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ANALYSIS OF THE PRESENCE AND DIVERSITY OF DIAZOTROPHIC ENDOPHYTES

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Abstract

The initial isolation step of endophytic diazotrophs is a surface disinfection, followed by extensive washing and maceration of the tissue. Alternatively, bacteria can also be isolated from axenically derived plant sap, using vacuum devices or centrifugation steps. These materials are used to inoculate semisolid, nitrogen-free enrichment media. In addition, specific antisera for certain groups of diazotrophic bacteria can be applied to specifically enrich endophytic bacteria in a fast and efficient immunoadsorption procedure. The diversity of diazotrophic bacteria is certainly far from being approximately revealed, since new endophytic diazotrophs are being continuously discovered. Since the initial isolation step bears uncertainties of a true endophytic origin of the bacteria, inoculation and/or direct *in situ* localization studies are necessary to prove the endophytic character of the diazotrophs in a certain plant. New whole-cell binding 16S rRNA-directed oligonucleotide probes have been developed and are available for *in situ* localization studies of *Herbaspirillum* spp. and *Azospirillum* spp.. Highly specific polyclonal or monoclonal antibodies are additional important tools for the *in situ* identification of bacteria with or without combination with the oligonucleotide probes. Using fluorescently labeled probes or bacteria, which are tagged with the *gfp*- or *gus*-marker, confocal laser scanning microscopy provides a clear *in situ* identification of bacteria in optical sections down to a depth of about 30µm. In addition, immunogold-labelling and electron microscopy enables a localization with high resolution.

Introduction

We consider any bacterium as a plant endophytic diazotroph, if: (i) it can be isolated from surface-disinfected plant tissue or extracted from inside the plants; (ii) it proves to be located inside the plant, either intra- or intercellularly by *in situ* identification; and (iii) it does fix nitrogen, as demonstrated by acetylene reduction and/or $^{15}\text{N}_2$ -enrichment. This definition includes internal colonists with apparently neutral or

saprophytic behaviour, as well as symbionts. The endophytic localization of N₂-fixing bacteria is of high potential importance, because the intimate localization inside plant tissues provides the chance to develop high population densities in a protected and less competitive habitat. In addition, the supply of nutrients for e.g. an energy intensive process such as nitrogen fixation may be very efficient with an endophytic association. Specific interactions and metabolic activities of the bacteria may also determine a certain degree of specificity in these endophytic-plant associations.

The isolation procedure for endophytic bacteria is of key importance for the initial research with endophytic bacteria. The first step consists of an surface disinfection step, which should recover only the internal or physically shielded population. Secondly, diazotrophic bacteria need to be enriched using different nitrogen-free media or a specific immunoabsorption technique, because non-diazotrophic bacteria also reside inside plants (1). Finally, the pure cultures need to be characterized by phenotypical and molecular means, to get an insight into the diversity of diazotrophic bacteria in a given plant. However, because of incomplete surface disinfection and of the adsorption of bacterial cells to plant cell structures, the true endophytic localization needs to be proven by inoculation and/or localization studies of plants with bacterial diazotrophs. In contrast, the penetration of disinfectant solution into plant tissues may result in loss of some endophytes.

Isolation of Endophytic Diazotrophs

Several techniques have been employed for the isolation of endophytic bacteria with different advantages and disadvantages, as summarized by Hallmann *et al.* (2). The optimization has to be empirically performed in each plant tissue under study. The most common procedures for the isolation of endophytic bacteria are based on surface disinfection of plant tissues using various disinfectants, including sodium hypochlorite (3), ethanol (4), chloramine T (5), mercuric chloride (6,7), hydrogen peroxide (1), or a combination of two and more of these treatments, followed by several washings in sterile water or buffer solution. Surface detergents like Tween 20, Tween 80, or Triton X-100 (8) can be added to reduce the surface tension of the solvent, thereby allowing the disinfectant to reach protected sites and grooves beyond collapsed epidermal cells on the plant surface. Depending on the plant species, age, and plant parts (roots, stems, leaves, or seeds), concentrations of the disinfectant being used vary and need to be optimized. Another approach of surface disinfection involves dipping plant tissues into ethanol and flaming the surface (4,9). This has been applied for stems, before the internal sap was derived. To decrease the possibility of recovery of surface contaminants, sterility checks should be included to monitor the efficacy of the disinfection procedure for each sample. As compared to the untreated control, at least a tenfold reduction should be reached, indicating a reasonable surface disinfection.

After surface disinfection and thorough washing with sterile water, the plant tissue is macerated with a mortar and pestle or mechanical device (e.g. blender) in sterile water, buffer solution, or growth media. The macerate can be processed for

bacterial enumeration, specific enrichment of diazotrophic bacteria or immunoadsorption (see below). To circumvent problems concerning surface disinfection, alternative methods have been developed. A vacuum technique was successfully used to extract xylem sap from the roots of perennial plants like grapevine (10) and the Scholander pressure bomb was used for extracting root sap from cotton and other agricultural crops (2). Stoltzfus *et al.* (7) used a re-infection step of axenically grown plant seedlings to specifically enrich endophytic bacteria. After surface sterilization and maceration of root pieces, axenic rice seedlings were inoculated. After growth under axenic conditions, these rice seedlings were again surface sterilized, macerated, and the bacteria were isolated and tested for acetylene reduction activity after growth in nitrogen-free semisolid medium.

Nitrogen-free semisolid media have been systematically developed by Dr. Johanna Döbereiner and coworkers at the EMBRAPA-Institute in Seropédica, Rio de Janeiro, Brasil, since the early 1970s (11). This has pioneered the field of isolation and characterization of microaerobic, plant-associated nitrogen fixing bacteria. The semisolid media NFb, JNFb, LGI, and JMV differ in the type of carbon source, pH- and buffer conditions as well as vitamins (12) and enable the enrichment of a diversity of diazotrophic bacteria, such as *Azospirillum* spp., *Herbaspirillum* spp., *Burkholderia* spp., and *Acetobacter diazotrophicus*. These studies have been corroborated and extended through the years by the results of many research groups. For the isolation of diazotrophic bacteria, a series of vials with nitrogen-free semisolid medium is inoculated with a dilution series of the macerated plant tissue and incubated. A sharp and dense growth pellicle develops upon incubation for some days below the agar surface, where the bacteria seek low partial pressures of oxygen for nitrogen fixation (11). After several passages on nitrogen-free, semisolid media and nitrogen rich plates, the pure bacterial cultures should show acetylene reduction activity as measure of nitrogenase. Following this approach, a diversity of diazotrophic bacteria have been characterized recently from the C4-fiber plants *Miscanthus sinensis* and *Pennisetum purpureum*, including a new endophytic diazotroph *Herbaspirillum frisingense* (13) and *Azospirillum dobereineriae* (14).

As alternative to the conventional isolation procedures, species-specific polyclonal or monoclonal antibodies were successfully applied to isolate diverse bacteria of a certain group using the immunoadsorption approach. This technique is much faster and allows to obtain direct isolation of specific bacterial populations from environmental samples. The availability of isolates of a certain group of diazotrophs is necessary to raise the antisera resp. antibodies. An improved immunoenrichment protocol (15) uses microtiterplates, coated with protein A. After adsorption of the specific antibodies to protein A, plant extracts are added. Following intensive washing steps to remove the unbound bacteria, the specifically bound bacteria are detached by applying glycine buffer (pH 3.0) to loosen the protein A-antibody binding. Depending on the quality of the antibody or antiserum used, enrichment factors can vary very much and reach values of up to 1,000. This approach has been applied to investigate the *Herbaspirillum* population of *Saccharum notatum* and *Pennisetum purpureum*. The bacterial isolates which were enriched using a mixture of polyclonal antisera for *H. seropedicae* and *H. rubrisubalbicans* were characterized at the genotypic level by ERIC-PCR. Remarkably, the isolates from a

single plant species showed very low diversity; the isolates from sugar cane belong to the *H. rubrisubalbicans* cluster, while the isolates from *Pennisetum purpureum* could not be identified as *H. rubrisubalbicans* or *H. seropedicae*, and may represent a new *Herbaspirillum* species.

Identification of Diazotrophic Bacteria Using Phylogenetic, rRNA-directed Oligonucleotide Probes

Ribosomal RNA based identification and detection methods have become techniques routinely used in all disciplines of microbiology. Together with the phenotypic properties and the DNA- and RNA-similarity data, the sequence information of the 16S- and 23S-ribosomal DNA of diazotrophic bacteria made their phylogenetic characterization more stringent and allowed the development of specific and convenient identification tools. The 16S- and 23S-rDNA contain both highly conserved as well as highly variable sequence stretches, which allow the amplification of diagnostic sequences by PCR using flanking conserved primers and the development of genus- and species-specific oligonucleotide probes. The application of different software packages, such as the ARB-software (16), enable the alignment of rRNA-sequences and the design and development of phylogenetic oligonucleotide probes of desired specificity. Using different sequence stretches of the 23S-rDNA, Kirchhof *et al.* reported probes for the identification of different plant-associated diazotrophs, such as *Azospirillum* spp., *Herbaspirillum* spp., *Burkholderia* spp. and *Acetobacter diazotrophicus* (17). These probes are very useful for screening of new isolates by dot blot hybridization. The nucleic acids of the isolates can be used either directly as targets for the diagnostic hybridization probes, or after the amplification of the 16S or 23S rDNA by PCR. For the screening of immobilized nucleic acids, probes at different phylogenetic levels are available. While the application of the probe EUB388 (for eubacteria) confirms the presence and amount of DNA on the filters, the probes ALF1b and BET42a of Manz *et al.* (18) narrow the affiliation to a certain subclass of proteobacteria. The information gained from the higher level probes are used to select the probes tailored for the next lower taxonomic levels, for example genus- and species-specific probes. This screening method is described as top to bottom approach (19) and leads to a rapid taxonomic identification of the isolate, if a specific probe set is available. Hybridization failure with the probes for known species provides evidence, that a possible new type of bacterium is under study. In this case, it is necessary to determine the 16S rDNA sequence of the isolate. Primary structure signatures can then be used as target sites for the development of new diagnostic hybridization probes. This approach has been successfully used recently in the screening of diazotrophic isolates of the C4-grass *Miscanthus sinensis* (20), and led to the characterization of new *Herbaspirillum* and *Azospirillum* species.

Currently, the most attractive and convenient technique for specific probe based identification and detection is whole cell hybridization with fluorescently labelled oligonucleotide probes, targeted to the 16S and 23S rRNA (19). In contrast to the dot blot hybridization of nucleic acid, the ribosome content of the cells and the cell

Table 1. New rRNA-targeted Oligonucleotide Probes for Fluorescence *in situ* Hybridization of the Genus *Herbaspirillum* and of the *Azospirillum* Cluster. 16S rRNA Target Sites (*E. coli* Numbering, 22) and Optimal Hybridization Conditions.

Probe	Sequence (5'-3')	Position ^a	Stringency ^b	Specificity	Reference
Herb68	AGCAAGCTCTATGCTGC	68-85	35	<i>Herbaspirillum</i> spp.	13
Herb1432	CGGTTAGGCTACCCACTT	1432-1449	35	<i>Herbaspirillum</i> spp.	15
Hsrs445	GCCAAAACCGTTTCTTC	445-462	35	<i>H. seropedicae</i>	13
Hrbri445	GCTACCACC GTTCTTCG	445-462	60	<i>H. rubrisubalbicans</i>	13
Hfrs445	TCCAGAACC GTTCTTCC	445-462	50	<i>H. frisingense</i>	13
AZO440a + AZO440b	GTCAATCGTCCGCTGC GTCAATCATGTCGTGTGC	440-457	50 50	<i>Azospirillum</i> spp., <i>Complomentomonas</i> spp., <i>Rhodovivax</i> spp.	23
AZO1	CACGATCCTCTCCGGAAC	655-672	50	species cluster <i>A. lipoferum</i> , <i>A. brasilense</i> , <i>A. helopruferens</i> , <i>A. doebereineras</i> , <i>A. largomobile</i>	23
Abras1420	CCACCTTGGGGTAAAGCCA	1420-1438	40	<i>A. brasilense</i>	23
Aama1250	CACGAGTCTGCTGCCAC	1250-1267	50	<i>A. amazonense</i> ^c	23
Alfa1113	ATGGCAACTGACGGTAGG	1113-1130	35	<i>A. lipoferum</i> , <i>A. largomobile</i>	23
Adoeb387	ACTTCCGACTAAACAGGC	387-404	30	<i>A. doebereineras</i> cand.	14
Adoeb94	CGTGGCCACTGTGCCGA	94-111	30	<i>A. doebereineras</i> cand.	14

^a Target site in 16S rRNA according to *E. coli* numbering (22).

^b Amount of formamide (% v/v) in hybridization buffer.

wall permeability are important parameters for good hybridization signals. For rRNA-targeted oligonucleotide probes, the accessibility of the target sites within intact or partially denatured ribosomes is also an important criterion for probe design (21). Unfortunately, only some of the 23S rRNA directed oligonucleotide probes (17) can be used for successful whole cell fluorescence labeling by *in situ* hybridization.

In comparison with conventional hybridization assays, the whole cell *in situ* approach provides the advantage, that two or multiple probes can be used in one experiment, if they are labeled with different fluorescent dyes. The combined application of two or multiple probes with identical or overlapping specificities targeting different parts of the 16S or 23S rRNA become more and more significant. This so-called multiple probe concept enables a fast identification of isolates and single cells in plant material according to the "top to bottom" approach. In addition, it reduces the risk of misidentification of bacteria. The probes listed in Table 1 are examples for the multiple probe concept using newly developed, 16S rRNA directed *in situ* applicable oligonucleotide probes within the genera *Azospirillum* and *Herbaspirillum*. Sets of nested probes for the genera *Burkholderia* and *Acetobacter* are in development.

***In situ* Localization of Endophytic Diazotrophic Bacteria**

For a direct *in situ* identification and localization of endophytic diazotrophs, a variety of methods are available. On one hand, phylogenetic oligonucleotide probes as well as antibodies at different levels of specificity can be applied as identification tools. On the other hand, bacteria specifically labeled with molecular tags, such as *gfp* (coding for the green fluorescent protein) or *gus*, are frequently used for monitoring the fate and specific activity of inoculated bacteria (7,24).

Endophytic diazotrophs could be localized at the rhizoplane and inside plant tissues using whole cell *in situ* hybridization with fluorescence labeled oligonucleotide probes (19, 25). The application of confocal laser scanning microscopy which produces optical sections and merges the digital images to a three-dimensional picture (25) allowed the resolution of single bacterial cells with their specific fluorescence label, according to the probes applied (25, 26). Monoclonal antibodies against certain strains of *Azospirillum brasilense* (27) were successfully combined with fluorescence-labeled oligonucleotide probes to study the colonization of wheat roots by these bacteria (28). For the detection of fluorescence signals from deeper cell layers, it is important to improve the fluorescence signal. The method of tyramine signal amplification (TSA) results in a several fold increase in signal intensity (29). Although confocal laser scanning microscopy resolves single labeled bacterial cells in the plant tissue, only rather shallow sections (depth of about 30µm) can be studied for technical reasons. Therefore, in order to investigate deeper layers of roots, stems and leaves, semi-thin sections have to be produced, e.g. by cryosectioning.

For a highly resolving localization of bacterial endophytes in the plant interior, embedding in resin, ultrathin sectioning in combination with immunogold labeling techniques and electron microscopy is still a key technique (27, 30, 31). In addition,

immunogold/silver enhancement for light microscopy has also proven as a convenient technique to get an overview of the sample. Using strain-specific monoclonal antibodies, the localization of the *A. brasilense* strain Sp245 which colonizes the interior of wheat roots and *A. brasilense* strain Sp7 which only colonizes the rhizoplane, has been studied in detail (27).

The colonization behaviour of endophytic diazotrophs for certain plant tissues seem to be strain and species specific (27, 32). Usually, endophytic diazotrophs colonize intercellular spaces in roots, stems and leaves (24, 27, 32-39). Evidence for intercellular localization of endophytic bacteria in roots arose already from the work of Patriquin and Döbereiner in 1978 (40). However, at that time, unequivocal identification and localization criteria of the bacteria were missing. An intracellular localization of endophytic bacteria in plant cells is much less frequently reported (25, 29, 33, 35). This colonization pattern apparently depends on the bacteria and plant cultivar used. Colonization of the vascular system by diazotrophic bacteria was also reported (33, 37). This agrees with the findings, that in some plant systems certain endophytic diazotrophs belonging to *Azoarcus* spp., *Herbaspirillum* spp., *Burkholderia brasilensis*, or *Acetobacter diazotrophicus* are distributed systemically in the plant.

An open question remains, whether the invaded plant cell or tissue is still functional and alive at the time of investigation or whether it has already undergone lysis. In some studies highly active endophytic bacteria were found, because an intensive staining with r-RNA directed oligonucleotide probes was evident (25). However, in some cases of intracellular localization, bacterial activity was low. Probably, there is a dynamic state of endophytic association within the plant, with locations and times of high bacterial activity and multiplication as well as locations and phases of bacterial decline.

Conclusions

Excellent methods to assess the occurrence, diversity and localization of endophytic diazotrophs are available. It will be very interesting to learn, whether there is a specificity of colonization of certain plants by particular endophytic diazotrophs and whether these endophytes really fix nitrogen effectively *in planta*. Some endophytes may reach a special physiological state in the plant environment, as is suggested for *Azoarcus* (41). The investigation of new plant species for endophytic bacteria yields both wellknown and new types of bacteria. Non-gramineous plants, such as tropical fruit trees, appeared to be also populated by a high diversity of diazotrophic bacteria (42), although the true endophytic localization has not been resolved.

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