Chapter 3
In Situ Localization and Strain-Specific Quantification of *Azospirillum* and Other Diazotrophic Plant Growth-Promoting Rhizobacteria Using Antibodies and Molecular Probes

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Abstract A central issue in the understanding of the interaction and symbiotic function of diazotrophic bacteria with non-leguminous crop plants is detailed knowledge about the localization of the associated diazotrophic bacteria within the plant, their in situ activities in the plant-associated niches, and strain-specific quantification of inoculated bacteria. In addition to the colonization of rhizosphere soil and the rhizoplane, it has become apparent that an endophytic location of a diazotroph would provide it with a higher potential to interact more closely with the plant, particularly with respect to increasing the availability of carbon and energy nutrients derived from the plant, as well as the possibility, in return, of improving the transfer of bacterial-derived metabolites to the plant. Detailed localization of bacteria was successfully performed using fluorescence labeled ribosome-directed oligonucleotide probes in the fluorescence in situ hybridization (FISH) approach coupled to the use of confocal laser scanning microscopy (CLSM), and via immunolocalization with specific antibodies using transmission electron microscopy (TEM). Furthermore, the fate of inoculated bacteria could be traced by using specifically marked strains by applying the genes for the green or red fluorescent

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protein (GFP, RFP) and β-glucuronidase (GUS). Strain-specific quantification approaches for inoculants based on quantitative PCR using sequence characterized amplified regions (SCARs) and other genomic marker sequences have been developed and successfully applied. In this chapter major achievements and existing obstacles using these high resolution approaches to analyze bacteria in situ are presented together with some basic protocols.

3.1 Introduction

The challenge of high resolution in situ analysis of plant growth promoting diazotrophic bacteria in non-leguminous plants was successfully confronted by the combined application of molecular genetic and immunochemical methods together with high resolution microscopical techniques. These allow for resolutions at or below the dimension of individual bacteria in the micrometer, and in the case of transmission electron microscopy (TEM), the nanometer range. The clear aim of these techniques is to use methods which allow for the identification and specific labeling of the bacteria of interest at the species and even at the strain level. While the identification of bacteria at the species or higher phylogenetic level is possible by applying phylogenetic probes derived from 16- or 23S rRNA sequences of the bacteria of interest coupled to fluorescent dyes, which can be used in the fluorescence in situ hybridization (FISH) technique (see protocol below), the identification of bacteria at the strain-specific level needs other approaches. Two such examples are (a) it has been demonstrated in several cases (see below) that antibodies can be raised that allow for a strain-specific identification and (b) the introduction of marker genes, such as *gfp*, *gus* and *lux* into the bacterium under study by genetic transformation (see protocol below). As microscopical tools, confocal laser scanning microscopy (CLSM) is frequently used, as it can detect and resolve specific fluorescence against an autofluorescence background, which is often quite strong in plants. To resolve details of the localization of bacteria and their subcellular resolution below the micrometer range, electron microscope-based approaches such as TEM, often combined with immunocytochemistry, have been successfully applied (see protocol below). Finally, in order to monitor the success of inoculations, strain-specific quantification has been developed using sequence characterized amplified regions (SCARs) or other strain-specific genomic marker sequences in real time quantitative PCR measurements.

3.2 In Situ Localization Approaches

In the case of *Azospirillu*m, a variety of *A. brasilense* isolates were studied concerning their colonization of different plants. While the type strain of *A. brasilense*—strain Sp7—was isolated from rhizosphere soil, the strain Sp245 was derived from

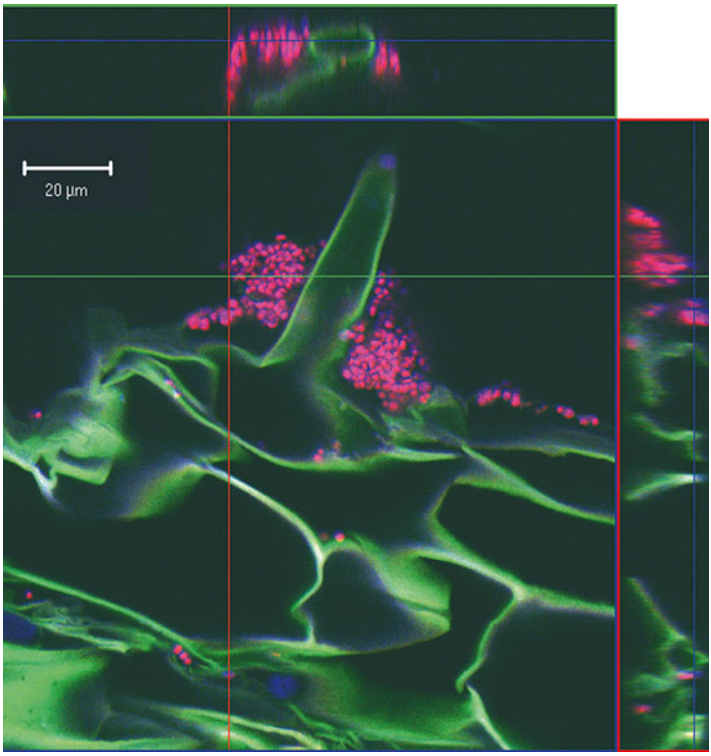

Fig. 3.1 *Azospirillum brasilense* Sp245 colonizing a wheat root. FISH staining using the species-specific probe Abras-1420-Cy5 (*blue*) and the general bacterial probe Eub-Mix-Cy3 (*red*) (image taken from Rothballer et al., Symbiosis 34, 261–279 (2003) with kind permission of Springer Science + Business Media)

surface disinfected wheat (*Triticum aestivum*) roots (Baldani et al. 1987). Upon inoculation of wheat plants, strain Sp245 was isolated in high numbers from surface disinfected roots, while Sp7 was more frequently recovered from rhizosphere soil (Baldani et al. 1987). Therefore, detailed localization studies were performed to identify unambiguously the different colonization properties of these two strains (Sp7 vs. Sp245) on wheat. Consequently, species-specific oligonucleotide probes were developed for *A. brasilense* to localize different *A. brasilense* strains after inoculation of wheat roots using FISH in combination with CLSM (Amann et al. 1990; Aßmus et al. 1995). It could be clearly shown in these studies that both strains (Sp7 and Sp245) colonized efficiently the root surface and that they produced pleomorphic forms. However, the strain Sp245 was able to colonize intercellular spaces in the epidermal layer and even occasionally the interior of root hairs or root cortical cells more frequently than strain Sp7, which was mostly found on the root surface (Figs. 3.1, 3.2 and 3.3).

Fig. 3.2 rgb-Color image

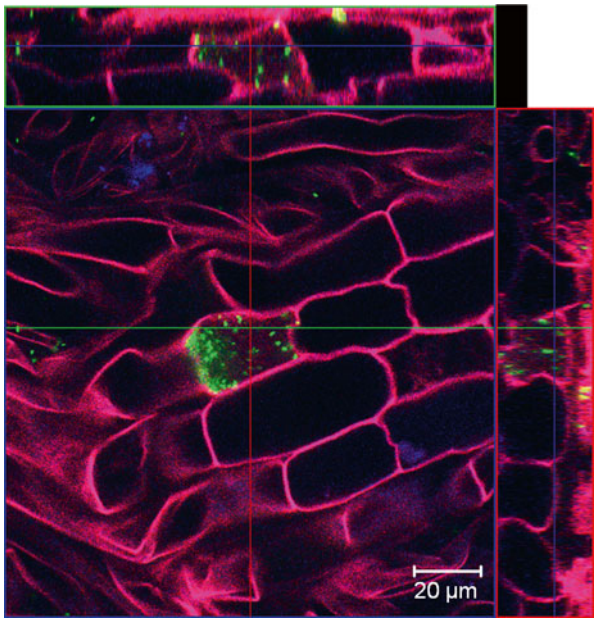

Fig. 3.3 *A. brasilense* Sp245 (gfpmut3 labeled) colonizing the interior of wheat roots (CLSM image) (image taken from Dr. M. Rothballer, Helmholtz Zentrum München, Neuherberg, Germany)

Using a light microscope-based approach and specifically CLSM the colonization of plants by bacteria (endophytically and rhizospherically) in combination with fluorescent oligonucleotide probes or antibodies and reporter genes (see below) has become an enormously powerful tool (Schloter et al. 1993; Aßmus et al. 1997; Alqueres et al. 2013). However, the resolution of the light microscope is limited to about 0.5 µm meaning that individual cells can be viewed but relatively little of their internal structure (e.g., organelles) can be discerned in any detail. In contrast, TEM utilizes a high energy beam of electrons which have a considerably smaller wavelength than light (c. 0.1 nm) and hence has a much greater resolution (<1 nm). These qualities make TEM an excellent technique for examining in detail the interactions between

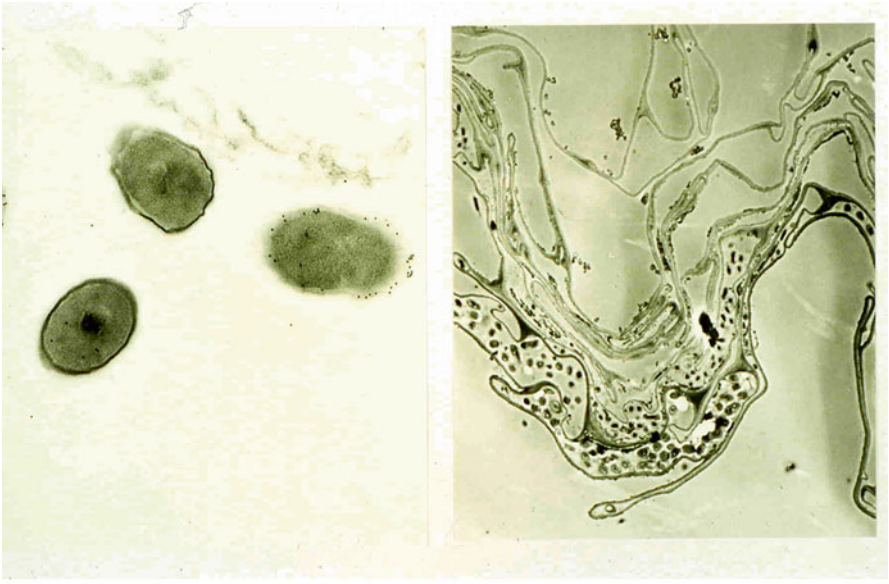

Fig. 3.4 *A. brasilense* Sp7 on the surface of wheat roots (TEM-immunogold image) (images taken from Schloter and Hartmann, Symbiosis 25, 159–179 (1998); with kind permission of Springer Science + Business Media)

beneficial/diazotrophic bacteria and their plant hosts. Of particular use over the last 30 years has been the combination of TEM with immunogold labeling (Levanony et al. 1989; Hurek et al. 1994; James et al. 1994, 1997, 2001, 2002; Gyaneshwar et al. 2001, 2002; Schloter and Hartmann 1998; Rothballer et al. 2008; Olivares and James 2008a, b) (Figs. 3.4, 3.5 and 3.6). In these cases, antibodies (monoclonal or polyclonal) that have been raised against specific bacterial species or even strains have been instrumental in confirming that they were genuinely endophytic within their economically important crop hosts, which were mainly grasses such as wheat, rice (*Oryza sativa*), sugarcane (*Saccharum* sp.), and sorghum (*Sorghum bicolor*). Other studies have used antibodies raised against particular bacterial proteins, such as nitrogenase (Hurek et al. 1994; Olivares et al. 1997; James et al. 2002; Gyaneshwar et al. 2002) and surface components (Schloter et al. 1994) to demonstrate specific gene expression in their specific plant habitat (Figs. 3.7 and 3.8).

3.2.1 Protocol: FISH Analysis

3.2.1.1 Fixation of Plant Samples

All samples need to be fixed for FISH analysis. Currently two different methods are widely applied: fixation with aldehydes (e.g., paraformaldehyde [PFA]) or with alcohols (e.g., ethanol). Paraformaldehyde (4 %) is best used to fix Gram-negative

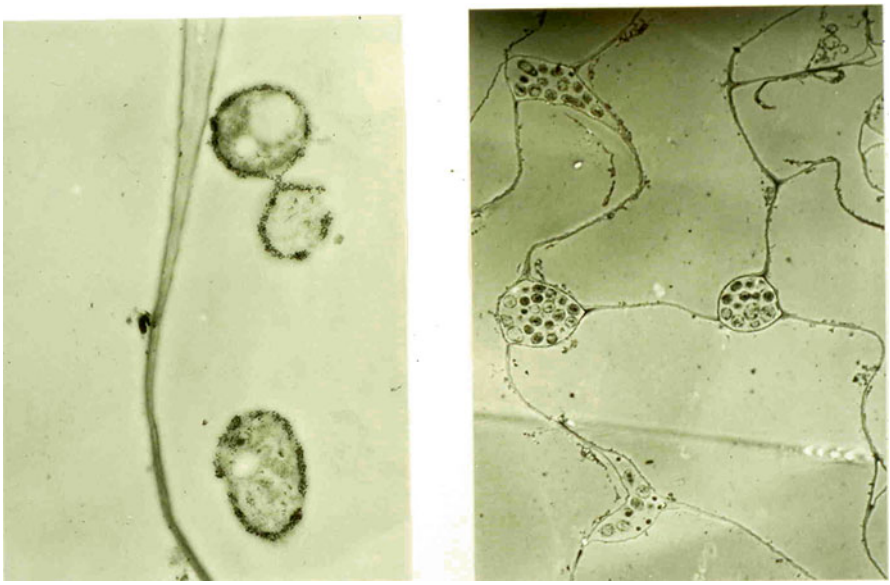

Fig. 3.5 *A. brasilense* Sp245 within intercellular spaces (apoplast) of a wheat root (TEM-immunogold image) (images taken from Schloter and Hartmann, Symbiosis 25, 159–179 (1998); with kind permission of Springer Science + Business Media)

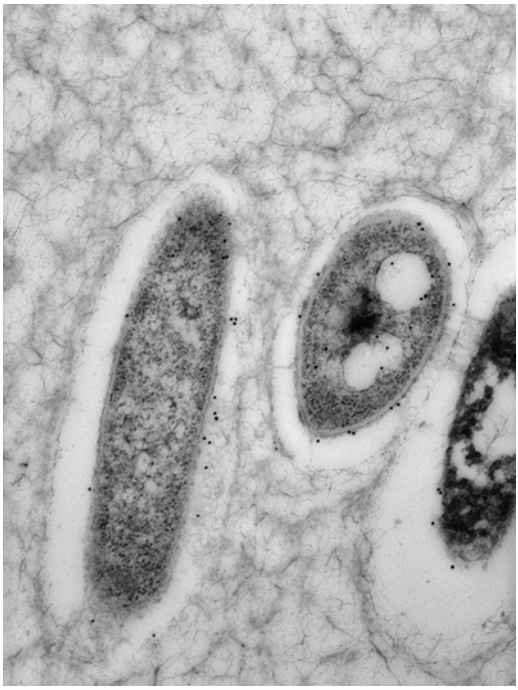

Fig. 3.6 *Gluconacetobacter diazotrophicus* PAL5 within a sugar cane leaf (TEM-immunogold image) (image taken by Dr. Euan James, The James Hutton Institute, Dundee, UK)

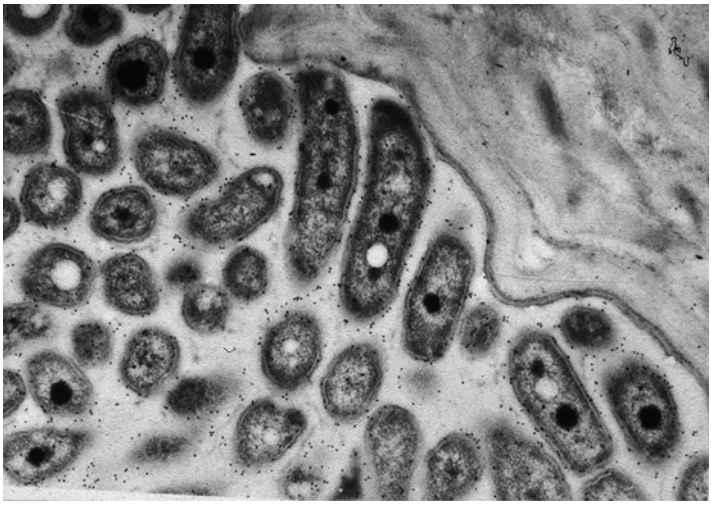

Fig. 3.7 *Herbaspirillum seropedicae* in a rice leaf (TEM-immunogold image) (image taken by Dr. Euan James, The James Hutton Institute, Dundee, UK)

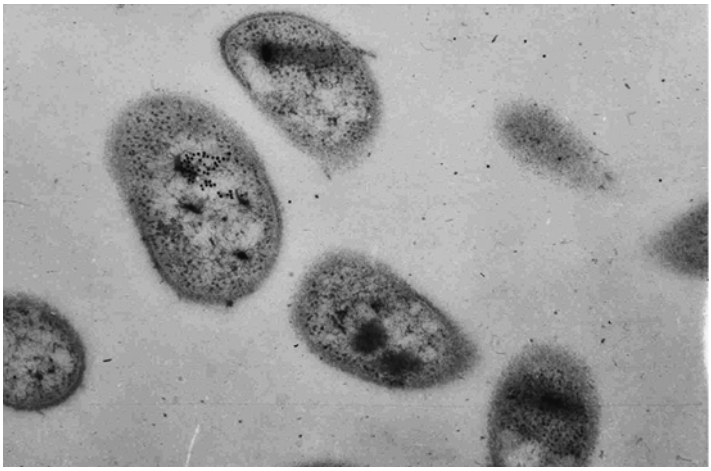

Fig. 3.8 *Herbaspirillum seropedicae* in rice roots (labeled with *nifH*-antiserum; TEM immunogold image) (image taken by Dr. Euan James, The James Hutton Institute, Dundee, UK)

bacterial cells because it causes cross-linking of the murein layer. The bacterial cell wall becomes denser and the shape of the cells is retained. The cell wall of Gram positive cells often becomes too dense for probe penetration during hybridization after they have been fixed using PFA, and therefore fixation with ethanol (50 %) is usually applied for successful FISH analysis of these bacteria.

3.2.1.2 Preparation of PFA Fixation Solution (50 mL)

- Heat 45 mL $H_2O_{ultrapure}$ to approximately 60 °C in a beaker on a magnetic stirrer with heating plate (visible condensation on the glass surface)
- Add 2 g PFA with caution; a milky suspension results (please wear protective mask while weighing the paraformaldehyde (Hazardous XN))
- Add 10 N NaOH dropwise until the solution becomes clear
- Add 5 mL 10× PBS
- Cool down to room temperature (pH is temperature dependent)
- Adjust pH to 7.2–7.4 (dropwise $HCl_{conc.}$, then dropwise 10 N HCl)
- Filter sterilize the solution using a 50 mL syringe with a 0.45 µm sterile filter
- Store on ice (stable for 1–3 days at 4 °C and up to 1 week at −20 °C)

3.2.1.3 Fixation of Roots

- Prepare a 3:1 (v/v) solution of PFA/1× PBS in a 50 mL Falcon Tube
- Harvest roots, remove adhering soil particles, wash roots with 1× PBS, and add roots gently to the fixation solution mentioned above. Incubate for at least 2 h or overnight at 4 °C
- To finish the fixation procedure, the fixation solution is discarded and the roots are washed 3× with 1× PBS. Store roots in 1:1 mixture of 1× PBS/$EtOH_{absolute}$ at −20 °C

3.2.1.4 Preparation of the Hybridization Buffer

Pipette into a 2 mL Eppendorf reaction tube (ERT) in the given order:

5 M NaCl (360 µL)
1 M Tris/HCl pH 8.0 (40 µL)
Formamide (deionized) and $H_2O_{ultra\ pure}$ depending on the chosen stringency (total volume 1,600 µL)
10 % (w/v) SDS (2 µL)

The hybridization buffers should be stored in closed Eppendorf caps on ice until use.

% Formamide	Formamide (deionized) [µL]	$H_2O_{ultra\ pure}$ [µL]
0	0	1,600
5	100	1,500
10	200	1,400
15	300	1,300
20	400	1,200
25	500	1,100

% Formamide	Formamide (deionized) [µL]	$H_2O_{ultra\ pure}$ [µL]
30	600	100
35	700	900
40	800	800
45	900	700
50	1,000	600
55	1,100	500
60	1,200	400
65	1,300	300
70	1,400	200
75	1,500	100
80	1,600	0

The Fluorescence in situ *hybridization* (*FISH*) *analysis is performed at 46°C*!
The hybridization oligonucleotide probes are coupled to fluorescent dyes.
Washing buffer, washing at 48°C !!! (*Probes labeled with fluorescent dyes*)
Preparation of the washing buffer
Pipette into a 50 mL Falcon Tube

- 1 M Tris/HCl, pH 8.0 1 mL
- 0.5 M EDTA, pH 8.0 (from 20 % formamide plus) 500 µL
- 5 M NaCl according to table below

% Formamide chosen in the hybridization buffer	[NaCl] in mol	[µL] 5 M NaCl from 20 % formamide plus add 500 µL 0.5 M EDTA
0	0.900	9,000
5	0.636	6,300
10	0.450	4,500
15	0.318	3,180
20	0.225	2,150
25	0.159	1,490
30	0.112	1,020
35	0.080	700
40	0.056	460
45	0.040	300
50	0.028	180
55	0.020	100
60	0.014	40
65	0.010	0
70	0.007	350 µL 0.5 M EDTA pH 8.0
75	0.005	250 µL 0.5 M EDTA pH 8.0
80	0.0035	175 µL 0.5 M EDTA pH 8.0

– Add 50 mL $H_2O_{ultra\ pure}$
– 50 µL 10 % (w/v) SDS

Preheat washing buffer in a water bath to 48 °C.

3.2.1.5 In Situ Hybridization (Samples on Slides)

– Place or drop fixed sample onto an epoxy resin-coated slide, dry it in an oven (about 5–8 min at 60 °C)
– Fix samples by treating them in an increasing ethanol series (3 min each in 50, 80, and 100 % ethanol)
– Drip 9 µL of hybridization buffer into each well
– Add 1 µL of probe without scratching the surface of the slide
– Prepare a hybridization tube (50 mL Falcon Tube) by folding a piece of tissue, putting it into the tube and pouring the rest of hybridization buffer onto it
– Transfer the slide into the hybridization tube and perform the hybridization in a hybridization oven (46 °C) for 1.5 h
– Remove the hybridization solution and incubate the slide within the washing buffer for 20 min in a preheated water bath (48 °C)
– Remove the washing buffer with distilled water and dry the slide in an air stream
– Embed the sample on the slide within an embedding media (e.g., Citifluor) and seal the slide with a cover slip

3.2.1.6 In Situ Hybridization (Roots in Eppendorf Caps)

– Treat roots in an increasing ethanol series (3 min each in 50, 80 and 100 % ethanol)
– Place dehydrated root pieces in a 2 mL Eppendorf cap
– Add hybridization buffer until the fixed roots are covered
– Add probes (add one-tenth of the hybridization buffer volume of each probe)
– Incubate for at least 1.5 h at 46 °C
– Discard hybridization buffer and add 2 mL washing buffer. Incubate for 15 min at 48 °C (water bath)
– Discard washing buffer, wash with distilled water, and place root pieces on a slide. Embed the samples on the slide with Citifluor and seal them with a cover slip

3.2.2 Protocol: Immunogold Labeling for Light Microscopy and TEM

The immunogold labeling method can be performed at both the light microscopy (>0.5 µm) and TEM levels, even on the same samples. However, it is necessary for this technique that the biological samples (e.g., plant roots) be sectioned on an

ultramicrotome; typically these sections are of the order of 0.5–2.0 μm thickness for light microscopy and 50–100 nm for TEM. In order to achieve uniform sections at these thicknesses the samples must be immobilized, either by freezing them or by chemical fixation and subsequent embedding in plastic/resin. Methods have been described in detail for freezing (i.e., cryo) techniques by Olivares and James (2008a, b), and so they will not be covered here. Chemical fixation of roots for immunogold labeling is similar to that described for FISH, but always involves aldehydes, usually PFA or glutaraldehyde, either singly or in combinations, as these allow for good ultrastructural preservation, with glutaraldehyde being particularly effective, as it very strongly cross-links proteins. The exact choice of fixative depends upon the antigen being detected; if it is a single epitope (e.g., for probing with a monoclonal antibody), or is delicate and/or easily damaged by cross-linking, then PFA should be used, but if there are multiple antigens, as is often the case with polyclonal antibodies raised against whole bacterial cells, then it may be possible to fix using glutaraldehyde only. Often, however, a fixative composed of a mixture is used, e.g., 4 % PFA to preserve antigenicity and a small concentration of glutaraldehyde to improve the ultrastructure. Further fixation for "classical" TEM, particularly in osmium tetroxide, should generally be avoided, as should embedding in epoxy resins, such as Spurrs, Araldite, and EPON, as although these treatments beautifully preserve ultrastructure they have a very deleterious effect on antigenicity. After dehydrating the samples in ethanol, the resin of choice for embedding of samples for immunogold labeling is normally an acrylic resin, such as LR White. After infiltration in the liquid resin, the samples can be solidified in the resin for sectioning by placing the sample + fresh resin in gelatin capsules and then "curing" them in an oven at 55 °C; this will allow the resin to polymerise and harden, and the sample can then be sectioned on an ultramicrotome.

3.2.2.1 Preparing Sections for Light Microscopy and TEM

- Cut fresh pieces of root into small pieces (2–3 mm in length) and immediately immerse them in a fixative solution, e.g., 2.5 % glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.0)
- Fix the samples for at least 24 h at 4 °C. If the samples are still floating after this period subject them to a weak vacuum for 5 s, release the vacuum, and then repeat the cycle until all the samples have sunk
- Dehydrate the roots in an ethanol series: 10 min each at 50, 70, 90, and 100 % (2×)
- Place the roots into a mixture (1:1, v/v) of ethanol and LR White acrylic resin for 2 h
- Place the roots into 100 % LR White for 24 h on a specimen rotator
- Place roots individually into gelatin capsules (Agar Scientific), cover them with fresh LR White resin, and seal the capsule with a cap
- Place the gelatin capsules into an oven at 55–60 °C for 48 h
- Remove the hardened capsules and allow them to cool in a fume hood

- Section the roots (1 μm) for light microscopy
- Collect the sections on Superfrost glass slides and dry them onto the slides by placing them on a hotplate
- Stain the sections using 1 % toluidine blue in 0.5 % sodium borate (pH 8.0) and view them under a compound microscope; if the sections contain roots and bacteria move on to the next steps (TEM and immunogold labeling)
- Trim the samples on the ultramicrotome, i.e., remove sufficient resin around the root so that the cross-sectional face of the resin-embedded root is approximately 1 mm^2 and that the edges of the rectangle/square are straight
- Section the samples for TEM (80 nm) and collect them on Pioloform (or Formvar or pyroxylin/collodion)-coated Cu or Ni grids (50–200 mesh)
- Proceed to immunogold labeling

Specific sites ("antigens") on TEM sections can be identified by incubating the sections in an antibody which has been raised in an animal (e.g., rabbit, rat, or mouse) against the specific antigen, and this is denoted the "primary" antibody. The primary antibody binds specifically to the corresponding antigen in the sample, and these binding sites can then be visualized under the TEM after incubation of the sample in a "secondary antibody" which has been conjugated to a gold particle (usually ranging in diameter from 5 to 20 nm). The gold particles are heavy metals and hence under the TEM are seen as distinct electron dense points on the surface of the section, and these points should then represent the location of the antigen(s) that the user is intending to identify. The gold-conjugated secondary antibody is not specific to the labeled antigen, but is raised in another animal against protein from the same animal as the primary antibody. For example, if the primary antibody is from a rabbit then the secondary antibody may be raised in goats against rabbit protein. An alternative to gold-conjugated secondary antibodies is to label the primary antibody with gold particles conjugated to Protein A, a protein from the fungus *Staphylococcus aureus* that binds tightly to a wide range of antibodies from different animal species, but most particularly to rabbit.

3.2.2.2 Protocol: Immunogold Labelling (Light Microscopy and TEM)

- Collect fresh sections on grids for TEM (see above)
- Incubate the grids for 1 h in a blocking buffer henceforth denoted as immunogold labeling or "IGL" buffer, which consists of 0.5 % bovine serum albumin (BSA) and 0.5 % Tween 20 in 0.1 M phosphate-buffered saline (PBS), pH 7.0
- Move the grids from the IGL buffer to drops of the primary antibody which has been diluted appropriately in the same IGL buffer. Incubate for 2 h at room temperature
- Remove grids from the primary antibody and wash them with IGL buffer by placing them onto two sequential drops of buffer for 5 min each
- Incubate the grids for 1 h on drops of the secondary antibody (e.g., goat anti-rabbit conjugated to 15 nm gold particles; BBI Solutions) which has been diluted 1:100 (v/v) in IGL buffer

- Remove the grids from the secondary antibody and wash them with IGL buffer by placing them onto two sequential drops of buffer for 5 min each, and then ten drops of sterile dH_2O for 30 s each. Dry the grids by placing them onto filter paper
- For light microscopy perform the above, but instead of grids place drops of the various reagents onto the slides with fresh sections
- After washing off the secondary antibody incubate the sections in a silver-enhancement kit (BBI Solutions) for 10–20 min or until the black silver precipitate is visible under a compound microscope
- Stop the reaction by washing off the silver solution with dH_2O. Dry on a hot plate
- Negative controls for the immunogold labeling must be included in parallel with the test samples. These typically include sections and grids that have been incubated as above, but with the primary antibody omitted or replaced by pre-immune serum from the same animal as the primary antibody was raised in

Evaluation of the results: View and photograph the light microscopy sections, with and without immunogold silver-enhancement (IGL-SE), under a high quality photomicrograph system and determine the presence and location of the bacteria on and within the roots; the bacteria will appear as purple-blue points (1–2 μm long) in the toluidine blue sections and as black points against a light blue background in the IGL-SE sections (also often seen as a "ring" of black around the perimeter of the bacterium, as the antibodies often bind to bacterial surface polysaccharides). Stain the TEM sections with 2 % aqueous uranyl acetate for 5 min, dry on filter paper, and view under a transmission electron microscope, with and without immunogold labeling; the location of the bacteria and their ultrastructure will be clear, and if they have been successfully immunogold labeled the gold particles will appear as uniform electron-dense spots on the surface of the bacteria.

3.2.3 *Protocol: Labeling of Cells with Fluorescence (e.g.,* **gfp-***Genes and* **rfp-***Genes) or Enzymatic (e.g., Glucuronidase,* **gusA-***Genes) Markers*

The major advantage of the application of fluorescently labeled or GUS-marked strains for bacterial colonization studies is that no fixation and other time-consuming and artifact-producing treatments have to be performed upon the sample, except for fluorescence or light microscopy after enzymatic reaction for gus activity. The limitations, however, are occasionally coming from the difficulty or even impossibility to introduce the marker gene or to get the marker gene to be expressed in the target cell at a sufficient rate. Therefore, different vectors, constructs, or variants of the marker genes should be used. It should be noted that the resulting bacteria are genetically engineered and hence cannot be applied outside the lab and must be used only in specifically licensed glasshouses.

In the case of *A. brasilense* gfp- and gusA-labeling were achieved and the colonization of wheat roots by the strain FP2 could be followed at the single cell level (Ramos et al. 2002). To use gfp as a biosensor for expression analysis of a specific operon to follow in situ gene expression with fluorescence monitoring at the single cell level, Rothballer et al. had to apply the stable variant gfpmut3 to get sufficient brightness of the fluorescence labeling in cells induced for the *ipdC*-expression as a key step of IAA-biosynthesis (Rothballer et al. 2005). Endophytic colonization of sugarcane as well as of sorghum and wheat by *Gluconacetobacter diazotrophicus* was successfully followed using GUS- and GFP-labeled bacteria (Fuentes-Ramirez et al. 1999; Luna et al. 2010; Rouws et al. 2010), as was the endophytic colonization of wheat by *Klebsiella pneumoniae* 342 and *Herbaspirillum frisingense* GSF30 using GFP-labeled strains (Iniguez et al. 2004; Rothballer et al. 2008). In terms of specific gene expression, the endophytic colonization of rice plants and the in situ expression of nitrogenase genes were convincingly evaluated on a single cell level via gfp-labeled bacteria that were fluorescing simultaneously with the fluorescence of Td-Tomato from a transcriptional *nifH*::*tdTomato* fusion (Reinhold-Hurek and Hurek 2011). The most recent developments in CLSM to reveal microbe–plant interactions in situ at single cell resolution have been recently reviewed by M. Cardinale (2014).

Name	Basic replicon	Application	Reference
pKmobGII	R6K	Delivery suicide vector for chromosomal gene replacements	Katzen et al. (1999)
pEX18	R6K	Delivery suicide vector for chromosomal gene replacements	Hoang et al. (1998)
pUT-miniTn5, pUT-miniTn7	R6K	Delivery suicide vector containing mini-Tn5/mini-Tn7 transposon for chromosomal tagging	de Lorenzo et al. (1990), Choi et al. (2005)
pME6010	pVS1, p15A	Shuttle vector for stable, plasmid-based labeling in Gram-negative, plant-associated bacteria	Heeb et al. (2000)
pBBR1-MCS	ColE1	Shuttle vector for stable, plasmid based labeling with broad host range	Kovach et al. (1995), Obranic et al. (2013)
pHC60	pSW213	*gfp*-gene labeling	Cheng and Walker (1998)
pHRGFPGUS	pBBR1, pHR	*Gfpmut3*- and *gusA*-gene labeling	Ramos et al. (2002)
pCHRGFP1/2	pCHR	Gfp-bioreporter	Branco et al. (2013)
TnMod-OGm	pUT	Modular mini-transposon for rapid cloning and mapping	Dennis and Zylstra (1998)

Two protocols for transferring the vector pHRGFPGUS into PGPR are presented in the following subsections. Before, preliminary tests are described to determine resistance/sensitivity of the recipient strain to antibiotics to choose the adequate selection marker and vector.

3.2.3.1 Choosing a Vector Considering the Selection Mark: Testing for Antibiotic Resistance/Sensitivity of the Strain

Classically, minimal inhibitory concentration (MIC) is determined using a series of tube dilutions, in which different antibiotic concentrations are prepared in the culture medium. After incubation of the strain in this series, tubes are inspected, and visible growth (turbidity) is evaluated. The tube that contains the least antibiotic concentration able to inhibit completely the microorganism growth defines the MIC, and this approach is known as the tube dilution method. This approach can be adapted to 96-well plates to improve the analysis throughput, and turbidity of the resulting cultures can be measured using an ELISA microplate reader if appropriate filter or light wavelength is set. Care must be taken as MIC is not constant for a given antibiotic, as it may be affected by the inoculum size, composition of the culture medium, incubation time, and conditions, such as temperature, pH, and aeration. When the final objective is to transform the strain through electroporation, ideally the strain must be subjected to the preparation procedure of electrocompetent cells followed by the electric pulse and then plated on serial dilutions of the antibiotic. A few strains (e.g., *Herbaspirillum seropedicae* SMR1 and *Gluconacetobacter diazotrophicus* PAL5) get more resistant for certain antibiotics after the electroporation procedure, as colonies are formed in the negative control (without DNA added) under MICs of antibiotics. Once MIC is determined for a given antibiotic, it (or up to ~4× its concentration) can be used in the culture medium for selecting the marked strain. MIC determinations are also useful to identify antibiotics for selecting a given strain from a conjugation mixture (see below: strain selection marker).

3.2.3.2 Gene Marker Transfer Through Conjugation

A number of vectors available have an origin of transfer, *oriT* or "mob," and then can be mobilized to diverse Gram-negative bacteria, if *trans*-acting *tra-* and *trb-encoded* proteins are provided by a helper plasmid such as pRK2013 or an *E. coli* strain such as S17-1. As an illustrative procedure, *Nitrospirillum* (formerly *Azospirillum*) *amazonense* CBAmc (=BR11145) can be recipient of the mobilizable vector pHRGFPGUS:

- Grow donor (e.g., S17-1 bearing the vector) and recipient strains to an $O.D._{600} \cong 0.2$ in LB and DYGS (2.0 $g \cdot L^{-1}$ glucose, 1.5 $g \cdot L^{-1}$ peptone, 2.0 $g \cdot L^{-1}$ yeast extract, 0.5 $g \cdot L^{-1}$ K_2HPO_4, 0.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 1.5 $g \cdot L^{-1}$ glutamic acid, pH 6.0) media, respectively
- Centrifuge both cultures (1 mL) at 3,000 × *g*, 5 min, and discard the supernatant
- Wash the cell pellets once in sterile saline solution (0.9 % NaCl) and resuspend in the same solution
- Mix 5 μL of the donor strain with 50 μL of the recipient and plate (do not scatter) the mixture on DYGS:LB 3:1; prepare also appropriate controls (without donor, recipient, or both)

– After growth, scrape the (mixed) colony and resuspend in 1 mL of saline, and then plate (by scattering) 0.1 mL of this suspension on DYGS in the presence of 10 μg/mL kanamicin (the vector selection marker), 10–20 μg/mL tetracycline (the strain selection marker), and 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (or X-Gluc, for the vector enzymatic marker glucuronidase, *gusA* gene). The other vector selection marker, ampicillin, is not adequate for selection of *N. amazonense* CBAmc conjugates, as this strain is quite resistant to that antibiotic (CMI ≅ 1 mg/mL)

3.2.3.3 Gene Marker Transfer Through Electroporation

Another method for transferring the vector containing the gene marker into cells of a bacterial strain is through electroporation, in which an electrical field is applied to increase the permeability of the cell membrane, allowing the uptake of the vector. For example, *Gluconacetobacter diazotrophicus* PAL5 (=BR11281) can be transformed through electroporation with vector pHRGFPGUS. For that, electrocompetent cells must be prepared:

– Grow *G. diazotrophicus* PAL5 in C2 (10 $g \cdot L^{-1}$ peptone, 15 $g \cdot L^{-1}$ glucose, 5 $g \cdot L^{-1}$ NaCl, 5 $g \cdot L^{-1}$ yeast extract, pH 6.0) medium up to an $O.D._{600} \cong 0.6–0.7$
– Incubate the culture flask for ~30 min in ice bath; all the remaining procedure is conducted in ice or at 4 °C
– Centrifuge the culture at 4 °C, 10 min, 3,000 × *g*
– Wash cells twice with ultrapure cold water and once with cold 10 % glycerol, using the same centrifugation conditions, except that in the presence of glycerol time is 25 min
– Resuspend the final cell pellet in 1 mL of cold 10 % glycerol, prepare aliquots of 100 μL and store at −80 °C until the electroporation step

The electroporation of *G. diazotrophicus* PAL5 has been executed in a Gene Pulser Xcell™ Electroporation System (Bio-Rad):

– Mix 2 μL of the vector solution prep, let's say 4 μg, with the 100-μL aliquot of PAL5 cells in ice bath
– Transfer the mixture into a 2-mm electroporation cuvette
– Apply an electrical pulse of exponential decay, 25 μF capacitance, 200 Ω resistance, 2.5 kV
– Add 1 mL of DYGS medium into the cuvette, transfer the suspension into a microtube and incubate at 30 °C, >200 rpm, 2 h
– Plate 0.1 mL of the suspension on DYGS in the presence of 400 μg/mL kanamicin (the vector selection marker), and 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (or X-Gluc, for the vector enzymatic marker glucuronidase, *gusA* gene)

3.2.4 *Protocol: Strain-Specific Quantification of Inoculated PGPR by qPCR Using SCAR-Markers*

The availability of techniques to quantify inoculated diazotrophic PGPR in a *strain-specific* way in the rhizosphere or even within plant tissues is very important to be able to estimate how efficiently and pertinently an inoculant strain is established within the plant. Recently, two molecular approaches have appeared which use stretches of strain-specific sequences in the bacteria to be monitored using the real-time qPCR technique. One method uses SCAR markers to develop strain-specific, real-time PCR primers for strain-specific quantification (Couillerot et al. 2010a, b) on the basis of unique REP-amplificates, while the other method derives the strain-specific sequences from comparative whole genome sequence data of the inoculant and closely related bacteria (Stets et al. personal communication). Quantitative PCR approaches to detect individual strains in complex environments such as the rhizosphere were first developed as MPN-PCR or competition-PCR (for a review see Sorensen et al. 2009), but real time PCR (RT-PCR) has recently become the method of choice for quantifying bacterial populations in the rhizosphere (Sorensen et al. 2009). This method is based on the quantitative measurement of SYBR Green which binds to double-stranded DNA after each PCR cycle. The PCR cycle at which the fluorescence crosses the threshold line (the CT value) is directly proportional to the amount of DNA present in the sample.

This technique was applied to the detection and quantification of two *A. brasilense* and *A. lipoferum* strains (Couillerot et al. 2010a, b).

Protocol of basic methodology (for details see Couillerot et al. 2010a):

- Molecular comparison of *Azospirillum* strains by REP-PCR genomic fingerprinting (Rademaker et al. 2000) to distinguish trains and to identify SCAR bands
- Excise, clone, and sequence strain-specific bands
- Compare the sequences to those on the web by BlastN and select unique sequences
- Based on these unique sequences primers are designed and checked against other *Azospirillum* strains from the same species, other *Azospirillum* species as well as other prominent rhizosphere and soil bacteria
- Based on the selected primers, qualitative and quantitative PCR reactions should be performed and the PCR products then need to be checked on agarose gels
- Carry out RT-PCR reactions with the selected primers and generate standard curves with genomic DNA
- Generate standard curves for RT-PCR quantification with DNA isolated from the rhizosphere by inoculating tubes containing the desired soil sample with the strain of interest in different densities per tube (10^3–10^8). Incubate and extract DNA to carry out RT-PCR and generate standard curves for each replicate by plotting CT numbers vs. log CFU added per gram of soil

– Carry out RT-PCR quantification of the inoculated bacterial strain of interest in the rhizosphere or resp. root environment. Inoculation of seedlings should reach between 10^4 and 10^6 CFU g^{-1} root. The inoculated seedlings should be planted in a axenic (sterile) or non-axenic (non-sterile) root environment and the microcosms should be incubated under the desired environmental conditions. Isolate DNA from the sub compartment of interest (e.g., tightly root adhering soil) and then carry out RT-PCR on the isolated rhizoplane soil DNA
– Express colony counts as log CFU g^{-1} root system and determine the relationship between log CFU data and CT values by regression analysis for the comparison of RT-PCR data with CFU data

In the approach described by Stets et al. (personal communication), the whole genome sequence of an inoculant strain (*A. brasilense* FP2) was fragmented in silico and the fragments were blasted against the genome of a whole genome of another strain from the same species (*A. brasilense* Sp245) at the nucleotide level. Fragments with no BLAST similarity were subjected to a second BLAST search against general public DNA sequence database. Putative strain-specific sequences without any match were used to design primer pair sets for a strain-specific RT-PCR quantification.

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