Plant and Soil **204:** 1–19, 1998.

rRNA based identification and detection systems for rhizobia and other bacteria

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Key words: hybridization, *in situ* identification, phylogeny, probe design, rhizobia, rRNA

Abstract

Ribosomal ribonucleic acids are excellent marker molecules for the elucidation of bacterial phylogeny; they also provide useful target sites for identification and detection with nucleic acid probes. Based on the currently available 16S rRNA sequence data, bacteria of the rhizobial phenotype (plant nodulation, nitrogen fixation) are members of three moderately related phylogenetic sub-groups of the α-subclass of the *Proteobacteria*: i.e. the rhizobia group, the bradyrhizobia group, and the azorhizobia group. All rhizobia, azo-, brady-, meso- and sinorhizobia are closely related to and in some cases phylogenetically intermixed with, non-symbiotic and/or non-nitrogen-fixing bacteria. Especially in the case of *Bradyrhizobium japonicum* strains, the 16S rRNA sequence data indicate substantial heterogeneity. Specific probe design and evaluation are discussed. A multiprobe concept for resolving specificity problems with group specific probes is presented. *In situ* identification with group specific probes of rhizobia in cultures as well as rhizobia and cyanobacteria within plant material is shown.

Introduction

The capability of rhizobia and some other plant-symbiotic bacteria to convert atmospheric nitrogen to ammonia and to turn it over to the host is of great economic importance in agriculture. Cultivation of leguminous crops helps to improve soil by natural nitrogen fixation and protects ground water from nitrate contamination resulting from excessive application of N-fertilizer. Legumes are routinely inoculated with rhizobia in many countries. Major efforts are being made to improve rhizobia used for plant inoculation. The major targets of such efforts are the ability of the bacteria to fix nitrogen, nodulate the host plant, and compete with the indigenous bacteria in soil for colonization of rhizosphere and root. To screen natural resources for better strains, reliable identification and detection methods are needed.

During the past decade, ribosomal RNA based identification and detection methods have become techniques routinely used in all disciplines of microbiology. As molecular chronometers (Woese, 1987) these molecules have preserved their evolutionary history. Highly conserved portions carry the information on early evolutionary events and more recent changes are documented within less conserved positions or stretches. The degree of divergence of present day rRNA sequences gives an estimate of their phylogenetic distances. Phylogenetic trees based on rRNA sequence data roughly reflect the evolutionary history of the organisms as shown by comparative sequence analyses of alternative non-rRNA phylogenetic marker molecules (Ludwig and Schleifer, 1994; Ludwig et al., 1993). Comparative analysis of comprehensive databases of bacterial 16S rRNA sequences (De Rijk et al., 1996; Ludwig, 1995; Maidak et al., 1996; Van de Peer et al., 1996) with appropriate software allows rapid identification of unknown bacteria based on their rRNA sequence data.

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Plant and Soil is the original source of publication of this article. It is recommended that this article is cited as: *Plant and Soil* **204**: 1–19, 1998.

Besides the phylogenetic information rRNAs also provide targets for specific hybridization probes (Amann and Ludwig, 1994; Amann et al., 1995). These targets are diagnostic sequence stretches which are unique to defined phylogenetic groups. Specific nucleic acid probes can be designed complementary to sequence stretches containing the diagnostic residues. Mainly, synthetic oligonucleotides are used for target monitoring. The presence of the target is indicated by stable hybrid formation which is detected by measuring probe or target associated labels. The classical radioactive labels have been more and more replaced by non-radioactive labelling and detection systems (Schleifer et al., 1993). The rRNA based specific probe technology permits the detection of microbial nucleic acids or organisms even within complex samples. Specific *in situ* identification of individual cells can be achieved by whole cell hybridization (Amann et al., 1995). The so called rRNA cycle was the first technique to combine the phylogenetic analysis and *in situ* visualisation of so far uncultured bacteria (Amann and Ludwig, 1994; Amann et al., 1995). Ribosomal rRNA genes can be *in vitro* amplified from environmental samples and subsequently cloned and sequenced. The sequence data allow the phylogenetic positioning as well as specific probe design. *In situ* cell hybridization closes the cycle by assigning the sequence to a morphotype within the original sample.

With rhizobia, most of the validly described species have been phylogenetically characterized by comparative analyses of almost complete 16S rRNA sequences (Ludwig et al., 1995; Nour et al., 1994, 1995; Sawada et al., 1993; Willems and Collins, 1992; Willems et al., 1993; Yanagi et al., 1993). Many strains and isolates can be (roughly) assigned to phylogenetic groups by full (Dupuy et al., 1994; Wong et al., 1994) and partial 16S rRNA sequence analyses (Chen et al., 1995; Eardly et al., 1992; So et al., 1994; Van Berkum et al., 1994; Van Rossum et al., 1995; Xu et al., 1995; Young et al., 1991). Not many reports on the use of specific probe technology for the identification of rhizobia exist however (Springer et al., 1993).

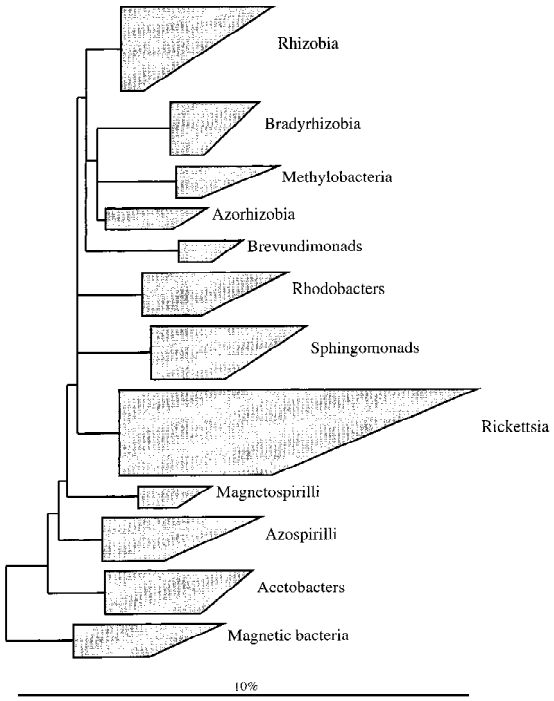

Figure 1. Major groups of the α-subclass of the *Proteobacteria*. The consensus tree is based on distance matrix, maximum parsimony and maximum likelihood analyses of all available (>90% complete) 16S rRNA sequences. Only alignment positions which share common residues in at least 50% of all available sequences from α-subclass proteobacteria were included for calculations. Multifurcations indicate branches for which a relative order cannot unambiguously be determined or a common order is not supported applying different treeing methods. The bar indicates estimated base changes (10%). The quadrilaterals indicate phylogenetic groups. The lengths of the horizontal sides indicate the overall shortest and longest branches within the cluster. The vertical extensions roughly indicate the number of sequences used. The brevundimonads group includes *Mycoplana bullata, M. segnis*, *Caulobacter crescentus* and *C. bacteroides*. Rhodobacters include *Paracoccus*, *Rhodovulum* and (less significantly) *Hirschia* and *Hyphomonas*. Rickettsia include *Ehrlichia*, *Cowdria*, *Neorickettsia* and (less significantly) *Caedibacter* and *Holospora*. Magnetospirilli include *Rhodospirillum fulvum* and *R. molischianum*. Azospirilli include *Rhodocista*. Acetobacters include *Gluconobacter*, *Acidiphilium* and *Roseococcus*. The magnetic bacteria group comprises magnetic non-cultured bacteria not related to the magnetospirilli. The phylogentic substructure of the rhizobia, bradyrhizobia and azorhizobia groups is shown in Figures 2–5.

The phylogeny of rhizobia

Currently, 10 species of the genus *Rhizobium* as well as 5 of *Mesorhizobium*, 6 of *Sinorhizobium*, 3 of *Bradyrhizobium*, and 1 of *Azorhizobium* have been validly described. Full and/or partial 16S rRNA sequence data are available in public databases (De Rijk et al., 1994; Ludwig, 1995; Maidak et al., 1994; Van de Peer et al., 1994) as specified in Table 1. According to 16S rRNA sequence data, all genera are members of the α-subclass of the proteobacteria. The class *Proteobacteria* represents one of the major lines of descent within the domain *Bacteria*. The separation of the initially described genus *Rhizobium* into the genera *Rhizobium* and *Bradyrhizobium* based on phenotypic

Table 1. Validly described rhizobia species. Accession numbers and references for 16S rRNA sequence data

Genus	Species	Accession	Reference
Azorhizobium	*caulinodans*	D13948, D11342, M55491, U12913, X67221, X77126, X94200	Rainey and Wiegel, 1996 Sawada et al., 1993; Van Rossum et al., 1995; Willems and Collins, 1993;
Bradyhizobium	*elkanii*	M55490, U35000	So et al., 1994
Bradyhizobium	*liaoningensis*	X86065	Xu et al., 1995
Bradyhizobium	*japonicum*	D11345, D12781, D13429, D13430, L23330, L23331, M55485–M55490, S46916, U12911, U12912, U50164, U50165, U69638, X66024, X71840, X87272, Z35330	Kuendig et al., 1995 Ludwig et al., 1995; Sawada et al., 1993; Springer et al. 1993; Van Rossum et al., 1995; Willems and Collins, 1992; Wong et al., 1994; Yanagi and Yamasato, 1993; Young et al., 1991;
Mesorhizobium	*ciceri*	U07934	Nour et al., 1994
Mesorhizobium	*huakuii*	D12797, D13431, S74905	Chen et al., 1995; Sawada et al., 1993; Yanagi and Yamasato, 1993;
Mesorhizobium	*loti*	D01270, D12791, D14514, U50166, X63825, X67229, X67230	Sawada et al., 1993; Willems and Collins, 1993; Yanagi and Yamasato, 1993 Young et al., 1991;
Mesorhizobium	*mediterraneum*	L38825	Nour et al., 1994
Mesorhizobium	*tianshanense*	S74904	Chen et al., 1995
Rhizobium	*etli*	L20762–L20766, U28916, U28939, U47303,	Hernandez-Lucas et al., 1995; Van Berkum et al., 1996
Rhizobium	*galegae*	D11343, D12793, X63823, X67226, Z79620	Huber and Selenska-Pobell, 1994 Sawada et al., 1993; Willems and Collins, 1993; Yanagi and Yamasato, 1993;
Rhizobium	*gallicum*	U86343, AF008127–AF008130	Anarger et al., 1997; Sessitsch et al., 1997
Rhizobium	*giardinii*	U86344	Anarger et al., 1997
Rhizobium	*hainanensis*	U71078	Chen et al., 1997
Rhizobium	*leguminosarum*	D01269, D14513, D12782, M55233, M55235–M55240, M55494, M63183, U09271, U29386, U29388, U31074, X67227, X67233, X77122, X91211	Breil et al., 1996; Eardley et al. 1992; Sawada et al., 1993; Segovia et al., 1991; Van Berkum et al. 1996; Willems and Collins, 1993; Yanagi and Yamasato, 1993: Young et al., 1991;
Rhizobium	*lupini*		Ludwig et al., 1995
Rhizobium	*tropici*	D11344, D12798, L20697, L20765, L21837, U38469, X67234, X77125, X77127	Hernandez-Lucas et al., 1995; Sawada et al., 1993; Van Berkum et al., 1994; Yanagi and Yamasato, 1993;
Sinorhizobium	*fredii*	D01272, D12792, X77123, X67231	Sawada et al., 1993; Willems and Collins, 1993; Yanagi and Yamasato, 1993

Table 1. Continued

Sinorhizobium	*meliloti*	D01265, D12783, D14509, D14516, M55242, M55243, M55495, X67222, X67231, X77121, X67231	Eardley et al., 1992; Sawada et al., 1993; Willems and Collins, 1993; Young et al., 1991;
Sinorhizobium	*medicae*	L39882	Rome et al., 1996
Sinorhizobium	*saheli*	X68390	Lajudie et al., 1994
Sinorhizobium	*terangae*	X68387, X68388	Lajudie et al., 1994
Sinorhizobium	*xinjiangensis*	D12796	Yanagi and Yamasato, 1993

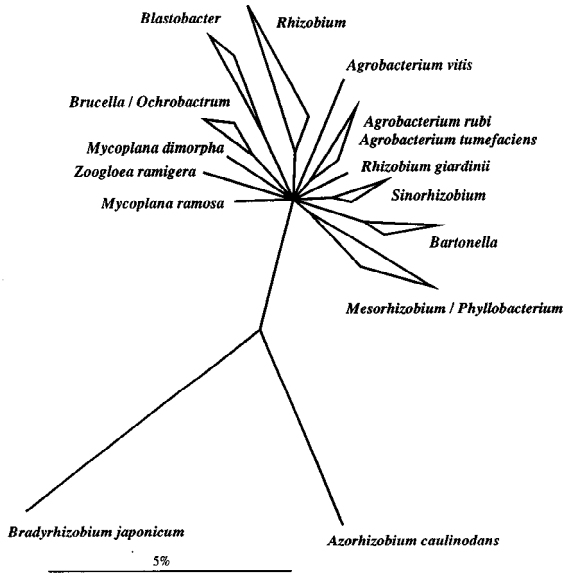

Figure 2. The rhizobia group. The tree is based on the results of a maximum likelihood analysis of all (>90% complete) 16S rRNA sequences from the members of the rhizobia, azorhizobia, bradyrhizobia groups and from selected representatives of other major groups of the α-subclass. The tree topology was evaluated and corrected according to the results of distance and maximum parsimony analyses of the complete sequence data set from α-subclass proteobacteria. Only sequence positions which share common residues in at least 50% of the members of the rhizobia group were included for calculations. The triangles indicate the phylogenetic subgroups. Multifurcations indicate branches for which a relative order cannot be unambigously determined or a common order is not supported by different treeing methods. The bar indicates estimated base changes (%).

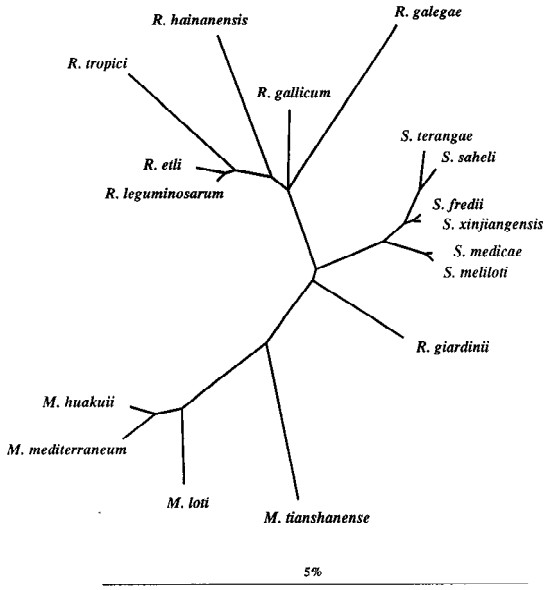

Figure 3. Phylogenetic relationships of *Mesorhizobium* (*M.*), *Rhizobium* (*R.*), and *Sinorhizobium* (*S.*) species. Only type strains are depicted. Tree reconstruction was as decribed for Figure 2.

characters and nucleic acid hybridization data (Jordan, 1982) reflects the phylogenetic situation as was later corroborated by comparative 16S rRNA sequence analysis (Figure 1). Later, the genus *Rhizobium* was further divided into *Mesorhizobum*, *Rhizobium* and *Sinorhizobium* (De Lajudie et al., 1994; Jarwis et al., 1997; Figure 2). With the exception of *Rhizobium lupini* all rhizobial, meso- and sinorhizobial species are members of one phylogenetic subgroup of the α-subclass of the proteobacteria (rhizobia group in Figure 1). This subgroup also comprises non-nitrogen fixing species of other genera associated (*Agrobacterium* and *Phyllobacterium*) or not associated with plants (*Blastobacter*, *Chelatobacter*, *Mycoplana* and Zoogloea) or even (facultative) pathogens such as *Bartonella*, *Brucella* and *Ochrobactrum* (Figure 2).

Species of the genus *Sinorhizobium* represent a coherent phylogenetic group (Figure 2). The genus has been amended based on the results of polyphasic taxonomic investigations (De Lajudie et al., 1994) to contain the renamed former *Rhizobium fredii* and *R.*

meliloti as well as several newly isolated and described species (*Sinorhizobium medicae, S. saheli, S. terangae* and *S. xinijangensis*) (Figure 3).

Another monophyletic group moderately related to *Phyllobacterium* comprises the species of the genus *Mesorhizobium* (Figure 2).This genus was recently created to harbour the former *Rhizobium ciceri, R. huakuii, R. loti, R. mediterraneum* and *R. tianshanense* (Jarvis et al., 1997; Figure 3).

The current genus *Rhizobium* (Figure 2) comprises *Rhizobium etli, R. galegae, R. gallicum, R. giardinii, R. hainanense, R. leguminosarum, R. lupini, R. phaseoli, R. trifolii* and *R. tropici* (Figure 3). From the phylogenetic point of view, however, the genus *Rhizobium* as currently defined is not a monophyletic group. According to 16S rRNA sequence data, the recently described *R. giardinii* (Amarger et al., 1997) does not cluster with the other *Rhizobium* species but rather represents a new line of descent within the rhizobia group (Figures 2 and 3). Furthermore, the genuine *Rhizobium* cluster (*Rhizobium etli, R. galegae, R. gallicum, R. hainanense, R. leguminosarum, R. lupini, R. tropici*) also contains probably misclassified *Agrobacterium tumefaciens* as well as *A. rhizogenes* strains.

There is a number of phylogenetic groups comprising non-rhizobial bacteria which are related to one another and to the mesorhizobia, sinorhizobia as well as the rhizobia groups at comparable levels. The type strains of *Agrobacterium rubi* and *A. tumefaciens* are related to one another and separated from rhizobial species. However, only part of other *Agrobacterium rubi* and *A. tumefaciens* strains group with their respective type strains. Some of the strains designated as *Agrobacterium tumefaciens* are only moderately related to the type strain but phylogenetically intermixed with *R. etli, R. leguminosarum, R. tropici* and *Agrobacterium rhizogenes* strains (Eardly et al., 1992; Sawada et al., 1993; Segovia et al., 1991; Willems and Collins, 1993; Yanagi and Yamasoto, 1993). In the case of *Agrobacterium rhizogenes*, the type strain is not related to the species of the present group but was reclassified as *Sphingomonas rosa* (Takeuchi et al., 1995). The *Sphingomonas* species are also members of the α-subclass of the proteobacteria, however, phylogenetically separated from the rhizobia and relatives (Figure 1). Species of the genera *Brucella* and *Bartonella* represent two entities which, with respect to the level of phylogenetic relatedness, are equivalent to the agrobacteria, rhizobia, sino- and mesorhizobia groups (Figure 2). *Ochrobactrum* apparently

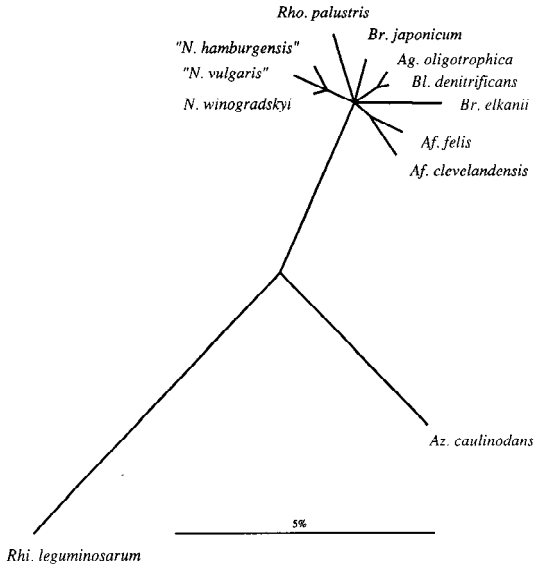

Figure 4. Bradyrhizobia group. Tree reconstruction and presentation is as described for Figure 2 except that only alignment positions which share common residues in at least 50% of the members of the brady- and azorhizobia group were included for calculations. Abbreviations: *Af – Afipia*; *Ag – Agromonas*; *Az – Azorhizobium*; *Bl – Blastomonas*; *Br – Bradyrhizobium*; *N – Nitrobacter*; *Rhi – Rhizobium*; *Rho – Rhodopseudomonas*.

shares a common origin with *Brucella*. A moderate relationship to this group is indicated for two *Mycoplana* species (*M. dimorpha* and *M. ramosa*; Yanagi and Yamasoto, 1993) applying different treeing methods. (The statistical significance is low, however.) Phylogenetically, other *Mycoplana* species are not members of this group but are related to *Caulobacter* and *Brevundimonas*, other proteobacteria of the α-subclass. Partial 16S rRNA sequence data indicate that *Chelatobacter heintzii* and *Chelatococcus asaccharovorans* also belong to the major rhizobia cluster (Auling et al., 1993). A remarkable example of mis-classification based on conventional taxonomic methods is the species *Zoogloea ramigera*. While the type strain (ATCC 19544) and another strain (ATCC 25935) cluster in two different groups of proteobacteria of the β-subclass, a third strain (ATCC 19623) clearly is a member of the rhizobia group (Rosselló-Mora et al., 1993; Shin et al., 1993). *Blastobacter aggregatus* and *B. capsulatus* emerge from a common branch rooting at the basal level of the rhizobia group. *Rhizobium lupini* is a mis-classified species and shares identical 16S as well as 23S rRNA sequences with the type strain of *Bradyrhizobium japonicum* (Ludwig et al., 1995).

Table 2. Ranges of overall 16S rRNA sequence similarities (% identical homologous bases) of non-*Bradyrhizobium japonicum* and *Bradyrhizobium japonicum* strains within the *Bradyrhizobium* group

Species	Similarity (%) with *Bradyrhizobium japonicum* strains
Bradyrhizobium elkanii	98.8–96.6
Blastobacter denitrificans	98.8–96.3
Rhodopseudomonas palustris	98.2–95.6
Nitrobacter hamburgensis	97.5–95.3
Nitrobacter vulgaris	97.5–95.2
Nitrobacter winogradskyi	98.5–95.5
Afipia clevelandis	98.5–95.5
Afipia felis	98.0–95.0

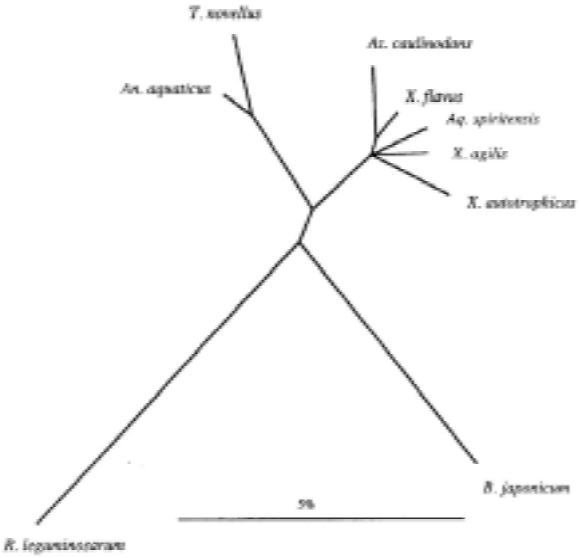

Figure 5. Azorhizobia group. Tree reconstruction and presentation is as described for Figure 2 except that only alignment positions which share common residues in at least 50% of the members of the brady- and azorhizobia group were included for calculations. Abbreviations: *An – Ancylobacter*; *Aq – Aquabacter*; *Az – Azorhizobium*; *B – Bradyrhizobium*; *R – Rhizobium*; *T – Thiobacillus*; *X – Xanthobacter*.

The bradyrhizobia together with *Rhodopseudomonas palustris*, *Blastomonas denitrificans* as well as *Afipia*, and *Nitrobacter* species represent a subgroup of closely related proteobacteria of the α-subclass which is rather distant from the rhizobia group (Figure 1). The current genus *Rhodopseudomonas* is phylogenetically diverse. All species analyzed so far are members of the α-subclass, however, only *Rhodopseudomonas palustris* is among the organsims of the bradyrhizobia group. The *Nitrobacter* species (*N. hamburgensis*, *N. vulgaris* and *N. winogradskyi*) share a common root in phylogenetic trees. However, a relative branching order of the *Bradyrhizobium* species, the *Agromonas / Blastomonas* line, *Rhodopseudomonas palustris* and *Nitrobacter* cannot unambiguously be determined (Figure 4). This is also reflected by overall similarity values of more than 95% for all available 16S rRNA sequences from members of the group including *Afipia*. The tree of Figure 4 is based on sequences from type strains (if available). A number of complete and partial sequences from *Bradyrhizobium japonicum* strains and related photosynthetic strains have been published (Ludwig et al., 1995; So et al., 1994; Swada et al., 1993; Van Rossum et al., 1995; Willems and Collins, 1992; Wong et al., 1994; Xu et al., 1995; Yanagi and Yamasoto, 1993; Young et al., 1991). No clearcut branching pattern can be reconstructed by applying different treeing methods. Furthermore, a cluster of *Bradyrhizobium* strains separated from the other genera is not significantly supported. The heterogeneity of these strains is also evident from a comparison of overall sequence similarity values. The lowest similarity values for the different *Bradyrhizobium japonicum* strains are 96.6%. As shown in Table 2, the corresponding overall 16S rRNA sequence similarity values resulting from comparison of the *B. japonicum* strains with non-*Bradyrhizobium* strains of the group are in the range of 98.6%–95.0% and thereby at least partly within the range of intra-*Bradyrhizobium japonicum* relationships. As discussed earlier, *Bradyrhizobium japonicum* could probably be reclassified as *Rhodopseudomonas palustris* (Young et al., 1991).

Later it was argued that this should not be done given the phylogenetic heterogeneity of the genus *Rhodopseudomonas* (Wong et al., 1994). It is evident from the sequence data, that major taxonomic revision of the whole group is needed. At this level of close relationships the resolution power of rRNA data is insufficient as a sole basis for reclassification however (Stackebrandt and Goebel, 1994). DNA–DNA hybridization studies and evaluation of chemotaxonomic and phenotypic data should be combined with the rRNA data in a polyphasic approach to resolve the taxonomic status of this group.

The closest relative of the single species genus *Azorhizobium* is currently *Xanthobacter flavus*. The genus *Xanthobacter* lacks phylogenetic coherence (Rainey and Wiegel, 1996). *Aquabacter spiritensis* and *Azorhizobium caulinodans* are intermixed with the Xanthobacter species (Figure 5). These organisms cluster phylogenetically with *Ancylobacter aquaticus* and *Thiobacillus novellus* (Figure 5). The azorhizobia group is only moderately related to the rhizobia and the bradyrhizobia groups, but is somewhat closer to the latter (Figure 1).

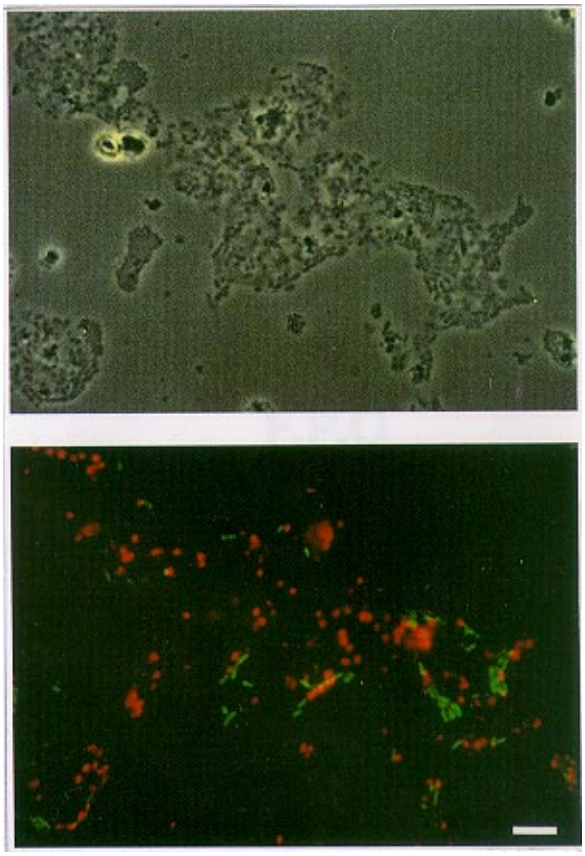

Figure 6. Artificial mixture of *Rhizobium etli* and *Escherichia coli* hybridized with probe GAM42a (green, specific for the γ-subclass of the *Proteobacteria*) and the rhizobia probe RHI1247 (red). Phase-contrast (upper panel) and epifluorescence (double exposure using filter sets for red and green fluorescence; lower panel) micrographs are shown for identical microscopic fields. Bar represents 5 μm.

Nucleic acid probe technology

Molecular background

Specific probe hybridization techniques are based on the capacity of complementary nucleic acid stretches to anneal by specific base pairing. Formation and stability of the formed hybrids depends on a number of physical and chemical parameters, e.g. temperature, ionic strength, denaturant concentration and pH. The crucial factor for specific probe hybridization is the degree of complementarity of a probe to target and non-target molecules as defined by the number of mismatches within the corresponding hybrids (Amann and Ludwig, 1994; Schleifer et al., 1993). Given a careful design of probe sequences and properly adjusted experimental conditions, single mis-matches in probe target hybrids of 15–30 base pairs can be sufficient to allow the differentiation of imperfect hybrids from perfectly matched ones. A variety of techniques can be used for specific probe hybridization (Schleifer et al., 1993). Three basic steps are typical for most of these methods: hybrid formation of probe and target, removal of non-bound probe or target molecules (washing), and detection of hybrids via probe or target associated labels. The classical radioactive labelling procedures have been replaced by a variety of non-radioactive labelling and detection methods based on coloured products of enzymic reactions, chemiluminescence, or fluorescence (Amann et al., 1995; Schleifer et al., 1993).

Design of specific rRNA targeted probes

As mentioned above, rRNA sequences contain sequence motifs which are diagnostic for phylogenetic entities and may be used as targets for taxon-specific probes. Successful design of reliable probes depends on the availability of comprehensive sequence information (De Rijk et al., 1994; Ludwig et al., 1995; Maidak et al., 1994; Van De Peer at al., 1994) and suitable software for database handling, target search and probe evaluation (Strunk et al., 1998). At first,

Table 3. Sequence and target positions of specific hybridization probes. The homologous regions are missing within the partial 16S rRNA sequences of *Mesorhizobium ciceri* and *M. tianshanense*

Probe	Specificity	Sequence	Target[1]
RHI1247	Rhizobia[2]	5′-TCGCTGCCCACTGTC-3′	1246
RHI820	Rhizobia[3]	5′-CCGACGGCTAACATTC-3′	820
BLA999	*Blastobacter*	5′-CTCCACTGTCCGCGACC-3′	999
RLM820	*Mesorhizobium loti*[4], *M. huakuii*, *M. mediterraneum*	5′-CCAACGGCTAGCTTCC-3′	820
RLP820	*Phyllobacterium*, *Mesorhizobium loti*[5]	5′-CCGACGGCTAGCTCTC-3′	820

[1] 5′-position, *Escherichia coli* numbering (Brosius et al., 1981).
[2] *Agrobacterium, Bartonella, Blastobacter aggregatus, B. capsulatus, Brucella, Chelatobacter, Mycoplana dimorpha, M. ramosa, Ochrobactrum, Phyllobacterium, Rhizobium, Mesorhizobium, Sinorhizobium* and *Zoogloea ramigera* strain ATCC 19623.
[3] *Agrobacterium, Bartonella, Brucella, Chelatobacter, Mycoplana dimorpha, M. ramosa, Rhizobium, Sinorhizobium* and *Zoogloea ramigera* strain ATCC 19623.
[4] *Mesorhizobium loti* IAM 13588.
[5] *Mesorhizobium loti* strains ATCC 33669, LMG 6125^T and LMG 4248.

all database sequences (currently >10.000) have to be checked for unique motifs for the respective phylogenetic group. These potential target sites then have to be evaluated with respect to base composition, length, intraprobe self complementarity, and optimal position and quality of hybrid destabilizing mis-matches in potential non-target hybrids. A major criterion for computer aided probe optimization is the experimental ease to clearly differentiate probe target hybrids from potential non-target hybrids. Central mis-matches in potential non-target hybrids are more destabilizing than lateral ones. A-A, A-C, C-C, T-T and C-T mispairings are more destabilizing than A-G, G-T and G-G. Types of base pairs next to mis-matches have also to be taken into account. The destabilizing effect of mis-matches can be partly compensated by the next neighbouring base pairs in correlation with the strengths of the latter. Probe length is usually chosen in a way that the difference of the predicted dissociation temperature of the (perfectly matched) target hybrid and that of the most stable potential non-target hybrids is sufficient (at least 2 °C). Mostly, probes are designed as oligonucleotides comprising 15–20 nucleotides. Self complementarities within probe and target sequences may result in competition of the formation of intra-molecular secondary structures with probe-target hybridization. A selection of target and the most similar potential non-target sites of a broad range probe (RHI1247, Table 3) specific for the rhizobia group as defined above is shown in Table 4.

In case the establishment of experimental conditions ensuring probe specificity is difficult, the simultanous use of competitor probes during the hybridization procedure often helps to keep the probe specific (Manz et al., 1992). Competitor probes are designed complementary to non target sites. The unlabelled competitor probes displace the labelled specific probes from the non-target sites and thereby improve the signal to noise ratio significantly.

In principle, the rRNAs or the corresponding genes are excellent targets for phylogenetic probes. In theory the alternating sequence of highly conserved to highly variable positions should provide potential probe target sites for many different phylogenetic levels or groups. Indeed, in many cases there are diagnostic sequence signatures (single nucleotides, sequence stretches, base pairs, deletions, insertions) common to all members of a given phylogenetic group which may provide unique group specific target regions. However, in praxis it is often difficult or even impossible to design specific probes exclusively targeting all organisms of a phylogenetic group. The reconstruction and current definition of phylogenetic groups is based on the comparative analysis of full 16S rRNA sequences whereas probe target sites comprise only short sequence stretches. Given that the groups are defined on the basis of the full information content of rRNA sequences, that means a number of positions or stretches which are informative for the particular level of relatedness, the existence of comprehensive signatures for the particular group based on one or a few diagnostic residues cannot be generally expected. Furthermore, evolution is progressing and signatures may be lost as a result of single base changes, while the phylo-

Table 4. Alignment of 16S rRNA regions homologous to the target sites of probe RHI1247 from a selection of α-subclass proteobacteria. The solid line separates target (rhizobia group; upper part) and non-target (organisms which are not members of the rhizobia group but contain the probe target site and a selection of organisms with one or two base differences within the homologous sequence region; lower part) organisms. Only base differences are shown. Identitites are indicated by =

Organism	Strain	Target 5′-GACAGUGGGCAGCGA-3′
Agrobacterium rubi	LMG 156^T	==========
Agrobacterium tumefaciens	NCPPB 2437	==========
Agrobacterium vitis	LMG 8750^T	==========
Bartonella bacilliformis	ATCC 35685^T	==========
Blastobacter capsulatus	–	==========
Brucella suis	ATCC 23444^T	==========
Mycoplana dimorpha	IAM 13154	==========
Ochrobactrum antropi	IAM 14119	==========
Phyllobacterium rubiacearum	IAM 13587	==========
Phyllobacterium myrsinacearum	IAM 13584	==========
Mesorhizobium huakuii	IFO 15243	==========
Mesorhizobium loti	LMG 6125^T	==========
Mesorhizobium mediterraneum	UPM-Ca36	==========
Rhizobium etli	USDA 9032	==========
Rhizobium galegae	ATCC 43677^T	==========
Rhizobium leguminosarum	LMG 8817^T	==========
Rhizobium tropici	LMG 9517	==========
Sinorhizobium fredii	ATCC 35423^T	==========
Sinorhizobium meliloti	LMG 6133^T	==========
Sinorhizobium saheli	LMG 7837	==========
Sinorhizobium terangae	LMG 6463	==========
Sinorhizobium xinjangensis	IAM 14142	==========
Zoogloea ramigera	ATCC 19623	==========
Zoogloea ramigera	IAM 12669	==========
Thermomicrobium roseum	ATCC 27502	==========
Azospirillum amazonenese	DSM 2787	==========
Blasrochloris viridis	ATCC 19567	==========
Rhodomicrobium vannielii	ATCC 51194	==========
Azospirillum lipoferum	ATCC 29707	=========A=====
Rhodospirillum salexigens	ATCC 35888	=====A=========
Rhodopseudomonas acidophila	ATCC 25092	====A====A=====
Rhodospirillum fulvum	ATCC 15798	==========C=
Azospirillum brasiliense	ATCC 29145	=========AU====
Acidiphilium rubrum	ATCC 35902	=========A===C=
Gluconobacter oxydans	DSM 3503	=========A===U=

Abbreviations: ATCC – American Type Culture Collection, Rockville, Maryland, USA; IAM – Institute of Applied Microbiology, University of Tokyo, Bunkyo-ko, Tokyo, Japan; IFO – Institute for Fermentation, Osaka, Japan; DSM – Deutsche Sammlung von Mikrorganismen und Zellkulturen, Braunschweig, Germany; LMG – Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; USDA – United States Department of Agriculture, Beltsville, USA.

genetic position of the organisms is maintained. There are well defined phylogenetic groups for which single probes cannot be designed, e.g. the rhizobia group. Frequently, it is not possible to design single probes that are specific for the complete group or which exclude all non-group organisms. The rhizobia probe (RHI1247, Table 3) represents an example of a group specific probe that detects all organisms of the rhizobia target group but fails to discriminate a few non-target group organisms (Table 5). In many cases this problem can be resolved by the combined application of probes with overlapping or supplementary specificities. A second 16S rRNA targeted rhizobia group specific probe (RHI820, Tables 3, 5) was designed which matches 16S rRNA targets of the majority of the members of the group, but does not hybridize to some of them (*Blastobacter*, *Ochrobactrum*, *Phyllobacterium*, *Mesorhizobium huakuii*, *M. loti* and *M. mediterraneum*). Sub-group specific probes (BLA999, *Blastobacter aggregatus* and *B. capsulatus*; RLM820, *Mesorhizobium huakuii*, *M. mediterraneum* and *M. loti* strains; RLP820, *Phyllobacterium* and *Mesorhizobium loti* strains; Table 5) have been designed. The successful hybridization of probe RHI1247 and one of the complementary probes RHI820, BLA999, RLM820, and RLP820 allows reliable identifcation of organisms as members of the rhizobia group and their respective sub-groups.

A similar problem concerns the reliability of probe specificity. Modern probe design is based on large and fairly comprehensive sequence data sets. However, the probes can only be optimized with respect to the available sequence data, and experimental evaluation of probe specificity is restricted by experimental facilities and the availability of reference strains. Given that a single oligonucleotide probe targets a short sequence stretch often containing only one or two diagnostic positions, it is possible that the same target may occur within unknown rRNAs of organisms which phylogenetically are not members of the specificity group. This is especially the case with probes specific for lower taxonomic units (e.g. species). Nucleotides which are diagnostic at these phylogenetic levels occupy rapidly changing sequence positions. Thus, there is considerable risk that 'false' identities occur within rRNAs of unrelated organisms resulting from multiple base changes during the course of evolution. Consequently, the use of single specific probes may result in misidentification. The multi-probe concept outlined above for group specific probes targeted to more conserved regions can also be applied to validate highly specific probes. It is highly unlikely that 'false' identities evolved at multiple non-homologous rRNA sites in phylogenetically distant organisms. Using multiple probes of identical specificities targeting different sites, successful hybridization of all probes of the set ensures correct identification.

Given the availability of rapidly growing sequence databases and improved software (Strunk et al., 1998), the risk of improper probe design is substantially reduced. New probes should still be evaluated experimentally against targets from a representative selection of reference organisms however. This is of major importance when *in situ* techniques will be used for the analysis of complex environmental samples. The potentially vast number of so far uncultured bacteria in such samples (Amann et al., 1995; Brock, 1987; Staley and Konopka, 1985) holds many possibilities of potential false positive identification. Table 6 shows the results of probe evaluation using the broad range rhizobia probe (RHI1247) in combination with *in situ* colony and whole cell hybridization techniques and a selection of target and non-target organisms.

Hybridization techniques

A variety of solid and solution phase techniques for specific probe hybridization to various nucleic acid preparations are in use (Schleifer et al., 1993). Target nucleic acids may originate from pure cultures, mixed cultures, or complex samples. For several reasons rRNAs are the preferred target molecules. In comparison with 1–12 rRNA genes per cell, the high copy number of rRNA molecules provides a natural target amplification resulting in a higher sensitivity of the hybridization approach (Amann et al., 1995). Furthermore, potential non-specific binding to genomic DNA not encoding rRNA is eliminated by using purified rRNA. Most hybridization techniques can be used to determine the relative abundance of specific targets. Universal probes which are complementary to invariant or highly conserved rRNA sequence stretches and consequently hybridize to rRNAs and/or the corresponding genes of essentially all organisms are used as standards for hybridizations with specific probes (Stahl et al., 1988). Results obtained from such quantitative measurements do reflect the relative abundance of rRNA molecules or genes rather than relative cell numbers however. The number of rRNA molecules and therefore the number of specific targets varies, e.g. in relation to the physiological status of the cell

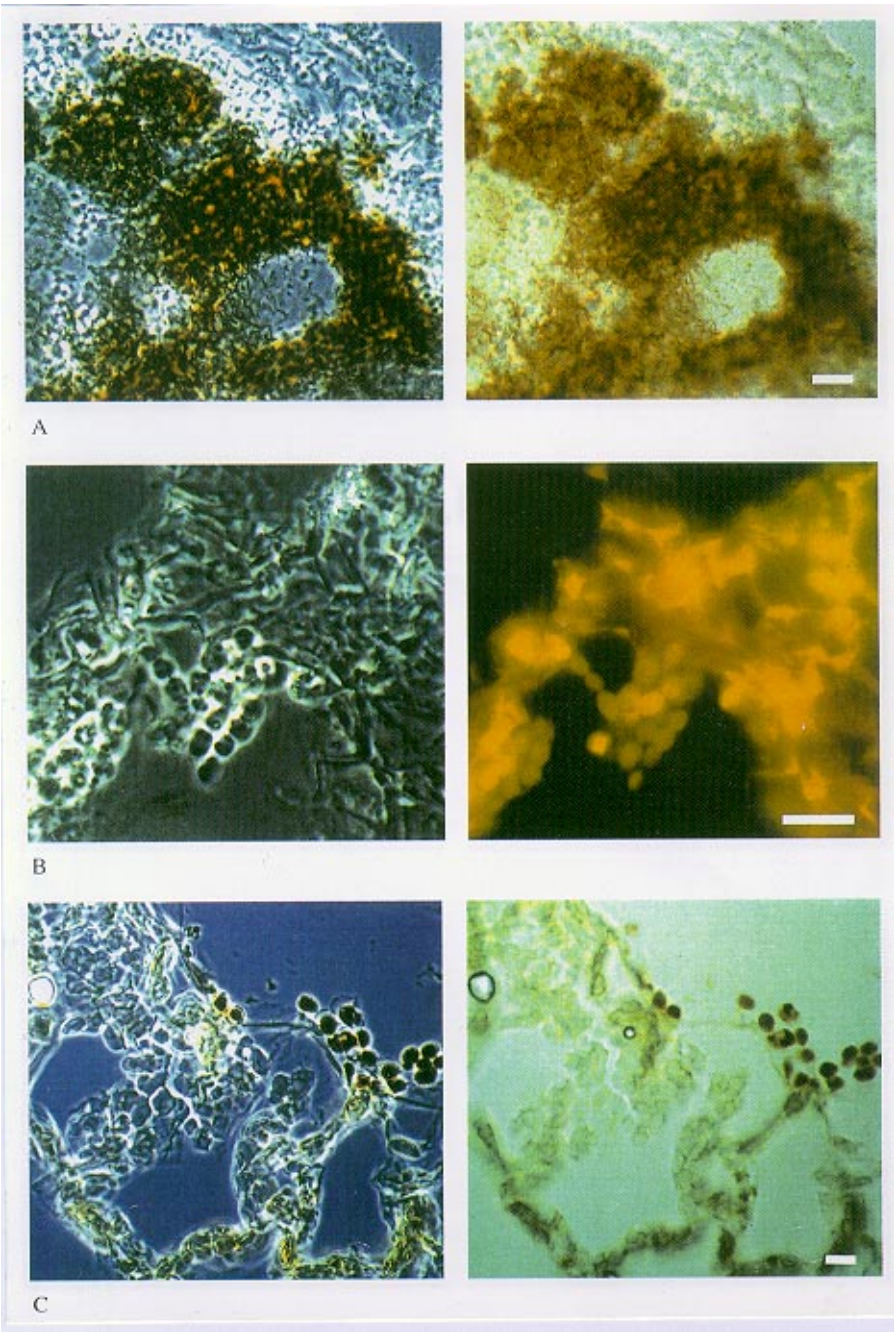

Figure 7. A: *In situ* hybridization of rhizobia within root nodules of *Trifolium* sp. Cryosections of root nodules were fixed in an increasing ethanol series and treated with methanol/H_2O_2 to block endogenous peroxidases (Amann et al., 1995). The peroxidase-labeled oligonucleotide probe RHI1247 was detected with diaminobenzidine which formed brownish precipitates in the cells. Left panel shows phase-contrast micrograph, right panel bright-field micrograph from one microscopic field. B: Autofluorescence of cyanobacteria and the surrounding plant material in cryosections of the waterfern *Azolla filiculoides*. Identical microscopic fields are shown in the phase-contrast (left) and epifluorescence micrographs (right, using a fluorescein-specific filter set). C: Cross-section of a dorsal leaf lobe from *Azolla caroliniana*. Cyanobacteria living in leaf cavities were hybridized with the eubacterial probe Eub338 labeled with peroxidase and detected as indicated in A. Phase-contrast (left) and bright-field (right) micrographs are shown. Bars represent 10 μm.

Table 5. Nested or supplementing specificities of probes RHI1247, RHI820, BLA999, RLM820 and RLP820. The complete data set of available small subunit RNA sequences was screened for the presence of the respective probe target sites. Only organisms which contain the target sites are listed. All other organisms which are represented in the database contain at least one base difference within the homologous sequence parts. The solid line separates target (rhizobia group; upper part) and non-target (organisms which are not members of the rhizobia group but contain the RHI1247 probe target site; lower part) organisms. Organisms for which only partial sequences are available lacking the target region (e.g. *Mesorhizobium ciceri* or *Rhizobium tropici*) are not included

Genus/species/strain	Hybridization with probe				
	RHI1247	RHI820	BLA999	RLM820	RLP820
Agrobacterium	+	+	–	–	–
Blastobacter aggregatus	+	–	+	–	–
Blastobacter capsulatus	+	–	+	–	–
Bartonella	+	+	–	–	–
Brucella	+	+	–	–	–
Chelatobacter heintzii	+	+	–	–	–
Mycoplana dimorpha	+	+	–	–	–
Mycoplana ramosa	+	+	–	–	–
Ochrobactrum	+	–	–	–	–
Phyllobacterium	+	–	–	–	+
Mesorhizobium huakuii	+	–	–	+	–
Mesorhizobium loti ATCC 33669	+	–	–	–	+
Mesorhizobium loti IAM 13588	+	–	–	+	–
Mesorhizobium loti LMG 4248	+	–	–	–	+
Mesorhizobium loti LMG 6125^T	+	–	–	–	+
Mesorhizobium mediterraneum	+	–	–	+	–
Rhizobium	+	+	–	–	–
Sinorhizobium	+	+	–	–	–
Zoogloea ramigera ATCC 19623	+	+	–	–	–
Zoogloea ramigera IAM 12669	+	+	–	–	–
Azospirillum amazonense	+	–	–	–	–
Azospirillum irakense	+	–	–	–	–
Blastochloris viridis	+	–	–	–	–
Rhodobium orientum	+	–	–	–	–
Rhodocista catenaria	+	–	–	–	–
Rhodomicrobium vannielii	+	–	–	–	–
Thermomicrobium roseum	+	–	–	–	–

(Schaechter et al., 1958). Also the number of rRNA genes may differ even between closely related species.

Classical hybridization formats, e.g. dot-, slot-, or spot-blot techniques, typically rely on membrane bound target nucleic acids. Alternatively, other solid supports such as micro-titer plates can be used for target immobilization. More sophisticated variations are the capture techniques (Morrissey and Collins, 1989; Schleifer et al., 1993). Two probes of different or identical specificities are used for immobilization of the target nucleic acids (capture probe) and for detection and quantification of targets (detector probe). The hybrids of capture probe and target nucleic acids may be immobilized on solid supports such as paramagnetic beads by different techniques (Morrissey and Collins, 1989; Schleifer et al., 1993). One possibility is the hybridization of capture probe tails and complementary nucleic acids bound to the support, another the interaction of capture probe associated haptens (e.g. biotin, digoxigenin) and binding proteins (e.g. avidin or antibodies) which are fixed on the support. In comparison with the direct hybridization methods, the capture probe assays provide higher sensitivity by specific enrichment of the targets prior to detection.

Table 6. Results of *in situ* colony and/or cell hybridization of probe RHI1247 to a selection of target and non-target organisms of the α-subclass of the *Proteobacteria*. The dotted line separates target (rhizobia group; upper part) and non-target (organisms which are not members of the rhizobia group; lower part) organisms. *In situ* colony hybridizations with peroxidase-conjugated probe RHI1247 were carried out in 5×SSC containing 35% (vv) formamide at 37 °C. *In situ* cell hybridizations with fluorescent or peroxidase-conjugated probe RHI1247 were performed in 0.9 *M* NaCl containing 45% formamide at 46 °C

Organism	Strain	*In situ* hybridization	
		Colony	Cell
Agrobacterium tumefaciens	ATCC 23308^T	+	+
Agrobacterium tumefaciens	CECT 4364	nd	+
Agrobacterium tumefaciens	At28[2]	+	nd
Agrobacterium tumefaciens	At4404[2]	+	nd
Agrobacterium tumefaciens	C58[2]	+	nd
Agrobacterium tumefaciens	GMI9023[2]	+	nd
Agrobacterium radiobacter	WS 1382	nd	+
Agrobacterium rhizogenes	WS 1383	+	nd
Agrobacterium rubi	WS 1384	+	+
"Agrobacterium ferrugineum"	LMG 128	nd	+
"Agrobacterium luteum"	LMG 134	nd	+
"Agrobacterium.kielense"	LMG 133	nd	+
"Agrobacterium stellulatum"	LMG 122	nd	+
Agrobacterium sp	KAg3[2]	+	nd
Agrobacterium sp.	CHAg4[2]	+	nd
Ochrobactrum anthropi	LMG 2136	+	nd
Ochrobactrum anthropi	LMG 3305	+	nd
Ochrobactrum anthropi	LMG 3331^T	+	nd
Ochrobactrum anthropi	IBÖ a	+	nd
Ochrobactrum anthropi	IBÖ b	+	nd
Ochrobactrum anthropi	IBÖ c	+	nd
Ochrobactrum anthropi	IBÖ d	+	nd
Ochrobactrum anthropi	IBÖ e	+	nd
Ochrobactrum anthropi	IBÖ f	+	nd
Rhizobium etli	Bra5[2]	+	nd
Rhizobium etli	F6[2]	+	nd
Rhizobium etli	Nitragin 8251[2]	+	+
Rhizobium galegae	625[2]	+	+
Rhizobium leguminosarum	127K80e[1]	+	nd
Rhizobium leguminosarum	127K12b[1]	+	nd
Rhizobium leguminosarum	127K44[1]	+	nd
Rhizobium leguminosarum	102K81-2[1]	+	nd
Rhizobium leguminosarum	102K102[1]	+	nd
Rhizobium leguminosarum	BRA-5b[1]	+	nd
Rhizobium leguminosarum	Vf39[2]	nd	+
Rhizobium tropici	299[2]	+	nd

Table 6. Continued.

Rhizobium tropici	BR833[2]	+	nd
Rhizobium tropici	BR864[2]	+	nd
Rhizobium tropici	CIAT 899	+	nd
Rhizobium tropici	LMG 9503	nd	+
Sinorhizobium fredii	NGR234[2]	+	+
Sinorhizobium meliloti	RMe41[2]	nd	+
Sinorhizobium meliloti	WS 1373	+	nd
Zoogloea ramigera	WS 1610	nd	+
Zoogloea ramigera	WS 1830	nd	+
Zoogloea ramigera	WS 1831	nd	+
Zoogloea ramigera	WS 1846	nd	+
Zoogloea ramigera	WS 1847	nd	+
Zoogloea ramigera	WS 11849	nd	+
Acetobacter liquefaciens	ATCC 14835^T	nd	–
Azorhizobium caulinodans	LMG 6465^T	nd	–
Azospirillum brasiliense	IBÖ SPT60	–	nd
Azospirillum brasiliense	DSM 1690	nd	–
Azospirillum lipoferum	IBÖ BR17	–	nd
Azospirillum lipoferum	IBÖ SP59b	–	nd
Beijerinkia indica	LMG 2817^T	nd	–
Blastobacter denitrificans	LMG 8483^T	nd	–
Bradyrhizobium japonicum	61A124[1]	–	nd
Bradyrhizobium japonicum	USDA 118	–	nd
Bradyrhizobium japonicum	61A152[1]	–	nd
Bradyrhizobium japonicum	A182[1]	–	nd
Bradyrhizobium japonicum	61A212[1]	–	nd
Bradyrhizobium japonicum	LMG 6138^T	nd	–
Bradyrhizobium japonicum	DSM 31031	nd	–
Brevundimonas diminuta	DSM 1635	nd	–
Enterobacter aerogenes	LMG 2094	–	nd
Enterobacter aerogenes	WS 1293	–	nd
Methylobacterium extorquens	DSM 1737^T	nd	–
Paracoccus denitrificans	DSM 1408	–	nd
Paracoccus denitrificans	DSM 1690	nd	–
Rhizobium lupini	DSM 30140	nd	–

[1]Pinero et al., 1988;
[2]Centro de Investigación sobre Fijación de Nitrogeno, Cuernavaca, México;
Abbreviations: CECT – Collection Espanola de Culturas Tipicos, Valencia, Spain; IBÖ – Institut für Bodenökologie, Forschungszentrum für Umwelt und Gesundheit, Neuherberg, Germany; WS – Bakteriologisches Institut, Süddeutsche Versuchs- und Forschungsanstalt für Milchwirtschaft, Weihenstephan, Freising, Germany; nd – not determined. Other abbreviations are as specified for Table 4.

The major advantage of reverse hybridization techniques (Ehrmann et al., 1994) is the simultaneous use of sets of probes of different or nested specificities bound to separate locations on solid supports such as membranes or micro-titer plates. The target nucleic acids are simultaneously hybridized to all probes of the set. If pure cultures are analyzed, the resulting hybridization patterns allow the rapid identification of isolates at various taxonomic levels, if complex mixtures of organisms, e.g. environmental samples are investigated, the composition of that part of microbial communities can qualitatively be elucidated for which specific probes are available. In the latter case, for rapid and sensitive screening of complex samples rDNA fragments containing the target or equivalent regions are amplified *in vitro* as potential hybridization partners. The principle of reverse hybridization is also the basis of a promising technique which is under development (Lipschutz et al., 1995). High density oligonucleotide probe arrays are prepared by light-directed chemical synthesis. Currently 20,000 sites on an area of 1.28 cm^2 can be used for *in situ* probe synthesis. The target nucleic acids are labeled with fluorescent reporter groups and hybrids are detected by epifluorescence confocal scanning.

The hybridization methods described above do not allow direct determination of viable or total cell counts. This can only be achieved by applying *in situ* probing techniques. *In situ* colony hybridization was used to evaluate the rhizobia specific probe (RHI1247) with a selection of rhizobial and non-rhizobial culture collection strains and isolates (Table 6). The bacterial strains of interest were first grown to small colonies on suitable nucleic acid binding membranes placed on the top of solid nutrient media. The cells were lysed *in situ* applying combinations of chemical (e.g. alkaline), enzymatic (e.g. lysozyme) and physical (e.g. micro wave) treatments (Schleifer et al., 1993). The released nucleic acids were fixed on the membrane and hybridized. Probes of different specificities marked with different labels for different detection systems can be used simultaneously. Alternatively, different probes can be applied in subsequent cycles of hybridization detection and denaturation. Depending on the growth and lysis behaviour of the strains, up to 2×10^3 minicolonies can be analyzed on one filter of the diameter of standard plates (9 cm) (Brockmann et al., 1996). Using appropriately diluted samples and properly designed specific probes, the bacteria of complex samples can be differentiated and enumerated as colony forming units. The advantages of this technique are the rapid detection, enumeration and identification of bacteria at defined taxonomic levels among a large number of non-target organisms. The major drawbacks are the demand for cultivation of the target organisms and the variability of growth and lysis behaviour. Fast growing organisms may inhibit or mask slow growing ones. This problem can be reduced by applying differently diluted samples and incubation of membranes in parallel at different conditions for bacterial growth. Nucleic acids of cells resistant to the lysis remain inaccessible for the probes. Excessive production of extracellular polymers may hinder immobilization of the released target nucleic acids or the hybridization procedure itself. This seems to be a common problem with a number of rhizobial strains. False negative results of specific probe hybridization can be recognized as such by performing hybridizations with a universal probe as a positive standard however.

Currently, the most attractive technique for specific probe based identification and detection is *in situ* whole cell hybridization. The procedure is based on specific probe hybridization to intra-cellular targets. Again rRNA has advantages as a potential target nucleic acid due to its natural amplification. An average bacterial cell contains somewhere between 10^3 and 10^5 ribosomes and as many copies of the 5S, 16S and 23S rRNA. These molecules can be made available in whole fixed cells for hybridization with specific probes by permeabilization of the cell periphery with aldehydes or alcohols. These compounds stabilize the cell structure and kill the cells (Amann et al., 1995). Microscopic identification of individual microbial cells is routinely achieved with fluorescently labeled rRNA-targeted oligonucleotide probes (Amann et al., 1990; DeLong et al., 1989). The technique allows identification of individual cells within the microscopic field and provides high spatial resolution. Even complex environmental material can be analyzed within few hours after sampling. As the technique is not relying upon prior cultivation of the cells of interest it differentiates not directly between viable and nonviable cells. Morphological integrity and the presence of high amounts of intra-cellular rRNA is a good indication for cell viability, however. As the majority of bacterial species has not yet been isolated in pure culture, the combination of direct rRNA sequence retrieval and fluorescent *in situ* hybridization allows completely cultivation-independent single cell identification and phylogenetic analysis (Amann et al., 1991; Spring et al., 1992). Fluorescent *in situ* hybridization with rRNA-targeted probes is a most

useful tool for microbial ecology since it allows to directly determine cell numbers of the target organisms in complex samples and to monitor strains with variable morphology. In case of immobilized communities, as in symbioses of nitrogen fixing rhizobia or cyanobacteria with plants, the spatial distributions can be determined. Furthermore, rRNA-contents of individual cells may be measured which may yield estimates on general metabolic activity of individual cells (DeLong et al., 1989; Poulsen et al., 1993; Wallner et al., 1993). Some drawbacks of this excellent technique have to be taken into account. Given that successful probe hybridization depends on permeabiltiy of the cell envelope and hybrid detection on the ribosome content, cells may escape their detection. Fixation inevitably causes shrinking or deformation of the sample material. Thus the spatial distribution of the detected cells cannot correctly reflect the situation in the original sample.

An example of single cell identification of rhizobia using fluorescently labelled oligonucleotide probes is given in Figure 6. An artificial mixture of *Rhizobium etli* and *Escherichia coli* was simultaneously hybridized with a fluorescein-labelled probe specific for γ-subclass Proteobacteria (Manz et al., 1992) and carboxytetramethyrhodamine-labelled probe RHI1247. After hybridization the two species could be clearly differentiated by the specific red (*R. etli*) and green fluorescence (*E. coli*) conferred by the two probes. On examination of the symbioses of nitrogen-fixing rhizobia with plants, the strong autofluorescence of the plant material interfered with the detection of fluorescent probes, however. This problem could be avoided by the use of a nonfluorescent hybridization assay in which horseradish peroxidase labelled oligonucleotides are used. The hybrids are detected by the formation of a coloured precipitate (Amann et al., 1992). Using this technique, rhizobia could be identified, e.g. in ethanol fixed cryosections of root nodules of *Trifolium* sp. with probe RHI1247 (Figure 7, panel A).

Autofluorescence is even more pronounced for the identification of nitrogen fixing cyanobacteria in the dorsal leaves of the water fern *Azolla caroliniana*. Here, not only the plant material but even the target bacteria strongly auto-fluorescence due to their chlorophyll content (Figure 7, panel B). Again specific detection of the cyanobacteria was achieved with the enzyme-linked assay using the bacterial probe EUB338 (Amann et al., 1995). This technique is advantageous not only for its ability to identify cells in fluorescent environments but also since it does not require an expensive epifluorescence microscope. The formation of precipitates can be seen under phase contrast (Figure 7, left side of panels A and C) but is best detected in a regular light microscope with brightfield illumination (Figure 7, right side of panels A and C). It should be noted, however, that the spatial resolution is inferior to that achieved with fluorescent probes so that dense formations of target cells are seen only as brown areas which makes enumeration almost impossible.

Limitations

The usefulness of highly variable positions for the design of highly specific probes needs further critical evaluation. Most bacteria contain multiple rRNA genes. Reports on base differences in multiple rRNA genes of the same organisms are available (Boros et al., 1979; Branlant et al., 1981; Carbon et al., 1979; Dryden und Kaplan, 1990; Fleischmann et al., 1995; Gazumyan et al., 1994; Moriya et al., 1985; Ogasawara et al., 1993; Shen et al., 1982), but no systematic investigations have been performed. Recent investigations (Nübel et al., 1996) indicate that interoperon differences exist at least in some phylogenetic groups at highly variable rRNA sequence positions. Various operons seem to be transcribed at different frequencies in closely related strains. The potential problems for single probe based identification are obvious: closely related strains missing the gene variant containing the correct target site may escape detection.

Another limitation of rRNA based identification and detection methods concerns their ability to resolve lower taxonomic levels and originates from the overall conserved character of the rRNA corresponding genes. Usually, strains are too closely related to contain diagnostic sequence differences in their rRNAs. In most cases, it is therefore not possible to gain phylogenetic information at this level or to design strain specific probes. Even if there are a few odd base differences that might be used for differentiation, these are of low significance for phylogenetic inference or probe based identification.

Another type of problem occurs when the current taxonomy does not agree with phylogeny and therefore taxon specific probes cannot be designed.

The quantification of microbial cells with specific probes is hampered by the demand for cultivation using *in situ* colony hybridization and possible problems

of cell permeability and ribosome content applying *in situ* whole cell hybridization.

In general, rRNA targeted probes are of only limited value when a phenotypic trait such as nitrogen fixation is of major interest. Application of rRNA targeted probes combined with probes specific for genes involved in the expression of the phenotypic trait might correlate phylogenetic identification with physiological capacity (see Perret and Broughton, this publication).

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