca*-

specifieke probe herkend werden. Een viertal typen *Frankia* sequenties en één niet-*Frankia* sequentie werden nader geïdentificeerd. Deze resultaten toonden aan dat de *Frankia* populatie in de onderzochte gronden uit meerdere verwante stammen bestaat.

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Curriculum Vitae

The author of this thesis was born on 10th of March 1958, in Rawalpindi District, Pakistan. In 1978 he joined the Department of Biological Sciences, Quaid-i-Azam University, Islamabad as a M.Sc. student. After completion of his M.Sc. studies, he initiated research in the field of Biological Nitrogen Fixation under the supervision of Professor A.H. Chaudhary and obtained a M.Phil. degree in 1984. From June 1989 to June 1993 he studied for his Ph.D. in the Department of Microbiology, Wageningen Agricultural University, The Netherlands. The results of this Ph.D. study are presented in this thesis.