Chapter 2

MOLECULAR PHYLOGENY AND ECOLOGY OF ROOT ASSOCIATED DIAZOTROPHIC α- AND β-PROTEOBACTERIA

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1. INTRODUCTION

The knowledge about the natural diversity of root associated diazotrophs is getting increasingly complex and fascinating, since more and more species of plant associated diazotrophs are successfully isolated and cultivated from a wide variety of plants especially from subtropical and tropical regions. The application of molecular genetic detection and identification methods greatly aids in clarifying the phylogenetic relationships of these bacteria. It is generally accepted, that only a combination of methods including classical cultivation techniques and cultivation independent techniques enable a comprehensive insight into the bacterial diversity in environmental habitats (Hartmann *et al*., 1997; Liesack *et al*., 1997). It has been demonstrated repeatedly, that a high bacterial diversity could be revealed using molecular techniques targeting directly either the diversity of the 16S-rDNA (Amann *et al*., 1995) as most used genetic marker for molecular phylogenetic studies or the diversity of the *nif*-genes (Ueda *et al.*, 1995). Concerning the rhizosphere environment, the degree of cultivability is assumed to be high due to the good growth conditions for microbes in the root environment. However, for the grass endophytic *Azoarcus* spp., an unculturable state has recently been demonstrated (Hurek *et al*., 2002).

The ribosomal RNA genes of bacteria, especially the 16S- and 23S-rRNA, are excellent molecular markers for phylogenetic studies, because of their functionally constancy, their ubiquitous distribution and elements rising from highly conserved

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to highly variable regions within the sequence (Ludwig *et al.*, 1998). This molecular phylogenetic approach can be applied to identify pure isolates and to assess the diversity of complex communities. Since powerful amplification and sequencing techniques became available in the last decade, now more than 16.000 complete and partial sequences are deposited in data banks like NCBI. Modern software, e.g. the widely used software package ARB has been developed as well to handle all this data for phylogenetic analysis (Ludwig and Strunk, www.arb-home.de). In addition, sophisticated software packages are available not only to use this information for phylogenetic evaluation but also for the development of discriminative oligonucleotide probes for diagnostic purposes. In cases of very close phylogenetic relationship, the higher information content of the 23S-rDNA or the intergenic spacer region of the rDNA-operon (IGS-region; Tan *et al*., 2001) offer additional very valuable molecular markers for phylogenetic studies and strain differentiation.

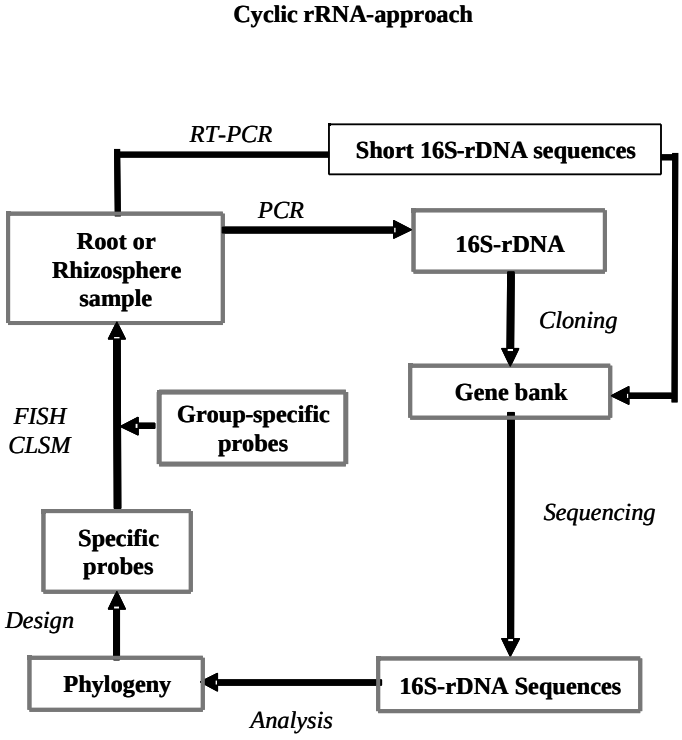


Figure 1: Cyclic rRNA-approach. Schematic representation of the different steps used for identification and in situ *localization of associated/endophytic nitrogen-fixing bacteria.*

The development of 16S-rRNA targeting fluorescence-labeled phylogenetic oligonucleotide probes enable to identify active bacterial cells in their natural habitat

by using the fluorescence *in situ* hybridization (FISH) technique (Amann *et al*., 1995), thereby closing the so called "cyclic rRNA-approach" (Fig. 1).

2. TOOLS FOR MOLECULAR PHYLOGENY AND *IN SITU* LOCALIZATION OF BACTERIAL ISOLATES AND COMMUNITIES

2.1. Use of 16-rDNA as Phylogenetic Marker

Due to its wide and successful use as phylogenetic marker, the 16S-rDNA provides an indispensable tool for the classification and identification of bacteria. In contrast to other cellular RNA-species, the ribosomal RNA´s occur in very high copy number per active cell (up to 16.000). This enables an efficient labeling of physiologically active cells with rRNA-targeting fluorescence labeled probes. For a first screening of bacterial isolates from any plant environment, a large set of phylogenetic oligonucleotide probes (Table 1) can be used in a hierarchical manner from the kingdom down to the genus and species level (Amann *et al*., 1995). Bacterial isolates of known species can be classified in a few hours and candidates of possibly not yet described phylogeny can be identified usually at least at the subphylum or genus level using the fluorescence *in situ* hybridization (FISH) technique (see 2. 4.). A more detailed phylogenetic analysis is necessary in the case of isolates with possibly new or uncertain phylogenetic classification by sequence analysis of the 16S-rDNA. Using PCR primers complementary to the highly conserved 5´- and 3´ends of the 16S-rDNA coding genes, ribosomal RNA sequences of pure isolates or of 16S-rDNA clones retrieved from complex microbial communities (see below) can be obtained. For a reliable identification, the complete 16S-rDNA sequence has to be used for phylogenetic analysis. As powerful software package, ARB (Ludwig, www link) offers several sequence analysis tools; e.g. for tree calculation. Specific oligonucleotide probes can be developed by the implemented PROBE DESIGN and PROBE MATCH tools. In order to have a cultivation independent analysis of the plant associated bacterial diversity, the 16S-rRNA or 16S-rDNA of the RNA / DNA, extracted from natural habitats needs to be PCR-amplified (in the case of RNA after a reverse transcriptase step) and cloned. The analysis of the 16S-rDNA clones is performed as described above, yielding insight into the natural diversity. After improving the set of probes for expected bacteria the *in situ* analysis with FISH can be performed. Closing the "cyclic rRNA-approach" (Fig.1). A similar approach has been performed using the *nif*-genes of natural plant associated communities (Hurek *et al*., 2002). However, an *in situ* labeling by FISH targeting *nif*-mRNA is much less efficient because of the lower copy number of mRNA as compared to rRNA.

2.2. Additional molecular markers

Due to the sometimes very close phylogenetic relationships, alternative molecular markers had to be used for a successful phylogenetic identification, such as the 23S-rDNA or the IGS-regions of the r-DNA operon. For a final decision of the

phylogenetic relationship of bacterial isolates, the DNA-DNA relatedness of the entire bacterial DNA´s has to be examined and a polyphasic identification approach has to be performed (Vandamme *et al.*, 1996) in addition to the 16S-rDNA similarity analysis; above the level of 70% DNA similarity, bacteria are defined to belong to the same species. Other biochemical markers support this type of classification, such as protein profiling, fatty acid analysis, DNA-fingerprinting techniques and could separate the diversity of bacterial isolates at the subspecies or microdiversity level (Rademaker *et al*., 2000, Schloter *et al*., 2000).

Table 1: Phylogenetic rRNA-targeting oligonucleotide probes

Probe	**Position**	**Sequence**	% FA	**Specificity**	**Ref**
		***In situ* probing of *Azospirillum* spp**.			
AZO-440a	16S, 440-457	GTCATCATCGTCGCGTGC	50	*Azospirillum* spp. *Skermanella*, *Rhodocista*	a
AZO-440b	16S, 440-457	GTCATCATCGTCGTGTGC	50	*Azospirillum* spp. *Skermanella*, *Rhodocista*	a
AZOI-655	16S, 655-672	CACCATCCTCTCCGGAAC	50	Species cluster: *A. lipoferum*, *A. brasilense*, *A. halopraeferans*, *A. doebereinerae*	a
Aama-1250	16S, 1250-1267	CACGAGGTCGCTGCCCAC	50	*A. amazonense*	a
Abras-1420	16S, 1420-1438	CCACCTTCGGGTAAAGCCA	40	*A. brasilense*	a
Adoeb-587	16S, 587-604	ACTTCCGACTAAACAGGC	30	*A. doebereinerae*	b
Ahalo-1115	16S, 1115-1133	ATGGTGGCAACTGGCAGA	45	*A. halopraeferans*	a
Ahalo-1249	16S, 1249-1266	GCGACGTCGCTTCCCACT	60	*A. halopraeferans*	a
Airak-1423	16S, 1423-1440	CACCGGCTCAGGTAAAG	10	*A. irakense*-cluster	a
Airak-985	16S, 985-1003	TCAAGGCATGCAAGGGTT	35	*A. irakense*-cluster	a
Alila-1113	16S, 1113-1130	ATGGCAACTGACGGTAGG	35	*A. lipoferum*, *A. largimobile*	a
Ahalo-1115C	16S, 1115-1133	ATGATGGCAACTGGCAGTA	45	Competitor	a
Ahalo-1249C	16S, 1249-1266	GCGACTTCGCTTCCCACT	60	Competitor	a
Abras-1420C	16S, 1420-1437	CACCTTCGGGTAAAACCA	40	Competitor	a
Alila-1113-C	16S, 1113-1130	ATGGCAACTGGCGGTAGG	20	Competitor	a
		***In situ* probing of *Herbaspirillum* spp**.			
HERB-1432	16S, 1432-1449	CGGTTAGGCTACCCAACTT	35	Genus *Herbaspirillum*	c
Hrubri-445	16S, 445-462	GCTACCACCGTTTCTTCC	60	*H. rubrisubalbicans*	c
Hsero-445	16S, 445-462	GCCAAAACCGTTTCTTCC	35	*H. seropedicae*	c
Hfris-445	16S, 445-462	TCCAGAACCGTTTCTTCC	50	*H. frisingense*	c

***In situ* probing of *Burkholderia* spp.**

Subu-1237	16S, 1237-1254	AATGGTCGGAACAGAGGG	60	Genera *Burkholderia* and *Suturella*	d
Bcv-13b	23S, 255-274	ACAGGGCACGTTCCGATGT	25	*B. cepacia*, *B. vietnamiensis*, Gruppe VI, *B. multivorans*, *B. stabilis*, *B. pyrocina*	d
Bglad-445	16S, 445-463	GCCCTCAGGATTTCTTTC	35	*B. gladiolii*, *B. glumae*, *B. vandii*, *B. plantarii*, *B. cocovenenans*	d
Bglad-465	16S, 465-482	GTCATCCCCGAAGGATAT	35	*B. gladiolii*, *B. glumae*, *B. plantarii*, *B. cocovenenans*	d
Bbras-636	16S, 636-653	CCAGCGCTGCAGTCACCA	60	*B. brasilense*	e
Bbras-62	16S, 62-79	AGCCCGCGCTGCCGTCCG	60	*B. brasilense*	e
Btrop-636	16S, 636-653	CAAGCGATGCAGTCACCA	55	*B. tropicalis*	f
Btrop-463	16S, 463-480	CATCCCCCGGCCATATTA	20	*B. tropicalis*	f

FA = % Formamide in the hybridization buffer; *a: Stoffels* et al., *2001; b: Eckert* et al., *2001; c: Kirchhof* et *al., 2001; d: Stoffels* et al., *1998; e: Baldani* et al., *unpublishedl; f: Reis et al., 2003.*

For diversity analysis targeting the functional genes of nitrogen fixation, the rather highly conserved *nifHDK* operon is used. There have been several studies to compare the 16S rDNA-based and the *nifH*-gene or *nifH*-protein sequences. This is necessary to confirm, that the evolutionary relationship are also applicable for this particular gene. Hennecke *et al.* (1985) investigated the relative few *nif*-gene sequences available at that time and showed that the *nifH*-sequence data support the 16S rRNA tree; this also holds true for the *nifD* and *nifK* sequences. Normand and Bousquet (1989) performed a similar study including *Frankia* and other Firmicutes showing a clustering in the Gram-positive bacteria. However, a lateral gene transfer especially in closely related species could not be excluded.

Evidence for lateral gene transfer emerged e.g. from studies of the *nifHDK*-genes of the β-proteobacterium *Azoarcus* (Hurek *et al.*, 1997). Recently, different primers and probes targeting the *nifH*-gene have been successfully used for phylogenetic analysis of diversity of diazotrophic bacterial communities in soil and rhizosphere environments (Ueda *et al.*, 1995; Zehr *et al.*, 1998; Widmer *et al.*, 1999; Mergel *et al.*, 2001).

Interestingly, these studies demonstrated a high diversity of environmental *nifH*-sequences, which clearly exceeds the sequence diversity of the hitherto known and cultivated diazotrophs. It is quite possible, that *nif*-genes are much more widely

distributed among bacteria known until now; *nif*-genes could frequently be present in the unculturable / not cultured fraction of environmental bacteria.

2.3. Diversity Studies Using Molecular Probing and Fingerprinting Techniques

In addition to the 16S- and 23S-rRNA oligonucleotide probes, a number of other molecular taxonomic tools have been developed and successfully applied such as RAPD-markers (Vaneechoutte, 1996), RFLP-analysis (Han and New, 1998) or rep-PCR analysis (Rademaker *et al*., 2000). These approaches allow the rapid molecular identification at the species and even subspecies down the individual strain level. The RFLP-analysis of the whole genome with rarely cutting restriction enzymes followed by pulsed field gel electrophoresis can be used for strain specific identification (Gündisch *et al*., 1993). These and related studies have also shown, that the 16S-rDNA genes of many bacteria do occur in multiple replicons (Gündisch *et al*., 1993; Caballero-Mellado *et al*., 1999).

2.4. Molecular Tools for in situ *Localization and Population Dynamic Studies*

Fluorescence *in situ* hybridization (FISH) using fluorescence labeled oligonucleotide probes provides a handy molecular tool, not only to screen and characterize the phylogeny of bacterial isolates, but even more to get insight into the localization of individual cells in their natural habitat, without the need of cultivation (Amann *et al.*, 1995, Wagner *et al.*, 2003). After a fixation step of the material usually in paraformaldehyde (3%) over night at 4^{o}C the material is fixed on glass slides and dehydrated with an increasing ethanol series. According to Wagner *et al.* (1993), the FISH protocol uses an incubation at 46°C for 90 min in an hybridization buffer containing 0.9 M NaCl and different concentrations of formamide according to the stringency conditions for the probes used (see Table 1). This step is followed by a stringent washing step at 48^{o}C for 15 min in a buffer with optimized NaCl concentrations and EDTA. After rinsing the slides with water the samples can additionally be counterstained with DAPI as general DNA-stain and mounted in antifading solution. Regular epifluorescence microscopy is usually not sufficient in environmental and root samples, because of the autofluorescence problem of the biological matrix. Confocal laser scanning microscopy, equipped with two lasers (Ar and HeNe, supplying excitation wave length at 365, 488, 543 and 633 nm) provides a much better resolution, as is reviewed by Hartmann *et al.* (1998).

An alternative very powerful fluorescence-based *in situ* detection method is the fluorescence tagging of bacteria with the *gfp*- (green fluorescence protein) or *rfp*- (red fluorescence protein) gene (Unge *et al.*, 1998). This molecular tagging is used for general cell tagging or in operon fusion constructs for expression studies of genes of interest (e.g. *nifH*-gene, Egener *et al.*, 1998).

If population studies do not focus on a high spatial resolution, *ex situ* molecular analyses of the bacterial diversity can be performed as is summarized by Hartmann *et al.* (2003). Genomic DNA and rRNA is isolated from the biological material following standard protocols (Miethling *et al.*, 2000). Usually a further purification

of the RNA/DNA extract is necessary. For amplification of the desired specific DNA range, either primer specific for 16S-rDNA coding genes, e.g. 616-F and 630-R (Juretschko *et al.*, 1998) or specific *nifH*-targeting primers (Ueda *et al.*, 1995, Widmer *et al.*, 1999) were used. The resolution of the diversity of PCR-amplificates can be resolved either by temperature gradient gel electrophoresis (TGGE), as described by Heuer and Smalla (1997), to separate high molecular DNA species according to their sequence. The separated DNA-amplificates are finally analyzed by sequencing to retrieve information about its affiliation and underlying diversity. If very high resolving molecular diversity analysis is needed, the method of choice is the development of clone libraries of long amplification products providing the basis for most comprehensive phylogenetic diversity analyses.

3. MOLECULAR PHYLOGENY AND ECOLOGY OF *AZOSPIRILLUM* AND OTHER NITROGEN-FIXING α-SUBCLASS PROTEOBACTERIA

3.1. Diversity of Diazotrophic α-Proteobacteria

The nitrogen-fixing bacteria of the α subclass of Proteobacteria mostly occur in six major groups. One cluster contains *Zymomonas, Rhizomonas* and *Sphingomonas*, with a diazotrophic bacterium isolated from rice characterized as *Sphingomonas paucimobilis.* A second group harbours the endophytic diazotroph *Gluconacetobacter* spp. with now three species *G. diazotrophicus* from sugar cane (Gillis *et al*., 1989) and *G. johannae* and *G. azotocaptans* from coffee plants (Fuentes-Ramírez *et al*., 2001). The former species *Acetobacter diazotrophicus* (Gillis *et al.*, 1989) was now reclassified to *Gluconacetobacter diazotrophicus* based on comparative sequence analysis of 16S-rRNA sequences (Yamada *et al.*, 1997). A small group comprises the genera *Rhodobacter* with the diazotrophic *Rhodobacter capsulatus* and *Paracoccus*. A fourth large cluster represents the symbiotic genera *Rhizobium, Sinorhizobium, Mesorhizobium* and *Ochrobactrum*. Very recently, a nitrogen-fixing *Ochrobactrum* sp. isolate from *Acacia* nodules was described, which belong to the Rhizobiaceae family, forming fully developed and functional nodules with roots of *Acacia* (A. Ngom, personal communication). The last two clusters are formed by the symbiotic bacteria *Bradyrhizobium* and *Azorhizobium* as well as *Beijerinckia*, *Xanthobacter* and *Rhodopseudomonas* and finally by the group comprising the nitrogen fixing genera *Azospirillum, Rhodospirillum, Aquaspirillum* and *Magnetospirillum* in the so-called α-1-subclass.

3.2. Diversity of Azospirillum spp.

In the rediscovery of *Azospirillum* in the 1970th by Dr. Johanna Döbereiner and her associates (Tarrand *et al*., 1978), the species *A. lipoferum* and *A. brasilense* were described, resembling *Spirillum lipoferum* originally described by Beijerinck in 1925. In the following years, *A. amazonense* (Magalhães *et al*., 1983), *A. irakense* (Khammas *et al*., 1989), *A. halopraeferens* (Reinhold *et al.*, 1987), *A. largimobile*

(Sly and Stackebrandt, 1999) were discovered (see Okon, 1994). The most recently identified *Azospirillum* species is *A. doebereinerae*, which was isolated from roots of the giant growing C4-grass *Miscanthus sinensis* (Eckert *et al*., 2001). The taxonomy, physiology and ecology of the genus *Azospirillum* were recently reviewed by Baldani and Hartmann (2003).

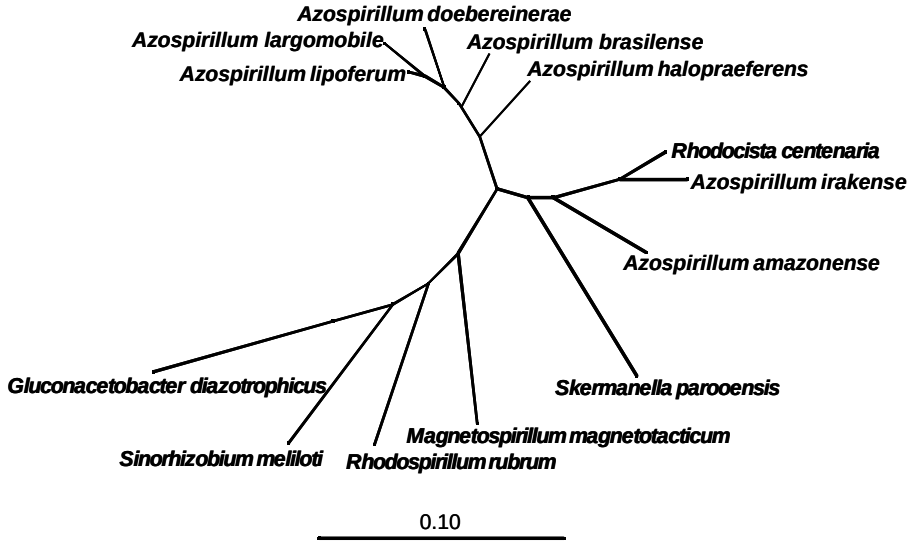
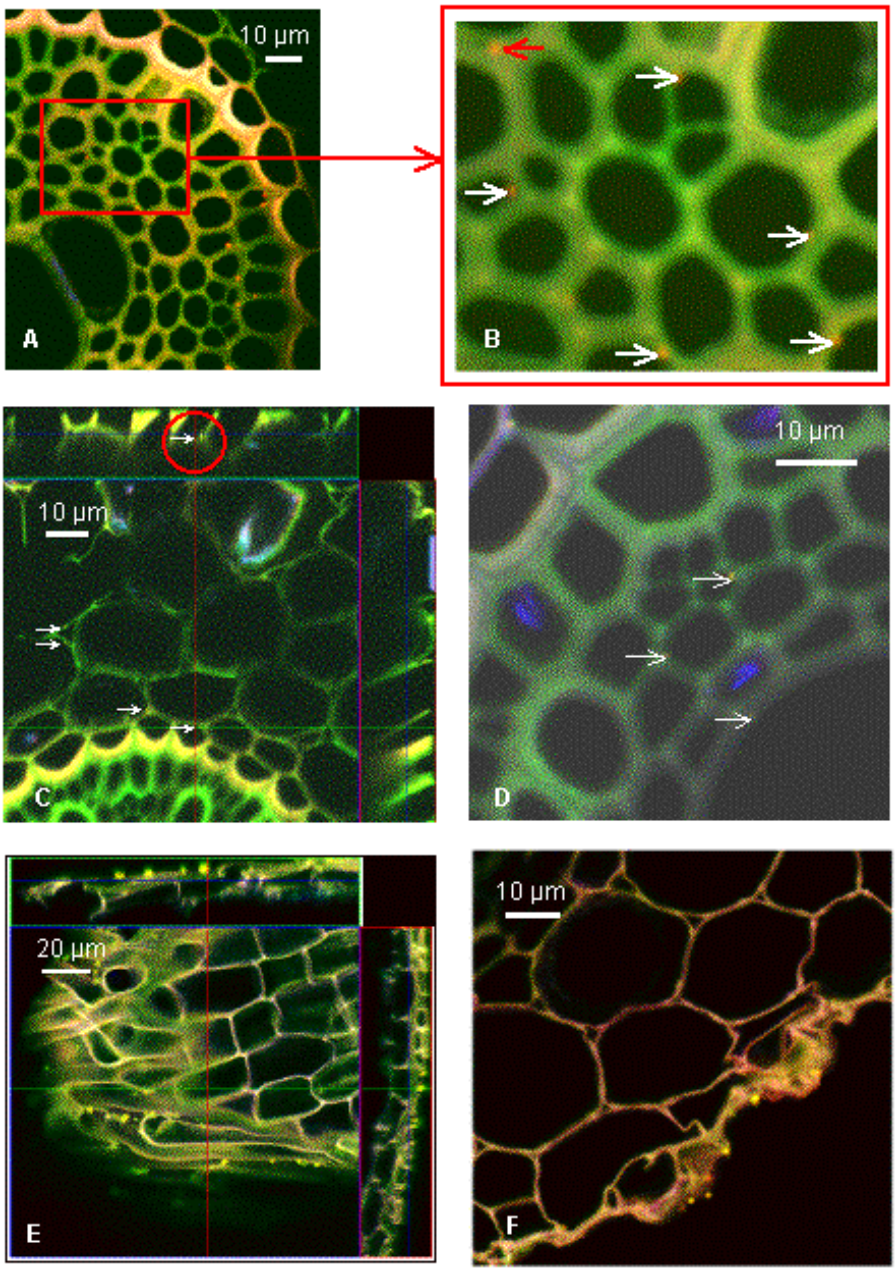


Figure 2: 16S rDNA phylogenetic tree of Azospirillum *spp. and related* α*-Proteobacteria*

Figure 3: In situ *localization of associated/endophytic nitrogen fixing bacteria.* ***A****: CLSM image of a radial slice of a barley root (magnification 400 x, scan zoom 1,5). Image shows endophytic colonization of the central cylinder with* Burkholderia cepacia *SXO702.* ***B****: Magnification shows bacteria in the intercellular space (apoplast). Some bacteria are attached to the inner surface of the cell wall.* ***C:*** *Orthogonal view of a radial slice of barley roots inoculated with* Herbaspirillum seropedicae *Z67 (magnification 400 x, Scan zoom 1,5). Cells were found in the root cortex (white arrows).* ***D:*** *Confocal image of a radial slice of barley roots (magnification 400 x, Scan zoom 2,0).* Herbaspirillum seropedicae *Z67 shows endophytic colonization of the central cylinder.* ***E:*** *Colonization of wheat roots (Cultivar Naxos) harvested after two weeks of cultivation in a monoxenic system inoculated with* Azospirillum brasilense *Sp7 (magnification 630 x, Scan zoom 1,4).* ***F:*** *Radial slice of a barley root inoculated with* Azospirillum brasilense *Sp7 under the same culture conditions as in E (magnification 400 x, Scan zoom 1,8).* ***A - D, F:*** *Bar indicates 10 µm,* ***E:*** *Bar indicates 20 µm.* ***A, B:*** In situ *hybridization was performed with the oligonucleotide probes Bcv-13b-Cy3 and EUB-338-I, II, III-FLUOS (Table 1).* ***C, D:*** In situ *hybridization was performed with the oligonucleotide probes Hsero-445-Cy3 and EUB-338-I, II, III-FLUOS (Table 1).* ***E, F:*** In situ *hybridization was performed with the oligonucleotide probes Abras-1420-Cy3, EUB-338-I, II, III- FLUOS (Table 1). Bacterial cells appear in orange/yellow after* in situ *hybridization according to the overlay of the Cy3 (red) and FLUOS (green) signal.*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3.2.1. 16S-rDNA Based Molecular Phylogeny

In a detailed 16S-rDNA-based molecular phylogenetic study, Stoffels *et al*. (2001) demonstrated, that the now known seven species of *Azospirillum* form a phylogenetic cluster together with *Skermanella* and *Rhodocista*. *A. brasilense*, *A. lipoferum*, *A. doebereinerae*, *A. largimobile* and *A. halopraeferens* constitute one subcluster while *A. irakense*, *A. amazonense* and *Rhodocista* form a second and *Skermanella* a third subcluster (Fig. 2). The DNA G+C content for these species is in the range of 64 - 71%. The 16S-rDNA-sequence similarity between the different species is in the range of 93.6 to 96.6% within one subcluster and 90.2 - 90.6% between the species members of the two subclusters. Accordingly, on the basis of different more or less conserved sequence stretches of the 16S-rDNA, it was possible to create a set of oligonucleotide probes with different degree of specificity from the whole cluster (probe AZO-440a+b) to subcluster (AZOI-665) and individual species levels e.g. probes Abras-1420, Alila-1113, Adoeb-587, Ahalo-1249, Aama-1250 and Airak-1423 (Stoffels *et al*., 2001) (Table 1). To block specifically cross-reacting 16S-rRNA species unlabeled oligonucleotide probes as competitors were suggested (Stoffels *et al*., 2001).

3.2.2. Use of Phylogenetic Probes for in situ *Localization*

Oligonucleotide probes with fluorescence labels like FLUOS, TRITC, Cy3 or Cy5 are applied in fluorescence *in situ* hybridization (FISH) of fixed bacterial cells and root samples for identification and *in situ* localization purposes. Since these probes were designed for different hierarchical levels, their application in a nested approach allows a very reliable identification (Fig. 3). The application of confocal laser scanning microscopy (CLSM) is necessary to reduce the out of focus fluorescence in root samples. Using this approach, single bacterial cells of *A. brasilense* Sp7 were identified and localized preferentially at the root surface, while the strain *A. brasilense* Sp245 was also found endophytically in the intercellular spaces of the root epidermis. Alternatively, a specific *in situ* monitoring of introduced bacteria could be performed using *gfp*- or *rfp*-labeled bacterial strains (Rothballer *et al*., 2003).

3.3. Diversity and Ecology of Gluconacetobacter *spp.*

Specific PCR-primers were developed to identify *G. diazotrophicus* (Kirchhof *et al*., 1998) and *G. johannae* as well as *G. azotocaptans* (Fuentes-Ramírez *et al*., 2001). Using the primers L927Gj and L923Ga, *G. johannae* and *G. azotocaptans*-specific amplification of a 400bp fragment can be used for a specific identification and semi quantitative estimation of the occurrence of these important endophytic diazotrophs. *G. diazotrophicus* was isolated not only from sugar cane, but also from coffee (Jiménez-Salgado *et al*., 1997) and pineapple plants (Tapia-Hernández *et al.*, 2000). The infection of sugar cane by *G. diazotrophicus* was investigated with electron microscopic techniques by James *et al*. (1994; 2001) and reviewed by James and Olivares (1997). The benefit of inoculation with *G. diazotrophicus* to sugar cane was carefully studied by Sevilla *et al*. (2001) using wild type and Nif$^-$ mutant strains,

demonstrating both an effect of nitrogen fixation and a probably phytohormone-mediated growth stimulation (see Chapter 10, this Volume).

4. MOLECULAR PHYLOGENY AND ECOLOGY OF *HERBASPIRILLUM*, DIAZOTROPHIC *BURKHOLDERIA* SPP. AND OTHER N_2-FIXING β -PROTEOBACTERIA

4.1. Diversity of diazotrophic β-Proteobacteria

Among the β-Proteobacteria, the number of known diazotrophic bacteria has increased much in the last decade. Young (1992) reported four diazotrophic genera in the β-Proteobacteria: *Alcaligenes, Rhodocyclus, Derxia,* and *Thiobacillus*. In the meantime, *Alcaligenes paradoxus* was group to the genus *Variovorax* and *Rodocyclus gelatinosus* to *Rubrivivax.* Nitrogen-fixing isolates of *Ideonella dechloratans* were obtained from rice (Elbeltagy *et al*., 2001). *Azoarcus* and related new diazotrophic genera (Reinhold-Hurek and Hurek, 1998; 2000; see also Chapter 9), several new *Herbaspirillum* species and an increasing number of diazotrophic *Burkholderia* species were newly described bacteria, originating from different plants. Since two years ago, true nodule-forming bacteria in *Leguminosae* were only known within the *Rhizobiaceae* (α-Proteobacteria). In 2001, Chen *et al*. (2001) isolated bacteria from root nodules on *Mimosa pudica* and *Mimosa diplotricha* and from cystic fibrosis sputum isolate as a novel *Ralstonia* species, *Ralstonia taiwanensis*.

The *R. taiwanensis* isolates from *Mimosa* nodules were proven to effectively nodulate the *Mimosa* species and were the first described β-proteobacteria capable of nitrogen fixation and root nodule formation. In the same year, Moulin *et al.* (2001) reported two *Burkholderia* strains STM678 and STM815, representing the new species *B. phymatum* and *B. tuberum* (Vandamme *et al*., 2002), isolated from two tropical legumes plants, *Aspalathus carnosa* in South Africa and *Machaerium lunatum* in French Guiana. It could be demonstrated, that nodules are formed by these cultures harbouring nodulation genes, which resemble the *nod*-genes in α-Proteobacteria. In addition, two other *Burkholderia* isolates, belonging to *B. caribensis* and *B. cepacia* genomovar VI were obtained from root nodules of *Mimosa* spp. in Taiwan and *Alysicarpus glumaceus* in Senegal, respectively (Vandamme *et al*., 2002). Therefore, the concept of "β-Rhizobia" arose, that some β-proteobacteria can nodulate legumes. The genetic capacity for this symbiotic trait may have spread e.g. by plasmid transfer to root-associated diazotrophs. A comparable observation was made among in rhizobial populations in the field by Sullivan *et al.* (1995). A symbiotic island was transferred to inefficient nodulating *Rhizobium loti* making them very efficient symbiotic strains. Many more not yet described nitrogen-fixing and nodulating β-proteobacteria may exist, which have not yet been tested for their nodulating behavior. Plant endophytic diazotrophs, which

were found in recent years in several genera of the β-proteobacteria, e.g. in *Herbaspirillum* and *Burkholderia,* could be ideal candidates for these new types of symbiotic bacteria.

4.2. Diversity and Ecology of Herbaspirillum spp.

The first species described of the genus *Herbaspirillum* was *H. seropedicae* (Baldani *et al*., 1986), which included bacterial strains isolated from roots of several cereals. *Pseudomonas rubrisubalbicans*, a mild pathogen in some sugar cane varieties was reclassified as *Herbaspirillum rubrisubalbicans* by Baldani *et al*. (1996). A third species, mostly harbouring strains from clinical origin was provisionally named *Herbaspirillum* species 3 had also to be included in the *Herbaspirillum* species because of its phylogenetic and biochemical close relatedness, although most of isolates do not fix nitrogen and are not derived from plant origin.

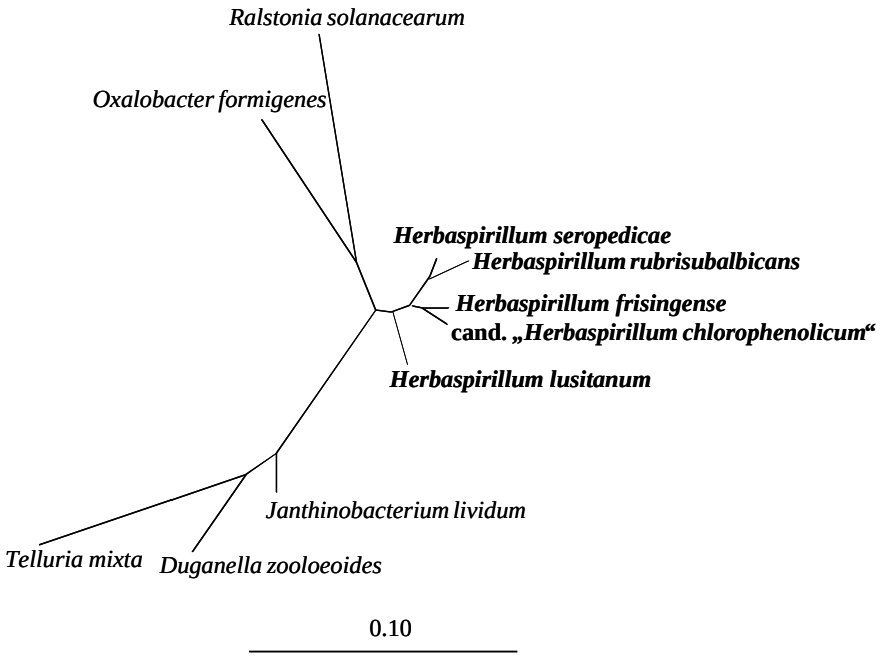

Figure 4: 16S-rDNA phylogenetic tree of Herbaspirillum *spp.*

H. frisingense was recently isolated from roots and stems of C4-fibre plants (*Pennisetum purpureum* in Brazil and *Miscanthus sinensis* in Germany) (Kirchhof *et al*., 2001). Most recently two more species of *Herbaspirillum* were suggested. Several isolates were obtained from the nodules of *Phaseolus vulgaris* plants from

Portugal, which showed close relatedness to *Herbaspirillum* (Valverde *et al*., 2003). The isolates showed 92-98% DNA-DNA relatedness amongst each other but only 29% DNA-relatedness to the other described species. Therefore, the new species *H. lusitanum* was suggested with the type strain P6-12^{T} (Valverde *et al.*, 2003). There is also 16S-rDNA sequence information about a fifth *Herbaspirillum* sp., named *H. chlorophenolicum*, which is able to degrade chlorophenol and was initially named *Commamonas testosterone* (Im *et al.*, unpublished). The 16S-rDNA-phylogenetic tree, based on maximum likelihood analysis, is shown in Figure 4. All five *Herbaspirillum* species form a monophyletic cluster with *Janthinobacterium lividum, Telluria mixta and Duganella zoogloeoides* as closest relatives.

With the exception of *H. chlorophenolicum* and *Herbaspirillum* species 3, all *Herbaspirillum* spp. are nitrogen-fixing bacteria and colonize plant roots. Some *Herbaspirillum* isolates can form a type of endophytic association with plant tissue, which has been investigated in detail in *Sorghum bicolor* (James *et al*., 1997) and sugar cane (Olivares *et al*., 1997). Recently, direct evidence for a nitrogen-fixing endophytic association has been obtained from the studies of *Herbaspirillum* sp. strain B501 in rice (*Oryza officinalis*) (Elbeltagy *et al*., 2001).

Based on the 16S-rDNA-sequences, a set of oligonucleotide probes were suggested by Kirchhof *et al*., (2001) which allow the identification and differentiation of *H. seropedicae, H. rubrisubalbicans* and *H. frisingense* by species-specific probes and FISH. Using these probes for a screening of new isolates from different sources, evidence for the presence of *Herbaspirillum* spp. came from many other plants, including pineapple, banana and rice (Weber *et al*., 1999; Cruz *et al*., 2001; Jha *et al.*, unpublished). 16S-rRNA-targeted oligonucleotide probes (Table 1) and FISH-analysis were used to localize these bacteria in the root environment. Figure 3 shows the *in situ* localization of an isolate of the proposed species *H. lusitanum* from barley roots colonizing the root endophytically.

An endophytic location of *H. seropedicae* was repeatedly described in roots, shoots and leaves in Gramineae by Olivares *et al.* (1996). The colonization behaviour and systemic spreading of *H. seropedicae* in the vascular tissue of *Sorghum bicolor* was carefully documented by James *et al.* (1997). In an axenic system with micro propagated *Miscanthus* seedlings, an efficient endopyhtic colonization and systemic spreading of *H. frisingense* was found (Eckert, unpublished results).

4.3. Diversity and Ecology of Diazotrophic Burkholderia *spp.*

An unprecedented high diversity of diazotrophic root-associated bacteria has been found in recent years within the genus *Burkholderia*. Among the presently 29 different *Burkholderia* species or genomovars of the genus *Burkholderia* are many plant and human-associated bacteria with partly high pathogenic potential, especially in the *B. cepacia* cluster. In addition, some *Burkholderia* are degraders of organic substances of anthropogenic origin or plant growth promoting bacteria, some with biocontrol activity. The first diazotrophic bacterial species within the genus *Burkholderia* was *B. vietnamiensis* (Gillis *et al*., 1995). This species was

isolated from the rhizosphere of young rice plants grown in Vietnamese soil (Tran *et al*., 1994). It also includes two clinical isolates, which are able to fix nitrogen and were misnamed as *Pseudomonas cepacia* (Yabuuchi *et al*., 1992; Palleroni, 1993).

Figure 5: 16S-rDNA phylogenetic tree of Burkholderia *spp. and related* β*-proteobacteria*

In a survey of root associated diazotrophs in sugar cane and rice in Brazil, a group of diazotrophic isolates were obtained using the LGIP semisolid nitrogen free medium, usually applied to isolate *G. diazotrophicus* (Reis *et al*., 1994), which were provisionally named "isolates E" (Oliveira, 1992). Application of phylogenetic oligonucleotide probes characterized these bacteria as β-proteobacteria of probably new phylogeny. Concomitant sequence analysis of 23S-rDNA coding genes indicated the affiliation of these bacteria to the genus *Burkholderia* (Hartmann *et al*., 1995). Among diazotrophic bacterial isolates obtained from banana and pineapple rhizosphere in Brazil, several isolates were found to belong to the new diazotrophic bacteria using 23S-rRNA oligonucleotide probing, ARDRA-pattern analysis and phenotypic techniques (Weber *et al*., 1999; Magalhaes-Cruz *et al*., 2001). Among N_2-fixing bacteria associated with maize and coffee plants grown in different climatic regions in Mexico a richness of *Burkholderia* species was characterized (Estrada-de los Santos *et al*., 2001). This finally led to the suggestion of a new bacterial species, *B. tropicalis* (Reis *et al*., 2003). It is closely related to *B. unamae*, another new diazotrophic bacterial species described by J. Caballero-Mellado and associates (personal communication). The "bacteria E"-isolates from rice plants turned out as separate *Burkholderia* species, for which the name *B. brasilensis* is suggested (Baldani *et al*., unpublished).

Surprisingly, this bacterium is very closely related to a diazotrophic bacterium isolated from a trichloroethylene-polluted groundwater, *B. kururiensis* (Zhang *et al*., 2000). Most interestingly, one of the β-rhizobia, *B. tuberum* strain STM678 (Moulin *et al*., 2001, Vandamme *et al*., 2002) is clustering to this group of mostly root associated endophytes (Figure 5). The other β-rhizobial species of *Burkholderia, B. phymatum* is closely related to *B. caribiensis* (Fig. 5).

For some of these new diazotrophic *Burkholderia* spp. 16S-rRNA-targeted oligonucleotide probes are available (Table 1). Using the FISH-analysis and confocal laser scanning microscopy, the ecology of these bacteria could be studied. An endophytic localization could be found in some of these isolates, like *B. cepacia* SXO (Fig. 3).

A *Burkholderia* sp. has also been found in association with the arbuscular mycorrhizal fungus *Gigaspora margerita* as non-culturable endosymbiont (Minerdi *et al*., 2001). It has been shown with molecular techniques, that this bacterium harbours the *nifH*-gene. In the association with plant roots and fungi, probably many more diazotrophic bacteria (culturable or non-culturable) are waiting to be discovered, which may surprise the scientific world with new symbiotic characters.

5 CONCLUSIONS AND PROSPECTS/FUTURE STUDIES

Using 16S rRNA-directed phylogenetic oligonucleotide probes, both the phylogenetic characterization of isolates and the *in situ* identification and localization of these bacteria in root and rhizosphere sample is possible. Due to the high fluorescence background of natural samples, the application of confocal laser scanning microscopy or other image analysis supported microscopic techniques

using e.g. the convolution method are necessary. Since new diazotrophic bacterial species are continuously described originating from different plants, a still high diversity of hitherto unknown diazotrophs can be expected. Therefore, the application of culture-independent approaches using primers for the 16S-rDNA and *nif*-genes are highly recommended in future diversity studies to get an even closer insight into the real diversity of plant-associated diazotrophs. Some of these diazotrophs may have acquired a very intimate state of coevolution towards a symbiotic life style in plants and even in fungi.

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