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Synthetic oligonucleotide probes for identification of *Frankia* strains

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Abstract

Reverse transcriptase sequence analyses of variable regions of 16S rRNA of the nitrogen-fixing (Nif^+) *Frankia* strain Ag45/Mut15 and the Nif^- strains AgB1.9 and AgW1.1 showed large differences in two of three variable regions between both *Frankia* groups. Synthetic oligonucleotides complementary to sequences in one of these different regions were used in hybridization experiments against isolated rRNA of several *Frankia* strains belonging to three compatibility groups. Ribosomal RNA of eleven effective *Frankia* strains obtained from different *Alnus* species strongly hybridized with the probe against the effective strain Ag45/Mut 15 (probe EFP), whereas ineffective strains and effective strains obtained from other hosts (*Elaeagnus*, *Comptonia*, *Coriaria*, *Hippophaë*, *Colletia* spp.) did not hybridize. Strong hybridization was also obtained with the effective *Casuarina* strain Ccl3. In the group of effective alder strains one strain showed weaker hybridization indicating small sequence differences. Different sequences were also found after hybridization with the probe against the ineffective *Frankia* strains AgB1.9 and AgW1.1 (probe IFP). Only these two strains showed hybridization. The same results were obtained by *in-situ* hybridizations with probe EFP, whereas hybridization with probe IFP showed crossreaction with several other strains. Tests of these probes against rRNA of several microorganisms indicate a high specificity.

Introduction

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

could be detected. Even in mixed cultures where target organisms may occur in low numbers this hybridization technique formed a powerful tool for detection of microorganisms, esp. for pathogens, symbionts and parasites, where isolation and cultivation were limiting factors (Lane *et al.*, 1988; Tenover, 1988).

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

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

Material and methods

Frankia *strains*

Pure cultures of *Frankia* strains described in Table 1 were grown in P + N-medium (Meesters *et al.*, 1985) for 2 weeks at 30°C. Cells were harvested by centrifugation (3000 × *g*), washed twice with PBS buffer (145 m*M* NaCl, 100 m*M* Na_2H-PO_4/NaH_2PO_4, pH 7.4) and stored at −70°C.

Extraction of RNA

About 2 g wet weight of cells of *Frankia* strains were resuspended in 10 ml cold (4°C) standard saline citrate (SSC, 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0). Lysis of strains Ag45/Mut 15, AgB1.9 and AgW1.1 used for sequencing was performed by shaking with 45 g glass beads (0.10–0.11 mm, Braun) for 35 s in a cell homogenizer (Braun)(Embley *et al.*, 1988). Lysis of strains used in dot blot experiments was performed by French pressure. Extraction of RNA was done according to the method of Embley *et al.* (1988).

Reverse transcriptase sequencing

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

Probe synthesis and labelling

Primers as well as oligonucleotide probes were synthesized by standard phosphoamidize methods using an Applied Biosystems 3801A or a New Brunswick Scientific Cyclone DNA synthesizer. 200 ng of oligonucleotides complementary to variable sequences of two different *Frankia* strains (Table 4) were 5′-labelled using phage T4 polynucleotide kinase (BRL) and 50 μCi of [τ-^{32}P]adenosine-5′-triphosphate (3000 Ci/mmol; Am-

ersham)(Maniatis *et al.*, 1982). The kinase mixture was used directly in hybridization experiments without separation of labelled oligonucleotides from unincorporated [τ-^{32}P]ATP.

Filter hybridization

RNA solutions were applied to nitrocellulose filters (Schleicher + Schuell) either by pipette or with a HYBRI.DOT manifold (BRL) and dried at 80°C for two hours; each spot contained 10 ng (*Frankia* strains) or 100 ng (other microorganisms) of rRNA. Filters were prehybridized in 10 ml of hybridization solution in plastic bags without labelled probe for 2 hours at 40°C. After the addition of the probe, hybridization was performed at the same temperature for 16 hours. The hybridization solution contained 1 × Denhardt solution [0.2 g Ficoll (Pharmacia; average M_w 400 000), 0.2 g of polyvinylpyrrolidone (PVP-360; Sigma) and 0.2 g of bovine serum albumine (Sigma, fraction V) per liter], 100 μg ml^{-1} denatured salmon sperm DNA (Sigma), 0.1% sodium dodecylsulfate (SDS; BRL) and 6 × SSC buffer.

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

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

In-situ *hybridization*

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

Results

Reverse transcriptase sequencing

Sequence analysis of three variable regions of 16S rRNA of the effective (Nif$^+$) *Frankia* strain Ag45/Mut15 and the two ineffective (Nif$^-$) and selectively infective strains AgB1.9 and AgW1.1 showed large differences in two regions between the effective and the ineffective strains. Differences in nucleotide sequence between the effective *Frankia* strain and the ineffective strains are illustrated in Figs. 1 and 2 showing secondary structures of variable regions at positions 1000–1040 and 140–210 (*E. coli* pos.). The third region was found to be identical. This region was found to be between nucleotides 430–500 (*E. coli* pos.)(Fig. 3). The sequences of both ineffective strains were completely identical.

Probe design

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

Filter hybridization

Temperature dependence and sequence specific-

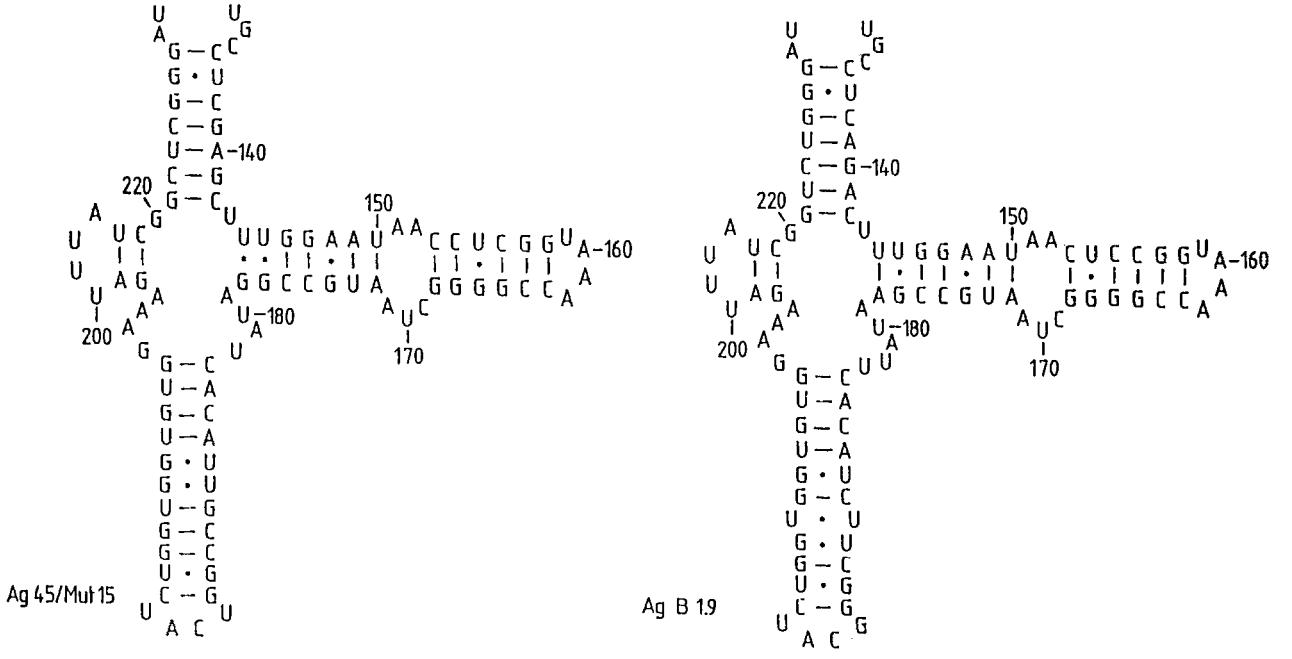

Fig. 1. Secondary structure of 16S rRNA region 140–220 (*E. coli* pos.) showing numerous differences between the effective *Frankia* strain Ag45/Mut15 and the ineffective strain AgB1.9.

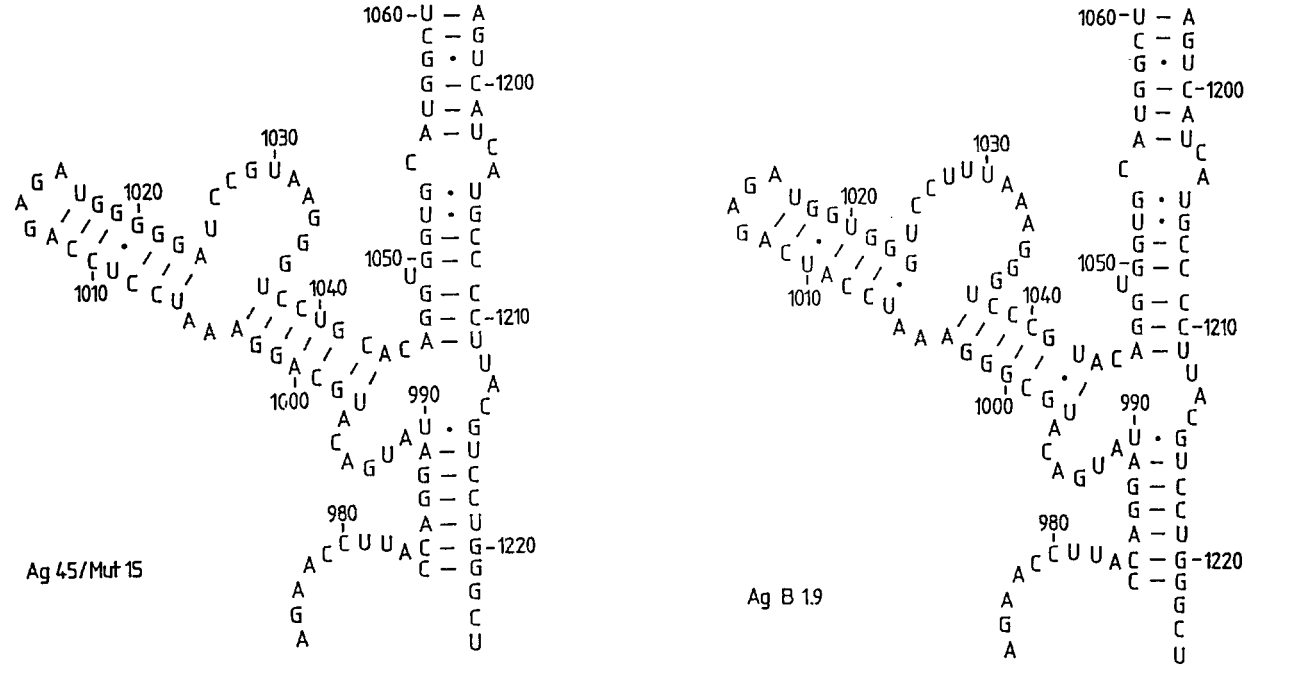

Fig. 2. Secondary structure of 16S rRNA region 980–1060 (*E. coli* pos.) showing numerous differences between positions 1000–1040 of the effective *Frankia* strain Ag45/Mut15 and the ineffective strain AgB1.9.

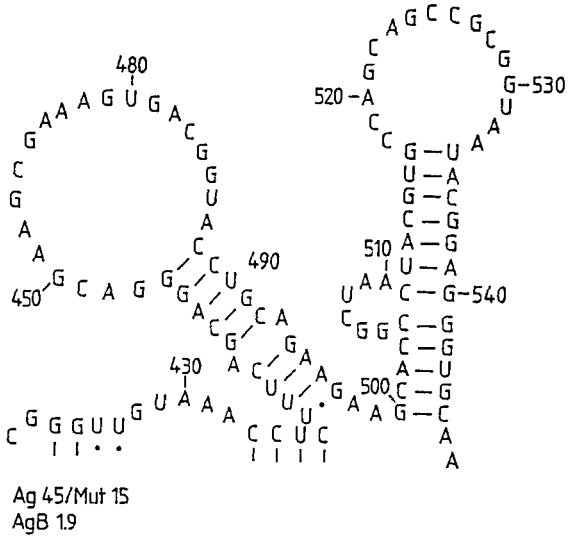

Fig. 3. Identical secondary structure of 16S rRNA region 430–540 (*E. coli* positions) of the effective *Frankia* strain Ag45/Mut15 and the ineffective strain AgB1.9.

ity of probe hybridization are illustrated in Fig. 5. Hybridizations were performed under low stringency conditions followed by washes at higher temperatures. Hybridization of the universal probe to various rRNAs immobilized on nitrocellulose filters indicated that rRNA was present in all RNA preps. Twenty-three *Frankia* strains of different origin were used in these experiments (Table 1). Ribosomal RNA of eleven effective *Frankia* strains obtained from different *Alnus* species strongly hybridized with the probe against the effective strain (probe EFP) whereas ineffective strains and effective strains obtained from other host plants (*Elaeagnus*, *Comptonia*, *Coriaria*, *Hippophaë*, *Colletia* spp.) did not hybridize, even not under low stringency conditions. Strong hybridization signals with probe EFP were also obtained with the effective *Casuarina* strain CcI3. Within the effective alder strains one strain AgKG'84/4 showed temperature dependent hybridization.

Specific hybridization was also found with the probe against the ineffective strains AgB1.9 and AgW1.1 (probe IFP). Only these two strains, but none of the other strains, neither the ineffective nor the effective strains showed hybridization. Additional tests of probe specificity to immobilized rRNA of different microorganisms did not show any significant hybridization, neither with probe EFP nor with probe IFP (Fig. 6).

In-situ *hybridization*

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

Discussion

Reverse transcriptase sequencing of 16S rRNA of two types of *Frankia* strains, the N_2-fixing strain Ag45/Mut15 and the ineffective strains AgB1.9 and AgW1.1 indicate the existence of two highly variable regions at positions 140–210 and 1000–1040

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3'  C C C T A G G - C A - T T C C C A G G A C G   5' probe EFP
5'  G G G A U C C - G U - A A G G G U C C U G C   3' Ag45/Mut15
5'  U G G G U C C U U U A A A G G G U C C C G U   3' AgB1.9
3'  A C C C A G G A A A T T T C C C A G G G C A   5' probe IFP
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Fig. 4. Sequences of synthetic oligonucleotide probes aligned with corresponding sequences of 16S rRNAs (*E. coli* pos. 1020–1042 (Brosius *et al.*, 1981)) of an effective (Ag45/Mut15) and an ineffective (AgB1.9) *Frankia* strain. Differences are in boldface type.

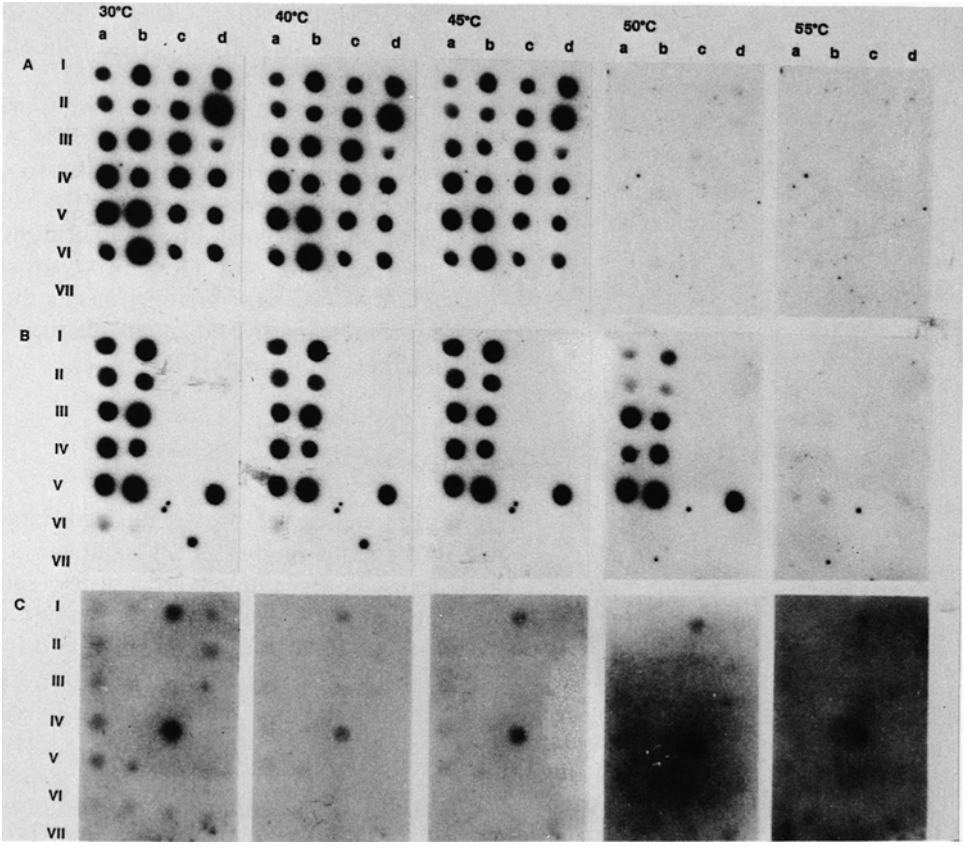

Fig. 5. Temperature dependence and sequence specificity of probe hybridization to nitrocellulose-bound rRNAs.

Each spot contains 10 ng of RNA. Spot positions are described in Table 1. Additional tests are carried out using RNA of *Streptomyes lividans* (pos. VIb) and DNA of *Alnus glutinosa* (pos. VIId). Abbreviations: **A**, probe primer 1115 (Embley *et al.*, 1988), **B**, probe EFP, **C**, probe IFP.

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

Specificity of both probes to other *Frankia* isolates is shown in hybridization experiments against rRNA of *Frankia* strains belonging to different compatibility groups (Baker, 1987). Hybridization of probe EFP only occurs with RNA of effective strains also obtained from alders and, surprisingly, to RNA of the *Casuarina* compatible strain CcI3 belonging to a separate compatibility group. Ineffective strains and strains belonging to the *Elaeagnus* compatibility group do not hybridize with probe EFP. The effective *Comptonia* strain CpI.2 belonging to the *Alnus* compatibility group also indicates sequence differences. Temperature dependent hybridization to probe EFP is obtained with RNA of the effective *Alnus glutinosa* strain

Table 1. Frankia strains

Pos.	Strain	N_2-fixation	Host species	Origin	Reference
Ia	Ag45/Mut15	+	*Alnus glutinosa*	F, Grossensee (FRG)	Hahn *et al.*, 1988
IIa	AgGS'84/45	+	*Alnus glutinosa*	F, Grossensee (FRG)	Hahn, unpublished
IIIa	AgN_2Cl_2	+	*Alnus glutinosa*	R AgN_2, Rijngeest (NL)	Burggraaf, 1984
IVa	AgN_3Cl_2	+	*Alnus glutinosa*	R AgN_3	Burggraaf, 1984
Va	AgP_1R_2C	+	*Alnus glutinosa*	R AgP, Hoogmade (NL)	Burggraaf, 1984
VIa	AgKG'84/4	+	*Alnus glutinosa*	F, Krems-Goels (FRG)	Hahn, unpublished
Ib	AgB16	+	*Alnus glutinosa*	F, Bad Bentheim (FRG)	Hahn and Starrenburg, unpubl.
IIb	AgB32	+	*Alnus glutinosa*	F, Bad Bentheim (FRG)	Hahn and Starrenburg, unpubl.
IIIb	ArI3	+	*Alnus rubra*	F, Oregon (USA)	Berry and Torrey, 1979
IVb	An2.24	+	*Alnus nitida*	R An2	Hafeez *et al.*, 1985
Vb	AvcI1	+	*Alnus viridis*	F, Ontario (Can)	Baker and Torrey, 1980
Ic	AgW1.1	–	*Alnus glutinosa*	F, Weerribben (NL)	Hahn *et al.*, 1988
IIc	AgB1.5	–	*Alnus glutinosa*	F, Bad Bentheim (FRG)	Hahn *et al.*, 1988
IIIc	AgB1.7	–	*Alnus glutinosa*	F, Bad Bentheim (FRG)	Hahn *et al.*, 1988
IVc	AgB1.9	–	*Alnus glutinosa*	F, Bad Bentheim (FRG)	Hahn *et al.*, 1988
Vc	AgB1.10	–	*Alnus glutinosa*	F, Bad Bentheim (FRG)	Hahn *et al.*, 1988
VIc	AgI5	–	*Alnus glutinosa*	F, Oostvoorne (NL)	Akkermans and van Dijk, unpubl.
Id	EuI1	–	*Elaeagnus umbellata*	F, Petersham (USA)	Baker *et al.*, 1980
IId	HrI1	+	*Hippophaë rhamnoides*	F, Petersham (USA)	Baker, unpublished
IIId	Cc1.17	+	*Colletia cruciata*	F,	Meesters *et al.*, 1985
IVd	CpI.1	+	*Comptonia peregrina*	R CpI.1,	Meesters *et al.*, 1985
Vd	CcI3	+	*Casuarina cunningham.*	F, Tampa (USA)	Zhang *et al.*, 1984
VId	CN_3	–	*Coriaria nepalensis*	F,	Mirza and Akkermans, unpublished

AgKG'84/4, indicating small differences in nucleotide composition of that region. Specificity of the probe against the ineffective strains AgB1.9 and AgW1.1 (probe IFP) is also demonstrated. Only these two but not heterologous strains can be detected by hybridization.

The probes described here are of relatively small size (20–22 nucleotides). This minimizes problems of cellular permeability and access to binding sites. *In-situ* hybridizations of fixed *Frankia* cells with probe EFP give the same results as hybridizations with immobilized RNA. In contrast, hybridization with probe IFP does not show the same specificity. Theoretically, this technique of *in-situ* DNA-RNA

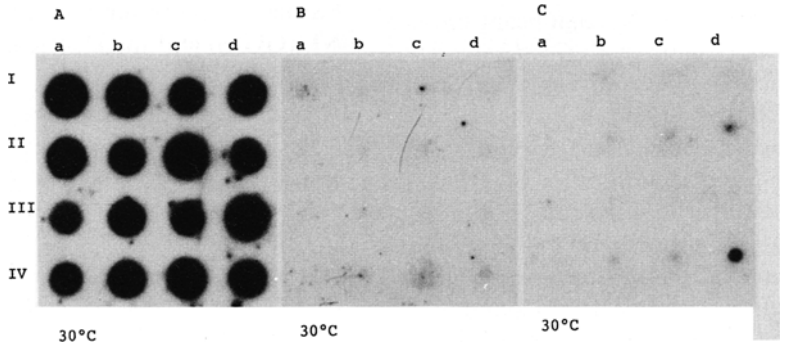

Fig. 6. Sequence specificity of probe hybridization to nitrocellulose-bound rRNAs of different microorganisms.

Each spot contains 100 ng of RNA. Spot positions: Ia *Streptomyces albus* (DSM 40313), Ib *Streptomyces cyaneus* (DSM 40108), Ic *Streptomyces kauaiensis* (DSM 43360), Id *Streptoverticillium baldaccii* (DSM 40845), IIa *Streptoverticillium luteoreticuli* (DSM 40509), IIb *Actinomyces bovis* (DSM 43014), IIc *Propionibacterium thoenii* (DSM 20276), IId *Nocardioides albus* (DSM 43109), IIIa *Pimelobacter simplex* (NCIB 8929), IIIb *Terrabacter tumescens* (NCIB 8914), IIIc *Tsukamurella* (NCTC 10741), IIId *Nocardiopsis dassonvillei* (DSM 43235), IVa *Casiobacter polymorphus* (NCDO 2097), IVb *Dermatophilus congolensis* (DSM 43037), IVc *Geodermatophilus obscurus* (DSM 43160), IVd *Frankia* AgB1.9. Abbreviations: **A**, probe primer 1115 (Embley *et al.*, 1988), **B**, probe EFP, **C**, probe IFP.

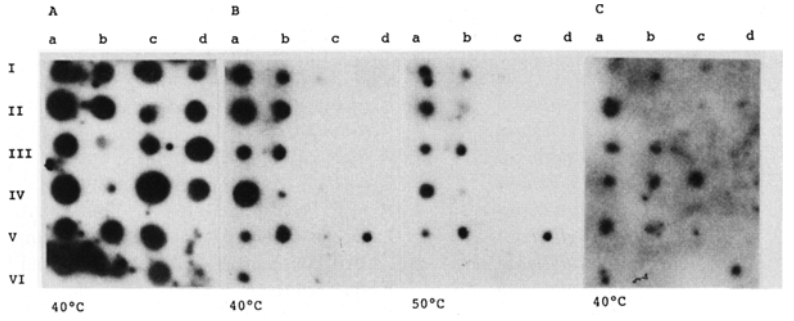

Fig. 7. In-situ hybridization of **A)** the universal probe primer 1115 (Embley *et al.*, 1988), **B)** the probe against the effective *Frankia* strain Ag45/Mut15 (probe EFP) and **C)** the probe against the ineffective *Frankia* strains AgB1.9 and AgW1.1 (probe IFP).

Each spot contains identical cell masses (2 μg protein). Spot positions are described in Table 1.

hybridizations can be used for taxonomical research because large amounts of strains can be tested in a short period of time. However, the results obtained from hybridization experiments with 23 *Frankia* strains from different compatibility groups indicate more strain specific sequences than group specific sequences so that the use of both probes in taxonomical investigations might be of limited value. Screening of much more *Frankia* strains should give more information about the real application of these probes in taxonomical research within the family Frankiaceae.

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

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

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

Acknowledgments

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