



Review

Assessment of the structural and functional diversities of plant microbiota: Achievements and challenges – A review



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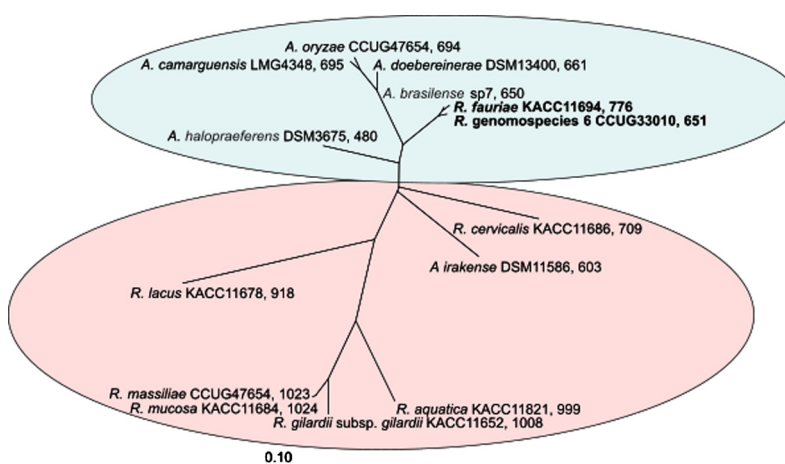
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HIGHLIGHTS

- History about the discovery of endophytes with the focus on *Azospirillum* and related diazotrophs.
- Contribution of approaches to reach highest resolution of microbial diversity assessment.
- Differentiation of beneficial *A. brasilense* and opportunistic human pathogen *R. fauriae*.
- Osmoadaptation and oxygen tolerance as major traits for endophytic bacteria.
- Bacteria-plant communication with focus on bacterial *N*-acyl homoserine lactones.

GRAPHICAL ABSTRACT



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ABSTRACT

Analyses of the spatial localization and the functions of bacteria in host plant habitats through *in situ* identification by immunological and molecular genetic techniques combined with high resolving microscopic tools and 3D-image analysis contributed substantially to a better understanding of the functional interplay of the microbiota in plants. Among the molecular genetic methods, 16S-rRNA genes were of central importance to reconstruct the phylogeny of newly isolated bacteria and to localize them *in situ*. However, they usually do not allow resolution for phylogenetic affiliations below genus level. Especially, the separation of opportunistic human pathogens from plant beneficial strains, currently allocated to the same species, needs genome-based resolving techniques. Whole bacterial genome sequences allow to discriminate phylogenetically closely related strains. In addition, complete genome sequences enable strain-specific monitoring for biotechnologically relevant strains. In this mini-review we present

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Opportunistic human pathogens
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N-acyl-homoserine lactones

high resolving approaches for analysis of the composition and key functions of plant microbiota, focusing on interactions of diazotrophic plant growth promoting bacteria, like *Azospirillum brasilense*, with non-legume host plants. Combining high resolving microscopic analyses with specific immunological detection methods and molecular genetic tools, including especially transcriptome analyses of both the bacterial and plant partners, enables new insights into key traits of beneficial bacteria-plant interactions in holobiontic systems.

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Introduction and historical aspects of the discovery of endophytes with focus on *Azospirillum* and related diazotrophs

More than one decade ago, the hologenome theory was introduced to express the tight interaction of microbes with animals and plants as basis for a better adaptation to changing environmental conditions with implications for co-evolution and speciation [1]. Holobionts are multicellular eukaryotic organisms living together in a symbiont-like manner with different types of external and internal microorganism (e.g. endophytes), which contribute essential life traits [2,3]. A more recent study concludes, that in order to understand speciation in the frame of the hologenome concept holobionts do not necessarily need to be viewed as units of selection, but it is sufficient to consider them as units of tight co-operation of eu- and prokaryotic organisms [4]. Looking back, it took a long time until this detailed view of omnipresent organismic interactions was established by firm evidence, because the appropriate methodological approaches had not been available. First evidences for bacterial endophytes, i.e. bacteria colonizing the interior of plants, were already published in the late 19th century. In 1887, M. L. V. Galippe reported the isolation of bacteria from the interior of different plants and postulated soil as origin of these bacteria [5]. Since he could not further prove their location and identity, these findings were heavily criticized. However, Hellriegel and Wilfarth demonstrated in 1888 the presence of endophytic bacteria within root nodules of legumes and their contribution of nitrogen for plant growth (reviewed by R.H. Burris) [6]. The general concept of the “rhizosphere” as the habitat where plant roots attract beneficial and pathogenic soil microbes by their exudates was finally coined by L. Hiltner in 1904 [7]. He found that microbes were enriched around the roots, but also recognized bacteria-like bodies within roots, which he called “bacteriorhiza” [8]. This term was coined in analogy to the term “mycorrhiza”, which had been defined in 1885 for filamentous organisms within roots by Albert Bernhard Frank, a German botanist and biologist. In 1893, Hiltner and Nobbe developed the first efficient Rhizobium-based inoculants, which they called “Nitragin”, based on their discovery of host specificities in Rhizobium-legume symbioses [9]. However, Hiltner was not successful to establish plant growth promotion by bacterial inoculation of non-leguminous plants. His quite early death in 1923 and the difficult post-world war situation in Germany contributed to slow down scientific progress in this field. For many decades no further major breakthrough on plant growth promoting bacteria was reported. Only in the 1970s new interest arose on plant beneficial bacteria after the isolation and introduction of *Azospirillum* spp. by Döbereiner and Day [10]. In the 1980s for example Baldani and coworkers [11,12], and Cavalcante and Döbereiner [13] from EMBRAPA-Agrobiologia, Seropédica, RJ, Brazil, isolated and characterized new diazotrophic bacteria from roots of different important crop plants. The high engagement and dedication for their science in combination with establishing a worldwide cooperation including sharing newest results as well as newly isolated strains by Johanna Döbereiner helped enormously in developing this field of research. Most recently, a book describes Johanna Döbereiner's life as highly engaged scientist [14]. In 1981, Walter Klingmüller, head of genetic

department at the University of Bayreuth, Germany, initiated a series of six biannual workshops entitled “*Azospirillum*: Genetics, Physiology and Ecology” bringing together the international research community on *Azospirillum* and related microorganisms – the last two workshops were organized by Istvan Fendrik, Madalena del Gallo and Jos Vanderleyden [15–20]. While the first four workshops were focused on research about *Azospirillum* spp., increasing interest and research activities also on other plant-growth promoting bacteria led to a broadening of the subject in the workshops V and VI. The articles in the corresponding proceeding books not only document the groundbreaking establishment of molecular genetic tools for *Azospirillum* by several research groups and discoveries of new endophytic diazotrophic bacteria, but also most interesting work by different groups on outstanding physiological properties, like e.g. the cyst-formation of *Azospirillum* spp., and early field application trials [15–20]. In parallel, an international symposium series “Biological Nitrogen Fixation with Non-Legumes” started in 1979; the XVIth symposium of this series was held in August 2018 in Foz de Iguaçu, Brazil, attracting more than 300 participants (www.mpcp2018.com.br). Quite recently, the current status of research on endophytic diazotrophic rhizobacteria was also summarized by Reinhold et al. [21] and Kandel et al. [22].

The challenge for pioneering research on endophytic diazotrophs and other plant growth promoting bacteria was not only to understand the biochemical and genetic processes characterizing the basis for plant growth promotion, but also the ecology of the interaction with their host plants as fundament of the beneficial action. The ultimate applied goal was to use these bacteria as so-called “biofertilizer” or “biostimulants” towards the establishment of sustainable agricultural management. In the 1980s, substantial agronomic applications were still far away on the horizon, while within the last ten years several *Azospirillum brasilense* strains [23] and other PGPRs including biocontrol-active Gram-positive bacteria [e.g. [24]] have been applied successfully in agro-biotechnology worldwide. However, in this mini-review, the vast development of Gram-positive inoculants has not been covered. It is still a key issue to provide unequivocal evidences for the colonization and localization of the bacteria as well as their *in situ* activities in the rhizosphere and within the plant. Serological and molecular genetic techniques suitable for these *in situ* analyses have been developed over the years, but always have to be adapted for successful identification, localization and quantification of bacteria in their specific association with plants. In addition, key functions in the beneficial interaction of rhizobacteria with plants needed to be identified. Moreover, the development of culture independent approaches was necessary to overcome the bias of studying only culturable members of the plant microbiome. In this review, a number of techniques and approaches are presented from a historical to current development perspective, which allows the detailed analysis of the composition of beneficial plant microbiota – even down to the level of monitoring specific inoculant strains – and their functions leading to plant growth promotion. Furthermore, a scientific based distinction of plant beneficial from opportunistic human pathogenic bacteria is addressed.

Techniques for resolving the diversity and function of the plant microbiome at highest resolution

Serological techniques coupled with confocal laser scanning microscopy (CLSM) as identification and quantification tools

The prerequisite of creating antibodies is the availability of bacteria in pure culture, which certainly is a limitation for the application of this approach, since many plant-associated bacteria are difficult to cultivate. After developing fluorescent-labeled monoclonal antibodies against *A. brasilense* Sp7 which are directed against EPS-cell surface compounds [25,26], confocal laser scanning microscopy (CLSM) was successfully used by Schlöter et al. in 1993 for the first time to produce clear images of these bacteria being embedded in the rhizoplane matrix [27]. Using the confocal technique as well as silver enhancement of the antibody detection, the root colonization pattern of the plant growth promoting *Rhizobium leguminosarum* bv. trifolii R39 was characterized in different gramineaceous plants in 1997 by Schlöter et al. [28]. In the same year, Yanni et al. [29] could also demonstrate the endophytic colonization of rice by the N₂-fixing symbiont *Rhizobium leguminosarum* bv. trifolii strain C6 in Egyptian berseem clover (*Trifolium alexandrinum*) applying immunofluorescence techniques. This demonstrated for the first time an intimate colonization also of rice by *Rhizobium*. In the case of *A. brasilense*, monoclonal antibodies against the putative endophytic strain Sp245, isolated from surface disinfected wheat roots [30,31], demonstrated a different colonization pattern of roots by the strains Sp7 and Sp245: strain Sp7 colonized wheat roots mostly at the root-surface, while strain Sp245 was able to enter the root, colonizing the apoplast tissue in wheat roots [32]. In addition, also quantitative colonization data of Sp7 and Sp245 in wheat plants could be obtained by the ELISA-technique, confirming the microscopic evidences of different colonization patterns [32]. Furthermore, *in situ* expression of specific enzymes (e. g. nitrogenase) in different rhizobacteria colonizing their host plant could be achieved using this technique [33].

Monoclonal or mono-specific polyclonal antibodies are also unique tools to easily enrich and cultivate a high diversity of root-associated bacteria of the same or closely related species from the root and the rhizosphere using the antibody based immunotrapping technique [34]. For example, antibodies against whole cells of a rhizosphere isolate of *Ochrobactrum anthropi* were coated on microtiter plates, followed by adsorption of soil extracts. After proper washing steps, the bound bacteria were desorbed with 0.1 M KCl-solution. This resulted in a more than 100-times enrichment of this specific group of bacteria and isolates of this particular species could be easily obtained. Thus, the influence of the crop plant, management practices, and ecotoxicological effects of applied agrochemicals on the micro-diversity spectra of *Ochrobactrum anthropi* communities in soils and the rhizosphere could be isolated and studied [35]. Even isolates of closely related new species could be retrieved using the immuno-trapping approach [36]. The application of this immuno-enrichment technique turned out to enable access to a hidden bacterial micro-diversity and should be applied more generally. In this straightforward approach, a greater diversity of saprophytic and beneficial rhizobacteria of specific species may be achieved.

Ribosomal RNA as identification marker with limitations to separate closely related strains

The establishment of a phylogenetically based natural system of organisms for the domains Archaea, Bacteria and Eucarya by Carl R. Woese, Otto Kandler and Mark L. Wheelis in 1990 [37] was the landmark for a molecular approach to the phylogeny of Bacteria and Archaea. The 16S rRNA genes of Bacteria rapidly became the

gold standard of molecular phylogenetic analysis, because the ribosomal RNA is present in all organisms and its sequence has highly conserved and variable regions. This facilitates the design of primers or oligonucleotide probes, usually 16–20 nucleotides long, with specificities to different taxonomic levels: probes complementary to conserved regions of the 16S or 23S rRNA will identify all bacteria of a high taxonomical rank, e.g. family or domain level, while for targeting bacteria on genus or in some cases - if a differentiation is possible - even species level, probes need to target highly variable regions of the rRNA specific to the taxonomic group of interest. In addition, the rRNA genes are expressed at very high levels in physiologically active cells (with copy numbers up and over 10,000), are more stable compared to mRNA due to their secondary structure and are therefore good targets for labelling the bacteria with fluorescent probes. Consequently, cells with low activity have usually low rRNA contents, resulting in low fluorescence labeling due to an insufficient number of target sites for the probes. This means on the one hand that positively labeled cells are very likely also functionally relevant for the analyzed habitat, but on the other hand also implies that this method is of limited use for targeting bacteria with low physiological activity. In addition, the cell wall penetration of applied probes has to be optimized, i.e. due to their differences in cell wall structure, Gram-negative and Gram-positive cells need to be treated with different fixation protocols to enable the phylogenetic probes to get into the cells [38]. Despite some obvious limitations of this approach, so-called “phylogenetic stains” became rather quickly a widely employed tool to identify single cells using the Fluorescence *In Situ* Hybridization (FISH) technique [39]. In combination with flow cytometry, FISH was successfully applied to quantify single cells [40] or to identify and localize bacterial consortia in complex natural habitats with the help of highly resolving confocal laser scanning microscopy and differentially labeled sets of oligonucleotide probes [41].

The first application of the FISH-technique coupled with CLSM-application to characterize plant microbiota was to identify and localize *A. brasilense* strains in the rhizosphere of wheat [42]. The inoculated *A. brasilense* bacteria colonizing the root surface and intercellular spaces in the epidermis had swollen cyst-like morphology harboring high ribosome content, which verified earlier evidences from light and electron-microscopic scanning [42]. The productive cooperation with the institute of Prof. Karl-Heinz Schleifer (TU München), coming from the “phylogenetic school” of Prof. Otto Kandler (LMU München), was very helpful to establish the FISH-technique for rhizosphere research. It could further be demonstrated that *A. brasilense* strain Sp245 could colonize wheat roots also endophytically. Some root hairs or intercellular spaces in the root cortex and even cortical cells were heavily colonized by the strain Sp245 showing high staining intensity with the rRNA-targeted oligonucleotide probes reflecting high physiological activity of the bacteria [42]. A combination of a differentially fluorescence-labeled monoclonal antibody against *A. brasilense* Wa3, and a species-specific oligonucleotide for *A. brasilense* revealed a different colonization profile of the strains Wa3 and Sp245 [43]. In the 1990s, when six *Azospirillum* species were known, all *Azospirillum* spp. could be clearly distinguished using a set of differentiating oligonucleotide probes [44]. At present, 19 different *Azospirillum* species are known and validly published, which makes it difficult to clearly allocate new isolates to one of these very closely related species by 16S rRNA sequences and 16S rRNA directed probes. Although the larger 23S rRNA gene and the 16S-23S rRNA intergenic regions provide higher separating power, these different species are impossible to separate with individual species-specific probes. The present solution of differentiation and even strain-specific identification is provided by the increasingly available whole genome sequences. Based on the comparison of the different available whole genome sequences within one species,

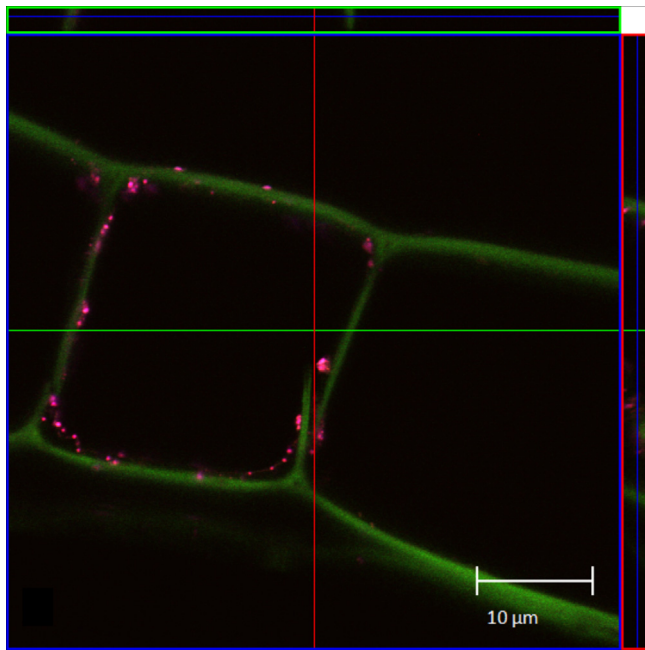


Fig. 1. CLSM-image with adult sugarcane (green) samples, viewing unspecific fluorescence signals in magenta ([46]).

strain-specific sequences could be found for e.g. *A. brasilense* strain FP2. Primers derived from these unique regions led to a specific and quantitative amplification of the target strain even from natural habitats like soil-grown wheat plants [45]. Thus, whole genome sequencing is becoming an ever more popular approach and currently only suffers from a lack of genome information for type and reference strains in the database.

There are also severe limitations for the application of the FISH-technique to identify and localize endophytic bacteria. In many environmental samples and also in adult field grown plants, like sugarcane, multiple auto-fluorescent objects in the sizes of bacteria are present in the tissue or within cells [46] (Fig. 1). Therefore, an alternative labelling method replacing fluorescence was necessary. Schmidt et al. [47] developed a modification of the CARD-FISH-protocol using gold-particles resulting in a specific bacterial identification using scanning electron microscopy as detection method for the deposited gold-particles. Nevertheless, this technique is limited to surface scans and therefore thin sections are required for the analysis of endophytic communities.

Fluorescent protein-tagging for *in situ* analysis of structural and functional aspects

A very powerful cell labelling method is the tagging with a constitutively expressed gene coding for a fluorescing protein, like the green-fluorescent protein (GFP). The basics and variations of this approach were reviewed by Crivat and Caraska [48]. Several applications for studying rhizosphere bacteria were reviewed by Reinhold-Hurek and Hurek [49]. Fig. 2 shows fluorescence-tagged *Herbaspirillum frisingense* cells located within root tissue. Alternatively the tagging gene can be inserted under the control of a promoter from a gene of interest to study its expression *in situ* [50]. Furthermore, a *GusA*-kanamycin reporter gene was inserted into the *nifH*-genes of an *A. brasilense* wild type and ammonium-excreting strains to facilitate an expression analysis in barley roots [51]. Quantitative data can be retrieved even from field samples, as was demonstrated by You et al. [52]. In a GFP-tagged *Herbaspirillum* the expression of *nifH* was quantified by

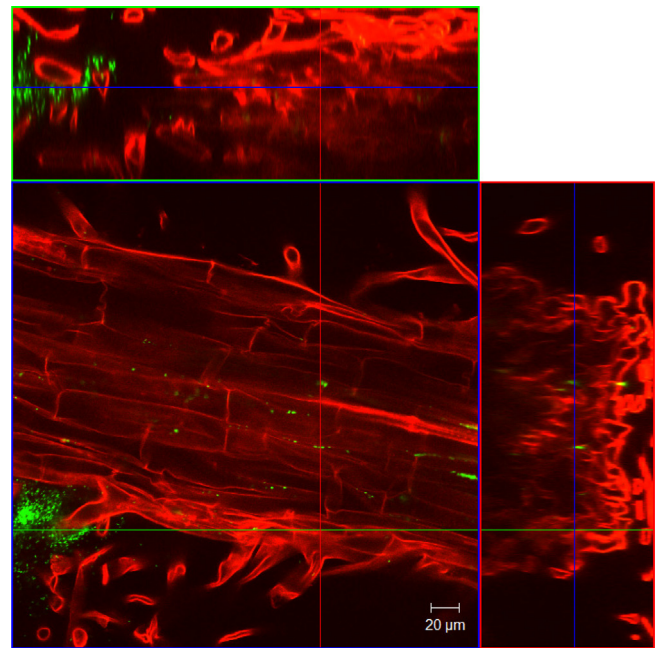


Fig. 2. Optical sectioning through intact barley (*Hordeum vulgare*, red) roots (19 days old) from a monoxenic quartz sand growth system colonized by inoculated *Herbaspirillum frisingense* GSF30 fluorescently tagged by a constitutively expressed chromosomal *gfpmut3* gene (green).

RT-qPCR and related to the amount of the tagged bacteria colonizing rice endophytically.

Concluding this phylogenetic and identification part, it can be stated that 16S rRNA-based phylogeny is still the prerequisite for powerful approaches of bacterial identification, including *in situ* localization by FISH as well as high-throughput amplicon sequencing based community analysis (discussed in the next section), but the applications are limited. Detailed resolution of diversity and functional aspects in a strain-specific resolution may also need molecular tagging approaches or advanced bioinformatic analyses based on whole genome sequence information.

Community metagenomics and functional transcriptomics of bacteria and plants

Undoubtedly, the culture-independent analysis of complex bacterial communities associated with plants would not be possible without using PCR-based amplification of different regions of the 16S rRNA gene. As prerequisite, DNA or RNA needs to be isolated from plant material and purified to remove plant substances inhibiting the PCR enzymatic reactions. While a proper quality of DNA/RNA is quite easily achievable from plant seedlings, especially, from soil free model experiments, it can be very challenging to obtain sufficiently pure DNA/RNA in enough quantity from field grown, adult plants. However, after optimization, this important initial step of microbial community analysis was achieved in several cases. For example, Fischer et al. [53] retrieved many bacterial 16S rRNA sequences from field grown sugarcane plants, which were not known from cultivation-based approaches. From their data it became obvious that a high diversity of diazotrophic bacteria colonized roots and stems and also a high diversity of *nifH*-genes was expressed. However, from the five inoculated strains of the EMBRAPA-inoculum (*Gluconacetobacter diazotrophicus* Pal5T-BR11281, *Nitrospirillum amazonense* Cbamc-BR11145, *Herbaspirillum seropedicae* HRC54-BR11335, *Herbaspirillum*

rubrisubalbicans HCC103-BR11504, and *Paraburkholderia tropica* PPe8T-BR11366), only *Gluconacetobacter diazotrophicus* Pal5 was found to be able to colonize sugarcane roots and stems for several months [53]. A high diversity of different active *Rhizobium* and *Bradyrhizobium* species was also found in these adult, field grown sugarcane plants, based on retrieved 16S rRNA. This clear demonstration of hitherto only rarely observed diversity of *Rhizobium* and *Bradyrhizobium* strains colonizing sugarcane and other non-legume plants triggered the attempt to isolate these bacteria in scavenging experiments with broad host range legumes [54], which resulted in the successful isolation of a diversity of *Bradyrhizobia*. The knowledge about the high diversity of uncultured bacteria within the plant microbiota also led to isolation approaches not aiming for single bacteria through specific enrichment procedures but for whole communities in non-selective complex media. Indeed, this yielded the growth of bacterial consortia, including species which could not be isolated from the plant microbial community before. This has been exemplified for the sugarcane community yielding complex plant growth promoting consortia [55]. However, as this approach is difficult and lacks reproducibility, it seems more straightforward to isolate members of the plant microbiota using plant derived cultivation media and subsequently combining these individual pure isolates based on functional criteria (so-called “syncoms”).

The crosstalk of beneficial endophytic bacteria and their plant hosts during the interactions is of key importance to understand holobiontic interactions and to optimize the efficiency of inoculation trials. Several highlights of important ecophysiological and interactive traits for plant microbiota and their hosts in a holobiontic context could be already identified by metagenomic and especially transcriptomic studies at both the bacterial and plant side [56–59]. Metagenome and transcriptome analyses on both bacterial and plant side during the interaction contribute very important functional information. However, to guarantee the reliability and reproducibility of these types of results principles for standardization have to be followed, as was learned from human microbiome research [60,61]. Based on frequently expressed genes during the interaction of plant endophytic bacterial communities in the holobiont context, functions like e.g. osmoadaptation, phytohormone

production, oxygen tolerance and quorum sensing are of particular relevance.

Discrimination of plant beneficial bacteria from closely related human pathogenic bacteria exemplified by *A. brasilense* and *Roseomonas fauriae*

The rhizosphere is a habitat, which is colonized by a phenotypically wide spectrum of bacteria: from symbionts to pathogens. This has been pointed out by Berg et al. [62] and more recently by Mendes et al. [63], who highlighted the presence of plant beneficial, plant pathogenic and human pathogenic microorganisms in the rhizosphere. Already Lorenz Hiltner had proposed that many “wanted or unwanted guests” are attracted by root derived nutrients [7]. Even within a particular rhizobacterial genus, species with plant beneficial and pathogenic phenotypes are known [64].

In recent years, isolates with almost identical 16S rRNA to *A. brasilense* type strain Sp7, which also have high root colonization potential [65], were retrieved from wounds and other human sources. These isolates had been originally classified as *Roseomonas fauriae* or *R. genomospecies* 6, but lately they were reallocated to the *A. brasilense* species [66], based on wet DNA-DNA-hybridization analysis using the re-association method according to Brenner et al. [67]. Also, the ITS region of 16S-23S rRNA genes and many household genes are almost identical (Fig. 3).

However, recent whole genome DNA-DNA hybridization analyses using a spectrophotometric determination of re-association kinetics [69] revealed only 61.2% and 54.4% DNA-DNA sequence identity between *A. brasilense* Sp7^T and *Roseomonas fauriae* and *R. genomospecies* 6 (measurements of DSMZ, Braunschweig, Germany, unpublished) (Table 1). This definitely argues for a phylogenetic separation of *A. brasilense* from these opportunistic pathogenic *Roseomonas* bacteria. These results were corroborated by *in silico* determinations of ANI-values (Average Nucleotide Identity) based on whole genome sequences [70]. Based on a concatenated phylogenetic analysis of *rpoD*- and 16S rRNA gene sequences [70], it was further proposed to separate the *A. brasilense* strains into three closely related species: *A. brasilense sensu stricto*,

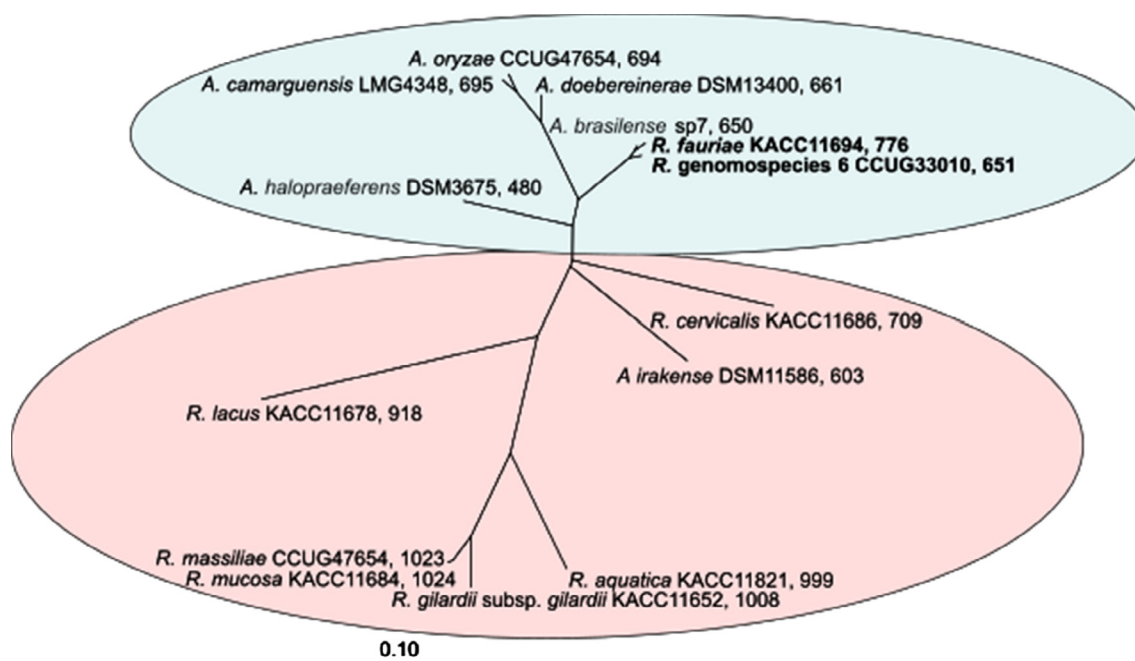


Fig. 3. Phylogenetic tree (ITS-region of the 16S-23S rRNA genes, maximum-likelihood method with 50% conservation filter) of *Azospirillum* spp. and *Roseomonas* spp. [68]

Table 1

Spectrophotometric DNA-DNA hybridization analysis, according to Huss et al. [69] of *A. brasilense* Sp7^T to several *A. brasilense* strains, *Roseomonas fauriae*, and *R. genomospecies* 6 (data from Deutsche Stammsammlung für Mikroorganismen und [68]).

	<i>Azospirillum brasilense</i> Sp7 ^T
<i>Azospirillum brasilense</i> FP2	96.5%
<i>Azospirillum brasilense</i> Sp245	54.0%
<i>Azospirillum brasilense</i> NH	56.0%
<i>Azospirillum brasilense</i> Az39	48.3%
<i>Azospirillum lipoferum</i> ^T Sp59b	28.7%
<i>Roseomonas fauriae</i> ^T KACC1694	61.2%
<i>Roseomonas genomospecies</i> 6 CCUG33010	54.4%
<i>Roseomonas mucosa</i> ^T KACC11684	12.5%

A. formosense [71] and *A. himalayense* [72]. Thus, it became apparent, that there is an unresolved micro-diversity within the species of *A. brasilense*. In addition, the plant endophytic *A. brasilense* strains Sp245, Az39, and strain NH, isolated from salt-affected wheat rhizosphere from Northern Algeria [73], were all shown to have DNA-DNA-hybridization values around 50% compared to the *A. brasilense* Sp7^T (Table 1). Therefore, further DNA-DNA hybridization studies and whole genome sequence analyses are necessary to clarify the relationship within *A. brasilense* and closely related species and their phylogenetic relationship to *R. fauriae* and *R. genomospecies* 6.

The application of whole genome-based comparative software tools together with the assessment of the pathogenic potential of each species [74], finally helped to clarify the difficult case of distinction between saprophytic or beneficial and pathogenic strains within the genus *Burkholderia*. This genus harbored a large number of species with human pathogenic or opportunistic pathogenic phenotypes as well as environmental and plant growth beneficial and symbiotic species. For a long time, there was a situation, when regulatory authorities banned every environmental release of a *Burkholderia* strain, including the beneficial and even symbiotic ones. Now, based on the available complete genome sequence data, conserved sequence indels (CSI) were successfully used as molecular marker for the demarcation of the *Burkholderia* groups [75]. Finally, there are at present three different genera within the *Burkholderia* cluster: (i) *Burkholderia*, containing the pathogens and opportunistic pathogens, (ii) *Paraburkholderia*, comprising the plant-associated and -beneficial species, and (iii) the *Caballeronia* cluster, a group of environmental species [76]. An even more complex situation is present within the species *Serratia marcescens*. Strains of environmental and nosocomial origins were intermixed without any handle to separate them based on a strict and efficient scientific approach. Whole genome multilocus sequence types (wgMLSTs) and core genome multilocus sequence types (cgMLSTs) were created with the PHYLIP program UPGMA algorithm creating two sectors representing strains with environmental or nosocomial origins [64]. Since there were even genomes identified, which reflected intermediary genomic situations, there is the chance to have even closer insights into steps of micro-evolution to optimize the fitness in an apparently altered habitat.

Major traits of rhizosphere bacteria for efficient root colonization

Osmoadaptation

Lack of available water is causing stress to each living organism, because all life processes and essential proteins and cellular structures are dependent in their native conformation on available water molecules. Due to their molecular structure, several small molecules, so-called osmolytes, like proline, glycine betaine,

ectoin, and trehalose are able to replace water molecules to some degree [77]. During osmoadaptation, organisms activate the synthesis or uptake of these and similar substances within their cells. Since these osmolytes are functional across different organisms, microbes and higher organisms can help each other out under water stress [78]. They also enable to protect salt-sensitive enzymes and stabilize cellular structures and functions by balancing the osmotic pressure in plant cells against the outside osmotic pressure caused by salt or water deficiency. In saline soils, osmo-tolerance mechanisms are omnipresent. For rhizosphere bacteria, osmoadaptation has selective power also in non-saline soils, because salt is being concentrated around the roots during the continuous uptake of water by the plant, resulting in an accumulation of ions in the rhizosphere. In addition, during daytime, the transpiration stream causes water deficiency in the rhizoplane, which may only be replenished during night time by slow diffusion of water from root-distant soil habitats. This water dynamics and the increasing salt-pollution of soils made osmo-adaptation and osmo-tolerance important traits in rhizosphere bacteria [79]. Moreover, the salt-tolerant IAA-producing rhizobacterium *A. brasilense* NH isolated from salt-affected rhizosphere soil of wheat in northern Algeria, can replenish specific phytohormones, like indole acetic acid (IAA, i.e. auxin), which are not sufficiently produced by salt-stressed root tissues [80]. In salt-affected soils, the 1-aminocyclopropane-1-carboxylate (ACC)-deaminase activity of rhizobacteria is of particular relevance, because due to this enzymatic activity, elevated levels of ethylene are reduced in roots, which would inhibit plant activities drastically [81,82]. It is remarkable that the occurrence of the ACC-deaminase gene is rather frequent in plant-associated bacteria from saline habitats and there are indications of horizontal gene transfer of this beneficial trait [83].

Among *Azospirillum* spp. different levels of osmotolerance can be found [84]. *A. halopraeferens* has the highest salt-tolerance and it could be shown that it is able to synthesize glycine betaine or take up and transform choline into betaine [85], while *A. brasilense* is only able to take up betaine glycine [86]. Trehalose is not significantly used as osmolyte by *A. brasilense*. However, when transformed with a plasmid harboring a trehalose biosynthesis gene-fusion from *Saccharomyces cerevisiae*, *A. brasilense* Cd accumulates trehalose under water stress and is able to grow up to 0.5 M NaCl. Furthermore, maize plants inoculated with this engineered bacterium were able to withstand drought stress and increase its biomass and grain yield [87]. The ability of salt-tolerant *A. brasilense* and *A. halopraeferens* strains to utilize proline and other amino acids as C-source for growth was only rather limited [88]. *A. brasilense* strains with increased NaCl-tolerance could be isolated which proved to be spontaneously resistant to the toxic proline antimetabolite dehydroproline under mild salt stress conditions [89]. Another relevant stress adaptation in *Azospirillum* is the cyst formation, which occurs when cells are challenged with nutrient deprivation or desiccation. In *Azospirillum* this regularly occurs, when cells are inoculated to roots as was shown in several independent techniques [42]. The induction of cyst formation can also be triggered by the application of fructose and nitrate as C- and N-sources in laboratory media. Malinich and Bauer [90] recently compared the metabolic and replicative gene expression by transcriptome analysis in vegetative and cyst states of *A. brasilense*.

Phytohormones and other growth enhancers

Besides IAA and derived substances with auxin activity, also nitrogen oxide (NO) is often found as plant growth regulating compound in rhizosphere bacteria. In the case of *A. brasilense*, which is a most successful and widely used PGPR, it is documented that

besides IAA also NO has a pronounced effect on the stimulation of root growth [91].

It has been shown in inoculation experiments of mutants, which produced only very low levels of NO, that root morphology was almost not changed in contrast to the inoculation with the NO-producing *A. brasilense* Sp245 wild type [92]. Similarly, IAA-deficient mutants lost the activity of root growth stimulation. The level of IAA-production could be increased in mutants of *A. brasilense* SpCd, resistant to the antimetabolite 5-fluor-tryptophan [93]. Inoculation of maize plants in an axenic system with the IAA-overproducing mutant FT326 showed root growth stimulation only at low inoculation densities and very low nitrate levels compared to the wild type inoculation [94]. In a similar way, mutants which show ammonium excretion could be selected from *A. brasilense* Sp7 by Machado et al. [95] using the antimetabolite ethylenediamine for ammonium assimilation. Using the ammonium-excreting mutant HM053 as inoculant for maize or wheat, nitrogen fixation and N-assimilation in inoculated plants were changed compared to the wild type inoculation [96,97].

Thus, the application of mutations resulting in drastically reduced or increased functions or the production of certain effector molecules are of central importance in the assessment of functional relevance of interaction traits. A detailed collection of physiological properties of *Azospirillum* spp. by Hartmann and Zimmer can be found in Yaacov Okon's book on *Azospirillum*/plant associations [98].

Oxygen tolerance

Induction of reactive oxygen species is a key element of defense reaction of plants. Thus, bacteria which approach plants need to be equipped with defense measures against these toxic oxygen species. In the case of the plant endophytic diazotroph *Gluconacetobacter diazotrophicus* Pal5, mutants devoid of catalase and superoxide dismutase were unable to colonize rice roots and to establish an endophytic life style [99]. Another oxygen defense mechanism uses O₂-diffusion protection by gum production. Consequently, mutants of Pal5 in gum-production lacked endophytic colonization too [100]. In the case of the interaction of the diazotrophic *Burkholderia australis* Q208 with sugarcane, a downregulation of reactive oxygen production of plants could be demonstrated by RNAseq during colonization by *B. australis* Q208 [59]. On the bacterial side, LPS- and flagella-production, which are well-known elicitors for pathogen-associated molecular patterns, were reduced in strain Q208 during the root colonization process. Since also strain Q208 harbors the QS-related genes for *N*-acyl-homoserine production [59], which are usually activated during biofilm production and root colonization, it is quite possible that they are involved in regulatory processes in the physiological changes occurring during root colonization and the interaction with plants (see below).

Bacteria-plant communication with focus on *N*-acyl homoserine lactones

Bacterial quorum sensing signals are involved in many important ecological functions, like biofilm formation, induction of antibiotic production and virulence. In Gram-negative bacteria *N*-acyl-homoserine lactones (AHL) were often found regulating these processes through an activation of the luxI/luxR-type regulatory circuit [101]. It has been shown using AHL-biosensor constructs that the production of AHL-molecules was heavily induced during the colonization of root surfaces by bacteria harboring the luxI/luxR-type auto-inducing system [102,103]. The auto-induction of AHL-synthesis can be activated already in microcolonies at the root surface due the spatial accumulation of the

AHL-compounds [103]. However, the excreted quorum sensing molecules are not only sensed by neighboring rhizosphere bacteria, but also by the plant hosts [104]. This *trans*-kingdom signaling induces different responses in the plants, depending on the type of AHLs (diffusile, water-soluble AHLs with short C-side chains or lipophilic, water-insoluble AHLs with C-side chains from 12 to 14 C-units). Water-soluble AHLs are taken up actively into the plant shoots inducing gene expression of antioxidative and xenobiotic degradation genes in roots and shoots as well as phytohormonal changes in the whole plant [105–107]. Also NO-accumulation and membrane hyperpolarization accompanied by increased K⁺ uptake are early events after AHL application to barley roots [108]. In contrast, water-insoluble AHLs prime the induction of systemic resistance response in the plant hosts [109] and finally confer increased resistance towards biotrophic and hemi-biotrophic pathogens in wheat and Arabidopsis [110,111]. The central involvement of QS-regulation in endophytic colonization of rhizobacteria could also be demonstrated, when mutants devoid of *luxI* or *luxR* homologous genes were tested for endophytic colonization. For example, a negative mutant for AHL synthesis of the beneficial root endophyte *Acidovorax radialis* N35 had reduced endophytic colonization abilities. In contrast to the wild type, the AHL synthesis mutant caused induction of the flavonoid biosynthesis genes, which are known to be part of the plant defense response [112]. Thus, the AHL-lacking mutant may not be recognized by the plant as beneficial bacterium. Furthermore, an AHL receptor mutant of *Gluconacetobacter diazotrophicus* Pal5 was also no longer able to colonize the plant host endophytically, since the QS-coordination was not functioning (Hofmann A and Baldani JI, unpublished results). Thus, QS-signaling in bacteria-plant interactions may not only act through direct interaction with the plant, but also by establishing and coordinating an adapted gene expression of traits like biofilm formation, necessary for endophytic colonization. *A. brasilense* strain Ab-V5 (originally derived from *A. brasilense* Sp7^T), applied in large scale for about 10 years in Brazilian agriculture, was recently shown to respond to *N*-acyl-homoserine lactones (especially 3-oxo-C8-HSL). It showed increased biofilm and exopolysaccharide formation as well as cell motility, because it harbours a *luxR*, but no *luxI* homologous gene [113]. Interestingly, while *luxI* homologues are missing in *A. brasilense*, they are present in most of the *A. lipoferum* strains [114]. Transconjugants of Ab-V5 carrying a plasmid with the *N*-acyl-homoserine lactonase gene abolished the PGPR effect of the wild type. The functionality of so-called *luxR*-solos reflect the release of AHL-mimic compounds by the plant host [115] or by the accompanying plant microbiome. As one important mechanism of stimulation of plant performance, AHLs induce priming effects, which are specific plant responses in the crosstalk of root-colonizing bacteria with their plant hosts leading to an alert state towards the attack of plant pathogens. It has been shown that a wide variety of molecules can induce priming, besides AHLs also including antibiotically active compounds, like lipopeptides of pseudomonads and bacilli, as well as certain volatile compounds [116,117]. The effects of priming are not visible in the absence of pathogens, but in the situation of pathogen attack, the defense responses are rapid and enhanced.

Conclusions and further perspectives

Thanks to the great methodological progress in the last two decades, there are now quite some “eye-opening insights” into many structural and functional details of the plant associated microbiome and key interactions between the plant microbiome and the host plant in the holobiont context [118]. However, the complexity of interactions is overwhelming and thus the collection and careful interpretation of further metagenome and transcrip-

tome data needs to be intensified for a deeper understanding. This should be supported by isolation approaches of novel bacteria leading to defined inoculation experiments and testing of functional hypothesis with mutant studies. In addition, the improvement of their environmental fitness and key interaction traits with the plant host (phytohormone production, ammonium excretion) by spontaneous selection or chemical mutagenesis of already established inoculation strains should be considered, since in some cases these appeared quite feasible. The final goal is to implement the knowledge about plant microbiome/host interactions under field conditions into practical applications. Ideally, this would mean to utilize synergistic effects in “synthetic” holobionts, where a specifically tailored set of beneficial microbes is introduced to plants which have been improved by selection, breeding or genetic modification in supporting the beneficial plant microbiome in a most productive manner. Within the “Plant Phytobiome” concept [119] aiming to integrate biological, soil, climate and agricultural management, a deeper understanding of key interaction and communication processes of the plant and its microbiome within the holobiontic context is urgently needed.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethical Requirements

This article does not contain any studies with human or animal subjects.

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biome, especially nitrogen fixation and the interkingdom communication based on quorum sensing signaling compounds were studied.



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identification of new diazotrophic genera and species, particularly *Herbaspirillum* with the species *H. seropedicae*, *H. rubrisubalbicans* as well as the *Nitrospirillum amazonense*. Strains belonging to these species are now being used as inoculants in sugarcane, maize and rice crops. More recently, his group has applied molecular approaches (genomic, transcriptomic and proteomic) to understand the plant-bacteria interaction of mainly those related to sugarcane inoculant strains. In addition, he has dedicated part of his research on the biocontrol area involving the use of endophytic bacteria with activity against phytopathogens (bacteria and fungi) and pest insects of economic importance to Brazilian crops.



Michael Rothballer studied Biology (Diploma) at the Technical University of Munich (1994-2000). He accomplished his PhD at GSF-Research Center for Environment and Health in Neuherberg in the working group Plant-Microbe Interaction of the Institute of Soil Ecology under supervision of Prof. Dr. Anton Hartmann. 2004-6 he was a scientific employee at the GSF-Research Center in the Department of Rhizosphere Biology in the Institute of Soil Ecology and from 2006 he was deputy group leader of the working group Molecular Microbial Ecology in the research unit Microbe-Plant Interactions at the Helmholtz Zentrum München.

From June 2016 until January 2017 he was acting as a head of the Research Unit Microbe-Plant Interactions. Finally, in February 2017 he became part of the newly founded Institute of Network Biology at the Helmholtz Zentrum München under Prof. Pascal Falter-Braun, where he leads the working group Molecular Microbial Ecology.