Fabricio Dario Cassán · Yaacov Okon
Cecilia M. Creus *Editors*

Handbook for Azospirillum

Technical Issues and Protocols

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Preface

The genus *Azospirillum* is one of the most widely studied and commercially used plant growth-promoting rhizobacteria (PGPR) in agriculture, especially in South America (MERCOSUR). It belongs to the class Alphaproteobacteria and comprises free-living, nitrogen-fixing, vibrio- or spirillum-shaped rods that exert beneficial effects on plant growth and on the yield of many agronomically important crops. Following inoculation of several grasses, cereals, and legumes with azospirilla, there are pronounced morphological effects on the roots, beginning with the proliferation of root hairs. The morphological effects are generally dependent on inoculum concentration and are consistent with the exogenous indole acetic acid (IAA) levels secreted by bacteria. The IAA/cytokinin ratio and nitric oxide (NO), a key signaling molecule involved in a wide range of functions in plants, are also important. One of the most important achievements obtained from research is the utilization of commercial azospirilla inoculants (the bacteria in a suitable carrier), mainly in Argentina, Brazil, Uruguay, Paraguay, Mexico, and South Africa. About 3.5 million ha of wheat, maize, sorghum, and legumes have been inoculated (as of 2014), and the use continues to increase.

The following research developments have been important to understand how these bacteria promote plant growth. The controversy on the potential contribution of biological nitrogen fixation to the system was clarified in 1977–1985. It is generally agreed today that the association of *Azospirillum* with plants such as maize, wheat, and sorghum could contribute only a few kilograms (kg) of fixed N per hectare (ha), whereas in modern cereal production fields are fertilized with 150–250 kg N/ha. The effect of inoculation on root proliferation resulted in yield increases of the order of 10–30 %. Field experiments in Israel in 1977–1985 very clearly demonstrated the improvement in crop yield. These early observations were extensively confirmed by continuous field experimentation and interest in the MERCOSUR and Mexico (1980 to present). Results from field experiments became more consistent with the commercial use of successful liquid inoculants containing osmoregulators for maintaining 10^9 viable cells/ml inoculant after 1 year of storage. The regulation of products (see Chap. 27 on development of protocols for the quality control of inoculants) in the past decade has contributed to system success.

Another important development (2000 to present) is the study of signals or dialogue between the bacterium and the plant such as auxins–indole acetic acid and nitrous oxide–NO involvement.

We are presenting a wide interdisciplinary effort written by authors with extensive research experience in the subject of PGPR, especially *Azospirillum* sp., but also other beneficial rhizobacteria such as *Herbaspirillum*, *Gluconacetobacter*, *Burkholderia*, *Pseudomonas*, and *Paenibacillus*. This handbook was conceived as a tool to be on the bench of all laboratories that investigate PGPR. It intends to bring to the lector a series of basic and advanced experimental approaches and protocols. Its content is grouped in four "toolboxes," each concerned with a different approach: (1) biochemistry and molecular biology, (2) physiology and metabolism, (3) biotic interactions, and (4) agronomic and industrial applications. The handbook covers these well-characterized bacteria that promote plant growth directly, not via biological control, and have good potential to be commercially applied for increasing the yields of agricultural crops, but others that are less well known could be also assayed by the protocols depicted in this handbook.

Each chapter presents the state-of-the-art methodologies for investigating *Azospirillum* and other plant–bacterial associations. In the introduction for each chapter, the authors present a brief description of the subject with the latest updates from the literature. The methodologies cover the subject at many levels and aspects, beginning with isolation, cultivation, fermentation, and proliferation in the laboratory of the associated bacteria, but also cultivation and storage for commercial inoculants (bacteria in a suitable carrier), production, and marketing. Methods for studying the biology, biochemistry, and physiology of the organisms including production of storage materials, biofilm formation, mechanisms for survival under stress, biological nitrogen fixation, the N cycle, and production of plant growth substances such as auxins and NO are presented. Methodologies for studying the genetics, genomics, DNA recombination, and genetic engineering of PGPRs are covered in detail in various chapters. We present also the ecological aspects and interactions with other beneficial organisms such as symbiotic nodule-forming nitrogen-fixing rhizobia and with arbuscular mycorrhiza.

The most important statistical evaluation of PGPR performance in field experiments is by parameters measured for plant growth: plant physiological aspects as influenced by inoculation and interactions with the bacteria and the way these are evaluated in the controlled greenhouse environment and in the field. Also, methodological aspects for commercialization of the products, marketing, and commercial companies are involved.

We wish to express our deep appreciation to all authors for sharing their basic, applied, and practical knowledge on this subject. We are confident that the material presented will facilitate the research and application of plant–bacteria interactions for improving sustainable agricultural crop production.

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Part II Physiology and Metabolism Toolbox

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Part I
Biochemistry and Molecular Biology Toolbox

Chapter 1
Isolation, Identification and Biochemical Characterization of *Azospirillum* spp. and Other Nitrogen-Fixing Bacteria

Veronica Massena Reis, Vera Lucia Divan Baldani, and José Ivo Baldani

Abstract *Azospirillum* is one of the most well-characterized genera and currently includes 15 N-fixing and one non-N-fixing species. *A. brasilense* is a bacterium used in agriculture in many countries since its discovery in 1978. It is found in association with many plants worldwide and can be isolated from soil as well. The main strategy used to isolate and count bacterium from this genus uses N-free semi-solid medium, and the recipe used by many authors over the past 34 years is the semi-solid NFb medium described in 1976. In this chapter, we present the procedures used for diazotrophic counting and identification either from rhizosphere soil or on the surface or within plant tissues. We also describe methods of measuring nitrogenase activity in vials containing semi-solid medium, as well as the use of carbon sources for species characterization and storage procedures.

1.1 Introduction

1.1.1 Azospirillum spp.

The genus *Azospirillum* is a member of the α-subclass of proteobacteria and includes a species previously named *Spirillum lipoferum* (Beijerinck 1925). The growth of a spirillum-like bacterium was first observed by Beijerinck in 1922, when a nitrogen-deficient malate- or lactate-based media was inoculated with garden soil. This new bacterium increased the nitrogen content of the malate medium and was named *Azotobacter spirillum*. Three years later, the genus was renamed as *Spirillum* and little attention was given to this organism until 1974, when root samples of several grasses (Digitaria, *Panicum maximum*, *Cynodon dactylon*), grain crops (wheat, maize, sorghum, millet, rice) and soil samples were used by Döbereiner and

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collaborators to isolate this organism. Several strains were later isolated from plants collected in several countries, including Peru, Nigeria, Colombia and Senegal, and also from other researchers, such as Shank (USA), Milam (Ecuador, Venezuela) and Eskew (USA), as described in the publication by Tarrand et al. (1978). A group of 61 isolates from several plants and locations was then analysed in a detailed taxonomic study, and based on DNA homology, the *Azospirillum* genus was described with two species: *A. lipoferum* (old group II) and *A. brasilense* (old group I). The medium used to obtain these isolates was a semi-solid nitrogen (N)-free medium containing 0.005 % of yeast extract, as described by Day and Döbereiner in 1976. This medium was later named NFb (Dobereiner et al. 1976); the small amount of yeast extract was omitted and a vitamin solution was added to benefit some strains that require biotin to grow.

Initially, the N-free NFb medium, with neutralized malic acid as a carbon source, pH near 7.0 and prepared with a low concentration of agar (1.75 g L^{-1}) to produce a semi-solid condition in tubes or vials, was the key to discover that plants from different soils, regions and plant parts possess diazotrophs. This semi-solid condition was important to produce the microaerophilic environment that allowed the organism to synthesize the nitrogenase complex and initiate N-fixation.

The description of *Azospirillum* species increased exponentially over the years and other groups adopted the NFb medium recipe and discovered many new species, genus and, consequently, interactions with several plants. This procedure, recognized as an enrichment medium, helps separate diazotrophs from a complex plant and environmental soil samples. From 1980 to 2014, 14 more *Azospirillum* species were described, but the N-free semi-solid medium was not used by all authors (Table 1.1). The importance of the recently described species is not yet known.

The use of semi-solid N-free medium with some modification, such as carbon source, phosphate buffer and final pH, allowed Magalhães et al. (1983) to describe the species *A. amazonense* isolated from palm trees collected in Amazonia and also from cereal tissues grown in Seropédica, RJ. Four years later, *A. halopraeferens* (Reinhold et al. 1987) was isolated from a Kallar grass (*Leptochloa fusca*) planted in saline soils in Pakistan using the semi-solid medium modified by increasing the NaCl concentration and final pH (Table 1.1). *Conglomeromonas largimobilis* subsp. *largomobilis* showed high phylogenetic similarity to *A. lipoferum* and therefore was renamed as a new *Azospirillum* species called *A. largomobilis*, which was later changed to *A. largimobile* (Dekhil et al. 1997).

In 2001, a new group of N-fixing bacteria was isolated from *Miscanthus* using the traditional NFb medium recipe and named *A. dobereinerae* in honour of the famous scientist Johanna Döbereiner (Eckert et al. 2001). *A. oryzae* was isolated from rice samples grown in China (Xie and Yokota 2005). Similarly, *A. melinis* was isolated from roots and stem of *Melinis minutiflora* grown in China after inoculation into semi-solid N-free NFb or LGI media (Peng et al. 2006). A modification of the N-free NFb semi-solid medium by increasing the pH contributed to the discovery of *A. canadense* (Mehnaz et al. 2007a) and *A. zeae* (Mehnaz et al. 2007b).

Other species of the genus *Azospirillum* were also described, but the N-free semi-solid medium or serial dilution technique (Döbereiner 1995) was not applied.

Table 1.1 List of *Azospirillum* species isolated from plant samples using different solid and semi-solid media

Media	*Azospirillum*	Carbon source	Conditions	Reference
Semi-solid				
NFb	*A. lipoferum*	Malate[a]	pH 6.5–6.8 + biotin	Tarrand et al. (1978)
	A. brasilense	Malate	pH 6.5–6.8 + biotin	Tarrand et al. (1978)
	A. doebereinerae	Malate	pH 6.5–6.8 + biotin	Eckert et al. (2001)
	A. melinis	Malate	pH 6.5–6.8 + biotin	Peng et al. (2006)
	A. formosense	Malate	pH 6.5–6.8 + biotin	Lin et al. (2012)
NFb	*A. irakense*	Malate	pH 7.0–8.5 + 0.3 % NaCl	Khammas et al. (1989)
SM	*A. halopraeferens*	Malate	pH 8.5 + 1.5 % NaCl. Incubation at 41 °C	Reinhold et al. (1987)
LGI	*A. amazonense*	Sucrose	pH 6.0–6.2	Baldani (1986)
	A. melinis	Sucrose	pH 6.0–6.2	Peng et al. (2006)
FAM	*A. amazonense*	Sucrose	pH 6.0 + NaCl, 100 mg L^{-1}	Magalhães et al. (1983)
M	*A. oryzae*	Malate	pH 6.8 + yeast extract and NaCl 100 mg each	Xie and Yokota (2005)
NFG	*A. oryzae*	Glucose	pH 7.3 + $CaCO_3$ 5 g	Xie and Yokota (2005)
M	*A. zeae*	Malate	pH 7.2–7.4 without biotin	Xie and Yokota (2005)
M	*A. canadense*	Malate	pH 7.2–7.4 without biotin	Xie and Yokota (2005)
NFB or LGI	*A. melinis*	Malate	Depending on the recipe	Peng et al. (2006)
MPSS	*A. thiophilum*	Sodium succinate	pH 7.5 + FeS suspension	Lavrinenko et al. (2010)
Solid				
TYB	*A. palatum*	Rich medium	Yeast–beef–tryptone extract	Zhou et al. (2009)
TSA	*A. picis*	Rich medium	Tryptone soya agar	Lin et al. (2009)
NB	*A. humicireducens*	Rich medium	pH 7.2	Zhou et al. (2012)
NB	*A. fermentarium*	Rich medium	Tryptone soya agar	Lin et al. (2013)

[a]Malic acid neutralized with KOH

The procedure used plates containing different rich media to obtain single colonies followed by molecular characterization to identify new species. For example, *A. rugosum* was isolated from discarded road tar soil collected in Taiwan using brain-heat infusion (BHI) agar and trypsoy soy agar plates (Young et al. 2008).

In 2009, two new species were described using solid medium: *A. palatum* (Zhou et al. 2009) and *A. picis* (Lin et al. 2009). *A. picis* was also isolated from oil-contaminated soil samples. Even though the N-free semi-solid media were not used by these authors, they also observed that the bacteria could fix nitrogen and exhibited nitrate reduction activity, differing from *A. palatum. A. thiophilum* was isolated from a sulfide spring sample collected in Russia (Lavrinenko et al. 2010). This new species was isolated using an old recipe of medium called MPSS, described by Caraway and Krieg (1974), with a freshly prepared solution of FeS described previously by Kucera and Wolfe (1957) with sodium succinate and hydrolysed casein. *A. formosense* was isolated from agricultural soil collected in Taiwan (Lin et al. 2012) using the N-free medium recipe used by Reinhold et al. (1987) to describe *A. halopraeferens*. The N-fixing *A. humicireducens* was isolated from microbial fuel cells (Zhou et al. 2012) using a complete new medium, and *A. fermentarium* was isolated from a fermentative tank in Taiwan using the standard tenfold dilution plating technique (Lin et al. 2013).

Most of the studies involving the interactions *of Azospirillum* with plants are based on the species *A. brasilense*. The other *Azospirillum* species described in the last century, such as *A. lipoferum*, *A. irakense* and *A. amazonense*, are known to predominantly colonize the root surface, and only a few strains of *A. brasilense*, *A. lipoferum* and *A. amazonense* are detected inside plant tissues after inoculation (Patriquin and Döbereiner 1978; Patriquin et al. 1983; Assmus et al. 1997; Oliveira et al. 2009). The physiological basis for the observed invasiveness of *A. brasilense* is unknown. Even for species such as *A. irakense* that possesses enzymes that degrade the host cells, this model is not established (Khammas et al. 1989). Normally the bacteria enter the inner part of the plant using opportunities such as disrupted cortical tissues at the lateral root junction, lysed root hairs or natural cracks on the plant tissues (Steenhoudt and Vanderleyden 2000).

1.1.2 Other Genus and Species of Diazotrophs

Many diazotrophic bacteria including new genera were described using the semi-solid medium modified by the addition of plant macerates or extracts (Table 1.2). For example, in 1988, the group of Johanna Döbereiner modified the LGI medium, traditionally used to isolate *A. amazonense*, by the addition of cane juice to isolate the first species of the Acetobacteraceae family that could fix nitrogen (Cavalcante and Dobereiner 1988). Reis et al. (1994) later reduced the amount of cane juice from 250 to 5 mL and adjusted the final pH to 5.7 with acetic acid. The initial name of the bacterium described with this new medium was *Saccharobacter nitrocaptans* (Cavalcante and Dobereiner 1988) and was later modified to *Acetobacter diazotrophicus* (Gillis et al. 1989) and finally to *Gluconacetobacter diazotrophicus* (Yamada et al. 1997, 1998).

The root has been traditionally used as the target plant tissue to isolate diazotrophs. This assumption was based on the fact that rhizobia fix nitrogen in nodules

Table 1.2 Media used to isolate other diazotrophs from plant tissues and soil samples

Media	Bacterial species	Carbon source	Conditions	Reference
LGI-P	*Gluconacetobacter diazotrophicus*	Cristal sugar	pH 5.7 + cane juice for isolation	Reis et al. (1994))
JMV	Diazotrophic *Burkholderia*	Manitol	pH 4.5 or 5.5	Reis et al. (2004)
Baz	Diazotrophic *Burkholderia*	Azelaic acid	pH 5.7	Estrada-de-los-Santos et al. (2001)
Bac	Diazotrophic *Burkholderia*	Azelaic acid + L-citrulline	pH 6.0	Estrada-de-los-Santos et al. (2001)
SM	*Azoarcus*	Malate	pH 7.0	Reinhold et al. (1986)
JNFb	*H. seropedicae*	Malic acid	pH 5.8	Baldani et al. (1986)
	H. rubrisubalbicans	Malic acid	pH 5.8	Baldani et al. (1996)
	H. frisingense	Malic acid	pH 5.8	Eckert et al. (2001)
	Sphingomonas	Malic acid	pH 5.8	Videira et al. (2009)

localized mostly in this plant part. However, sugarcane possesses unlimited carbon sources, especially in the aerial plant tissues, and therefore the entire sugarcane plant, including the xylem sap, has been used to isolate several strains of *Gluconacetobacter diazotrophicus* using the new recipe of LGI-Pc medium (Reis et al. 1994). Based on these new findings, another type of plant relationship was established. These microorganisms localized in plant tissues were called endophytes, as they live inside the plant without causing disease symptoms. The more simplistic, but clearly more accurate, definition for bacterial endophytes was proposed by Kado (1991), describing endophytes as "bacterial living in plant tissues without causing any substantive harm or gaining benefit other than residency." This definition excludes bacteria that cause disease symptoms or detrimental effects on host plants. *G. diazotrophicus* was one of the first true endophytes described at that time, since its survival outside of plant tissue is low (Reis et al. 1994).

After this description, several bacterial species and genera joined the list of endophytes that can improve plant growth. Good examples of this group are the two species of *Herbaspirillum*, *H. seropedicae* (Baldani et al. 1986) and *H. rubrisubalbicans* (Gillis et al. 1991; Baldani et al. 1996), that were isolated using the semi-solid JNFb medium, which is a very similar recipe to NFb medium (Table 1.2). One aspect that differentiates the two species is the infection of sugar cane leaves. *H. seropedicae* has never been isolated from naturally occurring cane leaves, although roots and stems are often highly infested. Olivares et al. (1997) and James and Olivares (1998) compared the inoculation of sorghum and sugarcane leaves with

H. rubrisubalbicans and *H. seropedicae*. When a suspension of *H. rubrisubalbicans* was injected into the leaves of a cane variety susceptible to mottled stripe (B-4362), these bacteria completely blocked some of the xylem vessels and colonized the intercellular space of the mesophyll cells. At this particular site, the presence of nitrogenase in the centre of the microcolonies was observed using an antiserum against the FeMoCo subunit of the enzyme (James and Olivares 1998). In sorghum, the metaxylem was also colonized by this bacterium and the nitrogenase antigen was also associated with this bacterium (Olivares et al. 1997). In the genotype resistant to mottled stripe disease, SP 70-1143, the bacterium also colonized the xylem, but formed clusters of 10–20 cells encapsulated by membranes, probably of plant origin.

In Germany, the group of Reinhold and Hurek identified grass-associated and toluene-degrading diazotrophs using a modified SM medium (Reinhold et al. 1993; Hurek and Reinhold-Hurek 1995). Based on the partial 16S rDNA sequence, a new genus called *Azoarcus* was created to include these N-fixing bacteria with description of three species: *A. communis*, *A. indigens* and *Azoarcus* spp. (included only a single strain BH72) (Reinhold-Hurek et al. 1993) (Table 1.2). The strain BH72 is the most studied from this genus, and even with the genome sequenced, the species name has not been proposed yet. *Azoarcus* spp. BH72 showed no common characteristics with the two other species described. *A. indigens* can grow on medium containing *p*-amino-benzoate, while *A. communis* can metabolize citrate and D-ALANINE. New genera and species were later isolated from Kallar grass and named *Azovibrio restrictus*, *Azospira oryzae* and *Azonexus fungiphilus* (Reinhold-Hurek and Hurek 2000). In 2013, a new species of *Azoarcus*, called *A. olearius*, was isolated from oil-contaminated soil and showed 100 % homology with the BH72 strain (Chen et al. 2013) and the ability to grow in semi-solid NFB medium.

Another huge group of diazotrophs that also belongs to the betaproteobacteria subclass is the *Burkholderia* genus, which includes more than 70 species isolated from remarkably diverse ecological niches, including environmental samples and human pathogens (Vial et al. 2011). This genus was created in 1992 with the transfer of the *Pseudomonas* homology group II to this new genus (Yabuuchi et al. 1992; Gillis et al. 1995). This large genus can be divided into two sub-lineages: one including human, animal and plant pathogen (*Burkholderia cepacia* complex, BCC) species and some environmental species, and the second group with more than 25 related environmental species, which in most cases are non-pathogenic and have been found to be associated with plants (Suárez-Moreno et al. 2012). Bacteria of this genus have a large genome size (around 8–9 MBp) and several properties that improve plant growth, including N-fixation (with or without nodule formation), phosphate solubilization, internal colonization of plant tissue, and degradation of aromatic compounds among others (Paganin et al. 2011).

It is difficult to indicate a better recipe for the isolation of *Burkholderia* species, considering that they vary in so many aspects. However, common medium recipes have been used in Brazil for isolating, counting and cultivating of non-symbiotic *Burkholderia* species such as *B. kururiensis* (Zhang et al. 2000). The medium called JMV contains mannitol as a carbon source and a final pH from 4.5 to 5.5

(Baldani et al. 1996). The LGI medium, used for the enrichment of *A. amazonense*, can also be applied to isolate species of N-fixing *Burkholderia. Burkholderia tropica*, which includes strains from sugarcane plants collected from Pernambuco (Brazil), can be isolated using LGI or LGI-P medium with 10 % sucrose (routinely used for the isolation of *G. diazotrophicus*) (Table 1.2). *B. unammae* (Caballero-Mellado et al. 2004), *B. silvatlantica* (Perin et al. 2006) and others also use semi-solid medium with recipes based on azelaic acid and a mixture of three carbon sources (Estrada-de-los-Santos et al. 2001) (Table 1.2).

The common aspect of all these finding is the strategy used to separate bacteria with a special feature. In the case of diazotrophs, the use of media without addition of nitrogen is one strategy that easily allowed the isolation of these bacteria from soil and plant samples. The use of tubes or vials containing semi-solid media is another strategy when part of the population does not grow in this condition, such as some strains of the genus *Rhizobium* (Perin et al. 2006). For this genus, the approach is based on the nodule as a source of the bacterium, and therefore a classic solid medium is the base of the strategy (Vincent 1970). For anaerobic bacteria, a chamber that reduces the oxygen gradient is another strategy. This chapter will not include all these recipes and steps. It will focus on the experience of isolating diazotrophs from plants using semi-solid medium and basic methodologies used to isolate, identify and store *Azospirillum* species.

1.2 Methods

1.2.1 Steps for Counting Culturable N-Fixing Bacteria

1.2.1.1 Roots, Stems and Leaves and Soil

Roots of harvested field plants should be washed in tap water to clean off rhizosphere soil, and then cut into 10 cm pieces, dried on a paper towel and weighed (10 g per sample or less when the amount of tissue is insufficient). The samples are then ready for the serial dilution procedure.

Usually, rhizosphere soil (10 g) is also homogenized for 30–60 min in 90 mL of saline solution (eg. salts of NFb medium) or sucrose solution (4 %), using a rotary shaker at 150 rpm. In this case, it is used directly in the serial dilution procedure.

1.2.1.2 Surface Sterilization

In the case of surface-sterilized tissue (roots), the roots are immersed into 1 % chloramine T solution ($CH_3{\cdot}C_6H_4{\cdot}SO_2{\cdot}NNaCl{\cdot}3H_2O$). The time in this solution is determined by age and type of plant: roots from maize and sorghum at the flowering stage are usually immersed for 30 min to 1 h, while rice and wheat are immersed only for 5–15 min in the same solution. After the surface sterilization, roots are

placed in sterile distilled water for 1/3 of the time that they were in the sterilization solution. This procedure is repeated for the same amount of time in phosphate buffer (50 mM, pH 7.0) and again in distilled water, with the total time equal to the Chloramine T sterilization step. Roots that are not to be sterilized should be soaked in distilled water for the same amount of time as used in the sterilization procedure (Baldani et al. 1986). For aerial plant tissue, another method of sterilization is recommended depending on the tissue. For sugarcane stems, the stem piece is flamed using alcohol. Sodium or calcium hypochlorite is also better adapted for aerial plant tissue.

1.2.2 Counting of Diazotrophic Microorganisms

Sterilized or washed roots, stems and leaves (10 g) are blended in one of the above solutions for 1–2 min. After 1 h of incubation, aliquots of 1 mL are used for serial dilutions (10^{-2}–10^{-7}) in 9 mL saline or sucrose solution. The addition of Tween 80 (0.02 %) to this saline solution improves the dilution efficiency.

1.2.2.1 Estimating the Diazotrophic Population Using Semi-Solid Medium

In most cases, the numbers of N-fixing bacteria (free living, associative and endophytic) are estimated by the most probable number (MPN) method using McCrady's probability tables (Okon et al. 1977). Counts are made on N-free semi-solid medium by inoculating 0.1 mL of each dilution in three tubes (or five tubes, not presented here) containing the medium specific for the target organism, placing the inoculum from the bottom to the centre of the tube, as these bacteria are sensitive to O_2. These tubes are then incubated at 30–34 °C, without shaking, from 5 to 7 days, to allow pellicles to form. The method used to quantify the MPN is based on the presence or absence of pellicles in the semi-solid medium and the approximate numbers computed using McCrady's MPN Tables (Table 1.3—see example).

For example, the number 321 in the Table 1.3 corresponds to the positive dilutions. In this case, diazotrophic number is 15.0. This is multiplied by the first dilution on counting and then by 10 (for the 0.1 mL for inoculation):

MPN (cells mL^{-1}) = $15.0 \times 10^5 \times 10 = 1.5 \times 10^7$.

1.2.2.2 Bacterial Counting Using Solid Media

When dealing with pure cultures, it is possible to determine the number of cells by homogenizing 1 mL of the culture in 9 mL of saline solution, sterile water or sucrose solution (4 %) and proceeding to serial dilutions up to 10^{-9} as previously described. It is also possible to use Tween 80 detergent to disperse the organisms. By using

Table 1.3 McCrady's table for most probable number (MPN) bacterial counting using three tubes per dilution

Dilution with growth	Diazotrophic number	Dilution with growth	Diazotrophic number	Dilution with growth	Diazotrophic number
000	0.0	201	1.4	302	6.5
001	0.3	202	2.0	310	4.5
010	0.3	210	1.5	311	7.5
011	0.6	211	2.0	312	11.5
020	0.6	212	3.0	313	16.0
100	0.4	220	2.0	320	9.5
101	0.7	221	3.0	321	15.0
102	1.1	222	3.5	322	20.0
110	0.7	223	4.0	323	30.0
111	1.1	230	3.0	330	25.0
120	1.1	231	3.5	331	45.0
121	1.4	232	4.0	332	110.0
130	1.6	300	2.5	333	140.0
200	0.9	301	4.0		

Example of counting: take the three highest dilutions with growth (10^{-5}, 10^{-6}, 10^{-7})
10^{-5}: 3 positive tubes
10^{-6}: 2 positive tubes
10^{-7}: 1 positive tube

Drigalsky's loop, aliquots of 0.1 mL are spread onto petri dishes containing the specific medium amended with a nitrogen source in very low amounts, usually 50 mg yeast extract per litre. If using rich medium (potato agar, DYGS, Nutrient Broth or other synthetic media), the nitrogen source is not required. Calculation of the number of cells is obtained by multiplying the number of colonies formed on the plate by the corresponding dilution factor and the sample volume (in the case of 0.1 mL, this equals 10).

Example: Inoculated plate inoculated with 0.1 mL of 10^{-7} dilution shows 60 individual colonies. The number of cells per millitre will be $60 \times 10^7 \times 10 = 6.0 \times 10^9$

1.2.2.2.1 How to Isolate *Azospirillum*

Different media are used to isolate different bacterial species. For isolation of *A. brasilense* and *A. lipoferum*, the semi-solid NFb medium is inoculated with 0.1 mL of soil or root suspension and incubated at 30–34 °C. Pellicles will form within 4–7 days, and the veil pellicles should be transferred to new semi-solid NFb medium and incubated again for 4–7 days.

The culture is streaked on solid NFb medium supplemented with 50 mg yeast extract and incubated 3–5 days when colonies from both species are white, dry and small. Individual colonies should be transferred to semi-solid NFb medium and

incubated again for 4–7 days. After pellicle formation, cultures should be streaked on solid potato medium. Colonies formed in this medium are initially yellowish-white, and eventually becoming pinkish. These are transferred to new semi-solid NFb medium from which the bacteria can be identified in wet mounts under the microscope. *A. brasilense* cells are medium sized (1 × 3–5 μm), very motile, curved rods with spirilloid movement. *A. lipoferum* cells initially are indistinguishable from the former, but once the medium turns alkaline (blue colour), the cells change into large pleomorphic forms.

Purified colonies of *A. lipoferum* and *A. brasilense* should be incubated in semi-solid NFb medium in the same conditions cited above (48 h at 30–34 °C). *A. brasilense* cells are curved rods at 1 × 3–5 μm even after the medium becomes alkaline. *A. lipoferum* cells are identical, but when the medium turns alkaline, cells grow large, pleomorphic and immotile (size may grow up to 10 μm in length and 3–5 μm thick). These species may also be differentiated using medium containing glucose, as *A. lipoferum*, but not *A. brasilense*, is capable of metabolizing glucose as the sole carbon source (Table 1.6).

For other *Azospirillum* species, the recipe of NFb medium can be modified. *A. irakense* is characterized by growing in semi-solid NFb with up to 0.3 % NaCl and alkaline pH (7.0–8.5). When growing on Nutrient Broth, cells can be as large as 20 μm. *A. halopraeferans* is identified by its growth at high temperature (41 °C) and salt tolerance. Cells may grow to 1.2 μm length and 0.7–1.4 μm thick if the pH turns alkaline.

A. amazonense is characterized by its capability to use sucrose as the sole carbon source (some *A. lipoferum* strains also grow in presence of sucrose). In LGI medium, its cell size is 0.9 × 1.4 μm. In potato medium, colonies of *A. amazonense* are typically cream, light brown and the edges are wrinkled.

A. largomobile colonies grown on peptone yeast extract agar are 2 mm in diameter after 72 h of incubation at 28 °C, and cream to buff coloured, opaque, low, convex and round with an entire edge and smooth surface.

Azospirillum species may be distinguished phenotypically as they show several physiological and morphological differences (Table 1.6).

1.2.2.2.2 How to Isolate Other Diazotrophs

To isolate *Gluconacetobacter diazotrophicus*, N-free semi-solid LGI-P medium is used containing a high sucrose concentration (100 g L^{-1}) (recipe below). The recipe may also be amended with 5 mL L^{-1} sugar cane juice (LGI-Pc) to improve isolation direct from the plant tissue, but further purification steps do not require adjusted medium. Serial dilution aliquots from roots, leaves or stems are inoculated into semi-solid LGI-Pc medium and incubated at 30 °C. Seven to 10 days later, the medium shows orange pellicles and becomes colourless. The pellicle is then streaked onto plates containing solid LGI-P medium amended with 40 mg yeast extract and incubated for 7 days at 30 °C. Colonies will be small and orange. The purification step is made by streaking colonies onto potato-P medium, on which

colonies are initially moist and clear and change to chocolate-brown 7–10 days after incubation at 30 °C. They are stored as described above. Cultivation under liquid LGI-P medium requires addition of 50 mg yeast extract. To isolate *Gluconacetobacter* species from coffee plants, Jimenez-Salgado et al. (1997) used LGI medium supplemented with sugarcane juice containing cycloheximide (150 mg L^{-1}) to control fungi growth and a final pH 4.7. It can also be helpful for isolation of diazotrophic bacteria from sugarcane.

Another recipe based on NFb medium, named JNFB, was modified by Baldani et al. (1986) to improve the isolation of the *Herbaspirillum* genus. *Herbaspirillum seropedicae*, *H. rubrisubalbicans* and *H. frisingense* (Baldani et al. 1986, 1996; Kirchhof et al. 2001) form a veil-like pellicle in JNFb semi-solid medium similar to that of *Azospirillum* spp. These bacterial pellicles are generally streaked onto JNFb or NFb agar plates containing yeast extract (50 mg L^{-1}), on which colonies become small and white with a central blue point after 1 week incubation. This colour is more evident in NFb medium with three times the normal concentration of bromothymol blue (denoted NFb 3×), mainly for strains of *H. seropedicae* and *H. rubrisubalbicans* (for details, see Baldani et al. 2005). Purification on potato medium with sucrose and malate yields small wet raised colonies that become brownish in the centre, while they remain white and wet on BDA. This medium also can be used to isolate strains of the *Sphingomonas* genus (Videira et al. 2009). However, in contrast to *Herbaspirillum* species, white pellicles are initially formed, which later become yellow with loss of the blue colour of the JNFb medium.

Bacteria belonging to the *Burkholderia* genus and associated with grasses such as rice, maize and sugarcane can be easily isolated using JMV medium (Baldani 1996). Roots, leaves and stems are surface sterilized and macerated as previously described. The serial dilutions are inoculated into the JMV semi-solid medium and incubated for 4–7 days at 32 °C. On the fourth day, thick pellicles begin to form. Once these pellicles have migrated to the surface of the medium, they are streaked onto plates of JMV solid media containing 60 mg yeast extract and again incubated for 4–7 days at 32 °C. Another recipe described by Estrada-de-los-Santos et al. (2001) used a medium with azelaic acid as a carbon source and pH 5.7 and Bac medium containing two carbon sources, azelaic acid and L-citrulline, to isolate *Burkholderia* associated with several grasses.

1.2.3 Media

The isolation of *Azospirillum* spp. is based on the use of N-free semi-solid media, containing low concentration of agar. NFb medium for *A. lipoferum*, *A. brasilense*, and later with some minor modifications, was useful to isolate *A. irakense* and *A. halopraeferans*. The recipes are described in Table 1.4.

A. irakense (Khammas et al. 1989) can be isolated in semi-solid NFb medium containing up to 0.3 % NaCl with pH adjusted to 7.0–8.5 and incubation at 33 °C. *A. doebereinerae* (Eckert et al. 2001) can be isolated in NFb semi-solid medium

Table 1.4 Five semi-solid media used for enrichment and cultivation of some *Azospirillum* species

Ingredient (per litre)	NFb	LGI	FAM	SM	M
D,L-malic acid	5 g	–	–	5 g	5 g
Cristal sugar	–	5 g	5 g	–	
K_2HPO_4	0.5 g	0.2 g	0.12 g	0.13 g	0.1 g
KH_2PO_4	–	0.6 g	0.03 g	0.17 g	0.4 g
$MgSO_4{\cdot}7H_2O$	0.2 g	0.2 g	0.2 g	0.25 g	0.2
NaCl	0.1 g	–	0.1 g	1.2 g	0.1 g
$CaCl_2{\cdot}2H_2O$	0.02 g	0.02 g	0.02 g	0.22 g	0.02
$Na_2MoO_4{\cdot}2H_2O$		0.002 g	0.002 g	–	
Na_2SO_4	–	–		2.4 g	
$NaHCO_3$	–	–		0.5 g	
Na_2CO_3	–	–		0.09 g	
K_2SO_4	–	–		0.17 g	
FeEDTA, solution 1.64 %	4 mL	4 mL	4 mL	4 mL	
$FeCl_3$	–	–	–	–	10 mg
KOH—control pH:	4.5 g	v		4.8 g	4.8 g
Bromothymol blue (0.5 % in 0.2 N KOH)—pH indicator	2 mL	5 mL		–	
Microelements solution	2 mL	–	AD	AC	AE
$CuCl_2{\cdot}2H_2O$	–		–	0.002 mg	–
Vitamin solution	[a]1 mL	[a]1 mL	[a]1 mL	[b]10 mL	
Biotin[a]	–	–	–	0.1 mg	2 µg
Yeast extract	–	–	–	–	0.1 g
Final pH	6.8	6.0–6.2	6.0	8.5	6.8
Agar—semi-solid	1.75 g	1.75 g	1.75 g	1.75 g	N.D.
Agar solid media + yeast extract	17 g	15 g	15 g	15 g	15 g
Solution prepared separated from the medium recipe					
Microelements (sol.—1,000 mL)			AD	AC	AE
$CuSO_4{\cdot}5H_2O$	0.04 g		0.08 mg	–	–
$ZnSO_4{\cdot}7H_2O$	0.12 g		0.24 mg		–
H_3BO_3	1.4 g		2.8 mg	0.15 mg	–
$Na_2MoO_4{\cdot}2H_2O$	1.0 g		2 mg	2 mg	2 mg
$MnSO_4{\cdot}H_2O$	1.175 g		2.35 mg	0.2 mg	–
Vitamin (solution 100 mL)[a]					
Biotin[a]	10 mg				
Pyridoxal-HCl[a]	20 mg			–	
Vitamin solution (sol. 1,000 mL)[b]					
D-biotin[b]	–	–	–	40 mg	–
Calcium pantothenate[b]	–	–	–	40 mg	–
Myo-inositol[b]	–	–	–	200 mg	–
Niacinamide[b]	–	–	–	40 mg	–
p-Aminobenzoic acid[b]	–	–	–	20 mg	–

(continued)

Table 1.4 (continued)

Ingredient (per litre)	NFb	LGI	FAM	SM	M
Pyridoxine hydrochloride[b]	–	–	–	40 mg	–
Riboflavin[b]	–	–	–	20 mg	–
Thiamine hydrochloride[b]	–	–	–	4 mg	–
Incubation at (°C)	30	30	30	41	30

v—adjust the pH with a KOH solution 10 %
N.D. = Not described (Xie and Yokota 2005)
LGI and FAM medium (Magalhães et al. 1983)
[a]Vitamin solution described by Tarrand et al. (1978) for NFb medium
[b]Vitamin solution described by Reinhold et al. (1987)

after incubation for 3–5 days at 30 °C. Further purification is done on NFb (supplemented with yeast extract 50 mg L^{-1}). *A. halopraeferans* (Reinhold et al. 1987) cells grown in SM medium can reach 1.2 μm length and 0.7–1.4 μm thick if the pH turns alkaline. Another medium was also used, containing malic acid and 1.2 g of NaCl and biotin as a sole source of vitamins.

A. oryzae can be isolated using the M medium (Table 1.4). The other medium used was NFG medium composed of the following (g L^{-1}): glucose, 10.0; $CaCl_2 \cdot 2H_2O$, 0.020; $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 1.0; $CaCO_3$, 5.0; $FeSO_4 \cdot 7H_2O$, 0.050; $Na_2MoO_4 \cdot 2H_2O$, 0.001. The medium is completed to 1,000 mL with distilled water and the pH adjusted to 7.3. *A. zeae* and *A. canadense* can be isolated on M medium by omitting biotin and adjusting the pH to 7.2–7.4 (Xie and Yokota 2005). *A. melinis* (Peng et al. 2006) is isolated using semi-solid LGI medium or semi-solid NFB medium after incubation at 28 °C for 3–5 days. Purification can be done by repeatedly streaking the isolates on plates of solid LGI or NFb medium.

A. palatum (Zhou et al. 2009) did not use the minimal medium, and instead to obtain new species, TYB medium was used, containing 0.3 % yeast extract, 0.2 % beef extract, 0.6 % tryptone, 0.3 % NaCl and 0.001 % $FeCl_3$, pH 7.0.

Azospirilla do not harbour powerful oxygen-protective mechanisms for the oxygen-sensitive N-fixing system and are unable to grow on nitrogen as the sole nitrogen source in N-free agar plates or liquid media. Microaerobic diazotrophs are aerotactic and as a result the N-fixing population collects in zones of reduced oxygen concentration, where they form a thin pellicle or veil that moves upwards as it becomes thicker. After characteristic pellicles have formed, N-fixation can be checked by acetylene reduction activity, and active cultures are transferred to new vials containing the same medium. As soon as a new pellicle is visible, the cultures are streaked out on agar plates containing the same medium with yeast extract (20 mg L^{-1}) added. The small amount of yeast extract permits the growth of small colonies on the surface of plates. Characteristic individual colonies are then transferred again to N-free semi-solid media, and those that grow well are streaked out on potato agar for final purification.

Other N-free semi-solid media used for isolation of diazotrophs from plant samples are listed in Table 1.1 and described in detail below.

JNFb medium (*Herbaspirillum* spp.). Developed by J. Döbereiner from the NFb medium. Composition (in g L^{-1}): malic acid, 5.0; K_2HPO_4, 0.6; KH_2PO_4, 1.8; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.02; micronutrient solution (above), 2 mL; bromothymol blue (0.5 % in 0.2 N KOH), 2 mL; FeEDTA (1.64 %), 4 mL; vitamin solution (above), 1 mL; KOH, 4.5. Complete to 1,000 mL with distilled water. Adjust pH to 5.8 with KOH. For semi-solid and solid medium, add 1.8 and 17 g agar L^{-1}, respectively.

LGI-P medium (*G. diazotrophicus*). P stands for Pernambuco, the first location that *G. diazotrophicus* was isolated (Cavalcante and Dobereiner 1988). Particularly when used to isolate from plant tissue, 5 mL L^{-1} of sugarcane juice is necessary (medium called LGI-Pc described by Reis et al. 1994). Composition (in g L^{-1}): crystal sugar, 100; K_2HPO_4, 0.2; KH_2PO_4, 0.6; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; $Na_2MoO_4 \cdot 2H_2O$, 0.002; bromothymol blue (0.5 % in 0.2 N KOH), 5 mL; $FeCl_3 \cdot 6H_2O$, 0.01. Complete volume to 1,000 mL with distilled water. Adjust pH to 5.5–5.7 using acetic acid. Add 1.8 and 17 g agar L^{-1} for semi-solid and solid medium, respectively. LGI final pH 4.5 and cycloheximide (150 mg L^{-1}) can be used to isolate other diazotrophs, such as *Gluconacetobacter* (Jimenez-Salgado et al. 1997).

JMV medium (*Burkholderia* spp.). Developed by Baldani (1996) from the NFb medium. Composition (in g L^{-1}): mannitol, 5.0; K_2HPO_4, 0.6; KH_2PO_4, 1.8; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.2; micronutrient solution (above), 2 mL; bromothymol blue (0.5 % in 0.2 N KOH), 2 mL; FeEDTA (1.64 %), 4 mL; vitamin solution, 1 mL. Complete to 1,000 mL with distilled water. Adjust pH to 4.5 or 5.5 with KOH. For semi-solid and solid medium, add 1.8 and 25 g agar L^{-1}, respectively.

BAz medium and BAc agar plates as described previously (Estrada-de-los-Santos et al. 2001). BAz medium had the following composition (in g L^{-1}): azelaic acid, 2.0; K_2HPO_4, 0.4; KH_2PO_4, 0.4; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; $Na_2MoO_4 \cdot H_2O$, 0.002; $FeCl_3$, 0.01; bromothymol blue, 0.075; and agar, 2.3. The medium was adjusted with KOH to pH 5.7. *Media*. BAc medium (0.2 % azelaic acid, 0.02 % L-citrulline, 0.04 % K_2HPO_4, 0.04 % KH_2PO_4, and 0.02 % $MgSO_4 \cdot 7H_2O$) was also used for isolation and culture of *Burkholderia* species.

1.2.3.1 Rich Media

Potato medium (purification of *Azospirillum* spp. and *Herbaspirillum* spp.) contains 200 g of potato, 2.5 g of malic acid, 2.5 g of sucrose, 2 mL of micronutrient solution (above) and 1 mL of vitamin solution (above). The potato (200 g) is peeled, washed and boiled for 30 min, and then filtered using a large funnel and cotton wool. Malic acid and white sugar are mixed in 25 mL distilled water, and the pH is adjusted to 6.5–7.0 using KOH. This solution along with the vitamin solution (NFb medium) and micronutrient solution is added to the potato sample and completed to 1,000 mL by adding distilled water. *Potato-P medium* (*Purification of G. diazotrophicus*) is prepared the same way described, but 100 g white sugar is substituted for the malic acid.

For rapid multiplication, *Azospirillum* spp. can be grown in liquid media to which a combined nitrogen source has been added (NH_4Cl [1 g L^{-1}] or yeast extract [0.4 g L^{-1}]). Alternatively, complex media such as Nutrient Broth (NB) and DYGS medium (D,L-malate [1 g L^{-1}], yeast extract [2 g L^{-1}], glucose [1 g L^{-1}], glutamate [1.5 g L^{-1}], peptone [1.5 g L^{-1}], $MgSO_4 \cdot 7H_2O$ [0.5 g L^{-1}]) described by Rodrigues Neto et al. (1986) can be applied. In such media, with rapid stirring or shaking, cell concentrations of 10^8 per mL are reached after 24–48 h. To stabilize the pH at the desired value upon prolonged growth, the addition of 50 mM MOPS (3-[*N*-morpholino] propanesulfonic acid) buffer (pH 6.8) or MES (2-[*N*-morpholino] ethane-sulfonic acid) buffer (pH 6.0) is recommended.

Alternatively, the *Azospirillum* minimal medium of Okon et al. (1977), which also contains high phosphate levels, can be used. This medium was also modified by the micronutrient elements and limited amount of NH_4Cl to aid in initiating aerobic growth. The medium contains the following (per litre of distilled water) and prepared with two steps: (1) In 100 mL add 6.0 g of K_2HPO_4 and 4.0 g of KH_2PO_4, autoclaved separately from the other medium constituents and the phosphate solution is later. (2) In the rest of the solution (900 mL) add: $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; DL-malic acid, 5.0 g; NaOH, 3.0 g; $FeCl_3$, 10.0 mg; $NaMoO_4 \cdot 2H_2O$, 2.0 mg; $MnSO_4$, 2.1 mg; H_3BO_3, 2.8 mg; $CuSO_4 \cdot 5H_2O$, 0.04 mg and $ZnSO_4 \cdot 7H_2O$, 0.24 mg. The final pH is adjusted to 6.8. For semi-solid medium add 0.5 g of agar per litrE dissolved by boiling before autoclaving (900 mL) and after autoclave add the buffer. For liquid medium the authors recommended the addition of NH_4Cl (1.0 g) and yeast extract (0.1 g) with the objective to shorten the lag in growth. For solid medium add 2 % of agar. It is necessary to autoclave the buffer in a separate solution as the medium precipitated during autoclaving. Using fructose you can reduce the strength of phosphate buffer. But the autoclave in separate was useful (Okon, personal communication).

1.3 Physiological Tests

1.3.1 Carbon Source Utilization

The ability of the isolates to use different carbon sources for growth dependent on nitrogen fixation is one analysis necessary to describe new species, identify better medium for growth and nitrogenase activity, or even bacterial storage. Several commercial kits are used, such as API™ (bioMérieux), Biolog™ (Table 1.5) or medium prepared by changing the carbon source. In this case, the main carbon source is omitted and the carbon sources of interest are tested. Initially, the isolates are grown in rich media that can be prepared in the laboratory, as for example, DYGS media (Rodrigues Neto et al. 1986) and SYP liquid medium (Caballero-Melado and Martinez-Romero 1994), or sold by companies, such as Nutrient Broth. A smaller amount of bacterial cells (10–20 µL) is inoculated into the semi-solid medium

Table 1.5 *Azospirillum* species and their pattern of carbon sources using Biolog or API systems

Azospirillum	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A. lipoferum	+	+	−	+	v	+	−	v	+	+	v	−	−	+	v	−	+	+	−	−
A. brasilense	−	v	−	+	−	v	−	+	v	+	−	−	−	−	−	−	−	−	−	−
A. amazonense	v	+	+	+	+	+	+	−	+	−	+	v	+	−	+	v	+	−	+	+
A. halopraeferens	nd	v	nd	+	nd	−	nd	nd	−	+	nd	nd	nd	+	+	nd	+	−	−	nd
A. irakense	+	+	+	v	+	+	+	−	+	−	−	+	+	−	+	+	v	−	+	+
A. largimobile	+	+	−	+	−	+	+	−	+	+	−	−	−	+	−	−	+	+	nd	−
A. dobereinerae	−	nd	−	+	nd	v	−	v	v	+	−	−	−	+	−	−	−	+	−	−
A. oryzae	nd	+	−	+	nd	+	nd	nd	+	nd	−	−	−	−	nd	+	+	−	−	nd
A. melinis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	nd	+	+	+
A. canadense	−	−	−	−	−	−	−	nd	−	−	−	−	−	−	−	−	nd	−	−	−
A. zeae	v	+	−	+	+	+	−	nd	v	+	−	−	−	+	−	−	nd	+	−	−
A. rugosum	−	−	nd	+	nd	nd	nd	+	+	nd	nd	−	−	−	nd	nd	nd	nd	nd	nd
A. palatum	v	v	+	+	nd	nd	nd	nd	+	−	nd	nd	+	+	nd	nd	nd	−	+	+
A. picis	+	+	−	nd	−	+	−	+	+	nd	nd	nd	−	+	−	−	+	+	−	−
A. thiophilum	nd	+	nd	+	nd	+	nd	nd	+	+	nd	−	nd	+	−	−	nd	+	−	−
A. humicireducens	+	+	nd	nd	+	nd	nd	nd	nd	nd	+	nd	+	+	W	+	+	+	+	nd
A. formosense	nd	+	nd	+	nd	+	nd	nd	+	+	nd	−	−	−	nd	−	nd	−	nd	nd
A. fermentarium	nd	nd	nd	nd	+	+	nd	nd	nd	nd	+	nd	+	nd	nd	+	nd	+	+	nd

Data from Eckert et al. (2001), Sly and Stackebrandt (1999), Ben Dekhil et al. (1997), Khammas et al. (1989), Reinhold et al. (1987), Xie and Yokota (2005), Peng et al. (2006), Mehnaz et al. (2007a, b), and Lavrinenko et al. (2010)

Carbon sources: (1) *N*-acetylglucosamine, (2) L-arabinose, (3) D-cellobiose, (4) D-fructose, (5) L-fucose, (6) D-galactose, (7) Gentiobiose, (8) D-gluconate, (9) D-glucose, (10) Glycerol, (11) Myo-inositol, (12) Lactose, (13) Maltose, (14) D-mannitol, (15) D-mannose, (16) L-rhamnose, (17) D-ribose, (18) D-sorbitol, (19) Sucrose, (20) D-trehalose

Symbols: +, positive; −, negative; v, variable or inconsistent; nd, not determined

(5 mL medium containing the carbon source in a defined concentration). The culture flasks are incubated in a dark room at 30 °C for up to 10 days until formation on the surface of the medium of the pellicle characteristic of the tested diazotroph, indicating that the sugar was used as carbon source.

Bacteria of this genus also use several carbon sources, such as sugar, amino acids and sugar alcohols (Table 1.5), and the pattern of carbon utilization has been used for discriminatory purpose between species of the genus. The versatile carbon and nitrogen metabolism in this genus make it well adapted to the rhizosphere and soil samples, and is also important as a plant growth promoter bacteria, particularly *A. brasilense* and *A. lipoferum* strains.

The genus *Azospirillum* is widely known to contain N-fixing plant growth-promoting rhizobacteria, and the carbon source and N-metabolism within the genus makes it well adapted to several soil conditions and competent to colonize the rhizosphere and in some cases the inner plant tissues, as described above. Representatives of the genus *Azospirillum* are known to use tricarboxylic acid as the sole carbon source, but there is variability among the species in the use of sugars and sugar alcohols (Table 1.5) and these carbon sources can be used in the discrimination of species. Other sugars, such as myo-inositol, lactose and L-rhamnose, are used for only three species described so far, while *A. canadense* is the only species that does not use any of the carbon sources tested and presented in Table 1.5. This species used malic acid, potassium gluconate, acetic acid and pyruvic acid, among others, as a sole carbon source.

1.3.2 Measurement of N-Fixing Using Acetylene Reduction Activity

Nitrogenase activity is estimated using the acetylene reduction assay (ARA) (Boddey 1987) as an indirect method of nitrogenase, as acetylene is a competitive inhibitor of the enzyme activity. Based on this assumption, small vials (10–13 mL) containing 5 mL of modified N-free semi-solid media without yeast extract, vitamins and indicator of pH changes, for example bromothymol blue (necessary for Lowry protein measurements), are inoculated with 20 μL of adjusted cell suspension. After growth at 30 °C for the period necessary to grow the pellicle (such as 48 h for *Azospirillum*), the vials are sealed with a rubber stopper (SubaSeal®) and 1 mL of acetylene gas is injected to a final concentration of 10 % (v/v) of the flask volume. Samples are removed after a 1 h incubation period and assayed for ethylene using a gas chromatograph, equipped with a flame ionization detector. Ethylene concentration is determined using a standard calibration curve and a digital integrator.

For the quantification of protein in cultures grown on semi-solid medium, a 1 M NaOH solution is used for cell lysis. Total cell extracts are diluted twice and used for Lowry reaction. The cell sediments of these cultures are used for protein quantification by the Lowry method (Lowry et al. 1951). The cultures (10 mL) are centrifuged (5,000 × *g*, 15 min), re-suspended in an equal volume of sterilized water and

diluted (100 µL cell suspension to 400 µL water). This cell suspension (500 µL) is mixed with NaOH 1 M (500 µL) and boiled (100 °C, 5 min) to disrupt the membranes and release the total cellular contents. Next, 2.5 mL of fresh solution (100:1:1) of Na_2CO_3 (5 %):$CuSO_4$ (1 %):$KNaC_4H_4O_6{\cdot}4H_2O$ (2 %) is added. After 10 min of reaction, 500 µL Folin-Ciocalteu reagent (1 M) is added. After 30 min of incubation in the dark, the protein content of each sample is measured at 750 nm using a spectrophotometer (PE Lambda Bio, Model 11). A standard curve of serum albumin is used for calibration of the protein method and protein quantification.

By dividing the amount of acetylene reduced by the amount of protein in one vial, the specific activity is obtained. Using this procedure, it is possible to compare strains and species in a quantitative way. If the protein measurement is not performed, the ARA activity can be considered a qualitative assessment (positive or negative). In some strains/species, it is not possible to perform the acetylene reduction activity, as the growth on the semi-solid medium is not characteristic (pellicle formation).

It is also possible to measure acetylene reduction activity in plates (Wilcockson and Werner 1978; Pan and Vessey 2001) or liquid medium (Reis and Döbereiner 1998), but normally the activity is very low. Wilcockson and Werner (1978) used 2–6 h of incubation to measure ARA activity of *Bradyrhizobium japonicum*. Pan and Vessey (2001) used a modified atmosphere to measure nitrogenase activity in plates. They utilized the same method to measure nitrogenase activity and respiration rate in *G. diazotrophicus*; colonies grown 5–6 days on solid LGI-P medium were transferred to a chamber, in which various atmospheric pO_2 were applied. Gas mixtures fed into an assay chamber were composed of various partial pressures of O_2 in N_2 (N_2-O_2) or in Ar (Ar-O_2). Respiration rate was measured as the rate of CO_2 evolved from the colonies with N_2-O_2 as the input gas. Nitrogenase activity was measured as H_2 evolution in N_2-O_2 and in Ar-O_2

Reis and Döbereiner (1998) measured nitrogenase activity in liquid cultures of *G. diazotrophicus* by acetylene reduction in closed batch assays and found that activity was maximal when the culture was at equilibrium with 0.2 kPa of O_2 in the gas phase.

1.3.3 Other Physiological Tests

Bacteria belonging to the genus *Azospirillum* are Gram-negative, motile and generally vibroid in shape and contain poly-β-hydroxybutyrate (PHB) granules. Three species were classified as facultative anaerobic: *A. melinis*, *A. thiophilum* and *A. humicireducens*. Almost all strains were classified with one single polar flagellum and only *A. palatum* was not described as a N-fixing species (Table 1.6). Based on several new species obtained from soil and other unusual environments, it could be affirmed that this genus is widespread in nature. Azospirilla have a typically aerobic metabolism, with O_2 as the terminal electron acceptor. In *A. brasilense* and *A. lipoferum*, NO_3 can replace O_2 in respiration and under alkaline conditions N_2O

Table 1.6 Physiological differences among species of the genus *Azospirillum*

Azospirillum species	Cell size	PC	Biotin use	Optimal *T* (°C)	pH range	NaCl 3 %	Denitr	Nitrate reduction	Indole production
A. lipoferum	1.0×1.7	+	+	37	5.7–6.8	–	+	+	+
A. brasilense	1.0×1.2	–	–	37	6.0–8.0	v	+	+	+
A. amazonense	0.9×1.0	+	–	35	nd	–	–	v	+
A. halopraeferens	0.7×2.4	+	+	41	6.8–8.0	+	+	+	+
A. irakense	nd	+	–	30–33	5.5–8.5	+	+	v	+
A. largimobile	0.7×3.0	+	–	28	nd	–	–	+	+
A. dobereinerae	1.0×2.0	+	–	30	6.0–7.0	–	+	+	+
A. oryzae	1.0×1.5	nd	+	30	6.0–7.0	–	–	+	nd
A. melinis	0.7×1.0	nd	–	20–33	4.0–8.0	+	nd	+	
A. canadense	0.9×1.8	nd	–	25–30	5.0–7.0	–	nd	+	–
A. zeae	0.9×1.9	nd	–	30	5.0–7.0	–	+	+	–
A. rugosum	nd	+	nd	22–37	nd	–	nd	+	–
A. palatum	0.6×2.0	nd	nd	30–47	6.0–8.0	nd	nd	–	–
A. picis	nd	nd	–	37	6.5–9.0	–	nd	+	–
A. thiophilum	nd	+	–	37	6.5–8.5	+	–		
A. humicireducens	2.5×1.2	nd	+	30	5.5–8.5	–	–	–	–
A. formosense	2.5×0.8	–	–	30	5.0–9.0	–	nd	+	nd
A. fermentarium	2.4×1.0	nd	–	30	6.0–8.0	+	nd	+	nd

(1) Size—minor number in long and wide (μm); (2) PC=pleomorphic cells; (3) biotin requirement; (4) optimal *T*=optimal temperature of growth; (5) pH range; (6) growth with 3 % NaCl; (7) denitrification; (8) nitrate reduction; (9) indole production. nd not determined

and N_2 are formed (Stephan et al. 1984). Under these conditions, anaerobic growth and N_2 fixation have been shown to occur (Nelson and Knowles 1978; Scott et al. 1979). Weak fermentative ability has been observed with *A. lipoferum* grown on glucose or fructose (Tarrand et al. 1978).

1.3.4 Preservation

Preservation of the cultures for many years at −80 °C or in liquid N_2 is also possible after adding 50 % glycerol or dimethylsulfoxide (DMSO) to an exponentially growing culture. The cells can also be preserved by lyophilization according to the protocol described by Döbereiner (1995). The cultures are grown to late log-phase in the following medium: K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; DL-malate (for *A. brasilense*) or glucose (for *A. lipoferum*), 5 g; yeast extract, 0.4 g; and 1 L of distilled water. The cells must then be collected by centrifugation and re-suspended to a dense cell suspension with 10 % sucrose solution containing 5 % peptone. Then, 0.1 mL portion is transferred into lyophilization ampoules, which are frozen and lyophilized according to the procedures recommended for *Rhizobium* spp. (Vincent 1970). Another variation is to grow the bacteria in rich medium, collect the cells by scraping and mixing with glycerol 10 % and transfer to cryotubes.

The simplest and cheapest way to preserve bacteria is in their own isolation medium or potato agar. Tubes may contain slopes of medium, which facilitates inoculation with a loop and also enhances the area for bacterial growth, although flat medium can be used. After inoculation of the organism and incubation for 2–3 days, it is possible to either seal the flask with rubber cap or add 2–3 mL sterile mineral oil, glycerol or liquid glycerine before sealing with a cotton wool or rubber cap. Several tubes should be prepared for each bacterium, as once a tube is opened, the bacteria will usually die. This happens more frequently when cultures are stored without any protection over the medium surface and the tube is not completely sealed. *A. amazonense* should be stored on medium with sucrose as a carbon source otherwise the pH will rise over 6.5 when the substrate is an organic acid and it will induce death of the bacteria within a few days. *A. lipoferum* is relatively sensitive to alkaline pH, and *G. diazotrophicus* also does not survive a long time in potato agar, storage is performed in SYP medium.

After growing the microorganism 48–72 h at 30 °C in tubes containing inclined specific medium, or a variation of the carbon source related to the species to be lyophilized (*A. brasilense* grows using malic acid; *A. lipoferum* and *Herbaspirillum* spp., glucose; *A. amazonense*, sucrose; *G. diazotrophicus*, medium SYP), SYP medium described by Caballero-Melado and Martinez-Romero (1994) can be used (quantities per L): sucrose (10 g), yeast extract (3 g), K_2HPO_4 (1 g), KH_2PO_4 (3 g), with bromothymol blue as an indicator, pH 6–6.25. Cultures are re-suspended in 3 mL of lyophilization solution. Aliquots of 0.2 mL are distributed into lyophilization ampoules that are dehydrated as described for *Rhizobium* species (Vincent 1970). Lyophilization solution is prepared with 10.0 g of sucrose plus 5.0 g of peptone and completed to 100 mL with distilled water.

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Chapter 2
Molecular Tools for Identification and Characterization of Plant Growth Promoting Rhizobacteria with Emphasis in *Azospirillum* spp.

Chiu-Chung Young, Shih-Yao Lin, Fo-Ting Shen, and Wei-An Lai

Abstract *Azospirillum* is considered an important genus among plant growth promoting rhizobacteria (PGPR). After the recent reclassification of *Azospirillum irakense* to *Niveispirillum irakense* and *Azospirillum amazonense* to *Nitrospirillum amazonense* based on their polyphasic taxonomic characteristics, at present this genus encompasses 15 valid species. In this chapter, the identification and characterization of the genus *Azospirillum* through genotypic, phenotypic or chemotaxonomic approaches were reviewed. Under the given set of PCR condition, the genus specific primers Azo494-F/Azo756-R were sufficient to differentiate *Azospirillum* and other closely related genera such as *Rhodocista* and *Skermanella*. Along with PCR—denaturing gradient gel electrophoresis (PCR-DGGE) or real-time quantitative PCR (qPCR), the specific primers were useful to detect and identify *Azospirillum* in a short time no matter pure cultures or environmental samples were used. The minimum detection limit in real-time quantitative PCR analysis is 10^2 CFU g^{-1} in the seeded soil sample. Cells of the genus *Azospirillum* are Gram-stained negative, spiral or rod-shaped and non-spore-forming diazotrophic. Poly-β-hydroxybutyrate granules were observed after few days of incubation. The major fatty acids were $C_{16:0}$, $C_{16:0}$ 3-OH, $C_{18:1}$ 2-OH, $C_{14:0}$ 3-OH/$C_{16:1}$ iso I, $C_{16:1}$ ω7c/$C_{16:1}$ ω6c and $C_{18:1}$ ω7c/$C_{18:1}$ ω6c; the predominant polar lipids included phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidyldimethylethanolamine (PDE) and unidentified aminolipid (AL) and phospholipids (PL); the common major respiratory quinone was

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ubiquinone Q-10 and predominant polyamines were *sym*-homospermidine and putrescine. These features are also useful to provide bases in the description of members belonging to the genus *Azospirillum*.

2.1 Introduction

The genus *Azospirillum* which comprised two species namely *Azospirillum lipoferum* and *Azospirillum brasilense* was first described from the reclassification of *Spirillum lipoferum*, the root-associated nitrogen fixer appearing as vibrioid cells with single polar flagellum (Tarrand et al. 1978). The members of nitrogen-fixing genus *Azospirillum* are distributed mainly from soils and frequently associated with grasses, cereals and crops (Döbereiner and Day 1976; Ladha et al. 1987; Kirchhof et al. 1997). Besides, some species are found from other environmental resources. Currently, the validly described species belonging to the genus *Azospirillum* were recorded in LPSN website (Euzeby's nomenclature list, http://www.bacterio.net/index.html) and are demonstrated in Fig. 2.1. Among them, *Azospirillum irakense* and *Azospirillum amazonense* were reclassified as *Niveispirillum irakense* and *Nitrospirillum amazonense* recently by Lin et al. (2014) based on phylogenetic, physiological, chemotaxonomic and phenotypic characteristics. The occurrence of *Azospirillum* species, their isolation source and etymology information are listed in Table 2.1.

The versatile genus *Azospirillum*, which is widely known as free-living nitrogen-fixing plant growth promoting rhizobacteria (PGPR) (Okon and Vanderleyden 1997; Okon and Itzigsohn 1992), belongs to the Gram-stained negative *α-proteobacteria*. Among the free-living nitrogen-fixing PGPR, *Azospirillum* strains have been well recognized as biofertilizers owing to their plant growth promoting activities such as nitrogen fixation, phosphate solubilization and production of the phytohormones indole 3-acetic acid (IAA) (Bashan et al. 2004), cytokinin (Hartmann and Baldani 2003; Steenhoudt and Vanderleyden 2000), abscisic acid (ABA), ethylene, gibberellic acid (GA3) and zeatin (Tien et al. 1979); plant growth regulatory substances such as polyamines (Thuler et al. 2003), osmotic stress response in plants (Aziz et al. 1997), phosphate solubilization (Seshadri et al. 2000) and siderophore production (Saxena et al. 1986) make *Azospirillum* species an attractive biofertilizer and is commonly used in field tests. Besides, the versatile C- and N-metabolism in this genus makes it well adapted to the rhizospheric environment and show higher competitiveness (Steenhoudt and Vanderleyden 2000).

According to the polyphasic features within *Azospirillum*, cells are Gram-stained negative, spiral or rod-shaped and non-spore-forming diazotrophic. Poly-β-hydroxybutyrate (PHB) granules were observed after few days of incubation. The major fatty acids were $C_{16:0}$, $C_{16:0}$ 3-OH, $C_{18:1}$ 2-OH, $C_{14:0}$ 3-OH/$C_{16:1}$ iso I, $C_{16:1}$ ω7c/$C_{16:1}$ ω6c and $C_{18:1}$ ω7c/$C_{18:1}$ ω6c (Table 2.2). The predominant polar lipids included phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidyldimethylethanolamine (PDE)

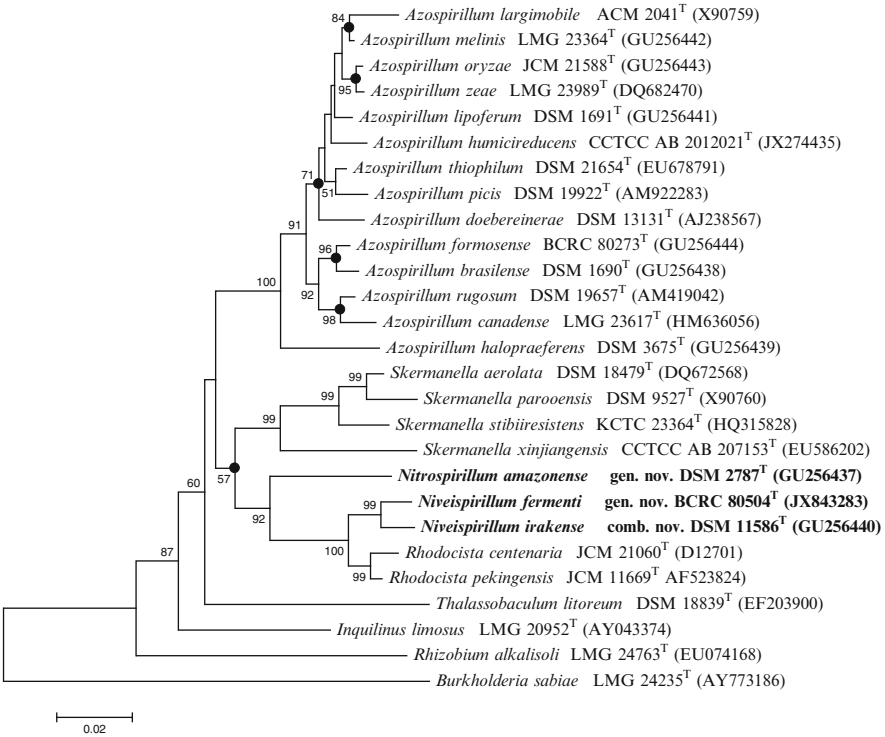

Fig. 2.1 Phylogenetic analysis of *Azospirillum* spp. and other representatives of the family *Rhodospirillaceae* based on 16S rRNA gene sequences (Lin et al. 2014). Distances and clustering were performed by using Neighbor-Joining method with the software package MEGA 6. Filled circles indicate that the corresponding nodes were also recovered in the tree constructed based on Maximum Likelihood and Maximum Parsimony algorithm. Bootstrap values (>50 %) based on 1,000 replications are shown at the branching points. *Rhizobium alkalisoli* LMG 24763T and *Burkholderia sabiae* LMG 24235T were used as outgroups. Bar, 0.02 substitutions per nucleotide position

and unidentified aminolipid (AL) and phospholipids (PL) (Table 2.3). The common major respiratory quinone was ubiquinone Q-10 and predominant polyamines were *sym*-homospermidine and putrescine.

On the other hand, the introduction of rRNA-targeted oligonucleotide probes is a milestone for microbial ecological studies (Stahl et al. 1988). Molecular markers such as 16S, 23S rRNA sequences or intergenic sequences are useful for distinguishing microorganisms among various genera, species and even to differentiate among strains (Woese et al. 1990; Shen and Young 2005). By using the PCR-based techniques the detection, identification and quantification of microorganisms can be performed and complemented with culture-dependent and biochemical methods. The development of *Azospirillum* species-specific probes labelled with fluorescence has been worked out by Stoffels et al. (2001) and used in fluorescence in situ

Table 2.1 Occurrence of *Azospirillum* spp.

Isolation source	Etymology	References
Azospirillum amazonense (roots of *Digitaria decumbens*)	N.L. neut. adj. *amazonense*, pertaining to the Amazon region of Brazil, South America	Magalhães et al. (1983), Falk et al. (1985, 1986)
Azospirillum brasilense (roots of *Digitaria decumbens*)	N.L. neut. adj. *brasilense*, pertaining to the country of Brazil, South America	Tarrand et al. (1978), Falk et al. (1985, 1986)
Azospirillum canadense (corn rhizosphere, Canada)	N.L. neut. adj. *canadense*, pertaining to Canada, the region of isolation, referring to its isolation from Canadian soil	Mehnaz et al. (2007a)
Azospirillum doebereinerae (plant Miscanthus, Germany)	N.L. gen. fem. n. *doebereinerae*, of Döbereiner, in honour of Johanna Döbereiner, who isolated and characterized many *Azospirillum* spp., and other diazotrophic plant-associated bacteria	Eckert et al. (2001)
Azospirillum fermentarium (fermentative broth, Taiwan)	L. neut. adj. *fermentarium* pertaining to fermentation, reflecting the fact that the type strain was isolated from a fermentative tank	Lin et al. (2013)
Azospirillum formosense (agricultural soil, Taiwan)	N.L. neut. adj. *formosense*, pertaining to Formosa, Taiwan	Lin et al. (2012)
Azospirillum halopraeferens (roots of kallar grass, Pakistan)	Gr. n. *hals halos*, salt; L. v. *praefere*, to prefer; N.L. part. adj. *halopraeferens*, salt preferring	Reinhold et al. (1987)
Azospirillum humicireducens (humic substances, China)	N.L. adj. *humicus* (from L. n. *humus*, soil) humic; L. part. adj. *reducens* leading back, bringing back and in chemistry converting to a different oxidation state; N.L. part. adj. *humicireducens* converting humic substances to a reduced oxidation state	Zhou et al. (2013)
Azospirillum irakense (rhizosphere of rice, Iraq)	N.L. neut. adj. *irakense*, pertaining to the country of Iraq	Khammas et al. (1989)
Azospirillum largimobile (fresh lake water, Australia)	N.L. adv. *largo* (from Italian adv. *largo*; from L. adj. *largus*), in a very slow tempo (musical); L. adj. *mobilis -is -e*, movable, mobile; N.L. neut. adj. *largimobile*, moving in a very slow manner	Ben Dekhil et al. (1997)
Azospirillum lipoferum (root of wheat)	Gr. n. *lipos*, animal fat, lard, tallow; L. suff. *-ferus -a -um* (from L. v. *fero*, to carry), bringing, bearing; N.L. neut. adj. *lipoferum*, fat bearing	Tarrand et al. (1978)
Azospirillum melinis (molasses grass, China)	N.L. n. *melinis*, genus name of stinkgrass, *Melinis minutiflora* Beauv.; N.L. gen. n. *melinis*, from stinkgrass, referring to its frequent occurrence in association with molasses grass	Peng et al. (2006)

(continued)

Table 2.1 (continued)

Isolation source	Etymology	References
Azospirillum oryzae (roots of the rice, Japan)	L. gen. n. *oryzae*, of rice, from where the type strain was isolated	Xie and Yokota (2005)
Azospirillum picis (discarded tar, Taiwan)	L. gen. fem. n. *picis*, of pitch, tar	Lin et al. (2009)
Azospirillum rugosum (oil-contaminated soil, Taiwan)	L. neut. adj. *rugosum*, wrinkled, as the form of the colonies on the agar changes to a wrinkled appearance	Young et al. (2008)
Azospirillum thiophilum (sulfide spring, Russia)	Gr. n. *theion* (Latin transliteration *thium*), sulfur; N.L. neut. adj. *philum* (from Gr. masc. adj. *philon*), friend, loving; N.L. neut. adj. *thiophilum*, sulfur loving	Lavrinenko et al. (2010)
Azospirillum zeae (corn rhizosphere, Canada)	L. gen. n. *zeae*, of spelt, of *Zea mays*, referring to its isolation from rhizosphere soil of corn (*Zea mays*)	Mehnaz et al. (2007b)

Table 2.2 Fatty acid profiles of *Azospirillum* spp. and other closely related species

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12
Saturated												
$C_{10:0}$	–	–	–	–	–	–	–	–	–	–	1.2	3.5
$C_{12:0}$	–	3.6	–	–	–	tr	–	–	–	tr	4.2	–
$C_{14:0}$	–	1.4	–	tr	–	1.6	1.1	tr	–	1.9	1.2	2.4
$C_{15:0}$	–	–	–	1.4	–	–	–	–	–	–	–	–
$C_{16:0}$	4.1	13.6	4.4	4.3	5.2	7.7	5.7	6.2	6.2	7.3	2.1	10.0
$C_{18:0}$	–	–	–	tr	–	tr	–	tr	–	tr	–	1.5
Unsaturated												
$C_{16:1}$ ω5c	–	–	–	–	–	–	–	–	–	8.6	5.2	2.6
$C_{17:0}$ cyclo	–	–	–	–	–	–	–	–	–	1.5	tr	tr
$C_{17:1}$ ω6c	–	–	–	7.1	–	tr	–	1.5	–	–	–	–
$C_{17:1}$ ω8c	–	–	–	3.4	–	–	–	tr	–	–	–	–
$C_{18:1}$ ω5c	–	–	–	–	–	–	–	–	–	tr	1.3	–
$C_{19:0}$ cyclo ω8c	–	5.8	–	1.6		6.8	–	7.4	–	14.8	6.2	14.1
Hydroxy												
$C_{14:0}$ 2-OH	–	–	–	–		–				4.5	5.9	4.8
$C_{15:0}$ iso 3-OH	–	1.3	3.4	–	2.3	–	4.8	4.8	3.8	–	–	–
$C_{16:0}$ 2-OH	–	–	–	–		–				tr	–	1.5
$C_{16:0}$ 3-OH	3.7	4.2	4.2	4.3	7.6	9.6	6.6	5.4	4.7	7.3	11.6	15.5
$C_{18:1}$ 2-OH	5.9	1.4	6.9	5.5	9.4	1.0	7.9	1.3	7.0	6.5	11.6	8.5
Summed feature 2	4.8	5.5	5.8	5.9	10.6	10.9	8.3	4.9	5.6	1.0	10.4	2.1

(continued)

Table 2.2 (continued)

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12
Summed feature 3	13.1	12.5	15.1	6.5	14.4	6.6	11.4	21.5	16.4	4.8	2.6	1.3
Summed feature 5	–	–	–	–	–	–	2.7	–	–	–	1.1	–
Summed feature 8	60.2	46.7	56.4	53.4	44.5	51.83	45.3	42.3	53.4	36.4	31.3	27.6

Taxa: (1) *A. brasilense* BCRC 12270^T; (2) *A. fermentarium* BCRC 80505^T; (3) *A. formosense* BCRC 80273^T; (4) *A. lipoferum* BCRC 12213^T; (5) *A. oryzae* JCM 21588^T; (6) *A. picis* DSM 19922^T; (7) *A. rugosum* DSM 19657^T; (8) *A. thiophilum* DSM 21654^T; (9) *A. zeae* LMG 23989^T; (10) *Niveispirillum fermenti* BCRC 80504^T; (11) *Niveispirillum irakense* BCRC 15764^T; (12) *Nitrospirillum amazonense* BCRC 14279^T

tr <1 %, – not detected

Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2: $C_{14:0}$ 3-OH/$C_{16:1}$ iso I; Summed feature 3: $C_{16:1}$ ω7c/$C_{16:1}$ ω6c; Summed feature 5: $C_{18:0}$ ante/$C_{18:2}$ ω6,9c; Summed feature 8: $C_{18:1}$ ω7c/$C_{18:1}$ ω6c

Table 2.3 Comparison of polar lipid profiles of *Azospirillum* strains and other representatives of the family *Rhodospirillaceae* (Lin et al. 2014)

	Polar lipid										
Organism	PC	PE	PG	DPG	PME	PDE	APL	AL	PL	GL	L
Azospirillum brasilense BCRC 12270^T	+	+	+	+	–	+	–	+	+	–	–
Azospirillum picis DSM 19922^T	+	+	+	+	–	+	+	+	+	–	–
Azospirillum rugosum DSM 19657^T	+	+	+	+	–	+	–	+	–	–	–
Azospirillum formosense BCRC 80273^T	+	+	+	–	–	+	–	+	+	–	–
Azospirillum oryzae JCM 21588^T	+	+	+	+	–	+	–	+	+	–	–
Niveispirillum fermenti BCRC 80504^T	+	+	+	+	+	+	+	+	+	+	–
Niveispirillum irakense BCRC 15764^T	+	+	+	+	+	+	+	+	+	–	–
Nitrospirillum amazonense BCRC 14279^T	–	–	–	+	–	–	–	+	+	–	–
Rhodocista pekingensis JCM 11669^T	+	+	+	+	+	+	+	+	–	+	–
Rhodocista centenaria JCM 21060^T	+	+	+	+	+	+	+	+	–	+	–
Skermanella aerolata DSM 18479^T	+	+	+	+	–	–	+	+	–	–	+

Symbol: + positive, – negative

PC phosphatidylcholine, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *DPG* diphosphatidylglycerol, *PME* phosphatidylmethylethanolamine, *PDE* phosphatidyldimethylethanolamine, *AL* aminolipid, *GL* glycolipid, *APL* unidentified aminophospholipid, *PL* unidentified phospholipid, *L* unidentified lipid

hybridization (FISH) analysis. However, this method does only detect physiologically active bacteria and fails to detect all members belonging to the genus *Azospirillum* in the sample.

Recently, an *Azospirillum*-specific primer pair Azo494-F/Azo756-R was successful in differentiating the genus *Azospirillum* from other genera under the given set of PCR conditions (Lin et al. 2011). The primer set, which amplifies a 263-bp fragment of the 16S rRNA gene of *Azospirillum* species, can also be used to detect the presence of *Azospirillum* species in soil samples either in DGGE or in qPCR analysis. The qPCR can be used to detect *Azospirillum* species when the cell number is low in the soil samples, providing higher resolution in the detection of this specific genus from soil or aquatic environments. This genus-specific PCR-based technique will aid in the rapid detection and identification of members of the genus *Azospirillum* from pure cultures or soil samples, and can be used in the studies of the diversity of *Azospirillum* or exploration of novel species in the ecosystem.

2.2 Methodology

Approaches used in the study of *Azospirillum* spp. from pure cultures or environmental samples are illustrated in Fig. 2.2. The traditional approaches include bacterial enumeration, biomass or activity determination. In polyphasic approaches,

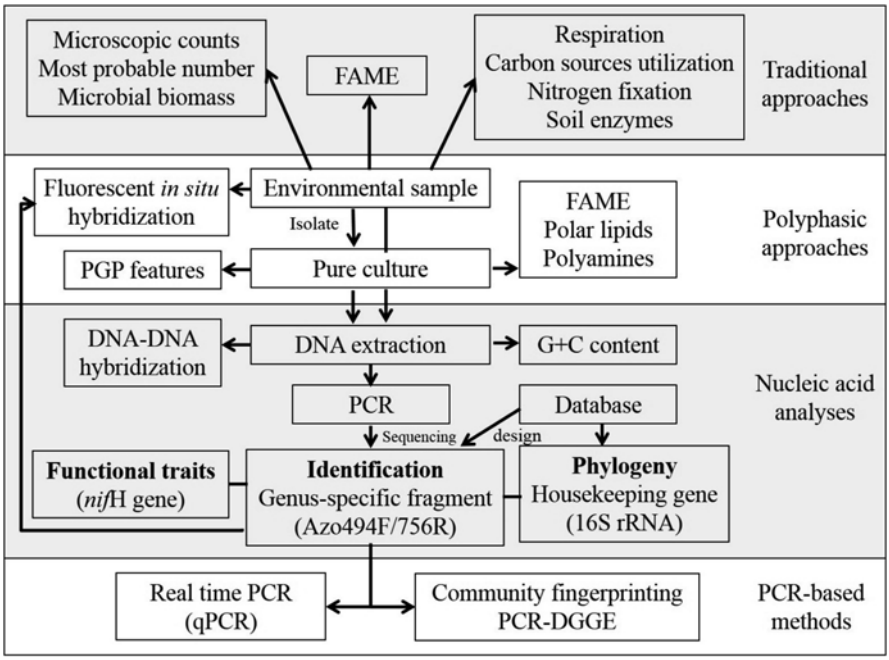

Fig. 2.2 Approaches used in the study of *Azospirillum* spp. from pure cultures or environmental samples

genotypic or phenotypic traits were demonstrated. The nucleic acid analyses part encompass technologies such as DNA extraction, DNA–DNA hybridization, G+C content determination and PCR amplification. Primers specific to genus, functional gene or housekeeping gene were useful in the identification, studies of functional traits or phylogeny of bacteria. Further, the genus-specific primers can be used along with PCR-DGGE or qPCR to resolve the community structure regarding *Azospirillum* in a variety of environments such as rhizosphere. The details of the methodologies mentioned in this chapter are given below.

2.2.1 Preparation of Genomic DNA from Pure Culture/Environmental Samples

Bacterial genomic DNA was isolated using UltraClean® Microbial Genomic DNA Isolation Kits (MO BIO, USA) according manufacturer's instructions. Briefly, the collected microorganisms were stabilized and homogeneously dispersed to lysis (detergent solution SDS and heat). Then, thought the protein precipitation, bind DNA to the silica filter membrane and wash with an ethanol-based solution. DNA was released and recovered with centrifugation. DNA from various soil samples (250 mg) was extracted using a bead beating method with the PowerSoil® DNA Isolation Kits (MO BIO, USA). It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications. It should be noted that when protocols fail, the phenol/chloroform prep will usually work on any remainder of the sample. Briefly, add one volume of phenol/chloroform/isoamyl alcohol (25:24:1) to the lysed sample (with 10 % SDS, lysozyme and proteinase K) and shake by hand thoroughly for approximately 20 s. Centrifuge at 4 °C for 1 h at 16,000×*g*. Transfer the upper aqueous phase to a fresh tube. Be sure not to carry over any phenol during pipetting. Then, proceed to ethanol precipitation, below: add 3 M NaOAc (1/10× volume of sample) and homogeneous mixing, add 100 % ethanol (2× volume of sample) or isopropanol (1× volume of sample) and keep at 4 °C for hours, the genomic DNA will be precipitated after mixing well. Seventy percent ethanol was used to wash and the DNA was finally resuspend in appropriate volume of Tris–EDTA buffer (20 mM Tris, 0.5 mM EDTA, pH 8.0). The quality of the extracted DNA was checked by agarose gel electrophoresis (0.8 % (w/v)) after staining with ethidium bromide (EtBr). DNA quantity was measured according to the absorbance of a sample at 260 nm in a spectrophotometer.

2.2.2 PCR Amplification and Sequencing of 16S rRNA Gene for Phylogenetic Analysis

An almost full length of 16S rRNA gene was amplified by using bacterial universal primers 1F (5′-GAG TTT GAT CAT GGC TCA GA-3′) and 9R (5′-AAG GAG GTG ATC CAA CCG CA-3′); the related primers 3 (5′-CCT ACG GGA GGC AGC

AG-3′), 5F (5′-AAA CTC AAA TGA ATT GAC GGG G-3′) and 4R (5′-TTA CCG CGG CTG CTG GCA C-3′) were used for sequencing reaction (Edwards et al. 1989). DNA extracted by the above methods was used as template for PCR amplification. The PCR reactions were performed in a final volume of 25 µL containing 0.2 mM each of the four dNTPs, 20 pmol of each primer, 50 ng of DNA and 1 unit of Taq DNA polymerase with appropriate reaction buffer. The PCR conditions were as follows: initial denaturation for 2 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C and 1.5 min at 72 °C, with a final extension of 7 min at 72 °C. The expected size of the product was about 1,500 bp. Gene sequencing was performed by using the Bigdye terminator kit (Heiner et al. 1998) and nucleotide sequence of PCR product was determined by a genetic analyser (ABI 3730, Applied Biosystems, CA, USA). The DNA fragments were assembled using the Vector NTI 9.0 software (IBI, USA). Subsequently, closely related 16S rRNA gene sequences were retrieved and aligned by using the CLUSTAL_X (1.83) program (Thompson et al. 1997). The phylogenetic analysis was performed using MEGA 6 software (Molecular Evolutionary Genetics Analysis, version 6.0) (Tamura et al. 2013) and the topology of the resultant trees was evaluated by Neighbor-Joining (Saitou and Nei 1987), Maximum Likelihood (Felsenstein 1981) and Maximum Parsimony (Fitch 1971) methods. The bootstrap values (Felsenstein 1985) were computed after 1,000 replications.

2.2.3 PCR Amplification and Sequencing of nifH Gene for Phylogenetic Analysis

Genes of nitrogenase reductase were amplified to confirm the existence of sym genes. The *nifH* gene was amplified by PCR using the primer set Zehrf (5′-TGY GAY CCN AAR GCN GA-3′)/Zehrr (5′-AND GCC ATC ATY TCN CC-3′) described by Zehr and McReynolds (1989). Another primer set PolF (5′-TGC GAY CCS AAR GCB GAC TC-3′)/PolR (5′-ATS GCC ATC ATY NTC RCC GGA-3′) was described previously by Poly et al. (2001). The PCR conditions were as follows: initial denaturation for 2 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, with a final extension of 7 min at 72 °C. The expected size of the product was about 360 bp. The amplified patterns were screened by electrophoresis in 1.0 % (w/v) agarose gel, stained with EtBr, visualized under UV radiation and photographed. PCR amplicons were sequenced and translated the nucleotide sequence to a protein sequence using translate tool (http://web.expasy.org/translate/, ExPASy). The phylogenetic analysis was performed using MEGA 6 software described in the previous section.

2.2.4 Design of Genus-Specific Primers for Azospirillum spp.

The development of *Azospirillum* species-specific probes labelled with fluorescence has been worked out by Stoffels et al. (2001) and used in FISH analysis. However, this method does only detect physiologically active bacteria and fails to detect all members belonging to the genus *Azospirillum* in the sample. A new primer pair defined Azo494-F/Azo756-R with sequences flanking to the conserved regions in *Azospirillum* 16S rRNA gene was designed in our previous study (Lin et al. 2011). By conducting PCR amplification the robustness of the primer set proved to rapidly identify members of this genus from either pure isolates or from soil samples.

The forward primer Azo494-F, 5′-GGC CYG WTY AGT CAG RAG TG-3′ (corresponding to 494–513 bp in *Azospirillum picis* IMMIB TAR-3^T) and the reverse primer Azo756-R, 5′-AAG TGC ATG CAC CCC RRC GTC TAG C-3′ (corresponding to 732–756 bp in *Azospirillum picis* IMMIB TAR-3^T) were used to amplify a fragment 263 bp in length (Lin et al. 2011). Cycling conditions for primers Azo494-F/Azo756-R were: initial denaturation for 2 min at 95 °C followed by 35 cycles of 1 min at 94 °C, 1.5 min at 68 °C and 0.5 min at 72 °C, with a final extension of 7 min at 72 °C. Amplification products were separated on 1 % agarose gels and stained with EtBr.

The *Azospirillum*-specific 16S rRNA gene fragment (263 bp) was successfully amplified for all the reference *Azospirillum* species with the designed primer pair. No amplification was noted for closely related species from other genera (*Skermanella* or *Rhodocista*). However, two strains assigned earlier as *A. amazonense* (DSM 2787^T) and *A. irakense* (DSM 11586^T) failed to produce an *Azospirillum-specific* fragment with this primer pair. Further, polyphasic taxonomic evidences of these two type strains indicated that these two strains belong to other genera rather than *Azospirillum*. Therefore, the names *Nitrospirillum amazonense* gen. nov. and *Niveispirillum irakense* comb. nov. were proposed by Lin et al. (2014).

2.2.5 Screening and Identification of the Genus Azospirillum from Pure Culture

In the screening of *Azospirillum* isolates, bacteria were isolated by using the standard tenfold dilution plating technique. After 3 days of aerobic incubation on nutrient agar, R2A or nitrogen-free agar plate (Reinhold et al. 1987) at 30 °C, the colony PCR was adopted. Briefly, colonies were picked and suspended in PBS buffer (pH 6.8). After treating with 200 μL lysozyme buffer (100 mM Tris, 50 mM EDTA, pH 8.0; lysozyme final concentration is 4 mg mL^{-1}) at 37 °C for 30 min, colony PCR was carried out by using 3 μL of bacterial suspensions as templates. Polymerase chain reaction was carried out using primer set Azo494-F/Azo756-R and followed the further described conditions.

2.2.6 *Detection of the Genus Azospirillum in Environmental Samples Using PCR in Combination with DGGE Analysis*

DNA from various soil samples (250 mg) was extracted using a bead beating method with the PowerSoil™ DNA Isolation Kits (MO BIO, USA) according to manufacturer's instructions. Briefly, soil sample was loaded into a tube containing a buffer that will help disperse the soil particles, begin to dissolve humic acids and protect nucleic acids from degradation. SDS and other disruption agents were added for complete cell lysis using vortex and/or 70 °C water bath. Non-DNA organic and inorganic materials (humic substances, cell debris and proteins) were precipitated to remove. Then, bind DNA to the silica filter membrane and wash with an ethanol-based solution. DNA was released and recovered with centrifugation. Genus-specific primer pair GC-Azo494-F/Azo756-R was used to downstream PCR reaction. The forward primer used in the PCR-DGGE is clamped with 40 mer GC at the 5′ end (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCG CGG GGG G-3′). PCR was conducted using an annealing temperature of 68 °C to improve the detection of this group from environmental samples and to minimize sample contaminant interference (Lin et al. 2011).

The PCR amplicons obtained from the above reactions were purified from agarose gel using the QIA quick gel extraction kit (Qiagen, Inc., Chatsworth, California, USA) and further used for DGGE analysis. The PCR-generated amplicons were separated on a DCode universal mutation detection system (Bio-Rad Laboratories Inc., USA); 10 % (w/v) polyacrylamide gel with a 40–60 % parallel denaturing gradient was prepared with a Hoefer SG100 gradient maker. Denaturant (100 %) contained 7 mol L^{-1} urea and 40 % (v/v) deionized formamide. Approximately 30 μL of PCR products of the expected size was loaded in each well. The gel was run for 17 h at 60 V in 1× TAE buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA, pH 8.0) at 60 °C, stained with EtBr or SYBR Green, visualized under UV radiation and photographed. Selected bands were cut from the gel and recovered by using electro-elutor and sent for sequencing (Lin et al. 2011).

2.2.7 *Quantitative Analysis of the Genus Azospirillum by Real-Time PCR*

Bacterial culture with known cell number (CFU μL^{-1}) was seeded into the non-sterile soil, mixed thoroughly and further used for DNA extraction. Soil DNA was extracted using the bead beating method with the PowerSoil™ DNA Isolation Kits (MO BIO, USA). The DNAs after serially diluted were used as templates in real-time PCR detection; meanwhile DNA concentrations were determined by using spectrophotometer and OD_{260} value was calculated to obtain the actual DNA concentration. The PCR reactions were performed in a final volume of 25 μL containing

20 pmol of each designed genus-specific primer, 1 μL extracted soil DNA and 1× GoTaq® qPCR Master Mix with the SYBR Green as fluorescence dye. Real-time PCR reaction was performed in a smart cycler® system II (TaKaRa Bio Inc., Japan) with the following cycling conditions: hot-start activation for 2 min at 95 °C followed by denaturation and annealing/extension 40 cycles of 15 s at 95 °C, and 1 min at 68 °C, with a final dissociation from 60 to 95 °C. The determination of the detection limit was based on the appearance of C_t (cycle threshold) value and recognizable linear correlation between all the data (C_t value) obtained (Lin et al. 2011). All the tests were done in triplicate.

2.2.8 Morphological and Physiological Characterization

(a) Cell morphology was determined by placing the cells (1–2 days old) on a carbon-coated copper grid followed by staining with aqueous solution of 0.2 % (w/v) uranyl acetate for 5–10 s, brief air-drying and observation under a transmission electron microscope (JEOL JEM-1400). For thin sections, the cells were prefixed with 2 % (v/v) glutaraldehyde and 3 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h at room temperature and washed three times in the same buffer. Thin sections were prepared and examined. For scanning electron microscopy, 50–200 μL of culture was placed onto a Millipore filter (0.45 μm) and fixed with 2.5 % (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h. Then the samples were rinsed with buffer, dehydrated in a graded series of ethanol and critical-point-dried. The filters were coated with gold palladium and observed with field emission scanning electron microscope (JEOL JSM-7401F, Japan) (Lin et al. 2012).

(b) Gram staining was performed as described by Murray et al. (1994). Briefly, fix the fresh culture to the slide by heat or by exposure to methanol. Heat fix the slide by passing it through a flame to warm the glass. Crystal violet (a basic dye) is then added by covering the heat-fixed cells with a prepared solution. Allow to stain for approximately 1 min. Briefly rinse the slide with water (the heat-fixed cells should look purple at this stage). Add iodine (Gram's iodine) solution (1 % iodine, 2 % potassium iodide in water) for 1 min. After briefly rinse with water, decolorize the sample by applying 95 % ethanol or a mixture of acetone and alcohol (the important aspect is to ensure that all the color has come out that will do so easily). Rinse with water to stop decolorization. Then, rinse the slide with safranin reagent which stains all cells red. Blot gently and allow the slide to dry. Observe the samples using a light microscopy.

(c) Catalase activity was determined by assessing bubble production by cells in 3 % (v/v) H_2O_2 and oxidase activity was determined by using 1 % (w/v) *N*,*N*,*N*,*N*,-tetramethyl-1,4-phenylenediamine reagent (bioMérieux, France). Intracellular PHB granules were detected with different methods, including Sudan Black staining (Schlegel et al. 1970) and Nile blue A staining (Ostle and Holt 1982), which result in dark blue or fluorescent granules.

(d) Carbon source utilization pattern was determined by using Biolog GN2 MicroPlate (bioMérieux, France). Nitrate reduction, indole production, activity of β-galactosidase and urease, hydrolysis of esculin and gelatin and assimilation of 12 substrates were tested with API 20 NE strips (bioMérieux, France). The activities of various enzymes were determined by using API ZYM system (bioMérieux, France).

2.2.9 Biochemical and Chemotaxonomic Characterization

(a) Fatty acid methyl esters (FAME) were prepared, separated and identified according to the standard protocol (Paisley 1996) of the Microbial Identification System (MIDI) (Sasser 1990) by gas chromatograph (Agilent 7890A) fitted with a flame ionization detector. The cultures were grown under the same condition on R2A plates at 30 °C. After 48 h cells were harvested from the plate and subjected to saponification (reagent 1 contains: NaOH 45 g, methanol 150 mL and d.d. H_2O 150 mL), methylation (6.0 N HCl 325 mL and methanol 275 mL) and extraction (hexane 200 mL and methyl-*tert*-butyl ether 200 mL) as described by Miller (1982). Identification and comparison were made by using the Aerobe (TSBA6/RTSBA6) database of the MIDI System (Sherlock version 6.0).

(b) Polyamines were extracted as described by Scherer and Kneifel (1983), analysed by high performance liquid chromatography (HPLC). Bacterial cells were hydrolysed in 0.2 M perchloric acid ($HClO_4$) at 100 °C (30 min) with shaking once after 15 min. After centrifugation, 200 µL of the supernatant was incubated with 300 µL of Na_2CO_3 solution (100 mg mL^{-1} in water) and 800 µL of dansyl chloride solution (7.5 mg mL^{-1} in dry acetone) in 0.5-dram vials with Teflon-lined caps (20 min at 60 °C). One hundred microlitres of a proline solution (50 mg mL^{-1} in water) was added to bind excess dansyl chloride (10 min at 60 °C). After cooling in a refrigerator (to 5 °C), the mixture was shaken with 100 µL of toluene. Ten microlitre of the extracted sample was applied on TLC plate (Silica gel 60 F_{254}, 20×20 cm, Merck 5554, Germany) and running solvent was used by ethyl acetate/cyclohexane (2:3 v/v). The dansyl derivatives were separated by using a Hitachi L-2130 chromatograph equipped with a Hitachi AS-4000 injector, Hitachi L-2485 fluorescence detector (excitation at 360 nm and emission at 520 nm) and a reverse-phase C18 column (Phenomenex® Synergi 4 µ Fusion-RP80 250×4.60 mm). A linear gradient (40–80 %) of acetonitrile-water at 40 °C with the flow rate of 1 mL min^{-1} was used.

(c) Polar lipids were extracted and analysed by two-dimensional thin layer chromatography (TLC), the method was described by Minnikin et al. (1984). Briefly, approximately 50 mg of dried biomass was placed into an 8.5 mL tube with a PTFE-lined cap. Add 2 mL of aqueous methanol (10 mL of 0.3 % aqueous NaCl added to 100 mL of methanol) followed by 2 mL of hexane and shake in

a tube rotator for 15 min. Centrifuge at low speed to break the emulsion and remove the upper layer to a small vial (this contains non-polar lipids). Repeat the extraction with a further 1 mL of hexane and add the second supernatant to the first. The pooled supernatants contain menaquinones which can be further purified and analysed. Heat the remaining biomass and aqueous methanol in a boiling bath for 5 min and cool to 37 °C in a water bath. Add 2.3 mL of chloroform–methanol–aqueous 0.3 % (w/v) NaCl (90: 100: 30, v/v) to the biomass lower layer and mix on a tube rotator for 1 h. Centrifuge at low speed to pellet biomass, remove supernatant (monolayer) to a clean tube. Add 0.75 mL of chloroform-methanol-0.3 % (w/v) NaCl (50:100: 40, v/v) to the cell residue and mix for 30 min. Centrifuge to pellet cells, remove supernatant and add to previous supernatant; repeat last step. Add 1.3 mL of chloroform and 1.3 mL of aqueous NaCl to the pooled supernatants and mix thoroughly; centrifuge; remove top layer and dry lower layer (contains polar lipids) using a flow of nitrogen while heating at 37 °C.

(d) For the analysis of DNA G+C content, DNA samples were prepared and degraded enzymatically into nucleosides as described by Mesbah et al. (1989). In the standard method, 25 µL of a solution containing 2–25 µg of DNA in a 1.5 mL eppendorf tube was heated in a boiling water bath for 2 min and rapidly cooled in an ice water bath. Then 50 µL of 30 mM sodium acetate buffer, 5 µL of 20 mM $ZnSO_4$ and 3 µL of P1 nuclease were added. The sample was incubated for 2 h at 37 °C. Five microlitres of 0.1 M glycine hydrochloride buffer (pH 10.4) and 5 µL of bovine intestinal mucosa alkaline phosphatase (200 U/mL in glycine buffer) were added. With these additions, the pH of the sample was between 7.5 and 8.5. The sample was incubated for 6 h at 37 °C, centrifuged at 10,000×*g* for 4 min and stored at −20 °C until it was chromatographed. The nucleoside mixtures obtained were then separated and analysed via HPLC (Hitachi L-2130 chromatograph equipped with Hitachi L-2200 autosampler, Hitachi L-2455 Diode array detector and a reverse-phase C18 column (Phenomenex® Synergi 4 µ Fusion-RP80 250×4.60 mm)).

(e) DNA–DNA hybridization assay was conducted (when 16S rRNA gene similarity more than 97.0 % was considered) using DIG DNA labelling and detection kit (Roche Diagnostics; Cat. No.11 093 657 910) according to the manufacturer's protocol. Briefly, chromosomal DNA (200 ng per µL) was used to construct hybridization probes by labelling with digoxigenin-11-dUTP (DIG) using a mixture of random hexamers, a dNTP mix containing alkali-labile DIG and labelling grade Klenow enzyme. DIG-labelled DNA probes are generated according to the random primed labelling technique and used for hybridization to membrane blotted nucleic acid to standard methods. DNA samples from different strains were loaded on to positively charged membranes as described by Seldin and Dubnau (1985). Fix the nucleic acid to the membrane by cross linking with UV light (1,200×100 µJ/cm^2) for 2 min. Preheat an appropriate volume of hybridization buffer to hybridization temperature. Pre-hybridize filter for 30 min with gentle agitation in an appropriate container. Denature DIG-lablled DNA probe (25 ng mL^{-1}) by boiling for 5 min and rapidly cooling in ice/water.

Add denature DIG-lablled DNA probe to preheated buffer and mix well but avoid foaming. Incubate at least 6 h to overnight with gentle agitation at hybridization temperature. After hybridization, the blots were subjected to stringent washing steps (wash 2×5 min in ample 2× SSC, 0.1 % SDS at room temperature under constant agitation; then wash 2×15 min in ample 0.5× SSC, 0.1 % SDS at 65–68 temperature under constant agitation) and incubated for 30 min in blocking solution (dissolve blocking reagent in maleic acid buffer: 0.1 M maleic acid, 0.15 M NaCl; adjust with NaOH to pH 7.5). Antibody solution was added and incubated for 30 min, then wash 2×15 min in washing buffer (0.1 M maleic acid, 0.15 M NaCl; pH 7.5; 0.3 % (v/v) Tween 20). Equilibrate 2–5 min in detection buffer (0.1 M Tris–HCl, 0.1 M NaCl, pH 9.5). The nylon membrane is subjected to immunological detection with anti-digoxigenin–AP conjugate and the freshly prepared colour substrate solution. When desired spot is achieved, stop the reaction by washing the membrane for 5 min with sterile double dist. water or with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Levels of relatedness were determined by scanning the X-ray membranes and slot intensities were determined by using the UVP Vision Acquisition and Analysis Software version 6.8 (BioSpectrumR imaging system). Self-hybridization values were considered to represent 100 % of the maximal achievable signal and values obtained with the other strains were compared with this standard. The experiments were carried out in triplicate for each sample.

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

Anton Hartmann, Euan K. James, Frans J. deBruijn, Stefan Schwab, Michael Rothballer, and Michael Schmid

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

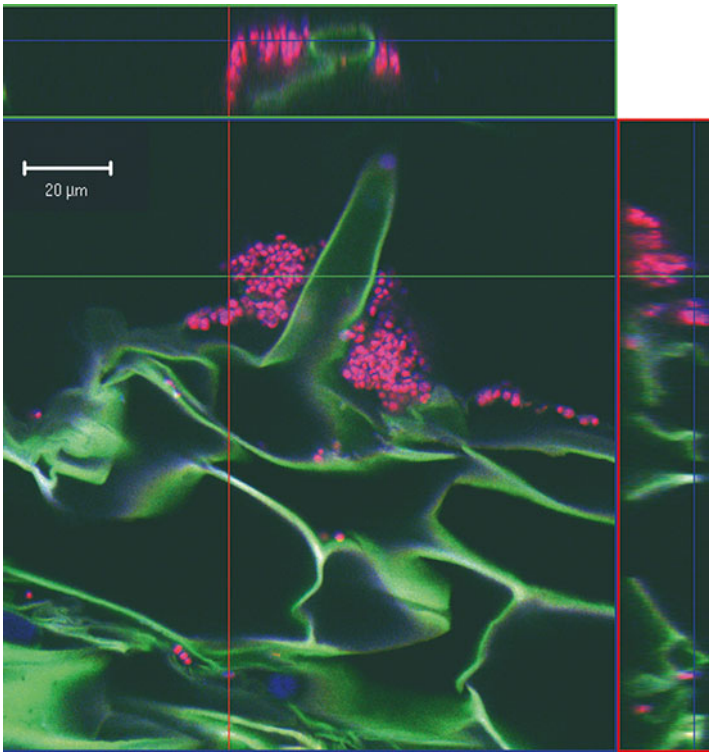

Fig. 3.1 *Azospirillum brasilense* Sp245 colonizing a wheat root. FISH staining using the species-specific probe Abras-1420-Cy5 (*blue*) and the general bacterial probe Eub-Mix-Cy3 (*red*) (image taken from Rothballer et al., Symbiosis 34, 261–279 (2003) with kind permission of Springer Science + Business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

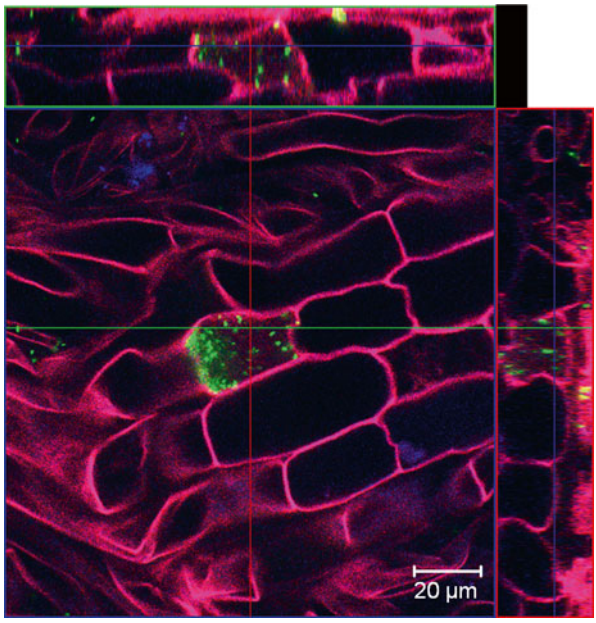

Fig. 3.3 *A. brasilense* Sp245 (gfpmut3 labeled) colonizing the interior of wheat roots (CLSM image) (image taken from Dr. M. Rothballer, Helmholtz Zentrum München, Neuherberg, Germany)

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

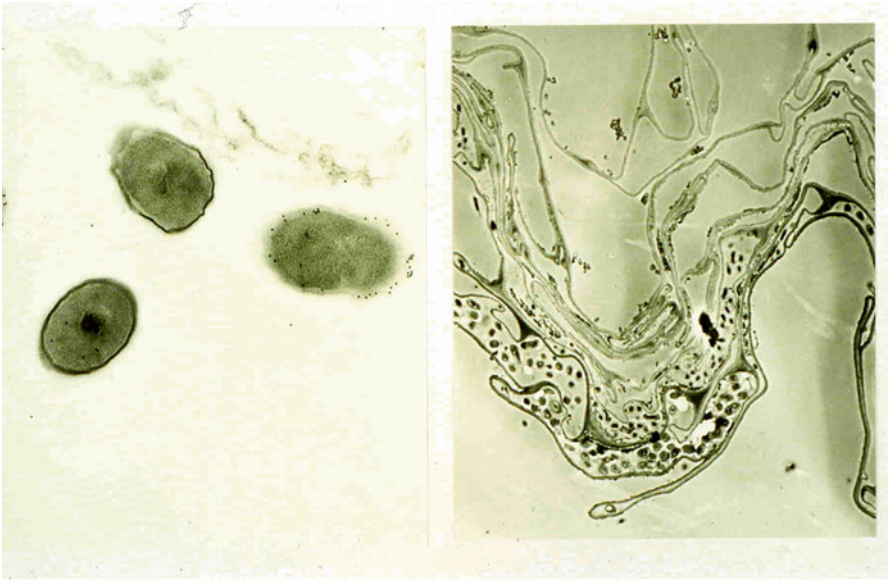

Fig. 3.4 *A. brasilense* Sp7 on the surface of wheat roots (TEM-immunogold image) (images taken from Schloter and Hartmann, Symbiosis 25, 159–179 (1998); with kind permission of Springer Science + Business Media)

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

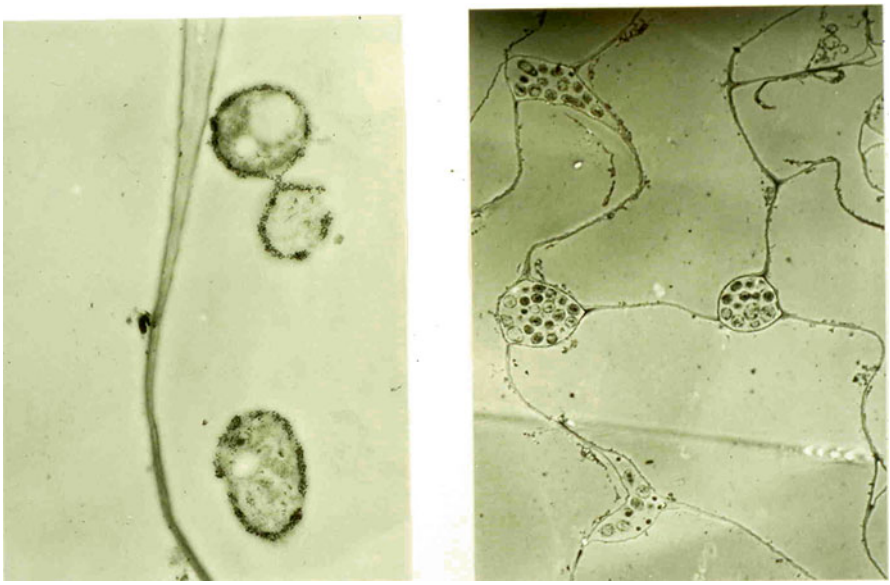

Fig. 3.5 *A. brasilense* Sp245 within intercellular spaces (apoplast) of a wheat root (TEM-immunogold image) (images taken from Schloter and Hartmann, Symbiosis 25, 159–179 (1998); with kind permission of Springer Science + Business Media)

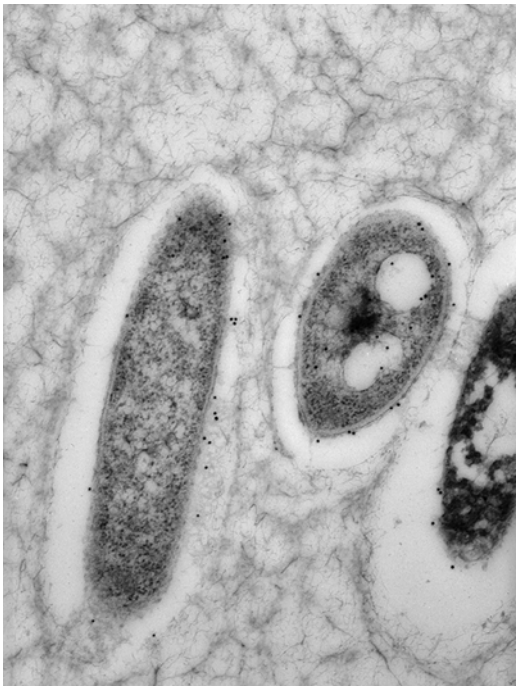

Fig. 3.6 *Gluconacetobacter diazotrophicus* PAL5 within a sugar cane leaf (TEM-immunogold image) (image taken by Dr. Euan James, The James Hutton Institute, Dundee, UK)

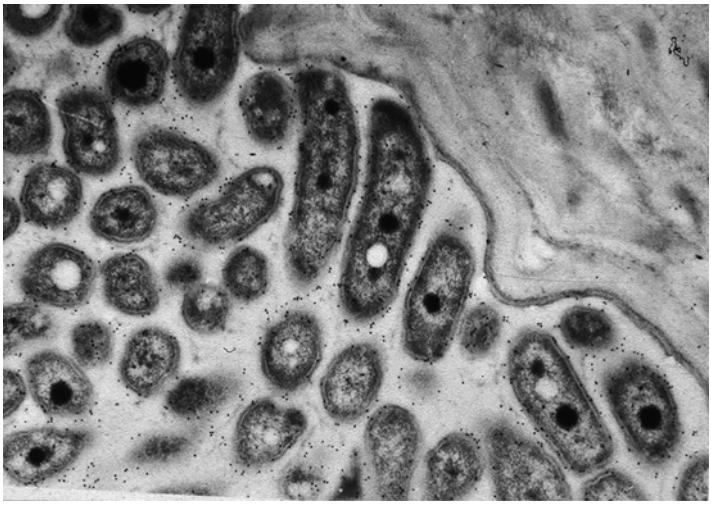

Fig. 3.7 *Herbaspirillum seropedicae* in a rice leaf (TEM-immunogold image) (image taken by Dr. Euan James, The James Hutton Institute, Dundee, UK)

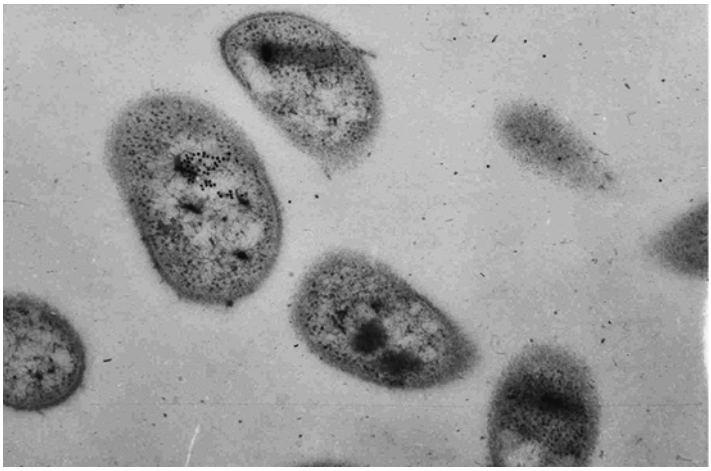

Fig. 3.8 *Herbaspirillum seropedicae* in rice roots (labeled with *nifH*-antiserum; TEM immunogold image) (image taken by Dr. Euan James, The James Hutton Institute, Dundee, UK)

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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Chapter 4
Molecular Tools to Study *Azospirillum* sp. and Other Related Plant Growth Promoting Rhizobacteria

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Abstract Molecular methods have been used in the study of *Azospirillum* and other related PGPRs to carry out gene functional analysis, create gene knockouts, generate genetically engineered strains, and carry out gene expression studies. Genetic transformation has routinely been carried out using conjugation, while chromosomal modifications have been performed using unstable, suicide plasmids, or more stable, broad host-range vectors. Gene expression studies are often carried out using promoter-bound reporter genes; however, quantitative methods such as reverse transcribed polymerase chain reaction can now be used to directly study gene expression. In this chapter we describe the common types of vectors used in *Azospirillum*, as well as methods for transformation and mutagenesis. We also describe the use of promoter-bound reporter genes and the applications of quantitative RT-PCR for *Azospirillum* gene expression studies. Methods for the isolation of DNA and RNA from *Azospirillum* for use in molecular and gene expression studies are also described.

4.1 Mutagenesis and Genetic Transformation

Genetic transformation has been used in *Azospirillum* for gene functional analysis employing random and site-directed transposon-induced mutagenesis, gene knockout and genetic exchange, gene expression studies using promoterless reporter gene translation cassettes, genetic engineering by introducing new genes/traits, and for genetic labelling by inserting constitutively expressed reporter genes.

Genetic transformation in *Azospirillum* spp. has been mainly performed by conjugation using various groups of plasmid vectors. Generally, chromosomal modifications have been performed using unstable, suicide plasmids, while stable broad host-range vectors have been used in applications requiring the maintenance of plasmids.

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In this section we discuss the main vectors used with *Azospirillum*, transformation by conjugation, gene replacement, and transposon mutagenesis. The analysis that follows mutagenesis requires the extraction of genomic DNA, and while nowadays there are commercial kits suitable for this purpose, a cost-effective method for the isolation of large amounts of *Azospirillum* DNA is described.

4.1.1 Vectors

Among other vehicles, such as the cosmid pLAFR3, used for the construction of *Azospirillum* genomic libraries (Revers et al. 2000), DNA elements can be transformed into recipient cells on plasmid vectors. The selection of a vector depends on the intended use, method of genetic transformation, and user preference/tool availability. Vectors available for *Azospirillum* can be divided into stable, broad host-range vectors, and unstable suicide vectors.

4.1.1.1 Broad Host-Range Vectors in *Azospirillum*

Until the early 1980s genetic tools for *Azospirillum* chromosome mobilization included the IncP1 plasmid R68-45 (Haas and Holloway 1976), adopted from use with *P. aeruginosa* (Elmerich and Franche 1982). Michiels et al. (1985) tested plasmids belonging to the incompatibility groups (Inc) P1, Q, and W, but only IncP1 plasmids pRK290, pRK252, and BIN19 were stable in *Azospirillum* (all derivatives of RP4). The most widely used stable vectors in *Azospirillum* have been pRK290 (Ditta et al. 1980) and its derivatives the cosmid pVK100 (Fig. 4.1) (Knauf and Nester 1982) and pLA2917 (Fig. 4.2) (Allen and Hanson 1985). These low copy, broad-host range vectors, from the IncP1 group with RK2 replication factors, are not self-transmissible but can be mobilized if supplied with the plasmid transfer elements in trans (see Sect. 4.1.2). They can be transferred to *Azospirillum* recipients by conjugation and are stably maintained. These vectors contain a number of unique restriction sites (Figs. 4.1 and 4.2) to enable selection and analysis following cloning. Such restriction sites exist within either the kanamycin or tetracycline resistance markers, which will be inactivated if disrupted with cloned DNA. Other vectors stable in *Azospirillum* include the cosmid pLAFR1, originally used with *Rhizobium*, and its derivative pLAFR3 (Milcamps et al. 1996; Kadouri et al. 2002); a pRAJ275 derivative, namely, pFAJ21 (Revers et al. 2000) as well as pBBR1MCS-2 vector (Kovach et al. 1995), which was shown to be stable even without selective pressure (Rothballer et al. 2003).

Stable vectors can be used to clone genes for complementation (Pedrosa and Yates 1984; Pereg Gerk et al. 1998), create genomic libraries (Fogher et al. 1985), study gene expression (Fani et al. 1988; Liang et al. 1991; Vieille and Elmerich 1992; Pereg Gerk et al. 2000; Pereg Gerk 2004; Revers et al. 2000), and carry reporter genes for cell visualization (Arsène et al. 1994; Pereg Gerk et al. 1998).

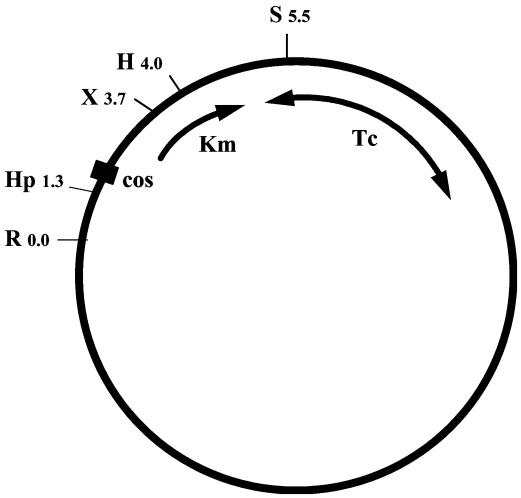

Fig. 4.1 Map of the plasmid vector pVK100. This plasmid is a derivative of pRK290 with a *Sal*I-*Eco*RI fragment of the cosmid pHK17. It contains the *cos* site of phage λ and it is a broad host vector, stable in *Azospirillum*. Unique restriction sites: (H) *Hind*III (S) *Sal*I (R) *Eco*RI (Hp) *Hpa*I (X) *Xho*I. Numbers are given in kb. There are neither *Pst*I nor *Bam*HI sites in the vector. The plasmid codes for tetracycline (Tc) and kanamycin (Km) resistance (Knauf and Nester 1982)

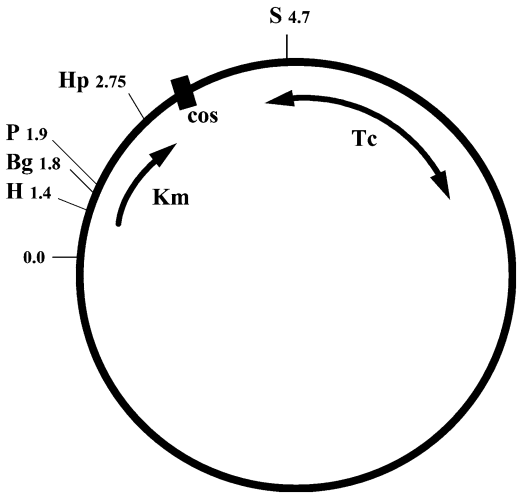

Fig. 4.2 Map of the plasmid vector pLA29.17. It contains the *cos* site of phage λ and it is a broad host vector, stable in azospirilla. Unique restriction sites: (H) *Hind*III (S) *Sal*I (Bg) *Bgl*II (P) *Pst*I (Hp) *Hpa*I. Numbers are given in kb. There are neither *Xho*I nor *Bam*HI sites in the vector. The plasmid codes for tetracycline (Tc) and kanamycin (Km) resistance (Allen and Hanson 1985)

They have been used in gene discovery, analysis of genetic regulation, and in studying *Azospirillum*–plant association. Table 4.1 summarizes the most common stable vectors used in *Azospirillum* transformation.

Table 4.1 Stable vectors and suicide vectors used in *Azospirillum*

Vectors	References	Examples of use in *Azospirillum*
Stable vectors		
pR68-45	Haas and Holloway (1976)	Cloning of *ntrB&C* by Pedrosa and Yates (1984) for gene complementation
pRK290	Ditta et al. (1980), Michiels et al. (1985)	Regulation of *nif* gene expression by Liang et al. (1991); promoter identification by Fani et al. (1988)
pVK100	Knauf and Nester (1982)	Gnomic library and cloning of *glnA* by Fogher et al. (1985); *flcA* cloning for complementation by Pereg Gerk et al. (1998)
pLA2917	Allen and Hanson (1985)	A constitutively expressed pLA-*lacZ* fusion by Arsène et al. (1994), for direct observation and quantitative measure of wheat root colonization Pereg Gerk et al. (1998); *flcA-lacZ* for gene expression studies by Pereg Gerk (2004)
pGD926 (pRK290 derivative, lacYZ)	Liang et al. (1991)	*nif-lacZ* gene expression cassettes by Liang et al. (1991) also used for gene expression in association with plants by Arsène et al. (1994), Katupitiya et al. (1995), Pereg Gerk et al. (2000); *nodG-lacZ* cassette by Vieille and Elmerich (1992)
pLAFR1	Milcamps et al. (1996)	Construction of genomic DNA libraries
pLAFR3	Milcamps et al. (1996)	Cloning and analysis of the *rpoN* gene (Milcamps et al. 1996) and *phbC* (Kadouri et al. 2002)
pFAJ21 (pRAJ275 derivative)	Revers et al. (2000)	*nif-gusA* cassette to study *nif* gene expression
pBBR1MCS-2	Kovach et al. (1995)	*ipdC* translational promoter fusions with *gfp* by Rothballer et al. (2005) to study gene expression; labelling *Azospirillum* for plant interaction assays by Rothballer et al. (2003)
Suicide vectors		
pGS9	Selvaraj and Iyer (1983)	Random Tn5 mutagenesis in *A. brasilense* and *lipoferum* by Vanstockem et al. (1987)
pSUP2021	Simon et al. (1983)	Random Tn5 mutagenesis in *A. brasilense* and *lipoferum* by Vanstockem et al. (1987)
pSUP202	Simon et al. (1983)	Identification of nif regulatory genes by Liang et al. (1991); Tn5-induced, site-directed mutagenesis of *flcA* by Pereg Gerk et al. (1998) and *mreB* by Biondi et al. (2004)
pCIB100	Van Rhijn et al. (1990)	Tn5-*lacZ* random mutagenesis for gene discovery and expression study

4.1.1.2 Suicide Vectors in *Azospirillum*

Suicide vectors are useful for transposon mutagenesis, gene knockout, and chromosomal exchanges since they allow mobilization of DNA into *Azospirillum* without stable integration of the whole vector. Instead, double recombination events replace host DNA with vector-borne DNA. Suicide vectors for gene replacement in Gram-negative bacteria may carry a conditional lethal gene that can discriminate between the integration of the entire vector and double recombination events. The suicide vectors pJQ200 and pJQ210, carrying P15A origin of replication, have been used successfully with *Rhizobium* (Quandt and Hynes 1993). The plasmid pGS9, originally developed as a suicide plasmid for insertional mutagenesis in *R. meliloti* (Selvaraj and Iyer 1983), is composed of p15A-type replicon and N-type bacterial mating system.

The suicide vector pSUP2021 is a derivative of pSUP202 carrying a Tn5 mobilizable transposon (Simon et al. 1983). The plasmid pSUP202 (Fig. 4.3) is derived from the commonly used *E. coli* vector pBR325, with a ColE1 replicon and a IncP-type Mob region, which is unable to replicate outside the enteric bacteria—it can be mobilized into, but not stably maintained, in *Azospirillum*. Therefore pSUP202 and pSUP2021 are good transposon carriers for random or site-directed transposon insertion. However, note that not all other derivatives of pBR325 can be used

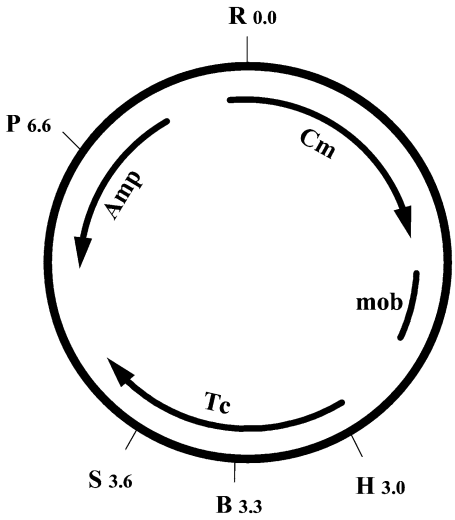

Fig. 4.3 Map of the vector pSUP202. It is a pBR325 (E. coli vector) derivative carrying the IncP-type transfer genes (Mob site from plasmid RP4) and can be mobilised with high frequency from the donor strains. It is unable to replicate in strains outside the enteric bacterial group thus, it is not stable in *Azospirillum* and can be used as transposon carrier replicon for random transposon insertion mutagenesis. This vector and members of its family, such as pSUP101, 201, 203, are especially useful for site-directed transposon mutagenesis and for site-specific gene transfer in a wide variety of Gram-negative organisms in any strain into which they can be mobilised but not maintained (Simon et al. 1983). Unique restriction sites shown: (H) *Hind*III (S) *Sal*I (B) *Bam*HI (P) *Pst*I (R) *Eco*RI. Numbers are given in kb. The plasmid codes for tetracycline (Tc), chloramphenicol (Cm) and ampicillin (Amp) resistance

effectively for this purpose in *Azospirillum*, as is the case with pSUP5011 (Vanstockem et al. 1987).

The plasmid pCIB100 (ColE1 replicon) has also been used as a donor for Tn5 random mutagenesis in the isolation of motility and chemotaxis mutants. The transposable element in this case included a promoterless *lacZ* reporter gene (Tn5-*lacZ*) enabling gene expression studies (Van Rhijn et al. 1990). Table 4.1 summarizes the most common suicide vectors used with *Azospirillum*.

4.1.2 Transformation Techniques: Electroporation and Conjugation

Genetic transformation techniques including the use of heat shock competent *A. brasilense* cells (Fani et al. 1986) and electroporation have been published. Vande Broek et al. (1989) reported on electroporation of *A. brasilense* but not *A. lipoferum* cells with plasmid pRK290 and concluded that optimal conditions for electroporation probably vary with the *Azospirillum* strain. Nevertheless, the most common procedure for the transformation of *Azospirillum* with plasmid DNA over the last three decades has been conjugation.

Transformation by biparental conjugation requires donor and recipient strains. To mobilize plasmids that are not self-transmissible, the donor strain, often *E. coli*, has to carry the transfer functions of the broad host range IncP-type plasmid RP4 integrated in its chromosome. Examples of plasmid-mobilizing *E. coli* donors that can utilize many Gram-negative bacteria as recipients include the strains SM10 and S17-1 (Simon et al. 1983).

In triparental conjugation, when plasmid DNA is mobilized into *Azospirillum* from another *E. coli* strain which does not contain the RP4 transfer elements, a third parent strain is required as a helper to supply the transfer gene in trans. *E. coli* HB101 containing the helper plasmid pRK2013 (*tra*$^+$) (Ditta et al. 1980) is often used as a helper strain.

4.1.2.1 Conjugation Protocol

In a typical conjugation protocol, overnight liquid cultures of donor and recipient strains (and helper strain if required) are mixed on nonselective nutrient agar (NA) plates. These can be mixed prior to applying, or applied to plates in stages, beginning with the donor, then the recipient on top, with drying in between. The mixture is allowed to dry and incubated overnight at 28–30 °C. A quantity of the mating mix can be spread on selective medium and incubated for 48 h or longer at 30 °C to observe for transformants (Pereg Gerk et al. 1998). In some cases 10 mM $MgSO_4$ is used in the donor and recipient cell cultures and added to the selective medium (Vanstockem et al. 1987).

The selective medium is a medium on which only the transformed or mutated recipients should grow; control cultures of the parent donor, recipient, and helper

strains should not grow. Other selective pressure in addition to antibiotics can be applied for the elimination of some parent strains. For example, the *E. coli* S17.1 donor is an auxotroph for proline and thus cannot grow in proline-free minimal lactate medium (Table 4.2, Dreyfus et al. 1983) or Nitrogen-free medium (Nfb, Table 4.2, Katupitiya et al. 1995). While proline-free minimal media can be used to select against

Table 4.2 Proline-free minimal medium for *Azospirillum* transformation

Minimal lactate medium[a]	Nitrogen-free medium (Nfb)[a]
Prepare minimal medium supplemented with 6.3 mL/L of sodium lactate	Into 800 mL of DW add:
Before use add 10 mL of $CaCl_2$ solution (7 g/L), 10 mL of trace element solution, 1 mL of $FeCl_3 \cdot 6H_2O$ solution (10 g/L), 1 mL of $Na_2MoO_4 \cdot 2H_2O$ solution (0.8 g/L), and 1 mL of vitamin solution	$CaCl_2$ 0.02 g (always add $CaCl_2$ first and mix!)
Each of the solutions should be autoclaved separately	Malic acid 5 g
Minimal medium supplemented with 6.3 mL/L of sodium lactate	$K_2HPO_4 \cdot 3H_2O$ 0.5 g
100 mL phosphate solution	$MgSO_4 \cdot 7H_2O$ 0.2 g
10 mL $MgSO_4$/NaCl solution	NaCl 0.1 g
500 mL DW	4 mL Fe-EDTA (1.64 % aqueous, w/v); 2 mL of trace element solution
6.3 mL sodium lactate	Adjust pH to 6.8 with KOH and make up to 1 L with DW
Complete with DW to 1 L	Sterilized by autoclaving at 120 °C for 20 min
Autoclave at 121 °C for 20 min	Add 1 mL vitamin solution per liter medium (after autoclaving)
Phosphate solution pH 6.8–7	*Trace element solution*
K_2HPO_4 16.7 g/L	In 1 L:
KH_2PO_4 8.7 g/L	$MnSO_4 \cdot H_2O$ 250 mg
MgSO4/NaCl solution	$ZnSO_4 \cdot 7H_2O$ 70 mg
$MgSO_4$ 29 g/L	$CoSO_4 \cdot 7H_2O$ 14 mg
NaCl 48 g/L	$CuSO_4 \cdot 5H_2O$ 12.5 mg
Trace element solution	H_3BO_3 3 mg
In 1 L:	$Na_2MoO_4 \cdot 2H_2O$ 200 mg
$MnSO_4 \cdot H_2O$ 250 mg	*Vitamin solution*
$ZnSO_4 \cdot 7H_2O$ 70 mg	In 100 mL:
$CoSO_4 \cdot 7H_2O$ 14 mg	Biotin 1 mg
$CuSO_4 \cdot 5H_2O$ 12.5 mg	Pyridoxine 2 mg
H_3BO_3 3 mg	Filter sterilize
Vitamin solution	
In 100 mL:	
Biotin 1 mg	
Pyridoxine 2 mg	
Filter sterilize	

[a]For solid media add 16 g/L Agar. For aerobic growth add also 2.5 mL of 20 % NH_4Cl as nitrogen source

the donor and helper strains, antibiotics are often used to select against the parent *Azospirillum* recipient strain.

When selecting for recipients transformed with a stable plasmid antibiotic resistance encoded by the plasmid, and not by the recipient parent, will be used for selection. In the case of suicide vectors the selection pressure will be dependent on the marker integrated into the recipient chromosome; for example, Tn5 often encodes for antibiotic resistance (often kanamycin), GFP for green fluorescence, or *lacZ* for β-galactosidase activity (blue-white selection on X-gal). Other morphological traits can be used in the selection of transformants, for example, Pereg Gerk et al. (1998) selected for white, nonencapsulating Tn5-induced mutants, against a background of red colonies on minimal medium containing kanamycin and Congo-Red. In the case of suicide vectors, it is important to check that a double and not single recombination event has occurred, to avoid the integration of the entire plasmid (Simon et al. 1983).

4.1.3 Transposon Mutagenesis and Gene Knockout

Classical methods of bacterial mutagenesis such as chemical treatment or UV irradiation have been successfully employed in *Azospirillum* (examples are given in Elmerich 1983; Del Gallo et al. 1985; Holguin et al. 1999). However, mutated genes are more easily and confidentially analyzed in genetically defined transposon-induced mutants or those produced by chromosomal site-specific exchanges.

Tn5 is a DNA transposable element, which carries an antibiotic resistance gene, often encoding kanamycin resistance. Similarly to Tn10 it is bracketed by the same insertion sequence IS50 (Reznikoff 1982). Vanstockem et al. (1987) performed transposon mutagenesis and generated Tn5-induced mutants of *Azospirillum brasilense* Sp7 and *A. lipoferum* Br17 by mating with *E. coli* strains carrying suicide plasmid vectors pSUP2021 and pGS9. These Tn5-carrier plasmids were developed for use with any Gram-negative bacteria not closely related to *E. coli* (Simon et al. 1983; Selvaraj and Iyer 1983). This random mutagenesis system is based on the following: (1) the vector plasmids are mobilized with high frequency into non-*E. coli* hosts by the broad host range transfer functions of the donor strain, (2) the vector plasmids are unable to replicate in these hosts, since their basic replicon displays a very narrow host range, and (3) transposition events can be isolated simply by selecting for transposon-mediated drug resistance while the initial transposon carrier plasmid is eliminated (Simon et al. 1983).

For random mutagenesis, the suicide plasmid containing the self-mobilized transposon is inserted into the recipient cells with the expectation that the transposon will be randomly mobilized into the host genome and that the suicide vector will not be maintained in the next generation. The final selection step is therefore critical and there are two main strategies that can be applied: (1) collect a large number of [transposon$^+$/vector$^-$] cells and screen them for different traits and (2) if direct screening for specific trait is possible, use selective media to identify [transposon$^+$/vector$^-$/mutation$^+$] mutant strains.

The development of the pSUP family of suicide plasmids (Simon et al. 1983) also promoted the possibility of site-directed Tn5 mutagenesis. This is based on homologous recombination between vector-borne and specific genomic DNA sequences. Site-directed mutagenesis is achieved by cloning the gene of interest, disrupted by a transposon (e.g., Tn5 derivative), onto a suicide plasmid and inserting the plasmid by conjugation into *Azospirillum*. To increase the chance of double homologous recombination between the chromosome and the plasmid it is important to ensure that there are sufficiently long *Azospirillum* gene sequences bracketing the Tn5 carried on the plasmid (Pereg Gerk et al. 1998). It is recommended to include at least 500 bp of chromosome-homologous sequences on each side of the plasmid-borne Tn5 for optimal results in *Azospirillum* (Pereg, unpublished). Similarly to random transposon mutagenesis, the selection stage is important in the generation of specific mutants. Pereg Gerk et al. (1998) directly selected for mutants that cannot undergo morphological transformation to cyst-like cells by identifying white colonies on selective medium containing kanamycin (selected for Tn5) and Congo-red (binds to exopolysaccharides, wild-type appears red). White, kanamycin resistant colonies were then tested for chloramphenicol sensitivity, indicative of double homologous recombination and the elimination of the pSUP202 vector (Pereg Gerk et al. 1998). Kadouri et al. (2003) used a derivative of pSUP202 for site-directed mutagenesis and characterization of *Azospirillum phaZ*.

De Lorenzo et al. (1990) and Herrero et al. (1990) constructed a selection of mini-Tn5 and Tn10 transposon delivery, R6K-based, suicide plasmids with antibiotic resistance and nonantibiotic selection markers for chromosomal insertion of DNA into Gram-negative bacteria. The system was used successfully with *Rhizobium* for insertion mutagenesis and gene expression studies using the *gusA* and *lacZ* reporter genes (Reeve et al. 1999). Elements from these plasmids have been used in mutagenesis of specific *Azospirillum* genes in IAA synthesis (Carreno-Lopez et al. 2000) and genetic labelling of *Azospirillum* with a fluorescence marker (Rodriguez et al. 2006).

Gene knockout and other chromosomal gene replacements can be achieved by double homologous recombination in a similar manner to site-directed Tn5 mutagenesis. In these cases no transposon is used, and either a truncated gene or gene sequences bracketing a marker gene, such as antibiotic resistance, are cloned into the suicide plasmid (Hou et al. 2014). Other *Azospirillum* mutants isolated globally using gene replacement with a marker include *nif*, *nodPQ*, *glnB*, *DraT*, *DraG*, *rpoN*, *NtrBC*, *recA* gene mutants, and others (summarized in Holguin et al. 1999).

4.1.4 Genomic DNA Extraction

Genetically transformed *Azospirillum* strains are often analyzed by techniques such as Southern blotting and PCR amplification. PCR amplification requires only a small amount of genomic DNA and extraction methods such as the "Freeze-boil" method can be used to obtain a sufficient amount of template. However, Southern blotting analysis requires a large amount of genomic DNA which can be purified using a commercial DNA extraction kit, or the more cost-effective protocol outlined below.

4.1.4.1 Freeze-Boil Method

A loop-full of fresh cells is resuspended in 50 μL of sterile milli-Q water. The cell suspension is frozen at −70 °C for 30 min; then boiled at 100 °C for 2 min; spun down at high speed for 3–4 min; and the debris-free supernatant used immediately (preferably) or kept frozen at −20 °C.

4.1.4.2 Large Scale Azospirillum Genomic DNA Extraction Protocol

(As used by Pereg Gerk et al. (1998) The protocol was provided to L Pereg by C. Elmerich.)

Prepare 5 mL of a late logarithmic phase culture of *A. brasilense* in nutrient broth. Centrifuge for 10 min and wash the pellet twice with 1.5 mL of $T_{50}E_{20}$ buffer (Tris 50 mM, EDTA 20 mM, pH 8). Resuspend in 400 μL of $T_{50}E_{20}$ buffer. Lyse cells by adding 7 μL of Pronase E (50 mg/mL) and 50 μL of 10 % SDS, and incubate at 37 °C for 1 h. Gently pump the clear lysate several times up and down with a 1-mL syringe equipped with a wide needle (18G1.5, 1.2×40) to physically disrupt the DNA. Extract the DNA by adding 300 μL of phenol and 300 μL of chloroform. Repeat the phenol–chloroform extraction until the supernatant is clear. RNA can be eliminated from the solution by the addition of 3 μL of RNase (0.5 μg/μL) and incubation at 37 °C for 30–60 min. Perform a final extraction with one volume of chloroform and transfer the supernatant containing the DNA into a clean tube. Solubilize the DNA by adding 1:10 volumes of sodium acetate (3 M, pH 5.5) and 2 volumes of 100 % ethanol. Incubate the solution at −20 °C for 2 h or overnight. Precipitate the DNA by centrifugation for 15 min at 4 °C, then wash the pellet with cold 70 % ethanol. Allow pellet to dry then dissolve in 200 μL of TE buffer. The DNA can be examined by gel electrophoresis (0.8 % agarose mini gel) and stored at −20 °C for further use.

4.2 Gene Expression in *Azospirillum*

Regulation of gene expression in *Azospirillum* has been studied largely using expression systems consisting of promoterless reporter genes. With the availability of the genomic sequences, the study of gene expression using more direct methods, such as quantitative reverse transcribed polymerase chain reaction (qRT-PCR), is becoming more feasible. This technique eliminates the need for cloning the gene promoter and overcomes the problems associated with studying the expression of genes in the presence of foreign vectors.

In this section we describe the use of promoter-bound reporter genes for studying gene expression in *Azospirillum*. We also present a protocol for the extraction of total RNA from *Azospirillum* for use in gene expression studies. Considerations for the use of qRT-PCR are also described.

4.2.1 Using Promoter-Bound Reporter Genes

Promoter-bound reporter genes are constructed by fusing promoterless reporter genes, such as *lacZ*, *gusA*, and *gfp*, to gene regulatory elements (promoters). These gene expression cassettes may be plasmidborne, or integrated into the host genome, and can be introduced into *Azospirillum* by genetic transformation (see Sect. 4.1.2).

Fani et al. (1988) used the promoterless gene encoding for the enzyme chloramphenicol acetyl transferase and conferring Cm resistance as a reporter gene to test for active promoters in *Azospirillum*. Vande Broek et al. (1992) used the activity of β-glucuronidase encoded by the *gusA* gene to measure gene expression. The regulation and induction of *nifH* have been analyzed using a *nifH-gusA* fusion (Vande Brock et al. 1996). Liang et al. (1991) constructed plasmid-borne fusions of promoterless *lacZ* gene with several gene promoters, such as *nifA-lacZ*, *nifH-lacZ*, *nifB-lacZ*, and *ntrC-lacZ,* that were widely used in the analysis of nitrogen fixation regulation. De Zamaroczy et al. (1993) constructed a *gln-lacZ* and Arsène et al. (1994) have used these constructs to study the expression of *nif* genes in association with plants and further constructed pSUP202 derivatives of these fusions for recombination into the chromosome. Pereg Gerk (2004) constructed a *flcA-lacZ* fusion using pKOK5 (Kokotek and Lotz 1989) as the source of the *lacZ*-Km cassette and pVK100 as the carrier of the fusion. The constitutively expressed *lacZ* fusion on pLA-*lacZ* (Arsène et al. 1994) has been used as a control when studying gene expression using *lacZ* fusions.

The *lacZ* gene, encoding β-galactosidase, is widely used in reporter gene constructs in *Azospirillum*. A typical protocol for β-galactosidase assay is shown below. This assay is based on the ability of the enzyme to hydrolyze the β-galactoside bond of the *o*-nitrophenol-β-D-galactoside (ONPG) substrate to yield a yellow product, orthonitrophenol, which can be quantified using absorption spectrometry.

4.2.1.1 Typical Protocol for β-Galactosidase Assay in Culture or on Plants

Harvest cells from liquid culture by centrifugation. Resuspend cell pellet in 0.5 mL Z buffer supplemented with 5 µL of β-mercaptoethanol. Add 20 µL of 0.1 % sodium dodecyl sulphate (SDS) and 40 µL of chloroform and mix vigorously to lyse cells. Preincubate tubes in a water bath at 28 °C for 5–10 min, then add 100 µL of fresh ONPG solution (Table 4.3) and mix well ("start time"). Continue incubation at 28 °C until the samples start turning yellow. Stop the reaction by adding 250 µL of 1 M

Table 4.3 Phosphate buffer and ONPG solution

Phosphate buffer	ONPG solution
In 100 mL: K_2HPO_4, 1.05 g; KH_2PO_4, 0.45 g $(NH_4)_2SO_4$, 0.1 g; Tris sodium citrate, 0.05 g	In 10 mL phosphate buffer or in 10 mL Z buffer (pH 7.0) dissolve 40 mg of ONPG

Na_2CO_3. Record the start and stop times. Centrifuge for 5–10 min at 14,000 rpm and measure the absorbance of the supernatant at 420 and 550 nm, against a Z buffer blank (treated in the same way as bacterial samples). Express the results as Miller units/min/mg bacterial protein (Miller 1972). This assay can also be carried out on *Azospirillum* growing in association with plant roots, as described in Chap. 10.

4.2.2 RNA Extraction

Extraction of total RNA for downstream applications such as qRT-PCR can be carried out using a Trizol extraction protocol. Up to 1×10^8 cells for RNA extraction should be harvested from liquid culture by centrifugation at $6{,}000 \times g$ for 5 min at 4 °C. Cells may be used immediately or stored in an RNA preservation solution for later use. Resuspend the cell pellet in 1 mL Trizol reagent and incubate for 5 min at room temperature. Add 0.2 mL cold chloroform and shake vigorously, then incubate for 2–3 min at room temperature. Centrifuge at $12{,}000 \times g$ for 15 min. Transfer the colorless upper aqueous phase (~0.4 mL) to a fresh tube. Precipitate RNA by adding 0.5 mL cold isopropanol and mixing. Incubate for 10 min at room temperature. Centrifuge at $15{,}000 \times g$ for 10 min and carefully remove the supernatant. Resuspend the RNA pellet in 1 mL 75 % ethanol and vortex. Centrifuge at $7{,}500 \times g$ for 5 min. Discard the supernatant and allow RNA pellet to air-dry. Resuspend RNA pellet in 50 µL RNase-free water. Agarose gel electrophoresis of RNA should show clear 16S and 23S ribosomal bands. RNA concentration may be determined by spectrophotometry. Extracted RNA should be stored at −80 °C in aliquots to avoid repeated freeze-thawing.

4.2.3 qRT-PCR

Quantitative reverse transcribed polymerase chain reaction (qRT-PCR) has become the preferred method for the study of differential mRNA expression. Semiquantitative RT-PCR has been used in *Azospirillum* to analyze, for example, changes in expression of genes involved in CO_2 fixation (Kaur et al. 2009), quorum-sensing (Vial et al. 2006), and cell aggregation (Valverde et al. 2006). Quantitative RT-PCR has been less widely used in *Azospirillum* studies (see, for example, Kumar et al. 2012; Hou et al. 2014) but presents a much more sensitive system to detect changes in mRNA levels.

4.2.3.1 Reference Gene Selection

One important consideration in the application of qRT-PCR is the selection of internal reference (housekeeping) genes for the normalization of data. As no standard set of reference genes has been determined for prokaryotic cells such as *Azospirillum*, it is important to identify stable reference genes prior to undertaking qRT-PCR

Table 4.4 Reference genes identified in different bacterial species for qRT-PCR data normalization

Bacterial species	Reference genes	Reference
Azospirillum brasilense	*gyrA*, *glyA*	McMillan and Pereg (2014)
Clostridium ljungdahlii	*gyrA*, *rho*, *fotl*	Liu et al. (2013)
Lactobacillus casei	*gyrB*, *GAPB*	Zhao et al. (2011)
Xanthomonas citri	*atpD*, *rpoB*, *gyrA*, *gyrB*	Jacob et al. (2010)
Pectobacterium atrosepticum	*recA*, *ffh*	Takle et al. (2007)
Staphylococcus aureus	*Pyk*, *proC*	Theis et al. (2007)
Actinobacillus pleuropneumoniae	*glyA*, *recF*	Nielsen and Boye (2005)
Pseudomonas aeruginosa	*proC*, *rpoD*	Savli et al. (2003)

analysis (McMillan and Pereg 2014). Appropriate reference genes can be selected from a set of potential reference genes by analyzing expression of each gene in the target species under all different experimental treatments. Free software packages such as BestKeeper (Pfaffl et al. 2004), Normfinder (Andersen et al. 2004), and GeNorm (Vandesompele et al. 2002) can then be used to identify the most stable reference genes. Some reference genes identified for different bacterial species, including *A. brasilense*, are shown in Table 4.4.

4.2.3.2 Primer Design

qRT-PCR primers should be designed against the specific sequence of the gene of interest. For *Azospirillum* spp. these sequences may not always be available; however, the complete genome sequences of *Azospirillum* sp. B510 (Kaneko et al. 2010), *A. brasilense* Sp245 and *A. lipoferum* 4B (Wisniewski-Dye et al. 2011), are available and may be used to design specific primers for closely related species. Ideally, for optimal PCR efficiency, the amplicon length should be between 50 and 150 bases. Longer amplicons can lead to poor amplification efficiency. Primers should be between 18 and 25 bases (20 bases is standard). The primer melting temperature (T_m) of each PCR primer should be between 58 and 60 °C, and the T_m of both primers should be within 4 °C of each other. The GC content of primers should be within 40–60 %. To avoid the formation of primer dimers in the PCR reaction complementarities between primers should be avoided. Primer design software can be used to simplify the primer design process and select the optimal primer pair for a given sequence.

4.2.3.3 One-Step qRT-PCR

In one-step PCR the reverse transcription step is carried out in the same reaction tube as the PCR reaction. This has the advantage of being quicker, involving less pipetting than a two-step protocol, and eliminates the possibility of contamination between reverse transcriptase and PCR steps. Commercial one-step RT-PCR kits

Table 4.5 Typical two-step cycling protocol for qRT-PCR

Step	Temperature (°C)	Time
Reverse transcription	50	10 min
Enzyme activation	95	5 min
Two-step cycling (35–40 cycles)		
Denaturation	95	10–15 s
Annealing/extension	60[a]	30 s

Table 4.6 Typical three-step cycling conditions for qRT-PCR

Step	Temperature (°C)	Time
Reverse transcription	50	10 min
Enzyme activation	95	5 min
Three-step cycling (35–40 cycles)		
Denaturation	95	10–15 s
Annealing	55–60[a]	30 s
Extension	72	30 s

[a]Annealing temperature can be altered based on the T_m of primers used

include a mastermix containing Taq DNA polymerase, fluorescent dye (most commonly SYBR), dNTPs, and $MgCl_2$, to which reverse transcriptase and template RNA is added. A typical 25 μL reaction mix consists of 12.5 μL RT-PCR master mix, 1 μM each forward and reverse primer, 0.25 μL reverse transcriptase, 10–100 ng template RNA.

Most commercial qRT-PCR kits have been optimized for use in a two-step cycling protocol, with a combined annealing/extension step. Typical two-step cycling conditions are shown in Table 4.5. A no-template control (to test for primer dimer formation or contamination of reagents), a positive control, and a minus reverse transcriptase control (to test for genomic DNA contamination) should always be included.

A three-step cycling protocol may be used as an alternative to the two-step cycling protocol. Typical three-step cycling conditions are shown in Table 4.6.

4.2.3.4 Two-Step qRT-PCR

Two-step qRT-PCR is carried out with separate reverse transcriptase and PCR cycling steps. A two-step protocol may be more sensitive and allows for individual optimization of the reverse transcriptase and PCR steps. cDNA synthesis can be carried out using a commercial kit, and the composition of buffers and amounts of reagents used will vary with supplier. For *Azospirillum*, a typical reaction combines 0.1 ng–5 μg total RNA with 50 ng random hexamers in annealing buffer and is incubated at 65–70 °C for 5 min to denature RNA. The reaction is then chilled for 2–5 min to allow annealing of primers. Other components are added to the reaction including dNTPs (0.5–1 mM), reverse transcriptase (15–200 U/reaction depending on enzyme used), RNase inhibitor (40–50 U/μL), and $MgCl_2$ (5 mM). The reaction

is incubated for 5–10 min at 25 °C, followed by 50 min at 50 °C to allow for extension. The reaction is terminated by heating to 85 °C for 5 min. The cDNA can then be used as template for a qPCR assay.

The reaction mix and cycling conditions for the qPCR reaction are similar to those described for one-step qRT-PCR, with the omission of reverse transcriptase from the reaction mix, and the initial 50 °C reverse transcriptase step is eliminated from the PCR cycle.

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Chapter 5
Genomic Tools for the Study of *Azospirillum* and Other Plant Growth-Promoting Rhizobacteria

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Abstract Bioinformatics tools are essential for extracting valuable biological knowledge from bacterial genomes. Currently, there are many computational applications, algorithms, and programs to decipher the genomes in terms of structure, function, and evolution. Specialized databases to upload and retrieve genomic information have grown as well in the last past years. In this chapter, we highlight the basic bioinformatics procedures, databases, and web resources commonly used in bacterial genomics covering *Azospirillum* and related bacteria.

5.1 Introduction

The importance of *Azospirillum* as promoter of plant-growth promoting rhizobacteria (PGPR) is well known and is detailed elsewhere (Vacheron et al. 2013). Genomic approaches have shed light on important aspects on *Azospirillum* evolution, physiology, and niche adaptation through the comparison of the complete genomes of *A. lipoferum* 4B, *A. brasiliense* Sp245, *A. amazonense* Y2, *Azospirillum* sp. CBG497, and *Azospirillum* sp. B510 available from different databases (Kaneko et al. 2010; Rivera et al. 2014; Wisniewski-Dye et al. 2011, 2012). The *Azospirillum* genomes at Genoscope (AzospirilumScope) are among the most complete database and web resources for comparative genomics, metabolism, and phylogenomics for this organism. In this chapter, we provide the essentials for genomic study of *Azospirillum* taken into account that such methods are of more general application in bacterial genomics. Information about bioinformatics tools and web resources are presented and the reader could easily go to the original sources for more complete view of the methods.

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5.2 Genome Sequencing

Nowadays, bacterial genome sequencing has become affordable for small research laboratories worldwide. Since Sanger sequencing, new technologies collectively called Next Generation Sequencing (NGS) have improved the field lowering the cost per sequenced base (and per genome as a whole), and increasing the accuracy of the readings. A review of the current NGS technologies from a decade's perspective has been published by Mardis (Mardis 2011). Even Sanger capillary sequencing is still in use because its accuracy and long reads; pyrosequencing (454 Life Sciences-Roche) and sequencing by synthesis (Illumina) have dominated the field last years. Emerging technologies that produce very long reads, PacBio (Pacific Biosciences) (Eid et al. 2009), and Nanopore (Oxford Nanopore Technology) (Schneider and Dekker 2012) are not extensively used even their promissory future. To choose the most appropriate sequencing platform, it should be done through a careful balance of their performance: data amount, speed, flexibility, read length, and cost (Table 5.1) according to the genome it is working on.

Critical for any genomic project is to set up if genome sequences of reference organisms are available; if not, an experimental design for *de novo* sequencing should be devised. When the reference genome sequence of the microorganism to study is already in databases, a first effort should be to choose the NGS technology that allows for high coverage of the genomes (about 50× or more). Most used technologies include Illumina and 454 pyrosequencing; in both cases they produce short readings of about 100–300 and 400–500 bases, respectively, that can be easily mapped onto the reference genome with bioinformatics tools as MAQ, MUMmer (Kurtz et al. 2004), BOWTIE (Langmead et al. 2009), BWA, and TopHat (Fonseca et al. 2012). Before mapping, readings could be assembled in contigs but ordinarily

Table 5.1 Relevant features of selected NGS technologies

NGS technology[a]	Read length (bp)	Running time	Reads/runs (millions)	Error rate	Reference
3730×l (Sanger)	650	2 h	0.000096	Low (<1 %)	Mardis (2013)
ABI/Solid	75+35	8 days	>1,410	Low (<2 %)	Mardis (2013)
Illumina /Solexa	250+250	2 h	4–8	Low (<2 %)	Loman et al. (2012)
IonTorrent	200	2 h	1	Medium (<4 %)	Loman et al. (2012)
Roche/454	400–600	10–20 h	1	Medium (<4 %)	Loman et al. (2012)
PacBio	Up to 15,000	0.5–2 h	0.01	High (<18 %)	Eid et al. (2009)

[a]Actually, NGS technologies run a variety of platforms from relative small scale to very great scale. For instance, Illumina have versions like MySeq for small genomes and HiSeq for human genome scale, and a variety of intermediate apparatus and multiplex strategies to lower the costs and the time of running. Other companies have also diverse sequencing options

it is not necessary to invest computing time in assemblies since this strategy works well for re-sequencing of very related genomes. A good sequencing run, whatever technology employed, recovers more than 99 % of the gene content of a bacterial genome allowing for identification of single nucleotide mutations (SNPs) (Royce et al. 2013), or to map disrupted genes by the insertion of antibiotic cassettes or transposons in mutagenesis experiments (Held et al. 2012).

The *de novo* sequencing requires a variation in the experimental design as well as to include extensive bioinformatics processing for the assembly. It has been proved that the best strategy to *de novo* assembly is to combine the results of two classes of genome sequence readings. One is short-paired end readings from small fragment libraries (300–500 bp) to achieve high coverage, and the other from mate-pair readings from large insert libraries (3–8 kb) to solve the repeats and misassemblies (Ribeiro et al. 2012). Taken together, they give highly accurate assemblies even for the complex multi-replicon bacterial genomes of *Azospirillum*.

5.3 Assembly, Annotation, and Visualization

Genome assembly and finishing consist of the reconstruction of a superior structure based on small pieces of information, by closing gaps, correcting misassemblies, and improving the quality of the consensus bases. There are two levels at which an assembly can be evaluated: contigs and scaffolds. Contigs are groups of readings joined by overlaps, and scaffolds are groups of contigs not necessarily joined but with evidence (paired-end or mate pair sequences in two contigs) that they belong to the same genomic region. To assess how good is the assembly the measure of the contigs and scaffolds are referred to the N50 contig statistics that means that 50 % of the entire assembly is contained in contigs or scaffolds equal to or larger than this value.

There are many algorithms to assembly NGS readings that work at different levels of efficiency (Koren et al. 2014; Magoc et al. 2013). They are classified into three groups: greedy algorithms that use local overlaps to join reads as long as they do not meet conflict with the assembly itself, for instance the earlier assemblers Phrap (used in CONSED suite) (Gordon and Green 2013), the TIGR Assembler (Pop and Kosack 2004), and VCAKE (Jeck et al. 2007). Another kind of algorithms rely on over-layout consensus construction that means that the assembler first identifies all pairs, reads which overlap sufficiently well to produce a graph with nodes and edges onto which develop a map of contigs and scaffolds. Newbler (Margulies et al. 2005), the assembler dedicated to 454 pyrosequencing reads uses an algorithm like this. The third group of assemblers uses the de Bruijn graphs that depend on the construction of strings (sequences of k length) and substrings (of k-mers of $k-1$). This method results ideal for NGS that produces millions of short reads that can be equated to k-mers of small size doing the assembly computationally feasible. Velvet (Zerbino and Birney 2008), SOAP-de novo (Li et al. 2009), ABYSS (Simpson et al. 2009), ALLPaths (Butler et al. 2008), and SPAdes (Bankevich et al. 2012) are

programs that use the de Bruin graphs. It is not easy to decide what is the best assembler for a bacterial genome, but it is clear that the best should be one that cope very well with the repeats, combine short and large reads from different sequencing platforms, and be bioinformatically affordable in computing time and memory consuming (Koren et al. 2014; Lee and Tang 2012; Miller et al. 2010; Ren et al. 2012).

To understand and use the genome sequences, it is necessary to identify and assign functions to protein-coding genes, non-coding RNAs, regulatory sequences, repeats, and others features that make the genome more comprehensive for scientist. First step consists in predicting Open Reading Frames (ORFs) with computer programs that generally use statistical procedures like Markov chains. GeneMarkS and a training set of known ORFs, or long ORFs predicted from the genome itself (Borodovsky and Lomsadze 2014) and Glimmer (Delcher et al. 1999) are earlier programs used to predict ORFs; they are still among the most popular because they are easy and accurate. They can predict almost the complete gene content of a bacterial genome with about 88–98 % of accuracy. Among the problems commonly found with these programs is the prediction of very small ORFs, overlapping and missing ORFs, and the incorrect identification of the translation start site. There are other gene prediction programs available that improve the accuracy at expense of the sensitivity. For instance, Prodigal make more accurate translation site predictions based on the strength of the translation binding site (Hyatt et al. 2009).

ORFs can be represented in graphic interfaces with their associated features by genome browsers like Artemis Comparison Tool (ACT) (Carver et al. 2012) and mVISTA (Frazer et al. 2004; Nielsen et al. 2010). Artemis can be used to perform manual annotation since it can also function as editor of the sequences and ORFs, allow to write directly into the features, correct start codons, add or remove ORFs, send BLAST searches to databases, and generate the Genbank file; all in a friendly platform.

5.4 Reference Databases and Integral Web Resources

All the annotation procedures are based on the comparison with genomes and genes already annotated in databases, with previous predictions or with experimental evidence of the gene function. BLAST searches are the most used bioinformatic tools to compare the predicted protein-coding genes with databases of the International Nucleotide Sequence Database: GenBank-RefSeq (Benson et al. 2014), DDBJ (DNA Data Bank of Japan) (Kosuge et al. 2014), and EMBL–EBI (Kulikova et al. 2007). These databases provide the complete genome sequences in FASTA format or in other format (GenBank, DDBJ, EMBL) both in nucleotides and proteins together with the submitted annotations that can be downloaded by FTP. Additional resources in these databases include the taxonomic distribution of orthologs, links to Pubmed, summary tables of the annotation, COGs, and BLAST resources to look for homologs in other organisms, and structural domains in the Conserved Domain Database (NCBI-CCD). Another resource database widely used

due to the quality of its annotations and minimal redundancy is UniProt (Dimmer et al. 2012; Magrane and Consortium 2011). This consortium formed by the Swiss-Prot, TrEMBL, and PIR (Protein Information Resource) provide manually curated annotations and experimental information extracted from the scientific literature; therefore, annotations are enriched in aspects like subcellular location, protein interactions, alternative products, cross-reference databases, and more (UniProt Consortium 2014).

Additional resources to investigate for protein domains in protein family databases give strong support to the functional annotation of the predicted genes. The InterPro (Hunter et al. 2012) is the most used resource of protein families, domains and motifs, which can be accessed by web server or downloaded by anonymous FTP. Several protein family databases are joined in InterPro so with a single query using the InterProScan (Jones et al. 2014) could analyze the PFAM (Protein Families and Domains), TIGRfam, and COG (Clusters of Orthologous Genes) (Chen et al. 2013).

There are automatic annotation pipelines that alleviate much of the annotation work as SEEDRAST (Overbeek et al. 2014), xBASE (Chaudhuri et al. 2008), or integrated databases with annotation pipelines like IMG (Markowitz et al. 2014), Microscope (Vallenet et al. 2013), and KEEG (Kyoto Encyclopedy of Genes and Genomes). The Integrated Microbial Genomes (IMG) at JGI records primary genome information, finished or draft genomes, with functional annotation, provided from other databases (GenBank) or produced by the JGI itself. The complete pipeline, once the complete sequence of the organisms is submitted to IMG include the annotation of ribosomal and tRNA as well as protein-coding genes. The protein-coding genes are compared with protein families in the COG database with Blast, and TIGRfams with HMMER. They are also mapped to the KEGG database with KO (Kegg Orthology) terms and MetaCyc by EC (enzyme commission numbers) to associate them to metabolic pathways. One interesting feature at IMG is the design of biosynthetic clusters of genes associated to metabolic pathways involved in the production of secondary metabolites. Access to IMG is provided under request as user or submitter, providing agreement with the policies of the institution.

The KEGG database (Kanehisa et al. 2012) is one of the most complete references on metabolic pathways and cellular process that organize experimental knowledge and predictions. KEGG integrate 15 main databases divided into four categories: genomic, systems, chemical, and health information. KEGG has three main reference knowledge databases that consist of KEGG PATHWAY, BRITE, and MODULE. These databases are in close relation with the KEGG Orthology (KO) system, which defines sequence similarity groups that facilitate the identification of pathways in new sequenced genomes. To do this, KEGG has implemented genome analysis tools as KAAS (Moriya et al. 2007), an Automatic Annotation Server (KAAS) uses BLAST comparisons against the manually curated KEGG GENES database to generate functional annotation of genes for complete or partial genomes. The KAAS output contains KO assignments for the genes and automatically generated KEGG pathways list for the genome under study.

The genome sequences of *Azospirillum* are available from different public databases. For instance, in Genoscope it can be do diverse comparative analysis, metabolic reconstruction, and phylogenomics, using single-site web resources for AzospirilumScope through the MicroScope platform. Users get access as a guest with read rights or by an account created by petition to the project leader that give rights to explore the database deeply and participate directly in the annotation process. MicroScope allows setting up one of the *Azospirillum* genomes as your reference organism. This action will upload the web page that displays the data for the genome that the user has selected. Using the MaGe (Magnifying Genomes Tool) by clicking at the navigation bar it can be displayed the genetic map of the reference genome, the synteny map with respect to other *Azospirillum* genomes, and the expert annotation summary if available. Most of the analysis described in this chapter can be done at MicroScope since it integrates most of these applications.

At the end of the annotation process, a variable percentage of genes (about 30 % or less) remain described as hypotheticals of unknown function. To give important insight into the function of those hypothetical genes, context techniques that use the occurrence of genes in the same context (operon, synteny), or the phyletic co-occurrence of several genes are used. Databases like STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) (Franceschini et al. 2013) and KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa et al. 2012) use these approaches to infer the functional association among genes and provide deep genome annotations.

5.5 Whole Genome Alignments

Complete genomes allow deep analysis of structural variations and the degree of chromosome conservation among related bacterial species and strains. There are computer programs to perform alignments of whole genomes like MUMmer (Kurtz et al. 2004), VISTA (Frazer et al. 2004), and MAUVE (Darling et al. 2004). The first allow pairwise alignment both in terms of nucleotides and proteins to estimate synteny, rearrangements, and inversions. MAUVE permits alignment of multiple genomes, estimation of structural variations, and synteny blocks. In addition, MAUVE provides the basis for recombination analysis with ClonalFrame and Clonalorigin (Didelot and Falush 2007; Didelot et al. 2010) and losses and gains of genes with Genoplast (Didelot et al. 2009).

5.6 Genomic Islands, Phages, and Insertion Sequences

Bacterial genomes contain clusters of genes, called Genomic Islands (GIs), that were acquired by horizontal gene transfer. The presence of GIs can be addressed with computational programs which take advantage of the differential compositional

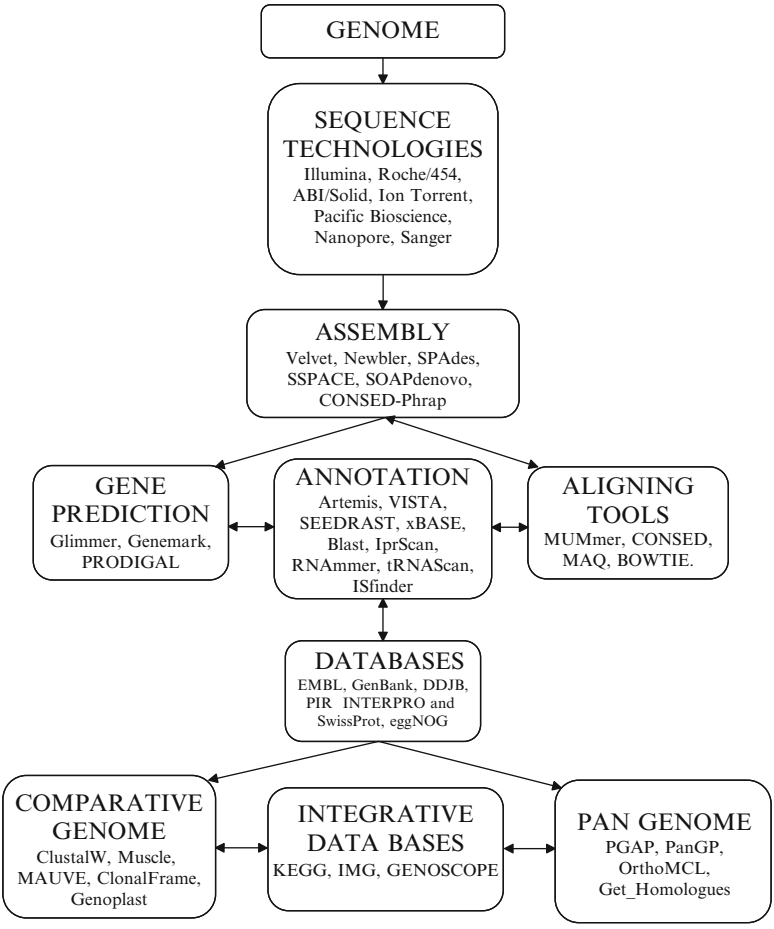

Fig. 5.1 Flow chart of genomic strategies, bioinformatics tools, and databases

features of the genome itself like GC content, frequency of di- or tetranucleotides, codon usage, their localization near to tRNA genes, and the presence of genes related to virulence, antibiotic resistance, or plasmid mobility. IslandViewer gets together three different genomic islands predictors (IslandPick, IslandPath, and SIGI-HMM) in an integrated web interface that allows easy viewing of the genomic island location in the chromosome, as well as to download annotations and coordinates of the island genes in different formats (Excel, FASTA, GenBank) (Langille and Brinkman 2009). An example of GIS prediction for *Azospirillum* 4B and B510 chromosome made with IslandViewer is given in Fig. 5.1.

Prophages are common genetic elements found in bacterial genomes. The computational programs PHAST (Zhou et al. 2011), Phage-finder (Fouts 2006), ProFinder (Lima-Mendez et al. 2008), and Prophage Finder (Bose and Barber 2006) combine similarity searches in databases and composition-based strategies to infer

the presence of phages integrated in the genome. The annotation of ORFs identified in phages is difficult because a great percentage of them (about 70 % in average) encode for hypothetical proteins, without homologs in the databases. One effort to systematize and cluster the phage proteins is the creation of Phage Orthologous Groups (POGs). This is composed by protein families involved in different phage processes like lysis, attachment-integration, replication, structure and assembly of the virion, and others (Kristensen et al. 2011a). The POG database also has concern for identifying phage genes as remnant of old infections, as well as active prophages in the bacterial genomes. In pathogenic *E. coli*, the percentage of POG-related genes are about 10 % whereas in the soil bacteria, such *R. etli* is about 5 % (Santamaria et al. 2014), and 2 % in *Azospirillum* .

A third class of mobile elements commonly found in the genomes is the insertion sequences. As more complete genomes become available reconstruction of their evolutionary history could be helped by tracing the evolution of the IS (Lozano et al. 2010). The most thorough compilation of IS families is the ISfinder (Siguier et al. 2006) that represent the reference database for the bacterial and archaea insertion sequences. Searches are exclusively done via comparative BlastN or BlastX under certain e-value that returns the best hit of candidate IS as well as the family features as repeats, ORFs number, and original host. However, IS are poorly annotated in the genomes providing limited information about the transposase and the inverted repeats. One effort to facilitate the annotation of IS in genomes has been done by Wagner et al. (2007), that survey bacterial insertion sequences in multiple genomes using the ISscan with a curated version of the ISfinder.

5.7 Orthologs and Protein Families

Underlying to all comparative genomic tools are the biological concepts of homology, orthology, and paralogy (for review, see (Kristensen et al. 2011b)). Homology means a gene that is related to other by descent from a common ancestral gene. Homologous genes of different species that diverge by speciation are called orthologs. Generally, orthologs retain the same function in the course of the evolution; therefore, orthologs identification is critical for reliable functional annotation, comparative genomics, and phylogenomic analyses. When homologous genes are duplicated within a genome or an evolutionary branch, they are named paralogs. They constitute gene families within the same genome, and they might have diverse functions.

To identify orthologs in genomes, there are two general methods: phylogenetic (tree-based) and bioinformatic (graph-based) (Kuzniar et al. 2008). Owing to the biological roots of the concept, phylogenetic methods are more accurate to get homologs. There are databases of orthologous genes used as reference to annotate the orthologs of a new genome by BLAST comparison. Among the phylogenetic

databases that provide genome-wide orthology and paralogy predictions are PhylomeDB (Huerta-Cepas et al. 2008), PhyloFacts (Datta et al. 2009), EnsemblCompare Gene Trees (Vilella et al. 2009), PANTHER (Mi et al. 2007), and HOGENOM (Dufayard et al. 2005). Since phylogenetic reconstruction at genomic level is computationally expensive these databases include only representative organisms of some taxonomic group. That is why most of the scientists use heuristic bioinformatics approach based on the pairwise sequence similarities between all the proteins and their posterior clusterization to construct homologous groups by reciprocal or bidirectional best hits. The OrthoMCL (Li et al. 2003), software based on the Markov Cluster algorithm (MCL), is used for grouping a large set of proteins in orthologs and recent paralogs from two or more genomes after BLASTp comparison of the whole proteomes. The OrthoMCL-DB (Chen et al. 2006), the database generated using OrthoMCL, is limited to 150 genomes representing phylogenetically diverse lineages. A more complete database is OrthoDB (Waterhouse et al. 2013) that uses 1,367 genomes from which 1,115 belong to prokaryotic organisms. This database provides functional annotation through mapping to other database like Gene Ontology (GO) and InterPro, as well as cross-references to UniProt and NCBI. In some cases is important to distinguish between genes duplicated after the speciation event (in-paralogs) and genes duplicated before the speciation event (out-paralogs). InParanoid (Ostlund et al. 2010) and QuartetS-DB (Yu et al. 2012) are able to separate these two classes of paralogs.

The Cluster of Orthologous Groups of proteins (COGs) at NCBI (Tatusov et al. 2003) in its updated version was constructed with the reciprocal best hits of proteins of 66 organisms (63 prokaryotic and 7 eucaryotic). The orthologous groups have one homolog at least in three distinct evolutionary branches and correspond to 17 functional categories. This classification system is widely used despite of the NCBI COG database has not been recently updated. Nowadays, the most complete and comprehensive compilation of clusters of orthologs is the EggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) (Powell et al. 2014) database that covers 3,686 organisms from which 3,496 prokaryotic genomes selected from the RefSeq-NCBI (Pruitt et al. 2012) repository. The coverage of the selected bacterial genomes in the EggNOG database is closely to 80 % that means that the majority of the protein-coding genes of one bacterial species belong to a cluster of orthologs (Koonin and Wolf). Both COGs and EggNOGs can be mapped in the course of the annotation process using the COGNITOR method (Makarova et al.).

5.8 Phylogenomics and the Pangenome Model

Phylogenomics is an area that includes several of the problems of phylogenetics at whole genome scale. It addresses the phylogenetic reconstruction of the organisms by whole genome phylogenies to evaluate the genomic coherence of the species tree

(Koonin and Wolf 2008), as well as the impact of the horizontal gene transfer in the ecological adaptation and species differentiation (Wisniewski-Dye et al. 2011). One aspect of the bacterial phylogenomics is the determination of the pangenome of the species. Comparison of multiple genomes of the same bacterial species led to discover a large reservoir of genes present only in individual strains, and a common core of genes shared by all the individuals. The primordial housekeeping functions are encoded by the core genome, whereas the dispensable functions often represent adaptations to particular ecological niche (Wisniewski-Dye et al. 2012). This component is the variable or accessory genome that together with the core constitutes the pangenome of the species. Since *Azospirillum* is free-living organisms with a high number of genes, it could be expected a large pangenome. Insights on the pangenome of *Azospirillum* with a limited set of genomes have been published (Wisniewski-Dye et al. 2012).

There are several programs implemented to model the pangenome. For instance, Pan-Genome Analysis Pipeline (PGAP) (Zhao et al. 2012), PanGP (Zhao et al. 2014), and GET_HOMOLOGUES (Contreras-Moreira and Vinuesa 2013) can perform different analytic functions: cluster all the genes, generate pan-genome profile, detect genetic variation in each gene cluster, and construct phylogenetic trees. In particular, GET_HOMOLOGUES is very flexible customizable and robust pipeline that calculate the pangenome from dozens bacterial species at different phylogenetic distances. It performs well in relative modest computing time and is accessible for non-trained people in bioinformatics. The design allows download the genome sequence and annotations, running BLAST and HMMER to find homologous genes and domains, including three distinct clustering procedures (OrthoMCL, COGs, and BDBH). The same program produces phylogenetic trees of the pangenome based on loss and gain of genes, as well as core phylogenies. Graphic representations of the pangenome components and phylogenetic trees can be obtained given the appropriate command lines.

5.9 Concluding Remarks

Along with the ease to obtain genome sequences, bioinformatics resources have also grown exponentially. A summary or resources and methods cited in this chapter are provided in the Fig. 5.2. Critical bottlenecks remains in the bioinformatic analysis concerning to search for significant biological meaning to that plethora of data. Experimental biologist needs friendly programs and computer interfaces to interact with most of the data generated by genomic procedures. Learning basic programing languages (PERL, PHYTON, R) could contribute to integrate the better of two worlds: genomic big data and experimental findings.

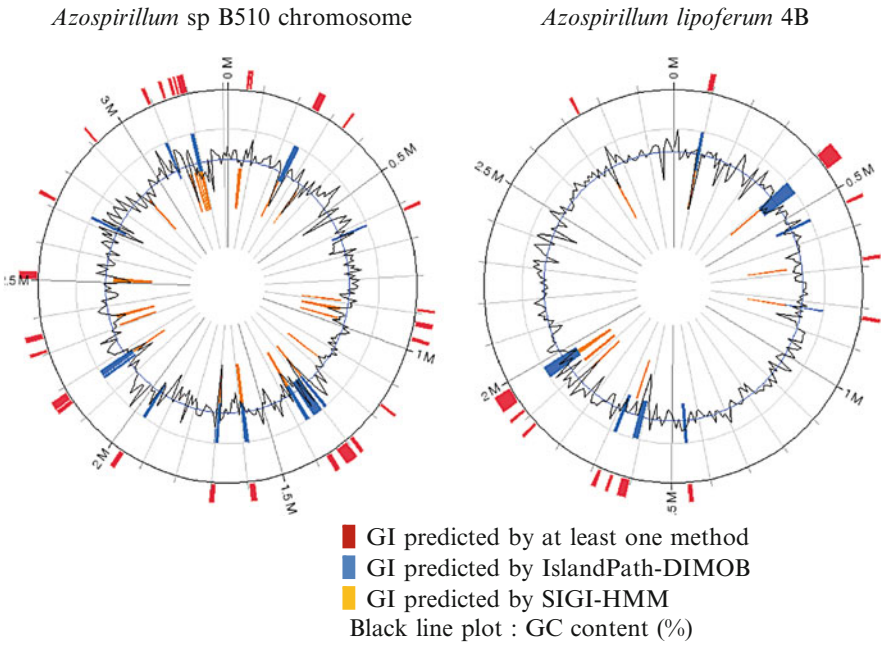

Fig. 5.2 Genomic islands predicted in two *Azospirillum* genomes according to IslandViewer

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Part II
Physiology and Metabolism Toolbox

Chapter 6
Chemotaxis in *Azospirillum*

Gladys Alexandre

Abstract The ability of *Azospirillum* to colonize the roots of plants depends on motility and chemotaxis. *Azospirillum* cells are motile and capable of chemotaxis toward organic acids, sugars, and some aminoacids. *Azospirillum* is also able to navigate gradients of oxygen, alternative electron acceptors, and redox active compounds. Most attractants and repellents described thus far for this bacterial genus include compounds that affect intracellular metabolism, leading to the suggestion that most taxis responses correspond to energy taxis in *Azospirillum* spp. Several spatial and temporal gradient assays that can be implemented as quantitative methods are available to characterize taxis responses in *Azospirillum* species. The analysis of complete sequence genomes of several *Azospirillum* species reveals that taxis responses are coordinated by multiple chemotaxis pathways. All genomes also possess a chemotaxis pathway that is predicted to regulate alternative cellular functions other than flagellar motility. Genome analyses indicate that all *Azospirillum* spp. sequenced to date encode for an extremely large repertoire of putative chemotaxis receptors, which is likely contributing to explaining their ubiquitous distribution.

6.1 Chemotaxis: An Overview of Paradigm

Microorganisms have evolved a plethora of strategies to adapt to spatial and temporal changes in the environment. Some of these strategies involve changes in patterns of gene expression, and thus the implementation of long-term adaptive responses. In addition, many bacteria are able to rapidly and transiently respond to changes in the environment. In motile bacteria, these transient adaptive responses include the ability to navigate toward more favorable conditions or away from deleterious ones. This behavior is referred to as chemotaxis when motile bacteria respond to gradients of chemical effectors, aerotaxis when they respond to gradients of oxygen,

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phototaxis when they respond to gradients of light, pH taxis when they respond to gradients of pH, etc. (Wadhams and Armitage 2004). Because of their small sizes, bacteria experience low Reynolds number environments, where inertia is negligible and where viscosity is a major force that constrains movement of particles and bacterial cells. As a result, motile bacteria use an undulatory behavior via helical flagella that rotate as propellers to move the cell forward or backward. Furthermore, bacteria behave as particles in a solution and they are subjected to physical forces, such as Brownian motion, that do not impact the behavior of larger organisms (Berg 1993). Brownian motion is essential to randomly reorient swimming bacteria in new directions, and this physical force is particularly significant for the reorientation of singly polarly flagellated bacteria (Mitchell 2002), such as bacteria of the genus *Azospirillum*. Furthermore, because of their small size, motile bacteria do not detect gradients spatially. Instead, they use temporal sensing in which current conditions are compared with the ones encountered a few milliseconds before. In the absence of a gradient, motile bacteria move about randomly and thus not in any particular direction. When moving up a gradient of an attractant, motile bacteria suppress the changes in swimming direction in the direction of the attractant and swim longer toward the attractant. As a result, their net movement is up the attractant gradient. Conversely, in a gradient of a repellent, bacteria tend to change swimming directions more frequently in the direction of the repellent but less when moving away from it. This behavior allows them to run longer away from the repellent (Sourjik and Wingreen 2012).

The molecular mechanism of bacterial chemotaxis has been studied in great detail in the model organism *Escherichia coli*, a peritrichously flagellated bacterium (Wadhams and Armitage 2004; Sourjik and Wingreen 2012). The *E. coli* proteins that comprise the chemotaxis signal transduction pathway are encoded within a single operon, with additional chemotaxis receptors located elsewhere on the genome. Environmental signals are detected by chemotaxis receptors (chemoreceptors or the so-called methyl accepting chemotaxis proteins or MCP) that are arranged in large arrays of allosterically coupled proteins located at the cell poles (Hazelbauer et al. 2008). *E. coli* possesses five different chemotaxis receptors: Tar, Tsr, Trg, Tap, and Aer. Trimers of receptor dimers are bound on their cytoplasmic side by a ring of CheA-CheW proteins (Briegel et al. 2012). CheA is the dedicated histidine kinase, while CheW is a docking protein that anchors CheA and the chemoreceptors within the array. Reception of a signal triggers a conformational change in chemoreceptors, thought of as a piston-like movement, that in turn modulates the activity of the CheA histidine kinase. Upon receiving a repellent signal, CheA becomes phosphorylated on a conserved histidine residue. Phospho-CheA then phosphorylates two response regulators: CheY and CheB. Phospho-CheY, but not unphosphorylated CheY, is able to bind to the flagellar motors with high affinity to trigger a change in the swimming direction. CheB is a methylesterase, which demethylates conserved residues in the cytoplasmic domains of receptors and counteracts the methylation of these residues by a constitutively expressed CheR methyltransferase. Changes in the methylation status of receptors reset them in a sensing mode. Because methylation and demethylation are catalytically slower than phosphorylation, CheB and CheR

affect receptors at a slower rate compared to the rate of phosphorylation of CheA and CheY. This time delay confers a short "memory" in bacterial chemotaxis. *E. coli* also possesses a phosphatase, CheZ which enhances CheY dephosphorylation and thus contributes to signal termination (Hazelbauer et al. 2008).

This mode of signal transduction represents a blueprint for bacterial chemotaxis. The molecular principles that govern chemotaxis in *E. coli* appear to be conserved in other bacterial and archaeal species, albeit with several differences (Wadhams and Armitage 2004; Briegel et al. 2009; Wuichet and Zhulin 2010). The *E .coli* chemotaxis system is also very simple in comparison to other bacterial genomes in terms of number of chemotaxis operons and additional chemotaxis genes, as well as the greater number of chemoreceptors present (Wuichet and Zhulin 2010). There is a need to characterize a broader diversity of chemotaxis systems to gain an accurate perspective of the role of this behavior in the ability of bacteria to respond and adapt to changes in their surroundings.

6.2 Types of Taxis Responses in *Azospirillum* spp.

Bacteria of the genus *Azospirillum* are motile by means of a single polar flagellum in liquid media (swimming) and several lateral flagella when moving across surfaces (swarming). The polar and lateral flagella are not only functionally different, but they also comprise a distinct set of structural proteins (Moens et al. 1995, 1996). While chemotaxis has been described to regulate polar flagellum mediated motility, i.e., swimming, in liquid environments, experimental evidence for chemotaxis regulating swarming motility has yet to be obtained.

Taxis behaviors have long been suggested to contribute to the ability of *Azospirillum* spp. to colonize the roots of plants, and experimental evidence in support of this assumption has been obtained (Reinhold et al. 1985; Bashan and Holguin 1994; Vande Broek et al. 1998; Greer-Phillips et al. 2004). Motility and chemotaxis have been best described in *A. brasilense* strain Sp7, which is thus the model for understanding taxis behaviors in this bacterial genus.

6.2.1 Chemotaxis

A. brasilense is capable of chemotaxis toward a large set of organic acids, sugars, sugar alcohols, and to a lesser extent, amino acids (Okon et al. 1980; Alexandre et al. 2000). By testing a range of chemoeffector concentrations in different types of chemotaxis assays (see below), it is possible to determine the relative strength of a chemoeffector. The chemotaxis response toward a particular chemical usually correlates with its concentration. True chemotaxis is optimum at a certain concentration and declines proportionally at higher and lower concentrations. A strong chemoeffector elicits a maximum chemotaxis response at low concentrations, while a weak

chemoeffector elicits a peak chemotaxis response at relatively high concentrations. The stronger attractants for *A. brasilense* are also the best growth substrates, and starving cells display an enhanced chemotaxis response (Alexandre et al. 2000). Organic acids that are also intermediates of the citric acid cycle, such as malate and succinate, are very strong attractants for *A. brasilense*. Pyruvate, citrate, oxalate, and fumarate also represent good attractants for this species.

Amongst the sugars, fructose is one of the strongest attractants but ribose, arabinose, and galactose are also good attractants for *A. brasilense*. As in many other bacterial species, chemotaxis to galactose is inducible, and it is not detected unless the cells are grown in the presence of galactose prior to measuring chemotaxis (Alexandre et al. 2000). Galactose metabolism is inducible in *A. brasilense* and includes expression of a specific ABC transport system, which uses a periplasmic binding protein named SbpA. In *A. brasilense*, galactose chemotaxis is mediated by a chemotaxis receptor that also uses SbpA and a mutant lacking SbpA is specifically impaired in galactose chemotaxis and metabolism (Van Bastelaere et al. 1999). It is noteworthy to indicate that *A. brasilense* is unable to display chemotaxis toward glucose. This observation makes sense when one considers that glucose is not a carbon source used by *A. brasilense*.

Amino acids such as glutamate, aspartate, alanine, glutamine, and asparagine are weak attractants for *A. brasilense*. The weak propensity of these amino acids to elicit a chemotaxis response likely results from the diazotrophic metabolism of *Azospirillum* spp. and thus the limited reliance on external organic nitrogen sources such as amino acids for nutrition. Glycine, histidine, isoleucine, threonine, valine, arginine, lysine, and methionine are not chemoeffectors for *A. brasilense* (Okon et al. 1980; Alexandre et al. 2000).

Chemotaxis toward phenolic compounds such as benzoate, catechol, and hydroxybenzoate was reported (Lopez-de-Victoria and Lovell 1993) but could not be reproduced by Alexandre et al. (2000). This discrepancy may be related to the different assays used by these authors. In particular, Lopez de Victoria and Lovell (1993) used the capillary assay, which is prone to false-positive results due to the strong aerotaxis response of *Azospirillum* in capillary tubes assays, which can mask the chemotaxis response measured. Several studies analyzing chemotaxis in *Azospirillum* spp. have concluded that the capillary assay was inadequate to assess chemotaxis in this species (Okon et al. 1980; Barak et al. 1982; Alexandre et al. 2000).

6.2.2 Aerotaxis

The strongest taxis response in *Azospirillum* is aerotaxis or the directed movement of bacteria in gradients of oxygen (Barak et al. 1982; Zhulin et al. 1996).

Azospirillum spp. have a microaerophilic oxidative metabolism that is optimum at about 0.4 % dissolved oxygen (Zhulin et al. 1996). *Azospirillum* thus grow best under low aeration conditions and when exposed to a spatial oxygen gradient, motile cells move away from elevated oxygen concentrations and toward low oxygen

concentrations. The physiological consequences of this behavior are readily observed when motile *A. brasilense* cells are inoculated in a tube containing a low agar concentration to permit motility: cells concentrate as a band that grows below the surface but never on top of the agar. Note that the formation of a dense area of growth in this assay does not solely represent an aerotaxis response. Indeed, the formation of this zone of growth below the surface depends not only on the motility of cells but also on their ability to navigate in oxygen gradients (i.e., aerotaxis) as well as to grow under these conditions. Aerotaxis requires that the cells have an active respiratory chain, suggesting that it is not sensing oxygen itself that triggers the movement in the oxygen gradients and that cells are sensing oxygen indirectly, by monitoring another parameter related to respiration with oxygen (Alexandre et al. 2000).

6.2.3 Taxis to Alternative Electron Acceptors

In the absence of oxygen, *Azospirillum* spp. are able to use alternative terminal electron acceptors, including nitrate, nitrite, and DMSO, for respiration. It is thus not surprising that chemotaxis in gradients of alternative electron acceptors has also been described in *A. brasilense* (Alexandre et al. 2000). These taxis responses are much weaker than the aerotaxis response. Consistent with the bacteria sensing nitrate, nitrite, or DMSO as terminal electron acceptors and not to fulfill other nutritional needs, taxis responses toward these compounds is only observed under anaerobic conditions and they are abolished when cells are incubated with oxygen. The taxis response is also not observed if cells are not provided with an electron donor in a form of a carbon substrate for growth.

6.2.4 Redox Taxis

Redox taxis is a motility behavior first described in *E. coli* (Bespalov et al. 1996) and it corresponds to the ability of bacteria to navigate gradients of redox active compounds. During redox taxis, bacteria monitor changes in redox, instead of change in the concentration of a specific compound. Redox taxis is determined by using substituted quinones, which are small lipophilic compounds able to diffuse through the membrane. Redox taxis can be convincingly established by analyzing the taxis responses of cells in gradients of quinones with different redox potentials (Bespalov et al. 1996; Alexandre et al. 2000). In order to be redox active, the quinones are maintained in an oxidized state using potassium ferricyanide. Redox taxis is best established using oxidized quinones which have redox potential values within the range of redox potentials that permit electron transfer in the electron transport chain. One such example is 1,4-benzoquinone (Bespalov et al. 1996). Quinones differ in chemical structure as well as redox potential; the lower the redox

potential of the quinone tested, the greater the affinity of the oxidized quinone for electrons, and thus the more likely it is this oxidized quinone will effectively compete for electrons. *A. brasilense* motile cells demonstrate a repellent response to oxidized quinones: the repellent response increases with the decreasing redox potential of the quinone tested (Alexandre et al. 2000). Redox taxis can be inferred when the repellent response displayed by motile *Azospirillum* cells correlates with the redox potential, rather than the chemical structure of the quinones tested.

6.2.5 *Energy Taxis as the Dominant Mode of Environmental Sensing*

Most but not all chemotaxis and other taxis responses in *A. brasilense* require a functional electron transport system, and these responses are enhanced when cells are starved (Alexandre et al. 2000). Further, the strength of the taxis response correlates with the extent to which chemoeffectors affect the integrity and efficiency of the electron transport system. Electron donors and electron acceptors are excellent attractants for *A. brasilense* while chemicals that block electron transport within the electron respiratory chain, such as quinones or myxothiazol trigger repellent responses. Taxis responses toward particular chemicals are also abolished if structural analogs of these chemicals, that cannot be metabolized, are used. Most taxis responses of motile *A. brasilense* cells appear to originate within the electron transport system, an observation that has led to the proposition that energy taxis dominates motile behavioral responses in this species (Alexandre et al. 2000). Energy taxis refers to the ability of motile cells to navigate in gradients of physicochemical parameters that affect the energy-generating cellular processes such as the electron transport chain (Alexandre 2010). Dedicated chemotaxis receptors proposed (Tlp1; (Greer-Phillips et al. 2004)) or demonstrated (AerC; (Xie et al. 2010)) to sense redox-related parameters have indeed been characterized in *A. brasilense* Sp7. While most taxis responses are related to energy taxis in *A. brasilense*, metabolism-independent taxis also functions in this species as suggested by the role of the periplasmic binding protein SbpA in chemotaxis toward galactose (Van Bastelaere et al. 1999)

6.3 Assays Suitable for Measuring Chemotaxis, Aerotaxis, and Redox Taxis in *Azospirillum* spp.

Several quantitative and qualitative assays to monitor all forms of taxis responses have been developed and optimized in *A. brasilense* and can be used in any of the *Azospirillum* spp. (Fig. 6.1).

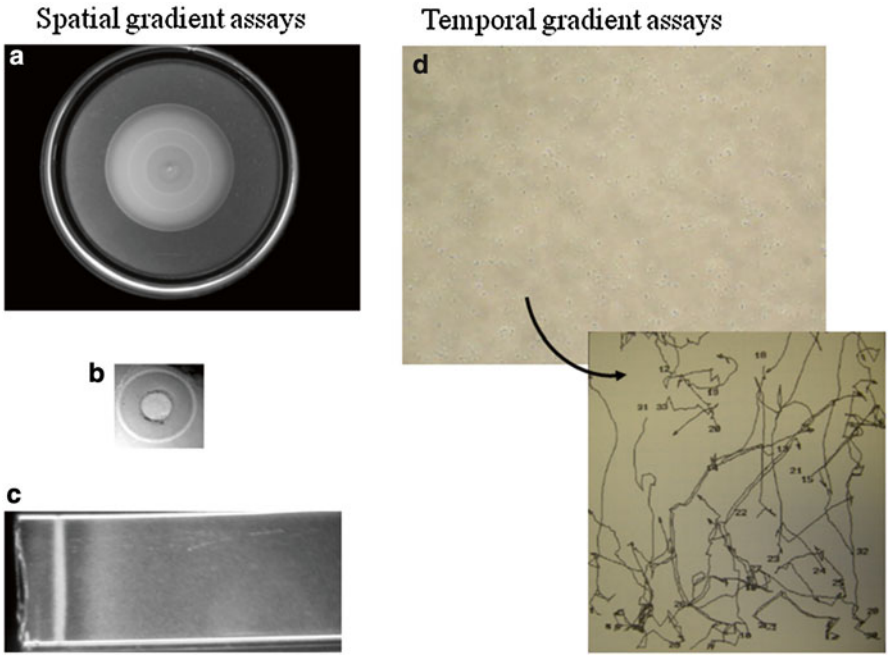

Fig. 6.1 Spatial and temporal gradient assays used to analyze taxis responses in *Azospirillum* spp. (**a**) Soft-agar plate assay; (**b**) mini-plug assay; (**c**) spatial gradient assay for aerotaxis; (**d**) temporal gradient assay. *Top*: example of a still image of a video captured to analyze by computational motion analysis. *Bottom*: example of tracks of individual cells detected by a computerized motion analysis system. See text for details

Regardless of the taxis response measured, using cell populations where at least 90 % of cells are motile and preferably grown in minimal medium to low density, is preferred to ensure homogenous results and avoid the potential interfering effect of the accumulation of intracellular granules of polyhydroxybutyrate (Alexandre et al. 2000). In addition, some taxis responses are inducible in *A. brasilense*. When testing taxis to compounds for which no previous experimental record exists, it is best to measure the taxis responses toward this compound in parallel assays that use cells induced with the compound to be tested as well as without the compound to be tested to ensure an accurate interpretation of the results.

Assays suitable for measuring taxis responses in *Azospirillum* spp. include spatial gradient assays (soft-agar plate, chemical-in-plug, aerotaxis assays) and temporal gradient assays. In spatial gradient assays, cells' metabolism interferes with the responses observed and thus, controls must be included to insure that taxis responses per se are measured. The most sensitive chemotaxis assays for *Azospirillum*, i.e., those able to detect the weakest behavioral responses are the temporal gradient assays while the least sensitive is the soft agar plate assay. Regardless of whether a spatial or temporal gradient assay is used, taxis responses are dose dependent and

show a maximum under specific conditions. A spatial or temporal gradient assay may thus be used in quantitative characterization of taxis behaviors by establishing a dose-dependent response. In this case, the stronger the chemoeffector, the lower the concentration at which it triggers a taxis response.

6.3.1 Soft Agar Plate Assay

The soft agar plate assay is carried out in media solidified with a low concentration of agar to allow cells swimming. This assay is often erroneously called a "swarm plate assay." Swarming is a form of motility across a surface and it requires different flagella and cell surface characteristics. The soft agar plate assay measures the ability of motile cells to swim down attractant gradients created via cellular metabolism. In this assay, cells from an overnight culture or a colony are inoculated in the center of a soft agar plate containing 0.3 % agar and a single carbon and nitrogen source that the cells can use for growth (Fig. 6.1, panel A). For this assay to be successful, the soft agar medium prepared with 0.3 % agar (w/v) must be boiled before autoclaving or pouring the plates to ensure uniform distribution and consistent results. Due to the sensitivity of *Azospirillum* spp. to oxygen gradients, it is also recommended that separate plates contain equivalent volumes of medium (e.g., 25 mL molten medium/plate).

Cells growing at the point of inoculation deplete the local environment of carbon and nitrogen sources. If these cells are motile and able to navigate chemical gradients (i.e., able of chemo-taxis responses), they will move away from this inoculation zone toward higher concentrations of nutrients and thus will spread outward. The presence of a chemotaxis ring, seen as a dense zone of cells with the cell density dropping before and after this zone, is indicative of chemotaxis or taxis to alternative electron acceptors, when performed under anaerobic conditions.

A variation of this assay consists of inoculating the cells in a soft agar plate made with a minimum buffer, and providing chemoeffectors "spots," distant to the inoculation point. The gradient of the chemoeffector tested will be established by diffusion, and the cells will preferentially move in the direction of the chemoeffector if it functions as an attractant. This latter assay has been used to demonstrate chemotaxis of *Azospirillum* toward the root exudates of various plants (Okon et al. 1980).

6.3.2 Chemical-in-Plug Assay

The chemical-in-plug assay is a spatial gradient assay that was first developed by Tso and Adler to detect chemotaxis responses toward repellents (Adler and Tso 1974). In *Azospirillum* spp., this assay can be used to measure chemotaxis, and it is also particularly well suited to analyze taxis responses to alternative electron

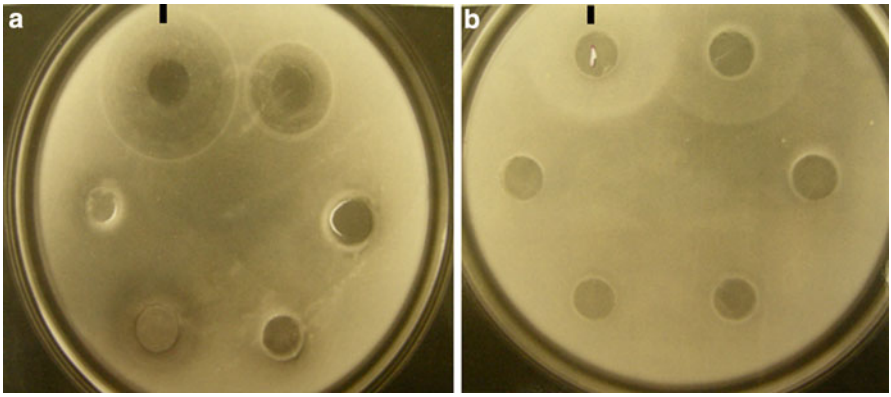

Fig. 6.2 Mini-plug assay used to detect repellent responses (**a**) and attractant responses (**b**). The observations were photographed after 1 h incubation at 28 °C. (**a**) Plugs contain the following described in clockwise direction from the mark on the plate: 1,4-benzoquinone (10 mM) and ferricyanide (2 mM), 1,4-benzoquinone (1 mM) and ferricyanide (2 mM), 1,4-benzoquinone (0.1 mM) and ferricyanide (2 mM), ferricyanide (2 mM) alone, acetate (10 mM), chemotaxis buffer. Note the clearing zone around the plugs in the repellents (1,4 benzoquinone and acetate). The size of the clearing zone depends on the concentration of the effector tested as seen with 1,4-benzoquinone. (**b**) Plugs contain the following described in clockwise direction from the mark on the plate: Malate (10 mM), Fructose (10 mM), Galactose (10 mM), Aspartate (10 mM), Proline (10 mM); Isoleucine (10 mM). Note the accumulation of cells around the plugs. The zone of accumulation is faint around Aspartate and Proline, and there is no accumulation around Isoleucine or Galactose. Cells were not induced with galactose prior to this assay

acceptors and to assess redox taxis (Figs. 6.1, panel B and 6.2). In this assay, the gradient of the chemoeffector to be tested is established spatially by diffusion from a plug of agar solidified with 2 % agar (w/v) placed into a soft agar (0.3 % agar) medium that also contains motile cells. The cells will form a ring around the plug if they respond tactically to it (Fig. 6.2). In the case of a repellent response, the ring will form around the plug but a clearing zone will be seen between the plug and the ring of cells. Confirmation of a taxis response and exclusion of growth as being responsible for the formation of the ring can include performing the assay in the presence of chloramphenicol or tetracycline to inhibit growth and by omitting essential nutrients (e.g., a carbon source) to prevent growth. The latter conditions may be achieved by using plugs and soft agar plates prepared in chemotaxis buffer (10 mM phosphate, 0.1 mM EDTA). Typically, a very dense cell suspension is mixed with an equal volume of 0.6 % agar in chemotaxis buffer to yield a soft agar at a final concentration of 0.3 %. Plugs of 2 % agar also prepared in chemotaxis buffer are cut out using large pipette tips as tools and placed in the still molten soft agar medium. Results are usually obtained between 1 and 4 h post-inoculation. A key parameter to consider in this assay is to use a dense suspension of motile cells which may be prepared from cultures grown to low density and concentrated

by centrifugation at low speed (to prevent shearing of the flagella). Different concentrations of chemoeffectors, ranging from 1 to 20 mM, can be introduced into the plugs in order to determine the threshold for the attractant or repellent response, which also makes the assay semiquantitative.

6.3.3 Mini-Plug Assay

A variation on the chemical-in-plug assay for use with a light microscope is the mini-plug assay. In this version of the assay, a small plug of 1.5 % low melting point agarose in chemotaxis buffer containing the chemical to be tested as a chemoeffector is placed in a microchamber. The microchamber is constructed using silicon grease to delineate a small volume the size of the coverslip to be used. A 100 μL aliquot of motile cell suspension, washed prior to being placed in chemotaxis buffer, is introduced into the microchamber and covered with a coverslip. A control plug containing only chemotaxis buffer must also be prepared. The formation of a chemotactic band away from (repellent effect) or near (attractant effect) the plug can be observed in real time. Chemoeffectors are tested in a wide concentration range, typically from 1 μM to 1 mM. Compared to the chemical-in-plug assay, the mini-plug yields results within a few minutes and there is no possible confounding effect of growth.

6.3.4 Aerotaxis Assay

Aerotaxis in *Azospirillum* can be analyzed using a spatial gradient assay where the oxygen gradient is established by diffusion of from air inside a suspension of highly motile cells. This assay is performed in optically flat microcapillaries (e.g., inner dimensions, 0.1 by 2 by 50 mm; Vitro Dynamics Inc., Rockaway, N.J.) and a light microscope. The microcapillaries are filled by capillarity by dipping them into a suspension of motile cells prepared by gentle washes and centrifugation at low speed and resuspension into fresh medium containing a carbon source and possibly, a nitrogen source. The formation of the aerotactic band is exclusively observed at the end of the microcapillary that was dipped into the cell suspension and not at the opposite end since conditions there are not controlled and consistent. Under these conditions, microscopic observations indicate that motile cells move from locations within the microcapillaries of higher and lower concentrations of oxygen toward a zone of preferred low oxygen concentration, forming an aerotactic band (Fig. 6.3). This aerotactic band forms within 2 min and remains stable over at least 30 min. The area or distance between the aerotactic band and the air-liquid meniscus can be measured for a semiquantitative use of this technique. However, the exact location of the aerotactic band with respect to the meniscus depends on cell motility, cell density, carbon source used as a substrate under these conditions, the respiration rate, and the stage of growth. Experimental conditions must therefore be calibrated to ensure reproducibility.

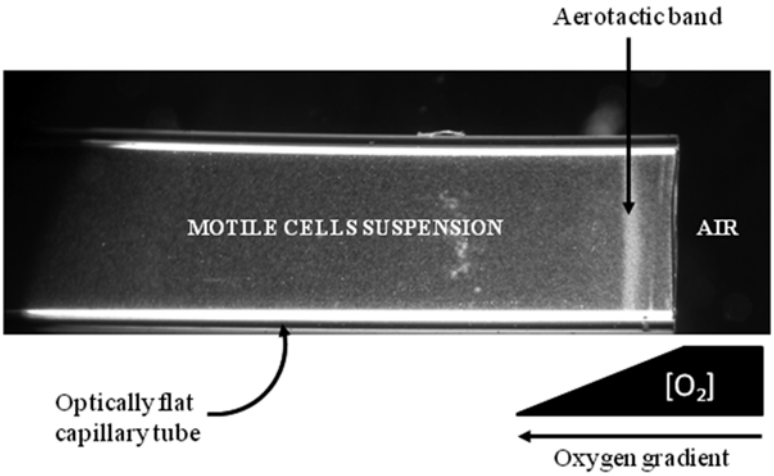

Fig. 6.3 Temporal gradient assay for aerotaxis in *Azospirillum* spp. using an optically flat capillary tube. This is a typical image taken 2 min after the capillary tube was filled with the cell suspension

6.3.5 Temporal Gradient Assay

The temporal gradient assay is the most direct assay to measure taxis responses in *Azospirillum* or any other motile bacterium because the ability of cells to temporally adjust their swimming motility bias (number of changes in swimming direction or reversals per unit of time) upon stimulation with an effector is measured (Fig. 6.1; panel D). Both attractant (transient suppression of reversals) and repellent (transient increase in the number of swimming reversals) responses can be measured. Furthermore, the response time (the time it takes for 50 % of the cell population to respond to the stimulus imposed) and adaptation time (the time it takes for the population to return to the pre-stimulus swimming bias) can also be measured using this method. Adaptation is the hallmark of bacterial taxis behaviors, and it cannot be measured in any of the other assay. In the temporal gradient assay, the chemoeffector (oxygen, air, or any chemicals) to be tested is directly added to a suspension of motile cells. Changes in the motility bias upon addition of the effector is recorded in real time and analyzed using a computerized motion analysis system (e.g., CellTrack, Hobson Tracker) to determine the reversal frequency or average number of changes in swimming direction per unit of time (Fig. 6.1; panel D). Video recording of the entire assay is required. A 9-μL drop of a diluted bacterial suspension (~10^7 cells/ml to facilitate tracking and downstream computerized motion analysis) in chemotaxis buffer is first placed on a microscope slide and equilibrated for at least 5 min. Next, the chemical compound to be tested (1 μL) is added to the suspension and video recording must be allowed for at least 5 min post-stimulation. Temporal assays can be performed under fully aerated conditions or under anaerobic conditions in a

microchamber (Zhulin et al. 1996; Alexandre et al. 2000), which allows the response in a temporal gradient of alternative electron acceptors or oxygen to be measured.

6.4 Chemotaxis in *Azospirillum* spp. Genomes

In contrast to the model organism *E. coli* which possesses a single chemotaxis pathway encoded within a single operon and only five different chemotaxis receptors, the genomes of *Azospirillum* species sequenced thus far indicate the presence of several chemotaxis signal transduction pathways encoded in operons and a very large number of receptors (Kaneko et al. 2010; Sant'Anna et al. 2011; Wisniewski-Dye et al. 2011, 2012).

The genome of *A. amazonense* Y2 encodes three chemotaxis pathways and 40 chemotaxis receptors, that of *A. brasilense* Sp245 encodes four chemotaxis pathways and 51 chemotaxis receptors, that of *A. lipoferum* 4B encodes five chemotaxis pathways and 63 chemotaxis receptors and the genome of *Azospirillum* sp. B510 codes for six chemotaxis pathways and 89 chemotaxis receptors. While most soil bacteria possess a large number of chemotaxis receptors, the average number is around 25–30. Bacteria of the genus *Azospirillum* have thus an extremely large repertoire of chemotaxis receptors, which further complicates the identification of their sensory abilities. Indeed, the sensory specificity of most chemotaxis receptors is not known (Krell et al. 2011). This complexity also suggests that chemotaxis signal transduction provides *Azospirillum* with a competitive advantage in the soil and the rhizosphere. Such a large repertoire of putative chemotaxis receptors suggests that *Azospirillum* spp. can monitor diverse environmental cues. This ability is likely contributing to explaining their ubiquitous distribution. However, the exact advantage that possessing multiple pathways provides cells with is not yet clear.

Of the multiple chemotaxis pathways encoded within the *Azospirillum* spp. genomes, most are predicted to control flagellar-based motility and thus probably direct swimming and/or swarming. One of the chemotaxis operons found in all *Azospirillum* spp. is not predicted to control flagellar motility but rather another cellular function (belonging to a larger group of ACF (Alternative Cellular Function)-like pathways) (Wuichet and Zhulin 2010). In *A. brasilense* Sp7, chemo- and aerotaxis are characterized by a transient change in swimming speed as well as a transient change in the swimming motility bias. Each of these behaviors is controlled by at least two different chemotaxis pathways (Stephens et al. 2006; Bible et al. 2008, 2012); one pathway controls transient changes in swimming speed during chemo- and aerotaxis (named Che1 (Bible et al. 2012)) and a second one (or perhaps more?), not yet identified, is implicated in controlling transient changes in swimming directions (Russell et al. 2013). In addition, signaling cross talk between Che1 and another chemotaxis pathway has been identified but is yet to be characterized at the molecular level (Stephens et al. 2006).

6.5 Conclusions and Outlook

Several genome sequences and a suite of quantitative and qualitative assays are available to study taxis behaviors in *Azospirillum* spp. The extended tool kit to assess taxis responses in this species provides an opportunity to use *Azospirillum* chemotaxis signal transduction as a model system for understanding how sensing and signaling are coordinated and integrated by multiple chemotaxis pathways. The complexity of chemotaxis signal transduction and the large number of chemotaxis receptors challenges the notion that chemotaxis could be manipulated to enhance the ability of bacteria to colonize the roots of plants and thus benefit plant health. However, deciphering the role of taxis responses in maintenance of *Azospirillum* spp. in the rhizosphere as well as the soil and in competing with other organisms could suggest avenues in which chemotaxis could be used in agricultural applications.

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Chapter 7
Phytohormones and Other Plant Growth Regulators Produced by PGPR: The Genus *Azospirillum*

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Abstract One of the first mechanisms proposed to explain the plant growth promotion due to bacterial inoculation has been the nitrogen biological fixation; however, this mechanism has been of less agronomic significance than was initially expected. In counterpart, other mechanisms have been proposed to explain these inoculation effects, such as production of phytohormones and other plant growth regulators. Nowadays, we know that benefic effect of inoculation with rhizobacteria can be correlated with production and metabolism of chemically defined compounds such as auxins, cytokinins, gibberellins, and other plant growth regulators, such as abscisic acid and nitric oxide. In this chapter, we highlight the analytical and biological methods used to identify and quantify the most relevant phytohormones and plant growth regulators produced by *Azospirillum* sp. and other benefic rhizobacteria.

7.1 Introduction

Growth and development of all living organisms is determined by the interactions between their genomes and the growing environment. Consequently, they evolved a multitude of complex signaling systems to respond to external and internal cues to regulate these processes. Hormones play critical regulatory roles in this process. Research on plant hormones has resulted in the discovery of a wide variety, which include auxins, gibberellins (GAs), cytokinins (CKs), abscisic acid (ABA), ethylene (ET), jasmonic acid (JA), salicylic acid (SA), brassinosteroids (BRs), nitric oxide (NO), and strigolactones (SLs) (Santner and Estelle 2009). They control various

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developmental events throughout the plant life cycle ranging from patterning, cell identity, and differentiation as well as coordinated growth. Also, the hormones act as regulators of plant responses to biotic or abiotic stress conditions (Feussner and Wasternack 2002). The hormone activity is determined by its availability, which is controlled at the level of metabolism and distribution, and by the efficiency of the hormonal signal perception and transduction, which could be an impact in gene expression or protein activity control. Furthermore, an additional level of complexity in the regulation of developmental process took place when interactions occur between hormones or between hormone producer organisms. Many genera of bacteria, including *Azospirillum*, *Bacillus*, *Achromobacter*, *Acetobacter*, *Azotobacter*, *Burkholderia*, *Herbaspirillum*, *Pseudomonas*, *Serratia*, *Xanthomonas*, *Klebsiella*, *Agrobacterium Enterococcus*, *and Enterobacter* (Tsavkelova et al. 2006), are producers of diverse phytohormones as auxins, CKs, GAs, ABA, and JA among others (Ping and Boland 2004). The aim of this chapter is to present the current knowledge on phytohormone metabolism and production as well as different extraction, purification, and quantification protocols in bacteria, particularly those belonging to genus *Azospirillum*.

7.1.1 Auxins

Auxin is the generic name that represents a group of chemical compounds characterized by their ability to induce cell elongation in the subapical region of the stem and to reproduce the physiological effect of the most abundant, naturally occurring auxin molecule, indole-3-acetic acid (IAA). These compounds have been associated with different plant processes such as (a) gravitropism and phototropism, (b) vascular tissue differentiation, (c) apical dominance, (d) lateral and adventitious root initiation, (e) stimulation of cell division, and (f) stem and root elongation (Tale et al. 2006).

7.1.1.1 Biosynthesis and Metabolism of Auxins in Plants and Bacteria

IAA is structurally related to the amino acid tryptophan (Trp), and the first studies on auxin biosynthesis hundreds of species proposed this compound as the main precursor in nature. However, this fact has proved difficult to demonstrate in plants. Nevertheless four pathways have been postulated in plant convert Trp to IAA including the indole-3-pyruvic acid (IPA), the tryptamine (TAM), and the indole-3-acetonitrile (IAN) pathway, but there is an amino acid-independent pathway from indole or indole-3-glycerol phosphate. In the case of bacteria, at least six metabolic routes for IAA biosynthesis have been proposed and most of them use Trp as a precursor, as the IPA, TAM, IAN, indole acetamide (IAM), and Trp side-chain oxidase (TSO) pathways. In addition, a tryptophan-independent pathway has been suggested. Despite this diversity of pathways to produce the active phytohormone, prokaryotic IAA biosynthesis seems to follow predominantly two major routes: the

IAM and the IPA (Lambrecht et al. 2000). Until now, at least four different pathways have been proposed/described for *Azospirillum* sp. three Trp-dependent pathways (via IPyA, IAM, and TAM) and one Trp-independent pathway (Cassán et al. 2014). *A. brasilense* Sp245 is one of the most studied strains world-wide and is considered as a type strain for this species, especially for its auxins biosynthesis. IAA production by *A. brasilense* Sp245 has extensively been studied with the IPA pathway, the main route for IAA biosynthesis. All genes involved in this pathway are found in the genome sequence of *A. brasilense* Sp245. Additionally, a nitrilase was also identified in the Sp245 genome sequence, which can catalyze the conversion of IAN to IAA.

7.1.2 Biosynthesis and Metabolism of Gibberellins

GAs, a large family of tetracyclic diterpenoid plant hormone, regulate many aspects of both vegetative and reproductive growth in plants, including seed germination, stem elongation, and leaf expansion; transitions from meristematic to shoot growth, and from vegetative growth to flowering, promotion of fruit set and subsequent growth, diseased resistance in rice and in response to different abiotic stress (Seo et al. 2009; Yang et al. 2013; Colebrook et al. 2014). Currently, approximately >130 GAs have been identified in plants, fungi, and bacteria, but only a small number are biologically active: GA_1, GA_3, GA_4, and GA_7. Many of the other GAs are biosynthetic intermediate or catabolites of bioactive GAs. They are biosynthesized from geranylgeranyl diphosphate (GGDP), a common C-20 precursor for diterpenoids and the plant biosynthesis can be divided into three steps according to the characteristics of the biosynthetic enzymes. In the first step, *ent*-kaurene, a tetracyclic hydrocarbon intermediate, is synthesized from GGDP by the *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) in proplastid. In the second step, *ent*-kaurene is converted to GA_{12} by two microsomal cytochrome P450 monooxygenase in the endoplasmic reticulum. The *ent*-kaurene oxidase (KO) catalyzes the sequential oxidation on C-19 to produce *ent*-kaurenoic acid, which is subsequently converted to GA_{12} by the *ent* kaurenoic acid oxidase (KAO). The reactions in the final step involved the formation of C-20 and C-19 GAs in the cytoplasm. Thereby, GA_{12} is converted to GA_4 (13 non-hydroxylated pathway), through oxidations on C-20 and C-3 by GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox), respectively. GA_{12} is also a substrate for GA13ox for the production of GA_{53}, which is a precursor for GA_1 in the 13 hydroxylated pathways. GA20ox catalyzes the sequential oxidation of C-20, including the loss of C-20 as CO_2 and the formation of γ-lactone and thereby, C19-GAs are produced. The bioactive GAs are formed by the introduction of a 3β-hydroxyl group in the inactive precursors. On the other hand, GAs are metabolically deactivated in several diversity of mechanisms; being the best-characterized deactivation reaction catalyzed by GA 2-oxidases (GA2ox) (Yamaguchi 2008). Also, the GAs can be converted into conjugates by conjugation with glucose through a hydroxyl group of GA to give a GA-*O*-glucosyl ether or via

the 6-carboxyl group to give a GA-glucosyl ester. Recently, two novel deactivation mechanisms were identified: epoxidation of non-13-hydroxylated GAs in rice and methylation of GAs in *Arabidopsis*.

7.1.2.1 Biosynthesis of Gibberellins in *Azospirillum* sp. and Other PGPR

Morrone et al. (2009) demonstrated that *Bradyrhizobium japonicum* encodes separate ent-copalyl diphosphate and ent-kaurene synthases. Thus, they provide the first evidence that this operon might indeed be involved in GAs biosynthesis in bacteria. Bottini et al. (1989) were the first to confirm the ability of *Azospirillum* sp. to produce GAs in chemically defined culture medium. They report the production of GA_1 and GA_3 on culture medium-free nitrogen of *A. lipoferum* Op33 by gas chromatography–mass spectrometry (GC-MS). Similar results were reported in *A. brasilense* by Janzen et al. (1992) and *A. lipoferum* AZm5 and *A. brasilense* VS9 by Esquivel-Cote et al. (2010). Additionally, it has been reported production of GA_{19} and GA_9 in chemically defined medium of *A. lipoferum* Op33, suggesting the existence of different routes of synthesis in those bacteria. The first pathways presumably include the metabolism of GA_{19} (and its metabolite, GA_{20}) to GA_1. On the other hand, the second path where GA_9 is (presumably) precursor of GA_3. These pathways were confirmed in subsequent reports for *A. lipoferum* Op33 by using minimal medium supplemented with deuterated precursors GA_{20} and GA_9 and subsequent identification as GA_1 and GA_3 by GC-MS. On the other hand, *A. lipoferum* Op33 can produce GA_{20} and GA_5 which suggest the existence of a second 13α-hydroxylation pathway for the metabolism of GA_{20} to GA_5 and GA_3 3β-hydroxylation; however, this route has not been confirmed unequivocally. GAs production, metabolism, and hydrolysis of conjugates by *Azospirillum* sp. were summarized by Bottini et al. (2004).

7.1.3 Biosynthesis and Metabolism of Cytokinins

CKs play an important role at all phases of plant development from seed germination to senescence such as maintenance of stem cell systems in shoots and roots, nodule organogenesis, leaf senescence, root vascular development, control of shoot branching in interaction with auxin and in the response to abiotic and biotic stress (Kouchi et al. 2010; O'Brien and Benková 2013). Structurally, CKs are adenine derivatives substituted in the N6-position and they can be divided into two groups based on their side chain: those with isoprene derived side chains, which are predominant in plants; and those with aromatic side chains that are found in plants at a lower abundance. In higher plants, the predominant CKs are isopentenyladenine (iP), trans-zeatin (tZ)-, cis-zeatin (cZ), or dihydrozeatin-type derivatives and they can exist as free bases and also in the form of nucleosides and nucleotides. In the past decade, CKs biosynthesis has greatly progressed due in large part to the identification of key pathway. A key enzyme in isoprenoid CKs biosynthesis in plants is

adenosine phosphate-isopentenyl transferase (IPT), which catalyzes N-prenylation of the adenosine 5′-phosphates (ATP, ADP, and AMP). The preferred donor of the isoprenoid side chain is dimethylallyl pyrophosphate (DMAPP) and the preferred acceptors are ATP and ADP. The primary products of synthesis are therefore either iP riboside 5′-triphosphate (iPRTP) or iP ribose 5′-diphosphate. The iP nucleotide is converted into tZ nucleotide by cytochrome P450 monooxygenases CYP735A1 and CYP735A2. To become biologically active, iP- and tZ-nucleotides are converted to nucleobase form by dephosphorylation and deribosylation. Recently, the existence of specific phosphoribohydrolase called LOG has been found in rice and *Arabidopsis* (Kuroha et al. 2009). An indirect pathway of CKs production involves the release of CKs by turnover of tRNA. CKs may be reversible or irreversible conjugated with sugars and amino acid, and they seem to serve as storage. Another way to control CKs content within tissue is by their irreversible degradation by the cytokinin oxidase/dehydrogenase action.

7.1.3.1 Biosynthesis of Cytokinins in *Azospirillum* sp. and Other Bacteria

CKs can be produced by bacteria, and they are used mainly as chemical signal either for communicating with the plant or as a tool for invading the plant host. In fact, CKs are also synthesized by phytopathogenic bacteria, such as *Agrobacterium tumefaciens* and *Pseudomonas savastanoi*, and is as a key factor in the formation of plant tumors. Barea et al. (1976) found that at least 90 % of the bacteria isolated from the rhizosphere of crops of agricultural interest were able to produce compounds type-CKs in defined culture medium. Horemans et al. (1986) modified the analytical procedure and were able to demonstrate that *A. brasilense* produces isopentenyl adenine (iP), isopentenyl adenine riboside (iPR), and zeatin (Z) in chemically defined culture medium. The most significant references CKs production by *Azospirillum* sp. was published by Strzelczyk et al. (1994), using a culture medium supplemented with different sources of carbon. They reported the production of isopentenyl adenine (iP), isopentenyl adenine riboside (iPR), trans-zeatin riboside (trans-Z), and zeatin (Z) in three strains of *Azospirillum* sp. isolated from sporocarps of ectomycorrhizal fungi *Rhizopogon vinicolor*, *Laccaria laccata* y *Hebeloma crustuliniforme*; however, they could just confirm iPR production in one of the three strains by gas chromatography (GC). An interesting case of synergism has been described for a mixed culture of *A. brasilense* and *Arthrobacter giacomelloi* showing a higher content of CKs, compared to those found in individual cultures of each microorganism (Cacciari et al. 1989). Tien et al. (1979) using different types of chromatography (HPLC and TLC) and a bioassay inoculation of pearl millet (Pearl millet) were the first to demonstrate the ability of *A. brasilense* to produce molecules of type-CKs; however, the partially purified compounds were not characterized. They reported that inoculation caused significant changes in the morphology of the root by increasing the number of lateral roots and root hairs density were similar to those obtained by the exogenous application of CKs. Similar results were found by Muralidhara and Rai (1986) in *A. lipoferum*.

7.1.4 Biosynthesis and Metabolism of Jasmonates

JA and its cyclic precursors and derivatives, collectively referred to as jasmonates (JAs), constitute a family of bioactive oxylipins. They function primarily in plant response towards biotic (insects, herbivores, and microbial pathogens) and abiotic (drought, salinity, ozone, UV light) stress, and a variety of plant growth and development processes, including flowering, fruit ripening, senescence, root growth, among others. JAs are generated via one specific branch of oxylipin biosynthesis, the AOS branch of the so-called LOX pathway, in an enzymatic process occurring in different subcellular compartments: plastid, peroxisome, and cytosol. The biosynthesis of JA has been extensively reviewed in recent years (Wasternack and Kombrink 2010; Kombrink 2012). The first step occurs in the membranes of chloroplasts, where the initial substrates α-linolenic acid (α-LeA; C18:3) or hexadecatrienoic acid (C16:3) are released from plastidial galactolipids by phospholipases such as DAD1 or GDL. In plants, generally, JAs synthesis occurs mainly from the C18:3 precursors through the octadecanoid pathway. Oxygenation of α-LeA is the initial step in JA biosynthesis. The oxygen has to be inserted in the C-13 position by the action of a chloroplastic 13-lipoxygenase (13-LOX) generating the 13-hydroperoxy derivative of linolenic acid (13-HPOT). The following step is the dehydration of the 13-HPOT into the allene oxide 12,13(*S*)-epoxyoctadecatrienoic acid (12,13(*S*)-EOT) by allene oxide synthase (AOS). This unstable allylic epoxide can decompose to different products, such as α- and γ-ketols, or spontaneously rearrange to racemic 12-oxophytodienoic acid, or it can be enzymatically cyclized by allene oxide cyclase (AOC) to optically pure *cis*-(+)-12-oxophytodienoic acid ((9*S*,13*S*)-OPDA). A small amount of hexadecatrienoic acid (16:3) can be converted by the same enzymes to dinor-12-oxo fitodienoic acid (dnOPDA), an OPDA structural homolog. Translocation of OPDA into peroxisomes, where the second half of JA biosynthesis occurs, has not been completely understood even though it depends on the carrier COMATOSE1/PEROXIMAL 1/PEROXISOME ABC TRANSPORTER (ABC CTS1/PXA1/PED3) and/or an ion trapping mechanism. OPDA reductase 3 (OPR3) catalyzes the reduction of OPDA and dnOPDA to 3-oxo-2-(2=[*Z*]-pentenyl)-cyclopentan-1-octanoic acid (OPC-8) and 3-oxo-2-(2=[*Z*]-pentenyl)-cyclopentan-1-hexanoic acid (OPC-6), respectively. JA synthesis proceeds with three rounds of β-oxidation that shorten the carbon side chain from the precursor molecule. Once synthesized, the (3*R*,7*R*)-JA is released into the cytoplasm from peroxisome by an unknown mechanism. JA can be further metabolized to different derivatives that take place mainly in the cytosol. The development of new, sensitive analytical techniques and isolation methods has led to an ever-increasing number of identified JA metabolites (Glauser et al. 2009). Among these compounds are the methyl ester and amino acid conjugates of JA, cucurbic acid, *cis*-jasmone, the glucose ester of JA, the 12-OH-JA and its sulfated, glucosylated and amino acid-conjugated derivatives; even the conjugate with the ethylene precursor aminocyclopropane carboxylic acid (ACC) and the glucose ester of OPC-4 were found. The conjugation of JA with Isoleucine (Ile) is an important step

because jasmonoyl-Ile (JA-Ile) has been identified as a biologically active jasmonate. Some plant pathogenic strains of *Pseudomonas syringae* produce a virulence factor called coronatine that is structurally similar to JA-Ile. OPDA is also biologically active without conversion to JA derivatives. So far differences have not been reported in the JA biosynthesis pathway between plants and bacteria.

7.1.4.1 Biosynthesis and Metabolism of Jasmonates in Bacteria

JAs have not been extensively investigated in bacteria. The presence of JA was identified in culture medium of the bacterium *E. coli* RC424 and RC-7 strains (Abdala et al. 1999). Also, JA was reported as produced by isolated endophytic bacteria from roots of *Prosopis strombulifera* (Piccoli et al. 2011). *Achromobacter xylosoxidans* (SF2) and *Bacillus pumilus* (SF3 and SF4) were selected as PGPR, among other characteristics, by the production of JA and OPDA (Forchetti et al. 2007).

7.1.5 Biosynthesis and Metabolism of ABA

ABA is an important phytohormone playing many physiological roles in plants including seed dormancy, stomatal movement, growth, and various environmental stresses (abiotic and biotic) (Jiang and Hartung 2008; Sreenivasulu et al. 2010). It is produced primarily in vascular tissues of roots and leaves, in guard cells and in seeds. Major steps of ABA biosynthesis take place in plastids, while the last two steps occur in the cytosol. ABA is an apocarotenoid synthesized via oxidative cleavage of epoxy-carotenoids in plants (Zeevaart and Creelman 1988). The epoxidation of zeaxanthin and antheraxanthin to form violaxanthin and neoxanthin is catalyzed by zeaxanthin epoxidase (ZEP/AtABA1). The products are isomerized to produce 9-cis isomers which are cleaved by nine-*cis*-epoxycarotenoid dioxygenase (NCED) to form xanthoxin. The later is subsequently converted to ABA by two oxidases, a short-chain dehydrogenase/reductase (SDR) and aldehyde oxidase 3 (AAO3). The level of active ABA is determined by its rate of biosynthesis, catabolism, transport between different organs and sensitivity of the cell/tissue to the hormone. When compared with biosynthesis pathway, ABA catabolism is much simpler. ABA can be hydroxylated at three different methyl groups in the ring structure (C-7′, C-8′, and C-9′), which leads to three pathways for ABA hydroxylation and produces three substantial biological activities metabolites. Although the hydroxylation does not reduce the biological activity of ABA thoroughly, it can trigger further inactivation steps. Among the hydroxylated products, only the 8′-hydroxy ABA can be changed into (phaseic acid) PA by spontaneous cyclization and then into dihydophaseic acid (DPA) by further reduction. ABA is inactivated at the C-1 hydroxyl group by different chemical which form different conjugates and accumulate in vacuoles or apoplastic space. Among them, ABA glucosyl ester (ABA-GE) is the most widespread conjugate which is catalyzed by ABA glucosyltransferase.

7.1.5.1 Biosynthesis of ABA in *Azospirillum* sp. and PGPR

In certain bacteria, ABA is synthesized as a C-15 terpenoid (sesquiterpene); however, more nothing is known about the biochemistry of ABA (biosynthesis and metabolism) or about a possible function of this molecule in bacteria. There are only a few reports on ABA production by *Azospirillum* sp. in chemically defined culture medium and in inoculated plants. Kolb and Martin (1985) were the first to report on ABA production by *A. brasilense* Ft326 in defined culture medium. *A. brasilense* Az39 and Cd have the capacity to produce ABA (75 and 6.5 ng mL^{-1} medium, respectively) in chemically defined medium as identified Perrig et al. (2007) by GC-MS. ABA was also characterized by gas chromatography with electron impact mass spectrometry (GC-EI-MS) in the supernatant of the model strain *A. brasilense* Sp245 from chemically defined media by Cohen et al. (2008). *B. pumilus* (SF3 and SF4) and *A. xylosoxidans* (SF2) produced JA and ABA in Luria Bertani (LB) culture media and increased the concentration when cultures were subjected to abiotic stress (Forchetti et al. 2007).

7.1.6 Biosynthesis and Metabolism of Salicylic Acid

SA is a phenolic compound, which is produced by a wide range of prokaryotic and eukaryotic organisms. Indeed, SA has been found to play a key role in various physiological and biochemical activities of plants such as thermogenesis, photosynthesis, stomatal closure, seed germination, plant growth (Abreu and Munné-Bosch 2009), response to local and systemic against pathogens, and response to abiotic stresses such as drought, chilling, heavy metal toxicity, heat, and osmotic stress (Hayat et al. 2010; Ng et al. 2011). SA is synthesized in plants through two distinct enzymatic pathways: the phenylalanine ammonia lyase (PAL)-mediated phenylalanine pathway in the cytoplasm, and the isochorismate synthase (ICS)-mediated isochorismate pathway in the chloroplast. Both of these pathways originate from chorismate, which is an intermediate of plant phenylpropanoid pathway. The common and initial enzymatic step of PAL pathway is the conversion of chorismate-derived L-phenylalanine to *trans*-cinnamic acid by phenylalanine ammonia-lyase. Subsequently, *trans*-cinnamic acid is hydroxylated to form *O*-coumarate followed by oxidation of the side chain to yield SA. Alternatively, the side chain of *trans-cinnamic* acid can be initially oxidized to give benzoic acid, which is then hydroxylated to produce SA. Thus, the difference between the two routes is the hydroxylation of the aromatic ring before or after the chain-shortening reactions. Most of the SA synthesized may undergo a number of biologically relevant chemical modifications including glucosylation, methylation, and amino acid conjugation. Glucose conjugation catalyzed by cytosolic SA glucosyltransferases, results in the formation of SA glucoside [SA 2-*O*-b-D-glucoside] and SA glucose

ester (Rivas-San Vicente and Plasencia 2011). The methylation converted SA to methyl salicylate (MeSA), this reaction is catalyzed by an SA carboxyl methyltransferase, and this volatile derivate is an important long-distance signal in tobacco and *Arabidopsis* systemic acquired resistance. Amino acid conjugation of SA is less well characterized, but may be involved in SA catabolism. Sulfonation is important for the activation or deactivation of various hormones and recently, SA has been shown to be sulfonated in vitro by members of the SOT family of sulphotransferases.

7.1.6.1 Biosynthesis and Metabolism of Salicylic Acid in *Azospirillum* sp. and PGPR

Several genera of bacteria such as *Pseudomonas aeruginosa* and *P. fluorescens* can synthesize SA from chorismate via two reactions catalyzed by ICS and isochorismate pyruvate lyase (IPL). ICS catalyzes the synthesis of isochorismate from chorismate and IPL catalyzes the conversion of SA from isochorismate (Mercado-Blanco et al. 2001). No gene encoding IPL has been cloned from plant species; whereby in plants, the conversion from IC to SA may be mediated by a route different from that characterized in bacteria. By contrast, SA synthesis in *Yersinia enterocolitica* and *Mycobacterium tuberculosis* is mediated by a single, bifunctional enzyme the SA synthase (SAS) that directly converts chorismate to SA via an isochorismate intermediate (Harrison et al. 2006). The presence of SA on *Azospirillum* sp. cultures was usually detected by not entirely suitable methodologies for SA determination, as silica TLC (Shah et al. 1992). Tortora et al. (2011) confirmed SA production by *A. brasilense* strains REC2 and REC3 under iron-starved conditions and isolated this compound from other catechols by the use of silica TLC coupled with fluorescence spectroscopy and GC-MS analysis. Siderophores produced by them showed in vitro antifungal activity against *Colletotrichum acutatum* M11. This fact was coincident with results obtained from phytopathological tests performed in plants, where a reduction of anthracnose symptoms on strawberry plants previously inoculated with *A. brasilense* was observed (Pedraza et al. 2010).

7.2 Methods

In the first part of the chapter, we offer several methods for the assessment (extraction and purification) of different hormones from bacterial cultures and plant tissues, with the aim that users can choose the most appropriate according to the availability of equipments and chemical reagents in their laboratories. The following section of the chapter describes analytical and biological methods for phytohormones identification and quantification. In addition, an introduction to Mass Spectrometry methodology is included as final remark.

7.2.1 Phytohormones Extraction and Purification

The methods described here are: (a) Ion exchange chromatography, which employs *Mini-column Sephadex A-25*; (b) *Anion exchange chromatography* including *Mini-column Amino* (*NH2*); (c) *Liquid-liquid partition* which employs double partition with ethyl ether; and (d) *Direct use of the sample* by centrifugation and filtering of supernatants.

7.2.1.1 Sample Assessment for JA Determination

This methodology is described by Gidda et al. (2003) and Andrade et al. (2005). For that, homogenize the lyophilized culture medium [approximately 0.2 g dry weight (DW)] with 10 mL 100 % methanol and add 50 ng (5 μL) of the corresponding deuterated standard (JA, OPDA, JA-Ile, 11 and 12-OH-JA). Filter the homogenate under vacuum through a column with a cellulose filter and collect the eluate and dry at 40 °C under vacuum in rotary evaporator. For the acetylation of endogenous hydroxylated-JAs, add a 400 μL of pyridine and acetic acid (2:1) and incubate overnight at 20 °C. Dry the extract at 40 °C under vacuum in rotary evaporator and prepare each cartridge of the column with a wash of 3 mL 100 % methanol. Discard this fraction and dissolve the dried extract in 10 mL of 100 % methanol and to load on columns filled with 3 mL Diethyl-aminoethyl (DEAE) Sephadex A25 or similar. Wash with 3 mL 0.1 M acetic acid in methanol and discard the eluate. Wash with 3 mL 1 M acetic acid in methanol and discard the eluate. Wash with 3 mL 1.5 M acetic acid in methanol and collect and evaporate the eluate under vacuum. Reconstitute the dried extract in 50 μL 100 % methanol (HPLC grade) and vortex briefly. For further jasmonates identification and quantification, inject 15 μL into LC-MS-MS.

Note: This methodology can be used for simultaneous determination of JA from bacterial culture medium or inoculated plant tissues. In the second case, approximately of 200 mg dry weight plant material must be ground in a mortar with liquid nitrogen and add 10 mL 100 % methanol.

7.2.1.2 Samples Assessment for ABA Determination

This methodology is described by Luna et al. (1993). For that, homogenize the lyophilized culture medium (approximately 0.2 g dry weight, DW) with 20 mL imidazole buffer (pH 7) plus 2,6-di-*tert*-butyl-*p*-cresol as antioxidant and add 50 ng (5 μL) of the corresponding deuterated standard (ABA). Incubate each sample overnight at 4 °C to allow extraction and standard equilibration. Centrifuge the sample for 15 min at 1,530 × *g*, then collect the supernatant and evaporate it under vacuum until to eliminate the isopropanol. An amino anion exchange minicolumn or similar is conditioned with 100 % methanol, deionized water, and imidazole

buffer 0.02 M. The aqueous fraction from step 4 is loaded onto the minicolumn. Wash sequentially the minicolumn with 6 mL each of hexane, ethyl acetate, and acetonitrile and discard these fractions. ABA is eluated with 6 mL of methanol:acetic acid (95:5, v/v) and the eluate fraction is evaporated to dryness under vacuum in rotary evaporator. For further ABA identification and quantification, inject 15 µL into high-performance liquid chromatography coupled to mass spectrometers (LC-MS-MS).

Note: This methodology can be used for simultaneous determination of ABA from bacterial culture medium or inoculated plant tissues. In the second case, approximately of 200 mg dry weight plant material must be ground in a mortar with liquid nitrogen and add 20 mL imidazole buffer (pH 7) plus 2,6-di-*tert*-butyl-p-cresol as antioxidant to the ground.

7.2.1.3 Samples Assessment for Simultaneous Determinations

For Simultaneous Determination of ABA, JA, IAA, and SA

This methodology is described by Durgbanshi et al. (2005). For that, centrifuge 20 mL of defined bacterial cultures, in exponential growth phase, separate at 8,000 rpm, 4 °C, for 15 min and add 50 ng (5 µL) of the corresponding deuterated standard (ABA, SA, GA_3, JA, and IAA). Acidify the supernatants at pH 2.5–2.8 with acetic acid solution (15 %, v/v) and partitioned two times with 20 mL of ethyl ether. Evaporate ethyl ether to dryness at 36 °C and resuspend dried samples in 1,500 µL methanol. Filter through a 0.22 µm cellulose acetate filter and dry methanol in a Speed Vacuum. Resuspend in 5 µL methanol (100 %) and injected into the analytical system.

Note: This methodology can be used for simultaneous determination of ABA, JA, IAA, and SA from bacterial culture medium or inoculated plant tissues. In the second case, approximately of 200 mg dry weight plant material must be ground in a mortar with liquid nitrogen, then add 5 mL ultra-pure water to the ground material.

For Simultaneous Determination of IAA, ABA, GA_3, and Z

This methodology is described by Perrig et al. (2007). For that, centrifuge 20 mL of defined bacterial cultures, in exponential growth phase, separate at 8,000 rpm, 4 °C, for 15 min. Add 100 ng of the corresponding deuterated standard (ABA, SA, GA_3, JA, and IAA) and acidify the supernatants at pH 2.5–2.8 with acetic acid solution (15 %, v/v). Keep the sample at 4 °C for 2 h and partition four times with the same volume of acetic-acid-saturated ethyl acetate (1 %, v/v). Evaporate ethyl ether to dryness at 36 °C and dried samples are diluted in 100 µL acetic acid/methanol/water solution (1:30:70, v/v) for ABA determination, acetic acid/acetonitrile/water solution (1:15:85, v/v) for IAA determination, and methanol/water solution (30:70, v/v)

for GA_3. Inject into a reverse phase octadecyl carbon chain (C18) column (300×3.9 mm) in an HPLC system coupled to a UV–Vis diode-array spectrometer.

Note: This methodology can be used for simultaneous determination of IAA, ABA, GA_3, and Z from bacterial culture medium or inoculated plant tissues. In the second case, approximately of 200 mg dry weight plant material must be ground in a mortar with liquid nitrogen, then add 5 mL ultra-pure water to the ground material.

Direct Use of the Bacterial Culture for IAA Determination

Centrifuge 1.5 mL of bacterial culture at 10,000 rpm for 15 min at room temperature and filter the supernatant using 0.22 μm cellulose acetate filters. Keep the sample in darkness conditions at 4 °C and use the clarified supernatant to colorimetric reaction with Salkowsky's reagent.

Note: This methodology can be used for determination of IAA and other related auxins from bacterial culture medium.

7.2.2 Phytohormones Identification and Quantification

7.2.2.1 Biological Methods

Biological activity of microbial phytohormones depends on their concentrations in culture supernatants and microbial ability to produce these molecules (Cassán et al. 2009a). Phytohormones may influence early seed germination, early seedling growth, plant colonization, and bacterial establishment. The methodology proposed in this section allows you to estimate the bacterial ability to produce cytokinins, gibberellins, and/or auxins in inoculated plants or in chemically defined culture medium.

Determination of Gibberellic Acid

A sensitive, easy, and fast bioassay is described by Cassán et al. (2009b) for detection and quantification of active GAs from bacterial solutions. For that, maize (*Zea mays* L.) or rice (*Oryza sativa* L.) seeds are surface disinfected by soaking 3 min in 1 % NaClO, and then washed with sterile distilled water to eliminate traces of this compound. Seeds are pre-germinated for 48 h at 30 °C and 80 rpm shaking in an Erlenmeyer flask containing sterile 80 μM uniconazole [S-3307, (±) (E)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol]. Almost five seeds are planted at 1 cm depth in individual pots, each one containing 200 mL of sterile perlite-sand (1:1) mixture. After full emergence, seedlings are thinned to three plants per pot. Plant pots are kept at field capacity by daily irrigation with

sterile distilled water, and once a week fertilized with 50 % of Hoagland's solution (Hoagland and Boyer 1936). Plants (three plants per pot, five replicates per treatment) are cultured in a growth chamber with daily cycles of 16 h at 25–30 °C with a light intensity of 450 μmol m^{-2} s^{-1} and 8 h at 20 °C in darkness for 7 days. Plants are grouped in the following treatments: (a) cell inoculated: each seedling is inoculated with 1 mL of bacterial culture medium with up to 10^9 cells mL^{-1}; (b) supernatant inoculated: each seedling is inoculated with 1 mL of supernatant of culture medium (10,000 rpm × 10 min at room temperature); (c) GA_3 treated: GA_3 is dissolved in pure ethanol to obtain 0.1, 0.5, 1.0, 5.0, and 10.0 μg mL^{-1} solutions and then 10 μL are applied individually at the first leaf with microsyringe for every seedling for the same treatment; (d) Control: seedlings individually treated with pure ethanol. At the end of the experiment the plants are carefully pulled out from the pots and rinsed for 5 s in the potassium phosphate buffer. The following parameters are measured as per plant basis: first internodes length and total shoot length.

Note: There are some dwarf mutants deficient in the production of physiologically active gibberellins, like the *dwarf-1* (*d1*) described in maize (*Zea mays* L.) by Fujioka et al. (1988) and the *dwarf-x* (dx or cv. Tan-ginbozu) described in rice (*Oryza sativa* L.) by Kobayashi et al. (1989). These mutants express the dwarf phenotype since young seedling stage, but the exogenous application of gibberellic acid (GA_3) or the inoculation with gibberellins-producer bacteria, allows expression of the normal phenotype. For this reason, the use of the described dwarf mutants is strongly recommended.

Determination of Indole Acetic Acid

A sensitive, easy, and fast bioassay is described by Epel et al. (1987) for detection and quantification of IAA in bacterial cultures. The bioassay consists of measuring the weight increase of hypocotyl sections from etiolated cucumber seedlings incubated in a simple growth medium. The sensitivity of the test is high with a significant response at an IAA concentration as low as 1 ng mL. The bioassay requires at least 3 h to evaluate and is easy to perform. Some advantages of this methodology are listed below: (1) the slope of the log-linear concentration-response curve is moderately steep; (2) the assay is insensitive to the pH of the media between 4 and 7 and can be performed in the presence or absence of buffer; and (3) the growth of the etiolated hypocotyl sections is insensitive to GA and to kinetin.

Cucumber seeds (*Cucumis sativus* L.) are germinated and grown in vermiculite subtract under darkness and watery irrigation conditions at 25 °C. When the etiolated seedlings were 5–8 cm in length (generally 4–5 days, depending on freshness of seeds) hypocotyl sections 10 mm in length are excised from the hypocotyl, starting about 2 mm below the hook, unless stated otherwise. The excised sections are immediately placed in tap water until sufficient sections had been cut. The sections are then washed in running tap water for about 30 min. The seedlings should be protected of direct light during this procedure. Washed sections, gently blotted with filter paper, are weighed in groups of 10–12 with a tared electronic balance with mg

precision (typical weight, about 250 mg) and placed in glass scintillation vials containing 3 mL standard growth medium (pH 7.0) containing 2 mM KCl, 0.1 mM CaCl and 10 mg L^{-1} chloramphenicol. IAA solutions are added individually into scintillation vials to produce a typical dose–response calibration curve. These solutions should contain a concentration of pure IAA between 0.1 and 100 μg mL^{-1}. The bacterial supernatants are also added in specific vials as problem treatment. After the treatment, the covered vials are gently shaken (80 rpm min^{-1}) at 30 °C between 3 and 12 h (linearity increases with the time of exposition) and sections removed, blotted, and grouped weighed. Each point should represent three replicates of 10–12 sections each. The growth response of IAA solutions is expressed as percentage of increase in weight [%] vs. log μg mL^{-1} IAA. The supernatant IAA concentration calculation is derived of that dose–response curve.

Determination of Cytokinins

The cucumber cotyledon greening bioassay reported by Fletcher et al. (1981) with modifications is used for detecting cytokinins from several natural sources, including bacterial cultures. The sensitivity of the methodology is extremely low and useful to detect a minimal of 0.0001 mg L^{-1} of *N′*-benzyladenine, zeatin, kinetin, or zeatin riboside in solution. Of the more frequent cytokinins tested, kinetin appeared to be the least active. Cucumber (*Cucumus sativus* L.) seeds are planted in vermiculite, in peat flats (20 × 15 × 7 cm) and germinated in the dark at 28 °C. The cotyledons from 5-days old plants are excised in the absence of green light, making certain that the hypocotyl hook is removed. The cotyledons are placed in 5-cm Petri dishes containing 3 mL of test solution which consisted of distilled H_2O, cytokinins at various concentrations to generate a dose–response curve or bacterial supernatant, and 40 mM KCl. The dishes are returned to the dark at 28 °C for 20 h. After incubation period, they are exposed to fluorescent light with an intensity of 12.9 W m^{-2}. After 3.5 h, cotyledons are homogenized, and the chlorophyll extracted in 8 mL 80 % acetone solution. The volume is brought up to 10 mL with acetone and then centrifuged at 2,500 × *g* for 10 min. The absorbance of the supernatants is read at 663 (chlorophyll A) and 645 nm (chlorophyll B) to obtain the partial and total concentrations according to Arnon (1949).

7.2.2.2 Analytical Methods

Determination of Indole-3-Acetic Acid by Spectrophotometry

The precise identification of auxin and related molecules can be obtained according to previous extraction, concentration, separation, and identification of the compounds by complex methodologies as high-performance liquid chromatography or GC-MS. Such methods, however, are time-consuming and cannot be used as routine assays. To solve these problems, various authors have used a colorimetric technique

derived from that of Salkowski (1885) for indole detection. This method has been used for years because it is simple, rapid, and cheap and allows the daily analysis of numerous bacterial supernatants. Glickman and Dessaux (1995) examined the sensitivity and the specificity of three versions of the Salkowski colorimetric technique. Two of these allowed the detection of indoleacetic acid over a low range of concentrations (0.5–20 mg mL^{-1}), while the third permitted the detection of IAA over a range of higher concentrations (5–200 mg mL^{-1}). These methodologies are shown to be specific for IAA, IPA, and IAM. The first colorimetric technique was performed according to Pilet and Chollet (1970), using reagent R1, which consisted of 12 g of $FeCl_3$ per liter in 7.9 M H_2SO_4. One milliliter of reagent R1 will be added to 1 mL of the sample solution, well mixed, deposited in a 3 mL spectrophotometer cuvette, and will be kept in the darkness conditions for 30 min at room temperature. The second and third colorimetric methods termed were derived from that of Tang and Bonner (1947). They used reagent R2, which consisted of 4.5 g of $FeCl_3$ per liter in 10.8 M H_2SO_4. An aliquot of 1 or 2 mL of reagent R2 will be added to 1 mL of sample solution, and the mixture will be processed as indicated for first technique. In all cases, mixtures will be examined at OD_{530} for determination of indolic compounds. The quantification of IAA will be performed by the use of a calibration curve of pure IAA solution.

Simultaneous Determination of IAA, ABA, GA_3 by HPLC

The chromatographic analysis is performed according to Kelen et al. (2004). For that chromatography system consists of pump with an auto injector and diode array detector, a column oven and a degasser system. The column used is C18 [250 mm × 4.6 I.D stainless steel analytical column with 5 μm particle size]. The electromotive force (e.m.f.) values used to evaluate the pH of the mobile phase are measured with a pH/ion analysis apparatus using an Ag/AgCl combination pH electrode. All solutions are externally thermostated at 25 ± 0.1 °C. Stabilize the electrode in appropriate acetonitrile water mixtures before the e.m.f. measurements. The mobile phases are acetonitrile-water (26:74 v/v) containing 30 mM p phosphoric acid with sodium hydroxide at PH 4.00. Equilibrate the C18 column for each mobile phase condition with a time limit of 30 min. Maintain the column temperature constant 25 ± 0.1 °C. The separation is carried out by isocratic elution at 0.8 mL min^{-1} of flow rate. An injection volume of 10 μL is used for each analysis. The standard solution of the individual acid are prepared in the mobile phase and chromatographed separately to determine the retention time for each molecule. The signal of the compounds is monitored at 208, 265, and 280 nm for GA_3, ABA, and IAA, respectively. Calculate the capacity factors from $k = (tR - to)/to$, where to is the hold-up time, and tR is the retention time of each hormone for each mobile phase. In this equation the hold-up time, to, is established for every mobile phase composition using potassium bromide solution [0.01 % (w/v) in water, μmax = 200 nm]. Determinate the retention time and capacity factor of solutes from three different injections. The identification of different peaks is based on retention time and spiking of the sample.

Mass Spectrometry

This technique works by ionization of compounds to generate charged molecules or molecule fragments. The mixture of electrically charged molecules is accelerated by a magnetic field, all in a high vacuum, this in order to prevent reactions occurring with other particles. The fragments of molecules which are obtained by mass spectrometry are the "fingerprint" of each analyte being tested; it can obtain identification with a very high precision. Compounds are separated by chromatographic methods prior to entering the mass spectrometer when hormones are determined. The gas chromatograph (GC) is a common combination with mass spectrometry (MS), where the molecules must be volatile and tolerate high temperatures. The ionization of molecules associated with gaseous chromatographer generally is produced by electron impact, where electrons are generated by through thermionic emission. The disadvantage of this technique of ionization is that they produce a substantial fragmentation of the molecules of the compound in evaluation. In general, the organic molecules are labile at high temperatures and in many cases non-volatile, by which such molecules must be modified by derivatization to improve their volatility, which not only is annoying to perform but also it is different according to family of plant hormones. The solution to such problem occurred after the appearance of high-performance liquid chromatography coupled to mass spectrometers (LC-MS), which allowed the separation of organic compounds without prior modification of such molecules at the entrance of the mass spectrometer (Fig. 7.1), and also the use of electrospray ionization (ESI), developed by Fenn et al. (1989). Micro-volume of solvent are required for the ESI technique. The HPLCs used possess flows in the order of microliters of solvent per minute (μL min^{-1}) or nano liter (nL m^{-1}) in the case of liquid chromatographs ultra performance (UPLC), which improves the definition of chromatographic peaks and ESI ionization. ESI is

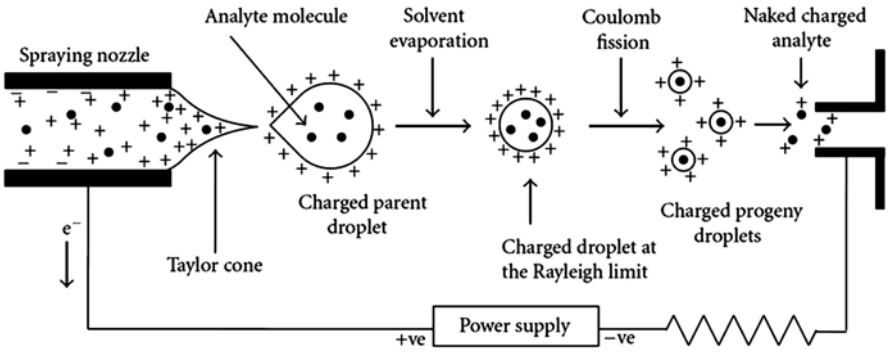

Fig. 7.1 Schematic representation of the electrospray ionization published by Banerjee and Mazumdar (2012)

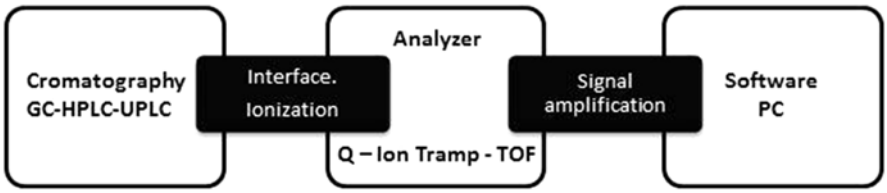

Fig. 7.2 The basic components of chromatography coupled to mass spectrometers

a soft ionization with very little fragmentation. The solvent with the molecules of interest pass through a capillary in an electric potential of 3–4 kV is applied. As a result highly charged droplets are produced, which are dried by the interface temperature and the gas stream nitrogen. The droplets are electrically overloaded and suffer a "coulomb explosion" and individual molecules charged "ionic form" positively or negatively, depending on the filler used in the ionization, are generated (Fig. 7.1). Analysis of these charged molecules is performed by different analyzers, for example, quadrupole (Q), ion trap (ionic tramp), or time of flight (TOF) and/or the combination of them (Fig. 7.2). Analyzers perform the separation of each molecule or fragment analysis by mass/charge ratio (*m*/*z*). When more than one analyzer is used, this is called tandem mass spectrometry MS/MS or MSn. In mass spectrometers in tandem, the parent ions with a specific *m/z* are selected in the first analyzer, after they are fragmented in the collision cell and the daughter ions with a specific *m/z* are selected in the second analyzer. A parent/daughter ions pair is often called to as a "transition" for example in salicylic acid: *m/z* 137/93. The identification and quantification in this case is performed by fragment molecular compound in the operating mode of Multiple Reaction Monitoring (MRM). In the mass spectrum, we can observe the relative abundance of ionized fragments and the *m*/*z*, where we can identify the "molecular ion" which is the molecular weight of the molecule in ionic form, and the other peak is called "Peak base," which is the analyte's relative abundance (100 %) and then find the different fragments obtained. Quantification is performed from areas that generate each of the molecules or fragments in a chromatogram that appear in the retention time (RT) for each compound. Such areas are associated with different concentrations generated by the endogenous and the internal standard (deuterated compounds), which have the same structure and different weight that the analyte being evaluated. These areas are associated with different concentrations generated by the synthetic and the internal standard (deuterated compound). For example, in the chromatogram of the ABA (Fig. 7.3) and ($^{2}H_{6}$)-ABA (Fig. 7.4) and their RT and related areas are observed. Such quantification of the endogenous compound can be done by direct relation: (peak area of endogenous compound/peak area of the deuterated compound) × amount of deuterated compound or through the use of a calibration curve between the area of the endogenous and deuterated compound and their concentrations.

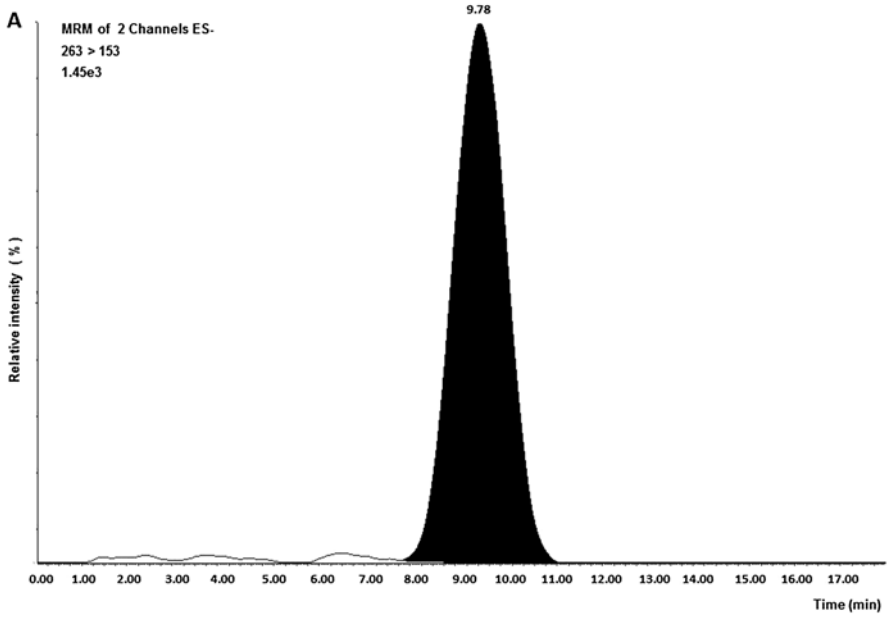

Fig. 7.3 Trace chromatograms in MRM mode of 263/153 (ABA)

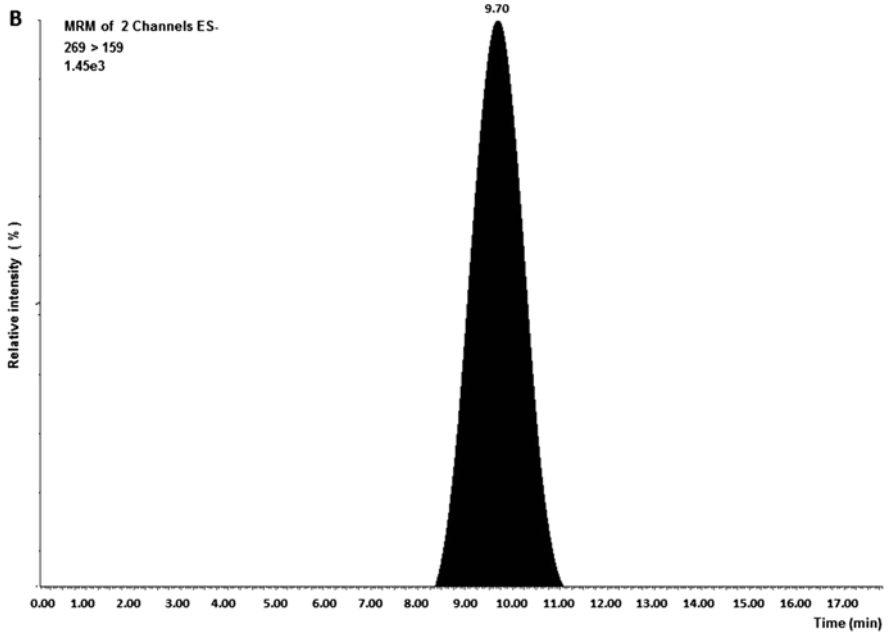

Fig. 7.4 Trace chromatograms in MRM mode of 269/159 ($^2H_6$-ABA)

Determination of Cytokinins by GC-MS-SIM

The chromatographic analysis is performed according to Timmusk et al. (1999). For that, HPLC system consists of a pump with a low-pressure gradient mixer (a 6-mL mixing volume between the pump and the sample injector), an online scanning spectrophotometric UV detector, and a 125 × 4 mm and a 75 × 4 mm column connected in series. The gradient profile is: 0 min, 1.0 %; 8 min, 3.0 %; 21 min, 16.6 %; and 25 min, 40.0 % acetonitrile in 2 % acetic acid and the low rate 2 mL min^{-1}. A standard run includes a mixture of the authentic cytokinins, zeatin, dihydrozeatin, and iP; their nucleosides, nucleotides, and 9-glucosides; and KR. Added dideoxyadenosine (ddAR, 100 pmol) before injection as a means to check the retention time. Store the fractions with putatives a cytokines at −20 °C. Evaporate the fractions with putative cytokinins to dryness and dissolve in 0.5 mL of methanol. Permethylation of standards and samples are performed according to Kovac (1993), except that chloroform that is used instead of dichloromethane and the organic phase are backwashed twice with water. After evaporation to dryness in a stream of N_2 at 60 °C, the samples are dissolved in 3 mL of chloroform and a 1 mL aliquot is injected into a g.c. coupled to a mass spectrometer. The mass spectrometer conditions are: EI ionizing voltage, 70 eV; source temperature, 300 °C; and interface temperature, 290 °C. Chromatograms are obtained by Selected Ion Monitoring (SIM) with a well time of 0.05 s and a mass range span of 0.3 amu. The gas chromatography system has a splitless injection port at 300 °C and a 15 m × 0.25 mm × 0.1 mm DB-5 ms column with He as the carrier gas at 27.6 kPa. The temperature program is started at 80 °C for 4 min, then is increased 10 °C min^{-1} to 290 °C, and finally is maintained for 10 min.

Simultaneous Determination of ABA, JA, IAA, and SA by HPLC-LC-MS

The chromatographic analysis is performed according to Durgbanshi et al. (2005) using an autosampler and a quaternary pump system. Inject aliquots (20 μL) on a Nucleosil ODS reversed-phase column (100 × 2 mm i.d., 5 μm). Elute the phytohormones with a gradient of methanol and 0.01 % CH_3COOH in water that started from 10:90 (v/v) and linearly reached 60:40 (v/v) in 10 min. In the following 4 min, increase the gradient to 80:20 (v/v). Retain isocratic conditions of 80:20 during the last 2 min of the run, restore the initial conditions, and allow equilibrating for 5 min, giving a total time of 21 min per sample. The solvent flow rate is 0.3 mL min^{-1} with working pressures around 70–100 bar. Introduce the effluents from the HPLC into a triple-quadrupole mass spectrometer. Drying gas, as well as nebulizing gas, is nitrogen generated from pressurized air in a nitrogen generator. The nebulizer gas flow is set to 80 L h^{-1} and the desolvation gas flow to 800–900 L h^{-1}. The collision gas for operation in the tandem MS (MS/MS) is 99.995 % pure argon mode, with a pressure of 2×10^{-3} mbar in the collision cell. The desolvation gas temperature is 350 °C, the source temperature 120 °C, and the capillary voltage 3 kV. The mass spectrometer is operated in multiple reactions monitoring (MRM) mode.

Simultaneous Determination of IAA, ABA, GA_3 by GC-MS-SIM

The chromatographic analysis is performed according to Perrig et al. (2007). For that, separate the bacterial cultures into several 20 mL fractions for determination of IAA, ABA, and GA_3. Centrifuge the culture fractions at 8,000 rpm for 20 min at 4 °C, acidify supernatants at pH 2.5 with acetic acid solution (1 %, v/v), and add 100 ng of corresponding deuterated internal standard (ABA, IAA, or GA_3) at the samples. Then keep the sample at 4 °C for 2 h. Partition each sample four times with the same volume of acetic-acid-saturated ethyl acetate (1 %, v/v). After the last partition, evaporate acidic ethyl acetate to dryness at 36 °C. Dilute the dried samples in 100 µL acetic acid/methanol/water (1:30:70) for ABA determination, acetic acid/acetonitrile/water (1:15:85, v/v) for IAA determination, and methanol/water (30:70 v/v) for GA_3 and Z determination. Inject into a reverse phase C18 HPLC column (300 × 3.9 mm) coupled to a UV-diode-array spectrometer. Elute each sample at 1 mL min^{-1} flow rate, fractions eluting at the retention time corresponding to each pure standard. IAA, ABA, and GA_3 are identified and quantified by GC-MS with selective ion monitoring (GC-MS-SIM). UV-absorbing fractions at 254, 262, and 220 nm are grouped for IAA, ABA, and GA_3 determination, respectively, and then methylated with ethereal diazomethane and silylated with 1:1 pyridine/BSTFA [bis (trimethylsilyl) trifluoroacetamide] plus 1 % trimethyl-chlorosilane to obtain methyl-trimethylsilyl derivatives of IAA, ABA, and GA_3. Inject aliquots of each sample directly into a capillary column (15 m × 0.25 mm, 0.25 µM methyl silicone) fitted in a GC system with a capillary direct interface to a 5970B Mass Selective Detector. The GC temperature program is 60–195 °C at 20 °C min^{-1}, then 4 °C min^{-1} to 260 °C. Carrier gas (He) flow rate is 1 mL min^{-1}, interface temperature is 280 °C, and data acquisition is controlled by an HP 300 Series computer. The amount of free ABA is calculated by comparison of peak areas of the ion at a mass/charge (*m*/*z*) 196 (molecular ion for [$^2H_6$] ABAMeTMSi) and the ion at *m*/*z* 190 (molecular ion for [^{1}H] ABAMeTMSi) at the corresponding time (Kovats 1958). Similarly, the amount of free IAA is calculated by comparison of peak areas for the parent ion (*m*/*z*) 194 and (*m*/*z*) 189 and amount of free GA_3 by comparison of peak areas for parent ion (*m*/*z*) 506 and (*m*/*z*) 504.

Simultaneous Determination of ABA, JA, IAA, GA_3, and SA by LC-ESI-MS-MS

The hormones are determined by liquid chromatography with electrospray ionization (LC-ESI). For that, perform the liquid chromatography and mass spectrometry analyses using a quaternary pump equipped with auto-sampler. A C_{18} column (2.1 mm × 100 mm, 5 m) is used at 28 °C, with injected volume 10 µL. The binary solvent system used for elution gradient consists of 0.2 % acetic acid in H_2O (solvent B), and MeOH (solvent A), at a constant flow rate of 200 µL min^{-1}. Apply a linear gradient profile with the following proportions (v/v) of solvent A [*t* (min), % A]: (0, 40), (25, 80), with 7 min for re-equilibration. Mass spectrometry/mass spectrometry (MS/MS) experiments are performed on a double quadrupole mass spectrometer. All analyses are performed using turboion spray source in negative

ion mode with the following settings for each hormone: capillary voltage −3,000 V, energy cone 35 V, RF Lens 1 (20), RF Lens 2 (0.3), source temperature 100 °C, solvation temperature 380 °C, gas cone 100 L h^{-1}, collision (50), and multiplier (650). Optimize the MS/MS parameters in infusion experiments using individual standard solutions of each hormone at a 50 ng μL^{-1} diluted in mobile phase A/B (40:60 v/v). MS/MS product ions are produced by collision-activated dissociation of selected precursor ions in the collision cell of the double quadrupole mass spectrometer, and mass is analyzed using the second analyzer of the instrument. For quantifying, inject the samples in MRM modes, since many compounds could present the same nominal molecular mass. The combination of parent mass and unique daughter fragment ions is used to selectively monitor hormones in plants extracts. MRM acquisition is performed using the 137/93 and 141/97 transitions for SA and ($^2H_4$)-SA; 263/153 and 269/159 for ABA and ($^2H_6$)-ABA; 209/59 and 215/59 for JA and ($^2H_6$-JA); and 174/130 and 179/135 for IAA and ($^2H_5$)-AIA, respectively, with dwell 1,000 ms for each transition. Acquire and analyze data using software, for example, MassLynx™ 4.1 and QuanLynx™ 4.1. For quantification, obtain the values from a calibration curve previously constructed using known amounts of each hormone and their pure standard/deuterated internal standard ratio.

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Chapter 8
Inorganic Nitrogen Metabolism in *Azospirillum* spp.

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Abstract Protocols for the study of the physiology of nitrogen fixation and nitrate metabolism in *Azospirillum* spp. are described in details. The protocols presented in this chapter have been used in our laboratory for many years and give reliable results. Small adaptations may be necessary for each particular bacterial strain and laboratory.

8.1 Introduction

Currently, the genus *Azospirillum* comprises 15 recognized species (http://www.bacterio.net/-allnamesac.html; July 9th, 2014). Two new species have been recently described in the Int J Syst Evol Microbiol. A third candidate species *Azospirillum massiliensis* was isolated from water using co-culture with *Acanthamoeba polyphaga*.

Most species have scarce metabolic characterization. The best-studied species are *A. brasilense*, *A. lipoferum,* and *A. amazonense*. All pathways of the nitrogen cycle, with the exception of nitrification, have been described in these species.

The following species had their genome sequenced completely or partially: *A. brasilense* strains Sp245, *Azospirillum* sp. B510, *A. brasilense* FP2 (Sp7 Nal^R,Sm^R), *Azospirillum lipoferum* 4B, *Azospirillum irakense* DSM 11586, *Azospirillum halopraeferens* DSM 3675, and *Azospirillum amazonense* Y2 (Wisniewski-Dyé et al. 2011; Sant'Anna et al. 2011; Kaneko et al. 2010; http://www.ncbi.nlm.nih.gov/genome/).

A. brasilense Sp245 was used to identify the pathways of nitrogen cycle since its genome has been completely sequenced and this strain has been characterized physiologically. The genes for nitrate/nitrite transport of the ABC-type (*nasFED*) and those for the assimilatory nitrite (*nirBD*), nitrate reductase (*nasA*), and uroporphyrin

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III C-methyltransferase (*nasG* or *cysG*) are in plasmid 1. Similar genes are also found in the genomes of *A. lipoferum*, *A. halopraeferens,* and *A. irakense*. Downstream from *nasFED*, the *nasT* gene encoding an antitermination protein is found. In the draft sequence of *A. amazonense* only *nasA* and *nasD* genes are found; this species was described as having only the assimilatory nitrate reductase.

In plasmid 2 of *A. brasilense* Sp245 genes for the periplasmic nitrate reductase, *napEFDABC* are present. Another set of *nap* genes are found in plasmid 4 which comprises *napEDABC*. The copper containing nitrite reductase gene *nirK* (*aniA*) is located in plasmid 3. These genes are also present in *A. lipoferum*, *A. halopraeferens,* and *A. irakense.*

Genes for nitric oxide reductase *norBCQD* and nitrous oxide reductase *nosZ* were found. Transport and regulatory genes are also present. Regulation of nitrate metabolism genes is complex and seems to be dependent on the interaction of several factors such as nitrate, nitrite, and oxygen levels (Döbereiner and Pedrosa 1987).

The *nif* gene cluster is present in all sequenced species. In plasmid 3 of the genome of *A. brasilense* Sp245, a second *nif* gene cluster is present, probably coding for an alternative iron or vanadium nitrogenase. However, the gene for alternative dinitrogenase reductase (*anfH* or *vnfH*) is not present in this cluster or elsewhere in the genome.

Ammonia assimilation in *Azospirillum* spp. occurs via two pathways, one involving glutamate dehydrogenase (*gdhA*) under high NH_4^+ concentration and the other involving glutamine synthetase (*glnA*) and glutamate synthase (*gltBgltD*) under limiting NH_4^+. Genes for these pathways are present in all species so far analyzed.

The protocols for studying inorganic nitrogen metabolism in *Azospirillum* spp. are described in the next section. These tools were developed mainly for the study of *A. brasilense*, *A. lipoferum,* and *A. amazonense,* but they can be applied to the other species.

8.2 Tools to Study Nitrogen Metabolism in *Azospirillum*

Azospirillum is a rhizobacterium with high potential as a nitrogen bio-fertilizer and plant growth promotion, thus the study of its nitrogen metabolism has attracted great interest. As in other diazotrophs, nitrogenase synthesis and activity is regulated by ammonium and oxygen. This regulation occurs both at the transcription (Pedrosa and Elmerich 2007) and translation levels (Huergo et al. 2012).

The tools described below for the study of nitrogen metabolism will deal only with in vivo systems. Physiological methods are still required even in the Omics era to assess the function of the genes and their products in defining metabolic pathways.

8.2.1 Determination of Nitrogenase Activity in Semi-Solid Medium

All *Azospirillum* are aerobic, but will only fix nitrogen in the presence of a constant supply of low oxygen (micro-aerobic) concentrations to prevent nitrogenase inactivation and *nif* genes repression. Johanna Döbereiner's group introduced the N-free semi-solid media for the study of nitrogen fixation in *Azospirillum* and other motile aero-tactic diazotrophs in the mid 1970s. This is the simplest approach to measure in vivo nitrogenase activity in *Azospirillum*. The bacteria is inoculated in N-free minimum growth media containing low agar concentration (usually 1.75 g/L) and incubated under static conditions. As the bacteria population increases, it forms a veil-like pellicle that continuously migrates to regions where the oxygen concentration allows the expression and activity of nitrogenase.

In order to detect maximum nitrogenase activity during semi-solid growth, time course measurements should be performed since at the early stages of growth the bacterial population is low for nitrogenase detection. At late stages, the bacterial population is too large and oxygen supply is too low to sustain energy production and full nitrogen fixation. The time point where nitrogenase activity is maximum depends on the initial inoculum concentration, growth medium conditions, and temperature. It is essential to determine the maximum nitrogenase activity when comparing different species, strains, and physiological conditions.

8.2.1.1 Time Course

Nitrogenase activity in semi-solid medium is determined by the reduction of acetylene, which is also a substrate for nitrogenase. The strains are grown in minimal liquid medium such as NFbHP (Machado et al. 1991) containing ammonium chloride (5 mM) as N source overnight at 30 °C in a rotary shaker (120 rpm). Ten microliters of the saturated culture are inoculated in bijou or penicillin bottles of 10 mL containing 4 mL of N-free NFbHP semi-solid medium. Inoculation is carried out with a micropipette and the inoculum is deposited in the semi-solid medium. The cultures are incubated 12, 24, 36, and 48 h at 30 °C. A veil-like bacterial growth should be visible just below the medium surface. For nitrogenase measurement at each time point, the bottles are tightly sealed with subaseals or rubber stopper with aluminum seals, and 0.6 mL of acetylene (10 % of gas phase) is injected in each bottle. The cultures are further incubated at the same temperature for 60 min and 0.5 mL of the gas phase is analyzed by gas chromatography to determine the amount of ethylene formed. The gas chromatograph is equipped with FID detector and a Porapak N (100–200 mesh) column (2 m, 1 mm diameter) operated at 110 °C. Ethylene (100 ppm in N_2) is used as standard and 0.5 mL contains 2.232 nmol of ethylene at 25 °C and 1 atm.

To calculate the specific activity of nitrogenase is necessary to determine the culture protein concentration. For this, the cultures in semi-solid medium are

quickly heated in boiling water to melt the agar and vigorously vortexed. Melted culture samples are then lysed in NaOH 0.1 M (final concentration) for 30 min at room temperature and protein concentration determined by standard Bradford procedure. The same procedure is used for liquid cultures.

Nitrogenase-specific activity is calculated by the following equation:

$$N_2\text{ase} = \left(\text{nmol}\, C_2H_4 \,\text{in}\, 0.5\,\text{ml}\right) \times 12 / \left(\text{mg protein} \times 60\,\text{min}\right)$$

Where 12 is the correction factor for the gas volume and mg protein is the total protein content of the culture in milligrams.

8.2.1.2 Effect of Inoculum Size

It is possible to determine the maximum nitrogenase activity using a different strategy, namely varying the inoculum concentration and measuring the activity at a fixed time point. The Azospirilla inocula are grown as described above and inoculated in increasing amounts (e.g., 1, 2, 4, 8, 16, 32, and 64 μL) into 4 mL of N-free semi-solid medium in 10 mL bijou or penicillin bottles. The cultures are incubated statically at 30 °C for 16–24 h and assayed for nitrogenase activity. A veil-like bacterial growth should be visible just below the medium surface. For nitrogenase measurement at each time point, the bottles are tightly sealed with subaseals or rubber stopper with aluminum seals, and 0.6 mL of acetylene (10 % of gas phase) is injected in each bottle. The cultures are further incubated at the same temperature for 60 min and the ethylene formed determined by gas chromatography as before.

8.2.2 Determination of Nitrogenase Activity in Liquid Medium

Azospirilla do not grow or express nitrogenase activity in N-free liquid media in batch cultures shaken under air since the dissolved oxygen concentration is too high for nitrogenase activity. However, nitrogenase activity expressed in liquid medium can be measured when the oxygen concentration (oxygen partial pressure) in the gas phase is decreased to levels compatible with nitrogenase activity. The introduction of glutamate (Pedrosa and Yates 1984) as a nitrogenase non-repressible nitrogen source for *Azospirillum brasilense* is made easier to study nitrogenase activity in liquid cultures under air. In this system, the bacteria grow using glutamate as N source until it reaches a cell density whose respiration decreases the levels of dissolved oxygen to those compatible with nitrogenase de-repression and activity. Low ammonium concentration can also be used as nitrogen source, but in this case all ammonium has to be consumed prior to nitrogenase de-repression. For a discussion on the effect of amino acids on growth and nitrogenase activity in *A. brasilense*, *A. lipoferum* and *A. amazonense,* see Hartmann et al. (1988).

8.2.2.1 Effect of Oxygen Concentration in Gas Phase on the Nitrogenase Synthesis and Activity in Liquid Medium

In order to determine nitrogenase activity in liquid medium culture, the experiment is carried out under different oxygen tensions and absence of ammonium ions. In the present experiment, nitrogenase is de-repressed in the presence of acetylene under increasing oxygen concentrations. A time course of nitrogenase synthesis and activity is obtained. The same type of experiment can be performed in cells actively fixing nitrogen.

The strains are first grown in 200 mL minimal liquid medium such as NFbHP containing ammonium chloride (10 mM) as N source in 500 mL erlenmeyer flask at 30 °C in a rotary shaker (120 rpm) until an OD at 600 nm of 1.5–2.0. The cells are collected by centrifugation (5,000 g, 10 min, 4 °C) and re-suspended in N-free NFbHP medium to an OD_{600} 1.0. Since these cells were grown in the presence of high NH_4^+, they are devoid of nitrogenase. The cell suspension (10 mL) is distributed in 60 mL penicillin bottles capped with subaseals or rubber stopper with aluminum seals. The gas phase of the sealed flasks is flushed with pure N_2 for 30 min using a gas manifold equipped with hypodermic needles. Hypodermic needles are inserted in each rubber seal to allow the gas phase to be exchanged. Air (20.1 % oxygen) or pure oxygen is then injected in flask to give zero, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10 % of oxygen in the gas phase, and a flask under air (20.1 % O_2). The oxygen concentration can be verified in a gas chromatograph equipped with Molecular Sieve 5A column (stainless steel; 2 m, 1 mm diameter) and a thermo-conductivity detector (TCD). Acetylene is injected to a final concentration in the gas phase of 10 % (5 mL of pure acetylene), and the flasks are incubated at 30 °C in a rotary shaker at 120 rpm. Two gas samples of 0.5 mL are collected with insulin-type hypodermic syringes after 1, 2, and 3 h and kept till the time of analysis by inserting them in a manifold of rubber corks. Samples of 0.5 mL of the gas phase are taken every 30 min and the ethylene produced is analyzed as described above. Since multiple gas samples are taken from the same flask, it is mandatory to normalize the amount of ethylene produced by the average acetylene peak. To calculate the specific activity of nitrogenase, it is necessary to determine the culture protein concentration. Protein concentration is determined by standard Bradford procedure as described above.

Nitrogenase-specific activity is calculated using the equation:

$$N_2\text{ase} = \left(\left(\text{nmol}\,C_2H_4 \text{ in } 0.5\,\text{mL}\right) \times 100\right) / \left(\text{mg protein} \times T\right)$$

Where 100 is the correction factor for the gas volume, mg protein is the total protein content of the culture in milligrams, and *T* is the incubation time in minutes.

Physiological studies with intact cells can be performed with *A. brasilense* grown in larger scale and kept in liquid nitrogen. Growth, harvesting, and maintenance of bacterial cells are described as follows. A 10 mL saturated *Azospirillum* culture is inoculated in 1,000 mL of NFbHP medium containing 10 mM NH_4Cl in a 2,000 mL erlenmeyer flask. Larger cultures can be set up as needed. The flasks are incubated at 30 °C, 120 rpm in a rotary shaker until OD_{600} 1.5–2.0 (about 24 h). The cells are harvested by centrifugation in a refrigerated centrifuge (5,000 g, 15 min, 4 °C).

The supernatant is discarded and the cell pellet is thoroughly re-suspended in 2.5 mL (approximately the same volume of the cell pellet) of N-free NFbHP medium. Using a 10 mL pipet, the cell suspension is dripped in a 250 mL becker flask containing about 150 mL of liquid nitrogen, forming small beads of concentrated cells. These beads are transferred to cryogenic flask or wrapped in aluminum foil and immediately stored in liquid nitrogen until use. To use the cells, remove about 2 mL of beads from the liquid nitrogen and transfer immediately (while still frozen) into 100 mL of N-free NFbHP medium at room temperature. Under these conditions, the cells are kept intact. The OD_{600} of the cell suspension is finally adjusted to the desired value, usually 1.0.

De-repressed cells can be obtained using the same system, but with 5 mM of sodium glutamate as the sole N source. However, the cell pellet is re-suspended with N-free NFbHP medium flushed with pure argon or nitrogen.

Manipulation of nitrogen-fixing cells and cell-free extracts must be performed under strict anaerobic conditions.

Larger amounts of cells can be obtained in a larger fermenter and processed as described.

When growing *Azospirillum brasilense* using organic acids as C source, the culture pH increases rapidly reaching values above 7.8. At these pHs, cells tend to lyse and the number of viable cells decreases. For physiological studies, NFbHP medium with high phosphate buffer concentration is recommended. However, *A. lipoferum* will not grow well in high phosphate.

8.2.2.2 Effect of Cell Density on Nitrogenase De-Repression in Liquid Cultures

It is possible to de-repress nitrogenase activity under air as long as the cell density is adjusted to one where respiration will keep the dissolved oxygen concentration at permissible levels under the experimental conditions employed. In this experiment, a series of cultures of increasing optical density is prepared in range of 0.6–2.0 (interval of 0.2 OD units) at 600 nm. The cells are grown in ammonium chloride as above, collected and re-suspended in 10 mL of N-free NFbHP medium (60 mL penicillin flasks) at the specified OD_{600}. The flasks are sealed with subaseals; acetylene (10 % of gas phase) is injected and incubated at 30 °C, 120 rpm. Gas samples (0.5 mL) for ethylene determination are collected in 30 min intervals and analyzed as described above.

8.2.2.3 Effect of the Nitrogen Source on Nitrogenase De-Repression

The introduction of glutamate as a sole nitrogen source for the growth of *Azospirillum brasilense* allowed the expression of nitrogenase activity in the presence of air (Pedrosa and Yates 1984). After an initial growth, dependent on glutamate as sole N

source, the respiratory rate of the culture decreases the dissolved oxygen concentrations to levels compatible with nitrogen fixation. It is worth noting that glutamate represses nitrogenase activity completely in *A. lipoferum* and partially in *A. amazonense*. Similarly to glutamate, histidine and serine are good non-repressible nitrogen source in semi-solid and may substitute glutamate under air (Hartmann et al. 1988).

In this experiment, nitrogenase is de-repressed under air and in the presence of different concentrations of sodium glutamate or ammonium chloride. The strains are grown in minimal liquid medium such as NFbHP-containing ammonium chloride (5 mM) as N source overnight at 30 °C in a rotary shaker (120 rpm).

One milliliter of the inoculum is inoculated in 20 mL NFbHP liquid medium containing increasing concentrations of ammonium chloride or sodium glutamate (0.1, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mM) as N source in 60 mL penicillin flasks. The cultures are incubated at 30 °C in a rotary shaker (120 rpm) overnight (16–24 h). The penicillin bottles are then capped with subaseals or rubber stopper with aluminum seals, acetylene is injected to a final concentration in the gas phase of 10 % (5 mL of pure acetylene), and the flasks are incubated at 30 °C in a rotary shaker at 120 rpm. Gas samples (0.5 mL) are taken after 60 min to determine the amount of ethylene produced by gas chromatography as described before. The expected results are: (a) For the cultures in ammonium chloride, the nitrogenase activity will be detected in cultures containing ammonium enough to reach high cell density, no residual ammonium ions, and a dissolved oxygen concentration compatible with nitrogenase de-repression and activity. Cultures with lower or higher NH_4Cl than this optimum will not reach those conditions; (b) For cultures in sodium glutamate nitrogenase will be detected in the cultures containing enough glutamate to reach a cell density where the dissolved oxygen concentration allows nitrogen fixation.

8.2.2.4 Effect of Inoculum Size on Nitrogenase De-Repression

It is possible to determine the maximum nitrogenase activity in liquid culture in the presence of 5 mM sodium glutamate by varying the inoculum concentration and measuring the activity at a fixed time point. Azospirilla inocula is grown in NFbHP containing 5 mM glutamate under the conditions described above and inoculated in increasing amounts (100, 200, 500, 1,000 μL) in 10 mL (final volume) of NFbHP medium containing 5 mM sodium glutamate in 60 mL penicillin bottles. The cultures are incubated at 30 °C in a rotary shaker (120 rpm) for 16–24 h. For nitrogenase measurement, the bottles are tightly sealed with subaseals or rubber stopper with aluminum seals, and 5 mL of acetylene (10 % of gas phase) is injected in each bottle. The cultures are further incubated at the same temperature for 60 min and the ethylene formed determined by gas chromatography and specific nitrogenase activity calculated as before. Alternatively, a time course of nitrogenase activity can be followed collecting samples at every 30 min. A switch-off/on experiment can be performed on the active cultures (see below).

8.2.2.5 Nitrogenase Activity Switch-Off /On by Ammonium Ions

Reversible inactivation of nitrogenase (switch-off/on) by ammonium occurs in several *Rhodospirillales*. An overall genomic analysis identified the presence of 29 different bacterial species of the Proteobacteria, Verrucomicrobia, Deferribacteres, Chysiogenetes, and some unclassified bacteria bearing the genes involved in nitrogenase switch off/on (Huergo et al. 2012). In the genus *Azospirillum,* this mechanism occurs in *A. brasilense*, *A. lipoferum,* and *A. halopraeferens*. There is no evidence for the genes coding for dinitrogenase reductase ADP-ribosylglycohydrolase (DRAG) and dinitrogenase reductase ADP-ribosyl transferase (DRAT) in *A. irakense* and *A. amazonense*. To perform a switch-off/on experiment, actively fixing nitrogen cultures of *A. brasilense* are challenged with ammonium ions, which will trigger inactivation of nitrogenase via ADP-ribosylation of dinitrogenase reductase by DRAT. Nitrogenase activity is restored by DRAG, which removes de ADP-ribosyl group from dinitrogenase reductase after ammonium exhaustion (Huergo et al. 2012). Switch-off/on experiments can be carried out in nitrogen-fixing cultures or following nitrogenase de-repression as described above. For *A. brasilense,* the simplest experiment is to de-repress four cultures for nitrogenase in N-free medium as determined above in the strict defined conditions. Acetylene (10 %) is added at zero time and gas samples (0.5 mL) are collected after 60 min to check for nitrogenase activity. If positive, gas samples are then collected at every 20 min. At time 80 min water (control), NH_4Cl 0.2, 0.4 or 1.0 mM (flushed with N_2 or argon to remove dissolved oxygen), is added to the cultures and gas samples are collected for additional 90 min at the same time intervals.

The results are plotted as nmol C_2H_4/mg protein as function of time in minutes.

The expected results are: (1) Addition of water does not change the rate of ethylene production; (2) Addition of NH_4Cl 0.2, 0.4 or 1.0 mM leads to inhibition of ethylene production after 5–10 min; (3) After ammonium consumption, nitrogenase activity will resume at the previous rate in the cultures added with NH_4Cl 0.2 mM and 0.4 mM, after approximately 20 min and 40 min, respectively; (4) No recovery of nitrogenase activity will be seen in the culture challenged with 1 mM NH_4Cl in the time period analyzed.

8.2.2.6 Immuno-Detection of ADP-Ribosylated Dinitrogenase Reductase

Four liquid cultures of *A. brasilense* are de-repressed for nitrogenase under conditions previously determined (see above). Acetylene (10 %) is added to check for nitrogenase activity. After 60 min 100 μL samples are collected with a Hamilton syringe and immediately transferred to a tube containing 50 μL of freshly prepared 3× SDS-PAGE loading buffer (for 10 mL mix 2.4 mL 1 M Tris–Cl pH6.8, 3 mL 20 % SDS, 3 mL glycerol 100 %, 1.6 mL β-mercaptoethanol, 0.006 g bromophenol blue). Water (control) or NH_4Cl to give the final concentrations of 0.2, 0.4 or 1.0 mM (flushed with N_2 or argon to remove dissolved oxygen) is added to the cultures. Liquid samples (100 μL) of the culture are subsequently taken from the flasks at 20 min interval

and mixed with 50 µL 3× SDS-PAGE loading buffer. The samples are immediately frozen in liquid nitrogen and stored at −80 °C. Nitrogenase activity is usually in the range of 10 and 25 nmol of ethylene produced per min per mg of total protein for *A. brasilense* strains.

The collected samples are lysed in a boiling water bath for 1 min, centrifuged at 13,000 g for 3 min, and 6–8 µL of the supernatant are loaded on a 12 % low cross-linked SDS-polyacrylamide gel (acrylamide:bisacrylamide of 172:1) in Tris-Glycine buffer pH8.3 (Kanemoto and Ludden 1984). Electrophoresis is run at 150 V in a mini gel electrophoretic system for 60–80 min. Protein spots in the gel are transferred to a PVDF membrane activated with methanol using a semi-dry electrophoresis transfer system. This is set up as follows: four layers of filter paper soaked in anode buffer (1) (300 mM Tris pH10, 20 % v/v methanol), (2) layers of filter paper soaked in anode buffer 2 (25 mM Tris pH10, 20 % v/v methanol), PVDF membrane, polyacrylamide gel, six layers of filter paper soaked in cathode buffer (25 mM Tris pH10, 40 mM caproic acid, 20 % v/v methanol). The system is closed and run at 80 mA for 1 h. After this, the membrane is washed briefly in TBST (25 mM Tris pH7.6, 150 mM NaCl, 0.05 % w/v Tween 20) and blocked with 20 mL solution containing 5 % w/v nonfat dry milk in TBST for 16 h at 4 °C. The membrane is then washed twice for 5 min with TBST and incubated with 10 mL primary rabbit antibody against *A. brasilense* dinitrogenase reductase (NifH) in TBST containing 1 % nonfat dry milk at the at the desired dilution (usually 1:5,000) for 90 min at room temperature. The membrane is washed once for 15 min and three times for 5 min with TBST and then incubated with 10 mL the secondary antibody anti-rabbit IgG coupled to horseradish peroxidase (1:5,000) in TBST containing 1 % nonfat dry milk for 90 min at room temperature. Again, the membrane is washed once for 15 min and three times for 5 min with TBST prior to the detection. For dinitrogenase reductase detection by chemiluminescence, the ECL Reagent is used as per the manufacturer's instructions. Excess substrate is removed, the membrane is covered with cling film, and the emitted light detected by exposing to X-rays film for 1.5–5 min or cooled CCD camera. As control, 1 µg of purified *A. brasilense* His-NifH can be used.

Expected results: The ADP-ribosylated dinitrogenase reductase is slower than the unmodified protein in this low cross-linking SDS-PAGE. When 100 % of the dinitrogenase is modified and inactivated, two bands of equal intensity are visualized, since only one subunit of the NifH homodimer is ADP-ribosylated.

8.2.3 Determination of Ammonium/Methylamonium Ions Transport

In *A. brasilense,* the transport of ammonium ions is dependent on energy and repressed by ammonium (Hartmann and Kleiner 1982). The same transport system is also capable of transporting methylammonium with low affinity. Ammonium,

methylammonium, or [^{14}C]methylammonium can be substrate for the ammonium transporter in *A. brasilense*. The ammonium transport system is coded by the *amtB* gene whose expression is regulated by the general nitrogen regulatory system (Ntr) (Van Dommelen et al. 1998). Cells are grown in 200 mL minimal liquid medium such as NFbHP containing low (2 mM), high (10 mM) ammonium chloride, or sodium glutamate (5 mM) as N source in 500 mL erlenmeyer flask at 30 °C in a rotary shaker (120 rpm), overnight or until an OD at 600 nm of 1.5–2.0. The cells are collected by centrifugation (5,000 g, 10 min, 4 °C) and re-suspended in N-free NFbHP medium to DO 600 nm of 1.0. The cell suspension (20 mL) is distributed in 60 mL penicillin bottles open to the atmosphere and starved for 30 min at the growth conditions. At zero time, ammonium chloride (200 μM final) is added and 0.6 mL liquid samples are removed at every 5 min during a period of 20 min, centrifuged in micro-centrifuge at 13,000 g for 20 s or filtered through a 0.2 μm filter. Residual ammonium present in 0.5 mL of the supernatant solution or filtrate is determined by the Chaney and Marbach (1962) procedure using 0.1 mL of concentrated solutions I (phenol 50 g/L, sodium nitroprusside 0.25 g/L) and II (sodium hydroxide 25 g/L, sodium hypochlorite 2.1 g/L) and incubated for 5 min at 50–60 °C. The blue color is read at 625 nm in a spectrophotometer. Ammonium sulfate (0.5–10 nmol of NH_4) is used as standard. Ammonium uptake rate is calculated as nmol of ammonium consumed per minute and mg of protein of the culture. Ammonium concentrations can be measured directly in the cultures using an ammonium electrode (Wood 1981; Van Dommelen et al. 1998).

For methylammonium uptake, the experiment is set up as described for ammonium uptake except that methylammonium replaces ammonium chloride. Fluorescamine (4′-phenylspiro[2-benzofuran-3,2′-furan]-1,3′-dione) is used to determine residual methylammonium. The reaction between methylamine and fluorescamine produces a blue-green-fluorescent derivative with excitation/emission maxima of 390/465 nm (Udenfriend et al. 1972) and a methylammonium solution is used as standard. The reaction system contains 0.5 mL of supernatant or filtrate solution, 1 mL borate buffer (0.5 M, pH8.5), and 0.5 mL of fluorescamine solution (30 mg in 100 mL acetone) and then vortexed vigorously. Alternatively, the product formed can be determined in a spectrophometer at 390 nm with a molar extinction coefficient of 4,000 M^{-1} cm^{-1}.

Methylammonium uptake can also be determined using [^{14}C] methylammonium (Van Dommelen et al. 1998). The culture is set up as described above except that the culture volume is 2 mL in a 10 mL bottle, and [^{14}C]methylammonium (2.1 GBq/mmol) is added to a final concentration of 10 μM. Samples of 100 μL are taken every 5 min during a 30 min period, and immediately vacuum-filtrated through a 0.2 μm filter in a filtration manifold. The filters are dried and radioactivity is measured in a liquid scintillation counter. The radioactivity counts reflect the amount of [^{14}C]methylammonium taken up by the cells. The specific activity is calculated in terms of nmoles of [^{14}C]methylammonium taken up per min per mg protein.

8.2.4 Nitrate Metabolism

Assimilatory and dissimilatory nitrate reduction occurs in the genus *Azospirillum*. Nitrate assimilation occurs via assimilatory nitrate and nitrite reductases with the production of ammonium ions, aerobically/microaerobically with no accumulation of extracellular nitrite. Nitrate supports aerobic growth of most *Azospirillum* strains aerobically using this pathway. Dissimilatory nitrate reduction or denitrification occurs via periplasmic dissimilatory nitrate reductase, nitrite reductase, nitric oxide reductase, nitrous oxide reductase with N_2 the end product. Denitrification occurs under microaerobic or anaerobic conditions in *Azospirillum* spp. with accumulation of nitrate, N_2O, and N_2 (Döbereiner and Pedrosa 1987, Steenhoudt et al. 2001). Under aerobic conditions, *Azospirillum brasilense* also produces low quantities of nitric oxide (NO), which has been shown to promote lateral root formation in tomato even in the absence of indole acetic acid (IAA) production (Molina-Favero et al. 2008). This observation may support previous suggestion by Zimmer et al. (1988) that nitrite or its reduction product (NO) was a factor involved in enhancement of grasses root growth.

8.2.4.1 Denitrification in Semi-Solid Medium

An easy way to detect in vivo denitrification in *Azospirillum* spp. is to grow the bacteria in 4 mL of semi-solid (1.75 g/L agar) minimum medium containing 10 mM KNO_3 in 10 mL penicillin bottle and observe the production of gas bubbles after mixing (Neyra et al. 1977; Magalhães et al. 1978). Prepare a control without nitrate. The culture vials are sealed with subaseals and injected with acetylene to a final concentration 1 % v/v and incubated statically for 24–48 h at 30 °C. Acetylene inhibits the nitrous oxide reductase allowing accumulation of nitrous oxide (N_2O), which is then measured by gas chromatography using Porapak Q column with He as carrier and a thermal conductivity detector (Neyra et al. 1977).

Denitrification can be qualitatively assayed by visualizing gas production in semi-solid cultures. For this, fresh cultures of the bacterial strains are inoculated in 4 mL of semi-solid (1.75 g/L agar) minimum medium containing 10 mM KNO_3 in 10 mL penicillin bottles. After 24–48 h incubation at 30 °C, the vials are shaken to mix the growth pellicle and gas formation (N_2O and N_2) is visually detected in 30–60 min intervals (Neyra et al. 1977). Nitrite concentration can be quantified in 0.1 mL samples of the same semi-solid cultures (vortexed) by adding 2.0 mL of nitrite reagent made by mixing equal volumes of solution A (0.02 g of N-(1-naphthyl)-ethylenediamine dihydrochloride in 100 mL HCl 1.5 M) and solution B (1 g of sulfanilamide dissolved in 100 mL of HCl 1.5 M) just before use (Neyra and van Berkum 1977). The reaction mixture is diluted with 2 mL of water and incubated at room temperature for 15 min. The absorbance of the purple-red colored solution is determined at 540 nm and compared with a standard curve in the range of 10–100 μM KNO_2.

8.2.4.2 Determination of the Effect of Oxygen on the Induction of Nitrate Reductase

Azospirillum brasilense will express both periplasmic and assimilatory nitrate reductases under microaerobic conditions. The optimum oxygen tension for this can be easily determined in experiments similar to that described for nitrogenase.

The strains are first grown in 200 mL minimal liquid medium such as NFbHP containing ammonium chloride (5 mM) as N source in 500 mL erlenmeyer flask at 30 °C in a rotary shaker (120 rpm) overnight until OD at 600 nm of 1.5–2.0. The cells are collected centrifugation (5,000 g, 10 min, 4 °C) and re-suspended in N-free NFbHP medium at an OD_{600} 1.0. Since these cells were grown in the presence of high NH_4^+, they are devoid of nitrogenase. The cell suspension (10 mL) is distributed in 60 mL penicillin bottles capped with subaseals or rubber stopper with aluminum seals. The gas phase of the sealed flasks is flushed with pure N_2 for 30 min using a gas manifold equipped with hypodermic needles. Hypodermic needles are inserted in each rubber seal to allow the gas phase to be exchanged. Air (20.1 % oxygen) or pure oxygen is then injected in flask to give 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10 % of oxygen in the gas phase, and a flask under air (20.1 % O_2). The oxygen concentration can be determined in a gas chromatograph equipped with Molecular Sieve 5A column and a thermo-conductivity detector (TCD). Potassium nitrate to a final concentration of 10 mM is injected and the flasks are incubated at 30 °C in a rotary shaker at 120 rpm. Liquid samples of 0.5 mL are collected with insulin-type hypodermic or a Hamilton type syringe after 1, 2, and 3 h, and the concentration of the nitrite formed is determined as described above. Protein concentration of the cultures is determined as described above.

8.3 Appendices

8.3.1 NFbHP Growth Medium

The NFb growth medium was originally described by Döbereiner et al. (1976). This medium has been modified along the years (Okon et al. 1977a) and the formulation that we use in our laboratory, named NFbHP (Machado et al. 1991), has the following composition per liter: KH_2PO_4 4 g, K_2HPO_4 6 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl 0.1 g, $CaCl_2$ 0.02 g, nitrilotriacetic acid (NTA) 0.056 g, $FeSO_4 \cdot 7H_2O$ 0.02 g, biotin 100 µg, Trace Elements Solution 10 mL, sodium lactate 5 g.

The Trace Elements Solution contains per liter: $NaMoO_4 \cdot 2H_2O$ 200 mg, $MnSO_4 \cdot H_2O$ 235 mg, H_3BO_3, 280 mg, $CuSO_4 \cdot 5\ H_2O$ 8 mg, $ZnSO_4 \cdot 7H_2O$ 24 mg. The final is adjusted to 6.8–7.0, with 1 M KOH.

Concentrated solutions of 1 M NH_4Cl or 1 M sodium glutamate are autoclaved separately and added at the moment of use to give a final concentration of 5 or 20 mM.

For N-free semi-solid medium, 1.75 g of Agar is added to the medium.

A 20 times concentrated phosphate solution is prepared, autoclaved separately, and added to cold sterilized medium.

For *A. lipoferum,* the phosphate concentration should be reduced five times.

A complete review of all growth media for *Azospirillum* spp. and other N-fixing associative/endophytic bacteria has been recently published by Baldani et al. (2014).

8.3.2 *Oxygen Concentrations in Gas Phase*

To calculate the amount of oxygen or air to be injected in the flasks to give different O_2 concentrations in the gas phase use the following equation:

$$V_2C_2 = \left(V_1 + V_2\right)C_1$$

$$V_2 = \left(V_1C_1\right) / \left(C_2 - C_1\right)$$

Where,

V_1 = volume of the gas phase of the flask.

V_2 = volume of pure O_2 (100 %) or Air (20.8 %).

C_1 = desire O_2 concentration in the gas phase.

C_2 = O_2 concentration of the injected gas.

After injecting oxygen, the gas phase internal pressure is equilibrated with the environmental pressure by puncturing the rubber cap with a hypodermic needle to release the pressure.

8.3.3 *Diazotrophic Growth of* Azospirillum *spp. Under Controlled Oxygen Concentrations in the Culture*

Diazotrophic growth and nitrogenase de-repression of *Azospirillum* can be obtained by growing in N-free medium using an O_2-stat system to control the dissolved oxygen concentration. In this system, a saturated culture of *Azospirilum* in inoculated in fresh N-free medium. A Borkowski-Johnson- type O_2 electrode measures the dissolved O_2 and the culture is sparged with sterile air or N_2 as needed to give optimum concentration of dissolved O_2 for nitrogenase de-repression.

The optimum dissolved oxygen concentration for diazotrophic growth of *Azospirillum brasilense* was determined to be in the range of 0.005–0.0075 atm (Okon et al. 1977b; Nelson and Knowles 1978).

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Chapter 9
Nitric Oxide in *Azospirillum* and Related Bacteria: Production and Effects

Melina Amenta, Celeste Molina-Favero, Cecilia M. Creus, and Lorenzo Lamattina

Abstract *Azospirillum* and other related plant growth-promoting rhizobacteria produce several phytohormones and signaling molecules. Among them, nitric oxide is now recognized as a key second messenger in plants and in beneficial and pathological plant-microorganism interactions switching on and off different processes. Nitric oxide has been associated to the signaling cascades leading to lateral and adventitious root development induced by *Azospirillum* in tomato, in the *Azospirillum* biofilm formation, and in the nodule development in rhizobia-legume symbiosis. As a central component of N cycle, nitric oxide is produced and consumed in different metabolic pathways such as denitrification and nitrification, and it is closely related to other N compound like nitrate and nitrite. The emerging importance of nitric oxide in the biology of bacteria-plant relation is a challenge for understanding the molecular and chemical basis underpinning the nitric oxide actions in the association of plant growth-promoting bacteria with roots. In this chapter we describe several techniques that allow detecting and quantifying endogenously produced and exogenously applied nitric oxide in bacteria cultures and inoculated plants, including real-time and/or in situ nitric oxide production. The most used methods—Griess assay, electron paramagnetic resonance, fluorescent probes, and electrochemical sensors—are described with detailed protocols, discussing their advantages and drawbacks. In addition, we remark factors affecting nitric oxide production like growth conditions, metabolic inhibitors, and others. Finally, pharmacological, genetic, and histochemical strategies to study the role of nitric oxide in the association of *Azospirillum* with plant roots are presented with examples and methodological procedures.

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9.1 Introduction

Rhizobacteria secrete metabolites into the rhizosphere which can act as signaling compounds perceived by neighboring cells within the same microcolony, by other bacterial species present in the rhizosphere, or by root cells of the host plant. Signals derived from changes in the soil environment trigger selective root and shoot responses. The mechanisms by which rhizobacteria elicit plant growth promotion, from the viewpoint of signal transduction pathways is a matter of debate. In this scenario, the interrelationships established between roots and the biotic components of the rhizosphere would have a strong impact not only on plant growth, but also on microorganisms. Nitric oxide (NO) is a small bioactive molecule produced either by eukaryotic and prokaryotic cells which in plants has a central role in the signaling pathway for growth and development of roots. The plant growth-promoting rhizobacteria (PGPR) *Azospirillum brasilense* has been proved to produce NO that participates in the signaling cascades inducing lateral and adventitious root formation in tomato (Creus et al. 2005; Molina-Favero et al. 2008) and also in the induction of bacterial biofilm formation (Arruebarrena Di Palma et al. 2013). Therefore, it would be useful and desirable to handle different approaches and techniques that deal with NO metabolism in PGPR and their interaction with plants.

The chemical properties of NO make it a versatile signal molecule that functions through interactions with cellular targets via either redox or additive chemistry. Its small Stokes' radius and neutral charge allows rapid membrane diffusion and, together with its short half-life, makes NO ideal as a near real-time signal between cells (Lamattina et al. 2003). In cells, NO can exist in the form of three interconverting compounds: a free radical nitric oxide ($NO^{\bullet}$), a nitrosonium cation (NO^{+}), and a nitroxyl anion (NO^{-}), each one with different chemical reactivities and properties. Furthermore, the existence of an unpaired electron makes NO highly reactive with oxygen (O_2), superoxide (O_2^{-}), N derivatives, and transition metals (Lamattina et al. 2003).

Nitric oxide can be produced by several pathways in bacteria: (1) Denitrification: is the stepwise dissimilative reduction of nitrate (NO_3^{-}) to nitrite (NO_2^{-}), NO, nitrous oxide (N_2O), and dinitrogen (N_2) by the corresponding N-oxide reductases. This pathway allows denitrifiers to grow under low-oxygen or anaerobic conditions, since NO_3^{-} is used instead of O_2 as a final electron acceptor in respiration (Cutruzzolá 1999). In addition to anaerobic denitrification, it is accepted that denitrification can also occur under fully aerobic conditions (Jetten et al. 1997); (2) Nitrification: is an aerobic ammonium (NH_4^{+}) oxidation pathway yielding hydroxylamine (NH_2OH), NO_2^{-}, and finally, NO_3^{-} (Wrage et al. 2001) in autotrophic conditions. NO is generated as an intermediary in the reduction of NO_2^{-} to N_2. This process can also be performed by heterotrophic microorganisms but without energy generation; (3) NO synthase: NO is produced by the oxidation of L-arginine to L-citrulline, in the presence of O_2 by a bacterial NOS-like enzyme (bNOS) harbored mainly by Gram-positive microorganisms (Stuehr 1997).

Since its discovery as an endogenous-free radical, NO has been proposed to be either cytoprotective or cytotoxic (Stamler 1994; Beligni and Lamattina 1999). The cytoprotection is based on NO's ability to regulate the level and toxicity of reactive oxygen species. NO-mediated toxicity is mainly generated by reaction with O_2^-, leading to the formation of the strong oxidant peroxinitrite, which can oxidize thiols and nitrate peptides and proteins at the phenyl group of tyrosine (Lamattina et al. 2003). These same reactions, along with the ability of NO to transiently bind to numerous sites of proteins—including heme, iron–sulfur clusters, and thiols—enable NO to impact cell activities from the transcriptional to posttranscriptional levels (Cohen et al. 2010). This dual behavior of NO reinforces the necessity of a proper knowledge in the designing, measurement, and results interpretation of experimental approaches to reveal NO actions in the study of plant–microbe interaction.

9.2 Production of NO by Beneficial Rhizospheric Microorganisms: Detection and Quantification

9.2.1 Methods for NO Measuring

9.2.1.1 Griess Assay

Nitric oxide production is often measured indirectly by quantifying the stable end products of its metabolism since NO is not stable and persistent in stored biological samples. Two of the more stable oxidation products of NO metabolism are inorganic nitrite and nitrate (Archer 1993). In 1879, the German organic chemist Johan Peter Griess described a colorimetric protocol for nitrite measurement, which is to date one of the most widely used assays for NO measurement because of its simplicity and commercial availability. In this method, nitrite is first treated with a diazotizing reagent, e.g., sulfanilamide (SA), in acidic media to form a transient diazonium salt. This intermediate is then allowed to react with a coupling reagent, *N*-naphthyl-ethylenediamine (NED), to form a stable azo compound. The intense purple color of the product allows assaying nitrite with high sensitivity and can be used to measure nitrite concentration as low as ~0.5 μM. The absorbance at 540 nm of the formed adduct is linearly proportional to the nitrite concentration in the sample (Xu et al. 2000). Through the years, many variations on the original reaction have been described. The most popular version seems to be the sequential method in which nitrite is mixed with SA first, immediately followed by the addition of NED. This method seems to give highest yield of the chromophore, and therefore, it is the most sensitive way to perform Griess Reaction assay (Verdon et al. 1995; Guevara et al. 1998). This approach is adopted in most commercial kits.

For a more accurate measurement of NO produced in a sample, the nitrate formed via oxidation of nitrite must also be measured. This is often accomplished by reducing nitrate to nitrite immediately prior to the addition of the Griess reagents to the initial

sample (Arita et al. 2007). The available kits use either cadmium or nitrate reductase to reduce nitrate into nitrite (Sun et al. 2003).

This approach was widely employed to identify denitrifying rhizobacteria in soil communities (Braker et al. 2010; Mora-Ravelo et al. 2013; Vercellino and Gómez 2013).

Materials and Reagents

- Reagent A: Sulfanilamide Solution (1 % (w/v) sulfanilamide in 5 % (v/v) phosphoric acid).
- Reagent B: NED Solution (0.1 % (w/v) *N*-1-napthylethylenediamine dihydrochloride).
- Standard Nitrite Solution: 0.1 M sodium nitrite.
- Polystyrene 96-well microtiter plate.
- Microtiter plate reader with 520–550 nm filter.

Griess Assay

- Add 100 μL of the cell-free culture or standard nitrite solution to a well of a microtiter plate in triplicate.
- Transfer 50 μL of reagent A to each well.
- Incubate 5–10 min at room temperature, protected from light.
- Add 50 μL of reagent B to the wells.
- Incubate at room temperature for 5–10 min, protected from light. A purple/magenta color will begin to form immediately.
- Record the absorbance at 540 nm with the microtiter plate reader.
- Compare the obtained values with the standard curve prepared with sodium nitrite (linear between 0 and 100 μM).

Considerations and Recommendations

- Sulfanilamide and NED compete for nitrite in the Griess reaction (Fiddler 1977), thus greater sensitivity is achieved when the two components are added sequentially.
- It is recommended to perform two-times reading to decrease the background variations between wells or samples (Xu et al. 2000).
- The final pH of a sample after addition of reagent A is critical for the Griess reaction. Lower final pH results in higher absorbance at 540 nm. When the pH is lower than 1.8, the absorbance at 540 nm is stable (Xu et al. 2000). For samples with a high buffer capacity, more acid should be added to reagent A.
- In a condition where there is low level of NO production, high amount of nitrate (or nitrite) in the media will make the measurement difficult due to the high

background (Arita et al. 2007), thus it is important to know minutely nitrite and nitrate concentration in the media broth used and also to minimize as much as possible the content of these compounds from water in the solutions.

9.2.1.2 Electron Paramagnetic Resonance

Electron paramagnetic resonance (EPR), also known as electron spin resonance (ESR), is a versatile technique specific for atoms, molecules, and complexes with unpaired electrons (free radicals). Among its several biological applications (for examples see García Rubio 2004), this method allows to identify and quantify a paramagnetic molecule as NO with high specificity and sensibility (detection limit ~10^{-9} M). The advantages of EPR are: (1) it is the only method that unequivocally discriminate NO from other related no free radicals, as nitrate and nitrite; (2) it can report NO levels in vitro and in vivo; and (3) it is possible to use non-transparent (i.e., turbid and/or opaque) and non-purified samples (Kleschyov et al. 2007). The main drawback of EPR is that specialized equipment and technical expertise are needed.

Unpaired electrons, as spinning charges, generate a magnetic field becoming a magnetic dipole. Similar to a compass, these dipoles align themselves in external magnetic fields. EPR is based in the property of these dipoles to take two possible orientations that show "resonance" as the electrons are flipped when energy in the microwave range is applied in presence of an external magnetic field. The amount of energy absorbed is proportional to the amount of unpaired electrons in the sample, and the value of the external magnetic field at which resonance occur, in a constant microwave frequency, is related to g, that is a spectroscopic factor characteristic of a given paramagnetic center. The shape and the hyperfine structure of the spectral line are also characteristic of a paramagnetic substance (Hogg 2010).

The transient nature and concentration of NO in biological samples prevent its direct detection with EPR. Instead, NO must be trapped to form stable paramagnetic adducts before being quantified. Several diamagnetic compounds have been used as "spin trap" of NO, among them are DETC (diethyldithiocarbamate), MGD (*N*-methyl-D-glucamine dithiocarbamate), and DTCS (dithiocarboxy sarcosine) (Venkataraman et al. 2002; Puntarulo et al. 2009).

EPR has been used to study NO metabolism in several microorganisms. As examples, the mechanisms for NO production by nitrite reductase (Nir) in *Pseudomonas* spp. (Radoul et al. 2012) and *Geobacillus stearothermophilus* (Davydov et al. 2009), and for NO consumption by respiratory NO reductase in *Paracoccus denitrificans* (Field et al. 2008) were assessed by this technique. In PGPR, EPR was employed to show the formation of nitrosylleghaemoglobin (LbNO) within soybean nodules of *Bradyrhizobium japonicum* (Meakin et al. 2007), the synthesis of NO by a Nir in *Sinorhizobium meliloti* (Ferroni et al. 2012), and to quantify the NO production by *A. brasilense* Sp245 wt and its IAA^- and periplasmic nitrate reductase (Nap^-) mutants (Creus et al. 2005; Molina-Favero et al. 2008). EPR methodology has also been used for assaying enzyme activity in *A. brasilense* (Vanoni et al. 1992).

Materials and Reagents

- Bacterial sample (a minimum of approximately 0.15 g is needed). Samples do not need to be purified and can simply consist in cells from cultures grown in the condition desired and harvested by centrifugation.
- Solution of 10 mM sodium *N*-methyl-D-glucamine dithiocarbamate (MGD) in 1 mM $FeSO_4$.
- Aqueous solution of 4-hydroxy-2,2,6,6-tetramethyl piperidine 1-oxyl, TEMPOL (a stable free radical used as standard to obtain the concentration of other free radical adducts).
- Bottom-sealed Pasteur pipettes.
- EPR spectrometer.

EPR Assay

- Add 220 µL MGD solution to 150 mg of bacterial sample.
- Transfer the sample with the spin trap to a bottom-sealed Pasteur pipette and introduce in the spectrometer cavity for measurements.
- Record the spectra at room temperature (~18 °C) in the EPR spectrometer operating at 9.5 GHz (X-band). Set the instrument at 200 G field scan, 83.886 s sweep time, 327.68 ms time constant, 5.983 G modulation amplitude, 50 kHz modulation frequency, and 20 mW microwave power. Different modulation amplitude and/or microwave power can be selected to improve the resolution of spectra depending on the spectrometer used.
- Record the spectra of the TEMPOL solution with the same spectrometer setting.
- NO concentration is quantified by double integration of the three-line spectra (Fig. 9.1a) and is referenced to the TEMPOL spectra, which have a known area to concentration ratio.

9.2.1.3 Fluorescent Probes: Diaminofluorescein Fluorescent Dyes

Diamine derivatives of fluorescein (DAF) are the most widely used NO fluorescent probes (Namin et al. 2013) and are one of the best options for NO detection due to their high sensitivity and simple procedure. Diaminofluorescein-2 (DAF-2) was the first product in the DAF series to become commercially available. It reacts with NO, in presence of oxygen, resulting in the formation of a triazolo-fluorescein analogue (DAF-2T) that exhibits a strong green fluorescence (Kojima et al. 1998a, b). DAF-2 DA, a diacetate analogue of DAF-2, was designed in particular for imaging of NO produced in living cells (Kojima et al. 1998a). The diacetate group is removed by cellular esterases, allowing the DAF-2 form to react with intracellular NO (Planchet and Kaiser 2006; Mur et al. 2011). Because of the membrane permeability

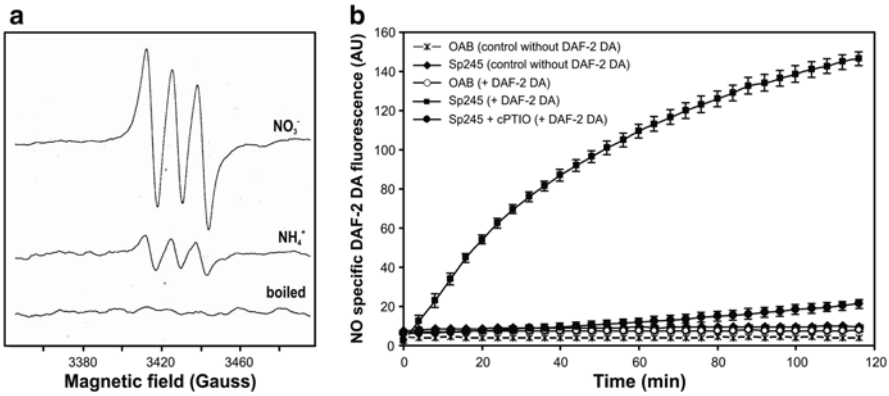

Fig. 9.1 Nitric oxide detection in *A. brasilense* cultures. (**a**) Three-line EPR spectra characteristic of the NO-MGD adduct recorded in pellets of *A. brasilense* Sp245 grown in liquid OAB medium with NO_3^- or NH_4^+ and in cultures boiled for 5 min. The readout resulting from the detector is a first derivative of the absorption spectrum, whose area is proportional to the concentration of unpaired electrons (in this case NO) and the intensity is measured in an arbitrary scale, so the *y* axis is omitted. (**b**) Kinetics of NO production by *A. brasilense* Sp245 determined with the fluorescent probe DAF-2 DA. Kinetics of controls without probe, non-inoculated OAB medium, and culture plus the scavenger cPTIO (0.5 mM) are indicated. The fluorescence is showed as arbitrary units (AU)

characteristic of DAF-2 DA, it is possible to monitor endogenous NO production within the cells in a real-time fashion, conferring an obvious advantage in efficient uptake of the fluorescent probe (Arita et al. 2007). DAF-FM has been developed as a more sensitive NO sensor than DAF-2 DA (~3 nM and ~5 nM, respectively; Murad 1999). Contrary to DAF-2 DA, it has also been suggested that the fluorescent signal of DAF-FM is not affected by pH above 5.

DAF-2 DA was employed to detect NO production by *A. brasilense* Sp245 growing under aerobic conditions (Fig. 9.2; Creus et al. 2005; Molina-Favero et al. 2008) and to establish the involvement of NO in the formation of lateral roots induced by *Azospirillum* (Creus et al. 2005). Schreiber (2006) made use of DAF-FM DA to image NO production by *Bacillus subtilis* and by a nitrifying biofilm. Using these probes, several authors have detected NO production by confocal microscopy in functional nodules during legume-rhizobia symbiosis (see Sect. 9.3.3. below).

Materials and Reagents

- NO-specific probes DAF-2 DA or DAF-FM DA (e.g., Calbiochem, La Jolla, CA, USA).
- DAF-2T or DAF-FMT as standard (e.g., Calbiochem, La Jolla, CA, USA).
- Polystyrene 96-well microtiter plate.
- Fluorescence plate reader (e.g., Fluoroskan Ascent; Thermo Electron) with the appropriate filters (excitation 480 nm; emission 515 nm).

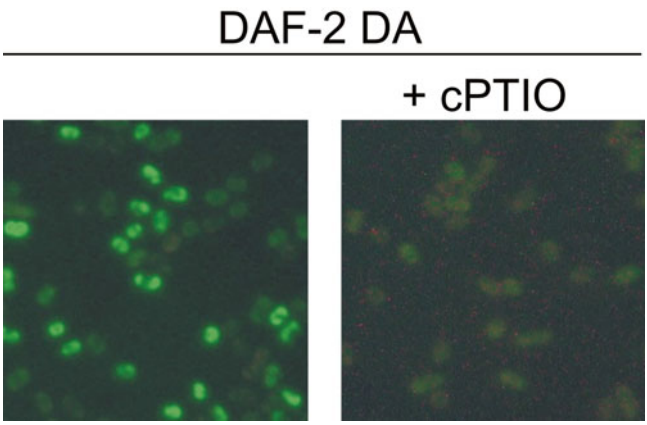

Fig. 9.2 *A. brasilense* Sp245 cells showing NO production examined by epifluorescence microscopy. The bacteria was resuspended in HEPES-NaOH with or without the scavenger cPTIO for 30 min. Bacteria was further incubated for 2 h with DAF-2 DA and examined at 1,000× magnification (Creus et al. 2005; with kind permission from Springer Science and Business Media)

Reagent Preparation

Prepare a 1× working solution of DAF-probe immediately prior to use; a 1:500 dilution in water or in 0.1 M phosphate buffer pH 7.4 is generally required. Do not store 1× solution for later use.

Observations: DAF-probes are very light-sensitive; protect the reagent and the stained samples from direct light. Bovine Serum Albumin (BSA) and phenol red in culture media may affect fluorescence.

Probe Assay

- Transfer 100 μL of the *Azospirillum* culture of each treatment to a 96-well microtiter plate. Do at least three replicates per measurement.
- Add 1× working solution of DAF-probe at 10 μM final concentration.
- Incubate 20 min at room temperature, in dark and with gentle agitation.
- Place the 96-well microtiter plate into a fluorescence plate reader. The fluorescence intensity must be measured every 2–4 min over a period of at least 2 h.
- Determinate NO concentration by comparison with the DAF-2T (or DAF-FMT) reference curve (linear between 0 and 1,000 nmol).

Considerations and Recommendations

- Three types of controls must be done in order to establish if DAF-fluorescence is actually due to NO detection and not caused by methodological artifacts: (1) culture media without the addition of DAF-probe as background fluorescence,

(2) non-inoculated media with DAF-probe to establish if the media cause any interference with the reaction, and (3) inoculated media with DAF-probe plus an NO scavenger (e.g., cPTIO, see Sect. 9.3.1. below) for NO specificity. Figure 9.1b shows kinetics of NO production by *A. brasilense* Sp245 in different conditions using DAF-2 DA and the controls described above.

- It is now established that simultaneous presence of a NO source and hydrogen peroxide (H_2O_2) augments the fluorescence (Balcerczyk et al. 2005), therefore it is important to determine if the treatment applied produces H_2O_2 by cellular lysis. Check this possibility using an inverted fluorescence microscope to visualize potential increases in background fluorescence.
- Only NO production kinetics can be measured with these probes because the reaction is cumulative. For NO consumption, other method must be used.

9.2.1.4 Electrochemical Sensors

Electrochemical methods are the most practical in measuring NO in biological samples due to small electrode size, in vivo capability, nondestructive properties, minimal or no reagents requirements, high sensitivity, simplicity, and the possibility to be operated with limited electrochemistry knowledge (Taha 2003).

Nitric oxide can be detected amperometrically using an NO-specific electrode, typically carbon- or platinum-coated, where the voltage of the electrode is held constant above the oxidation potential of NO (+900 mV against an AgCl reference electrode). The current generated is a linear function of NO concentration at the electrode surface. The available sensors are integrated, which means that there is no need for additional reference or counter electrode. They employ a gas permeable membrane for selectivity assurance, coupled with a controlled instrumental parameter. Membranes can be made from different compounds such as chloroprene rubber, cellulose acetate, collodion/polystyrene, polytetrafluoroethylene (PTFE), and phenylenediamine (Davies and Zhang 2008). These sensors are very sensitive and specific to NO because the gas permeable membrane eliminates all ions and other compounds except gases, and the applied electrical potential and electrode material eliminate interferences from other gases such as oxygen, carbon monoxide, carbon dioxide, etc. Detection levels are as low as 0.3–0.5 nM and a linear range can be achieved up to 25 μM NO (Taha 2003; Arita et al. 2007). Being electrochemical sensors, NO sensors are sensitive to temperature fluctuations (Taha 2003), but newer models are available with a temperature compensation option.

Electrochemical sensors have been extensively used in *Escherichia coli* (Gardner et al. 1998; Pathania et al. 2002; Bang et al. 2006) and in many other pathogenic bacteria (Ouellet et al. 2002; Arai et al. 2005; Lama et al. 2006; Avila-Ramirez et al. 2013) to study real-time NO consumption related to hemoglobin activity. In rhizobacteria, Arruebarrena Di Palma et al. (2013) demonstrated NO production in *A. brasilense* Sp245 biofilm formation employing this method.

Materials and Reagents

- Nitric Oxide Measuring System (e.g., inNO-T-II System, Innovative Instruments, Inc., Tampa, FL. USA).
- NO-specific sensor (e.g., amiNO-2000, Innovative Instruments, Inc., Tampa, FL. USA).
- 1 M sulfuric acid.
- Solid potassium iodide.
- 100 μM potassium nitrite as standard solution.

Sensor Calibration

- Before calibrating and using the sensor, it should have been polarized for few hours, preferably overnight and immersed in calibration solution or water.
- Prepare calibration solution adding 18 mL of water, 2 mL of 1 M sulfuric acid, and approximately 20 mg of potassium iodide to a 20 mL vial. A small stirring bar should be used for mixing and obtaining a uniform solution. When this solution becomes light yellow, due to the formation of iodine, prepare a new solution.
- Immerse the tip of the sensor in calibration solution. For the amiNO-2000, submerge 2–3 mm from the tip.
- Wait for the background to decrease to stable value and then zero the background.
- The in situ generation of NO is achieved by the addition of standard nitrite solution to an acidified solution in the presence of a reducing agent such as iodide ion. In this reaction the molar ratio of nitrite to nitric oxide is 1:1 and consequently, the amount of NO generated equals the amount of nitrite added.
- Add 10 μL of 100 μM nitrite standard solution to a 20 mL stirred calibration solution; this result in 50 nM NO.
- Before the second addition, wait until the current reaches its maximum potential and begins to decline. This normally takes a few seconds.
- Add 20 μL of nitrite standard solution. A final NO concentration of 100 nM will be obtained. Include more additions if you will. It is recommendable using calibration concentrations in the range of the test solution.
- Measure the peak height of the additions. Plot current *vs* concentration to make a reference curve.

Notes

- Calibration should be done at the same temperature of the sample to be tested.
- Other procedures can be chosen for calibration. For example, NO gas dissolved in aqueous solution or a NO-producing agent like *S*-nitroso-*N*-acetylpenicillamine (SNAP) can be used.

Real-Time *Azospirillum* NO Production

- Immediately previous to use, stabilize the microelectrode 15 min running in PBS buffer pH 7.2, followed by 15 min in fresh NFb-malic medium. If another medium will be used previously, make sure it does not generate interference in the measurement.
- Zero the background.
- Immerse microelectrode 3–4 mm in the bacterial culture and start recording changes on current potential. Usually, 30–40 min of recording time is needed per sample to measure NO production in *Azospirillum* cultures.
- Enter the obtained current value in the standard curve to establish NO concentration in the samples.

Considerations and Recommendations

- If you fail to observe the release of NO, you can also analyze for nitrite in free cell supernatants using the same solution employed for calibration. It is recommendable to make dilutions of the supernatant to avoid electrode and solution saturation. For *A. brasilense* Sp245 growing in NFb-NO_3^- media, 20 μL of a 1:25 dilution is sufficient for nitrite detection in 20 mL of calibration solution.

9.2.2 Factors Affecting NO Production in Azospirillum *Culture*

9.2.2.1 Culture Media and Growth Conditions

In order to set the conditions for different experiments, it is necessary to determine those circumstances in which NO production by rhizobacteria is favored.

One important issue to take into account is the bacterial growth phase as this represents a particular physiological stage in which some metabolic pathways are activated and other repressed. Therefore, NO production must be investigated in each phase to acquire a complete picture of the NO metabolism in the studied bacteria. For example, *A. brasilense* Sp245 produces NO throughout the exponential growth phase, with the highest concentration at the end of it (~16 h in OAB media).

On the other hand, the composition of the growth media is determinant of NO production and, depending on the pathway the bacteria use to biosynthesize NO, the media must provide the precursor for its synthesis. In denitrifying bacteria, media with NO_3^- and low O_2 tension must be supplied. If bacteria, as in the case of *A. brasilense*, harbors a periplasmic nitrate reductase not inhibited nor repressed by O_2, the agitation can be high. We have characterized aerobic NO production by *A. brasilense* Sp245 in OAB medium with NO_3^- as N source, in which bacteria produced ~120 nmol NO per gram, a concentration 25-fold higher than that observed

for NH_4^+ containing media (4.2 nmol NO per gram of bacteria) (Molina-Favero et al. 2008).

If one suspects that the major source of NO production in the studied bacteria is heterotrophic nitrification, that possibility must be tested by incubating in media with NH_4^+ as N source. In this media, the addition of hydroxylamine or nitrite should enhance NO production. A four-time increase in the NO production rate was observed when *A. brasilense* Sp245 was incubated in the presence of 0.1 mM hydroxylamine (concentrations up to 0.5 mM had no effect or were inhibitory; Molina-Favero 2014).

If the bacteria produce NO by the action of a bNOS, L-arginine, the substrate of this enzyme, must be provided in the media in concentrations about 10–15 mM.

9.2.2.2 NO Production Pathways: Inhibitors

The inhibition of key enzymes from metabolic pathways leading to NO production could help in the study of the biogenic origin of NO when specific deficient mutants are not available. In these cases, simple and cheap pharmacological application of inhibitors substances or analogues of substrates result in blocking the activity of key specific enzymes in a given pathway.

The denitrification pathway that accounts for the majority of the NO produced by *A. brasilense* when grown in nitrate is driven by nitrate reductase (NR), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) (Zumft 1997).

Two different dissimilatory NR are present in some strains of denitrifiers. The dissimilatory membrane bound nitrate reductase (Nar) generates ATP by proton motive force across plasma membrane, and the periplasmic nitrate reductase (Nap) which is not repressed nor inactivated by O_2 and is not involved in ATP generation.

The catalytic centre of NR joins a guanine molibdopterin dinucleotide cofactor. Tungstate can compete with molybdate for incorporation into the enzyme complex and results in the inactivation of molybdate-dependent enzymes (Harper and Nicholas 1978; Hille 2002). Tungstate is widely used as a NR inhibitor in plant NO research (Mendel 2007) and also in bacteria culture (Betlach and Tiedje 1981). In nitrogen fixing nodules of *Medicago truncatula*, the use of tungstate could inhibit NO production, showing that the NR is involved in the production of NO by the nodule, but not in roots and leaves as the inhibition by tungstate was not achieved in that tissues (Horchani et al. 2011).

However, evidence indicates that tungstate not only inactivates NR, but also inhibits other molybdate-dependent enzymes at least in plants (Xiong et al. 2012). In addition, a number of investigations have shown that tungstate also inhibits root growth, affects cortical microtubule formation, and induces programmed cell death in plants, just like other heavy metals do (Adamakis et al. 2008). Therefore, this inhibitor must be used with caution in analyzing plant microbe interaction experiments, keeping in mind that it is not completely specific (Xiong et al. 2012). The majority of the literature revised uses 1 mM of tungstate as final concentration in liquid bacteria broth (Kletzin and Adams 1996).

On the other hand, dissimilatory Nir is considered the major source of NO in bacteria. Its localization is periplasmic and two different types of Nir can be encountered in denitrifiers even though, so far, there is no report on bacteria harboring the two types (Zumft 1997). One of these types is cytochrome cd1-d Nir that contains heme c and heme d1 as prosthetic group. The other is a Cu-containing -d Nir (Ye et al. 1994).

A Cu-containing Nir from *Alcaligenes faecalis* strain S-6 was purified and crystallized. The purified enzyme was strongly inhibited by KCN, but only slightly by sulfhydryl reagents such as *p*-chloromercuribenzoate and *N*-ethylmaleimide (Kakutani et al. 1981). The use of this type of inhibitors is appropriate when testing purified enzymes, but not when pharmacological experiments are done with living bacteria that would result damaged by blocking other vital processes yielding confusing results and leading to misinterpretation. A combination of pharmacological and biochemical analysis with a genetic approach will be necessary in order to investigate the roles of NO in plants (Xiong et al. 2012), bacteria, and their interaction.

Many Gram-positive bacteria also harbor a specific bNOS which catalyzes the conversion of L-arginine to L-citrulline and NO (Stuehr 1997). Sequence analysis of the genes encoding bNOS reveals great similarity with the N-terminal NOSoxy domain of mammalian NOS, whereas the NOSred domain is completely absent in bNOS. Nevertheless, bNOS are functional and effectively synthesize NO from L-arginine, also showing inhibition by the mammalian NOS inhibitors (Chen and Rosazza 1994; Choi et al. 1997; Sari et al. 1998; Adak et al. 2002). The main used inhibitors for this enzyme are NG-nitro-L-arginine methyl ester hydrochloride (L-NAME) and L-N5-(1-iminoethyl)-ornithine dihydrochlorid (L-NIO). Both of them are selective competitors with L-arginine. They are used in a concentration range between 50 and 100 μM in liquid bacteria broth (Creus et al. 2005).

9.3 Study of the NO Functions in the Plant–*Azospirillum* Association

9.3.1 Pharmacological Approach: NO Donors and Scavengers

In order to fully understand the diverse bioregulatory functions of NO, several experimental strategies have been developed. The methodological approach mostly employed in NO research involves the application of exogenous NO. Such a relatively simple methodology has yielded numerous evidences for specific physiological functions of NO, although some of them seemed rather ambiguous and controversial (Floryszak-Wieczorek et al. 2006).

Nitrogen oxide donors are compounds which generate NO or related N-oxide species in a controlled manner. By definition, all NO donors release NO producing NO-related activity when applied to biological systems and thus are principally

suited to either mimic an endogenous NO-related response or substitute an endogenous NO deficiency (Feelisch 1998). The pathways leading to enzymatic and/or non-enzymatic formation of NO differ significantly among individual compound classes, as well as their chemical reactivities and kinetics of NO release.

Several chemical NO donors are currently being used in experimental studies. The most commonly used donor is sodium nitroprusside (SNP), a transition metal NO complex that generates mainly NO^+. Other donors frequently employed in NO research include S-nitrosothiols, being the main members within this group S-nitrosoglutathione (GSNO) and SNAP. The utilization of these compounds, along with other NO donors, allowed investigators to make a remarkable progress in the field of NO physiology. In bacteria, SNP and GSNO have been extensively used to produce nitrossative stress (Poole and Hughes 2000; Brandes et al. 2007; Avila-Ramirez et al. 2013), to mimic NO activity as signal molecule in biofilm formation (Barraud et al. 2006; Arruebarrena Di Palma et al. 2013; Barnes et al. 2013) and in plant biotic interactions (Creus et al. 2005; Molina-Favero et al. 2007; Scheler et al. 2013; Puppo et al. 2013), and also to complement mutations in NO production pathways (Arruebarrena Di Palma et al. 2013).

On the other hand, the use of different types, concentrations, and ways of application of the NO-releasing compounds has turned out into discrepancies between experimental results obtained with different NO donors. Hence, in order to make the obtained data reliable, it is necessary to complement with experiments using different inhibitors of NO synthesis or NO-scavengers and to monitor precisely the current concentration of NO in the biological sample (Neill et al. 2003).

There are several compounds available that can act as NO scavengers which differ in specificity and in their ability to gain access to the site of NO action. One of the first compounds used as NO scavenger is oxygenated hemoglobin, which in reaction with NO produces methemoglobin and nitrate (Doyle and Hoekstra 1984). This reaction is fast and stoichiometric, but with low specificity for NO (Arita et al. 2007) and non-suitable for intracellular scavenging (Feelisch 1998). Another class of compounds used as NO scavengers are the nitronyl nitroxides, which were introduced earlier for the quantification of NO by EPR (Yoshida et al. 1994). Among them, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (cPTIO) developed by Akaike and coworkers has proved to be very effective for NO scavenging because of its high specificity and stoichiometric reaction with NO (Akaike and Maeda 1996). The combination of DAF and cPTIO, although arguably (see Arita et al. 2007 for more information), has been frequently applied for proving in vivo NO production in animals (Pittner et al. 2003; Uruno et al. 2005; Negri et al. 2013), plants (Arita et al. 2007; Corpas et al. 2011; Tossi et al. 2012; Verma et al. 2014), and bacteria (Creus et al. 2005; Baudouin et al. 2006; Horchani et al. 2011). Additionally, the combined action of SNP and cPTIO treatments has been used to set up the participation of NO in several biological systems including the *Azospirillum*-promoted lateral root formation in tomato seedlings (Creus et al. 2005; Molina-Favero et al. 2007).

In order to make a proper experimental design using this methodology, the investigator should be aware of the key properties and differences between NO donor

classes, including the mechanism and kinetics of NO release from the donor, dependence on the external factors, toxicity of accumulated products, etc. Here are some considerations to take into account when using either SNP or GSNO as NO donors.

- In a biological system, the redox form/s of NO (NO^+, $NO^\bullet$ or NO^-) that is/are actually released makes a substantial difference to the NO donor's reactivity towards other biomolecules, the profile of by-products, and the bioresponse. For example, depending on the reaction conditions, GSNO can act as donors of NO, NO^-, or NO^+ (Singh et al. 1996).
- It is important to know the appropriate information about the susceptibility of a given NO donor to, e.g., oxygen, light, temperature, and changes in pH, in order to set appropriate conditions of the experiment and also to prevent decomposition occurring in the stock solution. In this regard, the release of NO from SNP has been shown to be caused by photochemical reactions (Leeuwenkamp et al. 1984). In contrast, GSNO was shown to release NO in the dark, even though light seems to be necessary to initiate the decomposition process (Floryszak-Wieczorek et al. 2006). In general, stock solutions should be made up fresh before use, kept on ice, and protected from light (SNP solution is prepared in water; for GSNO preparation see Hart (1985)). Final dilutions should preferably be prepared in assay buffer immediately before application and checked for pH.
- Formation of compound-specific by-products may arise during decomposition or metabolism, sometimes in amounts far exceeding those of NO released and even toxic. In the case of SNP, the reduction and subsequent decomposition of the nitrosyl complex is accompanied by cyanide release (Feelisch 1998), therefore controls with this anion should be included. Another negative control to check reactivity of the SNP degradation by-products can be made using an old SNP solution. On the other hand, photolytic and transition metal ion-mediated decomposition of GSNO can lead to GSH generation (Singh et al. 1996), a molecule with an important activity as antioxidant. Controls with this molecule should be included.
- Because of the duality nature of the NO (signal molecule vs. stressing agent), the effect of its action will depend to a large extent on its concentration. Hence, at too high NO donor concentration—instead of stimulation—an inhibition of the process may be observed. For instance, in *Azospirillum* Sp245 growing with nitrate as N-source, the addition of GSNO induces biofilm formation in a dose-dependent manner up to 50 μM GSNO, but higher concentrations produce in turn biofilm disaggregation (see Chap. 11 in this book; Arruebarrena Di Palma et al. 2013). Concentrations above 2 mM GSNO affect *Azospirillum* viability (own data not published). In addition, the efficiency of NO release by SNP is better than GSNO (Ederli et al. 2009) and consequently lower concentrations of SNP than GSNO are required to obtain similar biological effect.
- The temporal signatures of NO release differ significantly among NO donor classes. It is rapid and transient (between 30 and 60 min) for GSNO, while for SNP, a slow and sustained release of NO was registered, with a maximum peak at 24 h (Simontacchi et al. 2012; Grossi and D'Angelo 2005).

- The product of exposure time and concentration of the NO donor determines the quality and magnitude of the biological response to exogenously applied NO. Thus, short-lived NO donors may have to be administered as continuous infusions rather than in a bolus form in order to avoid the delivery of only a short burst of NO. In most cases, NO will have to be delivered continuously over the entire period of incubation in order to best mimic its biological activity.

9.3.2 Genetic Approach

Previously, we introduce the pharmacological approach to study NO, which is the most widely used strategy for plants and plant–microbe interactions (Delledonne 2005). However, it is highly recommended that the use of NO donors, scavengers, and inhibitors is complemented with a genetic approach to substantiate pharmacological findings and to overcome side effects of chemicals.

The genetic approach offers important tools to elucidate the biochemical pathways synthesizing NO and/or its signaling mechanism. The use of mutants produced by insertion of Tn5 transposon and lacking one or more activities has been used as strategy by several authors. Baudouin et al. (2006) inoculated *M. truncatula* seedlings with different *S. meliloti* 1,021 derivatives in order to identify the source of NO in fixating nodules. These mutants, impaired in N fixation ($NifH^-$) or denitrification ($NirK^-$ and $NorD^-$), produced nodules with an unaffected NO level, indicating that these genes are not involved in NO production. Going further, Horchani et al. (2011) used a nodule-targeted RNA interference (RNAi) strategy to specifically knockdown *M. truncatula* nitrate reductases NR1 and NR2 (*MtNr1/2* mutant), thus decreasing NR activity in the N_2-fixation zone without affecting nitrogen metabolism in the whole plant. By inoculating *MtNR1/2* with *S. meliloti* 2011 wt and denitrification mutants affected in *napA* and *nirK* genes, these authors showed that both plant and bacterial NR contribute to NO production in functional nodules. Similarly, we characterized NO production in *A. brasilense* Sp245 wt and two Tn5 derivative mutants impaired in IAA synthesis (*ipdC*$^-$, Faj009 strain, Costacurta et al. 1994) and in Nap activity (*napA*$^-$, Faj164 strain, Steenhoudt et al. 2001a), both in ammonia- and nitrate-containing medium. Subsequently, tomato seedlings were inoculated with these mutated *Azospirillum* strains and results demonstrated that bacterial root-growth-promoting activity is highly dependent on NO production, particularly by Nap (Molina-Favero et al. 2008). The use of both pharmacological and genetic approaches allows us to establish NO as a signaling molecule in the *Azospirillum*–root interaction and the cross talk of NO with auxin in the promoting effects (Creus et al. 2005; Molina-Favero et al. 2007, 2008).

Another genetic approach, successfully adopted by Pothier et al. (2007), involved the use of promoter traps technique. In this methodology short DNA fragments, harboring promoter sequences, are cloned upstream of reporter genes.

Screening a random promoter library of *Azospirillum*, these authors found that the NO-associated *nirK* gene is up-regulated by wheat seed extracts.

The post-genetic "omics" strategies can also be useful to understand NO function in PGPR–plant interactions. Boscari et al. (2013) showed the transcriptomic profiles during nodule development in *M. truncatula-S. meliloti* symbiosis and how the expression pattern is modulated by NO. In a similar manner, Van Puyvelde et al. (2011) studied the transcriptomic response of *A. brasilense* wt and Faj009 to exogenous and endogenous auxin. They found evidence of a putative connection between the IAA and NO biosynthetic pathways since the Nap operon was up-regulated in the *ipdC*$^{-}$ mutant.

Considering that genomes of *A. brasilense* Sp245 (Wisniewski-Dyé et al. 2011) and *Azospirillum* sp. B510 (Kaneko et al. 2010), and in a close future also the Az39 genome (Cassán 2014), are published and annotated, bioinformatics tools are now available. Using these tools and a PCR-based strategy, Arruebarrena Di Palma (2008) failed to show the presence of a bacterial NOS-like gene in *A. brasilense* Sp245, but he was able to show the presence of a putative ammonium monooxigenase gene possibly involved in NO synthesis. This gene is expressed during the growth of *A. brasilense* Sp245 wt and the mutant Faj164 (Molina-Favero 2014).

The following considerations should be kept in mind when a genetic approach is used:

- The conditions needed for a mutation to be expressed. As examples, NO production in *M. truncatula-S. meliloti* nodules rapidly increases in hypoxia and it is in this condition when the differences in NO levels between wt and mutants (both rhizobia and plant) are noticed (Horchani et al. 2011). Also, the knockout mutation of *A. brasilense* Sp245 Faj164 in the *napA* gene is only relevant when nitrate is present. Therefore, differences in NO synthesis and root growth-promoting effects due to inoculation are only recorded in incubation media with 2–10 mM nitrate (Molina-Favero et al. 2008).
- Mutations can have pleiotropic effects. As a case, some *A. brasilense* mutated in NR are reported to have a reduced colonization capacity (Steenhoudt et al. 2001b). For this reason, in inoculated tomato seedlings an obligated control is to check that the number of wt and Nap mutant Faj164 cells in roots are not significantly different (Molina-Favero et al. 2008). Specific controls must be performer in each case.
- The degree in which a single mutation affects a metabolic pathway and/or the number of genes which must to be mutated to completely knock out an activity. Analogous to IAA, several different NO biosynthetic pathways exist in bacteria (Schreiber et al. 2012) and in plants (Gupta et al. 2011). In the latter, NO null mutants has not been obtained so far (Gupta et al. 2011). In *A. brasilense* Sp245, there are also redundancies in metabolic pathways leading to NO production (Molina-Favero et al. 2007). As well, some compensation or a complementation can exist between plant and microorganism to produce NO (Horchani et al. 2011).

9.3.3 Histochemical Approach

So far in this chapter, we have introduced different methodologies to study NO production in *Azospirillum* and related bacteria, and diverse strategies to analyze the role of NO in bacteria and during beneficial plant biotic interaction by using pharmacological and genetic approaches.

To deeply understand NO functions in plant-rhizobacteria associations, the researcher should also analyze whether NO is actually produced differentially during the interaction and, if possible, identify its source, timing, and localization. These goals can be accomplished monitoring real-time NO production in root tissues of inoculated plants by using fluorescent probes under a microscope.

This technique allowed several authors to observe transiently NO production in *Lotus japonicus* and *Medicago sativa* roots 4 h after inoculation with their cognate symbionts, suggesting that NO production could result from the specific recognition of the plant and bacterial partners (Shimoda et al. 2005; Nagata et al. 2008). Furthermore, in *M. truncatula* roots, using DAF-2 DA, del Giudice et al. (2011) demonstrated that NO is produced at different sites during the infection process. Using DAF-FM DA, Baudouin et al. (2006) detected NO production by confocal microscopy in functional nodules during *M. truncatula-S. meliloti* symbiosis. Nitric oxide was specifically localized in the bacteroid-containing cells of the nodule fixation zone.

This approach was also employed by Creus et al. (2005) to analyze NO accumulation in *Azospirillum*-inoculated and control tomato roots grown in the presence or absence of 1 mM cPTIO (Fig. 9.3). Using DAF-2 DA, they demonstrated that: (1) roots from inoculated seeds displayed higher fluorescence intensity compared to non-inoculated ones and that this fluorescence could be partially blocked by cPTIO (Fig. 9.3a); (2) the fluorescence was located mainly at the vascular tissues and subepidermal cells (Fig. 9.3b). The protocol employed in this work is detailed below.

Materials

- 1× dye solution: 15 μM DAF-2 DA in 20 mM HEPES-NaOH pH 7.5.
- 20 mM HEPES-NaOH buffer pH 7.5.
- Bright-field and epi-fluorescence microscope with appropriate filters (e.g., Eclipse E 200, Nikon, Tokyo).

Tissue Sections Stain

- Choose the tissue section to analyze. Only fresh tissue sections should be used. Do not fix sections with organic solvents or formalin as this will denature enzymes and give false readings from fixatives.

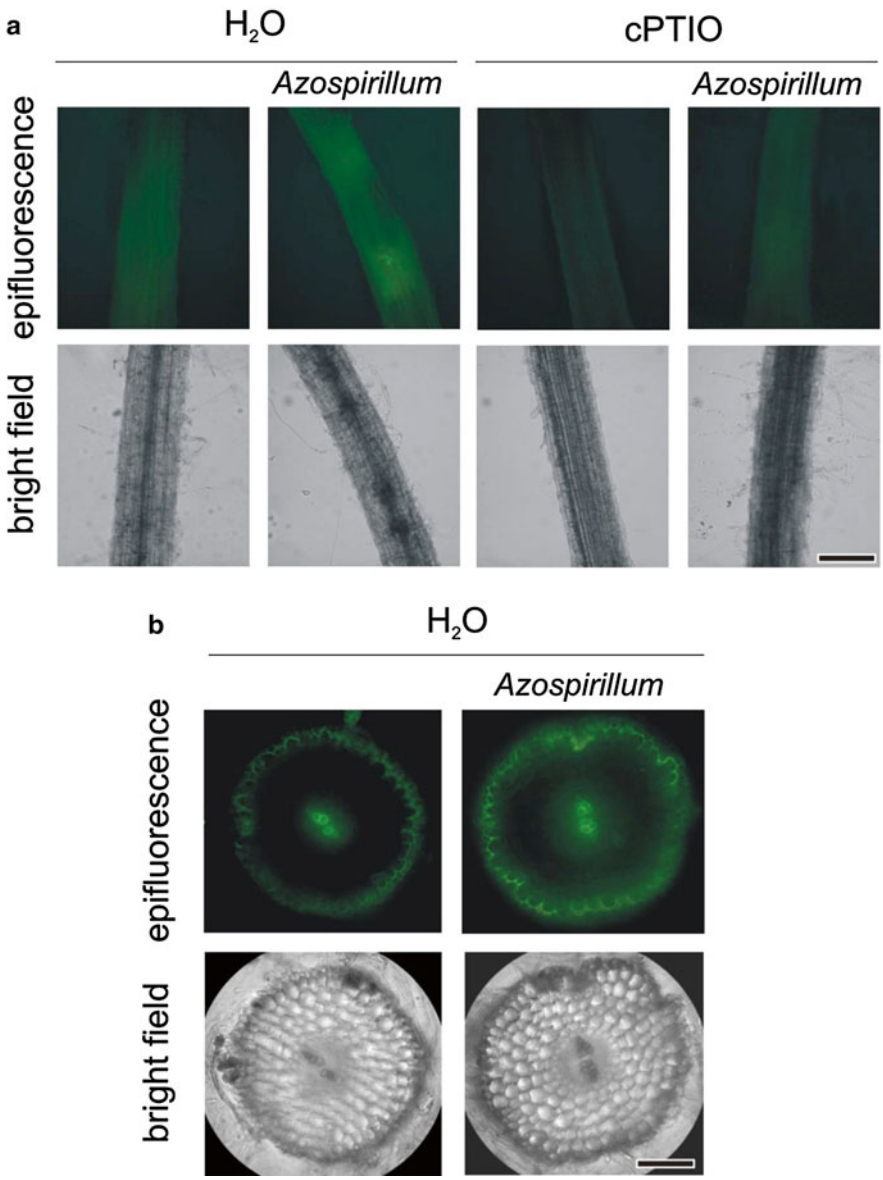

Fig. 9.3 Nitric oxide detection by fluorescence with DAF-2 DA in *Azospirillum*-inoculated tomato roots. Tomato seeds were inoculated with *A. brasilense* Sp245 and incubated in distilled water (H_2O) or cPTIO for 7 days. Control seeds were not inoculated (−). Root segments were incubated with DAF-2 DA and observed by epifluorescence (*upper panels*) or bright-field (*lower panels*) microscopy. (**a**) Longitudinal view of squashed roots. Bar = 0.3 mm. (**b**) Transversal sections of the primary root showing the green fluorescence corresponding to NO. Bar = 0.05 mm (Creus et al. 2005; with kind permission from Springer Science and Business Media)

– Wash the sections with 20 mM HEPES-NaOH pH 7.5 or other buffers free of serum, BSA, and phenol.
– Flood the sections with the 1× dye solution and incubate for 2 h in dark.
– Wash sections three times for 15 min each in the dark with fresh buffer to remove excess dye.
– Prepare the stained tissue sections to be examined by epifluorescence (excitation 490 nm; emission 525 nm) and bright-field microscopy (e.g., the stained root tomato segments shown in Fig. 9.3 were squashed (a) or transversally cut with a razor blade (b)).
– For relative fluorescence quantification, analyze the images using suitable software (e.g., IMAGE J).

Note

- Since increased NO accumulation in inoculated plants could be the consequence of NO production by PGPR itself and/or by the plant (Creus et al. 2005), it is recommended to use this methodology with genetically modified plants or PGPR strains displaying diminished NO generation in order to discriminate the origin of NO between both organisms.

9.4 Concluding Remarks and Perspectives

With the beginning of the twenty-first century, we have faced an impressive advancement of the knowledge in the field of the diversity, biochemistry, and influence of the rhizosphere in plant growth determination.

The results of improving our understanding of how the "interactome" works in the diversity of species inhabiting the microbiota in rhizosphere should help us in learning about the consequences of greatly disturbing the soil homeostasis. It is a challenge nowadays identifying significant plant–microbe interactions at the rhizosphere at individual level. Knowing the genomes composition of the association could lead to the incorporation of plant–microbiome interaction as a trait to be considered into plant breeding programmes (Lundberg et al. 2012; Peiffer et al. 2013; Wagner et al. 2014).

Soils contribute with almost 20 % of the global atmospheric NO budget (Conrad 1995). Nitric oxide is a product of denitrification, nitrification, and reduction of nitrate to ammonia through microbial activity in soils (Zumft 1997). On the other hand, there is a significant increase in the emission of NO from soils fertilized with either biological or inorganic products (IPCC 1995; Ruser et al. 1998), but, remarkably, no studies have been conducted to distinguish between the effects of NO emission from soils and the effect of N fertilization per se on the crops' yield. Besides, NO emission appears to be also dependent on crop species (Roelle et al. 2001), soil temperature, and water content, in addition to the level of total extractable nitrogen from soils (Davidson and Kingerlee 1997).

As a result, intriguing aspects concerning the significance of the NO produced by the biological activity in soils remains to be elucidated: (1) Is the contribution of the microbial activity in rhizosphere a source of NO important for the root perception of the rhizosphere composition and biodiversity, and for keeping the homeostasis required for the normal root growth and developmental processes?; (2) Is it possible to distinguish, among the increased NO fluxes emitted from the microbial activity in soils, between the NO that is acting as a signal for specific growth and developmental processes, from the NO derived as a by-product of N metabolism? (3) Is it possible to find a reliable method to distinguish the contribution of NO from microbiota in rhizosphere from the NO produced by plants?

These topics represent strong challenges to focus the attention of researchers working on plant–microbe interactions in soils in the coming decades.

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Chapter 10
Azospirillum Cell Aggregation, Attachment, and Plant Interaction

Lily Pereg

Abstract *Azospirillum* cellular and morphological transformation in culture as well as cyst formation, aggregation, and flocculation in response to nutritional limitations and increasing oxygen levels are discussed and typical protocols for flocculation and aggregation are presented. An overview of the mechanisms of attachment to plant roots and other surfaces is followed by protocols for labeling *Azospirillum* cells with reporter genes and using such genetically labelled cells in qualitative and quantitative assays of *Azospirillum*–plant associations. The potential of *Azospirillum* in plant pathogen and disease suppression is discussed.

10.1 Morphological Transformation in Culture, Aggregation, and Flocculation

Bacteria of the genus *Azospirillum* are Gram-negative, non-fermentative, vibrio- or spirillum-shaped, 1 μm in diameter, and 2.0–4.0 μm long; however, long filaments have been observed in liquid media that were supplemented with yeast or beef extract and in old cultures. *Azospirillum* species show distinct utilization patterns of organic acids and carbon sources (Table 10.1). Transition into non-motile, cyst-like cells (sometimes called C-forms) was also observed in older cultures, or in well-aerated cultures (Eskew et al. 1977; Tarrand et al. 1978; Hall and Krieg 1983; Becking 1985; Danneberg et al. 1985, 1986; Elmerich 1991; Del Gallo and Fendrik 1994).

Colonies of *Azospirillum* develop a hardened and dry surface after a few days of incubation in medium containing malate (Okon and Itzigsohn 1992). Pink colouration in old cultures, observed with *A. brasilense* and *A. lipoferum*, was attributed to carotenoid accumulation (Eskew et al. 1977). However, other species of *Azospirillum* are not pigmented (Elmerich et al. 1992). Hartmann and Hurek (1988) observed increased oxygen tolerance in carotenoid-overproducing mutants of *A. brasilense* Sp7 (as compared to the wild-type) under nitrogen fixing conditions. Colonies of

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Table 10.1 Carbon sources utilised by various *Azospirillum* species

Carbon source	*A. lipoferum*	*A. brasilense*	*A. amazonense*	*A. halopraeferens*	*A. irakense*
D-Glucose	+	−	+	−	+
Glycerol	+	+	−	+	−
D-Mannitol	+	−	−	+	−
Pectin	−	−	−	−	+
D-Sorbitol	+	−	−	−	−
Sucrose	−	−	+	−	+

The data are taken from Hartmann and Zimmer (1994)

several strains of *Azospirillum* can be readily distinguished from colonies of other diazotrophs by scarlet colouration in culture media containing the dye Congo red (Rodríguez-Cáceres 1982).

Azospirillum proliferates under aerobic and anaerobic conditions, but is preferentially microaerophilic in the presence or absence of combined nitrogen in the medium (Okon and Itzigsohn 1992). Bacteria of the genus *Azospirillum* are motile: they possess several peritrichous flagella used for swarming in semi-solid media and a longer, polar flagellum used for swimming in liquid media (Hall and Krieg 1983). The flagellin of the lateral flagella of *A. brasilense* Sp7 is encoded by *laf1*, and the derived protein Laf1 is extensively similar to the flagellin of *Rhizobium meliloti* and *Agrobacterium tumefaciens* (Moens et al. 1995). The structural gene *laf1* is expressed when cells are grown on solid media, but not in broth (Moens et al. 1996).

The characteristic formation of a pellicle of bacteria in semi-solid nitrogen-free media (Döbereiner and Pedrosa 1987) is due to aerotactic response of the motile bacteria towards the lower level of oxygen concentrations that allow nitrogen fixation and growth (Barak et al. 1983; Okon 1985; Okon et al. 1980). In this zone the concentration of dissolved oxygen permits optimal respiration rates without inhibiting nitrogen fixation (Day and Döbereiner 1976). This chemotactic attraction towards a specific oxygen tension was confirmed in semi-solid media with combined nitrogen. As growth continues and more oxygen is consumed, the pellicle moves towards the surface, where a dense pellicle forms. This growth pattern in semi-solid media is one of the most characteristic features of the growth of azospirilla and permits its tentative identification in enrichment cultures (Döbereiner and Pedrosa 1987).

Poly-β-hydroxybutyrate (PHB) is synthesised and accumulated by *A. brasilense* in the form of granules. PHB synthesis is favoured under limitation of respiratory electron acceptors, such as oxygen, nitrate, nitrite, and nitrous oxide, as well as by a high C/N ratio towards the end of exponential growth in batch culture. Accumulation of PHB, which under certain conditions can be as high as 70 % of *A. brasilense*'s dry weight, is considered a factor contributing to the better survival of the PHB-rich cells in the presence of stress conditions, such as desiccation, ultraviolet

radiation, and osmotic shock. It is also considered as an alternative carbon and energy source for growth and nitrogen fixation under "starvation" (Tal et al. 1990; Okon and Itzigsohn 1992; Zimmer et al. 1984).

Azospirillum displays high degree of pleomorphism with cellular and colony variations among the species as well as within each species depending on the strain, medium composition, and culture conditions (Becking 1985). Their ability to shift their metabolism quickly allows strains of *Azospirillum* to adapt well to swift changes in environmental conditions: under low oxygen tension, bacteria of the genus *Azospirillum* are vibrioid, nitrogen fixing, Gram-negative rods. They are highly motile by means of a long polar flagellum in liquid medium and additional peritrichous flagella on solid medium (Tarrand et al. 1978). Under aerobic conditions, particularly in aged cultures, vegetative cells undergo a transition to round, non-motile, encapsulated cyst-like forms (Sadasivan and Neyra 1985a, b, 1987). In minimal liquid media supplemented with certain carbon sources, such as fructose or β-hydroxybutyrate, heavy capsulation gives the cells a particular adhesive nature, so that they aggregate in a matrix of polysaccharide material, forming large macroscopic clumps (flocculate). During flocculation, the vegetative cells develop an outer layer coat of polysaccharides, lose motility, assume an enlarged spherical form, and accumulate abundant poly-β-hydroxybutyrate granules (Sadasivan and Neyra 1985a, b). The term cellular differentiation is sometimes used to describe the cellular shift from vegetative to cyst-like forms. However, it should not be confused with differentiation in multicellular organisms. The process in bacteria is not terminal, as with changing conditions, the cells may resume their original morphology. Therefore, the term morphological transformation will be used here.

The ultrastructure of *Azospirillum* cyst-like cells is different from that of *Azotobacter* cysts (Stevenson and Socolofsky 1966). Nevertheless, they have greater resistance to desiccation, osmotic pressure, and UV irradiation than vegetative cells (Lamm and Neyra 1981; Sadasivan and Neyra 1985a, b; Bashan et al. 1991a). The cyst-like cells are larger than the vegetative cells. They are non-motile and devoid of flagella. Lower nitrogenase activity, or no activity, was detected with cyst-like forms of *A. brasilense* and *A. lipoferum* in culture (Papen and Werner 1982; Bastarrachea et al. 1988). However, using in situ hybridisation with fluorescently labeled, rRNA-targeting oligonucleotide probes, cyst-like cells of *A. brasilense* were shown to be physiologically active in the rhizosphere of wheat, at least in the first stages of their morphological transformation (Assmus et al. 1995). In liquid media, cyst-like cells are embedded in a fibrillar matrix, forming flocs (Becking 1985). Flocculation in *A. brasilense* Sp7 and *A. lipoferum* Sp59b occurs under conditions of low nitrogen and high carbon concentrations, preferably nitrate (0.5 mM) and fructose (8 mM), and a high tension of oxygen (Sadasivan and Neyra 1987). The cyst-like cells of *A. brasilense* consist of a central body filled with poly-β-hydroxybutyrate (PHB) granules and a thick outer layer of polysaccharides (Sadasivan and Neyra 1985a, b, 1987). *A. lipoferum* strains form flocs when grown on a nitrogen-poor, PHB-rich, medium (Bleakley et al. 1988).

The response regulatory protein FlcA controls the shift of *Azospirillum* from vegetative state to cyst-like forms, both in cultures and in association with plants.

Tn5-induced *flcA*$^-$ mutants do not flocculate, do not transform from motile vibriod cells into non-motile cyst-like forms, and lack the exopolysaccharide material on the cell surface under all conditions (Pereg Gerk et al. 1998). This has strong effects on the colonisation efficiency of plant roots by *Azospirillum*, as they depend on the production of exopolysaccharides to firmly attach to the root surface (Katupitiya et al. 1995; Pereg Gerk et al. 1998, 2000; Pereg Gerk 2004).

Stress conditions induce aggregation, or flocculation, in broth cultures of *Azospirillum*. Phase-contrast microscopy was first used to show a transition from motile, vibriod cells to non-motile encystic forms during the formation of flocs, which also show higher resistance to desiccation than the vegetative cells (Sadasivan and Neyra 1985a, b). The extent of flocculation varies among species, and various strains of the same species differ in their extent of flocculation, e.g., *A. brasilense* Cd forms smaller and lesser flocs than Sp7 under several conditions tested (Burdman et al. 2000a). Some spontaneous flocculation mutants were isolated from the free cells suspension of a flocculating culture (Pereg Gerk et al. 2000) and by transposon mutagenesis of the *flcA* gene (Pereg Gerk et al. 1998). Interestingly, while flagella were shown to be involved in attachment to plants (Moens et al. 1995), non-flagelated mutants were not affected in their ability to aggregate (Burdman et al. 1998; Pereg Gerk et al. 2000).

The terms aggregation and flocculation are being used to describe the same phenomenon: the attachment of cells to one another in liquid cultures to form clumps (aggregates). These aggregates grow into macroscopic flocs, making flocculation observable by the naked eye. While aggregation has been considered to be a synonymous word for flocculation by some authors (Burdman et al. 2000b), there is a difference in the way they are measured experimentally and the way they are referred to in numerous publications: flocculation assays measure flocs that are left to occur spontaneously in specialised liquid media under agitation. Aggregation is often measured quantitatively using spectroscopy and a bioassay for aggregation has been developed with further processing of the cultures. Typical protocols for flocculation and aggregation assays are given below.

10.1.1 Flocculation

Flocculation is observed visually and is mostly qualitatively described, although there are reports of quantifying flocculation by filtering the broth culture through a filter that allows single cells to pass through (e.g., Whatman no. 1) trapping the flocs and weighing the dry or wet weight of the flocs (Sadasivan and Neyra 1985a, b). A typical protocol for observing flocculation is given below.

Azospirillum flocculation assays are performed in minimal medium supplemented with high ratio of carbon to nitrogen source, for example with 0.5 mM KNO_3 and 8 mM fructose or 20 mM β-hydroxybutyrate. Other nitrogen or carbon sources can be used, but nitrate and fructose were found to be the most effective for promoting flocculation in *A. brasilense* (Pereg Gerk et al. 2000).

A typical protocol for flocculation includes:

- Harvesting the cells from a log-phase culture grown in rich medium such as Nutrient Broth (NB) by centrifugation at 5,000 rpm for 10 min at room temperature.
- Washing (resuspending, centrifuging, and resuspending again) the cells with minimal medium.
- Inoculating 10 mL flocculation medium in 50 mL flasks to an absorbance (A) of 0.3–0.4 at 600 nm.
- Incubating with shaking (200 rpm) at 30 °C.

Flocculation can be determined by visual examination following 1–20 h of incubation and confirmed by stereomicroscopy. Clear flocks should be observed from 4 to 5 h with *A. brasilense* Sp7 and Sp245 (Pereg Gerk et al. 2000). Reduction in the absorbance reading at 600 nm, while avoiding the flocks, can be used as a quantitative measure of flocculation.

10.1.2 Aggregation

10.1.2.1 Quantitative Measure of Aggregation (from Burdman et al. 1999)

This assay was modified by Burdman et al. (1999) from the method of Madi and Henis (1989). In this assay suspensions containing aggregates are transferred to conical tubes and allowed to stand for 20 min at 24 °C. During that time, aggregates sink to the bottom of the tube and the free cells still float in the suspension. The turbidity of the suspension is then being measured at A_{540} (OD_1). The culture is then homogenised (for 1 min) and the total turbidity measured immediately (OD_2). The percentage aggregation (PA) is calculated according to the formula: $PA = (OD_2 - OD_1) \times 100/OD_2$.

10.1.2.2 Aggregation Bioassay (from Burdman et al. 1999, 2000a)

In this bioassay, cultures are being grown in high or low C:N medium (Burdman et al. 1999) and then centrifuged twice (4,000 g, 10 min) to yield approximately 0.3 g total bacterial dry weight. Harvested cells from high C:N medium are resuspended in 10 mM potassium phosphate buffer (pH 6.8) and then sonicated for 20 s on ice to temporarily disrupt the cell aggregates. The sonicated suspension is then centrifuged (5,000 g, 15 min) and the supernatant further filtered through 0.45 μm and made to 60 mL with phosphate buffer (designated sonicate extract). The pelleted cells from both high and low C:N media are also kept and resuspended in 10 mL of clean phosphate buffer (bacterial suspensions). Each bacterial suspension (0.5 mL) is then being treated with 10 mL of sonicate extract (or clean phosphate

buffer as control) and 4.5 mL of clean phosphate buffer and incubated under agitation (150 rpm) at 30 °C for 2–3 h. The extent of aggregation is then measured as described above.

Aggregation and flocculation in *Azospirillum* were found to be affected by a range of chemical and physical factors such as the sources of C and N, the ratio of C:N, pH, oxygen levels, and agitation (reviewed in Burdman et al. 2000a, b). Aggregation involves extracellular compounds such as fimbriae and pili (reviewed in Burdman et al. 2000b), proteins, including major outer membrane protein, *A. brasilense* Cd MOMP (Burdman et al. 2001), and polysaccharides, with arabinose content of exopolysaccharides playing a role in the ability of *A. brasilense* Sp7 cells to aggregate (Bahat-Samet et al. 2004).

10.2 Attachment to Plant Roots

The genus *Azospirillum* belongs to plant growth-promoting rhizobacteria (PGPR), a group of bacteria that displays beneficial effects on plant growth (Vande Broek and Vanderleyden 1995). Attachment of *Azospirillum* to roots is mainly dependent on two factors: the existence of a polar flagellum, allowing the bacteria to adsorb to the roots, and the production of exopolysaccharides (EPS), allowing the bacteria to firmly attach to the root surface (Michiels et al. 1991; Croes et al. 1993). Other root-associated microbes show similar traits. The production of β, 1-2 glucan is essential for the nodulation of legumes by the endosymbionts *Rhizobium meliloti* (Dylan et al. 1986) and *Bradyrhizobium japonicum* (Puvanesarajah et al. 1985, 1987), as well as for the induction of tumours following the attachment of the phytopathogen *Agrobacterium tumefaciens* to plant cells (Cangelosi et al. 1987; Matthysse et al. 1981). Several *cps* mutants of the pathogen *Erwinia stewartii*, impaired in capsular polysaccharide production, lost their virulence towards corn seedlings (Dolph et al. 1988; Coplin and Majerczak 1990).

Initially, the isolation of *Azospirillum* genes involved in plant interaction was complicated since plants do not develop an easily detectable phenotype following inoculation. Thus, it was impossible to isolate mutants of *Azospirillum* impaired in colonisation on the basis of deficiency of symbiosis or pathogenicity, as was initially done with *Rhizobium* and *Agrobacterium*. Two main approaches have been taken: (1) isolation of interaction genes by inoculation with mutants defective in phenotypes that are considered to have a role in plant association, such as the genes involved in the production of auxins and surface compounds, or in nitrogen fixation; (2) isolation of interaction genes on the basis of DNA homology with genes of other plant-associative bacteria, such as *nod* genes, which are homologous of *Rhizobium* nodulation genes (Onyeocha et al. 1990), *exo* genes, involved in exopolysaccharide production (Michiels et al. 1988; Petersen et al. 1992), and nitrogen fixation genes (*nif* and *fix* genes) (Vande Broek and Vanderleyden 1995).

Several genes involved in the effective nodulation of legumes by *Rhizobium* were found on plasmid DNA, such as nodulation genes and genes affecting EPS production (Hynes et al. 1986). The importance of plasmid DNA to the biology of

Rhizobium led to a great interest in the role of plasmid DNA in *Azospirillum*. The most studied is the p90 megaplasmid of *Azospirillum*, which carries genes involved in plant-bacterial interaction, such as: (1) *exoBC*, encoding for EPS production, (2) *nodPQ*, homologous to nodulation genes in *Rhizobium*, (3) *mot1*,*2*,*3* genes, involved in the production of the polar and lateral flagella, and (4) genes involved in IAA synthesis and in chemotaxis (Michiels et al. 1989; Katsy et al. 1990; Onyeocha et al. 1990; Van Rhijn et al. 1990; Vieille and Elmerich 1990; Elmerich 1991; De Troch et al. 1994). Croes et al. 1991, proposed to denote the p90 plasmid as a *rhizocoenotic* plasmid, pRhico. In contrast to p90, the plasmid p115 is easily lost in *A. brasilense* and its loss affects neither IAA production, free-living nitrogen fixation, motility, and chemotaxis, nor the attachment to plant roots (Vande Broek and Vanderleyden 1995).

Specific labelling of *Azospirillum* strains with fluorescently labelled phylogenetic oligonucleotide probes (Assmus et al. 1995) and with fluorescently labelled monoclonal antibodies (Schloter et al. 1993), using the confocal laser scanning microscope, was proved to be a useful tool for detection of endophytic as well as rhizospheric bacteria. Washing wheat roots colonised by *A. brasilense* Cd removed most of the root-external bacteria and revealed a smaller internal root population (Bashan et al. 1986). *A. brasilense* Cd was also detected internally, within the cortex, using the immuno-gold labelling (Levanony et al. 1989). Schloter et al. (1994b) examined colonisation of wheat by *A. brasilense* strains Sp7 and Sp245 using strain-specific monoclonal antibodies and found that both under axenic and field conditions, strain Sp245 colonised the root xylem, while Sp7 could only be detected on the root surface. Gough et al. (1997) have showed colonisation of the root interior of the dicot *Arabidopsis thaliana* by *Azorhizobium caulinodans*, following attachment to cracks at the lateral root emergence sites. *A. brasilense* Sp245 was also found at the sites of lateral root emergence, as well as at the root hair zone, during the first few days of association (Vande Broek et al. 1993). Although strain Sp7 was not found internally, it initially colonised the sites of lateral root emergence and the root hair zone (Katupitiya et al. 1995).

Detection of *Azospirillum* on plant roots lost its complexity with the recent development of genetic tools, such as the *lacZ* (Casadaban et al. 1980, 1983; Drahos et al. 1986; Pardy 1994) and the *gusA* labelled strains (Wilson et al. 1995). The *lacZ* fusion encodes the enzyme β-galactosidase, which hydrolyses β-galactoside bonds and can use both *o*-nitrophenol-β-D-galactoside (ONPG) and 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) as substrates, to give coloured products (yellow or blue, respectively). *GusA* encodes for the enzyme β-glucuronidase, which hydrolyses a variety of glucuronide substrates to give coloured or fluorescent products (Wilson 1995). Using simple and fast procedures, it is possible to follow the pattern of colonisation and to estimate the quantity of bacteria on roots inoculated with strains carrying a constitutive *lacZ* or *gusA* fusion (Arsène et al. 1994; Wilson 1995; Wilson et al. 1995). Since the *lacZ* and *gusA* fusions lack their own promoters, they can also be fused to the promoters of other genes (such as *nifH-lacZ* fusion), estimating their expression in cultures and in association with plants (Arsène et al. 1994; Vande Broek et al. 1993). A protocol typically used to observe and quantify root colonisation in vitro using a constitutively expressed *lacZ* fusion is described below.

10.2.1 Typical Protocol for Estimating Root Colonization by Azospirillum *In Vitro*

10.2.1.1 Seed and Plant Preparation

Sterilised seeds are individually transferred with sterile forceps onto YMA agar plates for germination and incubated at the appropriate temperature (depending on the plant) until germinated. Germinated seeds, from uncontaminated plates only, are transferred into sterile glass tubes (e.g., size 20×150 mm) pre-washed by ethanol and DW. The tubes contain a piece of folded filter paper (about 100×10 mm) to support the seedlings and nitrogen-free hydroponic solution (in the above tube size, typically 15 mL) and are covered by additional test tubes slightly larger in diameter (25 mm) as shown in Fig. 10.1a. The plants are grown in a controlled environment under light/dark cycle at the appropriate temperature for each plant species.

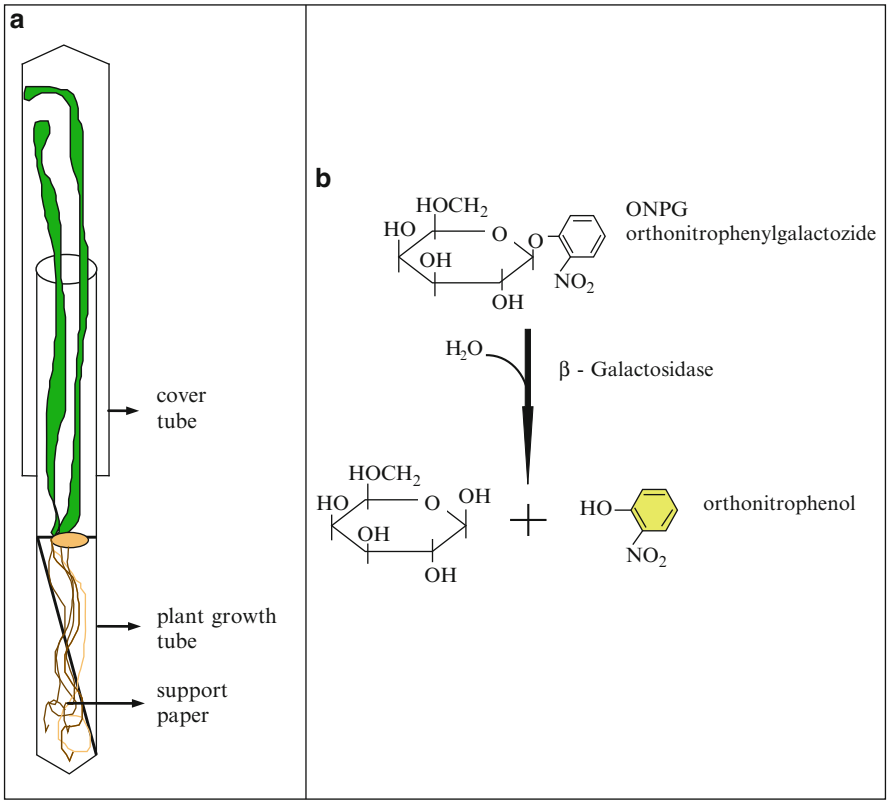

Fig. 10.1 (**a**) Plant growth system. (**b**) β-Galactosidase activity

Several days old seedlings (when root system is submerged in the solution, or as desired) are inoculated with 0.1 mL of *Azospirillum* culture (about 5×10^6 cells per mL of hydroponic solution). The *Azospirillum* cells contain pLA-*lac*Z, a constitutive *lac*Z fusion, which is expressed in all physiological conditions. The cultures are at late logarithmic phase and, in the case of *A. brasilense*, grown in minimal lactate medium supplemented with 10 mM of ammonium chloride, as described by Arsène et al. (1994). Periodically after inoculation, the plants are assayed for β-galactosidase activity (quantitative assay) or by X-gal in situ staining of bacteria (qualitative assay).

10.2.1.2 β-Galactosidase Activity in Roots Inoculated with Azospirillum

The pattern of colonisation is studied by in situ staining of roots with 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal), which allows examination of the spatial distribution of the bacteria (Fig. 10.2).

The extent of colonisation is estimated using the β-galactosidase activity assay, which is indicative of the number of bacteria associated with the root system (Arsène et al. 1994; Pereg Gerk et al. 1998). This assay is based on the ability of the enzyme to hydrolyse the β-galactoside bond of the *o*-nitrophenol-β-D-galactoside (ONPG) substrate (Fig. 10.1b), to yield a yellow product, orthonitrophenol, which can be quantified using absorption spectrometry.

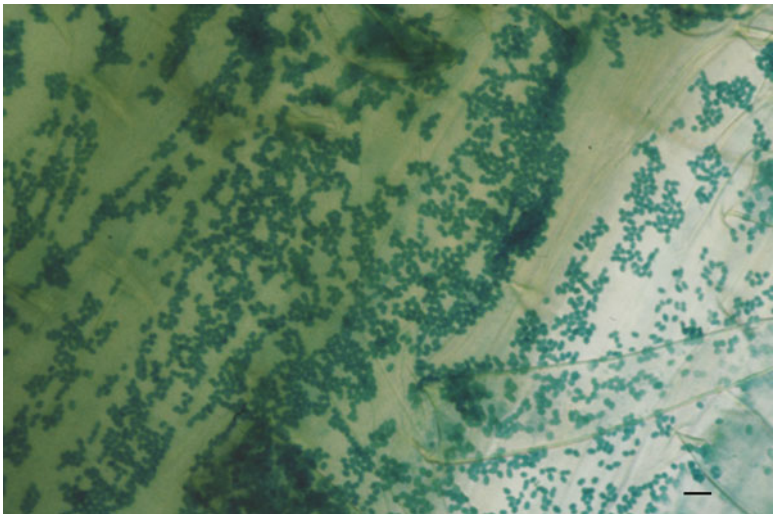

Fig. 10.2 Light micrograph of *A. brasilense* Sp7 [pLA-*lacZ*] cells on the surface of wheat roots. The *Azospirillum* cells contain pLA-*lac*Z, a constitutive *lac*Z fusion, which is expressed in all physiological conditions. They appear in shades of green to blue following X-Gal staining. Bar size is 5 μm

10.2.1.3 Glutaraldehyde Fixation and X-Gal Staining

Roots are harvested and placed in small vials containing 2 % glutaraldehyde in Z buffer (pH 7.4, Table 10.2). The samples are placed under vacuum for 30 min and then incubated for another 60 min without vacuum. The glutaraldehyde solution is then discarded and the samples washed twice for 15 min with Z buffer. Afterwards, each sample is covered with X-Gal solution (Table 10.2) and is then incubated for 24 h at room temperature in the dark. Then, the roots are washed three times in Z buffer and twice in sterile DW for 5 min. The samples can be stored at 4 °C if not used immediately for examination by light microscopy. Typical results are shown in Fig. 10.2.

10.2.1.4 β-Galactosidase Activity Assays

Intact roots are grinded in 2 mL of Z buffer (pH 7.0) and the solution incubated in small vials at 50 °C for 15 min to repress the background β-galactosidase enzyme produced by the plants. After cooling, each sample is supplemented with: 5 μL of β-mercaptoethenol (to stabilise the bacterial enzyme), as well as 20 μL of 0.1 % sodium dodecyl sulfate (SDS) and 40 μL of chloroform (to lyse the bacterial cells). The tubes are vigorously vortexed and duplicate portions of 500 μL transferred into clean tubes. Another portion of 200 μL is transferred into a separate tube for protein determination. The samples can be stored at 4 °C for a maximum of 48 h if they are not used immediately.

10.2.1.5 Plant Protein Determination

Freshly made 1 N NaOH solution (200 μL) is mixed well in a microfuge tube with 200 μL of the grinded roots in Z buffer (pH 7) prepared above. The samples are incubated for 5 min in a boiling water bath and, after cooling, neutralised with 400 μL of 0.5 N HCl. The tubes are centrifuged for 5 min (14,000 × g) to eliminate cell debris. A sample of the supernatant (200 μL) is mixed with 200 μL of Bio-rad reagent and 600 μL of DW and the A measured at 595 nm. For the preparation of a protein calibration curve, 200 μL of Z buffer (pH 7.0) containing β-mercaptoethanol (0.5 μL) and SDS (2 μL of 0.1 % solution) are supplemented with 0–30 μg of BSA and assayed in the same way as described above.

Table 10.2 Z buffers and X-Gal solution

Z buffer pH 7.4	Z buffer pH 7.0	X-Gal solution
In DW:	In DW:	Add:
$Na_2HPO_4 \cdot 7H_2O$, 70 mM	$Na_2HPO_4 \cdot 7H_2O$, 60 mM	20 μL X-Gal (20 mg/mL in dimethyl-formamide)
$NaH_2PO_4 \cdot H_2O$, 30 mM	$NaH_2PO_4 \cdot H_2O$, 40 mM	25 μL $K_4[Fe(CN)_6]$, 100 mM
KCl, 10 mM	KCl, 10 mM	25 μL $K_3[Fe(CN)_6]$ $3H_2O$, 100 mM
$MgSO_4 \cdot 7H_2O$, 1 mM	$MgSO_4 \cdot 7H_2O$, 1 mM	400 μL Z buffer (pH 7.4)

Table 10.3 ONPG solution and phosphate buffer

Phosphate buffer	ONPG solution
In 100 mL:	In 10 mL phosphate buffer or in 10 mL Z buffer (pH 7.0) dissolve 40 mg of ONPG
K_2HPO_4, 1.05 g; KH_2PO_4, 0.45 g	
$(NH_4)_2SO_4$, 0.1 g; Tris sodium citrate, 0.05 g	

10.2.1.6 β-Galactosidase Activity

The tubes containing 500 μL samples are pre-incubated at 28 °C for 5–10 min and then each tube is supplemented with 100 μL of freshly made ONPG solution (Table 10.3, Fig. 10.1b), mixed well by vortexing, and the time of the ONPG addition is recorded (start time).

The tubes are continuously incubated at 28 °C until yellow colouration is observed and then the reaction is stopped with 250 μL of 1 M Na_2CO_3 (stop time). The duplicate control samples should be treated in a similar way and incubated for the same period of time as the reaction tubes, except that the stop solution (Na_2CO_3) is added at the same time as the ONPG solution.

The plant debris in the reaction and in the control tubes are spun down by centrifugation for 10 min (14,000 × g). The absorbance of the supernatant is measured at 420 nm and at 550 nm, using the supernatant of the control sample as a blank for each reaction. The results are presented as Miller Units/mg plant protein/min.

10.3 Attachment to Soil Particles and Other Surfaces

A. brasilense Cd attaches to soil and peat by means of surface fibrillar material and could not be desorbed by washing (Bashan and Levanony 1988a, b; Bashan et al. 1991b), a factor which can assist the bacteria from being washed by soil flooding into deeper soil areas unfavourable for survival (Elmerich et al. 1992). The question may arise whether this feature could be a disadvantage in regard to plant root colonisation. However, adsorption to soil particles was decreased in the presence of plant exudates (Bashan and Levanony 1988a). The assays to examine *Azospirillum* attachment to soil, sand, and peat particles are described in Bashan and Levanony (1988a, b).

10.4 *Azospirillum* Disease Suppression

There are only a few reports of *Azospirillum*-mediated suppression of soil-borne plant diseases. *Azospirillum* sp. B510 induced resistance against the rice blast fungus *Magnaporthe oryzae* and the virulent bacterial pathogen *Xanthomonas oryzae* (Yasuda et al. 2010). *A. brasilense* showed moderate control of crown gall disease (Bakanchikova et al. 1993), bacterial leaf blight of mulberry (Sudhakar et al. 2000),

and bacterial leaf and vascular diseases of tomato (Romero et al. 2003; Bashan and de Bashan 2002a, b). *A. brasilense* Sp245 protects plants of *Prunus cerasifera* L. clone Mr. S 2/5 against *Rhizoctonia* spp., which caused total loss of plants, with a plant survival rate of nearly 100 % (Russo et al. 2008). However, the exact mechanisms involved in *Azospirillum* pathogen and disease suppression are not yet established and it is suggested that the protective mechanism may be explained by the plant growth promotion effects that are often associated with *Azospirillum* inoculation or by the mechanisms used by *Azospirillum* to outcompete other rhizospheric bacteria (Romero et al. 2003; Bashan and de Bashan 2002a; Pereg Gerk 2004).

Plant-associated *A. brasilense* strains enhance the growth of field crops via the production of plant growth factors, the promotion of root proliferation, and by improving water uptake (Sarig et al. 1988; Bashan and Levanony 1990). *Azospirillum* species have a highly versatile metabolism that allows them to survive under the widely variable conditions often occurring in soil. For example, several *Azospirillum* strains are equipped with a very efficient iron uptake system (Hartmann and Zimmer 1994; Shah et al. 1992). They produce low-molecular-weight chelating substrates, mainly phenolate compounds called siderophores with high affinity for Fe^{3+} (Vazquez-Cruz et al. 1992; Tapia-Hernandez et al. 1990). In principle, they can thus selectively deprive other microorganisms, including pathogens, of this essential element. *A. brasilense* strains REC2 and REC3 produce catechol-type siderophores, including salicylic acid (detected by thin layer chromatography coupled with fluorescence spectroscopy and gas chromatography–mass spectrometry analysis), with antifungal activity against *Colletotrichum acutatum* M11 and a reduction of anthracnose symptoms on strawberry plants (Tortora et al. 2011). In addition, many strains of *A. brasilense* and *A. lipoferum* have the capacity to produce bacteriocins, which inhibit closely related bacteria in vitro (Oliveira and Drozdowicz 1988). Somers et al. (2005) isolated antimicrobial compound from *A. brasilense* culture extracts, which was identified as the auxin-like molecule, phenylacetic acid (PAA).

A. brasilense Sp7 colonizes the root surface of cotton seedlings and protects them against the soilborne disease black root rot, with almost 100 % protection when the seedlings are first inoculated with the bacteria before planting into pathogen-infested soil (Lily Pereg and Jason Molynoux, unpublished). The fungal pathogen causing black root rot, *Thielaviopsis basicola*, has to come into contact with the root surface to progress to the biotrophic phase of the infection cycle (Mauk and Hine 1988; Hood and Shew 1997). Therefore, we suggest that, in addition to the mechanisms listed above, *Azospirillum* strains that colonize the root exterior may also mask the fungal–plant interaction zones on the root surface, creating a physical barrier between the pathogen and its host.

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Chapter 11
Methods for Studying Biofilms in *Azospirillum* and Other PGPRs

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Abstract To produce beneficial effects, plant growth promoting bacteria have to interact with the plant surface to form complex multicellular assemblies and aggregates named biofilms. The study of biofilm development and its physiology is an emerging topic in the knowledge of plant microbe interaction. Therefore, techniques to study its formation and functions are evolving from easy approaches to more complex, time consuming, and with expensive equipment requirement ones. The multiwell microtiter plate assay is the most widely used method due to its versatility to reveal the biofilm and that it allows the analysis of different stages of biofilm. Other more sophisticated protocols based on the use of fluorochromes are described. Care should be taken in analyzing results and their interpretation as differences among roots colonizing and biofilm formed on inert supports are normally to occur. Here, we make a presentation of the methods used to study biofilm and then describe in detail the most commonly used in investigations of rhizobacteria biofilms and its components with application to root colonization.

11.1 Introduction

The most frequent microbial lifestyle in natural environments is the occurrence of organized structures associated with surfaces known as biofilms (Watnick and Kolter 2000). Plant growth promoting rhizobacteria (PGPR) are not the exception to this rule. To produce beneficial effects, PGPR have to interact with the plant surface to form those complex multicellular assemblies (Bolwerk et al. 2003; Danhorn and Fuqua 2007; Bogino et al. 2013).

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Biofilms are defined as matrix-enclosed bacterial population adherent to each other and/or to surfaces or interfaces. This definition includes microbial aggregates and floccules and also adherent populations within the pore spaces of porous media (Costerton et al. 1995). These structured microbial cell communities can be composed of either, single or multiple species attached to living or inert surfaces.

The microorganisms in biofilms live in a self-produced matrix of hydrated extracellular polymeric substances that form their immediate environment. The polymeric substances, mainly polysaccharides, proteins, lipids, and nucleic acids, provide the mechanical stability for biofilms, mediate their adhesion to surfaces, and form a cohesive, three-dimensional polymer network that interconnects and transiently immobilizes cells (Ramey et al. 2004; Flemming and Wingender 2010). The shape of the biofilm is variable being as simple as multilayer of cells, or more complex with dome or mushroom shapes with channels or pores inside them through which water or fluids could run from one point to another. The spatially defined pattern of a biofilm is its architecture which is often specie-specific for pure cultures and substrate-specific for consortia (Costerton et al. 1995). Basic functional requirements underlie biofilm architecture, and structural diversity actually reflects the adaptation of unicellular organisms to communal mode of life.

The formation of a biofilm allows a lifestyle that is entirely different from the planktonic state. This mode of life is often crucial for survival of bacteria, as well as for the establishment of specific symbiosis with legume or actinorhizal host plants or nonspecific root colonization (Danhorn and Fuqua 2007; Rodríguez-Navarro et al. 2007).

Genetic studies of single-species biofilms have shown that they form in multiple steps, require intercellular signaling, and demonstrate a profile of gene transcription that is distinct from that of planktonic cells (Watnick and Kolter 2000).

The formation of biofilms begins when environmental signals trigger the planktonic cells to settle and establish microcolonies on a surface. In this step motility become lesser and bacteria repress the synthesis of flagella (Fujishige et al. 2006a, b) and in some cases fimbriae appear to connect cells to each other and to the supporting surface (Fig. 11.1a). Cells in the microcolonies produce abundant exopolymeric material (EPM) (Fig. 11.1b), and the colonies grow to form biofilms (Fig. 11.1c). Occasionally, whether fresh nutrients appear or when conditions became harsh within the biofilm, cells can become motile and return to the planktonic lifestyle, which can be envisaged as a mode of colonizing other sites (see Fig. 11.2, top part). The entire cycle may be viewed as a developmental process that shares some of the features of other bacterial developmental processes (Watnick and Kolter 2000).

Attachment of rhizobia (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and other related genera) to host roots is the first step required for infection and nodulation. This initial adsorption is a very early step in the symbiotic interaction during the complex host-specific infection process. This early step of attachment, mediated by lectins and/or rhicadhesin, is rather weak and also reversible (Matthysse and Kijne 1998). The second binding step would require the synthesis of bacterial cellulose fibrils, produced by the bacteria. These cellulose fibrils would cause a tight and irreversible binding and the formation of bacterial aggregates on

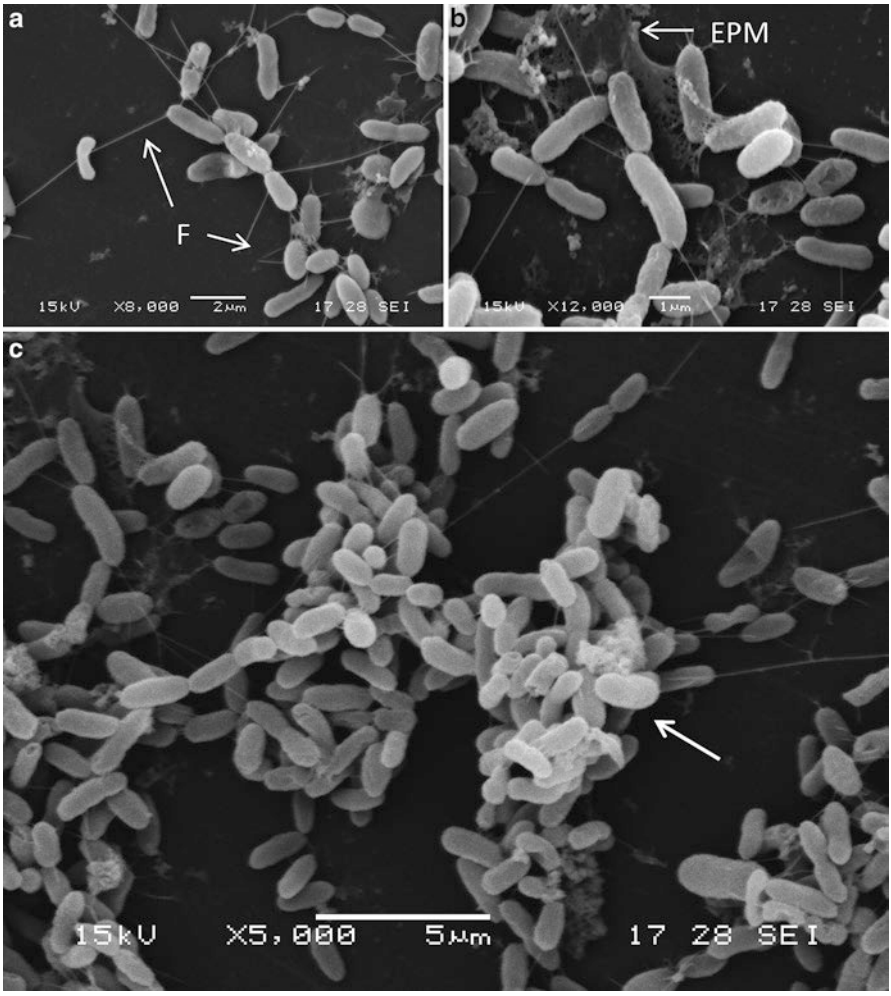

Fig. 11.1 Cells of *Azospirillum brasilense* attached to polystyrene surface visualized by SEM. (**a**) One day cultures of *A. brasilense* Sp245 in NO_3^- -Nfb media. Fimbrias (F) can be seen joining cells together and to the inert support. (**b**) Exopolymeric material (EPM) excreted by cells can be visualized. (**c**) Static cultured cells forming microcolonies some of them growing perpendicularly to the surface (*arrow*) and presumably will form structured biofilms

the host surface (Robertson et al. 1988). In contrast to these data, other reports did not demonstrate specific lectin-mediated binding of homologous rhizobia to legume root hairs (Smit et al. 1986; Vesper and Bauer 1986).

The attachment of *Azospirillum brasilense* cells to wheat roots also can be divided into two different steps (Michiels et al. 1991). The first phase is a weak, reversible, and unspecific binding governed by bacterial surface proteins, capsular polysaccharides, and flagella. Polar flagella of *A. brasilense* contain an adhesin

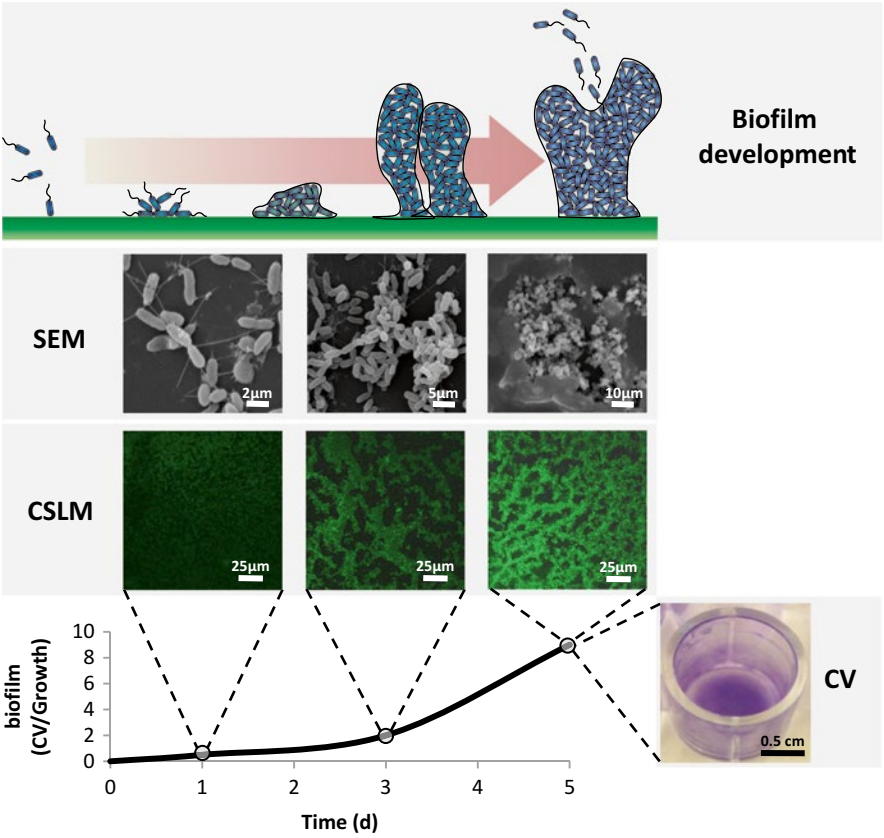

Fig. 11.2 Schematic representation of the different stages of biofilm (top part) paralleled with *A. brasilense* Sp245 tagged with *gfp* biofilm development as seen by SEM and CLSM (second and third lines of the scheme). The graph on the last line shows the timing of each stage that in *A. brasilense* corresponds to 1, 3, and 5 days of growth. The well in the inferior corner shows a crystal violet (CV)-stained biofilm after 5 days of growing *A. brasilense* Sp245 cells in multiwell plate in static conditions with Nfb-NO_3^- amended media

component that is involved in bacterial attachment to wheat roots. The second attachment phase appears to be irreversible and occurs up to 16 h after inoculation and is mediated by bacterial surface polysaccharides. Although azospirilla are general colonizers of plant roots showing not species-specificity for association, some grade of preference can be seen. *Azospirillum irakense* cells are mainly associated with rice root hairs, whereas *A. brasilense* cells are mainly located on root surfaces (Zhu et al. 2002). The involvement of extracellular fibrils was demonstrated in the irreversible anchoring of *A. brasilense* (Michiels et al. 1991). The nature of this fibrillar material has not been determined yet. Extracellular polysaccharide production has also been related to the process of flocculation of *Azospirillum* cells and might be similar to the fibrillar material produced during root association (Burdman et al. 1998; Skvortsov and Ignatov 1998). The major outer membrane protein of *A. brasilense* appears to be involved in cell aggregation and the first step of attachment.

Several factors like mechanical and nutritional stress, and quorum-sensing molecules among others, regulate biofilms assembly and disassembly (Karatan and Watnick 2009). Cell density-dependent quorum sensing is known to regulate many bacterial functions, including the colonization of the rhizosphere and the rhizoplane (Soto et al. 2006; Compant et al. 2010). The production of *N*-acyl-homoserine lactones (AHLs) has been demonstrated in both natural and cultured biofilms (McLean et al. 1997; Davies et al. 1998). AHLs are mediators of surface attachment in *Pseudomonas fluorescens* (Allison et al. 1998; Wei and Zhang 2006). However, other studies showed that AHLs mediating quorum sensing in Gram-negative bacteria, are not always required for plant colonization (Müller et al. 2009; Compant et al. 2010). The available information indicates that PGPR may employ an array of distinct mechanisms, either alone or in combination, to successfully colonize the root system (Compant et al. 2010). Intercellular communication between bacteria inside the biofilm is generally carried out by bacterial products that are able to diffuse away from one cell to another (Watnick and Kolter 2000). This way of intercellular signaling seems actually suited for bacteria in a diffusion-limited environment such as the biofilm.

A variety of cellular processes are mediated by receptor proteins and riboswitches specific to the bacterial secondary messenger cyclic diguanosine monophosphate (c-di-GMP). C-di-GMP controls flagellar motor speed (Boehm et al. 2010), and in *Vibrio cholerae* and *Shewanella oneidensis* it has been shown to upregulate gene clusters for biosynthesis of extracellular polysaccharides, which facilitate the adhesion between cells (Beyhan et al. 2006; Thormann et al. 2006; Krasteva et al. 2010).

The increase in c-di-GMP leads the transition from planktonic growth to a sessile state with biofilm formation (Jenal and Malone 2006; Hengge 2009).

Nitric oxide (NO) is a signaling molecule implicated in numerous processes in bacteria, including either formation or dispersion of biofilm, depending on genera and lifestyle. The synthesis of NO in Gram-negative bacteria relies mainly on denitrification pathway (See Chap. 9 in this Book). *Azospirillum brasilense* is able to produce considerable amounts of NO under aerobic conditions, and its production influences the lateral root formation in host plants (Creus et al. 2005; Molina-Favero et al. 2008). Recently, it was shown that in bacteria NO stimulates biofilm formation by controlling the levels of c-di-GMP (Plate and Marletta 2012). In *Azospirillum brasilense* Sp245 the production of NO derived from denitrification was shown to be a key regulatory step in biofilm formation (Arruebarrena Di Palma et al. 2013). Schmidt et al. (2004) proved that cultures of *Nitrosomonas europaea* treated with exogenous NO gas enhanced biofilm formation. Nevertheless, Barraud et al. (2006; 2009) showed that NO triggered the disassembly of *Pseudomonas aeruginosa* biofilms acting upstream of c-di-GMP signaling pathway. It seems that NO is a regulatory molecule that can modulate the biofilm formation or its dispersion depending on the lifestyle of the bacteria (pathogen or beneficial ones) and on its concentration in the media. Furthermore, encapsulation of bacteria into a biofilm provides a significant protective mechanism against harsh environmental conditions. At high concentration, the free radical NO and the related reactive nitrogen species, are active antimicrobials and detrimental to cellular growth, depending on its concentration and redox state of the environment. The occurrence of thick

biofilm layers and extracellular matrix encapsulation provide a protective diffusion barrier, avoiding damage to underlying cells.

When studying biofilms it is important to determine these messenger molecules since they are responsible for the different biofilm developmental stages and its regulatory mechanisms.

11.2 General Information About the Conditions to Study Biofilm Formation

11.2.1 Growth Conditions and Culture Media

The prevailing condition to form biofilm is the static growth. Nevertheless, some species can also form biofilm in agitated cultures but in less quantity.

Nutritional conditions were shown to be powerful modulators of the attachment of various bacteria species to surfaces (O'Toole et al. 2000; Rinaudi et al. 2006; Danhorn and Fuqua 2007). Siuti et al. (2011) studied the attachment of *A. brasilense* Sp7 and found that it was increased when the experiments were conducted under low aeration (i.e., nonshaking) conditions with cells transferred from culture in a rich medium (TY: 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl per L of distilled H_2O) to a minimal media (MMAB, on 1 L base: 3 g K_2HPO_4, 1 g NaH_2PO_4, 1 g NH_4Cl, 0.3 g $MgSO_4{\cdot}7H_2O$, 0.15 g KCl, 0.01 g $CaCl_2{\cdot}2H_2O$, 0.0025 g $FeSO_4{\cdot}7H_2O$, 5 g Na-malate, 0.005 g Biotin, and microelements). The type and ratio of Carbon (C) and Nitrogen (N) sources also affect the formation of biofilm. In an early report Burdman et al. (1998) showed that the strains Cd and Sp7 of *A. brasilense* cultivated in a media with high C:N ratio was induced to form aggregates, mainly if the C source is fructose. They demonstrated that both exopolysaccharides (EPS) and capsular polysaccharides (CPS) showed a positive correlation between aggregation and the relative amount of arabinose. When they were grown in a non-inducing aggregation media with low C:N ratio the extent of aggregation and arabinose in EPS was much lesser. Changes in adhesive cell surface properties that, in turn, affect cell-to-cell aggregation and flocculation could also be affecting biofilm formation. In this sense components of the media must be taken into account to induce biofilm. In general terms, nutritionally limited environment promotes the transition from a planktonic to a sessile mode of life. Biofilm formation may therefore represent a strategy for survival. In contrast, nutrient abundance in the medium seems to favor biofilm formation in *Pseudomonas* (Yousef-Coronado et al. 2008), possibly by increasing bacterial population size and accumulation of AHL, which promote biofilm formation (Rinaudi and Giordano 2009).

Arruebarrena Di Palma et al. (2013) reported that NO_3^- induced a higher and earlier biofilm formation in *A. brasilense* Sp245 than growing cells with NH_4^+ as N source. Also Siuti et al. (2011) found that biofilm formation in *A. brasilense* Sp7 was greater in media containing $NaNO_3$ compared to NH_4Cl or N-lacking media.

These results could be explained given that NO is produced in huge amounts in NO_3^- containing medium compared to NH_4^+ supplemented ones and, as it was previously mentioned, NO is required for biofilm formation. In our experience the recommended media for testing biofilm formation in *A. brasilense* is Minimal medium (e.g., Nfb or MMAB) added with L-malic acid and KNO_3 as C and N sources in a molar ratio of C:N = 2 (Nfb: 3.7 g Malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0,02 g $CaCl_2$ $2H_2O$, 0.065 g Fe-EDTA, 1.39 g KNO_3 or 0.7 g NH_4Cl, pH 6,5). However, other culture media have been described for different *Azospirillum* strains as useful to test biofilm formation. MMAB supplemented with NH_4Cl or $NaNO_3$ with fructose or sodium malate (Siuti et al. 2011) or LB medium (Sheludko et al. 2008) were used. Other PGPR, such as *Pseudomonas fluorescens*, form biofilm in LB or in minimal M67 medium and the following additions: glucose, citrate, glycerol, xylose, casaminoacids, glutamate, malate, or mannitol (O'Toole et al. 1999).

In some species of *Agrobacterium* genera the availability of phosphorus increases the attachment (Danhorn and Fuqua 2007). On the contrary, Siuti et al. (2011) found that no significant effect of varying the concentrations of either phosphorous or potassium, increased the attachment in *A. brasilense* Sp7, suggesting that subtle changes in experimental conditions could be defining the formation of biofilm in *Azospirillum*.

The effects of various nutrients and environmental conditions on the biofilm formation ability of *Sinorhizobium meliloti* were tested by Rinaudi et al. (2006). The concentrations of sucrose, phosphate, and calcium were positively correlated with biofilm formation, whereas extreme temperatures and pH values had a negative effect. These findings support the hypothesis that biofilm formation promotes the survival of non-spore-forming rhizobia in soil in the absence of a legume host.

Other factor to take into account is the timing of the biofilm formation. Generally, incubation time can vary from hours to days; particularly, for *Azospirillum*, the adhesion of cells in stationary phase is more evident after 24 h (Dufrêne et al. 1996). It is necessary not to exceed the moment in which the biofilm began to detach by aging or other factors, hence kinetics study is recommended.

In summary, to assure biofilm formation use static culture systems with low aeration (Green 2010) and for facultative anaerobes overlay the culture medium with mineral oil (O'Toole et al. 2000). It is important to determine the required conditions that promote biofilm formation and to provide a source of carbon, nitrogen- and phosphorus-containing compounds as they are potentially all EPS components crucial for biofilm formation (Flemming and Wingender 2010).

11.2.2 Types of Surfaces

The physicochemical properties of the surface exert a strong influence on the rate and extent of attachment (Stepanović et al. 2007). Most investigators have found that microorganisms attach more rapidly to hydrophobic, nonpolar surfaces such as teflon and other plastics than to hydrophilic materials such as glass or metals

(Donlan 2002). Plates made of polystyrene, polypropylene, polyvinyl chloride, or polycarbonate are most frequently used, but glass (usually coverslip), that is hydrophilic is also used as surface on which biofilms can be formed and studied (O'Toole et al. 1999). In addition to the physicochemical characteristics of the substrate, many other factors may affect bacterial attachment. Dufrěne et al. (1996) studied the adhesion of *A. brasilense* Sp7 to inert surfaces like polystyrene (hydrophobic) and glass (hydrophilic) in function of several factors like culture age, rinsing, contact time, temperature, and presence of tetracycline. They demonstrated that adhesion of cells harvested in exponential phase after 24 h contact time at 30 °C and mild rinsing, was clearly denser and thick on polystyrene than on glass. However, under the same conditions, cells harvested in stationary phase adhered with a similarity density on both supports. On the other hand, they showed that a decrease in temperature (4 °C) and in contact time (6 h), and the presence of tetracycline or a drastic rinse, significantly diminishes the adhesion of cells to the support.

Root surfaces are compatible with hydrophobic characteristics. Bashan and Holguin (1993) compared the anchoring (irreversible attachment) of *A. brasilense* Cd to hydrophobic polystyrene and to wheat roots. They showed that bacteria cells attached in significantly greater numbers to roots than to polystyrene. Some more recent studies showed that *A. brasilense* forms biofilm on polystyrene or polyvinyl chloride plates (Petrova et al. 2010; Combes-Meynet et al. 2011; Arruebarrena Di Palma et al. 2013), many studies suggest that the physical surface properties of glass could hamper the attachment for biofilm formation in *A. brasilense* (Siuti et al. 2011).

11.3 Methods for Biofilm Analysis

The systems to study biofilms in vitro can be divided into closed and open or continuous systems. Here we define closed and open systems and then the more suited and used systems will be described in detail.

In *closed systems* (usually called batch culture) bacteria grow under limited nutrients and aeration conditions. These are particularly useful for studying early stages of biofilm formation using short incubation times (hours to few days). Closed models allow researchers to easily vary multiple parameters including the composition of growth media, incubation temperatures, humidity, presence or absence of shear stress and O_2 and CO_2 concentration. They have the advantage of simplicity and are appropriate for high throughput analyses with direct and rapid quantification of biofilm mass. They include the use of 12, 24, or 96 microtiter wells (Coenye and Nelis 2010; Lebeaux et al. 2013; Macià et al. 2014).

In *open systems*, growth medium is continuously added, pumped through the chamber, and waste products are continuously removed (Coenye and Nelis 2010). Therefore biofilm is allowed to develop over a period of days to weeks. Generally, these methods permit the control of environmental parameters such as shear forces and have been therefore extensively used to study physical and chemical resistance of biofilms (Lebeaux et al. 2013). Furthermore, when using special microchambers these systems enable the analysis of mature biofilm in real time and allow the

observation of biofilm by microscopy (for example using fluorescent proteins and dyes). On the other hand, the open systems are less adapted to high-throughput analysis and often demand specialized equipment and technical skills (Coenye and Nelis 2010). They include the flow cell system, Kaudouri system, and microfermentors.

11.3.1 Multiwell Microtiter Plate Assay

The most widely used method for the study of biofilm formation is the multiwell microtiter plate assay. In this system, biofilms are grown on the bottom and on the walls of the wells of plate (flat-bottomed polystyrene microtiter plates are most frequently used). This protocol can be applied to any species to be studied, and it is a powerful method for the analysis of the different stages of biofilm. One of the advantages of this system is its low cost, as small volumes of reagents are required. This method allows performing a large number of tests simultaneously, enabling the study of factors that can influence the biofilm formation such as carbon or nitrogen sources, antibiotics, or the presence of stressful agents. Moreover, microtiter assay facilitates the screening of mutants affected in attachment to abiotic surface. One disadvantage of the microtiter system is that the incubation time is limited, because the nutrients are consumed and the wastes are cumulated. Depending on the staining technique used this method allows the differentiation between living and dead cells. Particularly for PGPR, this technique has been extensively used to study biofilm formation and factors that may affect this process (Fujishige et al. 2006a, b; Rinaudi et al. 2006, Sheludko et al. 2008; Lerner et al. 2009; Rinaudi and Giordano 2009; Petrova et al. 2010; Combes-Meynet et al. 2011; Siuti et al. 2011; Sorroche et al. 2012; Arruebarrena Di Palma et al. 2013). The microtiter plate assay consists in growing in static condition the bacteria on the plate with a culture medium appropriate for biofilm formation. Subsequently, planktonic cells are removed and attached biofilm is stained with a dye or treated with a substrate to see the adherence pattern of cells, or the dye may be solubilized to perform a semiquantitative analysis by spectrophotometric measurements (O'Toole et al. 1999; Merritt et al. 2005; Peeters et al. 2008) (see Sect. 11.4 in this same chapter).

These systems are used not only to monitor biofilm formation from initial attachment to development of the complex architecture characteristic of biofilms but also to study the transition from biofilm back to planktonic growth (O'Toole et al. 1999).

11.3.1.1 Procedure for *A. brasilense* Biofilm Formation Assay

- Streak *A. brasilense* on Agar Congo Red (ARC) medium (Rodríguez-Cáceres 1982), and incubate at 32 °C for 5 days.
- Choose 1 or 2 colonies and transfer to 125 mL flasks containing 25 mL of LB medium. Incubate for 16 h at 30 °C with orbital agitation (100 rpm) until the cells reach 1,1–1,4 DO_{540nm}.

– Harvest the cells by centrifugation, 5,000 rpm for 10 min, wash the pellet with phosphate buffer (66 mM), and resuspend to a final $DO_{540nm} = 2$.
– Dilute bacteria cultures 1/100 in fresh Nfb-NO_3^- malic medium. As control use fresh medium without bacteria. NOTE: the amount of fresh medium to be used depends on the type of plated used: for 24-well plate place 2 mL of medium per well, in the case of 96-well plate, 200 µL of medium per well.
– Incubate it at 30 °C without agitation for desired amount of time. NOTE: the incubation time depends of the objective of the experiment (hours to days).
– Determine total growth and biofilm formation (see the Sect. 11.4.4 "detection and quantification of biofilm" in this same chapter). NOTE: use four wells for growth determination and four wells for staining or quantification for each treatment, at least as repetitions of the same biological sample.

Considerations

- Controls must be placed in the same plate of the treatments.
- To avoid evaporation close the plate with its lid and seal with parafilm. Place the plate in a humidity chamber.
- Before putting the media with the bacteria in a well it is very important to homogenize with a low vortexing.

11.3.2 Colony Biofilm Assay

Principle: Colony biofilm systems consist in growing the biofilm on a semipermeable membrane (usually pore size of 0.22 µm) onto a solid culture medium (agar plate). This membrane is easily removed and allows the reallocation of the biofilm to a fresh culture medium. This technique is simple and reproducible, amenable to high-throughput screening, and maintains the basic characteristics of the biofilm such as structure or chemical gradients. The colony biofilm has been especially useful in assessing antibiotic or UV-light susceptibilities, and have become an appropriate model to visualize the morphotypes, extracellular components like polysaccharides, and gene expression profiles unique to biofilm growth.

11.3.2.1 Protocol for Colony Biofilm Assay (Based and Adapted from: Hamilton et al. 2003; Merritt et al. 2005; Peterson et al. 2011)

– Inoculate bacteria strain in 25 mL LB and grow overnight at 100 rpm, at 30 °C (e.g., to late exponential phase).
– Place 25 mm polycarbonate membrane with pore size of 0.22 µm, previously sterilized with UV for 10–15 min, onto agar plate with appropriate culture medium. Use sterilized forceps to manipulate the membrane.

- Dilute overnight cultures to an $OD_{600} = 0.05$ in the same medium used for biofilm growth and spot 5 μL of culture in the center of each polycarbonate membrane (up to six membranes per plate).
- Incubate at 30 °C for 24 h.
- Transfer the membranes (colony biofilm) to a fresh agar plate. Incubate for 24 h at 30 °C.
- After 48 h of growth, biofilm susceptibility can be assessed transferring the membrane to a fresh agar plate with or without antibiotics or another stressful agent.
- After incubation, remove the membrane from the agar plate with sterile forceps and transfer it to a tube containing 10 mL of dilution buffer or saline solution. Vortex the tube for 2 min at least, for removing and disaggregating the biofilm from the membrane.
- Serially dilute and plate the bacterial suspensions.
- Count bacteria as described in Sect. 11.4.1.1 in this chapter.

Considerations

- Bacterial strains that differ in surface motility will spread across the filter at variable rates.
- If bacterial biofilm completely covers the membranes, the membranes are difficult to handle, becoming flimsy and periodically folding on top of them, thus destroying the biofilm (Peterson et al. 2011).
- The number of membranes needed depends on the experimental design. Consider to use at least two membranes for each treatment (Merritt et al. 2005).

11.3.3 Other Used Methods

11.3.3.1 The Calgary Biofilm Device

This system consists of a polystyrene lid with 96 pegs that can be fit into a standard 96-well microtiter plate. Pegs are attached to the top lid of a microtiter plate and by closing the microtiter plate these pegs will be immersed in the media present in the wells, and biofilm forms upon the pegs. Following biofilm growth, the lid can be transferred to a second plate, or recovered from pegs by using low frequency sonication to disrupt the surface-adhered cells into a recovery medium. Generally used to determine antibiotic susceptibility (Ceri et al. 1999; Peeters et al. 2008; McBain 2009; Coenye and Nelis 2010) it is commercialized as the MBEC Assay: “Minimal biofilm eradication concentration” assay, by Innovotech Canada.

11.3.3.2 The Air–Liquid Interface

This method is useful mainly to microscopically visualize the biofilm. Twenty-four-well plates are accommodated in an angle of 30°–50° grades with respect to horizontal level. Bacteria in stationary phase are diluted in fresh media and put into wells. The tilt of plates allows the biofilm to be formed in the interface liquid–air (Merritt et al. 2005).

11.3.3.3 Continuous Flow System

Flow systems are often used when microscopic examination of biofilm is required. This system consists in a vessel through which fresh growth medium is pumped continuously and cells are attached to a surface where biofilms start developing. Transparent and not fluorescent microscopy coverslips are usually used for attachment surface (Palmer and Robert 1998; McBain 2009; Macià et al. 2014). The flow cell design is relatively simple and numerous works describe its construction of the flow cell (Wolfaardt et al. 1994; Christensen et al. 1999; Sternberg and Tolker-Nielsen 2006), or are available commercially (Biosurface Technologies Corporation.)

11.4 Detection and Quantification of Biofilm

11.4.1 Direct Enumeration of Cells

11.4.1.1 Viable Cell Quantification by Colony Forming Units

Principle: The traditional method of determining the number of adherent bacteria in biofilm is the measurement of viability by prior desorption through the use of sonication or vigorous agitation and subsequent plating on different agar plates (Hannig et al. 2007).

Procedure

The method is very simple. First the planktonic cells must be removed and then wash the biofilm three times with tap water. Biofilm has to be resuspended in buffer or saline solution by mechanically vigorous agitation. An aliquot of this solution is taken to be examined using the drop plate method. Briefly, a sample from this solution is subjected to serial dilutions 1/10 and then 10 μL is spotted onto Congo Red agar (CRA) (Rodríguez-Cáceres 1982) plates and the number of colony forming units (CFU) is counted after 5 days (Herigstad et al. 2001; Arruebarrena Di Palma et al. 2013).

Even if this technique has the advantage of determining the viable bacteria number, there is one important care to take into account in order not to underestimate the number of CFU and lead to misleading conclusions. All the cells in the biofilm have to be mechanically removed from the surface, and they have to be

completely separated and dispersed in the buffer or saline solution. Especially the latter could be difficult to accomplish, since there could be a tendency of the biofilm to get lumpy when it is scraped off the surface. This could result in that a lump of several microorganisms would be counted as one CFU. Despite the roughness of this method it is one of few methods that can estimate the amount of viable bacteria in biofilm (Nilsson and Otendal 2001).

11.4.1.2 Viable Cell Quantification by Using Staining Techniques: XTT Assay

Principle: This method is based on the use of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) as substrate which is reduced in the biofilm by cells. The intracellular XTT reduction releases a formazan compound that can be quantified by colorimetric estimation. Some authors question if really XTT reduction activity is proportional to biofilm biomass (Kuhn et al. 2003) as it is based on biofilm biological activity and not on biofilm biomass. In this context, it is suggested that this technique could be used on young biofilms because the most of biofilm cells will be biologically actives. By the contrary, the metabolism of some cell groups inside old biofilms will have less activity and could generate problems in the determination.

Procedure

- Fresh XTT solution 0.04 % (w:v) must be prepared in prewarmed (37 °C) physiological solution (NaCl 0.8 %; w:v).
- Add 100 μL of menadione solution (0.55 mg mL^{-1} in acetone) to 10 mL of XTT solution.
- Add 50 % v/v of XTT-menadione:PS in the wells.
- Incubate the plates in dark during 5 h at 37 °C.
- Supernatants obtained must be transferred to new eppendorf tubes; centrifugate at maximal speed for 5 min to remove residual cells.
- Use free cell supernatants to determine absorbance at 486 nm corresponding to the formazan compound that is a measure of viable cell quantification in biofilm (Peeters et al. 2008).

11.4.2 Total Staining: Crystal Violet Assay

Principle: Crystal violet (CV) staining was first described by Christensen et al. (1985) and has since then been modified to increase its accuracy and to allow the quantification of the total biofilm biomass in the entire well (O'Toole et al. 2000). CV is a basic dye, which binds to negatively charged surface molecules and polysaccharides in the extracellular matrix (Li et al. 2003). Because cells (both living and dead), as well as matrix are stained by CV, it is poorly suited to evaluate live or dead cells inside of biofilm (Pitts et al. 2003; Peeters et al. 2008).

Procedure

- Add 1 % (w:v) crystal violet on biofilm formed in each microtiter plate well, the final concentration of CV must be 0.1 % (v:v).
- Plates are incubated for 30 min at room temperature, and then washed carefully three times with tap water to remove the excess of CV.
- Dye attached to the wells is extracted with 200 μL of 33 % (v:v) acetic acid in water.
- Determine the absorbance at 590 nm using a micro plate reader.
- Data must be normalized by total growth estimated by measuring the absorbance at 540 nm as follows:

Calculations for the Assessment of Biofilm Formation

The following formulas can be used to assess the proportion of cells in the biofilm relative to total culture growth.

Total cell number (planktonic and biofilm bacteria) can be estimated by measuring the optical density at 540 nm (OD_{540}) or at 600 nm.

Adhesion can be calculated as the ratio between bacteria in biofilm and total bacteria:

$$\left(\text{O.D.}_{590\,\text{nm}}\right)/\left(\text{O.D.}_{540\,\text{nm}}\right).$$

11.4.3 Fluorometric Detection of Biofilms

When a molecule absorbs a photon, it becomes excited to a more energetic and unstable state. After a very short time (about 10^{-5}–10^{-8} s), the excess energy of the molecule could be emitted in the form of another photon, slightly less energetic than the previously absorbed one. This phenomenon is referred to as fluorescence, and its measurement is called fluorometry. Many organic molecules have the ability to fluoresce. This makes the fluorometry a good choice for the purpose of detecting biofilm (Nilsson and Otendal 2001).

11.4.3.1 Autofluorescence

In some cases, organisms or part of them are slightly autofluorescent and can be visualized in an unstained biofilm. However, autofluorescence is often weak, and normally only a small fraction metabolically active of a total biofilm population is autofluorescent.

A number of different fluorescing compounds have been found in bacteria. In fact, some of the most thoroughly examined organic compounds are important substances for the metabolism of the cell, and are therefore present in all organisms. That is the case of NAD(P)H with the ability to fluoresce when is excited to 340 nm and emitted 440 nm. It would be desirable if the fluorescence signal from certain compound is proportional to the biomass of the biofilm the fluorescent signal might reveal something about biofilm composition or metabolism. This is rarely the case though, taking a certain mass of biofilm, some compounds level can vary quite a bit from case to case, due to the enormous variations in biofilm composition (Nilsson and Otendal 2001).

11.4.3.2 Staining Fluorescence Techniques

Fluorescent dyes that stain either the cells in the biofilm or their immediate surroundings may also be used to label bacteria in biofilms and therefore analyze their structures. The main fluorescent dyes are currently used for monitoring biofilm formation, analyzing live/dead cells in biofilm or determining compounds in the matrix of the biofilm.

11.4.3.2.1 Based on the Use of Fluorescein Diacetate

Principle: Some stains (such as fluorescein) have a minimal effect on biofilm growth and may be used in a nondestructive way. Several viable bacterial stains are based on the formation of fluorescein as a result of intracellular esterase activity. It is a prerequisite that these substrates diffuse freely into the bacterial cells and yet remain within the interior of the cell after cleavage. As example Fluorescein Diacetate (FDA), one of the first dyes adopted, rapidly leaks from the cells and is rather unstable whereas carboxyfluorescein diacetate is better retained due to its negative charge (Leeder et al. 1989; Decker 2001). Despite this disadvantage, FDA has been used in biofilm study many times.

Procedure

- Dissolve FDA in acetone at 10 mg mL^{-1} concentration. Store this solution at −20 °C until its use.
- Stock solution should be diluted 1:50 in 3-(N-morpholino) propanesulfonic acid (MOPS) buffer 100 mM pH 7. Prepare it before each assay.
- Add 100 µL of MOPS buffer and 100 µL of working solution FDA to each rinsed well.
- Incubate in the dark at 37 °C for at least 2 h. The incubation time should be adjusted to each bacterial species.
- Measure fluorescence at λ_{ex}: 494 nm and λ_{em}: 518 nm (Peeters et al. 2008).

11.4.3.2.2 Based on the Use of 4′, 6-Diamidino-2-Phenyl Indole

Principle: 4′, 6-Diamidino-2-Phenyl Indole (DAPI) bind to the DNA, specifically to the AT-rich regions of double-stranded DNA of live and dead cells (Schwartz et al. 2003). However, no differentiation of different bacterial species within the biofilm is possible. However, most of living bacteria staining with DAPI seem to have adverse side effects and may inhibit the biofilm growth depending on the organisms. In general, these dyes seem to work better if the biofilm cells are fixed before applying the dyes (Hannig et al. 2010).

Procedure

- Rinse three times the biofilm with tap water carefully.
- Fix the biofilm with 2.5 % (v:v) formaldehyde in physiological solution for 2 h.
- Throw out the fixer solution and fill up the well with a solution of DAPI 4 μg mL^{-1}. The solution should be prepared in distilled water.
- Incubate 20 min at dark.
- Remove dye excess by soft washes.
- Depending on the objective of the study, the sample can be microscopically observed or quantified. The quantification of DAPI is carried out by the solubilization of DAPI with ethanol 95 % for 15 min.
- In each study case, the sample must be excited to 350 nm and measure the emitted fluorescence at 510 nm (Leroy et al. 2007).

11.4.3.2.3 Live/Dead Staining Techniques

Principle: The live/dead tests use mixtures of SYTO 9 green-fluorescent nucleic acid stain and propidium iodide a red-fluorescent nucleic acid stain. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 stain generally labels all bacteria in a population, those with intact membranes and those with damaged ones. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide. There are various kits in the market to do this technique and the protocols used are very similar among them. Considering the different metabolic conditions inside biofilm, this technique is very useful to establish the dynamics of global functionality in the biofilms, including cell turnover. However, the problem of several live/dead staining techniques is the stability of the stained material. The interaction of different bacterial species with certain dyes differs distinctly, yielding different patterns of vitality (Decker 2001).

11.4.3.2.4 Resazurin Assay

Principle: This technique is used to determine viability and is based on the reduction of resazurin by metabolically active cell. Resazurin (7-Hydroxy-3*H*-phenoxazin-3-one-10-oxide) is a blue dye, weakly fluorescent until it is irreversibly reduced to a pink resorufin, which is fluorescent. There are several kits to do this technique like live/dead assay.

Procedure

- Add 20 µL of commercial resazurin, CTB (CellTiter-Blue, Promega), in the well containing 100 µL of media. Also can be used 30 µL of 0.01 % resazurin salt dissolved in water or PS.
- Incubate at least 30 min at 37 °C.
- Determine the resorufin fluorescence formed by exciting to 560 nm and measuring emission to 590 nm (Peeters et al. 2008).

11.4.3.3 Tagging Bacteria with Fluorescent Protein to Study Biofilm

Principle: Monitoring occurrence and behavior of a specific bacteria strain may be achieved by molecular tagging of the organism with a reporter gene that encodes a fluorescent protein or an enzyme that catalyzes a reaction leading to a fluorescent product. Such reporter genes may not only be used for tagging, but also for monitoring specific gene activities nondestructively by the fusion of promoters of those specific genes to the reporter genes.

Green Fluorescent Protein and Its Derivatives: The *gfp* gene encoding the green fluorescent protein (GFP) was isolated from the jellyfish *Aequorea victoria*. GFP has several advantageous characteristics for studying gene expression in individual cells. GFP fluoresces without the addition of any external substrate, apart from low levels of oxygen needed for catalyzing an internal rearrangement of the protein into its fluorescent form. GFP has been mutated extensively in order to alter the properties of the wild-type protein. Random or directed mutagenesis of the *gfp* gene sequence has resulted in several spectral variants and mutants with enhanced fluorescence efficiency. One family of spectral mutants is the "red-shifted" group, Emission spectrum variants primarily fall into four groups: BFP (blue fluorescent protein), CFP (cyan), GFP (green), and YFP (yellow). By combining promoters that respond to specific environmental or internal signals with appropriate marker genes, it may be possible to tag specific organisms and monitor expression patterns of specific genes. For instance, it may be possible to construct strains that report the limitation of nutrients such as carbon, nitrogen, or phosphorus (Christensen et al. 1999).

GFP was used for tagging *Azospirillum* strains (Bacilio et al. 2004; Wisniewski-Dyé et al. 2011; Arruebarrena Di Palma et al. 2013). Biofilms formed with this strain can be softly rinsed with PBS and analyzed directly over the plates with a confocal laser scanning microscope (CLSM) at emission and excitation wavelength appropriate for the fluorescent protein used for tagging the bacteria.

11.4.4 Techniques to Determine Components of the Biofilm Matrix

Matrix quantification assays are based on the specific staining of each matrix component.

11.4.4.1 Calcofluor Assay

Principle: Calcofluor binds to β,1-3 and β,1-4 polysaccharides, such as those found in cellulose and chitin, and when excited with ultraviolet or violet radiation, will fluoresce with an intense bluish/white color (Harrington and Hageage 2003).

Members of the genus *Azospirillum* are characterized by the production of surface polysaccharides that bind calcofluor. The calcofluor-binding polysaccharides of *A. brasilense* are represented by EPS and CPS (Sheludko et al. 2008).

Procedure

- A concentrated stock of calcofluor must be prepared as a 0.2 % (w:v) solution in 1 M Tris HCl, pH 9.0, which is autoclaved to dissolve the reagent.
- Wash softly the biofilm formed to eliminate planktonic cells.
- Biofilm matrix is visualized by the addition of 200 μL of 0.2 % (w:v) calcofluor and incubation for 30 min at room temperature, followed by rinsing with deionized water.
- Determine the calcofluor-EPS binding fluorescence exciting the sample to 360 nm and measuring the emission to 460 nm (Bassis and Visick 2010).

11.4.4.2 The 1,9-Dimethyl Methylene Blue Assay

Principle: The dye 1,9-Dimethyl Methylene Blue (DMMB) forms an insoluble complexation product with sulphated polysaccharides in the biofilm matrix. The amount of dye released by adding a decomplexation solution is spectrophotometrically measured and reflects the amount of sulphated polysaccharides present in the biofilm matrix (Barbosa et al. 2003; Hannig et al. 2010).

Procedure

- Prepare a complexation solution containing 32 mg DMMB dissolved in 50 mL ethanol, and then add 200 mL 1 M guanidine HCl solution, 2 g sodium formate, and 2 mL formic acid (98 %; v:v) to the volume adjusted with MQ water to 1 L. This complexation solution (pH 3.0) is stable for 4 months when stored in the dark.
- Prepare a decomplexation solution to final volume of 400 mL: 1.64 g sodium acetate, 152.85 g guanidine HCl, and 40 mL propanol, adjust pH 6.8.

– First rinse softly the biofilm formed in the plate, add 200 µL complexation solution to each well.
– Incubate the plate in the dark on a rocking table (450 rpm, 25 °C, 30 min).
– Plates are rinsed to remove unbound DMMB.
– Add 200 µL decomplexation solution to each well and incubate in the dark for 30 min on a rocking table (450 rpm, 25 °C).
– The absorbance is measured at 620 nm (Peeters et al. 2008; Tote et al. 2008).

11.4.4.3 Lectins Assay

Principle: Lectins are proteins with sugar binding specificity. Some of the most known lectins are Concanavalin A with specificity for mannose and glucose residues, and wheat germ agglutinin (WGA) specific for *N*-acetylglucosamine (Sharon 2007). Other types of lectins are commercially available with different sugar-binding specificity. These lectins are covalent linked to fluorescence dye that allows the observation of biofilm matrix. The most used are Concanavalin A-Texas Red conjugate (ConA) (Villa et al. 2012), lentil lectin (LcH) conjugated to Fluorescein Isothiocyanat (FITC) that has affinity for α-mannose and α-glucose (λ_{ex} = 492 nm, λ_{em} = 517 nm) lima bean lectin (LBL) with affinity for *N*-acetyl galactosamine (Edwards et al. 2011), and lectin probe conjugated to tetramethyl rhodamine isothiocyanate (*Triticum vulgaris*-TRITC λ_{ex} = 568 nm, λ_{em} = 605/32 nm) (Lawrence et al. 1998).

Procedure

– Wash the biofilm with buffer PBS (7 mM Na_2HPO_4, 3 mM $NaHPO_4$ and 130 mM NaCl, pH 7.2).
– Dilute Fluorescent Labeled Lectin to desired concentration (between 50 and 100 mg/mL depending on fluorescent dye) using buffer PBS.
– Incubate tissue section with Fluorescent Labeled Lectin for 30 min in a moist chamber in dark.
– Wash biofilm with buffer three times.
– Examine biofilm with Fluorescent microscope. Use appropriate filter. For instance, to ConA-Texas Red conjugate the excitation wavelength is 592 nm, and emission at 615 nm (Villa et al. 2012).

11.4.5 Visualization of Biofilm by Electronic Microscopy

The great advantage of an electron microscope, as compared to an optical microscope, is the 10^3 increased resolutions of the images obtained. The resolution of an electron microscope is 10 nm, and samples could be studied in much greater detail. Two different types of electronic microscopy can be used to study biofilm structure: transmission electron microscope (TEM) and scanning electron microscope (SEM), each of them gives different details of biofilms.

11.4.5.1 Transmission Electron Microscope

Electrons are emitted from a cathode and accelerated by a potential difference. The electrons are formed to a parallel beam by a magnetic field before they pass through the very thin sample (usually in the range 10–100 nm) to be examined. The sample scatters the electrons that are passing through it, and then magnetic lenses focus them onto a fluorescent screen for viewing. The high resolution and details that TEM gives allow the study of a portion of the biofilm with its complexity.

11.4.5.2 Scanning Electron Microscope

An important variation is the SEM, where the electron beam is focused to a very thin line that is swept across the sample. As the beam scans the sample, electrons will be emitted from the surface of the sample. These secondary electrons will be collected and produce an image of the surface of the sample. Resolution is typically of the order of 10 nm (Hannig et al. 2010). A great advantage of SEM, as compared to TEM, is that the sample can be thicker or even it doesn't need to be sliced since it is only the surface that is examined. In addition, SEM images have a three-dimensional appearance. Thus, SEM is a good technique to study biofilm because it can be observed as the three-dimensional structure and the cell disposition in the biofilm as well as if cells are bound and how the binding occurs among them.

Due to the high vacuum required for evaluation of the samples and due to the fact that biological samples have nonconductive properties, fixation, dehydration, and coating with a conductive material are necessary. Therefore, many procedures are required to avoid destruction of the structure of the samples (Bergmans et al. 2005). After fixation with cacodylate buffer, the dehydration is carried out with a series of ascending concentrations of ethanol. In this way, the water is gradually replaced by the organic solvents. Samples have to be dried without destruction of the complex structures. This is achieved by reaching the critical-point drying without formation of any unreal structure. Thereafter, ethanol is replaced by a transitional fluid, usually consisting of carbon dioxide. Finally the samples have to be coated with a conductive material as gold (Bergmans et al. 2005; Hannig et al. 2010).

11.4.5.2.1 The Environmental Scanning Electron Microscope

This technique allows to study bacteria without any dehydration, fixation, or coating of bacteria in the natural state. This is a clear advantage, but three-dimensional visualization of the structures is sometimes limited and total resolution is more limited than conventional SEM (Danilatos 2012).

11.4.6 Fluorescent and Confocal Microscopy

Besides electron microscopic techniques such as TEM, SEM, and environmental scanning electron microscope (ESEM), fluorescence microscopic approaches based on fluorogenic dyes offer detailed insight into bacterial biofilms as confocal laser scanning microscopy (CLSM).

CLSM is the most versatile and nondestructive approaches for studying biofilm. It reduces greatly the need for pretreatments such as disruption and fixation, diminishing the perturbation of the bacterial relationships, the complex structure, and their organization in the biofilm. It allows the study of intact fully hydrated biofilm material creating images with enhanced resolution, clarity, and information content. As such, it is an ideal tool for studying spatial distribution of a wide range of biofilm properties. CLSM is a combination of traditional epifluorescence microscope hardware with a laser light source, specialized scanning equipment, and computerized digital imaging. Digital image analysis of the CSLM optical thin sections in each of the channels formed in biofilms and can be used to determine such parameters as biofilm depth, bacterial cell area (biomass), exopolymer area, and their locations inside biofilm (Lawrence et al. 1998).

CLSM in combination with a range of fluorescent probes and dyes offers an approach to quantitatively defining many aspects of biofilm communities (Lawrence et al. 1998).

To observe a sample with CLSM first requires the choice of an adequate staining that adjusts to obtain the desired information and then prepare the sample to observe.

Sample Preparation: CLSM can be used to study biofilm samples coming from materials adhered to natural surfaces without previous treatments. Biofilms can be formed in many different solid surfaces to be analyzed with this technique. To examine the fully hydrated living features of a biofilm, the sample is preferably used directly without any fixation procedures. Biofilm sample may be kept in a moist chamber until its observation with a microscope. In general, many staining procedures for CLSM study do not require fixation of the biofilm sample.

Staining Options: The type of staining or fluorescent probe/dye to be used must be selected. There are a wide range of stains and probes that may be used in conjunction with CLSM imaging to obtain information about the biofilm. Staining in CLSM may be either positive or negative in nature. Negative staining of biofilms achieves through the flooding of the sample with a fluorescent dye such as fluorescein. Positive staining encompasses nucleic acid stains as the SYTO 9, DAPI, protein stains, FITC, TRITC, or Texas Red. Other probes may be labeled using fluorescein, rhodamine, cyanins (CY2, CY3, CY5).

An important consideration is that unstained controls should be imaged with all samples using the same settings as for the stained materials to ensure that autofluorescence artifacts are not present in the resulting images (Lawrence and Neu 1999).

CLSM allows also the combination of different techniques described above as lectins and SYTO 9 to evaluate biofilm composition, through the visualization of EPS and bacterial biomass in the biofilm. In addition, this combination also shows a detailed quantitative examination of the three-dimensional structure of biofilms (Lawrence et al. 1998).

11.4.6.1 Raman Spectroscopy–CLSM Combination

Microscopic techniques cannot give the chemical information such as a precise identification of the biomolecules constituting the biofilm matrix. The combination of microscopic and spectroscopic methods allows the complete analysis of the production of EPS by sessile bacteria (Fahs et al. 2014). CLSM not only can be combined with fluorescence staining and probes but also with other techniques as Raman spectroscopy or Infrared absorption spectrometry. Compared to CLSM, Raman spectroscopy (RS) allows for a deeper insight into the chemical structure of EPS biofilm matrix (Wagner et al. 2009). No staining is required hence results can be obtained even from structures that are not substrate to the known staining techniques. Thus, no time-intensive or complex sample preparation is necessary, which reduces the risk of altering the biofilm or the creation of artifactual structures not desired. The possibility of measuring directly in the aqueous phase is one of the major advantages of RS and reveals information about the biofilm in its native state with spatial resolution in mm range. RS provides fingerprint spectra and allows characterization and identification of different substances and biological systems including single bacteria (Harz et al. 2005; Sandt et al. 2007). Although the Raman spectra of the biofilm matrix are complex superpositions of fingerprints of different polymers, it is possible to identify changes in structure and chemistry (Lawrence et al. 2003). Biofilms were analyzed directly on marked glass slides by means of a 63× water immersion objective (N.A. ¼ 0.9, laser spot diameter approximately 1 mm) with an exposure time of 100 s (Wagner et al. 2009).

When light passes through a transparent medium, the compounds that are present scatter some of the light in all directions. A fraction of the scattered light has a slightly different wavelength from that of the incident light. This shift in wavelength depends on the chemical structure of the molecules responsible for the scattering.

Raman spectra are acquired by irradiating a sample, in this case a biofilm, with powerful visible or near-infrared laser light. A rather powerful laser must be used, since the intensities of the Raman lines almost never exceed 0.001 % of the laser intensity (Fahs et al. 2014).

11.5 Methods for Studying Biofilm Formation on Roots

The following protocols described in this section have been used in the study of *Azospirillum*-root interaction by many authors and represent the conjunction of several available data. Although these methods are used in *Azospirillum*, they could also be used easily for other PGPR species.

11.5.1 Scanning Electron Microscopy

SEM has been used by numerous authors to study root colonization and the biofilm formed there by *Azospirillum* (Bashan et al. 1991; Bashan and Holguin 1993; Bacilio-Jimenez et al. 2001; Guerrero-Molina et al. 2012). This technique allows the visualization of sample previously fixed, dehydrated, and metalized. An interesting advantage of this technique is the focal depth that it possesses, where several points to different distances can be focused, allowing to obtain more realistic photographs.

Procedure

- Excise root from plants and fix for 4 h in 5 % (v:v) glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2, under vacuum.
- Wash twice in the same buffer and dehydrate by passing through increased ethanol concentrations (from 30 to 100 % (v:v) in steps of 5 %) at 4 ± 1 °C for 10 min each.
- Dry the sample in a critical-point dryer under CO_2 atm.
- Use forceps to transfer the sample to corresponding support and fix to this using double phases tape.
- Coat the sample with gold particles and observe it using scanning electron microscope.

Considerations

Dehydration steps are extremely important and must be followed carefully. Besides they must be adjusted to each type of root. If dehydration is incomplete the tissue could collapse and the observation of artifacts in the sample is possible, leading to a wrong analysis.

11.5.2 Fluorescent In Situ Hybridation

Principle: Fluorescent In Situ Hybridation (FISH)–CLSM is most frequently used for visualization of microbial colonization patterns and community composition (Moter and Göbel 2000; Amann et al. 2001), not for studying biofilm formed in vitro (Cardinale 2014).

A typical FISH protocol includes four steps: the fixation and permeabilization of the sample; hybridization; washing steps to remove unbound probe; and the detection of labeled cells by microscopy or flow cytometry.

The oligonucleotide probes used in FISH are generally between 15 and 30 nucleotides length and covalently linked at the 5′-end to a single fluorescent dye molecule. Common fluorophores include fluorescein, tetramethyl rhodamine, Texas red and, increasingly, carbocyanine dyes like Cy3 or Cy5.

This technique has been less used in *Azospirillum*, but in others organisms have generated good results.

It is interesting the application of FISH technique to complex samples, where inoculation conditions are not gnotobiotics and root could be colonized by other bacteria besides *Azospirillum*. For instance, a root plant sample that was grown in complex soils in greenhouse or in the field could be studied in this way and show the total biofilm using a general probe against bacteria and a specific targeted probe (e.g., against *Azospirillum*) on the same sample.

Procedure (Based and Adapted from Stoffels et al. 2001; Rothballer et al. 2003)

- Remove roots carefully and wash with PBS (7 mM Na_2HPO_4, 3 mM $NaHPO_4$, and 130 mM NaCl, pH 7.2).
- Cut 1 cm segments and transfer to 4 % paraformaldehyde fixation solution in PBS on ice. Incubate for 2 h.
- Wash roots carefully with PBS buffer and transfer to ethanol: PBS (1:1). Store at −20 °C until hybridization.
- Before hybridization, cut big roots (>500 μm diameter) longitudinal if it were necessary. Roots <500 μm in diameter could be processed without cut.
- Slices of the fixed root or entire root are placed on a gelatin (0.1 % w/v, 0.01 % w/v chromium potassium sulfate) coated microscope slides and fixed to the slide by adding one drop of warm, low-melt agarose solution (0.2 % w/v), and dried at 37 °C for 45 min.
- Samples are dehydrated by successive 50, 80, and 96 % ethanol washes (3 min each), then air dried.
- Incubate samples 15 min on hybridization buffer (0.9 M NaCl, 0.01 % w/v SDS, 20 mM Tris–HCl pH 7.2) with 45 % w/v formamide at 46 °C.
- Add fluorescent labeled probe Abras1420 (CCACCTTCGGGTAAAGCCA, Stoffels et al. 2001) and competitor Abras1420C (CACCTTCGGGTAAAACCA, Stoffels et al. 2001) to a final concentration between 5 and 20 μg/mL.
- Incubate for 2 h at 48 °C.
- Wash samples immediately with 1 mL of prewarm washing buffer (hybridization buffer) followed by immersion in washing buffer at 48 °C for 20 min.
- Roots are fast rinsed with deionized water to remove salts. After air drying roots are mounted with antifadent mounting media and observed under fluorescent confocal microscope.

11.5.3 Fluorescent Proteins

This method relies on tagged bacteria with one of the fluorescent protein (by plasmid or introducing onto bacterial genome) as was specified above (see Sect. 11.4.3.3).

The method implies to perform a plant inoculation with a PGPR, allow to grow the plant and the formation and establishment of the biofilm adequately in its roots.

An important consideration is that roots must be manipulated carefully to protect the biofilm integrity.

Roots must be placed on microscope slide and observe the biofilm with CLSM using the filters according to the fluorescent protein tagging the bacteria. Another important consideration during observation for obtaining good images is that root autofluorescence can generate benefits since it contrasts the fluorescence emitted by tagged bacteria. Nevertheless, it could bring some drawbacks in the case that both organisms emit fluorescence at the same wavelength. To avoid this, is recommendable to check the root autofluorescence at different exciting wavelengths. Thus, the selection of the fluorescent protein to tag the bacteria must consider the emission spectra of root autofluorescence in a way that allows the differentiation between the root and the biofilm.

11.6 General Conclusion

Plants support a diverse array of bacteria on or in their roots, vessels, stems, and leaves. These plant-associated bacteria have important effects on plant health and productivity. The interaction of these bacteria with plant roots is associated with symbiotic and pathogenic responses. In the so-called associative beneficial bacteria, that do not show species-specificity with plant host and neither specialized structures as nodules, the developing of biofilms on roots surfaces is a generalized way of colonization. For these bacteria the formation of appropriated biofilms on roots are of crucial importance to establish a colonizing population that would exert beneficial effects on plants.

Biofilm research is still a challenging field of interest and the techniques for quantification, visualization, and characterization of microorganisms have undergone rapid progress within the last 15 years. However, some of these promising techniques, such as CLSM or ESEM, require costly equipment while for others, such as TEM or SEM, exhaustive preparation of the samples is necessary (Hannig 1999; Bergmans et al. 2005; Dufrêne 2008). Ultrastructure, viability, and the metabolism of bacteria are quite variable. The same is true for the colonized substrates. In general, a combination of several methods is recommended when investigating adherent microorganisms as the different methods yield different information about these different aspects (Hannig et al. 2007). Figure 11.2 summarizes the stages of biofilm development paralleled with the most used techniques in studying biofilm formation (SEM, CLSM, and CV). The timing and photograph in Fig. 11.2 are from *A. brasilense* Sp245. The superior panel shows the initiation of a biofilm that starts with the attachment of some cells to a surface to the right the thick arrow indicates the gradual stages of biofilm development up to some cells detach the biofilm and presume to colonize other surfaces. The second and third lines describe each stage of biofilm formation as it is observed with SEM and CLSM (cells tagged with GFP), respectively. The graph on the last line shows the timing of each stage that in *A. brasilense* corresponds to 1, 3, and 5 days of growth. Finally, the well in the

inferior corner shows a CV-stained biofilm produced after 5 days of growing *A. brasilense* Sp245 cells in multiwell plate in static conditions with Nfb-NO_3^- amended media.

Unraveling the regulation of biofilm formation and disassembling, is essential to know its life cycle. The kinetics studies become critical in the understanding of signals inducing not only its formation and development but also the functions of different subpopulations of bacteria in the biofilms and the factors that induce its disassembly. The knowledge of this complex scenario would help in the understanding of root colonization and the dispersion of bacteria to colonize other roots or microsites and hence in the managing of inoculation technology.

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Chapter 12
Methods for Studying Phenotypic Variation in *Azospirillum*

Gal Reem, Saul Burdman, and Yaacov Okon

Abstract Phenotypic or phase variation has been demonstrated in many bacteria studied so far. It is an apparently adaptive process enabling bacteria to cope with changing environmental conditions, niche occupation, adaptive versatility, and optimized interaction with host organisms. Phase variation can be caused by genetic as well as epigenetic alteration. In this chapter, we focus on the methodology used to study phenotypic variations in rhizosphere bacteria of the genus *Azospirillum*. Phase variation can be detected under laboratory conditions, but also in bacteria isolated from the rhizosphere. The awareness of the possible occurrence of phase variation is important for the provision of high quality inoculum. Methods are described for selecting phase variants from cultures, and testing their stability after culture transferring, phenotypic analysis of phase variation includes colony morphology, production of polysaccharides and pigments, and genomic analysis of phase variation including DNA and plasmid profiles. Methods for assessing survival of phase variants and their plant growth promotion abilities in comparison to the parental strain are presented.

12.1 Introduction: The Phenomenon of Phenotypic Variation in *Azospirillum*

Phase variation—or phenotypic variation— (PV) is one of the mechanisms by which microorganisms adapt to environmental changes. This phenomenon is characterized by the presence of a subpopulation presenting a different phenotype from the major population. This subpopulation appears at a relatively high ratio, more than 10^{-5} (as compared with less than 10^{-6}, as for spontaneous mutations), but during appropriate conditions can become dominant. Phenotypic variation has been described for many different bacterial genera belonging to diverse taxonomic groups and displaying

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different ecological behaviors (e.g., pathogens, saprophytes and symbionts, free living or associated with plants and animals) (Van der Woude and Baumler 2004; Wisniewski-Dyé and Vial 2008; Wisniewski-Dyé et al. 2014).

Phenotypic variation is therefore used by several bacterial species to generate intra-population diversity that often increases bacterial fitness and is important in niche adaptation, or to escape host defenses (in the case of pathogens). Phenotypic variation can be associated with changes in various phenotypes, including alterations in pigmentation, motility, exopolysaccharides (EPS), synthesis of pili, and production of antifungal metabolites among others (Wisniewski-Dyé and Vial 2008).

The molecular mechanisms associated with phenotypic variation are diverse and include genetic and epigenetic changes, such as DNA inversions, duplications and deletions, transpositions, homologous recombination, slipped-strand mispairings, as well as differential methylation patterns (Vial et al. 2006). In phenotypic variation, the expression of a given factor is either ON or OFF and the changes (variations) are usually reversible (i.e., ON ↔ OFF), resulting in a heterogeneous population (Henderson et al. 1999).

In *Azospirillum*, bacterial surface components, such as extracellular polysaccharides and proteins, are involved in root colonization (Burdman et al. 2000a). Extracellular polysaccharides secreted by *Azospirillum* comprises lipopolysaccharides (LPS) and capsular polysaccharides (CPS), which form an adherent cohesive layer on the cell surface, and EPS that form an extracellular matrix that has little or no association with the cells. EPS properties contribute to cell protection against environmental stresses, attachment to surfaces, nutrient gathering, biofilm formation, and cell antigenicity. The relative monosaccharide composition and the molecular weight of the EPS and CPS in *Azospirillum* vary within species and strains, growth conditions, and physiological state of the culture (Burdman et al. 2000b; Bahat-Samet et al. 2004).

Several studies with some *Azospirillum* species and strains have identified and characterized phenotypic variants. First indications reported on frequently occurring spontaneous mutants in *Azospirillum brasilense* Sp7, with higher resistance to stress conditions like salt stress and iron acquisition (Hartmann et al. 1992). At continuation, a stable variant of *Azospirillum lipoferum* 4B, named 4V, which lost the ability to swim and had altered carbohydrate utilization abilities was reported (Alexandre and Bally 1999). The appearance of variants was correlated with the loss of a 750-kb replicon and changes in plasmid pattern (Vial et al. 2006).

Phenotypic variants of *A. brasilense* Sp7 were obtained under standard growth conditions, under prolonged starvation and stress conditions, and after re-isolation from maize roots. Some of the variants were found to produce significantly more and different EPS in terms of monosaccharide composition relative to the parental strain (Lerner et al. 2010). In these variants, genomic rearrangements were shown to be associated with phenotypic variation as demonstrated with the utilization of different DNA fingerprinting techniques (Lerner et al. 2010; Volfson et al. 2013).

The occurrence of phenotypic variation has to be considered, when bacteria are isolated from their natural habitat, like the root environment. While some bacterial isolates may keep their original properties under laboratory conditions, others may

undergo major changes, since genetic rearrangements may occur at high frequency. In laboratory mass production of inoculants for agriculture, genome plasticity should be taken into account and kept under control as much as possible. On the other hand, the genome plasticity could also be considered as a chance to preselect for strains optimized for specific niches and a possible way for strain improvement to specific plant and soil requirements (Wisniewski-Dyé et al. 2014). Extensive comparative genome-sequencing experiments of parental and phenotypic variants will bring much more insights into the underlying molecular and genetic mechanisms associated with phenotypic variation in *Azospirillum* species.

Here we review methodologies for studying phenotypic variation occurring mainly in plant growth-promoting bacteria of the genus *Azospirillum*, but could be relevant also in other associated rhizosphere bacteria.

12.2 Methods for Investigation of Phenotypic Variation in *Azospirillum*

12.2.1 Methods for Obtaining Variants and Assessing Their Stability

Azospirilla are routinely grown at 30–35 °C, pH near 7.0 in LB (Difco), nutrient broth (Difco) or in minimal medium, with varying carbon to nitrogen ratio (C:N) (Burdman et al. 1998) containing g L^{-L} D-fructose (6.67) or other appropriate carbon sources, $MgSO_4$ (0.2), NaCl (0.1), $CaCl_2$ (0.02), K_2HPO_4 (6.0), KH_2PO_4 (4.0), yeast extract (0.1), NH_4Cl (0.2), and microelements as described (Okon et al. 1977). For preparation of solid minimal medium, agar is amended at 15 g L^{-1}. Cell growth is determined spectrophotometrically at OD_{590}, by dilution plating or by drying the bacteria in an oven at 80 °C for 48 h, and by measuring the dry cell weight.

To assess the rate of appearance of phenotypic variants, a single colony of the parental strain is used to inoculate 5 mL of minimal medium. After incubation (24–72 h) with shaking at 35 °C (200 rpm), a $10^{4–5}$ dilution is plated onto minimal medium plates with a Drigalski spatula. The morphology of the colonies (altered pigmentation, slimy larger colonies producing more EPS, rough or smooth appearance, etc.) is examined after 2–3 or more days of incubation. At least 10,000 colonies are screened in each experiment. The stability of the variants is tested by transferring individual colonies to new minimal medium plates and recognition of the phenotypic variation.

Polymerase chain reaction (PCR) with *A. brasilense*-specific primers should be performed to confirm that colonies showing an altered morphology are phenotypic variants rather than contaminations. *Azospirillum* DNA can be extracted by standard methods for bacterial DNA extraction or by the utilization of commercial kits developed for such purpose. In the case of *A. brasilense*, the suspected variants can be tested for the presence of the *ipdC* gene, encoding indole-pyruvate decarboxylase,

a key enzyme in indole-3-acetic acid (IAA) synthesis. This gene, and the corresponding PCR primers, has been suggested as a reliable and specific indicator for *A. brasilense* (Shime-Hattori et al. 2011). As an example, each 25 μL PCR reaction contains 12.5 mL of Taq Master MIX (Lambda Biotech), $MgCl_2$ (1 mM), BSA (0.04 mg mL^{-1}), and 1.6 mM of each ipdC primer: A32f (forward), 50-CCCCTCCACAATTTCCG GCGCAT-30, and A42r (reverse), 50-CGCCACCCCTAGAGTGGAGCTGTA-30 (Shime-Hattori et al. 2011).

PCR amplifications are performed in an automated thermal cycler (Eppendorf Mastercycler Gradient Machine) with an initial denaturation (94 °C, 2 min) followed by 30 cycles of denaturation (94 °C,30 s), annealing (60 °C, 30 s) and extension (72 °C, 1 min), and a single final extension (72 °C, 5 min). PCR products are then electrophoresed directly on 1 % agarose gels, visualized by ethidiumbromide staining, excised from the gel with the HiYield Gel/PCR DNA Fragment Extraction Kit (RBC Bioscience) and sequenced.

12.2.2 Genomic Analysis of Variants

Phenotypic variants are compared with the parental strain by repetitive-PCR (Enterobacterial Repetitive Intergenic Consensus (ERIC)—and BOX-PCR) and random amplified polymorphic DNA (RAPD) analysis. Repetitive-PCR reactions are performed as described by Louws et al. (1994). Reaction mixtures (25 mL) contain 12.5 mL of Taq Master mix (Lambda Biotech), MgCl2 (3 mM), BSA (0.02 mg μL^{-1}), 2 mM of each primer [ERICIR, 50-GTAAGCTCCTGGGGATT CAC-30, and ERIC2, 50-AAGTAAGTGACTGGGGTGAGCG-30 for ERIC-PCR; BOXA1R 50-CTACGGCAAGGCGACGCTGACG-30 for BOXPCR) and 100 ng of template (genomic) DNA. PCR amplifications are performed in an automated thermal cycler with an initial denaturation (95 °C, 7 min) followed by 30 cycles of denaturation (94 °C, 30 s), annealing (42 and 53 °C for ERIC- and BOX-PCR, respectively, 1 min), and extension (65 °C, 8 min) with a single final extension (65 °C, 16 min). Samples of 8 mL from each reaction are separated by gel electrophoresis on 1.5 % agarose gels at 60 V for 3 h, and the gels are stained with ethidiumbromide. RAPD analysis is performed as described by Vial et al. (2006). Briefly, reaction mixtures (25 mL) contained 12.5 mL Taq master mix (Lambda Biotech), primer F1253 (50-GTTTCCGCCC-30) (2 mM), BSA (0.02 mg μL^{-1}), $MgCl_2$ (2 mM), and bacterial DNA (w 100 ng). Amplification conditions were: initial denaturation (95 °C, 5 min) followed by 35 cycles of denaturation (95 °C, 45 s), annealing (36 °C, 1 min), and extension (72 °C, 2 min) with a final extension at 72 °C for 7 min and 60 °C for 10 min. PCR products are analyzed as described for repetitive-PCR above.

Total DNA is isolated using the Wizard Genomic DNA Purification kit (Promega). For Southern blotting, DNA is digested with restriction enzymes purchased from New England BioLabs, electrophoresed, and blotted onto MSI nylon transfer membranes (Roche Diagnostics) by standard methods. Detection is performed using the DIG DNA Labeling kit (Roche Diagnostics). PCR amplifications are

performed on an automated Eppendorf Mastercycler. Sequence analyses are performed using the BLAST network service (Altschul et al. 1997) and Pfam (http://pfam.sanger.ac.uk/). Oligonucleotide primers are synthesized using the Primer3 program http://www.es.embnet.org/cgi-bin/primer3_www.cgi.

12.2.3 Phenotypic Analysis of Variants: Colony Morphology, Pigments Production, Exopolysaccharides, Lipopolysaccharides

Extracellular polysaccharides are involved in the *A. brasilense*–plant root interactions and in survival to environmental stresses (Lerner et al. 2009a, b). Therefore, comparison of LPS and EPS of the variants with those of the parental strain is recommended.

Azospirillum cultures either non-treated or exposed to starvation, high temperature (55 C) or other stresses, or isolated from inoculated plants (Lerner et al. 2010; Volfson et al. 2013) are grown and colonies are randomly collected after these treatments and assessed for phenotypic alterations. For recognition of variants, emphasis is put on change in pigmentation, gain of cell aggregation ability, and EPS overproduction. These phenotypes have been often associated with increased survival to different stresses in *A. brasilense* and in other bacterial species (Hartmann and Hurek 1988; Burdman et al. 2000b; Lerner et al. 2009a, b).

EPS, CPS and LPS extraction and determination are performed according to Lerner et al. (2009a, b). Briefly for LPS, 1 mL of overnight-grown cultures are centrifuged (13,000×*g*, 1 min, twice), and the resulting pellets are resuspended in 130 mL SDS sample buffer and then boiled for 5 min at 100 °C, and following removal of cell debris, proteinase K is added to a final concentration of 0.5 mg/mL. The samples are incubated at 37 °C for 1 h; afterwards, 20 mL aliquots are loaded onto SDS-PAGE gels containing 3 and 15 % polyacrylamide concentrations in the stacking and resolving gels, respectively. The samples are electrophoresed at 15 mA for 3.5 h, and following electroblotting (70 V, 1 h) to a nylon membrane (Rosch) to remove residual proteins, the gels are then silver-stained (Lerner et al. 2009a). LPSs are extracted also with an LPS extraction kit (Intron Biotechnology), and samples are run on a deoxycholic acid (DOC)-polyacrylamide gel according to Reuhs et al. (1998).

12.2.3.1 Extraction of EPS (Burdman et al. 2000b)

Sugar amount is evaluated by the anthrone method (Dische 1962). Microbial mass is determined by measuring the dry cell mass of pelleted cells at 80 °C until a constant weight is reached.

Identification of EPS monosaccharide composition is performed according to Albersheim et al. (1967), with minor modifications. Sugar composition of modified alditols is determined in a Hewlett Packard HP 5890 Series II gas chromatograph,

equipped with a DB-225 capillary column (30 m 6 0.25 mm) from J&W Scientific and a flame-ionization detector (FID) at 250 μC. Samples (1 mL) of mixed alditols are separated at 220 °C with helium as the carrier gas. Congo red and calcofluor staining, indicating polysaccharide composition in the cells, is performed for each strain, as described by Croes et al. (1991), respectively. Calcofluor binds predominantly to β 1–4- and β 1–3-linked glucans, congo red binds preferentially polysaccharides of certain bacterial species such as Azospirilla.

EPS is extracted from minimal medium liquid cultures, by fractionation with cold ethanol as described by del Gallo et al. (1989) and modifications by Lerner et al. (2009a). Briefly, following growth, bacterial cultures are centrifuged at 7,000×*g* for 10 min at 10 °C. The supernatant is collected and left to stand in three volumes of cold ethanol for 72 h at 4 °C, resulting in EPS precipitation. After ethanol evaporation, precipitated polysaccharides are suspended in and dialyzed against distilled water for 72 h at 4 °C, using a dialysis membrane with a molecular mass cutoff of 12–14 kDa, and lyophilized. Absence of proteins in the extracted EPS is confirmed by the Bradford method, following manufacturer conditions (BioRad). Evaluation of the sugar amount is done by weighing or by the Anthrone method using glucose as a standard (Dische 1962). For determination of EPS monosaccharide composition, EPS is hydrolyzed following lyophilization according to Lerner et al. (2009a). Briefly, sugar composition of modified alditols is determined in Hewlett Packard HP 5890 Series II gas cromatograph, equipped with a DB-225 capillary column (30 m×0.25 mm) from J&W Scientific and a flame ionization detector (FID) at 250 °C. Samples (1 μL) of mixed alditols are separated at 220 °C with helium as the carrier gas.

Pigment extraction and separation: Cells are resuspended in phosphate buffer and disrupted in an ice bath with an ultrasonic disintegrator and then centrifuged. Cells are extracted in acetone/methanol, after drying; the red pigment obtained is partitioned between methanol and chloroform and purified in chloroform. For details, see Nur et al. 1981. Bacterial carotenoids are determined using an absorption coefficient of 2,500 at 500 nm for 1.0 % W/V solution (Nur et al. 1981).

As an alternative, pigment extraction is carried out at room temperature, and all glassware is covered with aluminium foil to protect the pigments from light. UV–visible absorption spectra between 300 and 600 nm of the pigments dissolved in methanol are recorded with a Hitachi U-2000 spectrophotometer.

12.2.4 Survival of Variants Under Stress as Compared to Parental Strains (Lerner et al. 2010; Volfson et al. 2013)

Starvation experiments: Aliquots (10 mL) of overnight, high C:N medium cultures of wild-type and mutant strains at approximately 6×10^8 c.f.u. mL^{-1} are washed twice by centrifugation (4,000×*g*, 10 min). Cells are resuspended in 0.06 M

potassium phosphate buffer (pH 6.8) and incubated on a shaker at 200 rpm, 30 °C for 12 days under starvation, as described by Kadouri et al. (2002).

Bacterial viability is determined by dilution plating at the beginning and end of the incubation period. This experiment is repeated three times.

Stress endurance: In all experiments, 0.5 mL aliquots of LB-grown overnight cultures of wild-type and PV strains are used to inoculate 100 mL Erlenmeyer flasks containing 30 mL high C:N medium, for example, containing D-fructose 6.67 g L^{-1} and NH_4Cl 0.214 g L^{-1}, (low C:N contain the same components but 0.963 g L^{-1} NH_4Cl) and grown for 24 h at 30 °C with agitation at 250 rpm.

The resulting cultures are used to assess bacterial survival under various stresses. The percentage of viable cells is determined following dilution plating at the beginning and end of each experiment, and/or during the course of the experiment. In all experiments, the initial number of cells is approximately 5.0×10^8 c.f.u. mL^{-1}. All experiments are conducted three times as described by Kadouri et al. (2003) with the following modifications: in the heat resistance experiments, 10 mL of culture is incubated in a water bath at 55 °C for 60 min. Bacterial viability is determined every 15 min.

Bacteria are grown in D-fructose minimal liquid medium. The resulting cultures are used to assess bacterial survival under UV and heat stresses. In all experiments, the initial cell concentration are of approximately 5×10^8 CFU mL^{-1} (OD_{590} ~0.8). In heat resistance experiments, 10 mL of cultures are incubated in a water bath at 55 °C for 60 min. Bacterial viability is determined every 15 min. In experiments performed to assess resistance to UV radiation, 20 mL of cells in 90-mm Petri dishes are exposed to UV radiation (254 nm) using a VL-6LC ultraviolet lamp (Vilber-Lourmat) for 120 s, and bacterial viability is determined every 30 s. The percentage of viable cells is determined following dilution plating at the beginning and during the course of the experiment. In addition, the *A. brasilense* Sp7 variants are assessed for biofilm formation ability by the microplate method according to Lerner et al. (2010) and cell aggregation according to Burdman et al. (1998).

12.2.5 Effect of Variants on Plants as Compared to Parental Strains (Volfson et al. 2013)

Plant growth promotion experiments under controlled conditions in pots. Seeds of the tested plant such as maize or wheat are surface-sterilized by soaking them for 5 min in absolute ethanol followed by five washes with sterile-distilled water (DW). They are then placed in 50 mL tubes containing 30 mL bacterial suspension (Sp7 or PV) at $3–6 \times 10^7$ c.f.u. mL^{-1}. Controls are seeds incubated in 30 mL sterile DW. The tubes are shaken at 200 rpm for 3 h at room temperature. Then the seeds are collected, air-dried, and sown in pots filled with autoclaved vermiculite and covered with a 1 cm layer of autoclaved perlite. The pots are maintained in a greenhouse-appropriate temperature and light period for the particular plant species; the plants

are watered with 100 mL sterile DW every 4–5 days. It is recommended to utilize at least 10–15 plants per treatment. The experiment needs to be repeated at least three times. Because of the natural plant variation of plants, it is necessary to test as many plants as possible and to repeat the experiment in order to observe differences in inoculation when comparing the parental strain to the PV (Volfson et al. 2013).

It is necessary to follow during plant growth root and shoot wet and dry weight and root and shoot length.

Other possibility is to germinate seeds in Petri dishes (0.8 % agar) for 2 days. Pre-germinated seeds are then transferred aseptically to pots (two seeds per pot) containing sterilized and nitrogen-free sand, and immediately inoculated with *A. brasilense* strain Sp7 or tested PV at 1×10^7 CFU mL^{-1}.

Experiments with legumes include inoculation and co-inoculation (with *A. brasilense*, PV) and the symbiotic rhizobia (infective and effective strains), respectively. Forty eight hours after sowing, seedlings are thinned down to uniformity to one per pot and pots are arranged in a complete randomized block design, with five replicates per treatment. The following parameters are evaluated for legumes: shoot and root dry weights, total plant nitrogen content determined by the Kjeldahl method, the number of nodules per plant and nodule dry weight. For determination of nodulation parameters, plants are removed from the pots, the roots are thoroughly rinsed with water, blotted dry on filter paper, and nodules are collected.

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Chapter 13
Polyhydroxybutyrate in *Azospirillum brasilense*

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Abstract Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoate produced by several bacteria as carbon storage and reductive equivalents sink. The production of PHB has been reported for several plant-associated bacteria, and especially in *Azospirillum brasilense,* the production of PHB has been associated as a factor for stress endurance. In addition, bacterial cells colonizing plants are often shown to contain PHB granules, suggesting that both PHB production and accumulation may be an important factor for plant–bacteria association. Based on that, this chapter will present experimental protocols for screening of PHB-producer bacteria and quantification. Basically, the protocols address the bacteria growth conditions for PHB production, as well as different methodologies for PHB quantification using GC-FID, HPLC, and FT-IR. This information may be useful for researchers interested on PHB metabolism investigation, and additionally, for those interested in biotechnological applications concerning *A. brasilense*.

13.1 Introduction

Polyhydroxybutyrate, or poly-3-hydroxybutyrate (PHB), is an important polyhydroxyalkanoate produced and accumulated by several bacteria in conditions of carbon oversupply and low concentration of other nutrient, such as nitrogen (Madison and Huisman 1999). The PHB polymer is stored as intracellular-insoluble granules coated with proteins which may reach about 0.5–2 % of the granule weight (Grage et al. 2009; Jendrossek 2009). Previous reports have also indicated the presence of lipids-coating PHB granules (Grage et al. 2009); however, a recent report analyzing the granules by electron cryotomography suggested that they may be coated exclusively by proteins (Beeby et al. 2012). In addition to carbon storage, the accumulation of PHB has been related as a sinker for reducing equivalents, since the

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PHB biosynthesis consumes high titres of intracellular NAD(P)H (Anderson and Dawes 1990; Madison and Huisman 1999; Hauf et al. 2013). On the other hand, when the bacterium challenges an unfavorable condition for growth or survival, the stored PHB can be mobilized generating acetyl-CoA and NAD(P)H (Trainer and Charles 2006). Thus, the proper balance between PHB accumulation and mobilization has been described as an important factor to maintain fitness in bacteria (Kadouri et al. 2003; Ratcliff et al. 2008; Ratcliff and Denison 2010; Tribelli and Lopez 2011).

Specifically, in plant growth-promoting rhizobacteria (PGPRs), the PHB accumulation and mobilization seems to be important to sustain the metabolism, thus contributing for the success of plant–bacteria associations (Willis and Walker 1998; Jones et al. 2007). The role of PHB on plant–bacteria association is better described for symbiotic rhizobia, so far. Usually, the bacteroides found in nodules formed by Rhizobia have a high content of PHB deposits, which appear as electron-transparent granules in transmission electron microscopy (Trainer and Charles 2006; Trainer et al. 2010). The accumulation of PHB in the bacteroides is promoted by the uptake of carbon sources produced by plants, as C_4-organic acids, such as L-malate (Salminen and Streeter 1992; Poole and Allaway 2000). *Sinorhizobium meliloti* Rm1021-defective mutants *phaP1* and *phaP2*, which encode for the phasin proteins that coat the PHB granules, showed no PHB accumulation, as well as a decrease in the number of nodules per plant and in nitrogenase activity, in comparison to the wild-type strain (Wang et al. 2007).

Despite the numerous works carried out on PHB metabolism in rhizobia, the importance of this polymer on the association of nonsymbiotic bacteria and plants is less known. In this context, the better studied model is *Azospirillum brasilense*. The conditions for PHB production as well as genes encoding for the PHB biosynthesis enzymes were identified and a *phaC* mutant, defective in PHB accumulation, was produced in *A. brasilense* (Tal and Okon 1985; Tal et al. 1990a, b; Okon and Itzigsohn 1992; Itzigsohn et al. 1995; Kadouri et al. 2002). The *A. brasilense phaC* mutant has exhibited a decrease in starvation survival and higher motility as compared to the wild-type strain. In addition, production of exopolysaccharides (EPS) and capsular polysaccharides (CPS) in the *phaC* mutant was higher than the wild-type as well as root adhesion, which in maize and wheat was fivefold higher for the mutant (Kadouri et al. 2002). The authors suggested that the higher motility and extracellular polysaccharides production might favour the *phaC* mutant to adhere to cereal roots. On the other hand, under some stress conditions such as osmotic shock, desiccation, peroxide resistance, UV exposition, and survival in inoculant carriers, the wild-type strain showed higher survival rate than the *phaC* mutant (Kadouri et al. 2003). From these studies, it can be concluded that production of PHB by *A. brasilense*, and likely by other endophytic bacteria, may be important to improve or maintain shelf-life, efficiency, and reliability of commercial inoculants.

The PHB accumulation has been suggested as an important phenotype for adaptation and endurance of bacteria to different stress conditions. Some stress conditions may be present during plant–bacteria association or in storage of inoculants for long times. Therefore, bacteria producing high contents of PHB may have an improved fitness for competition and/or to resist for long periods of storage.

Thus, PHB screening and quantification methods may be useful for characterization of new isolates of *Azospirillum* and other PGPRs. This chapter aims to bring to the readers common protocols to screen for PHB-producing bacteria and quantify PHB production.

13.2 Related Methods and Protocols

13.2.1 Screening of PHB-Producing Bacteria by Staining with Lipophilic Dyes

The fluorescent dye Nile red (Spiekermann et al. 1999) and the Sudan Black B (Schlegel et al. 1970) have been applied as lipophilic dyes for staining intracellular PHB granules. Cells stained with Nile Red can be visualized by UV irradiation, or by fluorescence microscopy using a regular microscope or more sophisticated equipments as confocal microscopes. When using the Sudan Black B, bacterial colonies producing PHB become dark blue-stained and can be visualized by naked eye or by simple optical microscopy inspection. When applying this protocol for other microorganism than *A. brasilense*, or even new isolates of *Azospirillum* sp., one should certificate that the microorganism on analysis does not produce other kind of lipophilic material as triacylglycerol droplets, since Sudan Black and Nile Red can also stain lipid bodies.

13.2.1.1 Colony Staining with Sudan Black B

Culture cells are permissive for PHB accumulation, usually in high carbon-to-nitrogen ratio media. A common condition used is a mineral medium with excess of sugar or other carbon source and limiting concentration of an inorganic nitrogen source as ammonium chloride or ammonium sulfate. Specifically, for *A. brasilense* high amount of PHB has been reported using medium with an initial carbon-to-nitrogen ratio of 20 or above (Sun et al. 2000, 2002). The *A. brasilense* strain Cd accumulated 88 % of dry cell weight using 37 mM malate in a C/N ratio of 15 (Itzigsohn et al. 1995). Interestingly, in the presence of 0.2 mM $CuSO_4$, *A. brasilense* strain Sp7 accumulated PHB, even in a low C/N ratio (Kamnev et al. 2006).

- Grow bacteria onto an agar plate containing high carbon and limiting nitrogen source concentration to achieve C/N ratio above 15. Colonies with 1–2 mm of diameter are appropriate for staining. In the case of *A. brasilense,* this colony size may be reached within 48 h growth at 30 °C using mineral medium containing malate or lactate as carbon source and ammonium chloride as nitrogen source.
- Pour on the plates 10–15 mL of 0.02 % (w/v) Sudan Black B solution prepared in ethanol. It is important that the volume of solution be enough to cover all the colonies on the agar. Cover the plate with lid and incubate for 20 min at room

temperature without agitation. If interested in recovering colonies for further experiments, it is strongly suggested to have a replica plate that will not be exposed to Sudan Black since this solution can kill the bacteria, impairing cell recovery for further analysis.
– Remove the dye solution and incubate the plates for 1 min at room temperature with 10–15 mL of 96 % ethanol. PHB-containing colonies retain the dye showing a dark blue color. On the other hand, cell colonies with no PHB accumulation are colored light blue or gray due to dye loss during the ethanol wash. Stained colonies may be also resuspended in water and visualized using optical microscopy.

13.2.1.2 Colony Staining with Nile Red

– Prepare agar plates by adding 0.002 volume of 0.25 mg mL^{-1} Nile red in dimethylsulfoxide (DMSO), giving a final concentration of 0.5 mg dye per mL of medium.
– Grow bacteria onto the agar plates containing Nile Red. Colonies with 1–2 mm of diameter are appropriate to perform the visualization. This method can also be applied streaking the bacteria culture onto the agar plate with a sterile toothpick.
– Visualize the fluorescence by irradiating the plates with UV light λ365 nm.

Note that the UV radiation can potentially damage cells including DNA mutation, and hence, a replica plate of the analyzed colonies is strongly recommended.

13.2.2 Quantification of PHB in Whole Cells by Gas Chromatography

The following protocol was modified from previous works describing methanolysis of lyophilized cells and gas chromatography—flame ionization detector (GC-FID) to determine the amount of the methyl-3-hydroxybutyrate ester produced (Braunegg et al. 1978; Huijberts et al. 1994). The Me−3HB amount measured is used indirectly as the PHB amount of the sample.

– Grow bacteria in a PHB production permissive condition until the culture reaches the stationary phase. The maximum PHB accumulation is frequently achieved at this point of the growth. It is worthy to notice that an extended incubation after this point may lead to PHB degradation, especially if the carbon source in the medium is exhausted.
– Harvest cells from 10 mL of culture by centrifugation in 15 mL conical Falcon-type tubes (5,000×*g*, 4 °C, 10 min).
– Discard medium and add 1.5 mL of distilled water.

- Transfer cell suspension to a preweighted 2 mL microcentrifuge tubes and pellet cells by centrifugation (10,000×*g*, 4 °C or room temperature, 2 min).
- Freeze cell pellet using liquid nitrogen or by storing overnight at −20 °C.
- Lyophilize cell pellet by at least 16 h.
- Weight the tubes again and register the value to calculate the dry cell weight that will be used to normalize the PHB amount and to express the final result.
- Prepare methanolysis solution (85:15 methanol/H_2SO_4) by adding H_2SO_4 to methanol slowly drop-by-drop on an ice bath and in a fume hood. Add 0.1 mL of 50 mg mL^{-1} benzoic acid in methanol for each 10 mL of methanolysis solution, to give 0.5 mg of benzoic acid per reaction.
- Transfer the lyophilized dry cell pellet to a borosilicate glass tube with PTFE screw cap. Crush the pellet against the glass wall with a metal spatula. Wash twice the 2 mL tube with 1 mL each of methanolysis solution prepared in the previous step and transfer them to the glass tube. Add 2 mL of chloroform to each glass tube, vortex to mix properly, and close tightly with appropriate cap. Do not use rubber caps, since the solvent and acid will deteriorate this material.
- Incubate samples for 3.5 h at 110 °C using a dry heater block. For homogenization, gently invert the tube 2–3 times every 30 min.
- After cooling, add 1 mL of distilled water and mix by vortex during 60 s. After phase partition (about 30 min), remove the upper aqueous phase and dry the organic phase containing the resulting 3-hydroxybutyric methyl ester (Me−3HB) with solid Na_2SO_4 enough to not observe water droplets.
- Filter the dried organic phase using a 1 mL tip filled with a small piece of cotton.
- Analyze the ester content by GC chromatograph with a CP-Sil-5 CB column (10 m × 0.53 mm ID) or similar column, injecting 1 μL of the samples in a split ratio of 1:40. Nitrogen and argon can be normally used as carrier gas at a flow rate of 0.9 mL min^{-1}. In our laboratory, we have employed the following parameters: injector temperature at 250 °C and the detector at 275 °C. The oven temperature is programmed to initial temperature at 50 °C for 2 min, then from 50 °C up to 110 °C at a rate of 20 °C min^{-1}, and finally up to 250 °C at a rate of 20 °C min^{-1}. Chromatography parameters may vary due to the type and dimensions of the column. An initial optimization can be necessary to adjust the parameters for better resolution of peaks.
- Methyl-3-hydroxybutyrate (Me−3HB) amount in mg is determined by using the area of the corresponding Me−3HB peak related to the internal standard methyl ester from the benzoic acid (Me-3B). The retention time of Me−3HB and Me-3B can be determined using 1 mg of a 3-hydroxybutyric acid standard or 10 mg of a standard PHB sample in a methanolysis reaction and 1 mg benzoic acid, respectively. A positive reaction using standard PHB is strongly suggested to be performed in every set of samples, since it is useful to verify if methanolysis and/or GC apparatus are working properly.
- The internal response factor (IRF) of the internal standard must be determined in order to quantity Me−3HB. To determine the IRF, known amounts of internal standard (benzoic acid) and the analyte (the 3-hydroxybutyric acid or PHB) are

added in a methanolysis reaction. The IRF is calculated by the following equation:

$$\mathrm{IRF} = \mathrm{area}_{\mathrm{Me-Benzoic}} \times \mathrm{amount}_{\mathrm{3HB}} / \mathrm{amount}_{\mathrm{Benzoic}} \times \mathrm{area}_{\mathrm{Me-3HB}}$$

$\mathrm{area}_{\mathrm{Me-Benzoic}}$ = peak area of methyl benzoate.
$\mathrm{amount}_{\mathrm{3HB}}$ = amount (mg) of 3-hydroxybutyric acid added in the reaction.
$\mathrm{amount}_{\mathrm{Benzoic}}$ = amount (mg) of benzoic acid added in the reaction.
$\mathrm{area}_{\mathrm{Me-3HB}}$ = peak area of methyl 3-hydroxybutyrate.

Then, calculate the amount of the Me–3HB (in mg) in the samples using the following equation:

$$\mathrm{Me-3HB} = \mathrm{amount}_{\mathrm{Benzoic}} \times \mathrm{area}_{\mathrm{Me-3HB}} \times \mathrm{IRF} / \mathrm{area}_{\mathrm{Me-Benzoic}}$$

It is worthy to notice that the amount of Me–3HB measured corresponds to the injected volume (1 μL). To calculate the total Me–3HB in the sample, this value should be corrected to the total volume of chloroform phase, specifically 2 mL.

The PHB amount in each sample must be normalized by the weight of the lyophilized bacteria before the methanolysis. The result is expressed as % of PHB/dry cell weight (dcw).

13.2.3 Quantification of PHB in Whole Cells by HPLC

HPLC technique can also be applied to quantify the PHB content in bacterial cultures (Karr et al. 1983). This methodology is based on quantification of crotonic acid produced by PHB hydrolysis in concentrate H_2SO_4, which converts 3-hydroxybutyric acid into crotonic acid. An UV detector is normally used for detection.

Cell growth and harvesting conditions, following by lyophilization as described in the previous protocol, are also performed in this protocol.

- After lyophilization, transfer the dry cell pellet to a borosilicate glass tube with screw cap. Crush the pellet against the glass wall with a metal spatula and add 1 mL of concentrate H_2SO_4.
- Incubate the tubes for 30 min at 90 °C in a dry heater block.
- Cool the tubes on ice and add 4 mL of 7 mM H_2SO_4 and mix vortexing. Before analysis by HPLC, additionally dilute all the samples 5- to 100-fold with 7 mM H_2SO_4 containing 0.8 mg of adipic acid per mL as internal standard. It is strongly suggested to filter the samples through a 0.45 μm HAWP membrane filter to remove particulate material.
- Inject volumes ranged from 10 to 50 μL or sample concentrations from 0.2 to 560 μg mL^{-1} crotonic acid. These concentrations are suggested based on the original protocol (Karr et al. 1983). However, it is recommended that a standard curve be performed with known concentrations of crotonic acid in order to determine the limits of detection and quantification, since they may vary from one laboratory to another.

– Elute the samples with 7 mM H_2SO_4 at a flow rate of 0.7 mL min^{-1} from an Aminex HPX-87H ion exclusion organic acid analysis column (300 × 7.8 mm) or similar column for separation of organic acids. The use of an ion-exclusion pre-column is suggested to avoid clogging.
– Absorbance of crotonic acid is measured at 210 nm. Calculate the amount of crotonic acid produced from PHB in the samples from the regression equation derived from a standard curve obtained with crotonic acid standard solutions. The standard curve is constructed correlating the peak area of crotonic acid standard by concentrations.

13.2.4 Spectrophotometric Quantification of PHB

This protocol is also based on the conversion of 3-hydroxybutyric acid to crotonic acid, which can be monitored by UV absorption (Law and Slepecky 1961). However, this protocol requires PHB extraction and purification prior to quantification.

– Harvest cells from 10 mL of culture by centrifugation in 15 mL conical Falcon-type tubes (5,000×*g*, 4 °C, 10 min).
– Discard medium and resuspend cells in 10 mL of sodium hypochlorite 10–15 % (w/v).
– Incubate samples at 40 °C for 1 h.
– Centrifuge sample (5,000×*g*, 4 C, 10 min), and wash the insoluble material with 1 volume of water followed by 1 volume of acetone.
– Resuspend the washed material in 2 mL of chloroform, transfer to a screw cap vial, and incubate at 60 °C to complete dissolution.
– Evaporate the solvent by heating at 60 °C.
– Add 2 mL of concentrated H_2SO_4, cap the tubes and incubate at 100 °C for 10 min.
– After cooling, absorbance at 235 nm is measured using a sulfuric acid solution as a blank. The amount of crotonic acid is calculated using the molar extinction coefficient (ε) of 1.55×10^4 (M^{-1} cm^{-1}) (Slepecky and Law 1960). Considering that the ε value for crotonic acid may vary depending on the experimental conditions and the intrinsic characteristics of the spectrophotometer used, it is suggested to construct a standard curve with crotonic acid standard solutions in order to obtain an adequate ε value for the laboratory condition.

13.2.5 Quantification of PHB by Fourier Transform Infrared Spectroscopy

Recently, Kamnev and coworkers (2012) have applied the Fourier transform infrared (FTIR) spectroscopy to determine the PHB content in cultures of *A. brasilense* Sp7 and Sp245. The FTIR has been described to be a sensitive and convenient tool

for analyzing bacterial biomass samples, including PHB (Kamnev et al. 1996, 1997, 2006; Kansiz et al. 2000; Pistorius et al. 2009; Mayet et al. 2010, 2013; Hermelink et al. 2011). Although this technique is very simple and practical, because it requires only dried cells, a specific equipment to collect infrared spectra is imperative. The following protocol was adapted from the information previously described by Kamnev and coworkers (2012).

- Harvest cells by centrifugation using 10 mL of culture in 15 mL conical Falcon-type tube (5,000×*g*, 4 °C, 10 min).
- Discard medium and wash the cell pellet with 1.5 mL of phosphate-buffered saline (pH 7.1).
- Dry cells at room temperature (25 °C) up to constant mass.
- Powder the resulting dry biomass and place 0.5 mg in a sampling cup.
- Record DRIFT (Diffuse Reflectance Infrared Fourier Transform) spectra with a ±4 cm^{-1} resolution in triplicate on a FTIR spectrometer. The methodology described by Kamnev et al. (2012) has not employed KBr in sample preparation.
- The percentage of PHB in the dry biomass samples is determined from the spectra by calculating the intensity ratio of the υ(C═O) band of PHB around 1,730 cm^{-1} to that of the amide II band at *ca*. 1,550 cm^{-1}, which is due mostly to N–H bending vibrations (Thi and Naumann 2007; Hermelink et al. 2011). For calibration, it is suggested to use a recombinant PHB-accumulating *Escherichia coli*, or other PHB-accumulating bacteria as *A. brasilense* Sp7 cultivated in high carbon-to-nitrogen ratio, and correlated the data of FTIR spectra with gas-chromatography or HPLC analysis.

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Chapter 14
Siderophores Production by *Azospirillum*: Biological Importance, Assessing Methods and Biocontrol Activity

Raúl O. Pedraza

Abstract Siderophores are ferric ion-specific chelating compounds produced by bacteria and fungi growing under limited availability of iron. The function of siderophores is to scavenge iron from the environment and to make the mineral available to the microbial cell. Iron is an essential element for the growth of most bacteria because it is a component of enzymes with important roles in electron transfer, RNA synthesis, and resistance to reactive oxygen intermediates, among other biological processes. Different species of plant growth-promoting bacteria produce siderophores which can be a competitive advantage for plant, not only for growth, but also as biocontrol agent against phytopathogens. In this chapter, the siderophores production by *Azospirillum*, biological importance, assessing methods, and biocontrol activity are revised. Additionally, methods to detect and characterize siderophores, based on the universal Chrome Azurol Sulphonate (qualitative and quantitative) assay, as well as chemical analyses, thin layer chromatography coupled with fluorescence spectroscopy, and gas chromatography–mass spectrometry are described.

14.1 Introduction

Siderophores (from the Greek: "iron carriers") are defined as relatively low molecular weight, ferric ion-specific chelating agents produced by bacteria and fungi growing under limited availability of iron (Neilands 1995). Iron is the fourth most abundant element in the earth's crust and in most types of soil it is in excess. This element can exist in aqueous solution in two states: Fe^{2+} and Fe^{3+}; however, Fe^{3+} forms are not readily utilizable by plants and microorganisms because they frequently form insoluble oxides or hydroxides which limit its bioavailability (Desai and Archana 2011; Zuo and Zhang 2011). Siderophores form high-spin, kinetically

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labile chelates with ferric ion which are characterized by exceptional thermodynamic stability. The formation constant for typical molecules containing three bidentate ligands is 10^{30}, or greater. The affinity for gallium is also high, but the attraction for aluminum and for all divalent ions is substantially less. Therefore, the siderophore ligand can be specific for Fe^{3+} among the naturally occurring metal ions of abundance (Neilands 1995).

The function of siderophores is to scavenge iron from the environment and to make the mineral available to the microbial cell (Neilands 1995). However, not all microorganisms have need for iron, and siderophores; for example, some lactic acid bacteria are not stimulated to greater growth with iron as they have no heme enzymes, and the crucial iron-containing ribotide reductase has been replaced with an enzyme using adenosylcobalamin as the radical generator (Reichard 1993). In the other side, it was observed that many uncultured bacteria are chemically dependent on siderophores produced by other microorganisms, which enabled the isolation of rare bacteria (D'Onofrio et al. 2010).

14.2 Siderophores: Biological Importance

Iron is an essential element for the growth of most bacteria because it is a component of enzymes with important roles in electron transfer, RNA synthesis, and resistance to reactive oxygen intermediates (Braun 1997; Cornelis and Matthijs 2002). To maintain growth, bacteria have developed different strategies to obtain iron from the iron-limited environment, but siderophores-mediated iron uptake is probably the most common form of iron acquisition (Braun and Winkelmann 1987). In Gram-negative bacteria, the ferric siderophores are taken up in an energy-coupled process across the outer and cytoplasmic membranes (Braun 1985; Braun et al. 1991). Transport across the outer membrane requires receptor proteins that specifically recognize the diverse ferric siderophores. Outer membrane translocation of the ferric siderophores depends on TonB-ExbB-ExbD, a cytoplasmic membrane-localized complex that transduces energy from the proton motive force to receptors in the outer membrane (Braun 1995; Occhino et al. 1998).

More than 100 enzymes involved in primary and secondary metabolism have iron-containing cofactors, for instance, iron–sulfur cluster or heme groups. The reversible Fe^{2+}/Fe^{3+} redox pair is best suited to catalyze a wide spectrum of redox reactions and to mediate electron chain transfer (Miethke and Marahiel 2007). These enzymes and cofactors participate in diverse processes such as: respiration, activation of oxygen, degradation of hydrogen peroxide and hydroxyl radicals, amino-acid and pyrimidine biosynthesis, the citric acid cycle, DNA synthesis, nitrogen fixation, carbon fixation metabolism, photosynthesis, and oxygen binding (Andrews 1998). Additionally, several transcriptional and posttranscriptional regulators interact with iron to sense its intracellular level or the current status of oxidative stress to efficiently control the expression of a broad array of genes implicated mainly in iron acquisition or in the reactive oxygen species protection (Hantke 2001).

Azospirillum brasilense, a well known PGPB (Plant Growth-Promoting Bacteria) (Bashan and De-Bashan 2010; Reis et al. 2011) has a high necessity for iron because many enzymes implicated in N_2 fixation, including nitrogenase, contain Fe as a cofactor. It has been reported that in *A. brasilense*, four high-molecular mass proteins (87, 83, 78, and 72 kDa) in the outer membrane were induced (Bachhawat and Ghosh 1987a), and a catechol-type siderophore (spirilobacin) was secreted under iron deficiency (Bachhawat and Ghosh 1987b). However, not all the strains of *A. brasilense* reported so far were able to produce siderophores, as it is the case of *A. brasilense* Cd and Az39, two agronomically important strains (Perrig et al. 2007).

It was also found that *A. irakense* ASP-1 was able to degrade desferrioxamine-type siderophores, resulting in the production of various products that can serve as carbon and nitrogen sources (Winkelmann et al. 1999). In this line, the *fhu*E gene of *Escherichia coli* encodes the FhuE protein, which is a receptor protein in the coprogen-mediated siderophore iron-transport system (coprogen is a hydroxamate-type iron chelator, produced by *Neurospora crassa* and by certain *Penicillum* spp.) (Leong and Winkelmann 1998). A *fhu*E gene homologue from *Azospirillum brasilense* was cloned, sequenced, and characterized (Cui et al. 2006). The *A. brasilense fhu*E encodes a protein of 802 amino acids with a predicted molecular weight of about 87 kDa. The deduced amino-acid sequence showed a high level of homology to the sequences of all the known *fhu*E gene products; the *A. brasilense fhu*E mutant was sensitive to iron starvation and defective in coprogen-mediated iron uptake. The mutant failed to express one membrane protein of approximately 78 kDa that was induced by iron starvation in the wild type. However, complementation studies showed that the *A. brasilense fhu*E gene, when present on a low-copy number plasmid, could restore the functions of the mutant. With this, it was demonstrated that FhuE-mediated iron-uptake system exists in *A. brasilense* (Cui et al. 2006).

14.3 Siderophores: Plants and PGPB

Two strategies have been developed by plants to obtain iron from the soil. Strategy I involves acidification of the rhizosphere (the surrounding area of the root) followed by reduction of Fe^{3+} ions by membrane-bound Fe^{3+}-chelate reductase and subsequent uptake of Fe^{2+} into root cells. Strategy II refers to the plants which secrete low molecular weight phytosiderophores in order to solubilize and bind iron which is then transported into root cells via membrane proteins (Altomare and Tringovska 2011; Guerinot 2010). However, regardless these two strategies, extensive research have been addressed to correct chlorosis in plants (iron deficiency) by the application of available iron compounds to the soil and by selective plant breeding to produce Fe-chlorosis-resistant cultivars. In addition, the probable implication of siderophores produced by PGPB has been considered as a potential way to improve plant growth, nodulation, and N_2-fixation in iron-deficient conditions (Fernández-Scavino and Pedraza 2013).

Hence, siderophores can be also a competitive advantage for plant growth. It was reported that specific strains o the *Pseudomonas fluorescens-putida* group enhanced growth and yields when used as seed inoculants via siderophores production (Kloepper et al. 1980). Sharma and Johri (2003) reported that maize seeds inoculated with siderophore-producing pseudomonads positively influenced the crop productivity in calcareous soil system. Also, Pandey et al. (2005) observed that *Pseudomonas aeruginosa* GRC1, a prolific producer of hydroxamate siderophores in iron-deficient conditions enhanced the growth of *Brassica campestris* var Pusa Gold (Indian mustard) in field trials. Recently, Radzki et al. (2013) showed that siderophores produced by *Chryseobacterium* C138 efficiently provided iron to iron-starved tomato plants in hydroponics culture. There are some more examples on the contribution of bacterial siderophores to the plant growth, but at present, there is not information about the direct participation of *Azospirillum* siderophores' as PGPB in iron nutrition. As a particular case, it was reported that the community of endophytic siderophore-producing bacteria associated to rice (*Oryza sativa*) cultivated at field conditions is dynamic and diverse (Loaces et al. 2011). These bacteria were present in grains, roots, and leaves and their density fluctuated between $\log_{10}$ 3.44 and $\log_{10}$ 5.52 cfu g^{-1} fresh weight during the plant growth. Less than 10 % of the heterotrophic bacteria produced siderophores in roots and leaves of young plants, but most of the heterotrophic bacteria were siderophore producers in mature plants. Although siderophores production cannot be discarded as a mechanism that contributes to this constant association, other mechanisms may be involved since different bacteria able to sequester Fe^{3+} alternate in rice tissues (Loaces et al. 2011).

14.4 Methods to Detect and Characterize Siderophores

The detection of siderophores is most readily achieved in iron-limited media. The Chrome Azurol Sulfonate (CAS) assay has become widely used since it is comprehensive and remarkably responsive. It is based on the color change that accompanies the transfer of the ferric ion from its intense blue complex to the siderophore (yellowish-orange). A detergent must be present in order to achieve the intense color (Neilands 1995). Gram-negative bacteria, as in the case of *Azospirillum*, are resistant to detergents and hence the CAS reagent can be incorporated in the agar media. Before starting with the assay, it is important to clean all the glassware to be used to remove iron traces. This can be done with HCl 1 M and then by washing with bidistilled water pH 7.0. The following procedures to detect and characterize siderophores were adjusted for *A. brasilense*, using the NFb growth-culture medium (Tortora et al. 2011).

14.4.1 Chrome Azurol Sulphonate Agar Plate Assay

The method is based on the universal Chrome Azurol Sulphonate (CAS) assay, according to Schwyn and Neilands (1987). This detects color change of CAS-iron complex (from blue to orange) after the iron chelation by siderophores.

14.4.1.1 CAS 10× Preparation

Solution A: Dissolve 0.03025 g CAS (Sigma-Aldrich, USA) in 25 mL distilled water; it forms a reddish liquid. Then, add 50 µL KOH 10 % (w/v) (the liquid becomes a green-bluish color) and 5 mL $FeCl_3 \cdot 6H_2O$ 1 mM (dissolved in HCl 10 mM). Then add another 50 µL KOH 10 % (w/v), until the solution becomes a green-bluish color again.

Solution B: Dissolve 0.03645 g hexadecyltrimethyl ammonium bromide (HDTMA; Sigma-Aldrich, USA) in 20 mL distilled water pH 7.0 and mix until complete dissolution of solids (foam can be formed), stirring at 70 °C.

Then, add slowly Solution A to Solution B and mix (it becomes a reddish color again). Add about 600 µL KOH 10 % (w/v) (until it becomes green-bluish) and sterilize at flowing steam during 20 min.

Growth-culture medium: Prepare NFb liquid culture (150 mL) without Fe-EDTA and with the addition of 1 g L^{-1} NH_4Cl; adjust to pH 6.8 with KOH (Baldani and Döbereiner 1980). Then, add 4.575 g 1.4-piperazine diethane sulfonic acid (PIPES; Sigma-Aldrich, USA) to 120 mL of NFb. To dissolve PIPES, add KOH (pure powder) until pH 6.8 of the mixture and complete dissolution of PIPES. Finally, add NFb medium until final volume of 135 mL and add 2.3 g agar; autoclave at 121 °C for 20 min.

Agar-CAS preparation: Add 15 mL of CAS 10× solution to 135 mL of NFb medium with PIPES (after a short period from autoclaving) and mix. In case of needing to restore the green-bluish color, add about 50 µL KOH 5 % (w/v); then pour into sterile Petri dishes (about 20 mL) and cool down in sterile laminar flow chamber. Then keep them in refrigerator until use.

Azospirillum-siderophores assay: Spot inoculate the Agar-CAS medium with 10 µL of each bacterial strain and incubate 2–7 days at 30 °C. For the spot inoculation, bacteria are previously grown overnight in NFb liquid medium at 30 °C. Then, cells are collected by centrifuging at 8,000 × g 10 min; the pellet is washed three times with sterile bidistilled water, and the cell concentration is adjusted to 10^6 CFU mL^{-1} ($OD_{560} = 0.2$). Positive results are indicated by the formation of an orange halo around the colonies.

The different steps involved in the CAS-agar plate assay for *Azospirillum* are shown in Fig. 14.1. This assay can be performed by triplicate and determine the yield of siderophores production (%Ys) as [(halo diameter—colony diameter)/colony diameter) × 100].

14.4.2 Chrome Azurol Sulphonate Liquid Assay

The CAS solution prepared in 4.1 can be used to quantify siderophores activity in culture supernatant extracts by measuring the decrease in the absorbance of blue color at 630 nm (Schwyn and Neilands 1987). For that, mix 6 mL of HDTMA

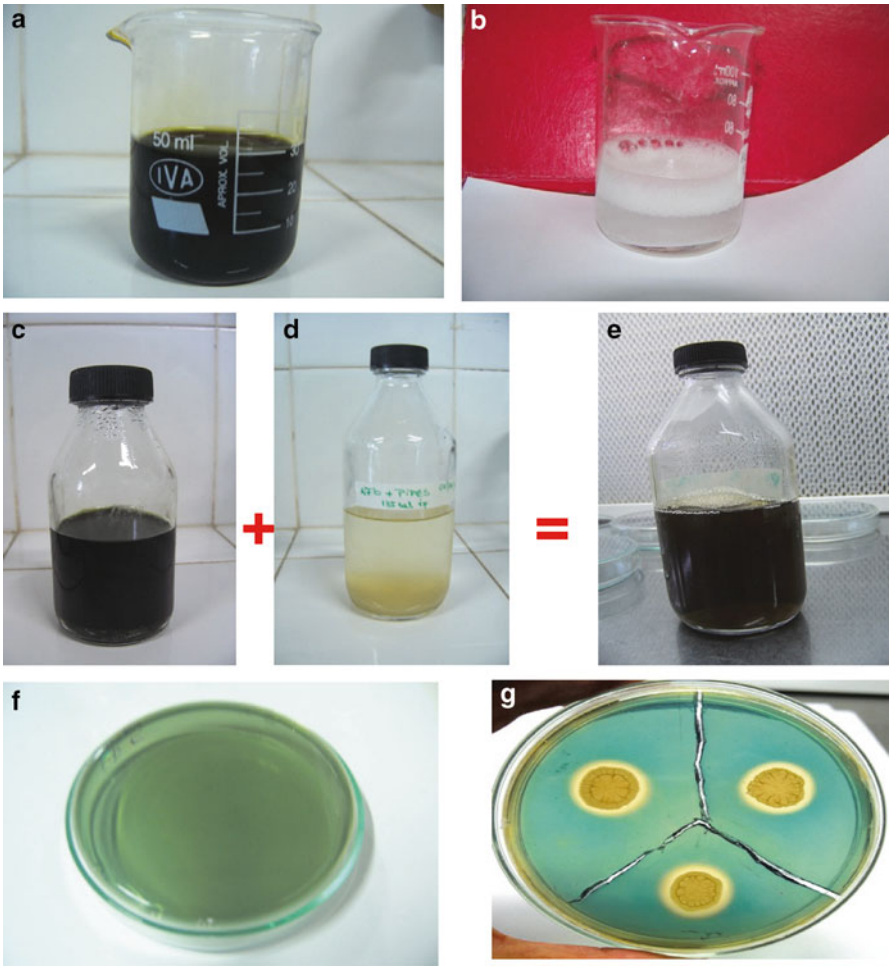

Fig. 14.1 Different steps of the CAS assay to determine siderophores production in *Azospirillum*. (**a**) Solution A, (**b**) Solution B, (**c**) CAS 10× solution, (**d**) NFb medium with PIPES, (**e**) mixture of CAS 10× solution and NFb medium, (**f**) Petri dish containing agar-CAS medium, (**g**) *A. brasilense* colonies showing siderophores production

10 mM diluted with water and mixed with 1.5 mL of Fe^{3+} solution (1 mM $FeCl_3 \cdot 6H_2O$ in HCl 10 mM) and 7.5 mL 2 mM aqueous CAS solution under stirring conditions. Separately, dissolve 4.307 g of PIPES in water and add 6.75 mL of 12 M HCl. Then, mix slowly this buffer solution (pH 5.6) with CAS solution and complete up to 100 mL with bidistilled water. A solution of 5-sulfosalicylic acid 0.2 M (Sigma-Aldrich, USA) has to be prepared to use as CAS shuttle solution to facilitate transfer of iron from the CAS complex to bacterial siderophores.

For quantitative assay, 150 µL of an overnight culture of *Azospirillum* in NFb liquid medium is adjusted to a final concentration of 10^6 CFU mL^{-1} and inoculated in

150 mL of the same medium and incubated 7 days at 30 °C. To increase the sensitivity of the CAS assay, the cell-free medium is concentrated 30-fold by lyophilization, resuspended in 5 mL of distilled water and desalted by using a Sephadex G-25 column (Sigma-Aldrich, USA) (Tortora et al. 2011).

The fraction containing siderophores is added to CAS assay solution (1:1) and mixed with shuttle solution (100:1). After 1 h at room temperature the absorbance ($A_{630\ nm}$) is measured. The percentage of siderophore units is estimated as the proportion of CAS color shift using the formula $[(A_r - A_s)/A_r] \times 100$, where A_r is the $A_{630\ nm}$ of the reference sample (medium plus CAS assay solution plus shuttle solution) and *As* is the $A_{630\ nm}$ of the sample (supernatant plus CAS assay solution plus shuttle solution).

14.4.3 Chemical Assays to Characterize Siderophores

Although siderophores vary greatly in chemical structure they are classified into two main groups: catechol and hydroxamate, according to the chemical group involved in iron Fe^{3+} chelation.

The chemical nature of siderophores can be investigated by analyzing the absorption spectra in a spectrophotometer. A peak between 420 and 450 nm and another at 495 nm after the addition of 1 mL of 2 % aqueous $FeCl_3$ to 1 mL of cell-free culture filtrate indicates the presence of ferric hydroxamates and ferric catecholate, respectively (Neilands 1981). The presence of catechol and hydroxamate type phenolates are assayed on ethyl acetate extracts of the culture supernatants. For that, the extracts are prepared by extracting 150 mL of supernatant twice with an equal volume of solvent at pH 2.0.

Catechol siderophores can also be assayed according to Arnow (1937). Briefly, 1 mL of each bacterial supernatant is mixed with 1 mL of HCl 0.5 N and 1 mL of nitrite molybdate reagent (10 g $NaNO_2$ and 10 g $NaMoO_4$, dissolved in 100 mL of distilled H_2O), and then mixed with 1 mL NaOH 1 N. After 15 min at room temperature the absorbance ($A_{510\ nm}$) is measured.

Hydroxamate siderophores can also be analyzed in culture supernatants according to the ferric perchlorate assay (Atkin et al. 1970). Catechin and hydroxylamine hydrochloride may be used as the standards. Perform each assay at least by triplicate.

For further analyses, the fraction containing siderophores is prepared by extraction of acidified (pH 2.0) supernatants with an equal volume of ethyl acetate; the solvent is eliminated by vacuum rotary evaporator at 37 °C and the residue is then resuspended in methanol for TLC analysis and in ethyl acetate for Gas chromatography–mass spectrometry (GC-MS) assay (explained below).

14.4.4 Thin Layer Chromatography (TLC) Coupled with Fluorescence Spectroscopy

The methanol-soluble siderophores fraction is analyzed by TLC using silica gel plates (e.g., Merck, $SiO_2 \cdot H_2O$ 20; 20 cm; G 60 F_{254nm}), and a mobile phase consisting in a solvent mixture of butanol/acetic acid/water [4: 0.5: 5.5 (v/v/v)]. The plate is dried at

room temperature and observed under long-wave UV light (365 nm) and short-wave UV (254 nm). For instance, salicylic acid PA grade or any other compound of reference can be used as standard. Then, bands with the same Rf as the standard can be scrapped out from the plate, dissolved in methanol 100 % (v/v), centrifuged, and concentrated by lyophilization. The residue is resuspended in methanol 20 % (v/v) and analyzed by fluorescence spectroscopy. Samples' emission spectra are evaluated at λ excitation of 296 nm.

14.4.5 Gas Chromatography–Mass Spectrometry

Following the method employed by Tortora et al. (2011), to confirm the presence of salicylic acid (SA) in bacterial supernatants, cultures were analyzed by GC-MS using a Thermo Electron equipment Polaris Q model associated to an electron-impact ionization source and an Ion Trap Mass Analyzer (Thermo Finnigan, USA). Separations were carried out on a DB-5 column (Agilent JW, USA) using He as carrier gas at 0.3 mL min^{-1} and an injection volume of 0.1 μL. The ion source is maintained at 200 °C; the GC oven is programmed with a temperature gradient starting at 50 °C for 1 min to 350 °C at 10° min^{-1}. Then MS analysis is carried out in the electron-impact mode at an ionizing potential of 70 eV. SA PA grade (1 μg mL^{-1}) was used as standard.

14.5 Siderophores of *Azospirillum* in the Biocontrol Activity Against Pathogens

Bacterial siderophores production is a biocontrol mechanism that has been barely studied in *Azospirillum* (Bachhawaat and Ghogh 1989; Saxena et al. 1986; Shah et al. 1992; Tapia-Hernández et al. 1990). Saxena et al. (1986) and Shah et al. (1992) reported the production of salicylic acid (SA) among siderophores produced by *Azospirillum lipoferum* in iron-starved conditions. SA besides being a compound with siderophore activity (Meyer et al. 1992; Visca et al. 1993) is a precursor in the biosynthesis of microbial catechol-type siderophores, such as yersiniabactin, pyoverdin, and pyochelin (Cox et al. 1981; Jones et al. 2007; Serino et al. 1995). Moreover, it was verified to play a central role as an endogenous regulator of localized and systemic acquired resistance against pathogen infection in many plants (Delaney et al. 1994). Therefore, SA-producing strains may increase defense mechanisms in plants. It was hypothesized that bacterial SA excreted to the medium was recognized by plant roots inducing signals for systemic resistance (Buysens et al. 1996; De Meyer and Höfte 1997; Maurhofer et al. 1998), although in some interactions, SA seems to be not the primary signal for ISR induction (Press et al. 1997) but other siderophores could be implicated (Leeman et al. 1996; Siddiqui and Shaukat 2005).

According to this information, Tortora et al. (2011) showed that under iron-limiting conditions, different strains of *A. brasilense* can produce siderophores, exhibiting different yields and rates of production according to their origin (isolated from root surface or inner tissues of stolons and roots). Chemical assays revealed that strains REC2 and REC3 secrete catechol-type siderophores, including salicylic acid, detected by thin layer chromatography coupled with fluorescence spectroscopy and gas chromatography–mass spectrometry analysis. Siderophores produced by them showed in vitro antifungal activity against *Colletotrichum acutatum* M11, the causal agent of anthracnose disease in strawberry plants. Furthermore, this latter coincided with results obtained from phytopathological tests carried out *in planta*, where a reduction of anthracnose symptoms on strawberry plants previously inoculated with *A. brasilense* was observed (Tortora et al. 2011). These results imply that some strains of *A. brasilense* could act as biocontrol agent, besides promoting plant growth (Pedraza et al. 2007, 2010; Tortora et al. 2011). Furthermore, Tortora et al. (2012) provided evidences that endophytic root colonization of strawberry plants with *A. brasilense* REC3 strain conferred systemic protection against *C. acutatum* M11 by the direct activation of some plant defense reactions, and also primed the plant for stronger elicitation of these defense responses following infection. The defense mechanisms induced by *A. brasilense* REC3 included the reinforcement of plant cell wall by increasing the content of total phenolic compounds and callose depositions, and the activation of a SA-mediated signaling pathway that concluded in the overexpression of defense-related genes, such as those encoding pathogenesis-related proteins like PR1, chitinases and glucanase. Based on these outcomes, it is proposed that the activation of systemic mechanisms for disease suppression together with the plant growth-promoting effect exerted by *A. brasilense* REC3 strain (Pedraza et al. 2010) and its capacity to inhibit in vitro fungal growth, via siderophores, could explain, in part, the increase of strawberry plants tolerance to anthracnose disease caused by *C. acutatum* M11. On these assumptions, some strains of *A. brasilense* could be considered as Biocontrol PGPB and raises the opportunity of using them as an alternative in the strawberry anthracnose disease management. However, further studies are necessary to obtain practical information for extending the spectrum of potential biocontrol strains of *A. brasilense* on different phytopathogen agents and agronomical important crops.

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Chapter 15
Cell–Cell Communication in *Azospirillum* and Related PGPR

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Abstract Quorum-sensing (QS) regulation based on *N*-acyl-L-homoserine lactones (AHL) is used to control various phenotypes that are often essential for interaction with a eukaryotic host, notably for plant-associated bacteria. Technical methodologies currently used to reveal AHL production in a specific strain and to decipher phenotypes under QS regulation are surveyed in this chapter. Analyses conducted on the genus *Azospirillum* and on other related PGPR are used to illustrate the different steps of the approach. Among the genus *Azospirillum*, a survey of 40 strains belonging to six species revealed AHL production for four strains belonging to the *lipoferum* species or to a close undefined species and isolated from a rice rhizosphere. Identification of genes mediating QS and regulating functions indicate that (1) distinct QS networks are present in some strains and seem to have been acquired independently by horizontal gene transfer; (2) QS regulation is strain specific with several phenotypes and numerous proteins being regulated by AHL-based QS in *A. lipoferum* B518, whereas no change is observed in *A. lipoferum* TVV3 deficient in AHL production; and (3) QS is dedicated to regulating functions linked to rhizosphere competence and adaptation to plant roots in *A. lipoferum* B518.

15.1 Introduction

Diverse bacteria, especially bacteria interacting with eukaryotes, possess the ability to regulate functions in a cell-density manner. In proteobacteria, this regulation involves the synthesis of signal compounds termed autoinducers, usually belonging to the family of *N*-acyl-L-homoserine lactones (AHLs) that differ in the length (from 4 to 18 carbons), saturation and substitution characteristics of the acyl chain (Fig. 15.1). The first AHL to be characterized was that of *Vibrio fischeri*, a bioluminescent marine bacterium living in symbiosis with the squid *Euprymna scolopes*, emitting bioluminescence only at high cell density; regulation of bioluminescence was shown to rely on a bacterial extracellular compound accumulating during growth

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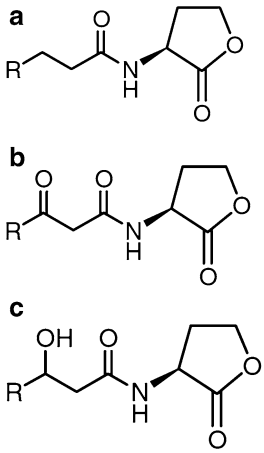

Fig. 15.1 General structures of *N*-acyl-L-homoserine lactones. (**a**) *N*-Acyl-L-homoserine lactone; (**b**) *N*-(3-oxoacyl)-L-homoserine lactone; (**c**) *N*-(3-hydroxyacyl)-L-homoserine lactone. R ranges from CH_3 to $(CH_2)_{14}CH_3$. All natural AHLs reported until now are characterized by a lactone ring that is *N*-acylated with a fatty acyl group at the α-position 1. The acyl chains have various lengths, saturation levels, and, in most cases, even number of carbons (from 4 to 18)

identified as *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C_6-HSL) (Eberhard et al. 1981). It was not until 1992 that production of 3-oxo-C_6-HSL was detected in other bacterial species, particularly in *Erwinia carotovora*, where the signal molecule regulates the biosynthesis of carbapenem, an antibiotic belonging to the β-lactams family (Bainton et al. 1992). Then the term "quorum sensing" (QS) was coined to describe this phenomenon that allows bacteria to assess their population density via the production of small molecule sensors ("sensing") and to initiate a coordinated response when a certain cell density is reached ("quorum") (Fuqua et al. 1994).

AHLs synthesis is based on the presence of one or more bacterial genes encoding AHLs synthases. Of the three families of AHLs synthases, the most common gathers LuxI-like proteins (the first member of this family being identified in *V. fischeri*) (Fuqua et al. 1994), the second family includes LuxM-type proteins identified in several species of *Vibrio*, and the third is represented by the enzyme HdtS identified in *Pseudomonas fluorescens* (Laue et al. 2000). LuxI-type proteins synthesize AHLs by catalyzing the amide bond between the fatty acid chain carried by a carrier protein of an acyl group (ACP) and the amino group of *S*-adenosyl methionine. Lactonization of the molecule then takes place with the release of 5′-methylthioadenosine. AHLs diffuse through cell wall and accumulate in the extracellular medium. When a threshold concentration is reached, AHLs bind to a receptor that is a transcriptional regulator of the LuxR family; the LuxR/AHL complex binds upstream target genes at sequences designated "lux" boxes and activates (or in some cases represses) target genes, such as the *lux* operon responsible for bioluminescence in *V. fischeri*. In many cases, the *luxI* gene is one of the targets of the LuxR/AHL complex, leading to a positive feedback loop of regulation.

QS regulation based on AHLs is used to control various phenotypes that are often essential for interaction with a eukaryotic host (Williams et al. 2007). Among plant-associated bacteria, QS regulates production of virulence factors (*Erwinia*, *Pantoea*), production of antifungal compounds (*Pseudomonas*), conjugative transfer of plasmids (*Agrobacterium*, *Rhizobium*), efficacy of nodulation (*Rhizobium*), etc.… (Barnard et al. 2007; White and Winans 2007; Sanchez-Contreras et al. 2007; Pierson and Pierson 2007).

This chapter aims at presenting the technical methodologies currently used to highlight autoinducer production in a specific strain and to decipher phenotypes that are under QS regulation. Analyses conducted by our team on the genus *Azospirillum* will be used to illustrate the different steps of the approach.

15.2 Technical Methodologies and Recent Developments on Quorum Sensing

15.2.1 Detection and Characterization of N-Acyl-homoserine Lactones (AHLs) Signalling Molecules

15.2.1.1 Detection of AHLs

The discovery that numerous unrelated bacteria have the ability to produce AHL molecules in response to a high cell density has been achieved by using bacterial biosensors (Steindler and Venturi 2007). These biosensors have a functional regulator protein (LuxR homologue) but lack the AHL synthase enzyme and consequently do not produce AHLs. Promoter activity of a gene or operon under QS regulation can be induced by the presence of exogenous AHLs. These biosensors have easily detectable phenotypes such as production of pigments, expression of β-galactosidase activity, light emission, or fluorescence (Table 15.1). Biosensors are either derivative of strains that naturally produce AHLs, or are entirely engineered plasmids that can be used in several bacterial backgrounds.

Among derivatives of natural strains, two are easy to implement and are widely used for AHL detection. The first, *Chromobacterium violaceum* CVO26, harbors a transposon into the *cviI* AHL-synthase gene; exposure of this strain to exogenous AHLs able to interact with CviR (the cognate LuxR) results in production of the pigment violacein. This biosensor is able to detect rather short acyl chain AHLs (4–8 carbons), the most active AHL being C_6-HSL, the natural *C. violaceum* AHL (McClean et al. 1997). It is noteworthy that *C. violaceum* CVO26 can also be used to detect long acyl chain AHLs or QS antagonists, by including C_6-HSL in the bioassay reaction mixture and screening for the inhibition of pigment production (McLean et al. 2004). The second is a derivative *Agrobacterium tumefaciens* strain NT1(pZLR4) that no longer produces its own signal molecule; this strain harbors a *traG-lacZ* fusion plasmid that can be induced when a cognate AHL is recognized by TraR. This second biosensor has the ability to detect a wide range of AHLs, including 3-oxo-HSLs and 3-hydroxy-HSLs (Luo et al. 2003).

Table 15.1 Biosensors commonly used for AHL detection

Strain or plasmid	Range of AHLs detected*	Phenotype triggered by AHL	Targeted LuxR and origin of LuxR	Reference
Chromobacterium violaceum CVO26	C_4 to C_8, oxo-C_4 to oxo-C_8	Production of violacein	CviR, *C. violaceum*	McClean et al. (1997)
Agrobacterium tumefaciens NTL1(pZLR4)	Most AHLs, except C_4	β-Galactosidase	TraR, *Agrobacterium tumefaciens*	Luo et al. (2003)
Agrobacterium tumefaciens KYC55(pJZ372)(pJZ384)(pJZ410)	Most AHLs, except C_4	β-Galactosidase	TraR, *Agrobacterium tumefaciens*	Zhu et al. (2003)
Agrobacterium tumefaciens (pAHL-Ice)	C_8 to C_{14}	Ice nucleation activity	TraR, *Agrobacterium tumefaciens*	DeAngelis et al. (2007)
Sinorhizobium meliloti Rm41 *sinI*::*lacZ*(pJNSinR)	12–16 carbons, including AHLs with an oxo substituent at C-3	β-Galactosidase	SinR, *Sinorhizobium meliloti*	Llamas et al. (2004)
pSB401	C_6 to C_{12}, oxo-C_6 to oxo-C_{14}	Luminescence	LuxR, *Vibrio fischeri*	Winson et al. (1998)
pSB403	C_6 to C_{12}, oxo-C_6 to oxo-C_{14}	Luminescence	LuxR, *Vibrio fischeri*	Winson et al. (1998)
pSB406	C_4 to C_{12}	Luminescence	RhlR, *Pseudomonas aeruginosa*	Winson et al. (1998)
pSB1075	C_{10} to C_{12}, oxo-C_{10} to oxo-C_{14}	Luminescence	LasR, *Pseudomonas aeruginosa*	Winson et al. (1998)
pAS-C8	C_6 to C_{10}, oxo-C_{10} to oxo-C_{12}	Fluorescence	CepR, *Burkholderia cepacia*	Riedel et al. (2001)
pKR-C12	C_{10} to C_{12}, oxo-C_{10} to oxo-C_{14}	Fluorescence	LasR, *Pseudomonas aeruginosa*	Riedel et al. (2001)
pJBA132	C_6 to C_8	Fluorescence	LuxR, *Vibrio fischeri*	Andersen et al. (2001)
pMHLAS	C_{10} to C_{12}	Fluorescence	LasR, *Pseudomonas aeruginosa*	Hentzer et al. (2002)
pSCR1	C_8 to C_{12}	β-Galactosidase	CepR, *Burkholderia cepacia*	Aguilar et al. (2003)
pMULTIAHLPROM	C_4 to C_{12}	β-Galactosidase	LuxR homolog of the host strain	Steindler et al. (2008)
RP4lux	C_6 to C_8, oxo-C_6 to oxo-C_8	Fluorescence	LuxR, *Vibrio fischeri*	Lumjiaktase et al. (2010)
RP4las	C_6 to C_{12}, oxo-C_6 to oxo-C_{12}	Fluorescence	LasR, *Pseudomonas aeruginosa*	Lumjiaktase et al. (2010)
RP4cep	C_8 to C_{12}, oxo-C_8 to oxo-C_{12}	Fluorescence	CepR, *Burkholderia cepacia*	Lumjiaktase et al. (2010)

*The numbers refer to the number of carbons contained in the acyl chain. In most cases, no data is provided about the sensitivity of the biosensor to 3-hydroxy-L-homoserine lactones, as these AHLs are not commercially available

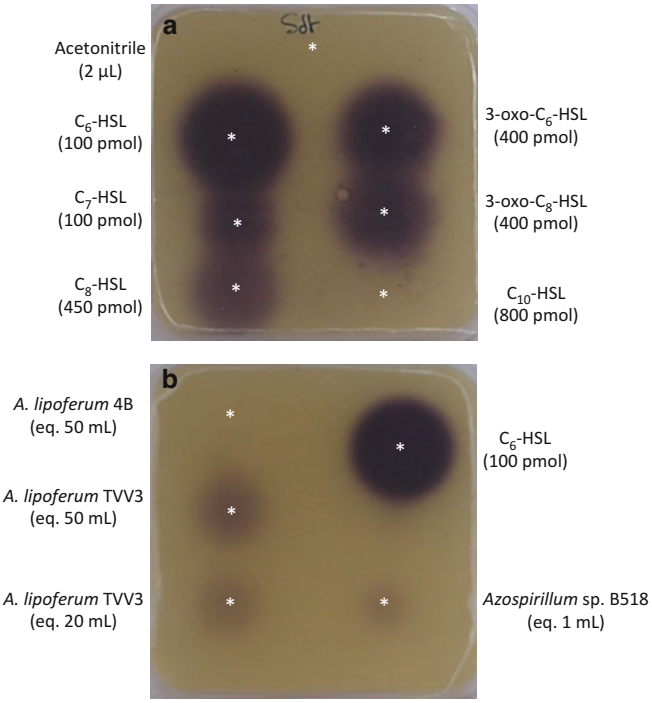

Fig. 15.2 Use of the biosensor *Chromobacterium violaceum* CVO26 to detect AHLs spotted on a plate assay. (**a**) Detection of synthetic AHLs standards showing the sensitivity of this biosensor to various AHLs; (**b**) Detection of AHLs present in extracts obtained from supernatants of some *Azospirillum* strains. *White stars* show the location of spotting. Note the quantity of extracts needed to trigger a positive response of the biosensor, that is correlated to the nature and quantity of AHLs produced by each strain. LC-MS and quantification using TLC revealed that *Azospirillum lipoferum* TVV3 produces 3-oxo-C_8-HSL (5 pM), C_8-HSL (1 nM), 3-oxo-C_{10}-HSL, 3-OH-C_{10}-HSL, and C_{10}-HSL (20 nM), whereas *A. lipoferum* B518 produced 3-oxo-C_6-HSL (50 nM), C_6-HSL, 3-oxo-C_8-HSL, 3-OH-C_8-HSL, and C_8-HSL (500 nM) (Vial et al. 2006). Consequently, only a tiny amount of *A. lipoferum* B518 extract that is enriched in C_8-HSL is needed to activate CVO26

These biosensors can be used in several ways: (1) by spotting a colony to be tested near a biosensor, i.e., cross-streak assay, that requires that both the sensor and the tested strain can grow on the same medium suitable for AHL detection; (2) by spotting a culture supernatant or an extract of supernatant onto a plate and overlaying with a sensor strain grown in the adequate medium (Fig. 15.2); and (3) by overlaying a thin layer chromatography where samples have previously been separated (see Sect. 15.2.1.3). After overnight incubation, a colored zone around the site of the spot will appear due to the presence of AHLs. In the case of a quantifiable phenotype (β-galactosidase activity, light emission), the biosensor can be incubated with the extract in liquid culture and the activity of the activated promoter can be measured.

As one biosensor can only detect a narrow range of AHLs with different sensitivities, more than one type of biosensors is generally needed to test a spectrum of

AHLs from one bacterium. Sensitivity of the biosensor can be affected by the medium used (Llamas et al. 2004). For plasmid-based biosensors, the range of AHLs detected may depend on the bacterial strain hosting the plasmid (Riedel et al. 2001; Lumjiaktase et al. 2010).

Biosensors are a convenient, fast, and effective tool for detecting the presence of AHLs. However, they cannot determine the structure, the number, and the concentration of AHLs, and most AHL-producing bacteria produce a set of AHLs in various proportions (Lithgow et al. 2001; Vial et al. 2006). In addition, biosensors are not always effective when used directly with a strain to be tested; indeed, several bacterial strains have been shown to produce AHL molecules, while normal biosensors yield negative results (Laue et al. 2000).

Using two different biosensors in a cross-streak assay, 40 *Azospirillum* strains were tested for their ability to synthesize AHLs. AHL production was detected for four strains belonging to the *lipoferum* species (TVV3, B52, B518) and to a closely related species (B510), all isolated from the rice rhizosphere (Vial et al. 2006).

15.2.1.2 Extractions of AHLs

In order to elucidate their structure, AHLs have to be isolated and purified from other molecules. To obtain enough material to perform characterization studies, signal molecules should be extracted from bacterial culture supernatants that contain numerous other components. Stationary cell growth phase is recommended for extraction as AHL accumulation is usually maximal at this growth stage. It is important to monitor the pH of the culture fluid as the lactone ring hydrolyzes at pH above 8; acidification of the culture fluid might be contemplated before extraction (Vial et al. 2006). Liquid–liquid extraction is the most common method used to isolate AHLs, but more recently the use of solid-phase extraction has been reported.

Liquid–liquid extraction uses organic solvents as extraction reagents. The solvents commonly used include dichloromethane, chloroform, acidified ethyl acetate (0.1 mL glacial acetic acid per liter of ethyl acetate), ethyl ether, and hexane. Previous studies show that the first two solvents produce the best extraction results with nearly the same yields, and that the extraction yield decreases with the polarity of the extracted AHLs (Morin et al. 2003). Samples are usually extracted twice with half or one third a volume of dichloromethane (Winson et al. 1995). The two organic phases are pooled and evaporated to dryness using a Rotavapor. The residue is then dissolved in a small volume of acetonitrile (usually 1/1,000th of the initial volume) and stored at −20 °C until analysis.

Use of solid-phase extraction has also been reported for AHLs (Frommberger et al. 2005; Schupp et al. 2005; Li et al. 2006), in combination or in replacement of liquid–liquid extraction. An improvement of the sensitivity by twofold to tenfold was reported compared with liquid–liquid extraction (Li et al. 2006). An optimized protocol has been proposed (Li et al. 2006): the sample (with 25 %, v/v ACN) is applied onto a Mega Bond Elut cartridge, which has previously been conditioned by 2 mL of water and 2 mL of methanol sequentially. The loaded column is then washed with 15/85 (v/v) methanol/water and the analytes are eluted with 25/75

(v/v) hexane/isopropanol. The elute is then dried and lately resuspended in the appropriate solvent for HPLC (high performance liquid chromatography) or UPLC (ultra-high-pressure liquid chromatography). For the washing step, applying methanol to the washing solvent (at a content lower to 15 % (v/v)) was shown to be useful to remove the highly polar solutes present in liquid culture media, especially in complex media like LB or NB. For elution, the presence of hexane favors the recoveries of long side chain AHLs (>C12) while maintaining the recoveries of shorter side chain AHLs (Li et al. 2006).

15.2.1.3 Separation by TLC and Detection

TLC coupled with biosensors produces a straightforward visual index of the AHLs synthetized by the tested bacteria. AHLs extracted from supernatants (see Sect. 15.2.1.2) and standards are loaded on a C_{18} reverse phase TLC plate. Once the solvent of the samples is evaporated, the plate is runned upright in a glass chamber containing a developing liquid solvent (usually a mixture of methanol and water). The AHLs migrate at different rates due to their difference in polarity (correlated to the length of acyl chain and the substitution at C-3). After migration and drying, the TLC plate is overlaid with soft agar containing the biosensor, and incubated overnight at 30 °C (Shaw et al. 1997). The color/light/fluorescence is emitted at the location to which the AHL has migrated onto the plate. The 3-oxo derivatives characteristically produce tailing spots with diffuse edges, whereas the 3-unsubstituted forms produce circular spots with sharp edges. The 3-hydroxy-substituted compounds migrate with the same mobility as their 3-oxo analogues, but the spots do not tail (Shaw et al. 1997). Quantification can be achieved by converting the area of spots into residual concentration of a given AHL via a calibration curve obtained from the standard synthetic AHL.

Although reasonably simple and fast, the TLC-biosensor assay must be used with caution. The signal molecule(s) must be recognized by the biosensor used and must be present at a level detectable by this biosensor. This implies that the absence of a positive response might be due to a concentration of signal molecules below the threshold of sensitivity of the biosensor, or to the presence of a specific acyl chain length or substitution that prevents the detection by the biosensor (Schaefer et al. 2008). It is noteworthy that the medium used for growing the strain to be tested may influence AHL production (Brelles-Marino and Bedmar 2001; Lithgow et al. 2001; Li et al. 2006).

Conversely, compounds present in the supernatant and co-extracted with AHLs can lead to false positive spots (Wisniewski-Dyé et al. 2002). Finally, if TLC provides a good index of the set of AHLs produced by a bacterium, one cannot ascertain the chemical structure of the molecules detected and other more precise and sensitive methods are required to provide definitive confirmation of their presence and allow formal structural confirmation and accurate quantification.

In the case of a low concentration of signalling molecules, an alternative method based on a radioactive incorporation of ^{14}C-carboxy-methionine assay has been developed (Singh et al. 2000). This assay allowed the detection of small amounts of

AHLs in complex matrices such as sputum or biofilms (Singh et al. 2000; Schaefer et al. 2001) or specific long-chain AHL, such as C_{16}-HSL identified in supernatants of *Rhodobacter capsulatus* and *Paracoccus denitrificans* (Schaefer et al. 2002).

15.2.1.4 Structural Identification of AHLs

Most procedures of AHLs characterization have been carried out by using a combination of reverse phase HPLC and mass spectrometry (MS). Because of the very large number of structurally related signalling molecules and the fact that other by-products present in the supernatant are co-extracted, a preliminary separation is indeed required using HPLC. Separation of AHLs by HPLC is performed on a C_8 or C_{18} reverse phase silica column using an isocratic or linear gradient elution of methanol-water or acetonitrile-water as the mobile phase, with a flow of usually 0.4–0.5 mL/min and a monitoring by UV at 200 or 210 nm (the only wavelength where AHLs can be hardly traced). Fractions eluting from the HPLC column with retention times identical to AHL standards or that are able to trigger activation of a biosensor can be rechromatographed; once a single active peak is obtained, it can be further analyzed for structural identification. Alternatively, a single active compound can be obtained via preparative thin-layer chromatography (TLC) and the active spot can be scraped off and extracted with dichloromethane, ethyl acetate, or acetone. After concentration, the active spot can be further analyzed (Shaw et al. 1997; Vial et al. 2006).

Mass spectrometry can make a record of charge/mass ratio of ionized compound. According to the molecular ion peaks and fragments, the molecular mass and structural features can be estimated. MS detects even picomoles of samples and can be combined with HPLC (Winson et al. 1995), gas chromatography (GC) (Zhang et al. 1993; Wagner-Dobler et al. 2005), UPLC (Li et al. 2006; Fekete et al. 2007), nano-LC (Fekete et al. 2007), and capillary zone electrophoresis (CZE-MS) (Frommberger et al. 2005). There are many types of ionization available, the most commonly used for AHLs being (reviewed in Wang et al. 2010) electron ionization (EI-MS) (Bainton et al. 1992), fast atom bombardment (FAB-MS) (Pearson et al. 1995; Winson et al. 1995), chemical ionization (CI-MS) (Gray et al. 1996), electrospray ionization (ESI-MS) (Morin et al. 2003), and atmospheric pressure chemical ionization (APCI-MS) (Vial et al. 2006). For quantification, the m/z 102 ion, specific for the lactone ring and detected with a good signal-to-noise ratio, allows low detection limits even in complex matrix samples (Morin et al. 2003; Ortori et al. 2007).

AHL molecules were structurally identified for two *Azospirillum* strains, using LC-MS (with the APCI mode of ionization on an Agilent Technologies 1100 MSD mass spectrometer): *Azospirillum lipoferum* TVV3 produces 3-oxo-C_8-HSL, C_8-HSL, 3-oxo-C_{10}-HSL, 3-OH-C_{10}-HSL, and C_{10}-HSL, whereas *A. lipoferum* B518 produces 3-oxo-C_6-HSL, C_6-HSL, 3-oxo-C_8-HSL, 3-OH-C_8-HSL, and C_8-HSL (Vial et al. 2006).

The structure of AHLs can be finally determined by nuclear magnetic resonance (NMR); elucidation of the structure of 3-oxo-C_6-HSL, which regulates carbapenem antibiotic production in *E. carotovora*, was achieved mainly by NMR (Bainton et al. 1992).

15.2.2 Identification of Genes Mediating AHL-Based Quorum Sensing

15.2.2.1 Transposon Mutagenesis

Before the availability of complete bacterial genome sequence, several strategies and molecular approaches have been developed to identify genetic determinants mediating AHL-based quorum sensing.

Random transposon mutagenesis of bacterial genomes is a classical genetic screen which allows the detection and characterization of bacterial genes encoding AHLs synthases and transcriptional regulators of the LuxR family. For instance, this strategy was applied on the phytopathogen *Erwinia chrysanthemi* known to produce detectable AHL signals (Hussain et al. 2008). Of the 15,000 Tn5 mutants screened with the biosensor *A. tumefaciens* NT1, transposon mutants showing altered AHL production were selected; sequencing of the regions flanking the transposon revealed insertion of the transposon in a *luxI* homologue, named *expI* (Hussain et al. 2008). However, this approach is time consuming, as it relies on the screening of several thousands of mutants; moreover, it involves searching for a negative phenotype (no activation of a biosensor), an approach often risky compared to a "gain-of-function" approach (Liu et al. 2007).

Searching for genetic determinants regulating the expression of phenotypes known or suspected to be controlled by cell density is often used for identifying *luxI* and *luxR* homologues. *cepI* and *cepR* were identified firstly as regulatory components involved in control of siderophore production in *Burkholderia cepacia* (Lewenza et al. 1999). Briefly, after random transposon mutagenesis, mutants producing more siderophore than the wild-type strain were screened and one revealed to contain an insertion in the *luxR* gene designated *cepR* (Lewenza et al. 1999). Transposon mutagenesis was not attempted on *Azospirillum* strains as a low efficiency of transposition was found in several *A. lipoferum* strains (Bally and Givaudan 1988; our unpublished results). Bacterial genes encoding LuxI and LuxR homologues have sometimes been discovered accidentally; characterization of a transcriptional activator that regulates elastase expression in *Pseudomonas aeruginosa* allowed the discovery of *lasR*, a *luxR* homologue essential for the virulence of this opportunistic pathogen (Gambello et al. 1991)

15.2.2.2 Heterologous Complementation Using a Biosensor

In the case of *Azospirillum*, a "gain-of-function" approach was successfully used to identify the genetic loci involved in AHL production (Vial et al. 2006). This strategy involves the construction of a cosmid library (or sometimes plasmid library) and the complementation of a biosensor strain. In the case of *A. lipoferum* TVV3, a cosmid library was constructed in *E. coli* by inserting DNA fragments of size ranging from 25 to 35 kb. The cosmid library was then transferred by conjugation into the biosensor strain *A. tumefaciens* NTL1 (pZLR4, *traG-lacZ*). After conjugation, if an AHL synthase gene is present in an *A. tumefaciens* clone, the signal molecules may bind

to TraR and activate the transcription of the *traG-lacZ* fusion. Thus, on a medium containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), only AHL-producing clones appear as blue colonies (β-galactosidase activity) on plates. Complete sequencing of the clone of interest is possible, and gene(s) involved in the production of AHL are generally identified by Blast homology search against Genbank database. An alternative to localize the AHL synthase encoding gene is to perform an in vitro transposition of the relevant cosmid (the biosensor is then no longer activated if the transposon insertion is located in the AHL synthase gene); once the transposon mutants of interest are isolated, sequencing of regions flanking the transposon allows the identification of the gene of interest. Thus, an ORF homologous to several genes encoding AHL synthases, and named *alpI* (for *Azospirillum lipoferum*) was detected and characterized in *A. lipoferum* TVV3 (Vial et al. 2006).

To identify the gene(s) responsible for AHL production in plant-associated *Pseudomonas*, similar approaches were undertaken, using other biosensors as recipient for the cosmid libraries, such as *C. violaceum* CV026 (Elasri et al. 2001) or the bioluminescent AHL biosensor pSB403 (Table 15.1) (Steidle et al. 2002). Interestingly, the gene *hdtS* encoding another family of AHL synthases was also revealed using this strategy (Laue et al. 2000). This type of approach has the major limitation of only being able to characterize synthases but not the transcriptional regulator. However, the regulator associated with the synthase is often easily characterized, as in most QS systems, the *luxI and luxR*-type genes are in close genetic proximity. Hence in *A. lipoferum* TVV3, the *alpR* gene encoding a *luxR*-type transcriptional regulator is located adjacently and in the same orientation than the *alpI* gene (Vial et al. 2006). However, it is not a rule, as *traI* and *traR* present on the tumor-inducing plasmid of *A. tumefaciens* are separated by approximatively 100 kb (Fuqua and Winans 1994).

15.2.2.3 Genome Analyses

Detection and characterization of gene mediating AHL-based QS are now greatly facilitated by the availability of complete genome sequences.

In bacterial genomes, LuxI homologues can be identified using homology searches but also by determining proteins matching the InterPro family IPR001690 (autoinducer synthase). AHL synthase proteins range from 190 to 226 amino acids in size and share 4 conserved sequence domains with an average of 37 % identity with each other (Watson et al. 2002). Only eight residues are conserved in the amino terminal portion of the LuxI family proteins (Watson et al. 2002). Identification of LuxR homologues using homology searches can be misleading since the C-terminal DNA-binding domain of LuxR is also present at the C-terminus of a large number of regulators (Whitehead et al. 2001). Indeed, 22 LuxR-like transcriptional regulators can be detected in the genome of *Azospirillum* sp. B510 but only one contains the InterPro domain IPR005143 which corresponds to the *N*-terminal autoinducer-binding domain (Kaneko et al. 2010). LuxR-type proteins (250 amino acids) can be subdivided into two functional domains: the amino-terminal region containing the

AHL-binding domain and the carboxy-terminal region containing a helix-turn-helix DNA-binding domain (Whitehead et al. 2001). LuxR proteins share only 18–25 % identity; however the two functional domains share much higher sequence conservation. Only five residues are completely conserved in all the LuxR homologues (Whitehead et al. 2001).

Recently, the genomes of five *Azospirillum* strains belonging to different species, isolated from various host plants, were sequenced and published: *A. lipoferum* 4B, *Azospirillum* sp. B510, *A. amazonense* Y2, and *A. brasilense* Sp245, CBG497, and Az39 (Kaneko et al. 2010; Sant'Anna et al. 2011; Wisniewski-Dyé et al. 2011, 2012; Rivera et al. 2014). The complete genome sequences of the 3 *A. brasilense* strains and that of *A. lipoferum* 4B reveal no *luxI* homologue. Even if many genes encoding LuxR homologues are present in these genomes, none of them possesses the InterPro domain IPR005143. In agreement with the detection of AHL in *Azospirillum* sp. B510, a gene encoding an AHL synthase (AZL_05890) is located on the 1.4-Mb megaplasmid (Kaneko et al. 2010). A gene encoding a LuxR transcriptional regulator with a *N*-terminal autoinducer-binding domain (AZL_06030) is located on the same replicon than the *luxI* gene but separated by 30 kb (Fig. 15.3).

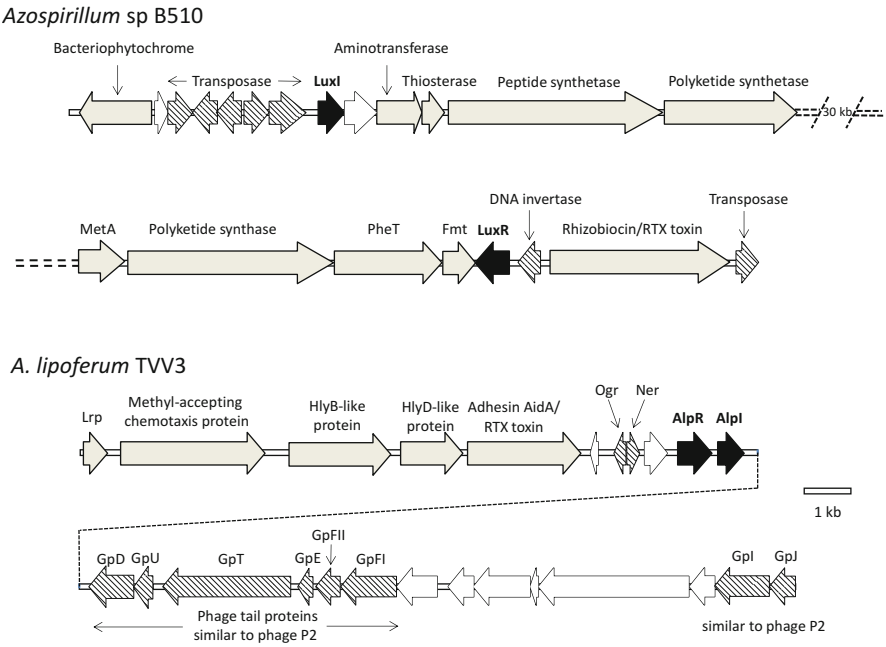

Fig. 15.3 Genetic organization of quorum-sensing genes in *Azospirillum*. The length of each *arrow* represents the relative ORF size and indicates the direction of transcription. The *black arrows* indicate *luxI* and *luxR* genes. The *dashed arrows* indicate ORF sharing sequence homology with transposase in the case of *Azospirillum* sp. B510 and with phage proteins for *A. lipoferum* TVV3. The *gray* and *black arrow* indicate, respectively, ORFs sharing sequence homology with genes in the databases and OFR encoding putative or unknown proteins

Analysis of the *A. amazonense* Y2 genome also revealed the presence of a gene encoding a LuxI homologue (AZOY2_3990004, labelled according to the MaGe annotation platform, http://www.genoscope.cns.fr/agc/microscope/mage/viewer.php?); a gene encoding a potential LuxR (AZOY2_3990005) is adjacent to *luxI* but transcribed in opposite direction (Fig. 15.3; Sant'Anna et al. 2011).

For strains belonging to the same species, high similarities of LuxI and LuxR proteins have been reported (Wisniewski-Dyé et al. 2002). However, it was also stated that the variation observed among LuxI and LuxR homologues from different strains of the same bacterial species was greater than the variation observed between closely related species (Gray and Garey 2001). Indeed, AlpI of *A. lipoferum* TVV3 is only 31 % identical (49 % similar) to the LuxI of *Azospirillum* sp. B510 and 39 % identical (56 % similar) to the LuxI of *A. amazonense* Y2. A weak conservation is also observed between AlpR and the LuxR of *Azospirillum* sp. B510 with only 26 % identity (Fig. 15.4). These observations explain the impossibility to detect by hybridization or by PCR *luxI* and *luxR* genes from *Azospirillum* sp. B510, and *A. lipoferum* B518 using probes and primers based on *alpI* or *alpR* sequences of *A. lipoferum* TVV3 (Vial et al. 2006; our unpublished data).

BLASTP search against NCBI Non-redundant Protein sequences database were performed, and it was found that the LuxI and LuxR homologues in *Azospirillum* displayed from 31 to 54 % identity to their closest matches (Table 15.2). Interestingly, AlpI and LuxI from *Azospirillum* sp. B510 share homology with putative autoinducer synthases from Rhodobacterales, whereas LuxI from *A. amazonense* Y2 shares homology with autoinducer synthases from species belonging to the Burkholderiales (Table 15.2).

The weak sequence homology of QS genes between the different strains of *Azospirillum* suggest that some of *luxI* and *luxR* genes may have been acquired by horizontal gene transfer. In the case of *A. lipoferum* TVV3, this hypothesis is supported by several features. The *alpI* and *alpR* genes are located on a 85 kb plasmid, a replicon that seems to be absent in other *A. lipoferum* strains (Vial et al. 2006; our unpublished results). The 2.6-kb containing these genes (accession number DQ439000) displays a GC content of 59.3 mol%, contrasting with the typical value of 69–70 mol% usually found for *A. lipoferum*. Sequencing of the entire cosmid clone revealed that several ORFs displaying high homology with ORFs encoding bacteriophage proteins frame *alpI* and *alpR*, an observation which is also consistent with an acquisition by horizontal gene transfer (Fig. 15.3; our unpublished data). Notably, the two ORFs located upstream of *alpI* share homology with the phage genes *ogr* and *ner*; Ogr is described as a positive regulatory factor for phage P2 late gene transcription while Ner is a negative regulator of Mu bacteriophage early gene transcription (Birkeland et al. 1991; Kukolj et al. 1989). The downstream region of *alpR* reveals several ORFs whose products share similarity with tail proteins of bacteriophage T2 particles and notably with the FETUD operon encoding the major structural components of the P2 contractile tail (Christie et al. 2002).

Interestingly, for *Azospirillum* sp. B510, several ORFs encoding proteins displaying homology with transposases are located close to *luxI* and *luxR*, suggesting the possibility of horizontal transfer of QS genes in this strain (Fig. 15.3).

a

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A. lip. TVV3 AlpI    MAPIQS---------------HQLRHQAFVDRLRYAVPSHDGLEWDQYDTPRASYIVVEH 45
A. sp B510 LuxI      MITVFSG-SDRTRHAELCRQMYRLRAQLFLNRRKWSVKVRDGEEIDCFDLQDPVYVCVTD 59
A. ama. Y2 LuxI      MSSLRVVNFSGPDSPELLAQAWRLRHRVFREGLGWDVASVDLLEFDGFDR--KAWHCAAL 58
                     * .:                  :** : * :   : *   *  * * :*     :  .

A. lip. TVV3 AlpI    -DGRCAAVCRLVPTTAPYMLEELFPQLLPYAPPKS-ADIWEATRIAVDAALPAPAR---- 99
A. sp B510 LuxI      PDQRLIGSLRLLPTMGPHMLSDVFPEVMGDARPIRRADTWESSRFCVDTQAARAFHPDGI 119
A. ama. Y2 LuxI      QGGRMVGYWRALPTTEPYLLERDFPMLLGDRPAPHAPDVWEISRFVICPDVANRRD---- 114
                        *  .  * :**  *::*.  ** ::    .   .* ** :*: : .  .

A. lip. TVV3 AlpI    DAALRALIVGVQRFGLENGIRHFLGLMPVAIFKRTLVRNGVTVRILDAAAQPIDGIATAA 159
A. sp B510 LuxI      NEVTRALLGGLFGSARALGLQSIVSVYDIFVER-ILQRAGCRFTRLG-PVHAYDGLNTVA 177
A. ama. Y2 LuxI      --VGKVLVQAIGAFGVARAANRLIAVVEPAFGR-FVRLCGLPVTEETAPIHVGQGHKGDI 171
                       . :.*: .:   .   . . ::.:    . :  :   *  .     . :  :*

A. lip. TVV3 AlpI    GEILVDPATVERLAPSMAVAA---------------------------------------  180
A. sp B510 LuxI      GHFPVEMNLPSRFVAPGAVKSTLTESDAADGAPIAGTLSAILPARSLSVAAATAAFPQGRA 237
A. ama. Y2 LuxI      HAVIIALDVPAYATSPSSRTTAGADPRAA-------------------------------  200
                       . :        ... :  :
```

b

```
A. lip. TVV3 AlpR     ------------------------------------------------MWQAIQTFVEQA 12
A. sp B510 LuxR       MPPSVVSVSDHTDPGCTLMGQEPPTAWEGGMRQGKFRILSRSGRLPRQDSVLYCRFMDYF 60
A. ama. Y2 LuxR1      ------------------------------------------------MILDEALNRL 10
A. ama. Y2 LuxR2      ------------------------------------------------MRKIEDMLSAF 11
                                                                        :.

A. lip. TVV3 AlpR     GDDTDEDALFRRFESAATELGYPFYAFGALWGDPDAIKLNPAPAVRLNYPAEWVAHYFST 72
A. sp B510 LuxR       DDFKRSKDKSHFLSRIIDSVGPFNVTY--FGTQCTSSP-DRHVNLLTTYSEKWRMRYFDR 117
A. ama. Y2 LuxR1      GQPDNPEDLFNTFSQLAQDLGYAAVSY--IDSRPGPAPGEPDLFVRTTVREDFLHTYLSE 68
A. ama. Y2 LuxR2      TSAGTLDDLKNALDRHVANLGFDAASY--VDVRRLPIAEEPVPFYQTSLRKDFLETYLAE 69
                       .    .   . :.    .:*    ::  .     .   :       .   .:   *:

A. lip. TVV3 AlpR     GYNKIDPVVLVAPYAQTSVTWDELRSYR-----------------------PEFFDDAA 108
A. sp B510 LuxR       RYENIDPVLSAARRATMPVDWDQLPKTD---------------------SGVEDFIADAL 156
A. ama. Y2 LuxR1      GYMSHDPLMVMAKRQAAPLTWADVPEFHASLQAPSKLDMPARGRAKQDAQRAIAVMTSAA 128
A. ama. Y2 LuxR2      DLLGYDPVVNRAATTNAPFTWADVPEFR-----------PARGGGRGARNRARQVMDIAR 118
                           **::  *     .. * :: .                          .:  *

A. lip. TVV3 AlpR     TFGLR-SGIAIPLRAIQGC----YVLCVASGDDRPIR---PKERARLELFAHGLFAAYQR 160
A. sp B510 LuxR       CYGIRLQGVTFSLRDMHQR----LAIVSLQSNDDPAVWRVRRPRIIPDFIHFSLALHSAM 212
A. ama. Y2 LuxR1      DHGYT-QGYVIPTHTVDPQGRLASAVVSLFWTQKPEG--LISPDSAPAWLRLSALYFHER 185
A. ama. Y2 LuxR2      DFGYR-QGFVLPLHAVDGHGFPASALISLFWRQEPDL--LDRPEHRPAWLRLAGSFYHER 175
                       .*   .* .:. : :.       .:      : *             :  .

A. lip. TVV3 AlpR     LRELP--------AQDHGLTANTVQVIRLSMAGLSTAEICSRLKLTMDGVHWCLKDARKK 212
A. sp B510 LuxR       VSSEPD----DAEEVPCKLSPREREVLDWASRGKTSWETGMVLGLSEKTVNFYLRNACQK 268
A. ama. Y2 LuxR1      VVSMR----LTPIAPPPTLTQREQEALVWASRGKTVEETGEIIGVSGRTVTFHLQNALEK 241
A. ama. Y2 LuxR2      ALNLRREVALPPLVPP-SLTDREERECLVWACRGKTRAETADIMGVSDRTVEFHLTNTMRK 234
                        .              *: .  : :  :  * :  *    : ::   * : * :: .*

A. lip. TVV3 AlpR     LKCANTAQVYLKAIQQGIVAVS 234
A. sp B510 LuxR       MNAATKVQAAAQAVREGLI--- 287
A. ama. Y2 LuxR1      LGCYSKIHAVAKAIQMGLIYP- 262
A. ama. Y2 LuxR2      LGVHSKYHAIAVAVQLRLIAP- 255
                      :   .. :.   *::  ::
```

Fig. 15.4 Multiple alignment of LuxI and LuxR proteins from *Azospirillum*. Alignments were performed using ClustalW program. *Asteriks* indicate conserved residues, *two dots* residues with high similarity, and *one dot* residues with low similarity. Residues usually conserved in LuxI or LuxR protein are indicated in *bold* (Watson et al. 2002; Whitehead et al. 2001)

Table 15.2 Closest matches to the LuxI and LuxR homologues identified in *Azospirillum*

Protein	% Identity of closest match	Closest match	Bacterial order of the species displaying the closest match
A. lipoferum TVV3 AlpI	43	Putative AHL synthase of *Bradyrhizobium* sp. ORS 285	Rhizobiales
Azospirillum sp. B510 LuxI	54	Putative AHL synthase of *Ruegeria* sp. PR1B	Rhodobacterales
A. amazonense Y2 LuxI	39	Putative autoinducer synthase of *Acidovorax radicis*	Burkholderiales
A. lipoferum TVV3 AlpR	46	Hypothetical protein of *Novispirillum itersonii*	Rhodospirillales
Azospirillum sp. B510 LuxR	39	Autoinducer-binding transcriptional regulator LuxR of *Sulfitobacter* sp. NAS-14.1	Rhodobacterales
A. amazonense Y2 LuxR1	39	Hypothetical protein of *Aureimonas ureilytica*	Rhizobiales
A. amazonense Y2 LuxR2 (“orphan LuxR”)	31	Hypothetical protein of *Methylocystis* sp. SB2	Rhizobiales

The closest match was obtained from NCBI nonredundant protein sequence database (BLASTP search)

As for *A. amazonense* Y2, the genetic environment of the *luxI-luxR* genes displays no striking figures (data not shown). Surprisingly, a second protein containing the InterPro domain IPR005143 was detected in the genome of *A. amazonense* Y2 (AZOY2_6130003). This potential LuxR shows 46 % identity (61 % similarity) with the other LuxR of *A. amazonense* Y2 and contains also several residues conserved in all LuxR homologues (Fig. 15.4); however, it is not associated with a gene encoding an AHL synthase and may be considered an orphan LuxR or LuxR solo (Case et al. 2008). Many bacterial species harbor multiple LuxI and or LuxR homologues for the production or detection of multiple signal molecules and the presence of orphan LuxR has been widely described in the recent years (Case et al. 2008). Investigations are necessary to decipher the QS regulation in the *A. amazonense* Y2 strain, and to precise the role of this potential orphan LuxR.

15.3 Identification of Phenotypes and Genes Regulated by AHL-Based Quorum Sensing

15.3.1 Obtention of a Strain Deficient in Signal Production

Generally, the first step to identify phenotypes or genes regulated by QS relies on obtaining a strain deficient in signal production or unable to synthesize the cognate transcriptional regulator LuxR. The usual way involves disrupting ORF encoding *luxI* or *luxR* homologues. For a site-specific inactivation of a gene, the classical

approach, i.e., reverse genetic, consists on allelic exchange. A suicide plasmid that is unable to replicate in the studied strain is used to deliver an inactivated allele of the gene into the genome; a double crossing-over event leads to replacement of the wild-type copy of the gene with the disrupted copy carried by the plasmid. Although allelic exchange is easy to perform with several PGPR bacteria (*Pseudomonas*, *Bacillus*…), it remains tricky with *Azospirillum* probably due to the low frequency of double crossing-over events in several strains. However, a series of genetic tools was recently developed for *A. amazonense* (Sant'Anna et al. 2011). An alternative approach is to insert a suicide vector into the gene of interest (only one crossing-over event is needed). Several insertional mutants using the pKNOCK mutagenesis plasmids were successfully obtained in several *Azospirillum* strains but this technique was not applied for generating *luxI* or *luxR* mutants (Acosta-Cruz et al. 2012).

Several AHL-degradation enzymes (including lactonases, acylases, oxidoreductases, and paraoxonases) have been identified in a range of living organisms (Chen et al. 2013). Expression of these enzymes in AHL-producing strains efficiently quenches the microbial QS signalling. For example, lactonases cleave the lactone ring of AHLs which are no longer recognized as a signal and incapable of binding to the target transcriptional regulator.

Quorum quenching enzymes can be used as alternative tools to obtain strains impaired in AHL production, notably when genetic approaches are not available or when several genes encoding synthases are present in the studied strain. The quorum quenching strategy was for the first time reported in *Erwinia carotovora* where AiiA, a lactonase from *Bacillus*, inactivates AHL production and strongly attenuates the virulence of this phytopathogen (Dong et al. 2000). However, introduction of the *aiiA* gene from *Bacillus* in *A. lipoferum* TVV3 or B518 did not allow AHL inactivation, probably due to a high divergence in codon usage between *Azospirillum* and *Bacillus* (Boyer et al. 2008). In order to quench AHL accumulation in *Azospirillum*, a plasmid (pBBR1 with a constitutive promoter PntpII) with the *attM* gene encoding a lactonase from *A. tumefaciens* was introduced by conjugation in the two producing strains *A. lipoferum* TVV3 and B518 (Boyer et al. 2008). In these modified strains, AHL levels were undetectable (Boyer et al. 2008).

15.3.2 Screening of QS Regulated Functions by Phenotypic Assays

QS regulation is used to control various phenotypes, and it is rather hazardous to try to decipher the role of QS only by testing specific phenotypes. However, phenotypes regulated by QS are often essential for interaction with a eukaryotic host. It is thus possible to focus on functions essential in plant–bacteria interaction (and/or already shown to be QS-regulated in other plant-associated bacteria), such as motility (swimming and swarming), biofilm formation, root adherence, rhizosphere colonization, phytohormone production, synthesis of antifungal/antimicrobial metabolites, and phytostimulatory effects.

AHL inactivation in *A. lipoferum* B518 increases siderophore synthesis and reduces pectinase activity and indole acetic acid production. In *A. lipoferum* TVV3, none of the tested phenotypes appear to be regulated by QS (Boyer et al. 2008).

In vitro inoculation of rice with *A. lipoferum* TVV3 and B518 (as these two strains were isolated from the rice rhizosphere) revealed that AHLs were not implicated in regulation of phytostimulatory effects (Boyer et al. 2008). In the same way, endophytic colonization, germination, and plant-growth promotion of maize by *Burkholderia phytofirmans* PsJN is not impacted in a strain deficient in AHL production (Coutinho et al. 2013). On the contrary, the BraIR QS system of *Burkholderia kuriensis* has been shown to be important for endophytic colonization of rice (Suarez-Moreno et al. 2010). In biocontrol strains of *Pseudomonas*, colonization properties are often subjected to an AHL-based QS regulation (Zhang and Pierson 2001).

Among all the functions regulated by QS in plant-associated bacteria, two common points may be noted. Firstly, exopolysaccharide production is often subjected to QS regulation, as demonstrated in *Pantoea stewartii*, *Pseudomonas syringae*, and in plant beneficial *Burkholderia* (von Bodman et al. 1998; Quiñones et al. 2005; Suarez-Moreno et al. 2010). Secondly, QS is often involved in regulation of the production of antifungal/antimicrobial metabolites, notably in *Burkholderia ambifaria*, *Pseudomonas*, and *Serratia plymuthica* (Chapalain et al. 2013; Liu et al. 2007; Pierson et al. 1994).

15.3.3 Screening of QS-Regulated Genes by Molecular Approaches

Genes regulated by QS systems can also be identified using transposon mutagenesis approaches. This strategy was applied for several *Burkholderia* strains, and notably *B. ambifaria*, an effective biocontrol species (Chapalain et al. 2013). In order to identify genes whose expression is influenced by C_8-HSL (the major AHL produced by *B. ambifaria*), a *cepI* mutant was employed as background for a random whole-genome transposon-insertion mutagenesis with a *Tn*5 containing a promoterless *lacZ* (Chapalain et al. 2013). Only clones showing a difference in expression (revealed by *lacZ* transcription) between the medium with C_8-HSL and the medium without C_8-HSL could potentially contain promoter of genes regulated by the *cepIR* system. This strategy reveals that the production of antifungal/antimicrobial compounds is an important trait controlled by QS in this species (Chapalain et al. 2013). However, this approach is only applicable to identify positively regulated genes. Another strategy to identify genes controlled by QS was employed in *B. cenocepacia* K56. A random promoter library (constituted by DNA fragment with sizes between 0.5 and 3 kb) was generated in a plasmid which contains a promoterless *luxCDABE* operon as a reporter system; luminescence (reflecting gene expression) was compared between a medium supplemented with AHL and a medium without AHL. Not only the *luxCDABE* reporter system is more sensitive than the *lacZ* reporter, but negatively regulated genes can be detected by this approach (Subsin et al. 2007).

Even if several genes were identified by these methods, these are rather time consuming, fastidious, and above all, only a portion of QS regulated genes is revealed by these approaches.

15.3.4 Omics Approaches to Identify QS-Regulated Genes

In order to gain an overall view of QS-regulated functions in a specific strain, global changes in gene expression have also been investigated at the level of the transcriptome (microarray or RNA-seq) or the proteome. These global approaches can take into consideration the different lifestyles of microorganisms and can allow the study of QS regulation when bacteria are organized in biofilm or in interaction with the plant.

In the PGPR *P. putida*, the protein profiles of the wild-type strain and the *ppuI* mutant grown either in planktonic cultures or in mature biofilms were compared by two-dimensional gel electrophoresis. The expression of QS regulated proteins is strongly dependent on the lifestyle of the organism; indeed, half of the QS-controlled proteins identified in planktonic conditions were found to be oppositely regulated when *P. putida* was grown in biofilm (Arevalo-Ferro et al. 2005).

Transcriptome studies based on DNA microarray technology were recently conducted to identify genes regulated by QS in plant-beneficial *Burkholderia*, notably in *B. phymatum*, a species which forms symbiotic nodules and fixes nitrogen in association with legumes. The QS system of *B. phymatum* acts on the transcription of approximately 2.3 % of the protein-coding genes in this strain and was shown not to be involved in plant nodulation (Coutinho et al. 2013).

Direct sequencing of transcripts (RNA-seq) gives a very detailed gene expression level and now supplants microarrays for whole-genome transcriptome profiling. Only a few studies have used this powerful technology for the genome analysis of QS-dependent expression. RNA-seq was recently used to decipher the role of two AHLs synthases encoding genes (*traI* and *ngrI*) in the symbiotic bacteria *Sinorhizobium fredii* (Krysciak et al. 2014). A common set of 186 genes appears to be regulated by the TraI/R and NgrI/R regulon, with coregulated genes including 42 flagellar biosynthesis genes and 22 genes linked to exopolysaccharide biosynthesis (Krysciak et al. 2014).

These global omics approaches revealed that QS can affect several percent of the protein or gene content in bacteria, as observed for *B. phymatum* or *Rhizobium leguminosarum* (Coutinho et al. 2013; Cantero et al. 2006).

A global proteomic approach was undertaken in *A. lipoferum* TVV3 and B518 using the wild-type strain and the non-AHL-producing strain (containing the *attM* gene). No significant modification of the protein pattern was observed for *A. lipoferum* TVV3; this finding is consistent with the hypothesis of a recent acquisition by HGT of the QS system in this strain (see Sect. 15.2.2.3). On the contrary, two-dimensional polyacrylamide gel electrophoresis performed on soluble intracellular proteins and extracellular proteins revealed that numerous proteins are regulated by the AHL-mediated QS system in B518 (Fig. 15.5) (Boyer et al. 2008). Several QS-regulated proteins identified by MS-MS are implicated in

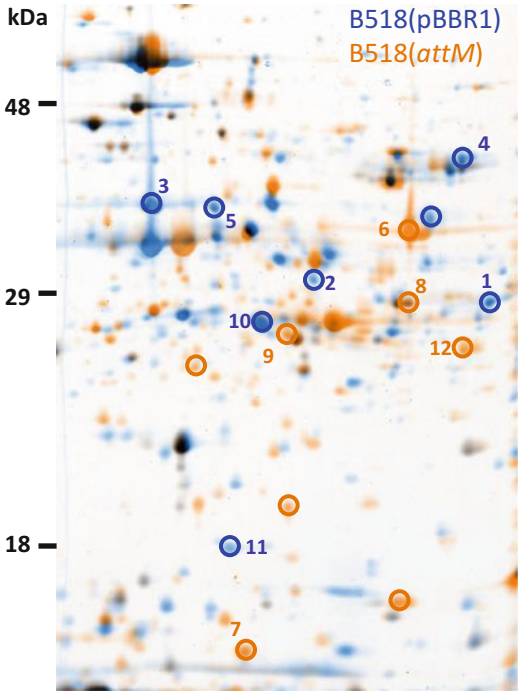

Fig. 15.5 2D-PAGE analysis of intracellular proteins of *A. lipoferum* B518(pBBR1) and B518(pBBR1-*attM*) strains. Isoelectric focusing in the first dimension was carried out on a linear pH gradient (4–7), followed by a second separation on 12 % acrylamide SDS-PAGE gels. Proteins were revealed by silver staining and the two gels were scanned and superimposed using the ProteomWeaver® software (Biorad). *Encircled blue spots* represent proteins absent from B518(pBBR1-*attM*) and thus positively regulated by QS. *Encircled orange spots* represent proteins absent from B518(pBBR1) and thus negatively regulated by QS. Subsequent analysis by LC-ESI/MS-MS lead to the following identification: (*1*) Succinyl-coA synthetase, alpha subunit; (*2*) Aspartate-semialdehyde dehydrogenase; (*3*) Major outer membrane protein OmaA-like; (*4*) High-affinity branched-chain amino acid ABC transporter; (*5*) Multiple sugar-binding periplasmic receptor ChvE; (*6*) Major outer membrane protein; (*7*) Putative endoribonuclease; (*8*) Dihydrodipicolinate synthase; (*9*) Triosephosphate isomerase; (*10*) Putative extracytoplasmic solute receptor; (*11*) Ribonuclease; (*12*) ABC transporter subunit. Adapted from Boyer et al. 2008

transport (the major outer membrane protein OmaA) and chemotaxis (ChvE), suggesting that QS in *A. lipoferum* B518 is dedicated to control functions linked to root colonization (Boyer et al. 2008).

15.4 Conclusion

Investigating cell–cell communication often begins with the evidence of a bacterial signal being produced. In the case of AHLs, various biosensors have been constructed to facilitate this task (Table 15.1). Identification of genes mediating

AHL-based QS can be performed by bacterial genetics and is now greatly facilitated by the availability of whole genome sequences. Most of the studies investigating QS in Gram-negative PGPR have focused on AHL signals that are classically detected by biosensors. Among the *Azospirillum* genus, AHL-mediated communication seems to be the exception rather than the rule (Vial et al. 2006; Boyer et al. 2008); indeed only a few strains have the ability to synthesize these molecules and the corresponding genes seem to have been acquired independently by horizontal gene transfer.

Given the range of other signalling molecules described so far and the ones that remain uncharacterized, one can assume that some PGPR might use other types of signals. Cell–cell communication might be achieved by aryl-homoserine lactones, as recently characterized in *Rhodopseudomonas palustris* and in some *Bradyrhizobium* (Schaefer et al. 2008), by diffusible factors as the one involved in regulating the plant growth-promotion properties of a *Stenotrophomonas maltophilia* strain (Alavi et al. 2013) or by still unidentified molecule(s) such as the ones regulating type IV pili in the grass endophyte *Azoarcus* (Hauberg-Lotte et al. 2012).

It is also noteworthy that plants seem to be able to detect and respond to bacterial signals; as such, AHLs are able to trigger systemic resistance to pathogens in different plants (Schuhegger et al. 2006; Schikora et al. 2011) and thus might be considered as playing an active role in the biocontrol activity of some rhizobacteria.

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Chapter 16
Methods to Study 1-Aminocyclopropane-1-carboxylate (ACC) Deaminase in Plant Growth-Promoting Bacteria

Clarisse Brígido, Jin Duan, and Bernard R. Glick

Abstract The lowering of plant ethylene levels by the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase is one of the key mechanisms employed by plant growth-promoting bacteria (PGPB) to facilitate plant growth. Since its discovery, it has been detected in both fungi and bacteria. It has been shown by a large number of workers in a wide range of bacteria that the activity of this enzyme in PGPB is important during normal plant development and also protects plants from the deleterious effects of a wide range of environmental stresses. ACC deaminase-containing PGPB bound to a plant act as a sink for ACC, thereby lowering ethylene levels in plant tissues. The result of the functioning of this enzyme is an increase in the growth of plant roots and shoots and a reduction of the inhibitory effects of ethylene synthesis especially during stressful conditions. This chapter briefly summarizes the current knowledge of various ACC deaminases emphasizing the use of ACC deaminase-containing bacteria in promoting plant growth under diverse biotic and abiotic stresses, and describes methods for the isolation and study of ACC deaminase-containing bacteria.

16.1 Introduction to ACC Deaminase

PGPB can help plants to grow by alleviating or avoiding the negative effects of both biotic and abiotic stresses on plant growth (Glick 1995a, 2012). PGPB exert beneficial effects on plant development by either direct or indirect mechanisms or by a combination of the two (Glick 1995a). The enzyme ACC deaminase (encoded by the *acdS* gene) is thought to be a key trait in the arsenal that PGPB use to promote plant growth. There are numerous literature reports describing this enzyme in

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taxonomically distinct bacteria, including PGPB, phytopathogens, opportunistic human pathogens (Blaha et al. 2006; Nascimento et al. 2014), and fungi (Jia et al. 2000; Viterbo et al. 2010; Singh and Kashyap 2012). In addition, based on sequence similarity, many microorganisms have putative ACC deaminase genes; however, whether all of these are bona fide ACC deaminases remains to be determined.

The term plant growth-promoting rhizobacteria (PGPR) refers to bacteria associated with the root surface (rhizospheric bacteria); however, other bacteria can enter into the root tissues establishing themselves as endophytes, or inside of specialized nodular structures of root cells, generally rhizobia (Rosenblueth and Martinez-Romero 2006). Thus, the more inclusive term PGPB is used here to include both rhizospheric and endophytic bacteria.

16.1.1 Biochemistry

ACC deaminase (E.C. 3.5.99.7) is a pyridoxal phosphate enzyme that is capable of cleaving ACC, the immediate precursor of ethylene in all higher plants, to α-ketobutyrate and ammonia (Honma and Shimomura 1978), subsequently lowering the ethylene levels in plant tissues (Glick et al. 1998, 2007a). In turn, decreased ethylene levels stimulate root growth and help the plant to be more resistant to a wide variety of environmental stresses, all of which induce the plant to increase its endogenous level of ethylene (Li et al. 2000; Glick et al. 2007b). It was first isolated from *Pseudomonas* sp. strain ACP (Honma and Shimomura 1978). Since then, ACC deaminase has been detected in a number of fungal and bacterial strains including some strains of *Azospirillum lipoferum* and *A. brasilense* (Blaha et al. 2006; Glick et al. 2007a, b; Glick 2014; Nascimento et al. 2014). Many bacteria that produce ACC deaminase have been identified by their ability to grow on minimal medium containing ACC as the sole nitrogen source.

Honna and co-workers have intensively studied the biochemical properties of ACC deaminase (Honma and Shimomura 1978; Walsh et al. 1981; Honma 1985; Honma et al. 1993a, b; Minami et al. 1998; Jia et al. 1999; Ose et al. 2003). In addition a few other studies on this enzyme and its biochemical properties have been conducted by other groups (Liu et al. 1984; Jacobson et al. 1994; Li et al. 1996; Zhao et al. 2003; Hontzeas et al. 2004).

ACC deaminase monomers are presumed to form a trimer in certain bacteria, e.g., *Pseudomonas* sp. strain ACP and 6G5; the size of the holoenzyme is approximately 104–105 kDa, and the subunit mass is approximately 36,500 Da (Honma and Shimomura 1978; Honma 1985; Jacobson et al. 1994) and it uses one molecule of the essential co-factor pyridoxal 5-phosphate (PLP) per subunit. However, in *H. saturnus* and *P. citrinum* the molecular mass of the holoenzymes and subunits are 69 kDa and 40 kDa, 68 kDa and 41 kDa, respectively, suggesting that these ACC deaminases are dimers (Minami et al. 1998; Jia et al. 1999).

Reported Km values for the binding of ACC by ACC deaminase range from 1.5 to 17.4 mM (Honma and Shimomura 1978; Jacobson et al. 1994; Hontzeas et al. 2004), indicating that the enzyme does not have a particularly high affinity for the

substrate ACC (Glick et al. 2007a). This has been interpreted as indicating that in order to compete with ACC oxidase for ACC, ACC deaminase must be present in much greater amounts, i.e., from 100- to 1,000-fold (Glick et al. 1998). Moreover, since plant ACC levels are usually in the micromolar range, the amount of substrate will nearly always be lower than the Km, and for every increase in the ACC concentration there will be a parallel increase in the rate of ACC cleavage (Glick 2005). The optimum temperature for the activity of ACC deaminase is typically around 25–35 °C and the optimum pH is 8.5 (Honma and Shimomura 1978; Jacobson et al. 1994; Jia et al. 1999).

Where it has been examined, bacterial ACC deaminase activity is localized only in the cytoplasm (Jacobson et al. 1994); it is not a secreted enzyme. The enzyme activity has been induced in both *Pseudomonas* sp. strain ACP and *P. putida* GR12-2 by ACC, at levels as low as 100 nM (Honma and Shimomura 1978; Jacobson et al. 1994); both bacterial strains were grown on a rich medium and then switched to a minimal medium containing ACC as its sole nitrogen source. The rate of induction of enzyme activity is relatively slow in that complete induction requires 8–10 h (Jacobson et al. 1994).

X-ray crystallographic analysis reveals that ACC deaminase folds into two domains, each of which has an open twisted α/β structure that is similar to the β-subunit of the enzyme tryptophan synthase (Yao et al. 2000). ACC deaminase is unique amongst PLP-dependent enzymes, since the ring cleavage catalyzed by ACC deaminase cannot precede through an α-carbanionic intermediate due to the lack of an abstractable α-hydrogen atom from the substrate ACC (Hontzeas et al. 2006). Despite the mechanism being not completely understood, the enzyme contains two key amino acid residues: a reactive thiol group at cysteine 162, located in the gap between the two domains of the molecule, and the PLP binding site at lysine 51 (Glick 2005). Notwithstanding the fact that the major activity of this enzyme is the cleavage of ACC, D-serine and D-cysteine can also act as substrates, although less efficiently than ACC. On the other hand, L-serine and L-alanine are effective competitive inhibitors of the enzyme (Glick 2005).

16.1.2 ACC Deaminase and Plant Stress

Ethylene, which is produced in almost all plants, mediates a range of plant responses and developmental stages (McKeon and Yang 1987). Moreover, ethylene levels in plant tissues increase as part of a plant's response to different types of stresses, such as extreme temperatures, water stress, ultraviolet light, insect damage, disease, and mechanical wounding (Abeles et al. 1992). Although ethylene is essential for normal growth and development in plants, at high concentration it can be harmful causing some stress symptoms as well as inducing defense responses in the plant, which may lead to reduced crop performance.

ACC deaminase activity is a key trait in PGPB because bacteria containing ACC deaminase can lower plant ethylene levels when the plants are exposed to different environmental stresses, thereby facilitating plant growth (Glick et al. 1998). Ethylene has long been recognized as a hormone that controls plant responses under

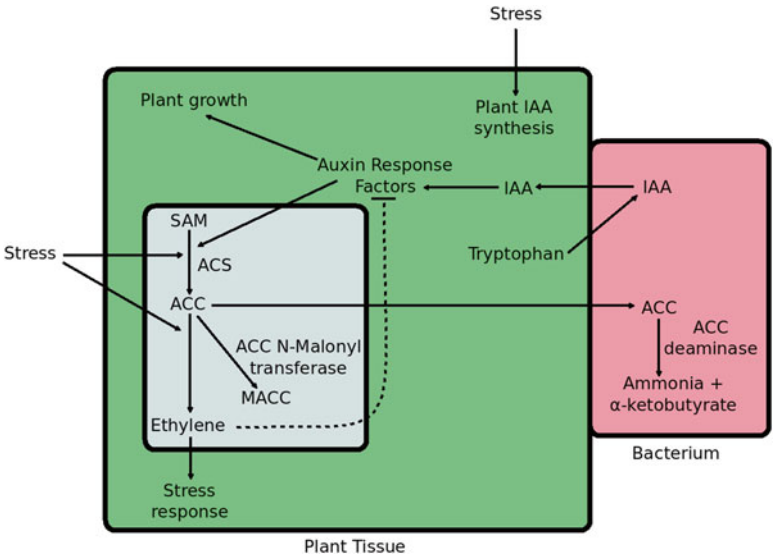

Fig. 16.1 A schematic model of how plant growth-promoting bacteria that both produce ACC deaminase and synthesize IAA may facilitate plant growth. The *grey rectangle* shows ethylene biosynthesis, where *S*-adenosyl-L-methionine (SAM) is converted to 1-aminocyclopropane-1-carboxylate (ACC) by the enzyme ACC synthase (ACS), ACC can then be converted to either the storage compound 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) by ACC N-Malonyl transferase, or to the end product, ethylene, by 1-aminocyclopropane-1-carboxylate oxidase. Indole-3-acetic acid (IAA) biosynthesis, both in bacteria and in plants is a complex multi-enzyme/protein process as is IAA signal transduction (modified from Glick 2014)

growth-limiting conditions (Abeles et al. 1992; Morgan and Drew 1997). It has been proposed that ethylene is produced in two peaks in response to environmental stresses (Stearns and Glick 2003; Pierik et al. 2006). The first peak is small and usually occurs a few hours after the onset of the stress. This small peak is thought to be beneficial to the plant because it may turn on the transcription of genes that are responsible for plant protection (Van Loon and Glick 2004; Van Loon et al. 1997; Glick 2004). The second ethylene peak is much larger and occurs 1–3 days after the stress. This second peak typically causes visible damage to the plant, such as senescence, chlorosis, and abscission (Glick et al. 2007a).

A model (Fig. 16.1) has been proposed to explain the mechanism of action of ACC deaminase in plant growth promotion (Glick et al. 1998, 2007a; Glick 2014). Generally, rhizobacteria attached to the seeds or roots of a developing plant can synthesize and secrete IAA in response to tryptophan and other small molecules in plant exudates (Patten and Glick 1996, 2002; Bayliss et al. 1997; Penrose et al. 2001). The IAA produced by the PGPB (some of which is taken up by the plant) together with plant IAA can stimulate plant growth and development or it can induce the transcription of the gene encoding the enzyme ACC synthase, which converts *S*-adenosyl-methionine (SAM) to ACC. The ACC can subsequently be converted to ethylene by the enzyme ACC oxidase but it can also be exuded to the rhizosphere and taken up by PGPB. Bacteria that produce ACC deaminase can

cleave the exuded ACC to α-ketobutyrate and ammonia. Since bacterial IAA induces the plant to synthesize more ACC, it also stimulates the exudation of ACC from the plant (as a consequence of plant cell wall loosening). Because the amount of ACC outside the roots or seeds is reduced, to maintain the equilibrium between internal and external levels, more ACC will be secreted, resulting in a lower amount of ACC available inside of plant tissues that is available for oxidation to ethylene. By decreasing the level of ACC inside the plant cells, the inhibitory effect of stress ethylene on root elongation (and plant growth in general) is reduced (Glick 1995a). Additionally, IAA can activate the transcription of ACC synthase, leading to more ethylene production (Kim et al. 1992; Kende 1993; Kende and Zeevaart 1997). However, it has been demonstrated that ethylene inhibits IAA transport and signal transduction (Stearns et al. 2012); therefore this feedback loop will eventually limit the ethylene levels when a plant is under environmental stress (Burg and Burg 1966; Morgan and Gausman 1966; Suttle 1988; Prayitno et al. 2006). With the presence of ACC deaminase-containing PGPB, the feedback loop breaks because a significant portion of the ACC that is produced is degraded in the bacteria, thus the repression of auxin response factor synthesis by ethylene is relieved, resulting in more IAA flux and more plant growth promotion (Glick et al. 2007b; Glick 2014).

ACC deaminase is usually present in bacteria at a constitutive but low level before it is induced, and the induction of enzyme activity is a slow and complex process (Li and Glick 2001; Glick et al. 2007a). Therefore, the first (small) peak of ethylene occurs right after an environmental stress, inducing a defense response in the plant, with very little impact on the ethylene peak from the presence of an ACC deaminase-containing PGPB. As the concentration of ACC increases, bacterial ACC deaminase is concomitantly induced, thus the second, deleterious, ethylene peak is reduced significantly by ACC deaminase activity (Glick et al. 2007a).

Many studies have shown that bacteria containing ACC deaminase promote plant growth when the plants are subjected to different environmental stresses including the presence of phytopathogens (Wang et al. 2000; Toklikishvili et al. 2010; Nascimento et al. 2013), high concentrations of salt (Saravanakumar and Samiyappan 2007; Cheng et al. 2007; Nadeem et al. 2007; Yue et al. 2007; Egamberdieva et al. 2008; Jalili et al. 2009; Siddikee et al. 2011; Ali et al. 2014), drought (Mayak et al. 2004; Belimov et al. 2009), flooding and anoxia (Grichko and Glick 2001; Barnawal et al. 2012; Li et al. 2012, 2013), heavy metals (Burd et al. 2000; Nie et al. 2002; Zhang et al. 2011), and organic compounds (Glick et al. 2007a, b; Glick 2014). Moreover, the *acdS* gene (encoding ACC deaminase) has become a widely used genetic marker for screening for PGPB functions. Here, we present a number of detailed methods to study ACC deaminase in PGPB.

Similar to the effects on plant growth by ACC deaminase-containing bacteria, the plant growth-promoting fungus *Trichoderma asperellum* T203 also induces beneficial effects on plant growth through the use of ACC deaminase (Viterbo et al. 2010). Additionally, transformation of bacterial strains lacking ACC deaminase activity with isolated *acdS* genes and their regulatory regions has been shown to improve their effectiveness. For example, the expression of an exogenous ACC deaminase gene increases the usefulness of *Azospirillum* strains that lack this enzyme (Holguin and Glick 2001, 2003).

16.2 Isolation of ACC Deaminase-Containing PGPB

A simple alternative to the previous (often tedious) approaches to the isolation of novel PGPB is to test the ability of microorganisms to grow on minimal medium containing ACC as its sole nitrogen source. The procedure of isolating ACC deaminase-containing PGPR involves five steps (Penrose and Glick 2003).

Day 1:
One gram of soil from a plant rhizosphere is added to 50 mL sterile PAF medium (per litre: 10 g proteose peptone, 10 g casein hydrolysate, 1.5 g anhydrous $MgSO_4$, 1.5 g K_2HPO_4, and 10 mL glycerol) in a 250-mL flask. The culture is incubated at a temperature between 25 and 30 °C for 24 h with shaking (200 rpm). Here, the PAF medium is for the enrichment of pseudomonads and similar bacteria. To isolate *Azospirillum* strains with ACC deaminase the PAF medium should be substituted by an *Azospirillum*-specific medium in this and the following steps (Bashan et al. 2011).

Day 2:
A 1-mL aliquot is removed from the growing culture, transferred to 50 mL of sterile PAF medium in a 250-mL flask and incubated at the same temperature as the first incubation for another 24 h with shaking (200 rpm).

Day 3:
A 1-mL aliquot is removed from the culture and transferred to a 250-mL flask containing 50 mL sterile minimal medium, DF medium (Dworkin and Foster 1958) (per litre: 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2 g glucose, 2 g gluconic acid, 2 g citric acid and trace elements containing 1 mg $FeSO_4 \cdot 7H_2O$, 10 μg H_3BO_3, 11.19 μg $MnSO_4 \cdot H_2O$, 124.6 μg $ZnSO_4 \cdot 7H_2O$, 78.22 μg $CuSO_4 \cdot 5H_2O$, 10 μg MoO_3, pH 7.2, and 2 g $(NH_4)_2SO_4$ as a nitrogen source). The ingredients should be dissolved in water one at a time to avoid precipitation. The trace elements and $FeSO_4 \cdot 7H_2O$ are prepared separately as stock solutions ($10^4\times$) in 100 and 10 mL sterile water, respectively, which can be stored at 4 °C for several months. When making 1 L of DF medium, all of the other ingredients and 0.1 mL of each of the stock solutions of trace elements and $FeSO_4 \cdot 7H_2O$ are dissolved in distilled water and then autoclaved for no longer than 20 min.

The culture is incubated at the same conditions as the first incubation for 24 h.

Day 4:
A 1-mL aliquot is removed from the culture and transferred to 50 mL sterile DF medium containing 3 mM ACC, which replaces $(NH_4)_2SO_4$ as the source of nitrogen. To prepare ACC, a 0.5 M stock solution (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) is filter sterilized, divided into small aliquots, and frozen at −20 °C until ready to use. The culture is incubated at the same conditions as the first incubation for 24 h.

Day 5:
Serial dilutions are prepared from the overnight culture and spread onto DF ACC (30 μmol $plate^{-1}$) agar medium for 48–72 h at the same temperature as the first incubation. After colonies form, single colonies are streaked onto new DF ACC plates for purification.

16.3 ACC Deaminase Assays

ACC deaminase activity is measured based on the amount of α-ketobutyrate produced as a function of time (Honma and Shimomura 1978; Penrose and Glick 2003).

16.3.1 Free-Living PGPB

Day 1:
After isolation of the ACC deaminase-containing PGPB, the bacterial culture is streaked onto solid rich medium, such as tryptic soy agar (TSA; Difco Laboratories, Detroit, Michigan, USA) and incubated overnight at its optimal growth temperature until colonies form.

Day 2:
A single colony from the agar plate is inoculated into 15 mL of liquid rich medium, such as tryptic soy broth (TSB; Difco Laboratories, Detroit, Michigan, USA) and incubated overnight with shaking (200 rpm) at its optimal growth temperature.

Day 3:
The bacterial culture is collected by centrifugation at ~5,000×*g* for 10 min at 4 °C and washed twice with 10 mL DF salts minimum medium (without a N source). The cell pellet is resuspended in 15 mL of DF salts minimum medium with 3 mM ACC. The culture is incubated for an additional 24 h with shaking at its optimal growth temperature.

Day 4:
The bacterial culture is collected by centrifugation at ~5,000×*g* for 10 min and washed twice with 5 mL 0.1 M Tris–HCl (pH 7.6). Then the cell suspension is transferred to a 1.5 mL microcentrifuge tube. All of the supernatant is removed after centrifugation at ~10,000×*g* for 1 min and the cell pellet is used for the enzyme activity assay.

The pellet is resuspended in 400 μL 0.1 M Tris–HCl (pH 8.0) with 20 μL of toluene. The suspension is vortexed at maximum speed for 30 s. Then, 50 μL of cell lysate is dispensed into each of three 1.5 microcentrifuge tubes. In two tubes, 5 μL of 0.5 M ACC is added whereas the third tube is a negative control (lysate without ACC). Another negative control is also prepared containing 50 μL of 0.1 M Tris–HCl (pH 8.0) and 5 μL of 0.5 M ACC. After adding ACC, the cell suspension is vortexed for about 5 s and all of the tubes are incubated at 30 °C for 30 min. After incubation, 500 μL of 0.56 M HCl is added into each tube and then vortexed for about 5 s. The cells are centrifuged for 5 min at ~10,000×*g* at room temperature.

Meanwhile, α-ketobutyrate standards are prepared as follows. First, a 100 mM α-ketobutyrate stock solution is diluted 10× with 0.1 M Tris–HCl (pH 8.0). Second, standards (in μmoles) including 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 are prepared in duplicate in 0.1 M Tris–HCl (pH 8.0).

Next, 500 μL of cell supernatant or α-ketobutyrate standards are added into each 13 × 100 mm glass test tube. After adding another 400 μL of 0.56 M HCl, 150 μL

0.2 % 2,4-dinitrophenylhydrazine (prepared in 2N HCl) is added and vortexed for about 5 s. Then, all of the test tubes are incubated at 30 °C for 30 min followed by the addition of 1 mL of 2N NaOH. The tubes are vortexed for about 5 s and the absorbance of the mixture is measured at 540 nm using a spectrophotometer. A color change from yellow to brown indicates a positive result.

16.3.2 Rhizobia

High concentrations of ACC or ethylene in root tissues negatively affect the nodulation process in legume plants (Ma et al. 2004; Middleton et al. 2007). Inhibitors of ethylene synthesis or its physiological activity enhance nodulation (Tirichine et al. 2006) demonstrating that ethylene acts as a negative regulator of nodulation, and a reduction in ethylene concentration has a stimulatory effect on the formation and development of nodules in legumes (Ding and Oldroyd 2009). Thus, rhizobial strains that express ACC deaminase are up to 40 % more efficient at forming nitrogen-fixing nodules than strains that lack this activity (Ma et al. 2003, 2004). Thus, a potential target root growth is to regulate or limit the biosynthesis of ethylene. This can be achieved by the use of rhizobia with the ability to reduce ethylene levels in plant roots through the cleavage of ACC by ACC deaminase enzyme.

The measurement of ACC deaminase activity for rhizobial strains was determined for the first time by Ma et al. (2003), using the methodology described above with some minor modifications. The level of ACC deaminase activity that is found in various rhizobial species is typically around 2–10 % of the level that is found in free-living bacteria. Therefore, the assay conditions must be adjusted accordingly. This generally means employing either a higher concentration of ACC or a longer incubation time or both.

16.3.3 Mesorhizobia

In contrast to other rhizobia, *Mesorhizobium* species only express ACC deaminase activity under symbiotic conditions, which makes it impossible to base their isolation on the activity of ACC deaminase. This is because the *acdS* gene from *Mesorhizobium* strains is under the transcriptional control of the *nifA* promoter that is responsible for activating the transcription of *nif*, nitrogen fixation genes (Kaneko et al. 2000; Sullivan et al. 2002; Uchiumi et al. 2004; Nukui et al. 2006; Nascimento et al. 2012). To improve the nodulation efficiency and competitiveness of *Mesorhizobium* sp. MAFF303099 in *Lotus* spp., plants were inoculated with the strain MAFF303099 transformed to constitutively express a second copy of the ACC deaminase gene (regulated like *acdS* genes from free-living PGPB) (Conforte et al. 2010). Additionally, expression of an exogenous *acdS* gene in *S. meliloti* strains and *Rhizobium* sp. strain TAL1145 increased their nodulation abilities in alfalfa and *Leucaena leucocephala*, respectively (Ma et al. 2004; Tittabutr et al. 2008).

16.4 Gnotobiotic Root Elongation Assay

The growth pouch assay is a quick and easy way to assess the effect of various bacterial strains on the growth of plant seedlings. The protocol described below (Penrose and Glick 2003) is used to measure the elongation of canola roots from seeds treated with different strains of bacteria or chemical ethylene inhibitors. Notwithstanding, the fact that the growth pouch assay has been largely performed with canola seeds, other seeds such as tomato are also effective as long as the different growth rate of each plant is considered.

Day 1:
The bacterial strain is inoculated into 15 mL of rich medium, such as TSB. The culture is incubated at its optimal growth temperature with shaking at 200 rpm until it reaches late log phase.

Day 2:
The bacterial culture is collected by centrifugation at ~5,000×*g* for 10 min at 4 °C and washed twice with 10 mL DF salts minimum medium (without a N source). The cell pellet is resuspended in 15 mL of DF salts minimum medium with 3 mM ACC and then incubated for an additional 24 h with shaking at 200 rpm at its optimal growth temperature.

Day 3 and so on:
The bacterial culture is collected by centrifugation at ~5,000×*g* for 10 min at 4 °C and washed twice with 0.03 M $MgSO_4$. Then, the cells are resuspended in 0.5 mL 0.03 M $MgSO_4$ in a microcentrifuge tube and subsequently diluted 8–10 times with 0.03 M $MgSO_4$ to a final OD_{600} of 0.15. The diluted cells are kept on ice until they are used to inoculate seeds.

The Seed-pack growth pouches (Northrup King Co., Minneapolis, MN, USA) are prepared as following. A clean plastic bin is used to hold three racks (Northrup King Co., Minneapolis, MN, USA). The growth pouches are placed upright in a rack and two empty pouches are placed at each end of the rack since plants at the end of a rack are usually subjected to extremes of light or air circulation. Then, 12 mL of sterile water are added to each pouch to prevent water loss. The plastic bin with three racks of pouches inside is wrapped with aluminum foil and autoclaved at 121 °C for 15 min.

Canola seeds should be disinfected immediately before use. The seeds are soaked in 70 % ethanol for 1 min. After removing the liquid by suction, the seeds are soaked in 1 % sodium hypochlorite for 10 min. Sodium hypochlorite is washed off by rinsing the seeds with sterile water at least five times. The seeds are incubated at room temperature for 1 h with the appropriate treatment including a negative control (sterile 0.03 M $MgSO_4$ only) and bacterial suspensions (in sterile 0.03 M $MgSO_4$).

Sterilized forceps are used to place six seeds in each growth pouch, and nine to ten pouches are typically used for each treatment.

Sterile water is added into the plastic bin to a depth of approximately 3 cm. Then, the bin is covered loosely with aluminum foil to prevent dehydration. After the seeds germinate, the aluminum foil is changed to plastic wrap.

The pouches are incubated in a growth chamber (Conviron CMP 3244: Controlled Environments Ltd, Winnipeg, MB, Canada) maintained at 20 ± 1 °C with a cycle beginning with 12 h of dark followed by 12 h of light (~18 μmol m^{-2} s^{-1}).

On the fifth day of growth the primary root lengths are measured. Typically, the root lengths of treated plant seedlings are 40–60 % greater than the lengths of untreated seedlings, with standard errors generally around 5 % of the length of the untreated seedling root. About 30–60 seeds are used for each treatment.

Seeds that fail to germinate two days after they are sown are marked and the roots that subsequently develop from these seeds are not measured.

16.5 HPLC to Measure ACC Levels

In order to measure ACC levels in plant tissue, the Waters AccQ·Tag Method™, which is designed to measure amino acids, may be utilized. This procedure involves the derivatization of ACC with the Waters AccQ·Fluor reagent, and then the stable derivatives are separated by reversed phase HPLC and quantitated by fluorescence (Penrose et al. 2001).

16.5.1 Preparation of Plant Extract

Sixty canola seedling roots (4~5 days old) grown for the growth pouch assay are excised at the juncture between roots and shoots, immediately frozen in liquid nitrogen, and then stored at −80 °C until use. On the day of extraction, the frozen tissue samples are ground in 2.5 mL of 0.1 M sodium acetate (pH 5.5) in a prechilled mortar and pestle, then kept on ice for 15 min. The suspension is centrifuged at 17,500×*g* for 15 min at 4 °C. The supernatant is clarified by centrifugation at 100,000×*g* for 1 h at 4 °C. Next, the clarified supernatant is transferred into glass vials and stored at −80 °C. Since ACC degrades ~5 % per day at 4 °C, precautions should be taken to avoid prolonged storage other than at −80 °C during the preparation of the extracts. Although this method is routinely conducted for canola seedlings in our lab, it can readily be adapted for use with other plant tissues.

16.5.2 Preparation of Standard Solutions and Derivatization

Standard solutions are prepared as stock solutions at 2.5 mM in 0.1N HCl. The components of the standards include ACC, α-aminobutyric acid, β-aminobutyric acid, γ-aminobutyric acid, cysteine, isoleucine, leucine, lysine, methionine, proline, tyrosine, valine, and a protein hydrolysate containing a mixture of 17 amino acids.

Table 16.1 Gradient table for ACC elution

Time (min)	Flow rate (mL/min)	%A	%B	%C
0	1.0	100.0	0	0
0.5	1.0	99/0	1.0	0
3.0	1.0	91.0	9.0	0
13.0	1.0	88.0	12.0	0
14.0	1.0	83.0	17.0	0
16.0[a]	1.0	0	60	40
18.0	1.0	100.0	0	0
23.0	1.0	100.0	0	0

[a]From this point, the column is being washed and conditioned for the next sample
A Waters AccQ·Tag acetate-phosphate buffer concentrate (50 mL diluted with 500 mL Milli-Q water), *B* HPLC-grade acetonitrile, *C* Milli-Q water

These solutions are diluted with sterile distilled water to a final concentration of 0.1 mM. Then, the 0.1 mM solutions are further diluted to generate concentration ranges from 5 to 25 pmol·20 μL^{-1} injection. The standard solutions are divided into 0.5 mL aliquots and frozen at −20 °C; they are used only once then discarded.

Derivatization of the standard solutions and plant tissue extracts are prepared based on the Waters AccQ·Fluor™ reagent Kit manual (Waters Chromatography Inc.).

16.5.3 HPLC

An AccQ·Tag Column (Waters Chromatography Inc.) is used to separate amino acid derivatives and a Hewlett Packard HPLC system is used to detect and measure the amino acid derivatives. A modification of the gradient normally used to separate amino acid mixtures is adapted to enhance the separation of ACC from other amino acids as well as to avoid background levels (Table 16.1) (Penrose et al. 2001). The amount of ACC in a particular plant tissue extract is quantified by comparison with an ACC standard curve that is linear between 1 and 25 pmol per sample.

16.6 Probes to PCR Amplify ACC Deaminase Genes

Once the ACC deaminase activity is known to exist in a bacterial strain, the ACC deaminase gene (*acdS*) can be isolated using the polymerase chain reaction (PCR). Examples of primers that have been used to PCR amplify *acdS* genes are shown in Table 16.2. In general, a practical strategy for identifying *acdS* genes in a strain can be described as following:

– If the genus of the strain has been determined, a search of known *acdS* genes (Nascimento et al. 2014) in the same genus may help with designing a pair of specific primers for this particular strain.

Table 16.2 Primers that have been used to PCR amplify *acdS* genes

Species	Sequences	References
Pseudomonas	F1: TGAAQQTNCAPJGNTTQCCNJG	Sheehy et al. (1991)
	R1: KNMLCATQTGKATPTTNCCNAC	
Achromobacter, *Acidovorax*, *Enterobacter*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, *Serratia*, *Variovorax*	F: GGBGGVAAYAARMYVMGSAAGCTYGA	Hontzeas et al. (2005)
	R: TTDCCHKYRTANACBGGRTC	
Rhizobium	F1: GGCAAGGTCGACATCTATGC	Duan et al. (2009)
	R1: GGCTTGCCATTCAGCTATG	
	F2: GCGAAACGCCCGAAACTG	
	R2: ATCGGGATTGTCCGTTGC	
Burkholderia	F1: ATGAAYCTSCARCGHTTY	Onofre-Lemus et al. (2009)
	R1: TYARCCGTYSCGRAARRT	
	F2: ATGAAYCTSCARMRHTTY	
	R2: TYARCCGTYGCGRAARATV	
Bacillus	F: GGBGGVAAYAARMYVMGSAAGCTYGA	Kumar et al. (2014)
	R: TTDCCHKYRTANACBGGRTC	

– If the specific primers fail to amplify an *acdS* gene or no *acdS* genes have previously been found in that genus, a pair of degenerate primers that target the conserved region of the *acdS* gene may be used.

It is important to mention that another enzyme, D-cysteine desulfhydrase, which is closely related to ACC deaminase, based on DNA sequence data alone, is often annotated as a putative ACC deaminase. In fact, it has been demonstrated that the two enzymes can be interconverted by altering only two amino acid residues (Todorovic and Glick 2008). However, D-cysteine desulfhydrase uses D-cysteine as a substrate rather than ACC. An amino acid sequence alignment between ACC deaminase and D-cysteine desulfhydrase genes indicated that there are several conserved residues that are only present in ACC deaminase (Nascimento et al. 2014), therefore degenerate primers that are designed for amplifying *acdS* gene should include those conserved residues to facilitate isolation of true *acdS* gene.

16.7 Construction of ACC Deaminase Minus Bacterial Mutants

In order to study the effect of ACC deaminase on plant growth promotion, ACC deaminase deficient mutants are often constructed and subsequently used to treat plants in parallel with the wild-type strain (Li et al. 2000; Ma et al. 2003; Sun et al. 2009; Ali et al. 2014). Once the *acdS* gene sequence has been determined, two strategies can be utilized to make *acdS* minus mutants. One strategy involves insertion of a tetracycline resistance gene within the *acdS* gene sequence, while the other strategy involves deletion of a portion of the DNA sequence within the *acdS* structural gene. For the first strategy, a tetracycline resistance gene is utilized because it

is less likely than other antibiotic resistance genes to create a debilitating metabolic load in the mutant strain (Glick 1995b). In either case, a replacement vector containing the disrupted ACC deaminase gene needs to be constructed first.

16.7.1 Strategy 1

- The PCR product of the *acdS* gene is cloned into a cloning vector, such as pGEM-T Easy vector (Promega, Madison, WI, USA), to generate pGEMACC. To interrupt the *acdS* gene on pGEMACC, a tetracycline resistance gene is obtained from the vector pBR322 and inserted within the *acdS* coding region to make pGEMACC^{-}.
- The fragment that contains the *acdS* gene with the tetracycline resistance gene insertion is either digested with restriction enzymes or PCR amplified, and then cloned into a broad host range mobilizable vector, such as pK19mobsacB (Schäfer et al. 1994). The *sacB* gene on the vector codes for levansucrase and confers sucrose sensitivity to Gram-negative bacteria, and is used as a conditional lethal gene to help to select for double cross-over events.

16.7.2 Strategy 2

- If there are appropriate restriction enzyme sites within the *acdS* coding sequence, a fragment of DNA can be removed from the *acdS* gene and then rejoined by T4 DNA ligase (Promega, Madison, WI, USA).
- If there are no ideal restriction enzyme sites within the *acdS* coding sequence, two primers can be designed to amplify a fragment containing the entire pGEM-T easy vector backbone and the N- and C-terminal ends of the *acdS* gene (without the center region of the sequences). Then the fragment is rejoined by T4 DNA ligase (Promega, Madison, WI, USA).
- Next, the fragment containing the mutant *acdS* gene is cloned into pK19mobsacB for subsequent transfer by conjugation.
- After the replacement vectors are constructed, they are transferred into the wild-type strain by conjugation, using a helper strain such as *E. coli* MT616 containing pRK600 (Finan et al. 1986).

16.8 Treating Plants with ACC Deaminase-Containing PGPB

Besides the gnotobiotic root elongation assay, where plant seedlings are grown in growth pouches, ACC deaminase-containing PGPB may also be used to treat plants grown in pots or in the field (Ali et al. 2014; Farwell et al. 2006).

Plant seeds, such as tomato seeds, are surface sterilized with 70 % ethanol for 1 min, then with 1 % commercial bleach for 10 min, and subsequently washed with sterile Milli-Q water. Bacteria that contain ACC deaminase are grown in a rich medium until the OD_{600} reaches ~0.25 ± 0.01. The seeds are then incubated with the bacterial cell suspension at room temperature for 1.5 h. For a negative control, sterile Milli-Q water, rather then the bacterial culture is used to treat the seeds.

For a 9.5 cm × 11 cm plastic pot, four tomato seeds preinoculated either with ACC deaminase-containing bacteria, ACC deaminase mutant bacteria, or Milli-Q water are planted in wet peat-based Sunshine4™ mix aggregate general-purpose growth medium (Premier Horticulture, St. Catharines, Ontario, Canada). After the seeds are placed on the soil, 1 mL of bacterial suspension is added on the top of each seed, and then the seeds are covered with the soil. All of the pots are placed in a green house for up to several months without fertilizer applications. Typically, a total of 24 pots (96 plants) are set for each treatment and the experiment is repeated at least twice.

The (tomato) plants are watered with tap water as needed. At week 7, the plants are transferred to 25 cm × 30 cm pots containing the same growth medium. At week 11 (or whenever it is appropriate for the particular experiment in question), plants are harvested and the fresh biomass is measured. Next, the plants are dried in an oven at 85 °C until there is no further decrease in the plant weight, and the amount of the dried biomass is recorded (Ali et al. 2014).

16.9 Summary and Conclusion

In the last two decades, the interaction between ACC deaminase-containing bacteria and plants has been studied in considerable detail. However, in order to effectively utilize these organisms in the field, it is necessary to develop an even greater understanding of the relationship between plants and ACC deaminase-containing bacteria at a basic level. The gaps in our knowledge notwithstanding, soil bacteria expressing the enzyme ACC deaminase are able to provide a significant measure of protection to a wide range of plants from many different biotic and abiotic stresses, so many researchers have become interested in using the activity of this enzyme as an adjunct to agricultural and horticultural practice as well as in phytoremediation protocols. In this sense, the procedures described herein are an important starting point in these endeavors.

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Chapter 17
Production of Volatile Organic Compounds in PGPR

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Abstract Bacteria affect plant growth through several mechanisms. A recently described mechanism involves the production of microbial volatile organic compounds (mVOCs), which are gaseous molecules capable of interacting with plants in the soil environment. mVOCs may promote plant growth directly, through induced resistance systemic (ISR), or indirectly through suppression of phytopathogens (biocontrol). In this chapter, we describe several experimental designs for evaluation of mVOCs effects on plant growth, ISR or biocontrol mechanisms; potential problems with the methodologies and possible solutions. To date, relatively few mVOCs have been identified and their effects on plant growth characterized. Generally, the effect observed on a particular plant–bacterium interaction was attributed to the pool of mVOCs produced, like an evidence of synergism among chemical compounds.

17.1 Introduction

Bacteria interact with host plants in many ways. These interactions have positive effects that vary depending on the participating species. The chemical compounds (constitutive or induced molecules) synthesized by soil microorganism are either nonvolatile (e.g., phytohormones, siderophores) or gases that diffuse through gaps between soil particles. The latter group is collectively termed microbial volatile organic compounds (mVOCs) (Kai et al. 2009). Microorganisms may also produce inorganic volatile compounds that play roles in the microbial food chain by being assimilated and incorporated into organic matter (bioconversion), or functioning as electron acceptors or donors in metabolic reactions. Such compounds include carbon dioxide, carbon monoxide, hydrogen, ammonia, hydrogen sulfide, and hydrogen cyanide. Their functions in general metabolism are well established but there is not clear proof to date that they participate in communication mechanisms or plant physiological process (Effmert et al. 2012).

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mVOCS are secondary metabolites, derived mainly from fermentation pathways that volatilize because of their low molecular weight (<300 g mol^{-1}), high vapor pressure, and low boiling point (Vespermann et al. 2007; Kai and Piechulla 2009). Studies to date have identified 346 distinct mVOCs released by bacteria. They include 75 fatty acid derivatives, 50 aromatic compounds, 74 nitrogen-containing compounds, 30 sulfur compounds, 96 terpenoids, and 18 compounds containing halogen, selenium, tellurium, or other metal (Schulz and Dickschat 2007). Data about origin, chemical proprieties, and applications for all mVOCs discovered have been deposited in database, such as Super-Scent and DOVE-MO (Dunkel et al. 2009; Effmert et al. 2012). mVOCs analyzed to date have been isolated from species of the genera *Stenotrophomonas*, *Serratia*, *Pseudomonas*, *Bacillus*, *Burkholderia*, *Erwinia*, *Agrobacterium*, *Staphylococcus*, and *Xanthomonas* (not *Azospirillum*). Emission from a given strain is complex blends of odors comprising up to 60 compounds in some cases (Kai et al. 2009). Rhizobacteria VOCs have profiles that vary depending on genomic content, metabolic capability, growth stage and availability of nutrients in the environment (Groenhagen et al. 2013). Different strains of the same rhizobacterial species can be distinguished on the basis of VOCs profile, illustrating the high specificity of their production (Thorn et al. 2011).

The effectiveness of a mVOC depends on its release, emanation/distribution through the environment, and perception by a target organism. mVOCs can move through the soil pore network because they are active in both gas and liquid phases and are capable of revolatization after passing through water-saturated pores. However, they move primarily by vapor diffusion because of their high vapor pressure. Adsorption and desorption of mVOCs are affected by its own chemical properties and the physicochemical properties of the surrounding soil. Humidity in moist or wet soils reduces the adsorption of nonpolar mVOCs to mineral surfaces and increases their adsorption to organic matter. mVOCs sometimes undergo abiotic degradation; mineral surfaces may serve as catalysts for chemical reactions or free radical oxidation with polar functional groups (Effmert et al. 2012).

Functions of mVOCs in beneficial plant–bacteria interactions have been examined by many groups. mVOCs can promote plant growth by increasing biomass and strengthening defenses against pathogenic microorganisms through ISR (Ryu et al. 2004; Ryu et al. 2005; Banchio et al. 2009; Radruppa et al. 2010; Santoro et al. 2011; Farag et al. 2013). ISR generated by mVOCs in the host plant *Arabidopsis thaliana* functions like bioprotector (Ryu et al. 2003). Certain species belonging to the genera *Bacillus* (Ryu et al. 2004) and *Pseudomonas* (Han et al. 2006) produce a short chain organic compound (C4) capable of activate an ISR through an ethylene pathway that protects tobacco (*Nicotiana* spp.) and *A. thaliana* from *Pectobacterium carotovorum* and *Pseudomonas syringae* (Radruppa et al. 2010). Similarly, *Bacillus* spp. produce short chain compounds (C4-C5) that activate ISR through a jasmonic acid pathway that protects cucumber (*Cucumis sativus*) from *P. syringae* and has a repellent effect against *Myzus persicae* (Song and Ryu 2013). Long chain compounds (C11, C13, C16) were recently found in *Paenibacillus polymyxa* that generate ISR through salicylic acid and jasmonic acid pathways that protect *A. thaliana* from necrotrophic and biotrophic phytopathogens (Lee et al. 2012; Park et al. 2013).

mVOCs have been demonstrated to improve biomass of *A. thaliana* by triggering several signaling pathways based on a variety of hormones including auxin, salicylic acid, gibberellins, cytokinins, and brassinosteroids (Ryu et al. 2003, 2005; Zhang et al. 2007, 2008a). Approximately 600 genes have been tested by microarrays analysis for direct effects on plant morphogenesis. Variation of expression was observed in cell wall, primary and secondary metabolism, stresses responses, and auxin homeostasis genes (Zhang et al. 2007; Ortiz-Castro et al. 2008). In addition, mVOCs were found to control sugar/abscisic acid signaling leading to an increase in chlorophyll content and the photosynthetic rate (Zhang et al. 2008a; Farag et al. 2013).

Furthermore, VOCs emitted by *Streptomyces*, *Pseudomonas*, *Burkholderia*, *Bacillus*, and *Serratia* spp. reduced vegetative growth and sporulation of phytopathogenic fungi. This effect confirmed biocontrol activity mediated by gaseous compounds (Kai et al. 2009; Groenhagen et al. 2013).

17.2 Technical Methodologies

17.2.1 Functional Analysis of mVOCs

mVOCs can act over a wide range of distance because their ability to diffuse in aqueous solutions and permeate through the atmosphere. Thereby, they are able to exert physiological effects on other organisms in the absence of physical contact and play important roles both above ground and below ground. In the last years, several studies have documented mVOCs produced by many bacterial species as bacterial metabolite capable of plant growth-promoting rhizobacteria (PGPR) activity. A variety of methodologies, briefly described below, have been developed to study the function of these VOCs.

17.2.1.1 Exposure of Plant to mVOCs

Divided plates method. Generally Petri plates (diameter 90–100 mm) with a central partition are used to avoid contact, because they share only the head space above the culture medium. Bacteria are inoculated on one side and plants on the other side of the partition. Prior to exposure of plants to bacterial VOCs, bacterial strains are cultured on rich liquid medium until reaching stationary phase. After repetitive washes with distilled water of bacterial cultures, 20 µL of the suspension ($OD_{600} = 1$) are applied dropwise on one side of the partition conditioned with mineral solid medium for bacteria growth. The other side of the plate, conditioned with solid medium for plant growth—Hoagland (Hoagland and Arnold 1938) or more commonly MS solid medium (Murashige and Skoog 1962)—is inoculated with seeds, pre-germinated plantlets, or micropropagated plant nodes. Plates are sealed with Parafilm® to minimize air and VOC exchange with the environment and finally incubated in growth cabinets under controlled conditions of light, temperature, and

relative humidity (Ryu et al. 2004; Zhang et al. 2007, 2008a, b, 2009; Radruppa et al. 2010; Blom et al. 2011; Santoro et al. 2011; Lee et al. 2012; Park et al. 2013).

Ring method. To evaluate the effects of bacterial VOCs on plant growth and development, an autoclaved stainless steel ring is placed at the bottom of a square Petri plate (120 × 120 mm), and the compartment is filled with mineral solid medium for bacterial growth. The surrounding space is filled with MS solid medium for plant growth. Twenty microliters of a bacterial overnight culture ($OD_{600} = 1$) is inoculated as a single drop inside the ring. Seeds, pre-germinated plantlets, or micropropagated plant nodes are placed at the top of the plate. The plates are sealed with Parafilm®, and incubated under controlled conditions of light, temperature, and relative humidity (Groenhagen et al. 2013).

Microtiter plate method. To test the effect of bacterial VOCs on plant growth at various distances from the bacterial inoculation site, a 24-well microtiter plate system is used. Seeds, pre-germinated plantlets, or micropropagated plant nodes are placed into each well, conditionated with 1 mL of half-strength MS medium. With the purpose of avoid physical contact between the bacterial inoculation and plants, the two wells of the first row are inoculated with a bacterial suspension at $10^{8–9}$ colony-forming units (CFU) mL^{-1}. Then, plates are incubated under controlled conditions of light, temperature, and relative humidity (Lee et al. 2012).

Magenta box method. The effects of bacterial VOCs are evaluated using Magenta boxes (75 × 75 × 100 mm) coupled in pairs by plastic collar (75 × 75 × 20 mm). Sterilized seeds, pre-germinated plantlets, or micropropagated plants nodes are planted in the boxes conditioned with half-strength MS solid medium. A separate glass vial (5 mL) containing solid medium for bacterial growth is placed within the box. Bacteria cells are harvested from TSA plates in double distilled water to yield 10^9 CFU mL^{-1} as determined by optical density. Two days after plant growth, bacterial suspension culture (50 μL) is added to each vial. Boxes are placed in a growth room under controlled conditions of light, temperature, and relative humidity (Xie et al. 2009).

Plastic square plate method. To evaluate the effect of bacterial VOCs on fruit ripening green (unripe) fruit are collected from field-growth plants and surface sterilized. A bacterial suspension (100 μL; $OD_{600} = 0.5$) is smeared on solid medium in plastic Petri plates (60 × 15 mm). The fruit and Petri plates are placed together in plastic square plates (118.7 × 118.7 × 16.5 mm) and sealed with Parafilm®. The square plates are incubated at 28 °C under fluorescent light at 16 h/day. The percentages color change from green (unripe) to red (ripe) fruit is assessed at day 8 and day 10 after incubation. Fruit ripening is also assessed using a scale of 0–4, where 0 = 100 % green, 1 = breaking green color, 2 = <50 % red color, 3 = 50–89 %, red color, and 4 = ≥90 % red color (Sang et al. 2011).

17.2.1.2 Exposure of Phytopathogens to mVOCs

Divided plate method. To evaluate the biocontrol activity, bacterial PGPR inocula are streaked or inoculated as a single 20 μL drop ($OD_{600} = 1$) on one half of a divided plate containing mineral solid culture medium. The plates are incubated at a controlled

temperature to allow bacteria. After 24 h, a disc (diameter 5 mm) of mycelial plugs of a phytopathogen fungus or 100 μL of an overnight culture ($OD_{600} = 1$) of bacteria phytopathogen is placed on the other half of the plate, containing solid medium (e.g., PDA or malt agar), and the plate is immediately sealed with Parafilm® to prevent volatiles diffusion. The plate is incubated for a sufficient period to allow bacterial and fungal mycelia growth. After that period, bacterial or radial mycelial growth are measured (Fernando et al. 2005; Groenhagen et al. 2013).

Sealed plate method. In order to test biocontrol activity, PGPR bacteria were streaked on the bottom of a Petri plate conditioned with solid medium. A 5 mm diameter mycelial plug is cut from the margin of an actively growing culture and placed in the center of the bottom of a second Petri plate conditioned with solid medium for fungal growth. The bottom of the fungal plate is inverted over the bottom of the bacterial plate and the plates jointly sealed with Parafilm® and incubated at a controlled temperature. After a sufficient incubation period, diameter fungal growth is measured (Fernando et al. 2005; Tonelli et al. 2010; Ting et al. 2011).

17.2.2 Chemical Analysis of mVOCs

Most emitted mVOCs are species specific, suggesting that VOCs profiles or individual compounds can be used as biomarkers (Kai et al. 2009; Thorn et al. 2011). Gas chromatography-mass spectrometry (GC-MS) is a sensitive method that allows determination of the number of different compounds and their relative proportions in the VOCs mixture. Novel compounds that cannot be found in existing databases can be structurally elucidated by chemical methods such as nuclear magnetic resonance (NMR) analysis (Kai et al. 2009).

17.2.2.1 Collection of mVOCs

Many techniques for extraction and collection of mVOCs have been investigated. Two systems have become generally accepted and are frequently used. Within these systems, various research groups have refined them by altering the conditions of stirring, heating (e.g., 25–80 °C) and/or collection timing (e.g., 10–30 min). In addition, saturated solutions of various salts (e.g., KCl, NaCl, Na_2SO_4, NaH_2PO_4) have been applied to improve the quantity and quality of compounds discovered by GS/MS analysis (Valduga et al. 2010). All studies have shown that the mechanism of mVOCs production is extremely specific, depending on not only the microbial strain but also the culture medium and analysis and the analytical techniques used.

Filter headspace collection method. This technique use adsorbent material (e.g., tenax, super Q, charcoal) that favors the binding of compounds with specific chemical proprieties. An Erlenmeyer flask containing the bacterial culture is equipped with an inlet/outlet device and constant airflow. A sterile cotton plug is placed in the inlet, and air is forced to pass through adsorbent traps in the outlet. This method typically involves extraction with organic solvents (e.g., dichloromethane, pentane, hexane) which may result in an inadequate resolution of highly volatile and early-eluting

compounds (Ryu et al. 2004; Kai et al. 2009; Kai and Piechulla 2009; Xie et al. 2009; Blom et al. 2011; Nawrath et al. 2012).

Head space-solid phase microextraction (HS-SPME). In this well-established alternative method the analytes present in the sample are adsorbed on an extracting phase. This methodology has several advantages over (1) the amounts of analyzed compounds is almost twofold higher solvent extraction; (2) it is rapid and requires only sample volumes. Therefore it is suitable for analysis of VOCs present in fermented broths and in samples with low analyte concentration. The nondestructive application of a thin polymeric film immobilized on a fused silica core allows evaluation of samples under varying experimental conditions and can be used to resolve small inorganic or organic gaseous molecules such as CO, CO_2, NH_3, HCN, and ethylene (Kai et al. 2009; Valduga et al. 2010). Head space extractions of mVOCs are typically performed on a magnetic stirrer. SPME fiber (stable flex divinylbenzene/carboxen/PDMS); (DCP, 50/30 μm) are inserted into the headspace above the bacterial sample contained in sealed sample vials with 75 % culture medium. The adsorption period is usually 30 min. The fibers are then desorbed at 210 °C in the injection port of a GC-MS. The fibers are cleaned up leaving them in the injection port for 10 min after each run (Farag et al. 2006; Valduga et al. 2010; Lee et al. 2012).

17.2.2.2 Analysis and Identification

Operating parameters for chromatographic analysis have been developed for optimal separation of compounds in a DB-5 column (30 m × 0.25 mm × 0.25 μm). In the case of SPME, the injection port is operated in splitless mode with a constant He flow of 1.0 mL min^{-1}. The initial oven temperature of 33 °C, is held for 3 min, ramped up at 10 °C min^{-1} to 180 °C, with a final ramp at 40 °C min^{-1} to 220 °C, and held for 5 min. Compounds are considered to be identified only if they demonstrate identical Kovats indices on two columns of different polarity and display mass spectra consistent with those found in the reference library (most commonly, the NIST-GC-MS library). Positive identification of a particular chemical constituent is made by comparison of its retention time and mass spectrum with those of an authentic standard (when available). The majority of VOCs studied today cannot be identified unequivocally using GS-MS libraries, suggesting that in most cases, their structures remain to be elucidated (Farag et al. 2006; Kai et al. 2009; Thorn et al. 2011).

17.2.3 Problems with the Methodologies

17.2.3.1 Sealed System

The above-described systems for evaluating mVOCs production are subjected to various problems and controversies. Researchers often attempt to capture VOCs by sealing the experimental setup, which is obviously in contrast to natural situations. Some experimental systems have been designed or adapted to demonstrate that

exposure to mVOCs increases plant biomass or inhibits phytopathogens. In some co-incubation experiments with partitioned Petri plates a third compartment filled with charcoal to trap VOCs has been used as a negative control. Researchers have found that in the presence of charcoal, fungal mycelial size was close to the control value and *A. thaliana* plants displayed normal growth. In these studies, mVOCs were proved to be responsible for the observed effects (Fernando et al. 2005; Vespermann et al. 2007).

The role of CO_2 in plant growth promotion must be considered. In experimental systems totally sealed with Parafilm® for evaluation of mVOCs effect, CO_2 produced by bacteria metabolism enhances photosynthesis, leading to increased plant biomass harvested. Kai and Piechulla (2009) performed "trapping" experiments using tri-compartment Petri plates. In co-incubation of mVOCs-producing bacteria and plant, the third compartment was filled with $Ba(OH)_2$ which captured atmospheric CO_2 and precipitated it as $BaCO_3$. Even in the presence of $Ba(OH)_2$, bioproducers of active mVOCs were able to promote plant growth. Observed difference in growth between systems with and without $Ba(OH)_2$ may be due to elimination of total CO_2 produced by tested plants, or active mVOCs-producing strain. It is unlikely that enhanced growth promotion is mediated only by bacterial CO_2 emission levels. Others studies, on partitioned Petri plates, have examined bacterial strains (e.g., *E. coli* DH5α) whose CO_2 emission levels are comparable to those of rhizobacteria that produce active mVOCs (Farag et al. 2006; Lee et al. 2012) or that belong to the same species and come from the same location (our group; unpubl. data). Data on plant biomass suggest that only bioproducers of active mVOCs enhance plant growth, and that this effect is not due to CO_2 capture in the experimental system.

17.2.3.2 Culture Medium

mVOCs are meanly products of fermentation pathways and their production is therefore highly dependent on the culture medium and the presence of O_2. Bloom and colleagues studied the production of active mVOCs in various culture media by strains of the bacterial genera *Pseudomonas*, *Burkholderia*, *Bacillus*, *Serratia*, and *Stenotrophomonas*. The media they used included Luria–Bertani (LB) medium, MR-VP medium, which favors production of butanediol, the principal active compound for *Bacillus* spp. (Ryu et al. 2004), MS medium, which had been used in previous studies (Ryu et al. 2003), and Angle medium, which was designed to mimic the soil environment (Angle et al. 1991). The effect of 3 weeks' exposure to mVOCs on *A. thaliana* plants grown in two-compartment Petri plates were evaluated using shoot fresh weight as an estimator of biomass. Most of the bacterial strains displayed plant growth promotion when grown on MR-VP, although few of them produced butanediol. In general, the strongest grown-promoting effects were observed for strain grown on LB plates. On the other hand, certain strains caused reduced growth or death of plants when grown on the nutrient-rich media, LB and MR-VP. The effect of mVOCs was much less notable on the relatively nutrient-poor media MS and Angle (Blom et al. 2011). Others researchers examined mVOCs production on culture media supplemented with mannitol or glucose, and found that

the mVOCs composition and quantities of various compounds varied depending on the carbon source (Kai et al. 2009; Valduga et al. 2010). In conclusion, mVOC production is closely related to culture media composition.

Blom et al. (2011) also examined the effects of the inoculum size (number of bacteria) after observing apparent correlation of bacterial colony sizes and distance from the plant with PGPR observed effects. The relationship between inoculums amount and plant growth response to mVOCs was assessed by dose–response experiment on two-compartment Petri plates. Clear dose-dependence was observed in nutrient rich LB and MR-VP media. In nutrient-poor MS and Angle media, increasing the inoculum amount had no effect on plant growth. The promoting effect on MR-VP medium was observed only when a one drop of inoculum was applied. Application of higher drop numbers or spreading the inoculums on MR-VP medium led to loss of the promoting effect, but not to deleterious effects as those observed on LB medium.

17.3 Applications

Many studies during the past decade have addressed applications of mVOCs produced by PGPR strains on various plants and phytopathogens. Relatively few of these studies (summarized in Table 17.1) have included characterization of the effect and identification of the specific compound responsible for the observed effect.

In the only study to date involving the genus *Azospirillum*, we examined the effects of mVOCs on peppermint (*Mentha piperita*) growth (Santoro et al. 2011). The effects of three PGPR strains (*Pseudomonas fluorescens* WCS417r, *Bacillus subtilis* GB03, *Azospirillum brasilense* SP7) were assessed using two-compartment Petri plates (90 × 15 mm) conditioned with 50 % strength MS solid medium. A single node removed aseptically form a sterile cultured *M. piperita* plantlet was placed in one compartment of the plate and 20 µL of PGPR suspension culture ($OD_{600} = 1$) was applied dropwise to the other compartment of the Petri plate. The plates were sealed with Parafilm® and kept in a growth chamber under controlled conditions of light (16/8-h light/dark cycle), temperature (22 ± 2 °C), and relative humidity (~70 %). Plants were harvested on day 30 and the growth-promoting effects of PGPR treatments were assessed on the basis of biomass and essential oils (EO) production. The effects of mVOC emission on plant development differed for the three PGPRs. *P. fluorescens* and *B. subtilis* mVOCs caused significant ($p < 0.05$) increases in shoot and root biomass and in as EO production. *A. brasilense* mVOCs had no significant effect on the biomass parameters but notably increased EO production, particularly the levels of menthone and menthol.

In summary, the effects of bacterial mVOCs on plant growth and development appear to be species specific. The mVOCs emitted by a particular bacterial strain do not cause the same effects, or to the same degree, in all plant species. Each plant–bacteria combination has its own characteristic responses. Future studies of plant–bacteria interaction should include as many combinations as possible. The results will be both variable and unpredictable.

Table 17.1 Summary of mVOCs identified and their effects

mVOC	Producer	Target	Effect	Reference
Tridecane	*Paenibacillus polymyxa* E681	*Arabidopsis thaliana*	PGPR activity mediated ethylene pathway	Lee et al. (2012)
			ISR mediated by salicylic acid, jasmonic acid and ethylene pathway effective against *Pseudomonas syringae* pv. *maculicola* ES4326	
2*R*,3*R*-Butanediol	*Pseudomonas chlororaphis* O6	*Arabidopsis thaliana*	Systemic tolerance to drought interceded by reducing stomatal aperture this effect is mediated by salicylic acid, jasmonic acid, ethylene an abscisic acid	Cho et al. (2008)
Hexadecane	*Paenibacillus polymyxa* E681	*Arabidopsis thaliana*	ISR against both *Pectobacterium carotovorum* and *Pseudomonas syringae* stimulating salicylic acid pathway	Park et al. (2013)
2*R*,3*R*-Butanediol	*Bacillus subtilis* GB03	*Arabidopsis thaliana*	PGPR activity mediated by cytokinin pathway	Ryu et al. (2004)
	Bacillus amyloliquefaciens IN937a		ISR status mediated by ethylene and effective against *Erwinia carotovora* subsp. *carotovora*	
Acetoin	*Bacillus subtilis* FB17	*Arabidopsis thaliana*	ISR mediated by salicylic acid and ethylene pathway effective against *Pseudomonas syringae* DC3000	Radruppa et al. (2010)
2,4-Di-tert-butylphenol	*Flavobacterium* sp. GSE09	Pepper	Stimulated pepper fruit ripening	Sang et al. (2011)
	Lysobacter enzymogenes ISE13	*Colletotrichum acutatum*	Inhibited mycelial growth, sporulation, spore germination, or appressorium formation	
		Phytophthora capsici		
Cyclohexanol	*Pseudomonas chlororaphis*	*Sclerotinia sclerotiorum*	Inhibition of mycelial plug/sclerotia	Fernando et al. (2005)
Decanal	*Pseudomonas fluorescens*			
2-Ethyl-1-hexanol	*Pseudomonas corrugate*			
Nonanal	*Pseudomonas aurantiaca*			
Benzothiazole				
Dimethyl trisulfide				

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Chapter 18
Stress Physiology in *Azospirillum* and Other PGPRs

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Abstract Rhizobacteria are constantly faced with environmental stimuli stresses and should be responding to a wide range of factors through signal transduction pathways that convert extracellular information into intracellular forms. The cytoplasmic membrane of bacteria is permeable to water but forms an effective barrier for most solute present in the cytoplasmic. To survive osmotic stresses, rhizobacteria need to adapt by accumulating specific solutes under hyperosmotic conditions and releasing them under hyposmotic conditions. These solutes are referred to compatible solutes and glutamate, K^+, trehalose, betaine, among others solutes, are used to response. But new studies to proteins, phospholipids, and polysaccharides and their pattern in response to stress are necessary.

18.1 Introduction

Improvement in agricultural sustainability requires optimal use and management of soil fertility and soil physical properties, and relies on soil biological processes and soil biodiversity (Choudhary et al. 2011). The continuous use of chemical fertilizers and manures to enhance soil fertility and crop productivity often results in unexpected harmful environmental effects, including leaching of nitrate into ground water, surface run-off of phosphorus and nitrogen run-off, and eutrophication of aquatic ecosystems. All plants are known to perceive and respond to stress signals such as drought, heat, salinity, herbivory, and pathogens (Hirt 2009). Soil grown plants are immersed in a sea of microbes and diverse beneficial microorganisms such as plant growth-promoting bacteria (PGPB) as well as plant growth-promoting

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fungi (PGPF) can stimulate plant growth and/or confer enhanced resistance to biotic and abiotic stresses (Lugtenberg and Kamilova 2009).

Soil microorganisms may comprise mixed populations of naturally occurring microbes that can be applied as inoculants to increase soil microbial diversity. Investigations have shown that the inoculation of efficient microbial community to the soil ecosystem improves soil quality, soil health, growth, yield and quality of crops. These microbial populations may consist of selected species including plant growth-promoting bacteria, N_2-fixing microorganisms, plant disease suppressive bacteria and fungi, actinomycetes, and other useful microbes. Microbial inoculants are promising components for integrated solutions to agro-environmental problems because inoculants possess the capacity to promote plant growth, enhance nutrient availability and uptake, and support the health of plants (Adesemoye and Kloepper 2009).

Bacteria belonging to different genera including *Rhizobium*, *Bacillus*, *Pseudomonas*, *Pantoea*, *Paenibacillus*, *Burkholderia*, *Achromobacter*, *Azospirillum*, *Microbacterium*, *Methylobacterium*, *Enterobacter*, among others, provide tolerance to host plants under different abiotic stress environments (Egamberdieva and Kucharova 2009). Rhizobia (*Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*) can also be considered as a soil bacteria with PGPR activity, where root colonization and growth promotion of rice, cereals, and other non-legumes have been reported (Chabot et al. 1996). Use of these bacteria (rhizobacteria) per se can alleviate stresses in an agriculture thus opening a new and emerging application of microorganisms (Choudhary et al. 2011).

Rhizobacteria are constantly faced with environmental stimuli stresses and should be responding to a wide range of factors through signal transduction pathways that convert extracellular information into intracellular forms. The rhizosphere, the soil zone influenced by plant roots is dynamic. Its extent and properties are influenced by soil physical and chemical properties, weather and plant-induced changes in soil water content, the composition and density of soil microbial populations, and the metabolic activities of plants and microbes (Miller and Wood 1996). To survive in different conditions, rhizobacteria need to adapt by the accumulation or the release of specific solutes and changes in their membranes. Rhizobia are distinguished by their existence as both free-living soil bacteria and nitrogen-fixing root endosymbionts. Among the rhizobia, the osmoadaptive mechanisms of the relatively salt-tolerant species *Sinorhizobium meliloti* have been examined in great detail (Miller and Wood 1996; Vriezen et al. 2012). Although *Azospirillum* is also a nitrogen-fixing Gram-negative bacterium found in the rhizosphere, it does not induce differentiated structures on the roots of its plant hosts. Instead, this bacterium colonizes the mucigel sheath layer or spaces between the root epidermis and cortex, thus forming an "associative symbiosis" with a wide variety of plants (Okon 1985; Miller and Wood 1996). Among azospirilla, the osmoadaptive mechanisms of *Azospirillum brasilense* have received the greatest attention.

The main purpose of the chapter is to analyze how different types of abiotic stress affect the physiology of *Azospirillum* and plant growth-promoting rhizobacteria (PGPRs) and how to study the system.

18.2 Stress Tolerance of Rhizobacteria

Rhizobacteria are constantly faced with environmental stimuli stresses and should be responding to a wide range of factors through signal transduction pathways that convert extracellular information into intracellular forms (Paulucci et al. 2012). To survive in different conditions, rhizobacteria need to adapt by the accumulation or the release of specific solutes and changes in their membranes. In nature, there are organisms that can survive desiccation by entering a dormant stage where apparent metabolism is not observed. When rehydrated, these organisms quickly restore their metabolic processes and often show an extremely high tolerance to various types of stress, including radiation, extreme temperatures, pressure, and organic solvents when in the dry state (Vilchez and Manzanera 2011).

Microorganisms possess a positive turgor, and maintenance of this outward-directed pressure is essential since it is generally considered as the driving force for cell expansion. Exposure of microorganisms to high-osmolality environments triggers rapid fluxes of cell water along the osmotic gradient out of the cell, thus causing a reduction in turgor and dehydration of the cytoplasm. To counteract the outflow of water, microorganisms increase their intracellular solute pool by amassing large amounts of organic osmolytes (Kempf and Bremer 1998). These solutes are often referred to as compatible solute because they can be accumulated to high levels by de novo synthesis or transport without interfering with vital cellular processes (Poolman and Glaasker 1998). Osmoprotectans are solutes that alleviate the inhibitory effect of hyperosmotic stress on the microorganisms when they are added to the medium, but often this term is also used for any solute that can overcome osmotic inhibition, but it is preferable to use only compatible solute for any compound that to accumulate either by uptake from the medium or by de novo synthesis and compatible solutes can protect from stress (Poolman and Glaasker 1998).

Osmoprotectants are solutes have been examined for compatible solute production by means of HPLC (high-performance liquid chromatography), NMR (nuclear magnetic resonance), and GC (gas chromatography) methods. The composition of the culture medium is important to study these molecules. The media must have a definite composition and media supplements such as yeast extract, peptone, and trypticase contain considerable amounts of protecting solutes or precursors. It can lead to false results (Galinsky 1995). Thus, the study of the use of each solute compatible as carbon or nitrogen source must be carried out prior to any study of stress.

These osmoprotectants are highly congruous with the physiology of the cell and comprise a limited number of substances including K^+, glutamate, trehalose, proline, glycine betaine, proline betaine, ectoine, and choline. The Gram-negative enteric bacteria are model organisms to study stress physiology but similarities and notable differences exist between the osmoadaptive responses of this group and the Gram-negative rhizobacteria.

Bacteria use lipid diversity to alter their membranes in response to environmental stress ensuring that the cell membrane remains within optimal membrane state and continued functionality (Finean and Michell 1981; Hazel and Williams 1990).

In general, perturbation of membrane fluidity by extrinsic chemical agents or other factors initiates an active response based on intrinsic chemical changes such as the modification of existing lipids and the de novo synthesis that tends to counteract the perturbation (Soltani et al. 2005; Denich et al. 2003).

In bacteria, the membrane lipid composition has been relatively well defined with predominant polar lipids including phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) with varying arrangements of chain length, degree of saturation, isomer conformation, branching, and cyclization of fatty acid (FA) (Denich et al. 2003). The phospholipid (PL) is arranged with the polar heads oriented externally with the lipid acyl chains directed toward the interior of the bilayer (Singer and Nicolson 1972; Rilfors et al. 1984). PE, PG, and cardiolipin (CL) are characteristic PL of most Gram-negative bacteria. Phosphatidylcholine (PC) has also been found in bacteria, and it is more widespread than originally thought, although its role is unclear (López Lara and Geiger 2001).

Phospholipid fatty acids are commonly extracted from samples by use of modifications of the Bligh and Dyer method followed by purification with techniques of chromatography (Bligh and Dyer 1959). The most widely used modifications of this method include use of a buffer (rather than water) in the extraction mixture. PL changes have been reported in response to environmental stress such as that produced by temperature (Russell 1992) and salt (Sutton et al. 1991). In addition, low-oxygen conditions have been shown to increase PE and PG amounts and also to decrease PC amount (Tang and Hollingsworth 1998). It was speculated that PC might serve a special function during host–pathogen/symbiont interactions. The importance of PC for the establishment of successful interactions with eukaryotic hosts is exemplified by the fact that *Agrobacterium tumefaciens* mutants lacking PC are unable to form tumors in susceptible plants (Wessel et al. 2006).

18.3 Tolerance and Osmoregulation in *Azospirillum*

Azospirillum genus is capable of stimulating growth in graminaceous as well as in nongraminaceous plants. *Azospirillum*–plant association is accompanied by biochemical changes in roots which, in turn, promote plant growth (Pereyra et al. 2006). A better understanding of the mechanisms of osmo-adaptation is expected to contribute to the long-term goal of enhancing plant–microorganism interactions under arid and semi-arid conditions (Bashan and Holguin 1997).

Miller and Wood (1996) resume the known patterns of compatible solute used by the most extensively characterized rhizobacteria, *S. meliloti* and *A. brasilense*. Although few of the osmoregulatory mechanisms present in rhizobacteria have been fully defined, some mechanistic predictions can be based on our existing knowledge of the enterobacterial systems. For example, organic compounds that are participants in intermediary metabolism (carbon or nitrogen sources) usually accumulate via the modulation of metabolic pathways and do not serve as compatible solute. On that basis, osmoregulatory mechanisms can be anticipated by determining whether

a particular compound is able to serve as a compatible solute and/or a carbon or nitrogen source (C/N).

Glutamate is accumulated in azospirilla during growth at elevated osmolality, and it is the predominant intracellular amino acid when *A. brasilense* is grown in the presence of moderately high NaCl concentrations (e.g., 0.3–0.5 M NaCl) (Madkour et al. 1990). In *Azospirillum* spp., the degree of osmotolerance is positively correlated with lower efficiencies to use amino acids and betaines for growth as sole carbon and nitrogen sources and the ability to take up and accumulate choline or glycine betaine as osmoprotectants (Hartmann 1988; Riou and Le Rudulier 1990). Hartmann et al. (1992) showed that mutants of *A. brasilense* Sp7 and Cd resistant towards 3,4-dehydroproline (DHP), exhibiting improved osmotolerance, have been selected under NaCl-stress in the laboratory.

Nabti et al. (2007) reported that *A. brasilense* NH showed maximum growth at 200 mmol/L NaCl, in the absence of osmoprotectants, while growth and IAA production was retarded at 1.7 mmol/L NaCl. It can be characterized as a halophilic and moderately halotolerant bacterium because *A. brasilense* NH appears to be more osmotolerant than *A. brasilense* Sp7 (type strain) with optimal growth at 100–150 mmol/L NaCl and optimal growth at very low NaCl concentrations. In other studies, seven azospirilla were growth under 300 mM NaCl stress and showed variability in salt tolerance from the most salt-sensitive strain Cd to the most salt-tolerant strain MTCC4036, and also a wide variation in the ability to utilize glycine betaine as osmoprotectant, with only three of seven showing an osmoprotective effect (Chowdhury et al. 2007). Under salt stress, all five isolates showed considerably higher growth than Sp7 and Cd. Strain MTCC4036 was the most salt-tolerant strain. When 1 mM of glycine betaine was provided together with 300 mM NaCl, strains MTCC4037, MTCC4038, MTCC4039, and Cd did not show any notable improvement in growth, whereas strains MTCC4035 and Sp7 showed osmoprotection by glycine betaine. Surprisingly, strain MTCC4036 showed notable growth inhibition by glycine betaine. Thus, neither salt tolerance nor the ability to utilize glycine betaine as osmoprotectant seems to be a species-specific characteristic, but rather it is dependent on the strain (Chowdhury et al. 2007).

A. brasilense did not tolerate osmotic stress or accumulate significant levels of trehalose. Rodríguez Salazar et al. (2009) have transformed *A. brasilense* with a plasmid harboring a trehalose biosynthesis gene-fusion from *Saccharomyces cerevisiae* and were able to grow up to 0.5 M NaCl and to accumulate trehalose. When this transformed bacterium was used to interact with maize plants, *A. brasilense* caused an increased drought tolerance and a 73 % increase in biomass compared with inoculation with the wild-type strain. Therefore *A. brasilense* modified could be used to increase the biomass, grain, yield, and stress tolerance in other relevant crops.

Kamnev et al. (1999) characterized phospholipid composition of *A. brasilense* Sp245 cell membrane, and emphasized the importance of growth phases. It was reported that the cells grown in two phases showed little changes in phospholipid content and that phosphatidylethanolamine was most abundant phospholipid and indicated the importance of the fatty acids of the PL. When *A brasilense* Az39 was growth in the presence of PEG6000 15 mM (−0.28 MPa), the wet biomass and viability

were reduced by 35 %, increased PC (51 %) while PE decrease (67 %) (Dardanelli et al., date not published). This means that bacteria subjected to such stresses may have changed its molecular components influencing the molecular dialogue and early events of interaction.

18.4 Responses of Rhizobacteria to Abiotic Stress

Since soils are subjected to high temperature stress in summer, saline concentration also increase, which may have detrimental effects on the introduced rhizobacteria. Temperature can affect rhizobial persistence in inoculants during shipment or in storage. Also temperature and salt stress can influence survival in soil and can limit both nodulation and nitrogen fixation (Vriezen et al. 2007; Paulucci et al. 2011). An understanding of the growth of *Rhizobium* is likely when the physiology of these organisms has been carefully studied under these suboptimal conditions (Abdelmounmen et al. 1999; Kulkarni and Nautiyal 2000).

Little is yet known about the biochemical and physiological basis of saline and temperature tolerance by rhizobia nodulating *Arachis hypogaea* (peanut) roots. In peanut rhizobia (*Bradyrhizobium* sp. ATCC10317, SEMIA6144, and TAL1371 strains) high growth temperature (37 °C) provoked a slightly reduced biomass production, an increase in the cellular content of low molecular weight oligosaccharides and fully suppressed the synthesis of neutral glucans (Dardanelli et al. 1997). The FA composition profiles of *Bradyrhizobium* and *Rhizobium* are quite different, and effects of growth phase (Boumahdi et al. 1999; 2001) and low temperature (Drouin et al. 2000; Théberge et al. 1996) on FA synthesis and composition in these genera have been studied, but not the effects of high temperature or high salinity. In peanut rhizobia (*Bradyrhizobium* sp. ATCC10317, SEMIA6144, and TAL1371 strains) high growth temperature (37 °C) provoked a slightly reduced biomass production, an increase in the cellular content of low molecular weight oligosaccharides and fully suppressed the synthesis of neutral glucans (Dardanelli et al. 1997). Salt and combined conditions affected negatively the viability of SEMIA6144 and the TAL1000 viability. Both rhizobia survived under stress conditions and they showed different saline tolerance, 50 mM SEMIA6144 and 300 mM TAL1000. It was observed that viability of salt-tolerant TAL1000 was unaffected by high temperature, consistent with studies that indicate a relationship between salt tolerance and temperature tolerance in rhizobial strains (Paulucci et al. 2011, 2012). In certain genera of rhizobia, tolerance to salinity has been associated with tolerance to high temperatures. Previous studies have shown that strains of *S. meliloti*, salt tolerant can grow well at elevated temperatures (Zhang et al. 1991) while *Rhizobium leguminosarum*, salt sensitive is much less tolerant to higher growth temperatures (Lindstrom and Lehtomaki 1988). Previous studies relating to the ability of rhizobial strains to grow in presence of salts have demonstrated a marked variation in tolerance. Some strains such as *Bradyrhizobium*

japonicum are inhibited by concentrations below 100 mM (Elsheikh and Wood 1990). By contrast, the growth of various strains of *S. meliloti* can occur at concentrations above 300 mM NaCl (Bernard et al. 1986), *Rhizobium* spp. isolated from plants *Hedysarum* nodules, *Acacia*, *Prosopis*, and *Leucaena* can tolerate up to 500 mM NaCl (Zhang et al. 1991).

In peanut rhizobia, alterations in membrane lipid composition in response to salinity and variation in the trehalose content have been detected in response to osmotic stress (Dardanelli et al. 2000, 2008, 2009; Medeot et al. 2007). At 400 mM NaCl, the synthesis and accumulation of this compatible solute in the salt-tolerant strain *Bradyrhizobium* sp. ATCC 10317 increased while in TAL169 diminished, indicating a strain dependent response. However, the growth of these peanut rhizobia under osmotic did not induce changes in their trehalose content (Dardanelli et al. 2009). An explanation for this response can be the genetic variability of peanut symbionts or the use of other compatible solutes to modulate their response to stress. Under combined stress, temperature and salinity, TAL1000 and SEMIA6144, the changes in FA percentages led to alteration of the ratio between unsaturated to saturated FA (U/S), (Table 18.1), which decreased in all experimental conditions for TAL1000 and SEMIA6144, but in SEMIA6144 it was not as remarkable as in TAL1000 (Paulucci et al. 2011).

Like many other microorganisms, *Pseudomonas* species respond to abiotic stress by accumulating compatible solutes. *P. halosaccharolytica* and *P. halophila* accumulated betaine present in the medium during osmotic stress conditions and accumulated ectoine and hydroxyectoine in a glucose mineral medium (Monteoliva-Sanchez et al. 1993; Severin et al. 1992). *P. aeruginosa* produced and accumulated a carboxamide of glutamine, *N*-acetylglutaminylglutamine amide (NAGGN), when subjected to osmotic stress in the absence of betaine (D'Souza-Ault et al. 1993).

In response to reduced water activity, *P. putida* S12 accumulated *N*-acetylglutaminylglutamine amide (NAGGN) simultaneously with mannitol and both solutes could be synthesized de novo. The intracellular amounts of each solute varied with both the type and amount of osmolyte applied to induce osmotic stress in the medium. Addition of betaine to the medium resulted in accumulation of this compound and depletion of both NAGGN and mannitol. Both solutes were accumulated when sucrose instead of salts was used to reduce the medium water activity and when glucose was substituted by other carbon sources (Kets et al. 1996).

The role of the new molecules in stress physiology is currently being investigated to PGPRs. *P. putida* (NBAII-RPF9) was identified as an abiotic stress-tolerant bacterium capable of growing at 45 °C as well as in 1 M NaCl. The analysis revealed involvement of heat stress responsive molecular chaperones and membrane proteins during heat stress. During salt stress, proteins involved in metabolic processes were found to be upregulated to favor growth and adaptation of the bacterium. Heat shock chaperones viz., DnaK and DnaJ were expressed under both saline and heat stress. Also, GroES and GroEL proteins were expressed under both tolerance and shock, indicating the importance of proteins with other molecules in stress tolerance (Rangeshwaran et al. 2013).

Table 18.1 Effects of temperature and salinity stress on trehalose content, fatty acid composition of two peanut-nodulating rhizobia

Molecules	Strains	Growth conditions			
		28 °C	28 °C + NaCl	37 °C	37 °C + NaCl
Trehalose (μmol/mg proteins)	TAL1000	0.68 ± 0.09	3.44 ± 0.05*	2.88 ± 0.06*	3.78 ± 0.08*
	SEMIA6144	0.46 ± 0.04	2.89 ± 0.04	0.95 ± 0.07	3.00 ± 0.06
Fatty acid type (%)					
Saturated					
Stearic acid (18:0)	TAL1000	12.6 ± 1.5	14.6 ± 1.6*	24.0 ± 2.7*	29.0 ± 0.9*
	SEMIA6144	1.40 ± 0.3	1.66 ± 0.3	2.00 ± 2.16	2.16 ± 0.9
Palmitic acid (16:0)	TAL1000	8.40 ± 1.7	9.30 ± 1.5	20.0 ± 2.3*	16.1 ± 1.8*
	SEMIA6144	11.0 ± 1.2	12.6 ± 2.7	18.6 ± 3.9*	19.7 ± 3.5*
Unsaturated					
Palmitoleic acid (16:1n-7)	TAL1000	ND	ND	ND	ND
	SEMIA6144	0.42 ± 0.0	0.90 ± 0.4	0.65 ± 0.2	0.56 ± 0.2
cis-Vaccenic acid + oleic acid (18:1)	TAL1000	63.3 ± 5.4	55.8 ± 2.7	8.20 ± 0.4*	4.20 ± 0.0*
	SEMIA6144	84.0 ± 2.2	82.4 ± 2.9	73.5 ± 3.4*	63.5 ± 6.8*
Eicosatrienoic acid (20:3)	TAL1000	6.30 ± 1.9	9.20 ± 1.2	15.8 ± 1.6*	19.0 ± 4.7*
	SEMIA6144	ND	ND	ND	ND
Cyclopropane $19:0_{cyclo}$	TAL1000	3.40 ± 0.7	3.85 ± 0.3	10.0 ± 1.5*	14.5 ± 1.7*
	SEMIA6144	ND	ND	ND	ND
Others	TAL1000[a]	6.70 ± 1.6	7.10 ± 0.8	23.2 ± 2.6*	17.1 ± 1.9*
	SEMIA6144	1.10 ± 0.0	2.55 ± 0.7	5.00 ± 0.2	5.30 ± 1.3
U/S[b]	TAL1000	3.3	2.7	0.5	0.5
	SEMIA6144	7.0	6.1	3.6	3.3

Trehalose was extracted and analyzed by anthrone reagent. Lipids were extracted, and fatty acid of total lipid were converted to methyl esters and analyzed by GC. Percentage of each fatty acid is relative to total fatty acids defined as 100 %. Values represent means ± SEM of three independent experiments

ND not detected

*Difference from control (28 °C) value statistically significant at $P < 0.05$ level

[a]Correspond to two peaks of retention times of 36 and 34.7 min. Such peaks could correspond to FA of more than 18 carbon atoms

[b]Ratio between sum of unsaturated and sum of saturated fatty acids

Fatty acids from the phospholipidic fraction are usually transformed into their less polar methyl ester derivatives (FAMEs) by mild alkaline methanolysis for analysis by gas chromatography. In the extraction of lipids from *Azospirillum*, the lipid extract may be slightly discolored. This is due to the ability of pigment synthesis that *Azospirillum* has but that does not interfere with the handling of the sample or separation of various lipid fractions. Table 18.2 shows how water stress affects fatty acid composition of the *A. brasilense* Az39. These changes have also been observed in the phospholipid composition which varies from this type of stress and these changes may be the cause of the bacterium presenting lower adhesion to the roots of maize and peanut (data not shown).

Table 18.2 Effects of hydric stress on fatty acid composition of *A. brasilense* Az39

Fatty acid	Control (−0.07 MPa)	Hydric stress (PEG6000 −0.28 MPa)
Saturates		
Stearic acid (18:0)	2.14 ± 0.2	5.42* ± 0.4
Palmitic acid (16:0)	8.19 ± 0.4	10.2* ± 1.9
Myristic acid (14:0)	0.44 ± 0.2	0.50 ± 0.01
Lauric acid (12:0)	1.54 ± 0.4	1.31 ± 0.4
Unsaturates		
cis-Vaccenic acid (18:1Δ^{11})	63.60 ± 2.2	59.2* ± 2.4
Palmitoleic acid (16:1)	22.50 ± 2.5	23.80 ± 2.9
ND	1.20 ± 0.4	2.06* ± 0.8
U/S	7	4.75

Lipids were extracted, and fatty acids of total lipid were converted to methyl esters and analyzed by GC (Paulucci et al. 2011). Percentage of each fatty acid is relative to total fatty acids defined as 100 %. Values represent means ± SEM of three independent experiments
ND not detected
*Difference from control value statistically significant at $P < 0.05$ level. U/S ratio between sum unsaturated and sum saturated fatty acid

18.5 Outlooks

Plants are frequently exposed to adverse environmental conditions where salinity, desiccation, and drought are considered the most important abiotic factors limiting plant and rhizobacteria growth. Plant growth-promoting bacteria residing in the rhizosphere frequently encounter desiccation, salinity, temperature, among other stress factors, and require strategies to persist during times of stress. On one hand, one strategy to survive in different conditions is intracellular amassing of compatible solutes accumulate, during growth in media of diverse osmotic strength. On the other hand, osmoregulatory mechanisms are designed to adjust compatible solute levels by modulating their biosynthesis, catabolism, uptake, or efflux. Bacterial survival depends on membrane lipid homeostasis and an ability to adjust lipid composition to acclimatize the bacterial cell to various environments.

New studies to proteins, phospholipids, and polysaccharides and their pattern in response to stress are necessary. Based on our observations to peanut rhizobia, it is tempting to speculate that trehalose and phospholipids may be involved in some of those regulatory mechanisms.

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Part III
Biotic Interactions Toolbox

Chapter 19
Alleviation of Abiotic and Biotic Stresses in Plants by *Azospirillum*

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Abstract In the face of global changes, plants must adapt to a wide range and often combined biotic and abiotic stresses that seriously impaired plant growth and development. Plants develop complex strategies to deal with water stress conditions, soil fertility losses, soil pollutions, pests, and disease. Emerging evidence suggest the involvement of common hormonal players in plant defense signaling pathways triggered in response to biotic and abiotic stresses. Besides plant strategies, plant growth-promoting rhizobacteria (PGPR), which colonize the root system and establish cooperative interactions with plants can improve their growth and help them to adapt to and cope with multiple stresses including drought, salinity, heavy metal pollutions, and parasites. Accordingly, PGPR supply added values to the plant defense strategies by expressing many relevant functions for modulating the plant hormonal balance, increasing nutrients supply to the plant, improving the functional and physical properties of protective barriers against plant parasites. Among PGPR, *Azospirillum* strains were long viewed as biofertilizers and less as biocontrol agents. It is becoming evident that *Azospirillum* is able to protect plants against a myriad of detrimental conditions. This review provides an update of works regarding the ability of *Azospirillum* strains to alleviate plant stress and brings out the relevant involved plant-beneficial functions. Developing PGPR-based bio-inoculants is a promising strategy to improve the growth and health of crops and develop sustainable agriculture.

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19.1 Introduction

As principal producers of organic matter, plants play a major role in ecosystem dynamics at both micro- and macroscopic scales. Through their roots, plants strongly influence the physical structure and chemical composition of the soil compartment (Hiltner 1904). The region of soil directly under the influence of roots, called the rhizosphere, is colonized by a huge abundance and diversity of microorganisms in continuous interactions with plants (Hartmann et al. 2008). A root system releases a large quantity of amino acids, sugars, and organic compounds by a phenomenon called root exudation (Haichar et al. 2014). Root exudates constitute a real "buffet" for rhizospheric microorganisms (López-Guerrero et al. 2013), and according to their chemical compositions they modulate the structure, abundance, and diversity of microbial communities in the rhizosphere. These microorganisms associated to the roots, including fungi, protozoa, nematodes, and bacteria, constitute the rhizo-microbiote. These microorganisms can establish a wide range of ecological interactions with plants, ranging from parasitism to symbiosis, and passing by commensalism (Hartmann et al. 2008).

Plants and their rhizo-microbiote must cope with environmental condition changes and can suffer from different biotic (diseases, competition for resources) and abiotic (drought, cold, acidification) stresses. In a context of global changes caused by human activities (urbanization, pollution and deforestation), stresses to which plants must adapt are more diverse, important, and frequent (IPCC 2001). Since the middle of the twentieth century, synthetic fertilizers and pesticides have considerably improved crop yields. This "green revolution" has allowed the world population to increase. However, chemical inputs and intensive use of soils caused environmental pollutions and soil fertility troubles (Gerhardson 2002; Ramirez et al. 2012; Eisenhauer et al. 2012). The world population still continues to grow rapidly. Increasing agricultural production is thus desirable, but the potential for expanding agriculture area is limited and chemical inputs are harmful to animals and humans and can accumulate in the environment. Environmentally friendly solutions, based on the use of microorganisms as biofertilizers and biocontrol agents, exist to improve crop yields (Berg and Smalla 2009; Babalola 2010). However, these solutions are rarely used by farmers due to their small commercial scale. Developing these solutions requires better understanding of the interactions between plants and their rhizo-microbiote, especially under biotic and abiotic stress conditions.

Modifications of environmental conditions can dramatically affect plant development and deeply modify the structure and diversity of root-associated microbiotes (Compant et al. 2010). Plants set up various physiological responses to limit the effects of stress on their development. The rhizo-microbiote can be directly affected by biotic and abiotic stresses, but also indirectly through the physiological modifications induced by stresses in plants. Conversely, the rhizo-microbiote can also modify plant physiology and help it to tolerate stresses. Indeed, cooperative microorganisms have plant-beneficial properties like inducing plant systemic defense, improving mineral nutrition, and controlling plant pathogen development

(Vacheron et al. 2013). Plant-beneficial properties are recovered especially in plant growth-promoting rhizobacteria (PGPR), which do not belong to a unique phylogenetic clade (Bruto et al. 2014). PGPR establish associative symbiosis with plant and colonize their roots without inducing the formation of a symbiosis-dedicated organ.

Several studies demonstrate the ability of PGPR to alleviate biotic and abiotic stresses affecting plants (Dimkpa et al. 2009). The most studied PGPR belong to the genera *Bacillus*, *Pseudomonas*, and *Enterobacter* (Babalola and Akindolire 2011). Bacteria of the genus *Azospirillum* are less known as being able to protect plants against stresses, but they have several plant-beneficial functions potentially efficient to alleviate biotic and abiotic plant stress (Wisniewski-Dyé et al. 2011). These include (1) modification of the plant hormonal balance (auxin production, ethylene modulation) resulting in root system augmentation; (2) improvement of mineral nutrition by siderophores production and fixation of atmospheric nitrogen; (3) production of antimicrobial compounds and inducing plant systemic defenses.

This chapter focuses on the technical methods that bring to light the beneficial traits of PGPR and, in particular, of bacteria from *Azospirillum* genus for their role in alleviating biotic and abiotic plant stresses.

19.2 Plant Responses to Biotic and Abiotic Stresses

19.2.1 Responses to Biotic Stresses

Mechanisms of plant defense against biotic stresses are complex and consist of several layers of defense. The recognition of pathogen-associated molecular patterns (PAMPs) can lead to the induction of protective responses in plants, such as callose deposition, oxidative bursts, production of antimicrobial compounds, and programmed cell death. This response corresponds to PAMP-triggered immunity (PTI). If pathogens are able to secrete effectors that suppress PTI, disease occurs resulting in effector-triggered susceptibility (ETS). If pathogens secrete effectors that are recognized by the plant, plant disease resistance occurs resulting in effector-triggered immunity (ETI). The latter involves the recognition of an avirulent (Avr) factor from the pathogen by a plant resistance protein (R). This leads to a hypersensitive response (HR), corresponding to a localized cell death that prevents root invasion of plant tissue by the pathogen.

Plants respond to pathogen attack by synthesizing pathogenesis-related (PR) proteins. They encode enzymes like chitinases and glucanases that can hydrolyze the cell walls of fungal pathogens (Mauch et al. 1988). In addition, plants also use the proteasome system to degrade proteins impaired by cellular stress. Ubiquitin is used to trigger this response (Dreher and Callis 2007). It acts as a covalent molecular tag to target proteins that must be degraded. After delivery to the proteasome, the poly-ubiquitylated substrates can be de-ubiquitylated and cleaved in small peptides to release free amino acids.

The induction of many defense-related genes is often linked to the increase of plant endogenous content of salicylic acid (SA), a key signaling molecule involved in plant defense against pathogens, and the establishment of the systemic acquired resistance (SAR), which renders the plant more resistant to subsequent attacks by pathogens.

Besides SA, reactive oxygen species (ROS) produced in part from mitochondria, and nitric oxide (NO) contents strongly increase following pathogen recognition. All of these signaling molecules coordinate defense responses in plant (Alvarez 2000; Neill et al. 2002; Laloi et al. 2004; Wendehenne et al. 2004; Delledonne 2005; Torres and Dangl 2005; Amirsadeghi et al. 2007).

Another key hormone, ethylene (ET), plays a major role in plant responses to pathogens. ET is both a plant growth regulator and a stress hormone. ET is produced endogenously by plants and in soil and plays a key role in inducing multifarious physiological changes in plants at molecular level. Under stress, the endogenous production of ET is accelerated substantially. This hormone stimulates the transcription of numerous defense-related genes (Dreher and Callis 2007). Transcription factors of the EIN3 (Ethylene-insensitive 3) family play a major role in the regulation of plant defense responses (van Loon et al. 2006). Jasmonate (JA) promotes resistance to microbial pathogens and to insects. JA and ET mostly operate synergistically to activate the expression of defense-related genes, and share a similar target gene network (Schenk et al. 2000). Contrariwise, SA and JA mostly operate in opposite ways. Certain key regulators play a pivotal role in the balance between SA and JA pathways (Li et al. 2006). For instance, it has been shown in *Arabidopsis* that the transcription factor WRKY70 acts as a positive regulator of SA-dependent defenses and a negative regulator of JA-dependent defenses whereas the Mitogen Activated Protein Kinase 4 (MPK4) acts inversely. The Nonexpressor of Pathogenesis-Related genes 1 (NPR1) also plays an important role in regulating the SA and JA pathways. It is important to note that the expression of PR1 is typically used as a marker of the induction of SA-dependent defense pathway (Martin et al. 2003), while the plant defensive PDF1.2 is one of the most useful markers for the induction of SA-independent defense pathway (Pieterse et al. 2002).

Numerous studies provide evidence that plant defense signaling networks are extremely complex. The involvement of auxin, abscisic acid (ABA), and gibberellic acid also act as important components of the signaling network involved in the regulation of defense responses against various pathogens (Bari and Jones 2009). Defense signaling networks activated by the plant depend on the life modes of pathogens. Briefly, SA is mainly involved in the activation of defense responses against biotrophic and hemi-biotrophic pathogens whereas JA and ET are usually involved in defense against necrotrophic pathogens. Since pathogens are able to produce phytohormones, numerous recent studies suggest that pathogens can manipulate the defense-related regulatory network of plants. Thus plant pathogens seem to manipulate components of hormone biosynthesis and signaling machinery leading to hormone imbalances and alterations in plant defense responses.

19.2.2 Responses to Abiotic Stresses

Plants have developed common strategies to cope with abiotic stresses like drought, flooding, salinity, chilling, or high temperatures. One common mechanism involves the accumulation of compatible solutes, like glycine betaine (amine), trehalose (sugar), and proline (amino acids) (Ashraf and Foolad 2007; Seki et al. 2007).

In many plant species, the quaternary amine, glycine betaine, is synthesized from choline at high level in response to various types of abiotic stresses, and protects plants against water deficiency, frost, and salinity (Chen and Murata 2002). It acts by stabilizing the quaternary structures of enzymes and proteins, and protecting cell membranes.

Other compatible solutes like mannitol, proline, and sorbitol are produced for scavenging ROS, which are produced by plants experiencing drought, salt, and temperature stresses. ROS can directly damage cellular components, and the accumulation of compatible solutes may protect cells against increased levels of ROS, thereby resulting in the protection of plants against stress-induced damages (Chen and Murata 2002). Accordingly, upregulation of anti-oxidative enzymes, like superoxide dismutase, is a general response to different abiotic stress conditions.

Polyamines such as putrescine, spermidine, and spermine also accumulate in response to abiotic stresses. Transcriptomic analyses in the model plant *Arabidopsis*, the utilization of polyamine-overproducing transgenic plants or, contrariwise, of mutants deficient in polyamine biosynthesis permit to evidence the involvement of polyamines in the tolerance of plants to different kind of stresses (Alcázar et al. 2006). It has been suggested that they may act as ROS scavenging molecules and as membrane protectors.

In response to water deficit or salinity stress, plants increase the synthesis of osmolytes (Farooq et al. 2009), thereby leading to increased osmotic potentials within cells and adaptation of the plant to drought. ABA plays a prominent role in plant responses to drought. It can directly affect ion transport in guard cells, thereby controlling stomatal aperture and plant transpiration (Roelfsema et al. 2004). In addition, it induces the expression of drought stress-related genes. The level of this hormone increases in response to drought and salt stresses (Seki et al. 2007).

The levels of several other hormones are induced in response to abiotic stresses. This is the case of the ET stress hormone, whose synthesis increases when plants are exposed to different types of abiotic stresses. As mentioned previously, the EIN3 transcription factor acts as a positive regulator that turns on a complex upstream network of signaling responses, thereby leading to the activation of numerous defense reactions (cell wall modification, oxidative burst). Increased levels of NO synthesis and subsequently the upregulation of NO-dependent defense genes are observed in response to drought, high or low temperature, salinity, heavy metals, and oxidative stress (Arasimowicz and Floryszak-Wieczorek 2007).

Increasing evidence suggests that crosstalk exists between plant responses to biotic and abiotic stresses. Signaling pathways regulated by ABA, and ET, play key roles in this crosstalk (Fujita et al. 2006). The generation of ROS appears as a

key convergent response mechanism between biotic and abiotic stresses (Mittler et al. 2004; Fujita et al. 2006). Transcriptomic analyses have thus revealed that a large set of genes that encode ROS-scavenging enzymes are commonly induced when plants are subjected to abiotic and biotic stress treatments (Fujita et al. 2006). Plants respond to biotic and abiotic stresses by changing their physiology and metabolism, in order to limit the negative effects of stresses on plant tissues. Root-associated microbial communities might also help them. Some rhizobacteria are indeed capable of alleviating biotic and/or abiotic stresses.

19.3 Plant Stress Alleviation by Rhizobacteria

19.3.1 Rhizobacteria Able to Reduce Stress in Plants

Rhizobacteria, including PGPR, dispose of a wide range of beneficial functions that may increase plant growth under stress condition (Dimkpa et al. 2009). A broad taxonomic and functional diversity occurs in the plant rhizosphere (Bouffaud et al. 2014) and may affect plant fitness under stress condition (drought, salinity, pollutions, parasite attacks, etc., Table 19.1). PGPR are found in all clades of Proteobacteria especially in Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria, and in Firmicutes such as in Actinobacteria. Hence, there are no specific media or a unique way to isolate PGPR able of alleviating stress in plant. Functional approaches can be developed to select potential PGPR.

One of the most common used methods is firstly to isolate bacteria and then to test whether they share biocontrol effects against plant pathogens and/or have plant growth stimulation properties under abiotic stresses like drought, salinity, or heavy metal stress. Mayak et al. (2004) screened bacteria that produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which is known for lowering the plant content of the stress hormone ET and enhancing plant growth under salt stress. *Achromobacter piechaudii* strains were isolated from salty soils on solid DF medium containing ACC as the sole source of nitrogen, and then tested for their capacity to reduce salt stress effects on tomato seedlings (Mayak et al. 2004). The same method was employed to isolate fluorescent pseudomonads, which alleviate salinity stress on canola (Jalili et al. 2009) and drought stress on pea (Arshad et al. 2008).

Another way to select bacteria that may have the potential to alleviate abiotic stress is to isolate strains, which are able to grow in extreme environmental conditions. For instance, a *Bacillus* sp. strain, isolated from desert soils, alleviates drought stress in lettuce by stimulating the symbiotic interaction between lettuce and arbuscular mycorrhizal fungi (Vivas et al. 2003). *Bacillus* and *Arthrobacter* strains, isolated from rhizosphere of wheat grown in saline soils, allow salt stress alleviation in wheat (Upadhyay et al. 2009). Numerous PGPR were also isolated from heavy metal polluted soils and are able to enhance both plant growth and plant development under heavy metal stress conditions, such as in the presence of arsenic (Reichman 2014), cadmium (Guo and Chi 2014), or both zinc and cadmium (Pereira et al. 2014).

Table 19.1 List of microbes identified for their ability to alleviate plant stress

Stress type	Inoculated bacteria	Plant species	Reference
Abiotic stresses			
Arsenic	*Bradyrhizobium japonicum* CB1809	Sunflower (*Helianthus annus*)	Reichman (2014)
		Wheat (*Triticum aestivum*)	
Cadmium	*Rhizobium* sp.	Italian ryegrass (*Lolium multiflorum*)	Guo and Chi (2014)
		Soybean (*Glycine max*)	
Cadmium and lead	*Arthrobacter mysorens* 7	Barley (*Hordeum vulgare*)	Belimov et al. (2004)
	Agrobacterium radiobacter 10		
	Flavobacterium sp. L30		
	Azospirillum lipoferum 137		
Cadmium and zinc	*Rhodococcus erythropolis* EC 34	Clover (*Trifolium repens*)	Pereira et al. (2014)
	Achromobacter sp. 1AP2		
	Microbacterium sp. 3ZP2		
Drought	*Achromobacter piechaudii*	Tomato (*Lycopersicon esculentum*)	Mayak et al. (2004)
Drought	*Pseudomonas* sp.	Pea (*Pisum sativum*)	Arshad et al. (2008)
Drought	*Bacillus* sp.	Lettuce (*Lactuca sativa*)	Vivas et al. (2003)
Drought	*A. lipoferum*	Maize (*Zea mays*)	Bano et al. (2013)
Drought	*A. brasilense* Sp245	Wheat (*T. aestivum*)	Creus et al. (2004)
Drought	*A. brasilense* AZ-39	Rice (*Oryza sativa*)	Ruíz-Sánchez et al. (2011)
Drought	*A. brasilense* Sp245	*Arabidopsis thaliana*	Cohen et al. (2014)
Drought	*P. mendocina* Palleroni	Lettuce (*Lactuca sativa*)	Kohler et al. (2008)
Drought	Consortium of *B. cereus* AR156, *B. subtilis* SM21, and *Serratia* sp. XY21	Cucumber (*Cucumis sativus*)	Wang et al. (2012)

(continued)

Table 19.1 (continued)

Drought	*Phyllobacterium brassicacearum* STM196	*Arabidopsis thaliana*	Bresson et al. (2013)
Nitrogen deficiency	*A. brasilense* VS9	Tomato (*L. esculentum*)	Esquivel-Cote et al. (2010)
	A. lipoferum AZm5		
Insoluble phosphate	*Bacillus* sp.	Maize (*Z. mays*)	Pal (1998)
		Fingermillet (*Elosine coracana*)	
		Amaranth (*Amaranthus hypochondriacus*)	
		Buckwheat (*Fagopyrium esculentum*)	
		Frenchbean (*Phaseolus vulgaris*)	
Salt	*P. fluorescens*	Canola (*Brassica napus*)	Jalili et al. (2009)
	P. putida		
Salt	*Arthrobacter* sp.	Wheat (*T. aestivum*)	Upadhyay et al. (2009)
	Bacillus sp.		
Salt	*Brevibacterium epidermidis* RS15	Canola (*B. napus*)	Siddikee et al. (2010)
	Micrococcus yunnanensis RS222		
	B. aryabhattai RS341		
Salt	*A. brasilense* NO40	Barley (*H. vulgare*)	Omar et al. (2009)
Salt	*A. lipoferum* JA4	Wheat (*T. aestivum*)	Bacilio et al. (2004)
Salt	*A. brasilense* Sp-248	Wheat (*T. aestivum*)	Alamri and Mostafa (2009)
Biotic stresses			
Botrytis cinerea	*B. thuringiensis* UM96	*Medicago truncatula*	Martínez-Absalón et al. (2014)
Colletotrichum acutatum	*A. brasilense* REC2 and REC3	Strawberry (*Fragaria x ananassa*)	Tortora et al. (2011)

Gaeumannomyces graminis	*P. fluorescens* 2-79	Wheat (*T. aestivum*)	Thomashow and Weller (1988)
	P. fluorescens 2-79	Wheat (*T. aestivum*)	Mazzola et al. (1995)
	P. aureofaciens 30-84		
Pratylenchus brachyurus	*A. brasilense*	Maize (*Z. mays*)	Dias-Arieria et al. (2012)
		Soybean (*G. max*)	
Pythium sp.	*P. protegens* CHA0	Cucumber (*Cucumis sativus*)	Maurhofer et al. (1994)
		Cress (*Lepidium sativum*)	
Pythium ultimum	*P. fluorescens* de Souza et al. (2003a)	Wheat (*T. aestivum*)	de Souza et al. (2003a, b)
Pythium sp.	*P. fluorescens* de Souza et al. (2003b)	Wheat (*T. aestivum*)	
Albugo candida			
Phytophthora infestans			
Ralstonia solanacearum	*Acinetobacter* sp. Xa6	Tomato (*L. esculentum*)	Xue et al. (2009)
	Enterobacter sp. Xy3		
Rhizoctonia solani	*P. fluorescens* HC1-07	Wheat (*T. aestivum*)	Yang et al. (2014)
	Azospirillum sp.	Tomato (*L. esculentum*)	Gupta et al. (1995)
	Azotobacter chroococcum		
Rhizoctonia sp.	*A. brasilense* Sp245	*Prunus cerasifera*	Russo et al. (2008)
Erwinia carotovora	*Pseudomonas* sp. B10	Potato (*Solanum tuberosum*)	Kloepper et al. (1980)
Fusarium oxysporum	*Pseudomonas* sp. WCS417r	Carnation (*Dianthus caryophyllus*)	van Peer and Schippers (1992)
Pseudomonas syringae			
Xanthomonas campestris	*A. brasilense* Sp7	Tomato (*L. esculentum*)	Romero et al. (2003)
	Azospirillum sp. BNM-65		
Agrobacterium tumefasciens	*A. brasilense* Sp7	Grapevines (*Vitis*)	Bakanchikova et al. (1993)
	A. brasilense 94-3		

(continued)

Table 19.1 (continued)

Pyricularia oryzae	*Azospirillum strains* A7, A18, A26 and A37	Rice (*O. sativa*)	Sankari et al. (2011)
Fusarium oxysporum	*A. brasilense* SBR	Cucumber (*C. sativus*)	Hassouna et al. (1998)
Rhizoctonia solani	*Azotobacter chroococcum* ZCR		
Pythium sp.	*Klebsiella pneumoneae* KPR		
Magnaporthe oryzae	*Azospirillum* sp. B510	Rice (*O. sativa*)	Yasuda et al. (2009)
Xanthomonas oryzae			
Thielaviopsis basicola	*P. protegens* CHA0	Tobacco (*Nicotiana tabacum*)	Voisard et al. (1989)
Meloidogyne incognita	*P. protegens* CHA0	Tomato (*L. esculentum*)	Siddiqui et al. (2005)

Certain plant-beneficial microorganisms can protect plants against diseases caused by fungi, and bacteria, such as against virus (Ryu et al. 2004), insects (Kupferschmied et al. 2013), and nematodes (Kerry 2000). Mechanisms involved in disease suppression by PGPR can be indirect through the activation of plant defenses or direct by competitive or antagonistic interactions against plant pathogens. PGPR can produce various compounds with detrimental effects on plant pathogens. Fluorescent *Pseudomonas* is one of the most widely studied bacterial lineages for antibiotic-based biocontrol activities. They can produce 2,4-diacetylphloroglucinol (DAPG), a well-known antibiotic involved in soil disease suppressiveness (Almario et al. 2013, 2014). Other *Pseudomonas* antimicrobial compounds are pyoluteorin active against *Pythium* (Maurhofer et al. 1994), phenazines against *Gaeumannomyces graminis* (Thomashow and Weller 1988), pyrrolnitrin against *Rhizoctonia solani* (Ligon et al. 2000), and cyclic lipopeptides (Clp) (Raaijmakers et al. 2006). Biocontrol strains can be isolated from suppressive soils on semi- or selective media (Stutz et al. 1986; Frapolli et al. 2010). Another way for PGPR to alleviate plant pathogen-induced stress is to produce lytic enzymes like chitinase, cellulase, and pectinase. Media containing colloidal chitin, methyl cellulose, or pectin can be used to isolate potential PGPR strains able to produce these enzymes, respectively (Siddikee et al. 2010).

Besides their ability to inhibit the growth of soil-borne pathogens, some PGPR are able to stimulate plant natural defenses, by a mechanism called induced systemic resistance (ISR) (Pieterse et al. 2014). ISR corresponds to a plant immune response that allows plants to express a stronger defense reaction when further exposed to pathogen attack. The bacteria trigger a plant-mediated resistance response in aboveground plant parts while inoculated on roots. Induced accumulation of pathogenesis-related (PR) proteins like PR-1, PR-2, chitinases, and some peroxidases are often observed in plants (Maurhofer et al. 1994; Pieterse et al. 1996; Park and Kloepper 2000; Ramamoorthy et al. 2001). However, certain PGPR do not induce PR proteins (Hoffland et al. 1995; van Wees et al. 1997) but rather increase accumulation of peroxidase, phenylalanine ammonia lyase, phytoalexins, polyphenol oxidase, and/or chalcone synthase (Van Peer et al. 1991; Ongena et al. 2000; Chen et al. 2000). They can stimulate callose deposit whereas no callose accumulation is observed in plants treated only with a pathogen (Tortora et al. 2012). Callose accumulation contributes to the reinforcement of the cell wall at the sites where the pathogen attacks. PGPR-elicited ISR has been demonstrated in many dicotyledonous plant species, including *Arabidopsis*, bean, carnation, cucumber, radish, tobacco, and tomato (van Loon et al. 1998) but less in monocotyledonous plants. PGPR strains belonging to the genera *Pseudomonas*, *Bacillus*, and *Azospirillum* have been reported to elicit growth promotion and ISR (Gutiérrez Mañero et al. 2001; Kloepper et al. 1980; Kloepper et al. 2004; van Peer et al. 1991). However, little information is available regarding the plant metabolic pathways involved in the systemic responses elicited by PGPR strains.

Among biocontrol rhizobacteria, *Azospirillum* was shown to be a good candidate for the mitigation of biotic and abiotic stresses.

19.3.2 Azospirillum *Strain Ability to Reduce Stress in Plant*

19.3.2.1 Alleviation of Biotic Stresses by *Azospirillum*

Some *Azospirillum* strains are able to suppress soil-borne pathogens. *Azospirillum* can reduce the incidence and severity of damping off caused by *Rhizoctonia solani* (Gupta et al. 1995), foliar diseases of tomato caused by *Pseudomonas syringae* pv. *tomato* (Bashan and de-Bashan 2002; Romero et al. 2003), crown gall disease (Bakanchikova et al. 1993), *Cucumis sativus* disease (Hassouna et al. 1998), bacterial leaf blight of mulberry (Sudhakar et al. 2000), *Prunus cerasifera* disease (Russo et al. 2008), anthracnose symptoms on strawberry plants (Tortora et al. 2012), and can enhance disease resistance in rice (Yasuda et al. 2009).

Different methods are employed to test the biocontrol activity of *Azospirillum* strains. These methods differ according to both (1) inoculum preparation protocols and (2) pathogen inhibition assays. *Azospirillum* inoculum may be prepared using complex media like Nutrient Broth (Russo et al. 2008) or synthetic media like Nitrogen free base or AB malate medium (Sankari et al. 2011; Tortora et al. 2011; Tortora et al. 2012). For inoculum preparation, a washing step is often used to remove culture medium residues and bacterial metabolites. This washing step is mostly realized with phosphate buffer (pH 6.8–7) (Tortora et al. 2011, Tortora et al. 2012; Sankari et al. 2011) or with water (Russo et al. 2008). Different protocols may be used to bring out the biocontrol activities of *Azospirillum*. First, the antagonistic action of *Azospirillum* against microbial plant pathogens can be evaluated in vitro by confronting *Azospirillum* and plant pathogen strains, on agar plate, and quantifying the growth inhibition of the plant pathogen (Russo et al. 2008; Tortora et al. 2011). In case of fungal pathogens, a plug of fungal mycelium can be laid on the center of an agar plate, and the biocontrol strain deposited few centimeters from the plug. At different incubation times, the mycelial growth rate can be recorded, both on the side where the bacterial inoculum was deposited and on the diametrically opposite side. The two growth rates can be compared in order to evaluate the ability of a strain to inhibit the growth of the fungus. In case of bacterial pathogens, they can be spread on an agar plate or grown in a soft agar layer and the biocontrol strain spotted on the surface of the agar. At different incubation times, growth diameters can be measured. Second, the antagonistic action of *Azospirillum* can be evaluated in plant assays performed in gnotobiotic or more complex conditions (Tortora et al. 2011). The timing, localization, and mode of application of *Azospirillum* inoculum, such as the density level of the bacterial inoculum, are key elements that influence the biocontrol activity of *Azospirillum* strains (Tortora et al. 2011, 2012). Disease symptom scales based on the number and size of necrotic area are established to evaluate plant health in biocontrol assays. The antifungal activity of *Azospirillum brasilense* strains REC2 and REC3 against *Colletotrichum acutatum* was evaluated on strawberry plants grown in hydroponic conditions. These two strains were mixed together and inoculated in the hydroponic system. Results showed that these two strains of *Azospirillum* confer a reduction of symptoms on

strawberry plants, which had previously been inoculated with them before the addition of the fungal pathogen (Tortora et al. 2011). Biocontrol activity had also been tested in sterile artificial substrate, and *Azospirillum* was inoculated by watering plants with bacterial solution (Tortora et al. 2012). Natural organic substrates or autoclaved soils were used to test the biocontrol activity of *A. brasilense* Sp245 and another strain of the same species against, respectively, *Rhizoctonia* spp. and *Pratylenchus brachyurus* (Russo et al. 2008; Dias-Arieria et al. 2012). *Azospirillum* was inoculated directly on the seed (Dias-Arieria et al. 2012) or by watering the plantlet (Russo et al. 2008). Bashan and collaborators (2014) recently reviewed inoculation methods and formulation technologies for *Azospirillum*.

19.3.2.2 Alleviation of Abiotic Stresses by *Azospirillum*

Environmental conditions and their fluctuation may be stressful for plant growth and development. Cereals require a large amount of water for their growth and development, and water can become the main factor limiting plant development. The inoculation of *Azospirillum* strains was shown to alleviate drought stress in maize (Bano et al. 2013), wheat (Creus et al. 2004), and rice (Ruíz-Sánchez et al. 2011). As noted above, water deficiency leads to the accumulation of free amino acids, proline, soluble proteins, and soluble sugars in plant tissues (Mohammadkhani and Heidari 2008). Bano et al. (2013) tested the capacity of an inoculated *A. lipoferum* strain to alleviate drought stress on maize, and the impact of the mode of application of *Azospirillum* on plant metabolite accumulation. *Azospirillum* supply was realized either by soaking seeds in *Azospirillum* cell suspension or by applying the bacterial inoculum in the close vicinity of roots. The water stress simulation was conducted by maintaining soil moisture content at 15 % for the drought stress condition against 19 % in the well-watered condition. Better drought stress resistance was obtained when *Azospirillum* was applied whatever the bacterial inoculation mode (Bano et al. 2013). Another way to apply water stress conditions on plants could be to decrease water irrigation by 50 % after 2 weeks of well-watered condition (Ruíz-Sánchez et al. 2011). Using this condition, the authors showed that beneficial effects of *A. brasilense* on most of the physiological and biochemical traits of rice plants were only clearly visible when the plants were mycorrhized (Ruíz-Sánchez et al. 2011).

Soil salinity may also strongly affect plant development. To simulate salt stress conditions, different protocols have been used. Bacilio et al. (2004) used autoclaved sand and watered wheat seedlings with either 80 or 160 mM NaCl. *A. lipoferum* JA4 was shown to enhance plant growth (higher height and dry weight of shoots and roots) under continuous irrigation with 160 mM NaCl compared to the un-inoculated control (Bacilio et al. 2004). In another study, the salinity stress was applied by watering barley plants grown in natural soil with either 250 or 350 mM NaCl (Omar et al. 2009). Omar and collaborators (2009) employed two different wheat cultivars, one sensitive and one resistant. *A. brasilense* NO40 addition was shown to enhance growth and salt tolerance of the sensitive barley cultivar by increasing photosynthetic pigment contents, reducing accumulation of the osmoregulator proline, and

the activities of antioxidant enzymes (Omar et al. 2009). Salinity stress can also be obtained using irrigation with diluted seawater solutions (Alamri and Mostafa 2009). These authors compared the effect of *Azospirillum* inoculation on wheat watered with seawaters at a final concentration of 4,650 and 9,300 ppm saline, and with tap water (140 ppm saline) as a control. Results demonstrate the ability of *A. brasilense* Sp-248 to reduce the deleterious effects of saline stress on wheat growth (Alamri and Mostafa 2009). In addition, this *A. brasilense* strain shares a relatively high tolerance to saline irrigation.

Soil chemical composition in terms of nitrogen content and heavy metal concentrations, etc. can also be stressful for plants. Esquivel-Cote et al. (2010) showed the potential of *A. lipoferum* to stimulate plant growth under different nitrogen levels (0, 170 and 340 kg $N \cdot ha^{-1}$). A better effect was observed when *A. lipoferum* AZm5 was inoculated in presence of a moderate nitrogen amount of 170 kg $N \cdot ha^{-1}$ (Esquivel-Cote et al. 2010). *Azospirillum* allows a better plant resistance to heavy metal stress (Belimov et al. 2004). Indeed, in pot and field experiments, *Azospirillum* seed inoculation improved the growth of barley plants in Pb- and Cd-contaminated soils. The presence of *Azospirillum* also prevented the accumulation of Pb and Cd in barley plants, thereby mitigating their toxic effects (Belimov et al. 2004).

Azospirillum is a good candidate to ensure the alleviation of biotic and/or abiotic stress. Mechanisms involved in the establishment of stress resistance in plants by *Azospirillum* and the molecular responses of the host plant are increasingly studied.

19.4 Main Mechanisms of Stress Alleviation

The principal mechanisms permitting alleviation of biotic stresses consist of antagonism, competition, and induction of plant defense against pathogens (Fig. 19.1). Mechanisms involved in alleviating abiotic stresses consist principally of modifications of the plant hormonal balance and increased nutrient availability for plants (Fig. 19.2) (Compant et al. 2005).

19.4.1 Mechanisms Recovered in Rhizobacteria

19.4.1.1 Against Biotic Stresses

Antagonism consists of the inhibition of plant pathogen development. In Rhizobacteria, this phenomenon is mediated by a large range of compounds including DAPG, phenazines, pyrrolnitrin, pyoluteorin, Clp, extracellular enzymes, and volatile compounds (Fig. 19.1).

One of the most studied and well-documented compounds is DAPG, which is produced by certain species of fluorescent pseudomonads (Bruto et al. 2014). It is a polyketide antibiotic efficient against bacteria, fungi, nematodes, algae, and protozoans

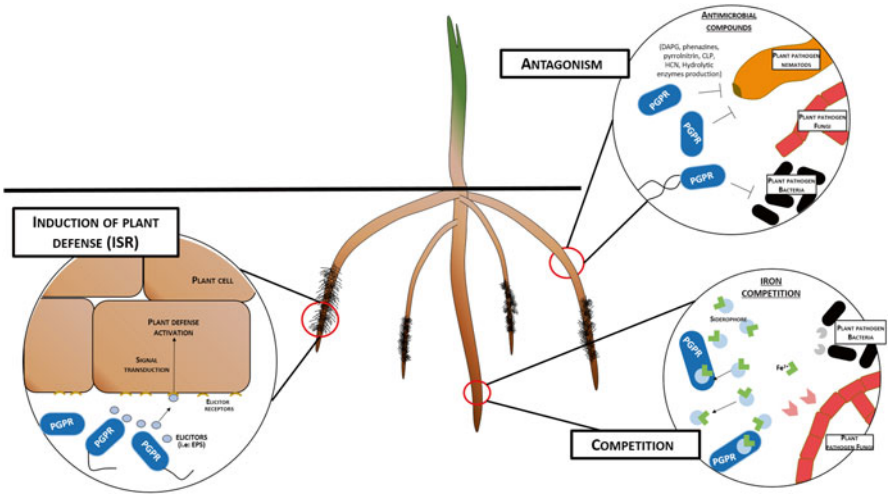

Fig. 19.1 Main bacterial mechanisms involved in alleviation of biotic stresses in plants by *Azospirillum* strains and other PGPR. The mechanisms involved in biotic stress alleviation in crops by PGPR can be separated in three main processes. Antagonism interactions can take place between PGPR and diverse plant pathogens like fungi, bacteria, nematodes. This involves the production of antimicrobial components and hydrolytic enzymes by PGPR. Competition for essential elements like iron through siderophore production by PGPR may lead to growth limitation of the plant pathogen. PGPR can also induce plant defenses (i.e., ISR) by producing plant defense elicitors like exopolysaccharides

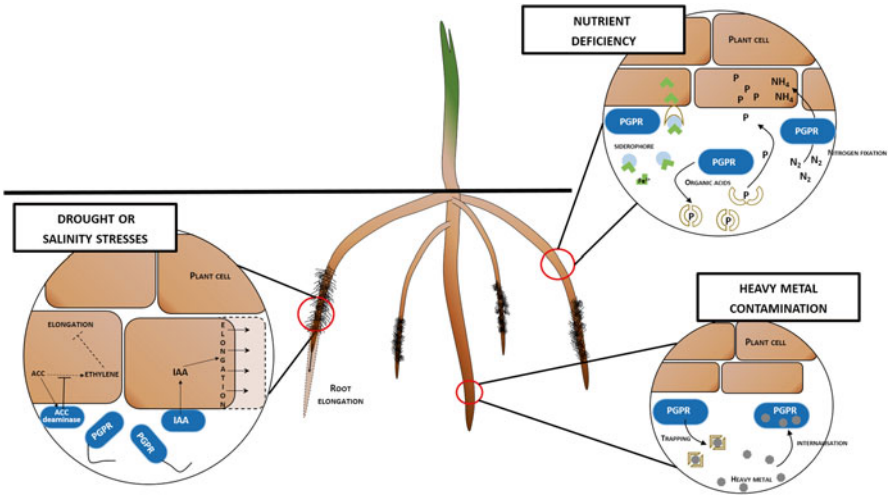

Fig. 19.2 Main bacterial mechanisms involved in alleviation of abiotic stresses in plants by *Azospirillum* strains and other PGPR. PGPR can modify the plant hormonal balance (in particular the ethylene or auxin signaling pathway), thereby favoring the host plant resistance to drought or salinity. PGPR may improve plant nutrient acquisition especially when nitrogen, phosphate, or iron are scarce. They can ensure phosphate solubilization by releasing organic acids, improve plant nitrogen inputs by fixing the atmospheric dinitrogen and/or facilitate iron acquisition through the production of siderophores. PGPR can play a role in alleviation of heavy metal stress in plants by trapping heavy metals outside the bacterial cell or by internalizing and detoxifying them

(Haas and Défago 2005; Jousset et al. 2006; Meyer et al. 2009). DAPG acts through membrane permeabilization and destabilization and triggers oxidative burst in the target cell. This oxidative burst causes inactivation of V-ATPase and the disturbance of cell respiration that leads to a loss of cell homeostasis (Kwak et al. 2011). Other PGPR belonging to *Pseudomonas*, *Bacillus*, and *Burkholderia* genera are able to produce hydrogen cyanide (HCN), a volatile compound with antimicrobial activity (Reetha et al. 2014; Ryall et al. 2008; Voisard et al. 1989). HCN may pass through the plant pathogen membrane and inhibits the cytochrome C oxidase and several other metalloenzymes (Blumer and Hass 2000). Phenazines have a wide spectrum of action. Their mechanisms of action, at the cellular level, are still not well resolved. Given the molecular structure of these compounds, it can be suggested that, after their diffusion through the membrane of the target cell, they can act as electron acceptors and shunt the respiratory chain. This interference leads to the production of hydrogen peroxide (H_2O_2) and superoxide ion (O_2^-), and cell death (Delaney et al. 2001). *Pseudomonas* phenazine producers are protected by their high superoxide dismutase activity (Delaney et al. 2001). Indeed, it has been demonstrated that the addition of sublethal doses of phenazines in the liquid cultures of *Mycosphaerella graminicola* induced a strong increase of the activity of catalase, superoxide dismutase and peroxidases, allowing the fungus to survive in the presence of these antibiotics (Levy et al. 1992). Pyrrolnitrin is an antimicrobial compound produced by numerous bacteria like *Pseudomonas* and *Burkholderia* (Costa et al. 2009). Pyrrolnitrin is produced from tryptophan and targets the mitochondrial electron transport system with the inhibition, among others, of succinate and NADH oxidase activities (Tripathi and Gottlieb 1969). Clp are composed of fatty acid tails linked to an oligopeptide, which is cyclized by forming a lactone ring between two amino acids. Clp like iturin and viscosin are surface-active agents causing membrane permeabilization in fungi, bacteria, oomycetes, protozoans, and nematodes (Raaijmakers et al. 2006; de Souza et al. 2003b; Ongena and Jacques 2008).

PGPR, especially *Bacillus* and *Pseudomonas* strains, can produce extracellular enzymes such as chitinase or glucanase, which degrade components of the membrane and cell wall of microbial pathogens (Siddiqui et al. 2005; Maksimov et al. 2011; Kim et al. 2014). Moreover, certain PGPR can quench quorum-sensing (QS) signals and affect QS-dependent pathogenicity in plant pathogenic bacteria by degrading or mimicking their acyl-homoserine lactones, thereby blocking the expression of virulence functions (Faure and Dessaux 2007; Boyer and Wisniewski-Dyé 2009; Helman and Chernin 2014).

The ability of rhizobacteria to produce insecticidal toxins is more and more sought-after (Wu et al. 2008; Péchy-Tarr et al. 2008; Lacey and Georgis 2012). Those rhizobacteria are taxonomically diverse. The most studied and used toxins for biological control of insects are the *Bacillus thuringiensis* toxins (Bravo et al. 2011). These toxins induce pore formation in the lipidic membrane of midgut insect cells (Vachon et al. 2012). Other biocontrol bacteria are known to be entomotoxin producers, like bacteria belonging to fluorescent pseudomonads (Kupferschmied et al. 2013, 2014; Ruffner et al. 2013). One of these toxins, fit, is increasingly studied. This insecticidal toxin is produced by some strains belonging to the *Pseudomonas*

protegens and *P. chlororaphis* subgroups (Péchy-Tarr et al. 2008, 2013; Kupferschmied et al. 2013, 2014). The mode of action of this toxin is still not entirely resolved.

Root surfaces and surrounding soil are areas where resources are abundantly available for microbial growth (Fig. 19.1). These various and suitable nutrients attract numerous microbes including PGPR and pathogens. Efficient competitiveness of PGPR for resources and ecological niches along roots is a fundamental mechanism by which they can protect plants against pathogens. The strong rhizocompetence of PGPR permits their fast and durable colonization of roots. Indeed, numerous PGPR can move rapidly along roots, thanks to their flagellar mobility (de Weert et al. 2002). Moreover they produce exopolysaccharides (EPS), lipopolysaccharides (LPS), pili or other fimbriae permitting root adherence and recognition between the host plant and the PGPR (Albareda et al. 2006; Rodríguez-Navarro et al. 2007). Furthermore, root-associated rhizobacteria share adequate enzymatic machinery to degrade toxic root exudate compounds (Bais et al. 2004). The ability of PGPR to efficiently colonize roots makes these microbes very well adapted to the rhizosphere life and competitive against pathogens. The rhizo-microbiote might act as a biological barrier against pathogens. Accordingly, parallels have recently been made with the intestinal microbiota and its barrier-protective function (Mendes et al. 2013; Ramirez-Puebla et al. 2013). Rhizobacteria can competitively use a great variety of molecules as carbon, iron, or nitrogen sources by producing high affinity transporters for these sources, and thereby scavenging nutrients away from plant pathogens. Competition for iron resources and production of siderophores by PGPR have been studied, revealing the importance of this property in biotic stress alleviation by PGPR (Crowley 2006; Dimkpa et al. 2009)

Rhizobacteria can reduce the activity of pathogens not only by antagonism or competition but also by activating plant defenses (Fig. 19.1). As previously mentioned, this phenomenon is called ISR. ISR has been widely described in literature (van Loon 2007). ISR induction is mediated by different kinds of elicitors such as LPS, flagellin (Meziane et al. 2005) or secreted molecules like DAPG or siderophores (Bakker et al. 2007). Elicitor recognition by host plant receptors triggers a local and systemic response, leading to the activation of JA and ET pathways (Iavicoli et al. 2003; Djavaheri 2007). PGPR-triggered plant responses depend on the type of pathogens (Vleesschauwer et al. 2008).

19.4.1.2 Against Abiotic Stresses

PGPR may interact with the plant-specific mechanisms related to abiotic stress resistance. PGPR effects involve multiple changes in plant metabolism and signaling networks (Lugtenberg and Kamilova 2009; Friesen et al. 2011). Modifications in phytohormone content and/or signaling have been reported (Dodd et al. 2010), such as decreased ET production via bacterial ACC deaminase activity (Glick et al. 1998; Belimov et al. 2009; Bresson et al. 2013), changes in cytokinin–ABA balance

(Figueiredo et al. 2008; Cohen et al. 2009) or changes in auxin signaling (Persello-Cartieaux et al. 2003; Contesto et al. 2010) (Fig. 19.2).

Multiple combinations of traits can participate in plant strategies for dealing with drought, including those that allow drought escape or drought resistance (Verslues and Juenger 2011). Several PGPR represent an added value to these strategies. For instance, some rhizobacteria help plants to maintain a favorable water status under water deficit (Creus et al. 2004), by enhancing the development of the root system (Marulanda et al. 2010). PGPR that produce ACC deaminase conferred resistance to drought stress in plants (Mayak et al. 2004; Glick et al. 2007). Under stress conditions, including drought, the plant hormone ethylene endogenously regulates plant homeostasis and results in reduced root and shoot growth (Figueiredo et al. 2008). However, degradation of the ET precursor ACC by bacterial ACC deaminase releases plant stress and rescues normal plant growth (Figueiredo et al. 2008). Some PGPR improve plant enzyme activity, such as catalase or superoxide dismutase, which alleviates the oxidative damage induced by drought (Kohler et al. 2008; Wang et al. 2012). Finally, PGPR have been shown to increase drought-response transcript abundances (Timmusk and Wagner 1999; Wang et al. 2005, 2012). Rhizobacteria often induce modifications in phytohormone signaling (Yang et al. 2009), which may mediate effects on meristem activity (Hayat et al. 2010). For example, the most noticeable phenological change observed on *Arabidopsis* plants inoculated with *Phyllobacterium brassicacearum* STM196 was a significant delay in flowering time corresponding with a prolonged vegetative phase of PGPR-inoculated plants under drought conditions. The intensity of changes was more pronounced under drought and led to better plant tolerance to drought (Bresson et al. 2013). Under drought stress, inoculated Arabidopsis showed increased ABA levels. An accumulation of ABA or enhancement of sensitivity to this hormone in the leaf cells is leading to the induction of plant genes inducing a reduction of transpiration through reduced leaf conductance following stomata closure (Harb et al. 2010). Moreover, a decrease in transpiration by stomatal closure can be followed on a longer timescale by a reduced plant growth rate (Westgate and Boyer 1985). Auxin plays a role in the regulation of leaf and floral initiation and of the position of lateral organs (Reinhardt et al. 2000). But, STM196 is not a high auxin producer (Contesto et al. 2010) and, thus, cannot supply plant roots with exogenous auxin. However, Bresson and colleagues (2013) observed that inoculation with STM196 changed auxin distribution within Arabidopsis roots towards apices, which probably explains the positive effect of STM196 on lateral root development (Contesto et al. 2010). In addition, other hormonal pathways are modified by STM196, including ET, which participates in root hair elongation in vitro (Contesto et al. 2008; Galland et al. 2012).

Phosphorus (P) is a major essential macronutrient for biological growth and development. Soluble P is often the limiting mineral nutrient for plant biomass production in natural ecosystems only taken up in monobasic (H_2PO^{-4}) or dibasic (HPO_2^{4-}) soluble forms (Glass et al. 1989), and the elevated levels of heavy metals in soil interfere with P uptake and lead to plant growth retardation (Zaidi et al. 2006). PGPR can either convert insoluble phosphates into available forms through

acidification, chelation, exchange reactions, and release of organic acids (Chung et al. 2005; Richardson et al. 2009) or mineralize organic phosphates by secreting extracellular phosphatases (Gyaneshwar et al. 2002; van der Heijden et al. 2008; Richardson et al. 2009) (Fig. 19.2). An increase in P availability to plants through the inoculation of phosphate-solubilizing bacteria has been reported in pot experiments and under field conditions (Pal 1998; Zaidi et al. 2003). In addition, fixation of atmospheric nitrogen is a metabolic activity of endophytes and rhizobacteria and their colonization offers benefit to the host (Dobbelaere et al. 2003).

For survival in metal contaminated soil, bacteria are coding an arsenal of functions, by which they can immobilize or transform metals rendering them inactive. The mechanisms are generally exclusion of metal by membrane permeability barrier or by active export of metal from the cell, physical sequestration of metal by binding extracellular polymers or extra cellular sequestration, detoxification where metal is chemically modified to render it less active (Rouch et al. 1995; Lièvremont et al. 2009). For instance, binding of metals to anionic functional groups from microbial origin (i.e., sulfhydryl, carboxyle, hydoxyle, sulfonate, amine, and amide groups) immobilizes the metal and prevents its entry into the plant roots. Similarly, metal-binding extracellular polymers, comprising polysaccharides, proteins, humic substances, may detoxify metals by chelating the heavy metals (Pulsawat et al. 2003). The bacterial siderophores and organic acids can also reduce the metal bioavailability and toxicity by chelating the metal ions (Tripathi et al. 2005; Dimkpa et al. 2008).

19.4.2 Mechanisms Recovered in Azospirillum

19.4.2.1 Against Biotic Stresses

Azospirillum is a good candidate to alleviate plant pathogen diseases as previously shown. The mechanisms involved in these biotic stress suppressions can be direct, by releasing secondary metabolites, which have an antagonistic activity against plant pathogens. *Azospirillum* can produce bacteriocins, which are generally considered as proteinaceous toxins (Tapia-Hernández et al. 1989; Bashan and de-Bashan 2002). These bacteriocins act on the bacterial membrane. They bind to membrane receptors and cause pore formations, thereby leading to cell lysis. The phenylacetic acid (PAA) produced by *A. brasilense* seems to exert antimicrobial activity against *Rhizoctonia solani*, *Pythium ultimum*, *Phytophthora capsici*, and *Pseudomonas syringae* (Somers et al. 2005). However, the molecular mechanisms involved are still not elucidated. Gonçalves et al. (1998) showed that certain *Azospirillum* strains might produce HCN as *Pseudomonas* biocontrol strains. Therefore, it is possible to consider the antibiosis action of this volatile compound against potential plant pathogen agents in *Azospirillum*. Antagonism mechanisms implemented by the secretion of secondary metabolites are not the only way used by *Azospirillum* to control plant pathogens.

Another way for *Azospirillum* to control soil borne diseases is to produce siderophores with high-affinity iron-binding activity. Some of these siderophores seem to have antimicrobial activity (Tortora et al. 2011). This property may be used by *Azospirillum* to compete with other microbes, including pathogens, for access to iron, and thereby to limit their growth (Shah et al. 1992; Tortora et al. 2011).

Azospirillum may also protect plant against pathogen agents by an indirect process involving the induction of plant defense, notably ISR. The EPS produced by *Azospirillum* strains A7, A18, A26, and A37 were described to induce ISR and protect rice plant against *Pyricularia oryzae* (Sankari et al. 2011). Recognition of *Azospirillum* EPS by plant pattern receptor (PRR) in plant cells might generate a regulation cascade inducing the transcription of plant defense genes (Boller and He 2009; Pieterse et al. 2014). Indeed, the protection of strawberry plants against *Colletotrichum acutatum* by endophytic *Azospirillum* PGPR is associated to enhanced content of plant phenolic compounds, a transient accumulation of SA, callose accumulation, and the induction of defense-related genes (Tortora et al. 2012). Regarding the protection effects of another endophytic strain, *Azospirillum* sp. B510, Yasuda and collaborators (2009) compared the responses of *Oryza sativa* cv. *Nipponbare* challenged with *Xanthomonas oryzae* in presence and absence of the PGPR. B510 had no antagonist activity on *Xanthomonas* in vitro, but activated the innate-immune system of the host plant. The authors analyzed, using real-time PCR, the effect of B510 on the expression of pathogenesis-related genes involved in the SA response (OsPR-1a, OsPR-1b and WRKY45) and in the JA response (OsPR-4). The results (i.e., B510 downregulation of OsPR-1a and OsPR-4 and no effect on OsPR-1b and WRKY45) suggest that B510 rather downregulated SA signaling pathway (Yasuda et al. 2009). Similar plant responses were observed with *Pseudomonas fluorescens* WCS417r, which triggers defense responses in *A. thaliana* through a pathway independent of SA and PR gene activation but dependent of JA and ET signaling (Pieterse et al. 1996). Ramos and collaborators (2008) also demonstrate that PGPR plant protection in pathogen-challenged plants is inversely related to SA production. Among the three PGPR studied, *Azospirillum brasilense* Sp7 caused the highest increase in SA, but showed the lowest level of defense of *A. thaliana* against *P. syringae* pv. *tomato* DC3000.

19.4.2.2 Against Abiotic Stresses

As described above, some PGPR harbor the *acdS* gene encoding ACC deaminase. This enzyme is involved in the biotic and abiotic stress alleviation in plant by modulating plant ET amount. This plant hormone has pleiotropic effects on different processes as on both plant growth and plant development (e.g., seed germination, root elongation, and fruit development), and response to environmental stresses (Glick 2014). A model was proposed to explain how bacteria harboring the ACC deaminase activity could lower ET amount in plant cells (Glick et al. 1998). This model is valid for certain *Azospirillum* strains that have ACC deaminase activity (Blaha et al. 2006; Prigent-Combaret et al. 2008). The ACC deaminase degrades ACC, the

ET precursor. Abiotic stresses induced the production of two peaks of ET in the plant (Glick et al. 2007). These authors suggest that ACC deaminase activity can lower the second ET concentration peak, also called the "deleterious" peak. In this way, ET concentration decreases allowing the plant to grow under abiotic stress (Jalili et al. 2009).

Azospirillum may also modify plant morphology, in particular through the production of hormones, to alleviate water stress by increasing xylem vessel area and stem hydraulic conductivity in tomato (Romero et al. 2014). Wider xylem vessels were found when *A. brasilense* Sp245 was inoculated on wheat seedling under water and osmotic stresses (Pereyra et al. 2012). Cell morphological changes often implicate a modification of plant hormonal balances. Auxins (especially indole-acetic acid—IAA) induce vascular differentiation in plant (Lovisolo et al. 2002). In this manner, the production of IAA by *Azospirillum* may lead to modifications of the vascular vessel morphology. Another plant hormone is involved in stress response, like ABA, which is involved in response to abiotic stresses like drought or cold stress (Cohen et al. 2008, 2009). During drought stress, stomatal closure is induced by ABA, thereby minimizing water loss by plant transpiration (Mishra et al. 2006). *Azospirillum lipoferum* and *A. brasilense* Sp245 strains produce ABA, and they might permit alleviation of water stress by inducing stomatal closure (Cohen et al. 2008, 2009, 2014).

Among the specific PGPR-mediated mechanisms identified is the enhancement of wheat growth by *Azospirillum* sp. strains under various drought intensities, which was associated with better maintenance of plant water status as a result of increased cell wall elasticity (Creus et al. 2004). The bacterial production of EPS that is a physiological response of *A. brasilense* to salt stress (Chowdhury et al. 2007) may protect the plant against drought stress. EPS of bacteria are highly hydrated compounds and can enhance water retention in area surrounding roots (Naseem and Bano 2014). However EPS overproduction in *A. brasilense* may not necessarily stimulate plant growth promotion in standard plant-growth conditions (Volfson et al. 2013).

Nutrients and/or mineral elements deficiency are responsible for plant developmental disorder and for reduced plant growth, as described above. The availability of some essential mineral elements such as iron, phosphorus or nitrogen can be insufficient to allow appropriate plant development. These elements may be in weak concentrations in soils, or they may be present in sufficient concentrations but not available to the plant roots. Nitrogen deficiency may be reduced by the process of biological nitrogen fixation (BNF), performed by nitrogenase activity harbored by diazotrophic bacteria like *Azospirillum* (Fig. 19.2). In the case of the inoculation of *Azospirillum amazonense*, a significant improvement of nitrogen nutrition in rice was observed through the use of a nitrogen isotope (^{15}N) (Rodrigues et al. 2008). The authors were able to show that the improvement in growth is mainly due to atmospheric nitrogen fixation made by the inoculum. Some works evidence that bacterial nitrogen fixation might improve nitrogen nutrition in plant, however, the accurate mechanisms involved are not yet well known (Richardson et al. 2009). Similar to nitrogen, phosphorus is an essential element for plants. However, its bioavailability is often low in soils due to its presence as insoluble and complexed

forms (Meyer et al. 2011). As mentioned above, one of the main mechanisms harbored by PGPR to solubilize phosphorus is based on soil acidification via the release of acid compounds as gluconic acid, citric acid, oxalic acid (Richardson et al. 2009). However, rhizosphere acidification could be obtained by indirect mechanisms. Inoculation with *A. brasilense* Cd increased rhizosphere acidification, and it was suggested that auxins produced by the *Azospirillum* PGPR may stimulate the plasma membrane H-ATPase, thereby leading to the transport of protons across the cell wall (Carrillo et al. 2002). Lastly, *A. brasilense* may increase root iron absorption by releasing bacterial siderophores that can be recognized by plant iron receptors and used to enhance iron content in plant (Barton et al. 1986).

19.5 Concluding Remarks

Plants adapt to fluctuating environmental changes by modifying their physiology and development. PGPR may help plants to cope with the biotic and abiotic stresses through the expression of a myriad of plant-beneficial functions. Important highlighted issues are:

- Crosstalk exists between plant signaling pathways induced in responses to biotic and abiotic stresses.
- PGPR including *Azospirillum* are able to mediate enhanced resistance to biotic stresses, as well as to increase tolerance to abiotic stresses in host plants.
- PGPR may elicit different plant pathways simultaneously, conferring additive responses that are more effective than single-elicited pathways.
- PGPR harbor co-occurring plant-beneficial properties. The identification of bacterial strains that have the potential to provide cross-protection against multiple stress factors in crops would be highly valuable for developing sustainable agriculture.

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Chapter 20
Interaction of *Azospirillum* spp. with Microalgae: A Basic Eukaryotic–Prokaryotic Model and Its Biotechnological Applications

Luz E. de-Bashan, Juan Pablo Hernandez, and Yoav Bashan

Abstract The interaction of the bacteria *Azospirillum* spp. with photosynthetic, single cell microalgae that are co-immobilized in alginate beads provides a significant shortcut for understanding the interaction of this plant growth-promoting bacteria (PGPB) with plants in general. This interaction is currently relevant for studying physiological, physical, biochemical, and molecular aspects. As an independent subfield of *Azospirillum* research, this interaction has some significant potential biotechnological applications, such as wastewater treatment, production of biofuel (ethanol and biodiesel), increased fertility of eroded soils combined with promoting growth of higher plants, production of pigments, and production of biomass. All of these applications have yet to be scaled up and evaluated for their true practical potential.

20.1 The Logic Behind Using This Interaction as a Model for Plant–Bacteria Interaction

A major obstacle in the study of interactions between *Azospirillum* spp. and plants is the complexity of the plant. Studies of basic plant–bacterium interactions of *Azospirillum* spp., done mainly with roots, are difficult because there are many

Dedication: This chapter is dedicated to the memory of the German/Spanish mycorrhizae researcher Dr. Horst Vierheilig (1964–2011) of CSIC in Spain.

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tissue functions and numerous possible interactions with plant roots and plant metabolism, as well as interference with the soil matrix. Plants with relatively small genomes, such as *Arabidopsis thaliana* (125-Mb genome) and rice (389-Mb genome), were sequenced and used as models for *Azospirillum* spp. interaction. However, larger plant genomes, in which *Azospirillum* spp. commonly interacts, such as maize (2.5 Gb), oat (11.4 Gb), and wheat (16 Gb), even though some are undergoing sequencing procedures, are unlikely to be understood in detail for some time. Green microalgae, on the other hand, have the smallest plant genome (~40 Mb). *Chlorella* spp. (Chlorophyceae) are simple, nonmotile, unicellular, aquatic green microalgae that have been intensively studied regarding metabolic functions of the cell. The *Chlorella* genome is the smallest eukaryotic, photosynthetic microorganism characterized so far, which makes it an alternative to higher plants with large genomes interacting with *Azospirillum* spp., with a specific aim of studying plant metabolism and molecular mechanisms affected by *Azospirillum* spp. The reason for co-immobilization of both microorganisms in a polymer bead is to keep them together in very close proximity to ensure that each affects the other's metabolism. Consequently the three basic components of the experimental model are cells of the microalgae *Chlorella* spp. and cells of *Azospirillum* spp. that have been co-immobilized in small (3–4 mm in dia.) alginate beads.

20.2 Co-immobilization Techniques

Co-immobilization techniques are detailed in Fig. 20.1. Alginate beads containing the two microorganisms are presented in Fig. 20.2, where 20 mL of axenic cultures (*C. vulgaris* and *A. brasilense*) are mixed with a 2 % alginate solution. Beads are formed using automated equipment (de-Bashan and Bashan 2010; http://www.bashanfoundation.org/beads/macrobead.html accessed 10 July 2014) or by drops from a large syringe (less recommended). To immobilize the two microorganisms in the same bead, each culture is washed and then each is resuspended in 10 mL 0.85 % saline solution. The two mixes are then mixed with the alginate before the beads are formed. Because immobilization normally reduces the number of *A. brasilense* in the beads, to increase the numbers of *A. brasilense* to its original level, a second 24 h incubation of the beads is necessary in OAB medium (Bashan et al. 1993, see also chapter on formation of inoculants) or in a diluted, rich media BTB-1 or BTB-2 (Bashan et al. 2011 see also Chap. 26 on formation of inoculants).

20.3 Applications

20.3.1 Basic Studies of Prokaryotic–Eukaryotic Interaction

This conceptual experimental and simple quantitative model offers a convenient and basic approach to studies of complex interactions between plants and bacteria. These interactions are mainly physiological, biochemical, and molecular mechanisms

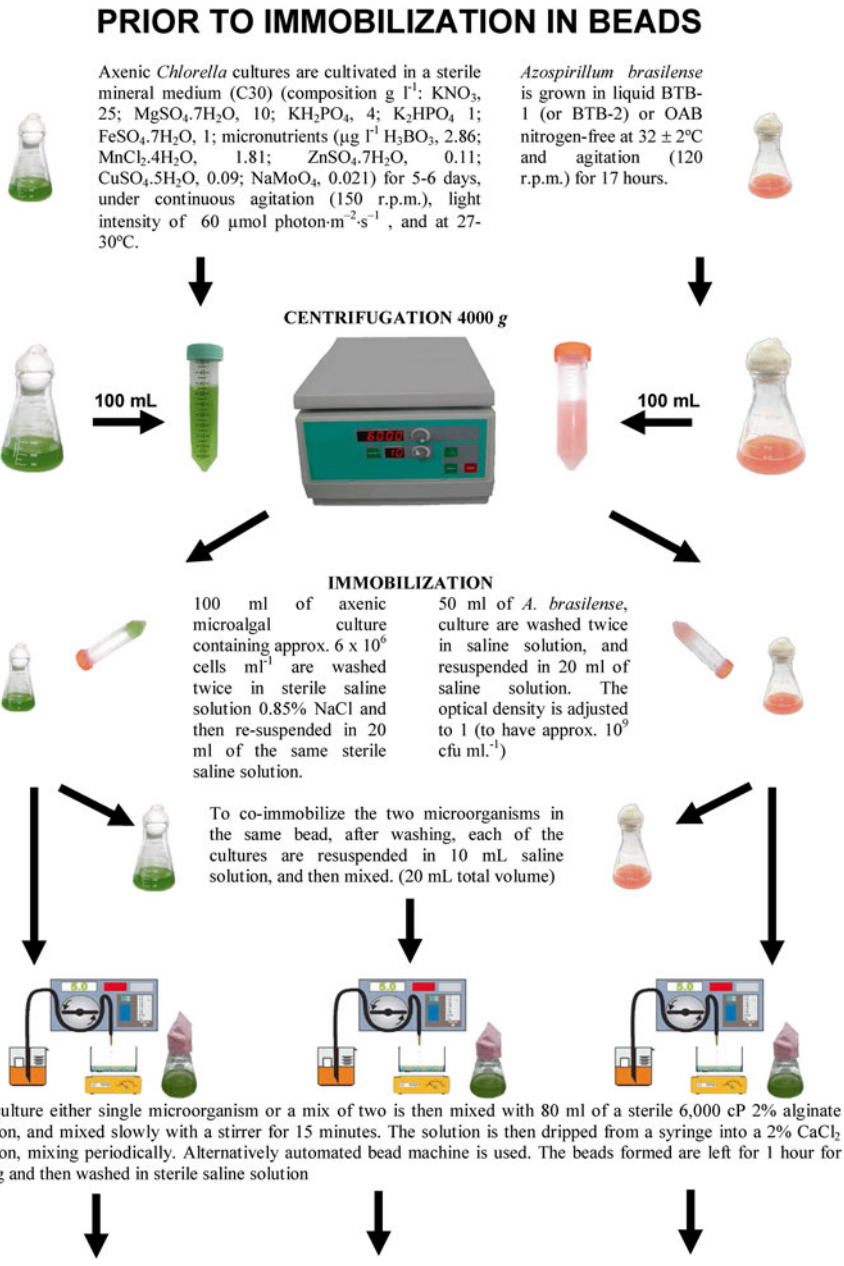

Fig. 20.1 Flow chart showing methods and techniques used to immobilize, co-immobilize, count, and cultivate microalgae and *A. brasilense* for various applications. Composition of media BTB-1, BTB-2, and OAB are given in this book (Bashan and de-Bashan, Inoculant preparation and formulations for *Azospirillum* spp.)

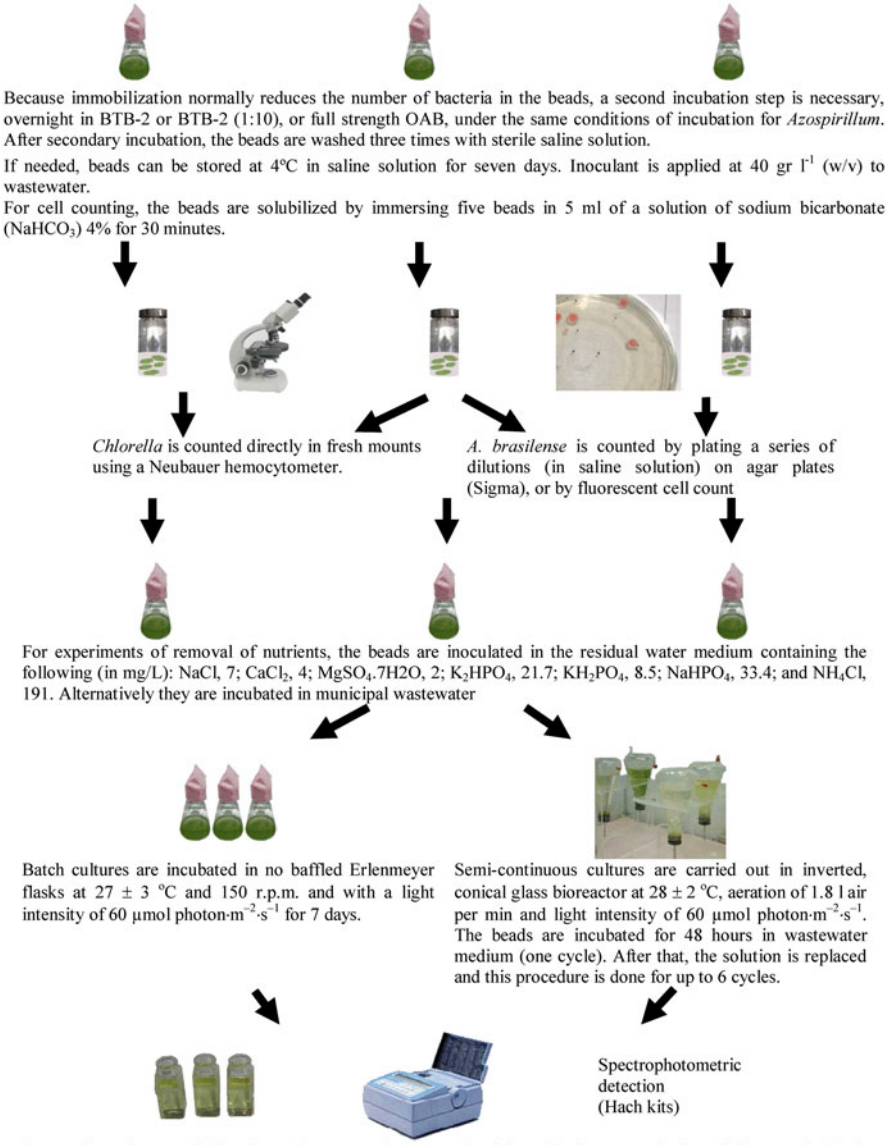

Fig. 20.1 (continued)

shared by higher plants and green microalgae (de-Bashan and Bashan 2008; de-Bashan et al. 2005). Many of the mechanisms proposed so far for *Azospirillum*–plant interactions (Bashan and de-Bashan 2010; Bashan et al. 2004) are relatively easy to study using this model. Apart from easy technical handling, the logic of choosing a microalga as the plant partner for *Azospirillum* spp. is straightforward.

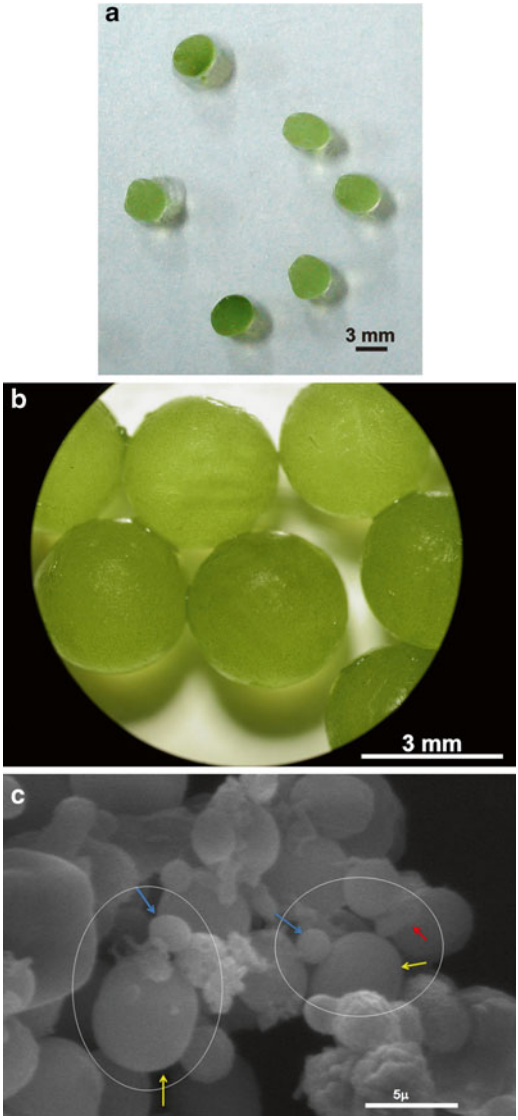

Fig. 20.2 (**a**) Beads containing *Chlorella vulgaris* co-immobilized with *Azospirillum brasilense* (**a**, **b**). (**c**) Scanning electron microscopy of the interaction between the two microorganisms inside the bead. *Circles* indicate interactions between *C. vulgaris* (*yellow arrows*) and cysts (*blue arrows*) and a vegetative cell (*red arrow*) of *A. brasilense*

The most basic definition of a green plant is that it contains chlorophylls *a* and *b*, starch as a storage material inside the chloroplast, and a cell wall made of cellulose. Higher plants and algae are part of the same group (Chlorobionta). There is 70–98 % genetic similarity between land plants and algae (Devereux et al. 1990). The size of the organism, the number of cells and differentiation into organs are not defining parameters of a plant. Consequently, single-cell microalgae are considered plants.

The following sequence of events occurs during the interaction between the two microorganisms within the polymeric bead. Initial immobilization is a random spread of particles inside a gel matrix (Gonzalez and Bashan 2000). Nutrients in the surrounding medium freely diffuse into the porous gel. Over time (6–48 h), depending on the pairing of microalgae and bacteria, both microorganisms are found in the same cavity within the bead, mainly just beneath the surface. Small parts of the internal structure of the bead matrix dissolve or split and separate as microcolonies develop and enlarge (Covarrubias et al. 2012; Lebsky et al. 2001; de-Bashan et al. 2011). The bacteria mainly excrete indole-3-acetic acid (IAA) and other undefined signal molecules that reach the nearby microalgal cells (de-Bashan et al. 2008a). At this stage, the activities of the microalgal enzymes (two were tested so far, glutamine synthetase and glutamate dehydrogenase) are not enhanced (de-Bashan et al. 2008c). At the next phase of interaction, beginning about 48 h after joint immobilization and continuing, glutamate synthetase and glutamate dehydrogenase activities are enhanced, photosynthetic pigment production is enhanced (de-Bashan et al. 2002a), nitrogen and phosphorus uptake into microalgal organelles is accelerated (de-Bashan et al. 2005), carbohydrates accumulation, especially starch, occurs (Choix et al. 2012a, b), as well as an increase in lipids and fatty acids (de-Bashan et al. 2002a; Leyva et al. 2015). At the same time, the co-immobilized system liberates oxygen produced by *Chlorella* spp. as a by-product of photosynthesis. The metabolic functions of this model, studied so far, are illustrated in Fig. 20.3. At the same time, the common phenotypic colonization of *Azospirillum* on roots, connection to the root surface by all sort of fibrillar material (Bashan et al. 1986; Levanony et al. 1989) are detected in the *Azospirillum–Chlorella* interactions (de-Bashan et al. 2011).

These favorable characteristics have biotechnological implications. The model is not restricted to *Chlorella vulgaris–Azospirillum brasilense* interactions that have comprised most of the studies done so far. Other PGPB, such as *Bacillus pumilus*, *A. lipoferum*, *Phyllobacterium myrsinacearum*, and other microalgae, such as *C. sorokiniana*, were successfully tested (de-Bashan et al. 2008b, c; Gonzalez-Bashan et al. 2000; Hernandez et al. 2009).

These options create opportunities for endless combinations of microalgae and PGPB and for many *Azospirillum* strains. Similarly, different alginates and derivatives from many macroalgae are commercially available (McHugh 2003) for entrapment and combination schemes, as needed. Because immobilization of microorganisms is also commonly used with other polymers (O'Reilly and Scott 1995), this model is not restricted to alginates; each polymer has its own advantages and disadvantages.

The practical and analytical aspects of this model are considerable. All ingredients are inexpensive, and the microorganisms are easy to cultivate and test in standard microbiology facilities. The results are available on a microbial time scale (days to a week). Reproducibility is very high, and replicates are merely Erlenmeyer flasks, allowing as many replicates as needed in a small space and in a soil-free system. Reviewing hundreds of published results using this system, it appears that the standard error is low and allows detection of minute effects between the interacting organisms. So far, we have not observed any disadvantages in experiments conducted over the past 15 years.

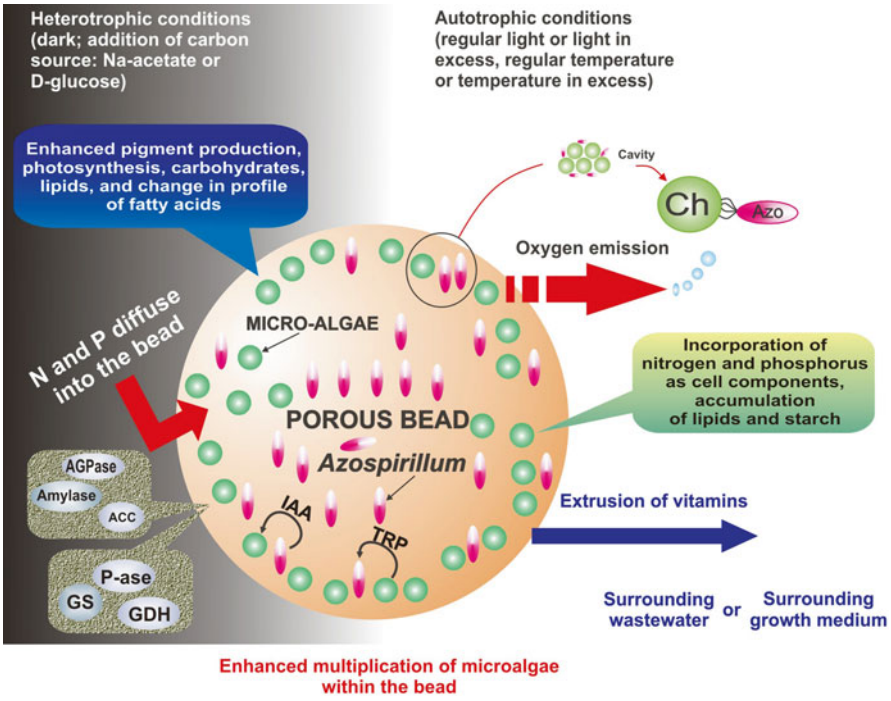

Fig. 20.3 A conceptual model of *Azospirillum* spp. co-immobilized with microalgae in alginate beads to study prokaryotic–eukaryotic interaction under autotrophic and heterotrophic conditions. *Azo Azospirillum* spp., *Ch Chlorella* spp., *GS* glutamine synthetase, *GDH* glutamate dehydrogenase, *IAA* indole-3-acetic acid, *P-ase* phosphatase, *Amylase* α amylase, *ACC* acetyl-CoA carboxylase, *AGPase* ADP glucose pyrophosphorylase. This is an updated version of a model previously published in de-Bashan et al. 2012. Applied Soil Ecology 61: 171–189

20.3.1.1 Methods

To observe the physical interaction and fibril formation between the two microorganisms during their association, the following techniques are used. These are common techniques that employ few small modifications to this bacterial species and are described in detail in the following references. However, when there are many small modifications, or when these small modifications have a significant importance for obtaining the expected results using this association, a detailed description of the method needs to be supplied.

- Scanning electron microscopy (SEM). There are standard techniques for SEM for plants, with modifications to adapt to the interaction with microalgae (Bashan et al. 1986; Covarrubias et al. 2012).
- Transmission electron microscopy (TEM) by conventional techniques (Lebsky et al. 2001).

- Fluorescent in situ hybridization (FISH), using one of the conventional techniques for FISH but adapted by many minor details specifically for this interaction. The images are observed by confocal laser microscopy (de-Bashan et al. 2011) or under fluorescent microscopy.
  - *Fixation and preparation of samples*. There are two ways to analyze the interaction inside the beads: dissolve the beads to free the microorganisms to measure the strength of attachment between the two partners in the model or slice the beads with a scalpel, in which case the physical distribution of the microorganisms inside the beads can be observed.
  - To dissolve beads (DB), at least ten beads are dissolved in 1 mL 4 % sodium bicarbonate for 30 min. One mL DB is centrifuged (14,000 × *g*); the pellet is washed twice in 1X PBS (15 % v/v 200 mM sodium phosphate buffer/130 mM NaCl at pH 7.4); it is then fixed with 4 % paraformaldehyde for 1 h at 4 °C. After fixation, the pellet is washed twice with 1X PBS and stored in a mix of 1X PBS/96 % ethanol (1:1 v/v) at −20 °C until used. Previous to hybridization, 10 μL of the sample is added to gelatin (0.1 % w/v, 0.01 % w/v chromium potassium sulfate)-coated microscope slide, air-dried, and dehydrated by successive 50, 80, and 96 % ethanol washes (3 min each). Samples are air-dried again (Daims et al. 2005).
  - For sliced beads (SB), each slice is mounted on gelatin (0.1 % w/v, 0.01 % w/v chromium potassium sulfate)-coated microscope slides, attached to the slide by adding 1 drop of warm, low-melt, agarose solution (0.25 % w/v), and dried at 37 °C for 45 min. The samples are then fixed with 50 μL 4 % paraformaldehyde and incubated at 4 °C for 1 h. Then the paraformaldehyde is removed by pipetting. The samples are washed with 0.85 % saline solution, dehydrated by successive 50, 80, and 96 % ethanol washes (3 min each), air-dried, and stored at 4 °C until hybridization.
  - In situ *hybridization*. This assay is based on the technique described by Assmus et al. (1995), with numerous small modifications. Hybridization is performed at 35 % formamide stringency at 46 °C for 2 h. Samples are washed at 48 °C for 5 min with 50 mL pre-warmed washing buffer. The slides are then rinsed for a few seconds with ice-cold, deionized water, and then air-dried. Slides can be stored at −20 °C in the dark until visualization. An equimolar mixture of probes is used: EUB-338 I (Amann et al. 1990), II, and III (Daims et al. 1999). These three probes, when combined, detected almost all bacteria. For *A. brasilense*, the specific probe Abras 1420 (Stoffels et al. 2001) is used. The EUB-338 I, II, and III probes are labeled with the fluorochrome FITC and the *Abras* 1420 probe is labeled with the fluorochrome Cy3. The final concentration of the probes is 30 ng·μL^{-1} for probes labeled with Cy3 and 50 ng·μL^{-1} for probes labeled with FITC. Before visualization, the slides are mounted in AF1 anti-fading reagent (Citifluor).

  - *Visualization*. With confocal laser scanning microscopy (CLSM), a LSM 510 META system with an Axiovert 100 M inverse microscope (Carl Zeiss, Oberkochen, Germany), or equivalent, can be used (Schmid et al. 2009). A helium neon laser provides the excitation wavelength of 543 nm (Cy3) and an argon ion laser provides the excitation wavelength of 488 nm (FITC). To distinguish between the fluorescence from Cy3 and FITC-labeled oligonucleotide probes, the specific signals are depicted in red and green, respectively. The third color channel (helium laser, 633 nm singular wavelengths) is used to visualize autofluorescence of the microalgae and is assigned a blue color. The three signals are combined and depicted as a red-green-blue (RGB) image. An Apochromat 63 X/1.2 water immersion lens is used for all analyses and acquisition of images. Analyses of images use LSM 510 4.2 software (Carl Zeiss).

- For epifluorescence microscopy, an Axioplan 2 (Carl Zeiss), equipped with a mercury lamp (HXP120, Osram) and Carl Zeiss filter sets for FITC/GFP (Emitter BP 525/50, Beamsplitter FT 495, Exciter BP 470/40), Cy3 (Emitter BP 605/70, Beam splitter FT 570, Exciter BP 545/25), and Cy5 (Emitter BP 690/50, Beam splitter FT 660, Exciter BP 640/30) excitation is used. An Apochromat 63 X/1.2 water immersion lens (Carl Zeiss) is used for all observations. Images are recorded with the CCD camera AxioCam MRm controlled by AxioVision Rel. 4.6 software (Carl Zeiss) and processed with Adobe Photoshop 8.0 software (Adobe Systems).
- A major technical difficulty observing microalgae–bacteria interactions by FISH is that autofluorescence of the microalgae is far stronger than the relatively faint FISH labeling of the bacteria. Consequently, it is impossible to obtain microalgae and bacteria in one sharp image. However, this does not affect the actual observation, since the laser's intensity can be manipulated. For precise observations, a technique used for solar photography is adapted, where the ultrabrilliant microalgae are obscured by a black circle, allowing observation of the nearby less-fluorescent bacteria. *A. brasilense* does not have autofluorescence. Consequently, after performing FISH with the probes described above, *A. brasilense* cells should exhibit fluorescence only in the green and red channels. Additionally, to enhance clarity of the images, exposure time is increased or decreased for each of the three channels, depending on the intensity of the observed autofluorescence and specific FISH signals. As a result, positive fluorescence signals from *A. brasilense* vary in their fluorescence color from yellow-green to orange, arising from different intensities of the separately recorded red and green channels. Similarly, microalgae show slightly different tones, ranging from magenta to light cyan. The major difference, however, is the presence of the blue color fraction, which is absent in *A. brasilense* signals.

  - *Quantification*. Cell counting and measuring populations and cluster size of the microalgae and bacteria in FISH images obtained from the confocal laser scanning and epifluorescence microscopies can be quantified using image analyzing software (Image Pro-Plus 4.1, Media Cybernetics).

20.3.2 Wastewater Treatment

A combination of microalgae *Chlorella vulgaris* or *C. sorokiniana* with *A. brasilense* strain Cd, co-immobilized in small alginate beads, was developed to remove phosphorus and nitrogen nutrients from municipal wastewater. Co-immobilization of the two microorganisms was superior to removal efforts by the microalgae alone, reaching up to 100 % ammonium, 15 % nitrate, and 36 % phosphorus within 6 days (varied with the source of the wastewater), compared to 75 % ammonium, 6 % nitrate, and 19 % phosphorus by the microalgae alone (de-Bashan and Bashan 2010; Covarrubias et al. 2012; Cruz et al. 2013). This happens in synthetic residual wastewater (de-Bashan et al. 2002b) or domestic wastewater (de-Bashan et al. 2004) at ambient temperature (~25 °C) or extreme temperature (>40 °C) and irradiation (up to 2,500 μmol m^2 s^{-1}), using microalgal strains that are resistant to these conditions (de-Bashan et al. 2008b) and under autotrophic and heterotrophic conditions (Perez-Garcia et al. 2010, 2011). Artificial, sterile (by autoclaving) wastewater used in some of these studies is prepared using the following (mg/L): NaCl, 7; $CaCl_2$, 4; $MgSO_4 \cdot 7H_2O$, 2; K_2HPO_4, 21.7; KH_2PO_4, 8.5; Na_2HPO_4, 33.4; and NH_4Cl, 191. For continuous and semi-continuous cultures, KH_2PO_4, at levels in the range of 12–15 mg/L, was used as the sole source of phosphorus.

Biological removal of phosphorus is a harder task than removing nitrogen. In domestic wastewater, phosphorus removed by *C. sorokiniana* was significantly enhanced after a starvation period of 3–5 days in saline solution, combined with co-immobilization with *A. brasilense* Cd. The best phosphorus removal treatment of a batch of synthetic or domestic wastewater was with tandem treatments of wastewater treatment first with pre-starved, co-immobilized microalgae and replacement of this culture after one cycle of removing phosphorus with a new, similarly starved culture. This sequential treatment with two cultures was capable of removing up to 72 % of the phosphorus from the wastewater (Hernandez et al. 2006). It appears that starvation periods, combined with co-immobilization with *A. brasilense* have synergistic effects on absorption of phosphorus from wastewater by microalgae.

The advantage of this technology is that microalgae that is co-immobilized with bacteria are always more effective at removing nitrogen and phosphorus than microalgae without bacteria. As the two microorganisms are immobilized in alginate beads that are easily and rapidly removed from wastewater by sedimentation, this technology could be a cost-effective alternative to chemical precipitation, which is the standard treatment of wastewater. It solves two problems in standard microalgal technology: increasing the population of microalgae to a level sufficient to clean the wastewater and using the waste biomass in soil remediation when the cleaning process is completed.

20.3.2.1 Methods

- Bioreactors of various sizes (Cruz et al. 2013).
- Water analytical methods (Eaton et al. 2005) for the following parameters: NH_4^+ (μM), NO_3^- (μM), NO_2^- (μM), PO_4^{3+} (μM), pH, conductivity (mS m^{-1}), salinity (‰), silicates (μM), total hardness (mg L^{-1}, $CaCO_3$), Cl (mg L^{-1}), SO_4^{2-} (mg L^{-1}),

acidity (mg L^{-1}), total suspended solids (mg L^{-1}), dissolved solids, and sediments (mg L^{-1}).
- SEM and FISH coupled with specialized image-analysis quantification software (Covarrubias et al. 2012; also see above)

20.3.3 Increased Fertility of Eroded Soil

A potential application of this model of interaction is that the biological residues from a biological wastewater treatment (described above) is a resource for improving quality of degraded soils and improved plant growth. After tertiary wastewater treatment (removal of nutrients), debris composed of alginate beads containing the co-immobilized microorganisms can be used as an amendment for eroded and infertile soils with low levels of organic matter, where the microalgae serves as organic matter and *A. brasilense* as a PGPB. *A. brasilense* survived in these used, dried alginate beads for at least 1 year. Three consecutive applications of the dry debris increased organic matter, organic carbon, and microbial carbon in the soil. Growth of sorghum in the amended soil was greater than sorghum grown in soil with low organic matter, untreated soil, or soil amended with beads containing other combinations of alginate, microalgae, or bacteria. The surface of plant roots growing in the amended soil was heavily colonized by *A. brasilense*, with no endophytic colonization; root tips were the preferred sites of colonization (Trejo et al. 2012). Application of this residue significantly changed the bacterial rhizosphere population of plants growing in these soils (Lopez et al. 2013).

20.3.3.1 Methods

- Extraction of DNA from degraded soil is a modification of the method described by de-Bashan et al. (2010a, b), using a kit (Fast DNA SPIN for soils, MP Biomedicals) and applied according to the manufacturer's instructions. To remove humic acids, the binding matrix–DNA complex can be rinsed with saturated 5.5 M guanidine thiocyanate (Fluka-Sigma-Aldrich). Each DNA extraction is performed with a 0.6 g soil sample.
- Polymerase chain reaction (PCR). A modification of PCR procedure described by de-Bashan et al. (2010a, b) is used. The V9 variable region of the 16S rRNA gene is amplified with the bacteria primers 1070F (5′-ATG GCT GTC GTC AGC T-3′) and 1406R (5′-ACG GGC GGT GTG TAC-3′) with a 40 bp GC clamp (Ferris et al. 1996). A modification of PCR for DGGE by Colores et al. (2000) is used. These modifications include: Each PCR mixture (25 μL) contains 1 × PCR buffer with 15 mM $MgCl_2$ (Qiagen Sciences), 200 μM of each deoxyribonucleoside triphosphate (Sigma), 0.2 μM each primer, 5 % dimethyl sulfoxide (Sigma), 0.4 μg L^{-1} bovine serum albumin (Sigma), 0.6 units μL^{-1} HotStarTaq DNA polymerase (Qiagen Sciences), and ~100 ng template DNA. PCR is run in a thermocycler (Eppendorf) at 95 °C for 15 min for 30 cycles (94 °C for 45 s,

55 °C for 45 s, 72 °C for 30 s, and an extension at 72 °C for 7 min). PCR products are viewed after electrophoresis by running a 2 % agarose gel (Sigma) with a gel stain (SYBR Safe, Molecular Probes). PCR products are quantified in a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific).

- Denaturing gradient gel electrophoresis (PCR-DGGE) analysis. A modification of DGGE of the 16S rRNA gene products by de-Bashan et al. (2010a, b) is performed using a D-code universal mutation detection system (Bio-Rad Laboratories). Acrylamide gels (6 %) are prepared with a 40–60 % urea-formamide denaturing gradient, according to the manufacturer's protocol. Lanes are loaded with 15 μL PCR product. The external reference ladder may consist of different known species of bacteria. Electrophoresis is run at 40 V for 10 min at 60 °C and subsequently at a constant 60 V for 16.5 h at 60 °C. Gels are stained with nucleic acid gel stain (SYBR Green I, Molecular Probes) and gel images are recorded with a gel documentation imaging system (Gel Doc XR, Bio-Rad Laboratories).
- Identification of *A. brasilense* in PCR-DGGE profiles. Presumptive bands of *A. brasilense* Cd are excised from DGGE gels (45–60 % gradient) using sterile razor blades under UV illumination. The excised bands are eluted in 300 μL ultrapure water and incubated at 37 °C for 1 h. Aliquots are diluted 1:10 in ultrapure water; 2 μL of this dilution is used as a template to re-amplify the replicon by using the same PCR conditions and DGGE primers described earlier. The size of the PCR product is confirmed on 2 % agarose gel after each round of amplification. Successive PCR-DGGE gels were run to verify the identity and purity of the excised bands by comparing the re-amplified PCR products to the profile of the external reference ladder containing *A. brasilense*. PCR products that exhibited the highest identity to the *Azospirillum* band in the DGGE gel are purified using the QIAquick PCR purification kit protocol (Qiagen Sciences), and then submitted for commercial sequencing using primer 1070F (Genewiz). The original *A. brasilense* inoculum and its corresponding band in the external reference ladder are also sequenced at the same time as the experimental samples.
- Statistical analysis of DGGE gels. Analysis of gels is incomplete without detailed statistics of the bands. The band profiles obtained from DGGE gels are analyzed for similarity using the Dice coefficient. A dendrogram is built either from the Weighted Pair Group Matching Average (WPGMA) or the Unweighted Pair Group Matching Average (UPGMA). Similarity varies from 0 to 1, where 1 indicates 100 % similarity. Additionally, the observed similarities between profiles of DGGE are analyzed by multivariate statistical analysis, such as Kruskal's non-metric multidimensional scaling (NMDS; Venables and Ripley 2002) using computing software (Statistica 8.0, StatSoft). The Kruskal stress coefficient was used to reflect goodness-of-fit of the model. Values of Kruskal stress <0.1 are considered a good fit. Canonical analysis is also used for that purpose (de-Bashan et al. 2010b).

  Bacterial richness considered each band as an individual Operative Taxonomic Unit (OTU) (Kisand and Wikner 2003). This is obtained from the Band Type Report of the Quantity One 4.6.7 imaging software (Bio-Rad Laboratories) that provides the number of bands detected in DGGE profiles. Bacterial diversity is

calculated by analyzing the relative intensity of each peak (corresponding to a defined band) in the densitometric profile with Shannon's Diversity Index (Iwamoto et al. 2000), calculated by the formula: $H = -\Sigma Pi \log_{10} Pi$, where Pi is the importance probability of the bands in a gel lane and is calculated as $Pi = ni/N$, where ni is the intensity of a peak and N is the sum of all peak intensities of bands (Iwamoto et al. 2000). Data is then analyzed by one-way ANOVA and then by Tukey's post hoc analysis (or any other post hoc analysis) at $P < 0.05$, using statistical software.

- Root colonization by FISH. The technical details are presented above. Colonization by *Azospirillum* spp. is counted from images of FISH with imaging software (Image Pro Plus 6.3.1.542, Media Cybernetics) (modification of Treiser et al. 2007). Using the software RGB color code definitions, the specific magenta color (or any other color that the bacterium was labeled for) of *Azospirillum* detects qualitatively by FISH in these images is composed R-255, G-000, and B-255. The software measures the number of pixels that harbor this specific fluorescence and ignores other colors. The coverage (in %) of this fluorescence per area of root (in μm^2) is measured; this reflects the presence and level of colonization of each of the ten segments measured for each root part. These ten segments cover the entire root tip.
- Microbial biomass (expressed as microbial carbon) of soil is determined with a combination of the fumigation-extraction-oxidation of dichromate techniques described elsewhere (Joergensen and Brookes 2005).

20.3.4 Increased Bulk for Animal and Human Feed

Co-immobilization of *C. vulgaris* and *A. brasilense* under autotrophic condition yield, under a variety of environmental conditions, a significantly increased growth of the microalga. Dry and fresh weight, total number of cells, size of the microalgal clusters (colonies) within the bead, number of microalgal cells per cluster, and cell size significantly increased (de-Bashan et al. 2002a, 2005; Gonzalez and Bashan 2000). An even higher cell yield can be induced under heterotrophic conditions with D-glucose or Na-acetate as carbon sources (Perez-Garcia et al. 2010). When the microalgae is growing under less than optimal conditions, co-immobilization with *A. brasilense* mitigates the effect of these adverse condition on growth and metabolism of the microalgae (de-Bashan and Bashan 2008; de-Bashan et al. 2008c; Choix et al. 2014). This system has not been scaled up for biomass production.

20.3.4.1 Methods

- *Microbial counts*. Beads are solubilized for cell counts by immersing five beads (one bead per milliliter) in a solution of 4 % $NaHCO_3$ for 30 min at ambient temperature of 25 ± 4 °C. *A. brasilense* is counted by plating a series of dilutions

(in PBS) on BTB agar plates (Bashan et al. 2011). Alternatively, *A. brasilense* cells are first stained with fluorescein diacetate (Sigma) (Chrzanowski et al. 1984) and then directly counted under a fluorescent microscope. *C. vulgaris* is counted using a Neubauer hemocytometer connected to image analyzer or manually under light microscopy (Gonzalez and Bashan 2000). Growth rate of *C. vulgaris* (μ) is defined as: $\mu = (\ln Nt_1 - \ln Nt_0)/(t_1 - t_0)$, where Nt_1 is the number of cells at sampling time and Nt_0 is the number of cells at the beginning of the experiment, t_1 is sampling time and t_0 the beginning of the experiment (Oh-Hama and Miyachi 1992).

- *Determining biomass*. Ten grams of beads containing co-immobilized microalgae and bacteria are dissolved in 100 mL, as described above. The suspension is then filtered through a 3 mm (pore size) plankton net, leaving a pellet of microalgae on the net. This pellet is suspended in 100 mL PBS. Aliquots (10 mL) are centrifuged for 3 min at $1{,}400 \times g$ in tubes containing filter paper at the bottom. The supernatant containing the bacteria is discarded. The dry weight of the microalgae is measured after extracting and drying the filter paper at 105 °C for 1 h that contains the microalgal pellet.

20.3.5 Increased Photosynthetic Pigments

Green microalgae are commonly used for production of pigments for food and cosmetics (Lebeau and Robert 2006). Pigment production of the four major microalgal pigments; chlorophyll *a* and *b*, lutein, and violaxanthin of *C. vulgaris* and *C. sorokiniana*, co-immobilized with *A. brasilense*, significantly increased (de-Bashan et al. 2002a). This is very similar to the increase of these pigments in wheat plants inoculated with *A. brasilense* (Bashan et al. 2006). This system has not been as yet scaled up for pigment production.

20.3.5.1 Methods

- Pigments other than chlorophylls are detected, analyzed, and quantified by a HPLC method used mainly for pigments in plants (Bashan et al. 2006).
- Determination of chlorophyll. To determine the quantity of chlorophyll *a*, (the major component of this molecule in the microalgae) extraction is done according to Sartory and Grobbelaar (1984), with small modifications. Quantification used the equation of Porra et al. (1989): Chl $a = 16.29\ (A_{665}) - 8.54\ (A_{652})$. Briefly, 10 mL 100 % methanol is added to 5 mL of freshly thawed beads and heated for 10 min at 70 °C. After cooling, the samples are incubated in the dark for 24 h at 4 °C. Then, the samples are centrifuged for 10 min (4 °C; $6{,}000 \times g$) and absorbance is recorded in the supernatant at 665 and 652 nm.

20.3.6 Increase Carbohydrate Production

The interaction of *Azospirillum* spp. with microalgae enhances accumulation of total carbohydrate and starch in microalgae, either under autotrophic conditions or in the dark under heterotrophic conditions when D-glucose or Na-acetate is supplemented as a carbon source. Cells of *Chlorella* accumulated the highest amounts of carbohydrate after incubation for 24 h. After incubation for 72 h, mainly under co-immobilization treatments of both microorganisms, the cultures reached their highest total carbohydrate content (mainly as starch). This coincides with enhanced activity of ADP-glucose pyrophosphorylase (AGPase) that regulates starch biosynthesis in higher plants and microalgae. This demonstrates the potential of *A. brasilense* to affect carbohydrates and starch accumulation in *Chlorella* spp. when both microorganisms are co-cultured. This can be an important tool for future applications of microalgae, as in biofuel production (Choix et al. 2012a, b, 2014).

20.3.6.1 Methods

- Extraction and determination of carbohydrates. One gram of alginate beads is washed in distilled water, dried at 80 °C for 12 h, and ground with a mortar and pestle to yield a 10 mg sample. This sample is resuspended in 5 mL 1 M H_2SO_4 and sonicated for 4 min at 22.5 kHz with an ultrasonic cell disruptor. Carbohydrates are extracted by acid hydrolysis of the slurry after 60 min at 100 °C. Total carbohydrates are quantified by the phenol–sulfuric method (Dubois et al. 1956), adapted to microplates, using glucose as the standard.
- Starch is quantified by the method described by Brányiková et al. (2011), which is based on total hydrolysis of starch by 30 % perchloric acid and quantified by colorimetric means of the liberated glucose.
- Uptake of D-glucose or Na-acetate from the growth medium by microorganisms is analyzed using the Megazyme D-glucose (glucoseoxidase/peroxidase) assay kit (K-GLUC, gopod format, Megazyme International) and a kit to measure acetic acid (K-ACETAF 12/07, acetyl-coA synthetase format; Megazyme International).
- Enzymatic activity of ADP-glucose pyrophosphorylase (AGPase):
  - *Extraction*: To determine enzymatic activity, 6 g alginate beads are dissolved in 30 mL 4 % $NaHCO_3$ solution and centrifuged at 2,000 × *g* for 6 min. The supernatant is discarded and the pellet is washed three times with sterile saline solution (0.85 % NaCl). Enzyme extraction is done in 3 mL 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 2 mM EDTA, 20 mM β-mercaptoethanol, 12.5 % (v/v) glycerol, and 5 % (w/v) insoluble polyvinylpolypyrrolidone-40 at 4 °C (Nakamura et al. 1989).
  - *Quantification*: Enzymatic activity of AGPase is measured by the method of Li et al. (2011), with modifications as follows: the reaction buffer contains (in mM): HEPES at pH 7.4 (100), ADP-glucose (1.2), sodium pyrophosphate (3), $MgCl_2$ (5), dithiothreitol (4; D0632, Sigma), in a final volume of 500 µL.

500 μL of the extracted enzyme is added to the reaction buffer. This reaction mixture is incubated at room temperature (26 ± 2 °C) for 20 min. The reaction is stopped by heating in boiling water for 2 min. Then, 600 μL distilled water is added, and the mixture is centrifuged at 13,000 × *g* for 10 min. The supernatant (1,000 μL) is mixed with 0.3 mg $NADP^+$. The activity is recorded as the increase in A_{340} after adding 2 μL of each of the two enzymes: phosphoglucomutase (0.8 U) and glucose-6-phosphate dehydrogenase (1 U). The enzymatic activity of AGPase is expressed as U mg^{-1} protein, where one unit is 1 nmol of ADP mg^{-1} protein min^{-1}. Proteins in the mixture are determined by the Bradford assay (Bradford 1976).

20.3.7 Increase Fatty Acids and Lipid Production

The interaction yielded more fatty acids and more lipids, mainly in the microalgae (de-Bashan et al. 2002a). Under autotrophic and heterotrophic growth conditions, co-immobilization always enhanced the activity of acetyl-CoA carboxylase (ACC), a key enzyme in de novo fatty acid biosynthesis, and yielded more lipids, when compared with immobilization of the microalga by itself. The highest lipid content under autotrophic conditions was obtained by also using an ammonium starvation period. Cultivation under heterotrophic conditions, without limitation of nitrogen, yielded a higher growth rate and accumulated more lipids than under autotrophic conditions (Leyva et al. 2014). Considering the major efforts to produce biodiesel from microalgae (Brennan and Owende 2010), this interaction has a significant, yet unexplored, biotechnological potential.

20.3.7.1 Methods

- Quantification and subsequent identification of fatty acids are done according to the method described by Sato and Murata (1988), with several small, but important, variations. The method is based on a direct transmethylation of fatty acids without previous extraction of total lipids. Freeze-dried bead samples (100–200 mg per sample) are placed in a screw-cap glass tube. Five mL of a mix of concentrated hydrochloric acid and absolute methanol (5:95: HCl:CH_3OH v/v) are added to each sample and the cap hermetically sealed with additional polytetrafluoroethylene (PTFE) film. The tubes are placed in a water bath at 90 °C for 2 h for transmethylation. These samples are cooled to room temperature (26–28 °C) and 2 mL pure hexane (HPLC grade, #650552, Sigma-Aldrich) and 0.5 mL MilliQ water (EMD Millipore) are added to each sample and gently mixed in a vortex. After 10 min incubation at room temperature, when the layers are separated, the top hexane layer is transferred to a clean tube and the water layer is discarded. The hexane is evaporated under nitrogen gas and the dry pellet was resuspended with a known volume of hexane (500 μL for *A. brasilense* and

1 mL for *C. vulgaris* alone or co-immobilized) and transferred to a crimp-top sealed vial (#5181-8801, Agilent Technologies) and injected into a gas chromatograph-mass spectrograph (HP-GDC1800B, Agilent Technologies) equipped with a 30 m × 0.25 mm × 0.25 μM column (Omegawax 250, Supelco). The latter dimension is the size of the particles in the column. Running conditions are specified by the manufacturer: 1 μL of injected sample, high purity helium as the carrier gas, flow rate of 0.9 mL·min^{-1}, and injections of the sample in the splitless mode. The temperatures of the injector and detector are 250 °C and 260 °C, respectively. Each run involved the following pre-programmed steps: initial temperature of 110 °C for 3 min, then an increase of 30 °C·min^{-1} to 165 °C for 2 min. Then, the temperature is increased at the rate of 2.2 °C·min^{-1} to 209 °C for 35 min. Identification of fatty acids is done by comparing the retention times of each methylated fatty acid with the corresponding fatty acid in the calibration curve of the gas chromatograph. Identification is confirmed by analyzing the mass spectrum of each fatty acid. The threshold of detection was set to 0.5 % of total fatty acids. The fatty acid analyses are based on 6 days of experiments and the samples were taken at the end of each experiment.

- Enzymatic activity of ACC.
  - *Extraction*: Frozen bead aliquots are dissolved in two volumes of 4 % $NaHCO_3$ solution for 40 min at room temperature. Each suspension is then centrifuged (5,000 × *g*, 10 min, 4 °C); the supernatant is discarded, and the pellet is washed twice in 0.85 % NaCl and centrifuged again. The pellet is frozen with liquid nitrogen and pulverized with pestle and mortar. For resuspension, 5 mL extraction buffer [100 mM Tris–HCl, pH 8.2, 4 mM ethylenediaminetetra acetic acid (EDTA), 10 mM dithiothreitol (DTT), and 1 mM phenylmethanesulfonyl fluoride (PMSF; #P7626, Sigma-Aldrich)] is added to the pellet. This is centrifuged for 30 min at 10,500 × *g* at 4 °C. The pellet is discarded and the supernatant is used as a crude extract for enzymatic reactions. The last steps are according to de-Bashan et al. (2008b).
  - *Quantification*: The reaction buffer is composed of 50 mM Tris–HCl pH 7.5, 6 μM acetyl-CoA, 2 mM ATP, 7 mM KHCO3, 8 mM MgCl2, 1 mM DTT, and 1 mg·mL^{-1} of bovine serum albumin (BSA; #B4287, Sigma-Aldrich). The crude extract is pre-incubated for 30 min at 25 °C with 10 mM potassium citrate and 2 mg·mL^{-1} BSA. Then, 500 μL crude extract is added to 0.5 mL of reaction buffer and the enzymatic reaction is incubated for 100 min at 30 °C. The reaction is stopped with 0.5 mL 10 % perchloric acid (PCA; #244252, Sigma-Aldrich). The total reaction mixture is filtered (0.22 μm membrane filter; EMD Millipore). Then 500 μL of this mixture are transferred to a 1.5 mL glass vial and injected into the HPLC according to the method described by Levert et al. (2002), using a 5 m × 150 mm × 4.6 μm column (Zorbax Eclipse Plus C-18, Agilent Technologies). The flow rate is 1 mL·min^{-1} and the UV detector is adjusted to 262 nm. Solution A is 10 mM KH_2PO_4 at pH 6.7 and solution B is absolute methanol. Using analytical software (ChemStation, Agilent Technologies), the peak areas are recorded and the quantity of acetyl-CoA is calculated with previously completed standard

curves of acetyl-CoA and malonyl-CoA; hence, measuring either the disappearance of the substrate (acetyl-CoA) or the formation of the product (malonyl-CoA). The specific activity is defined as nmoles of substrate transformed per minute per 1 mg of protein.

- Lipids.
  - *Standard curve for lipids*: The quantity of lipids is measured following the method described by Pande et al. (1963). Extraction of lipids follows the standard method described by Bligh and Dyer (1959), but with small, yet very important, modifications to adapt it to microalgae, which involves sonication to break down cell walls. Briefly, lipids are extracted by adding 4 mL methanol/chloroform solution (2:1, v/v) to dry beads. The beads are sonicated for 10 min (2 cycles of 5 min at 30 kHz) in an ice bath. Sonicated beads are then incubated at 4 °C for 24 h in the dark and this procedure (only sonication) is repeated under the same conditions. The sample is then centrifuged (5,000 × *g*, 20 min, 4 °C), and the supernatant is transferred to a clean tube. The rest of the analysis is done as originally described.
  - *Quantification of lipids*: Lipid assays, based on a potassium dichromate color change reaction, are done according to Pande et al. (1963), using a calibration curve with tripalmitin (#T5888, Sigma-Aldrich), as a standard. The concentration of lipids is determined in a microplate reader (Molecular Devices) at 590 nm, recording the intensity of the green color that is formed. Potassium dichromate has a yellow-reddish color before reaction with lipids and a yellow-green color after the reaction with lipids. The method quantifies lipids in the range of 70 μg to 1.33 mg.

20.4 Conclusions

The interaction of *Azospirillum* spp. with photosynthetic, single-celled microalgae provides an important shortcut for understanding the interaction of this PGPB with plants, in general. This interaction is relevant for studying physiological, biochemical, and molecular aspects of the interaction. As an independent subfield of *Azospirillum* research, this interaction has some important biotechnological applications; most are yet to be tried in larger scale production and evaluation of their potential.

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Chapter 21
Pseudomonas and *Azospirillum*

Claudio Valverde, Gustavo Gonzalez Anta, and Gustavo Ferraris

Abstract *Pseudomonas* strains are fast growing, genetically diverse and metabolically versatile bacteria. Many pseudomonad species are preferential inhabitants of the rhizosphere of plants, reaching up to 10^8 CFU/g of roots for crop species like soybean or maize in the field. Rhizospheric pseudomonads contribute to plant growth and health through a variety of plant probiotic mechanisms, including protection of roots against fungal pathogen attack. Due to their relative ease to isolate and cultivate in the lab, there is an enormous wealth of knowledge about physiological, biochemical, and genetic traits of pseudomonads. Based on their PGPR traits, several inoculant products are commercialized, either for seed, foliar, or post-harvest treatment of crops, vegetables, and fruits. Provided that pseudomonads share the rhizosphere niche with *Azospirillum* species, as well as with many other PGPR microorganisms, combined formulations have also become available for agronomic purposes. However, little information about interspecies and multispecies interactions is available. This chapter describes microbiological, genetic, and agronomic tools that may be applied to isolate and characterize novel *Pseudomonas* spp. from diverse source materials, to study their interaction with *Azospirillum* cells in the context of dual or multispecies inoculants, and to evaluate the quality and effectiveness of formulated products.

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21.1 Introduction

Members of the genus *Pseudomonas* (i.e., the pseudomonads) are ubiquitous Gram-negative γ-proteobacteria. In particular, many pseudomonad species are preferential inhabitants of the rhizosphere of plants, reaching up to 10^8 CFU/g of roots for crop species like soybean or maize in the field (Agaras et al. 2014). These root associated pseudomonads contribute to plant growth and health through a variety of plant probiotic mechanisms (Table 21.1). For instance, pseudomonads are one of the main prokaryotic taxonomic groups actively involved in protection of plant roots against fungal pathogen attack (Mendes et al. 2011; Spence et al. 2014). Many (but not all) species are proficient in secretion of siderophores (Cornelis 2010), which contribute to their competitive root colonizing ability (Lugtenberg and Kamilova 2009), and cause the appearance of fluorescent halos around colonies developed on iron limited media (i.e., "fluorescent pseudomonads").

Pseudomonas strains are fast growing, genetically diverse, and metabolically versatile bacteria. Due to their relative ease to isolate and cultivate in the lab, there is an enormous wealth of knowledge about physiological, biochemical, and genetic traits of pseudomonads (Ramos and Filloux 2007). This is reflected in the number of sequenced genomes available (www.pseudomonas.com). Based on their PGPR traits, several inoculant products are commercialized, either for seed, foliar, or post-harvest treatment of crops, vegetables, and fruits (Table 21.1). Provided that pseudomonads share the rhizosphere niche with *Azospirillum* species, as well as with many other PGPR microorganisms, combined formulations are available for agronomic purposes (Table 21.2). However, little information about interspecies and multispecies interactions is available. Here, we will summarize a series of microbiological, genetic, and agronomic tools that may be applied to isolate and characterize novel *Pseudomonas* spp. from diverse source materials, to study their interaction with *Azospirillum* cells in the context of dual or multispecies inoculants, and to evaluate the quality and effectiveness of formulated products.

21.2 Isolating and Characterizing Pseudomonads

There are many nonselective and semi-selective growth media for propagating pseudomonads in the lab and a few selective media for specific isolation of pseudomonads from complex samples like soil and plant materials. One of the most popular nonselective and differential medium is King's B (King et al. 1954). This medium is available as a pre-formulated commercial product (i.e., "*Pseudomonas* agar" or "F agar"). King's B medium allows fast growth of pseudomonads and provides iron-limiting conditions that induce siderophore production in fluorescent pseudomonad isolates.

Cetrimide agar (Brown and Lowbury 1965) and Gould's S1 (Gould et al. 1985) are two *Pseudomonas*-selective media that are based on their intrinsic resistance to detergents and to an antibiotic. In our experience (Agaras et al. 2012), Gould's S1

Table 21.1 Selected commercial products based on *Pseudomonas* strains

Product	Producer, country of origin	Strain	Claimed mechanism of action	Intended crop	More information
Amase®	Bioagri AB, Sweden	*P. azotoformans* (n.d.)	Not specified	Pine and spruce seedlings, cucumber, lettuce, tomato, peppers, eggplant, cabbage, and broccoli	www.bioagri.se
Biagro PSA liquid	Lab. Biagro SA, Argentina	*P. chlororaphis* subsp. *aurantiaca* (n.d.)	Root growth promotion	Wheat, barley, sunflower	www.biagro.com.ar
Biagro ProSol	Lab. Biagro SA, Argentina	*P. fluorescens*	Root growth promotion	Not specified	www.biagro.com.ar
Bio-Save® 10LP & 11LP	Jet Harvest Solutions, USA	*P. syringae* ESC-10 and ESC-11	Antibiosis	Citrus fruit, cherries, apples, pears, potatoes, sweet-potatoes (post-harvest)	www.jetharvest.com
Bio-Soil™	Jet Harvest Solutions, USA	*P. putida* (n.d.), *Bacillus* spp.	Not specified	Vegetables, ornamentals, grasses	www.jetharvest.com
BlightBan A506	Nufarm, USA	*P. fluorescens* A506	Antifreezing, antibiosis	Flowering fruit trees	www.nufarm.com
Cerall®, Cedomon®, Cedress®	Bioagri AB, Sweden	*P. chlororaphis* (n.d.)	Antibiosis, ISR, competence for niche	Wheat, triticale, barley	www.bioagri.se
Rizofos® Liq Maíz, Rizofos® Liq Trigo	Rizobacter Argentina SA, Argentina	*P. fluorescens* (n.d.)	Phosphorus solubilization, root growth promotion	Maize, wheat	www.rizobacter.com.ar

Table 21.2 Commercial products containing both *Azospirillum brasilense* and *Pseudomonas fluorescens* that are available in Argentina for agronomic application

Product	Contains[a]	Intended crops	More information
DEGfertil PGPR	Pf+Ab	Maize, sorghum, wheat, barley, sunflower	http://www.laboratoriosdegser.com/biofertilizantes/degfertil_m.html
DEGfertil PGPR Soja	Bj+Pf+Ab	Soybean	http://www.laboratoriosdegser.com/biofertilizantes/degfertil_m.html
PGPR® Biofertilizantes	Bj+Pf+Ab	Soybean, maize, sorghum, wheat, barley, sunflower	http://www.greenquality.com.ar/#!biofertilizantes/c13uf
Rhizoflo Premium	Pf+Ab	Wheat, sunflower, maize	http://www.ckc.com.ar/esp/index.html
BioPower	Pf+Ab	Maize, sorghum, sunflower	http://www.inoculantespalaversich.com/productos-biopower-info.html
Turbo (Soja)	Bj+Pf+Ab	Soybean	http://www.inoculantespalaversich.com/productos-turbo-info.html
Azomix Duo	Pf+Ab	Trigo	http://www.bionetsrl.com/inoculantes-azomix-duo.php
PGPR Soja	Bj+Pf+Ab	Soja	http://www.bionetsrl.com/inoculantes-pgpr-soja.php
Phoebus Biofertilizantes	Pf+Ab	Wheat, sorghum, maize, sunflower	http://agroat.com/new/catalogos/phoebus-biofertilizantes/
Phoebus Biofertilizantes	Bj+Pf+Ab	Soybean	http://agroat.com/new/catalogos/phoebus-biofertilizantes/

[a]*Ab Azospirillum brasilense*, *Bj Bradyrhizobium japonicum*, *Pf Pseudomonas fluorescens*

medium has proven effective to recover a wide variety of pseudomonad isolates from soil and rhizosphere material, without significant phylogenetic bias, and with a higher recovery and more colony morphotypes than the cetrimide agar medium commonly used in clinical laboratories for isolation of pseudomonads of clinical relevance. Both media are also differential because they induce siderophore production allowing distinction of fluorescent from non-fluorescent pseudomonad colonies.

21.2.1 Differential and Selective Media for Isolation of Pseudomonas *spp.*

All ingredients are indicated for 1 L of medium.

21.2.1.1 King's B Agar (King et al. 1954)

Proteose peptone #2 (DIFCO), 10 g; anhydrous K_2HPO_4, 1.5 g; glycerol, 15 g; $MgSO_4$ (1 M; sterile), 5 mL. Dissolve the first three ingredients in distilled water and bring volume to 1 L; adjust the pH to 7.0. Autoclave and then supplement with

sterile $MgSO_4$ (if added before autoclaving, the medium may become opaque). This medium is available as a pre-formulated commercial product (i.e., "*Pseudomonas* agar" or "F agar").

21.2.1.2 Cetrimide Agar (Brown and Lowbury 1965)

Gelatine peptone, 20 g; potassium sulfate, 10 g; magnesium chloride, 1.4 g; cetrimide, 0.3 g; agar, 15 g. As cetrimide agar is recommended for the isolation and differentiation of *P. aeruginosa* from different materials of clinical origin, there are many commercial suppliers of the formulated medium.

21.2.1.3 Gould's S1 (Gould et al. 1985)

Sucrose, 10.0 g; glycerol: 10.0 mL; casamino acids: 5.0 g; $NaHCO_3$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; K_2HPO_4, 2.3 g; sodium lauroyl sarcosine: 1.2 g; trimethoprim (5-[(3,4,5 trimethoxyphenyl)methyl] 2,4-pyrimidinediamine), 0.02 g; agar: 15.0 g. All ingredients can be dissolved in distilled water before autoclaving. The pH should be 7.4–7.6. After autoclaving, let the medium cool down and pour into Petri dishes. Once solidified, if not used immediately, S1 plates must be stored at room temperature and never kept in the fridge to avoid precipitation of lauroyl sarcosine. There is no commercial formulation of S1 medium, so every batch needs to be controlled for its selectivity by streaking positive (*Pseudomonas* strains) and negative controls (other Gram-negative and Gram-positive genera).

21.2.2 Isolation of Pseudomonads from Soil and Plant Material

We routinely isolate pseudomonads from soil and rhizosphere samples by plating aqueous suspensions on Gould's S1-selective plates (Fig. 21.1). The procedure described below (Agaras et al. 2012) may be readily adapted to isolate *Pseudomonas* spp. from other sources like plant leaf, fruit, or grain surfaces. Alternatively, endophytic strains may be also successfully recovered from root, shoot, or seed internal tissues, upon a proper surface disinfection procedure that has to be adjusted for every kind of sample.

Soil (1 g) or roots with tightly adhered soil (2 g) are thoroughly agitated (250 rpm, 20 min) in 50-mL conical tubes containing 10 volumes of saline solution (0.85 % w/v NaCl). Suspensions are then sonicated for 1 min in a water bath (40 kHz, 160 W, Testlab TB04, Argentina) to favor detaching of tightly adhered cells and to dislodge biofilm microcolonies. Tubes are briefly centrifuged at very low speed ($50 \times g$ for 1 min) to separate soil particles, and the supernatant is recovered in new tubes. Serial tenfold dilutions of soil or rhizosphere suspensions are plated in triplicate on Gould's S1 plates supplemented with cycloheximide (100 μg/mL) to inhibit growth

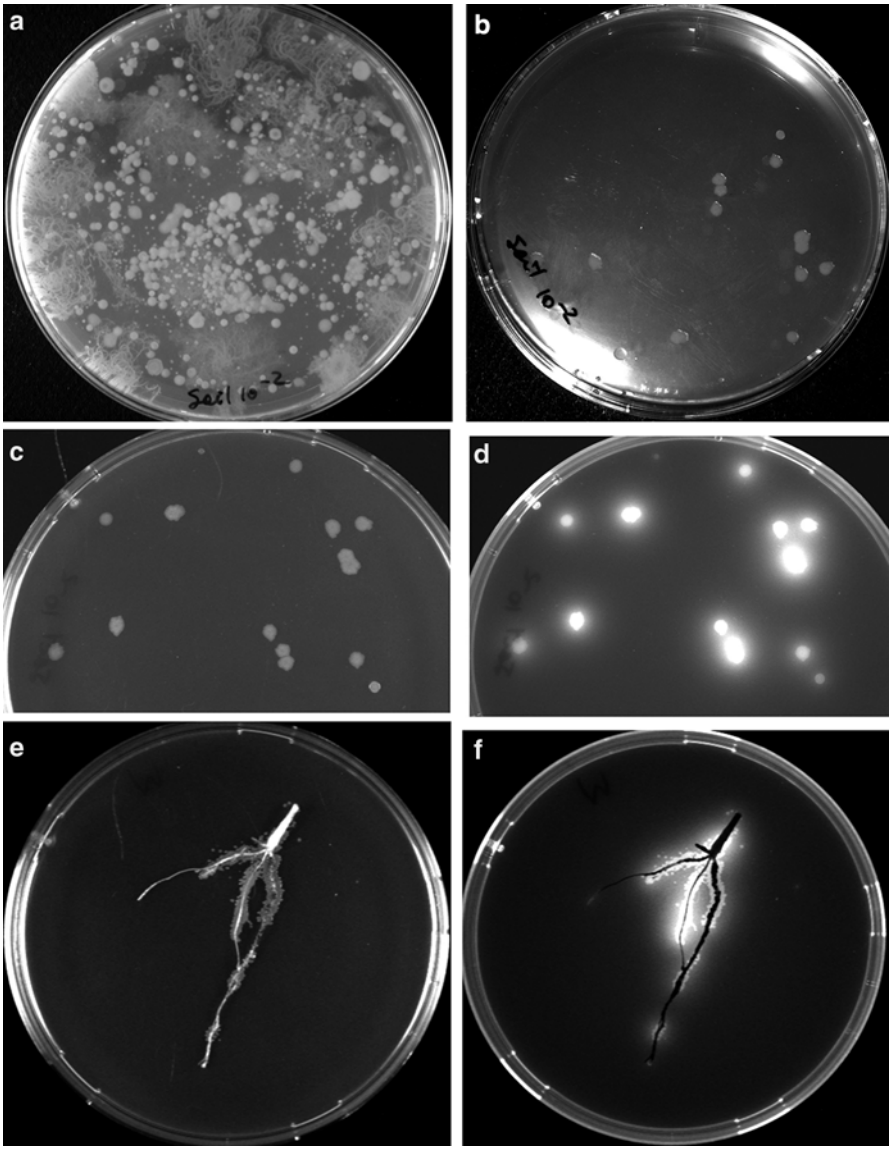

Fig. 21.1 Isolation of pseudomonads on selective agar plates (Gould's S1 medium) and observation of production of fluorescent diffusible pigments. (**a**) Bulk soil suspension plated on nonselective nutrient medium at a 1:100 dilution and incubated 72 h at 28 °C; (**b**) the same diluted soil suspension plated on Gould's S1 medium after incubation 72 h at 28 °C; (**c**) Colonies from a soil suspension on an S1 plate under normal white light; (**d**) the same plate as in (**c**), but under UV light; (**e**) a root system collected from soil and directly deposited onto a Gould's S1 plate, after incubation at 28 °C for 48 h and photographed under white light; (**f**) the same plate photographed under UV light

of fungi and yeasts. After 48–72 h of incubation at 28–30 °C, plates are inspected for the development of colonies under normal light and under UV light (260–290 nm) for individualization of fluorescent siderophore-producing colonies. Those colonies of interest must be re-streaked individually on S1 plates to confirm purity, and then they may be grown on rich complex media like nutrient agar or LB agar for short-term storage and further characterization.

21.2.3 Microbiological Confirmation of Isolates

Pseudomonads are highly motile Gram-negative rods that do not sporulate (Holt 1994). Metabolically, *Pseudomonas* strains are oxidase positive and do not ferment glucose. Other biochemical traits of the genus are: indole negative, methyl red negative, Voges–Proskauer negative, and citrate positive (Holt 1994).

21.2.4 Molecular Confirmation of Isolates

Isolated colonies from primary platings on Gould's S1 agar may serve as DNA source for a direct PCR detection procedure that is highly specific for the genus *Pseudomonas*, and that is based on the amplification of an internal fragment of the *oprF* gene encoding an outer membrane porin (Agaras et al. 2012). 16S rDNA sequencing confirmed that 100 % of the colonies developed on Gould's S1 plates are positive for the *oprF* PCR and are members of the genus *Pseudomonas* (Agaras et al. 2012). The typical protocol used in our lab is described below, and it may be subject to subtle adaptations depending on the available source of materials (Tables 21.3 and 21.4).

Table 21.3 PCR mixture for amplification of an internal fragment of the *oprF* gene, diagnostic for the *Pseudomonas* genus

PCR component	Stock concentration	Volume (µL)	Final concentration
Buffer 10×	10×	2.5	1×
dNTPs	2 mM	2.5	200 µM
$MgCl_2$	50 mM	1.0	2.0 mM
Forward (oprF-FW2)	10 µM	2.0	0.8 µM
Reverse (oprF-Rev2)	10 µM	2.0	0.8 µM
Taq DNA polymerase	5 U/µL	0.2	1.0 U
DNA template	–	See text	–
Deionized H_2O	–	Up to 25	–

Table 21.4 Thermal cycling program for amplification of an internal fragment of the *Pseudomonas*-specific *oprF* gene

PCR step	Temperature/time
1. Initial denaturation	94 °C/5 min
2. Denaturation	94 °C/1 min
3. Annealing	57 °C/45 s
4. Extension	72 °C/45 s
Back to step 2 (34×)	
5. Final extension	72 °C/5 min
Keep at 4 °C until analyzed	

DNA template: for rapid screening of an important number of colonies, we suggest doing direct colony PCR. For this, use a sterile toothpick to touch an isolated colony and dip into each reaction tube; gently twirl the toothpick 3–4 times; and remove it from the tube. Alternatively, you may scrape the whole colony with a toothpick and resuspend it into 100 µL of deionized water, then place the tube on a floating device in a boiling water bath and incubate for 10 min. Tubes are then centrifuged 1 min at 10,000 × *g* and 1–2 µL of the supernatant are pipetted into PCR reaction tubes. Make sure you include a positive control (a *Pseudomonas* spp. isolate or reference strain) and a negative control (a non-*Pseudomonas* isolate or reference strain).

PCR protocol: the sequences of the *oprF* primers are: Forward primer oprF-FW2: 5′-ATCGGYTACTTCHTBACHGA-3′; reverse primer oprF-Rev2: 5′-CCNACGGAGTCRGTRTGRCC-3′. Instructions for preparing the PCR mixture are shown in Table 21.3. Table 21.4 describes the *oprF* PCR program.

Amplicon detection: mix PCR reactions with loading buffer and run in 1 % agarose gels (in 0.5× Tris–borate–EDTA) at 10 V/cm for 50 min. Stain with ethidium bromide and visualize under UV light. *Pseudomonas* strains yield amplicons having 600–680 bp. Note that this amplicon size range is due to the presence of an internal deletion in the central domain of the OprF polypeptide of certain pseudomonad species (Bodilis et al. 2006).

21.2.5 Storage

Fresh colonies on different growth media may be safely stored as working material for up to 2 weeks at 4 °C without significant loss of viability. It is important to avoid repeated subculturing in solid or liquid media, as pseudomonads are well known for their ability to generate phase variants (see Sect. 21.2.6 and Chap. 12 for general information) and experience genetic rearrangements leading to adaptation to lab conditions. Instead, long-term storage under conditions of restricted growth is mandatory to preserve the genetic wealth and the corresponding physiological and PGPR traits. Thus, as a general rule, discard working cultures that have been subcultured up to three times, and go back to long-term storage samples to reinitiate fresh working plates.

Pseudomonas strains and isolates are routinely stored in ultrafreezers (at −80 °C or −130 °C) in their growth medium as saturated cultures containing glycerol as a cryoprotectant, from which they can be recovered after many years. Other long-term storage methods may be applied to preserve strains or even to formulate inoculants (e.g., freeze-drying and encapsulation into alginate beads), but they require adjusting conditions for ensuring acceptable survival rates.

21.2.5.1 Glycerol Stocks at Ultra-Low Temperatures

Pick a single colony of a fresh plate and grow it overnight in the appropriate liquid medium (e.g., Luria broth, nutrient yeast broth). Label a sterile screw cap microcentrifuge tube, and add 450 µL of the o/n culture and 150 µL of 80 % sterile glycerol solution. Vortex 1 min, let stand the tube 30 min on the bench, then freeze the glycerol stock at −80 °C. To streak out from the stock, put the tube on ice and take it to the place that you intend to streak the strain, flame a metal inoculating loop until it is red hot and once cooled down, scrape off a portion from the top of the frozen glycerol stock and streak it onto your plate. Return the strain to the ultrafreezer. Flame the metal inoculating loop a second time, cool it by inserting it into the agar of the plate and finish streaking out the strain so that it will be possible to isolate single colonies.

21.2.5.2 Freeze-Drying

This process requires the choice of an appropriate drying medium to increase recovery rates upon dehydration, and diverse substances are currently used as cryoprotectants (e.g., polyols, di- and polysaccharides). However, the protective effectiveness of a given additive will vary with the bacterial species or even strain, so conditions need to be adjusted for each isolate. A detailed procedure including different growth media (conventional or osmoadaptative medium) as well as different osmoprotectants has been recently reported (Cabrefiga et al. 2014). In this work, viability of lyophilized *P. fluorescens* EPS62e cells was improved to near 99 % when cells were osmoadapted during cultivation and lactose was used as cryoprotectant. Moreover, the dry powder remained active for 12 months and retained biocontrol activity similar to that of fresh cells (Cabrefiga et al. 2014).

21.2.5.3 Encapsulation into Alginate Beads

Alginate is an abundant, biodegradable, and renewable marine biopolymer, which is widely used as a material for encapsulation of microorganisms. The alginate beads are dry, easy to handle, uniform, nontoxic, and have proven useful to store viable cells of *P. fluorescens* F113 for up to 9 months (Power et al. 2011), or *P. fluorescens* strain 313 in combination with *A. brasilense* Cd for up to 14 years (Bashan and Gonzalez 1999). For a detailed procedure on preparation of alginate beads, we refer the reader to the pioneering work by (Bashan 1986). See also Chap. 26.

21.2.6 Phase Variation

As reported for *Azospirillum brasilense* and *Azospirillum lipoferum* (see Chap. 12), many plant-associated *Pseudomonas* species show colony phase variation (van den Broek et al. 2005) (Fig. 21.2). Colony phase variation is a genetic regulatory mechanism operating at the DNA level that results in highly frequent reversible switches between colonies with different phenotype (van den Broek et al. 2005).

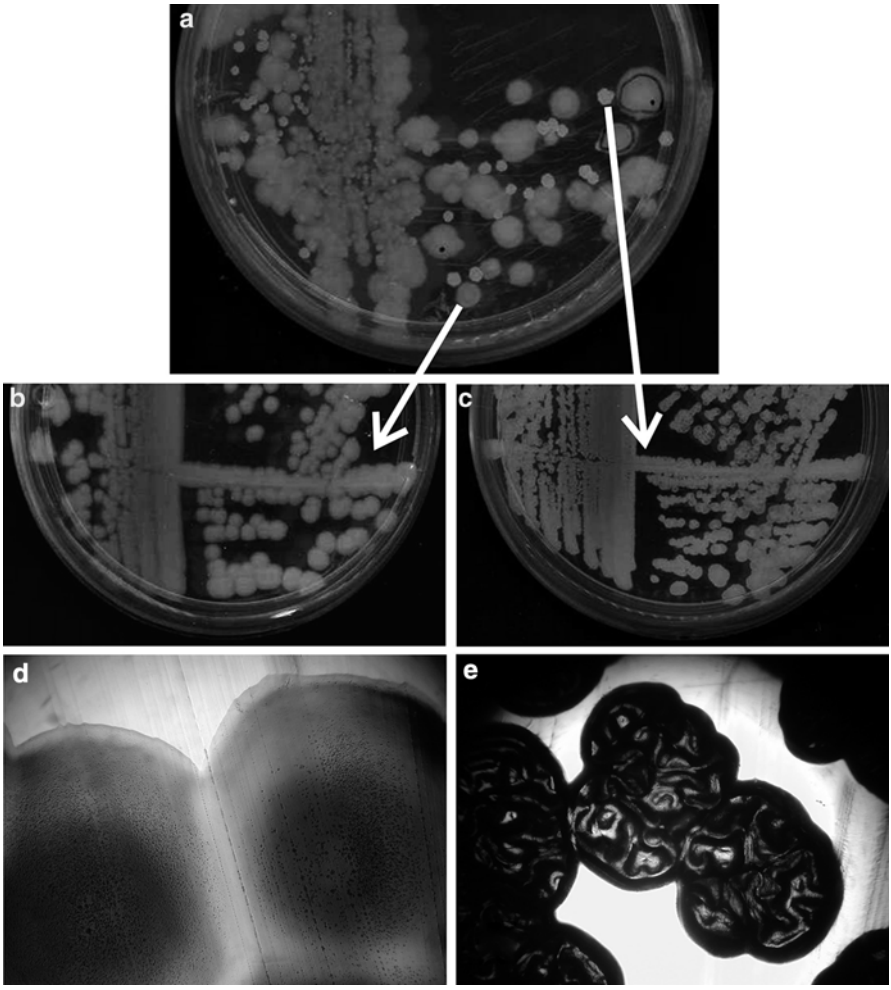

Fig. 21.2 Colony phase variation in pseudomonads. Mixed smooth and rough colony variants corresponding to the same 16S rDNA genotype are usually detected upon plating commercial inoculants (**a**) or lab cultures that were grown for several days. Both phenotypic variants, smooth (**b**, **d**), and rough (**c**, **e**) were purified on Gould's S1 *Pseudomonas*-selective medium

Phase variation often affects cell surface components, thus having a direct impact on colony morphotype (Sanchez-Contreras et al. 2002) and leading to the emergence of colonies with different aspect from the parental typical morphotype (Fig. 21.2). For instance, *P. fluorescens* F113 experiences phase variation during colonization of alfalfa roots, and this is caused by both the activity of a site-specific recombinase encoded by the *sss* gene (the main factor of phase variation) and by a point mutation in the *gacA* gene that alters the phenotype (Sanchez-Contreras et al. 2002). Provided the *gacA* gene is member of a global regulatory system controlling many phenotypes in different pseudomonads (Valverde and Haas 2008), phase variation may affect a plethora of other physiological and biochemical traits not as easily visualized as changes in colony aspect. For these reasons, we strongly suggest avoiding repeated subculturing of *Pseudomonas* isolates or strains, and to keep instead stock cultures under long-term preservation conditions (see Sect. 2.5). To discard contaminants upon sudden appearance of suspicious colonies from a pure culture, the atypical colonies may be streaked onto a *Pseudomonas*-selective medium like Gould's S1 and subjected to colony PCR targeting *oprF* gene (see Sects. 21.2.1 and 21.2.4). If both tests were positive, the endonuclease restriction pattern of the *oprF* amplicon may quickly allow distinguishing among two pseudomonad strains (Agaras et al. 2012).

21.2.7 Quantitative and Qualitative Culture-Independent Analysis of Pseudomonads

Due to their ease to isolate, cultivate and genetic manipulation, pseudomonads have been thoroughly characterized at the genetic and genomic level and a significant amount of molecular tools have been developed for their qualitative and quantitative study by culture-independent methods. Table 21.5 summarizes the culture-independent molecular methods available to specifically detect and quantify pseudomonads DNA and characterize its diversity in complex samples like soil, rhizosphere, or plant tissues.

21.2.8 Strain Tagging for Microscopy Detection and Follow-up in Complex Nonsterile Systems

The possibility to visualize cells in the presence of other microorganisms or to specifically recover them from complex bacterial habitats like the rhizosphere or the soil are valuable tools to study the root colonization ability of an isolate, its ability to establish as an endophyte, its persistence in the soil, and its interactions with other micro- or macroorganisms under both gnotobiotic and natural conditions. This can be simultaneously achieved by tagging bacteria with genes encoding fluorescent reporter proteins and antibiotic resistance markers. There are several available plasmids having these features, although plasmid instability in the absence of

Table 21.5 A summary of *Pseudomonas*-specific molecular methods for culture-independent analysis

Type of analysis	Method	Target gene	Sample type	References
Abundance	qPCR	Specific AFLP sequences	Soil	Xiang et al. (2010)
	qPCR	Specific SCAR sequences	Rhizosphere	Von Felten et al. (2010)
	qPCR	16S rDNA	Soil	Bergmark et al. (2012), Li et al. (2013)
Diversity	PCR + RFLP	*oprF*	Soil, rhizosphere	Agaras et al. (2012, 2014)
	PCR + DGGE	16S rDNA	Soil, rhizosphere	Marques et al. (2014), Schreiter et al. (2014)
	PCR + DGGE	*gacA*	Soil, rhizosphere	Costa et al. (2007)
	PCR + DGGE	*phlD*	Rhizosphere	Frapolli et al. (2008), Meyer et al. (2010)
	PCR + 454 GS FLX pyrosequencing	16S rDNA	Soil	Bergmark et al. (2012), Li et al. (2013), Marques et al. (2014)
	PCR + 454 GS FLX pyrosequencing	16S rDNA	Soil, rhizosphere	Marques et al. (2014), Schreiter et al. (2014)
Gene expression	qRT-PCR	*hcnC*, *phlD*	Soil	DeCoste et al. (2011)

antibiotic selection and horizontal transfer events are issues that complicate interpretation of results under natural conditions in the presence of many other bacterial species (Bloemberg et al. 2000). Thus, genetic tagging as single copy at one specific, neutral, chromosomal site is desirable. A versatile and efficient Tn*7* transposon-based toolset has been developed for stable integration of different reporter variants into a neutral chromosomal site (Lambertsen et al. 2004). The Tn*7*-delivery vectors carrying different fluorescent reporters and different antibiotic resistance markers can be introduced into the bacterium of interest by electroporation together with the Tn*7*-helper plasmid (encoding the required Tn*7* transposase) or can be mobilized by conjugation (Lambertsen et al. 2004). The tagged derivatives are confirmed by fluorescence microscopy, antibiotic resistance phenotypes, and by PCR to check the correct genomic insertion into the Tn*7* attachment site (Lambertsen et al. 2004). Tagged strains may then be applied to seeds or seedlings and examined under conventional or confocal fluorescence microscopy (Fig. 21.3), and also be selectively recovered from soil or rhizosphere samples by plating onto pseudomonas-selective medium (see Sect. 2.2) supplemented with the appropriate antibiotics. This method has proven useful for different pseudomonads (Jousset et al. 2006; Pliego et al. 2008), although it has not been tested yet for *Azospirillum* strains.

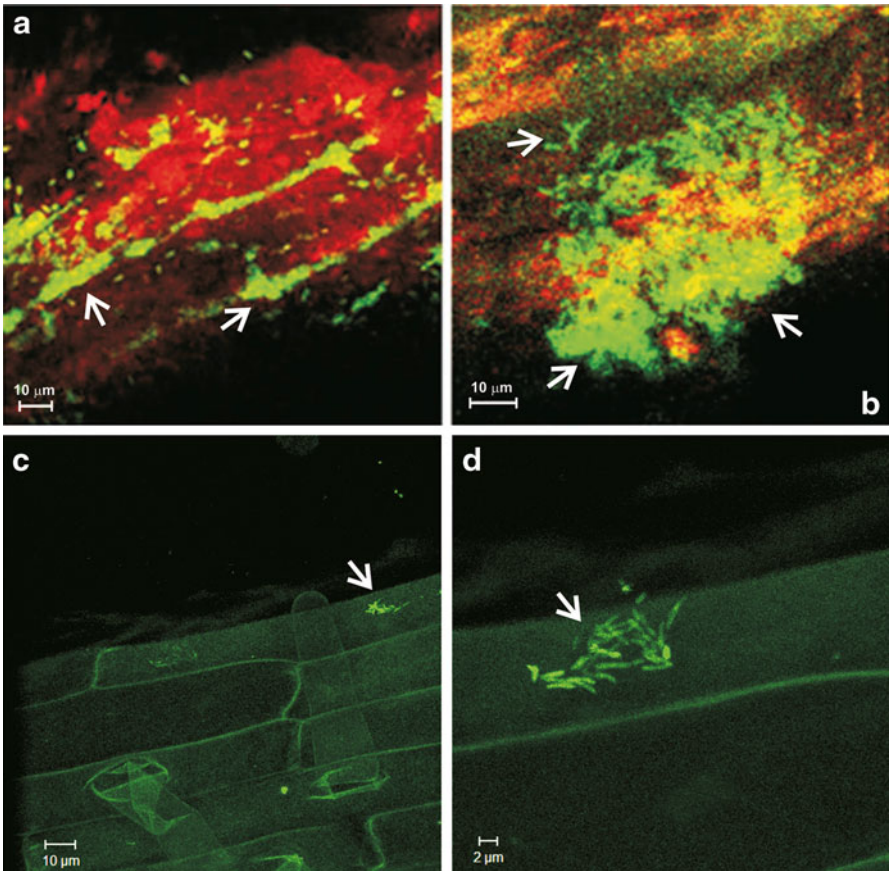

Fig. 21.3 *In situ* visualization of chromosomally GFP-tagged pseudomonads in the rhizosphere of avocado and wheat roots by scanning confocal laser microscopy. *P. pseudoalcaligenes* AVO110 (**a**) and *P. alcaligenes* AVO73 (**b**) in the rhizosphere of avocado (red). Arrows point to bacterial microcolonies (green) in epidermal intercellular spaces (a) or in a root wound (Pliego et al. 2008). Reproduced with permission from John Wiley & Sons. (**c**) A microcolony of *P. protegens* Pf-5 (arrows) in the rhizosphere of wheat, and a close up of the bacterial aggregate (**d**). Images were kindly provided by Romina Fox, Daniela Russo and Nicolás Ayub

21.3 Evaluation of Liquid Inoculants Containing *Pseudomonas* and *Azospirillum*

Quality control is a key step of the inoculant production chain. According to local regulations, inoculants must contain a minimal concentration of viable cells of the declared microorganisms, must be free of microbial contaminants, and must be effective in terms of their expected activities (Deaker et al. 2011). Stimulated by the synergism between a local industry demanding improved quality of inoculants and the academic institutions strengthening their links with inoculant producers, the

REDCAI network of public and private laboratories was established and committed to define standard protocols to assess the quality of inoculants in Argentina. The REDCAI network has recently published a Handbook with consensus protocols for evaluation of inoculants based on *Azospirillum* or *Pseudomonas* spp. (Albanesi et al. 2013) (for further details about quality control of *Azospirillum*-based inoculants, see Chap. 27). Such recommended procedures are addressed to single species inoculants and make use of RC medium (Rodriguez Caceres 1982) for *Azospirillum* spp. and F agar (King's B) for *Pseudomonas* spp., for determining the viable cell density of commercial inoculants (Manual de procedimientos microbiológicos para la evaluación de inoculantes 2013). However, mixed inoculants containing both *Azospirillum* and *Pseudomonas* cells cannot be readily analyzed with these protocols because both bacteria can grow on both media. For counting pseudomonads, Gould's S1 medium (see Sect. 21.2.1.3) provides the required selectivity to enumerate viable cells in mixed inoculants; however, highly selective solid media for *Azospirillum* species are not available. In this context, a plausible alternative to classical microbiological and culture-dependent methods is the estimation of genomic units by quantitative real-time PCR with specific oligonucleotides targeting both microorganisms ((Couillerot et al. 2013); Table 21.5). We refer the reader to Chap. 2 for established methods to estimate *Azospirillum* genomic units.

21.4 Interspecies Interactions

Both *Azospirillum* and *Pseudomonas* strains are natural members of the rhizosphere and endophytic compartments of diverse plant species, and many isolates display plant probiotic traits that have prompted the formulation of single or combined inoculants for agronomical applications (Tables 21.1 and 21.2). Whereas establishment of natural microbial communities of plant tissues is subject to diverse environmental, physical, and biological constraints that determine the spatiotemporal distribution of each microbial population, the artificial combination of pseudomonads and *Azospirillum* strains generates the forced coexistence at above-normal cell densities already in the inoculant formulation or on the seed surface upon bacterization. This may give rise to unexpected interactions among the strains (Couillerot et al. 2013; Loaces et al. 2011), which may be advantageous to study in detail before formulation. In this regard, the compatibility between wild type strains may be assayed first by dual culture in solid medium (Loaces et al. 2011; Vidhyasekaran and Muthamilan 1995). Ideally, the compatibility of both types of PGPR should be addressed in mixed liquid inoculants or in bacterized seeds, which is limited by the lack of fully selective media for enumeration of *Azospirillum* strains. To overcome this limitation, it may be useful to utilize antibiotic-resistant variants of the *Azospirillum* strain (either spontaneous or genetically modified derivatives) (see Chap. 4), allowing selective counts of viable cells in the presence of other microorganisms, as well as studying their colonization properties. We refer the reader to Chap. 11 for a description of *Azospirillum* tagging procedure for microscopic analysis of biofilm formation and root colonization in the presence of other bacteria.

21.5 Agronomical Evaluation of Inoculants Based on *Azospirillum* and *Pseudomonas* as a Single or Combined Formulation

There are several field experiments of seed inoculation with *Azospirillum* or *Pseudomonas* (reviewed in Puente and García 2009; Valverde and Ferraris 2009), but there is really very limited agronomical data regarding seed treatment with both microorganisms as a co-inoculant formulation (Faggioli et al. 2007). In order to generate incremental knowledge about the impact of dual inoculation of extensive crops, a series of field trials was conducted in Argentina during the seasons 2010–2011, 2011–2012, and 2012–2013. The performance of *Azospirillum brasilense* (hereafter Ab) as single formulations was compared to a combined formulation containing Ab and *P. fluorescens* (hereafter Pf), both in wheat and corn fields. The evaluation was done at three different levels of nitrogen (N) fertilization. Details of the experimental setup together with the quantified variables and representative results are described below to provide an overview of an agronomical design to assess the impact of PGPR inoculation.

A completely randomized block design was set up with three or four replicates depending on the properties of every soil location (Fig. 21.4). In all cases, seeds were pretreated with fungicides following recommended agronomical practices, i.e., mefenoxam + difenoconazole for wheat and mefenoxam + fludioxonil for maize. Seeds were treated sequentially, first with the fungicides, and then with the microbial inoculants. For wheat, three levels of N fertilization were assayed: natural soil fertility or no added N (0 N), +40 kg of N/ha (40 N), or +80 kg of N/ha (80 N), thus giving rise to the following treatments: (1) Non-inoculated or control plots (C 0 N, C 40 N, and C 80 N); (2) inoculated with Ab (Ab 0 N, Ab 40 N, and Ab 80 N); (3) co-inoculated with Ab + Pf (Ab/Pf 0 N, Ab/Pf 40 N, Ab/Pf 80 N). For maize, the N fertilization doses were natural soil fertility or no added N (0 N), +50 kg of N/ha (50 N), or +100 kg of N/ha (100 N), thus generating the following treatments: (1) Non-inoculated or control plots (C 0 N, C 50 N, and C 100 N); (2) inoculated with Ab (Ab 0 N, Ab 50 N, and Ab 100 N); (3) co-inoculated with Ab + Pf (Ab/Pf 0 N, Ab/Pf 50 N, Ab/Pf 100 N).

One key point when dealing with microbiological products in the field is that manipulation of the inoculant as well as seed handling has to be done avoiding exposure to sun, as ultraviolet radiation may compromise bacterial viability. It is also highly recommended to sow treated seeds the same day as they are bacterized with the inoculant in order to achieve the highest possible performance avoiding the loss of viability that occur on the dried seed surface. In these field trials, treated seeds were sown at the normal planting rate for wheat and maize at the different chosen locations. It is important to monitor and control other biotic factors that may interfere with crop development, such as weeds, insects, and diseases, as the aim of the experimental setup is to evaluate the impact of the microbial inoculants, and their interaction, with the fertilizers, thus minimizing any other interference.

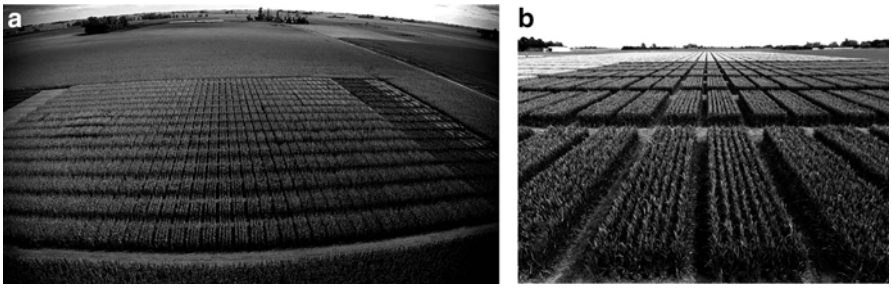

Fig. 21.4 Field trials for evaluation of inoculants containing *Azospirillum brasilense* and *Pseudomonas fluorescens*. (**a**) Panoramic view of the Ferré wheat experimental field. (**b**) Maize experimental field at Ferré

Table 21.6 Agronomic information of wheat fields

Field location	Planting date	Variety	Previous crop	Planting system
Junín	13 June 2010	K. Escorpión	Wheat/soybean	No till
Pergamino	18 July 2011	K. Chajá	Soybean	No till
Ferré	29 June 2012	K. Chajá	Soybean	No till
Pergamino	12 June 2012	K. Capricornio	Soybean	No till

21.5.1 Wheat Trials

The agronomical data of the different season trials is summarized in Table 21.6, whereas the chemical properties of each site are shown in Table 21.7.

For wheat, the number of spikes per m^2 is a good predictor of the potential yield. As shown in Table 21.8, the dual bacterization of wheat seeds (Ab/Pf) had a differential positive effect on the density of spikes, on top of the Ab effect. This was particularly evident when field plots were not fertilized with N (0 N).

Biomass production reflects nutritional and growth regulatory effects of the inoculated PGPR. In all cases, Ab alone or in combination with Pf, or both, had a positive effect on plant biomass at all three N fertilization levels (Table 21.9). Finally, the grain yield evaluation is the most important parameter that determines the adoption of an agronomical practice, such as seed bacterization with single or dual PGPR inoculants. In this case, the positive impact produced with this technology is significant as evidenced in Table 21.10.

21.5.2 Maize Trials

The agronomical data of the different season trials is summarized in Table 21.11, whereas the chemical properties of each site are shown in Table 21.12.

Table 21.7 Soil chemical analysis of wheat fields

Experimental site	pH	N-NO_3 (ppm)	P (ppm)	OM (%)	Total N (%)	C (%)
Junín	5.50	15.50	9.90	2.40	0.177	1.30
Pergamino	5.60	13.60	10.80	3.70	0.184	2.13
Ferré	5.40	11.90	10.10	2.90	0.144	1.68
Pergamino	6.10	13.60	12.90	2.90	0.144	1.70

N nitrogen, *NO_3* nitrate, *P* phosphorus, *OM* organic matter, *C* carbon

Table 21.8 Impact of dual treatment of wheat seeds with *A. brasilense* and *P. fluorescens* on the number of spikes per m^2

Experimental site	C 0 N	Ab 0 N	Ab/Pf 0 N	C 40 N	Ab 40 N	Ab/Pf 40 N	C 80 N	Ab 80 N	Ab/Pf 80 N
Junín	289	304	317	318	320	331	294	292	304
Pergamino	221	295	258	261	361	372	278	339	270
Ferré	422	445	452	440	518	452	425	511	444
Pergamino	247	304	361	241	268	255	283	276	397
Average	295	337	347	315	367	353	320	355	354

Table 21.9 Impact of dual treatment of wheat seeds with *A. brasilense* and *P. fluorescens* on plant biomass (kg/ha)

Experimental site	C 0 N	Ab 0 N	Ab Pf 0 N	C 40 N	Ab 40 N	Ab/Pf 40 N	C 80 N	Ab 80 N	Ab/Pf 80 N
Junín	555	561	689	616	617	723	676	632	690
Pergamino	741	785	735	916	913	987	827	866	845
Ferré	877	937	1,158	991	1,044	1,159	998	1,066	1,110
Pergamino	907	989	931	1,001	1,212	1,097	1,024	1,310	1,221
Average	770	818	878	881	947	992	881	969	967

Table 21.10 Impact of dual treatment of wheat seeds with *A. brasilense* and *P. fluorescens* on grain yield (kg/ha)

Experimental site	C 0 N	Ab 0 N	Ab/Pf 0 N	C 40 N	Ab 40 N	Ab/Pf 40 N	C 80 N	Ab 80 N	Ab/Pf 80 N
Junín	1,681	1,567	1,652	1,461	1,504	1,562	1,665	1,614	1,852
Pergamino	1,785	1,820	2,580	2,109	2,024	3,360	2,064	2,172	3,560
Ferré	3,814	4,248	4,111	3,923	4,226	4,270	4,038	4,509	4,257
Pergamino	2,378	2,783	2,700	2,904	3,167	2,867	3,009	3,015	3,163
Average	2,415	2,605	2,761	2,599	2,730	3,015	2,694	2,828	3,208

Table 21.11 Agronomic information of maize fields

Experimental site	Planting date	Variety	Previous crop	Planting system
Junín	24 October 2010	DR 684 RR2	Moha	No till
Pergamino	18 December 2011	DR 684 RR2	Soybean	No till
Ferré	09 October 2012	DR 747 RR2	Soybean	No till
Pergamino	30 September 2012	DR 747 RR2	Soybean	No till

Table 21.12 Soil chemical analysis of maize fields

Experimental site	pH	N-NO_3 (ppm)	P (ppm)	OM (%)	Total N (%)	C (%)
Junín	5.00	16.00	7.70	2.70	0.135	1.50
Pergamino	5.00	15.00	8.10	3.30	0.165	1.90
Ferré	5.90	13.00	5.50	2.30	0.123	1.33
Pergamino	6.10	4.30	8.50	2.70	0.134	1.60

N nitrogen, *NO_3* nitrate, *P* phosphorus, *OM* organic matter, *C* carbon

Table 21.13 Impact of dual treatment of maize seeds with *A. brasilense* and *P. fluorescens* on plant biomass (kg/ha)

Experimental site	C 0 N	Az 0 N	Az/Pf 0 N	C 50 N	Az 50 N	Az/Pf 50 N	C 100 N	Az 100 N	Az/Pf 100 N
Junín	533	589	675	566	644	668	547	579	670
Pergamino	432	462	482	360	355	381	285	295	298
Ferré	114	137	133	105	105	121	120	137	132
Pergamino	122	147	161	161	171	158	174	187	195
Average	300	334	362	298	319	332	282	299	324

Table 21.14 Impact of dual treatment of maize seeds with *A. brasilense* and *P. fluorescens* on grain yield (kg/ha)

Experimental site	C 0 N	Az 0 N	Az/Pf 0 N	C 50 N	Az 50 N	Az/Pf 50 N	C 100 N	Az 100 N	Az/Pf 100 N
Junín	8,668	9,198	9,403	8,859	9,310	9,548	9,126	9,742	9,565
Pergamino	8,817	9,293	11,639	10,308	10,222	10,529	10,832	11,263	11,847
Ferré	10,067	10,861	11,267	10,129	11,404	12,432	10,543	11,015	11,561
Pergamino	8,656	9,362	9,807	10,164	11,027	11,473	11,022	11,026	11,670
Average	9,052	9,679	10,529	9,865	10,491	10,996	10,381	10,762	11,161

Shoot biomass production did clearly reflect the positive impact of seed inoculation with either Ab or both Ab/Pf at all N fertilization levels (Table 21.13). Consistently with the increase in plant biomass, there was an incremental effect of Ab and Ab/Pf on grain yield at almost all treatments, reaching the highest yield with the combined Ab/Pf bacterization at almost all N fertilizer dose and locations (11 out of 12 treatments; Table 21.14).

The results of these experimental field trials support the use of combined inoculants containing the PGPR *A. brasilense* and *P. fluorescens*, and illustrate the importance of evaluating the performance of PGPR at different crops, different field locations and at different fertilization levels, while keeping constant the rest of the agronomical practices recommended at each productive region.

21.6 Conclusions and Perspectives

Pseudomonas and *Azospirillum* species are widely recognized inhabitants of the plant rhizosphere, with key traits that directly or indirectly promote plant growth, and that result of agrobiotechnological interest. Isolating and characterizing members of these taxonomic PGPR groups is critical for formulation of commercial inoculants for a variety of crops. In contrast to the rest of the book, this chapter focuses on the properties and methods routinely used to isolate, preserve, and characterize plant probiotic pseudomonads, and their interactions with *Azospirillum* strains.

With regard to the agronomical application of PGPR inoculants containing two or more microorganisms, its adoption for extensive crops is expanding at a relatively slow rate compared to legume inoculants. In this context, it is mandatory to significantly increase the number of field trials at a variety of soil types, climates, and crop management regimes. The use of multiple strain inoculants, including combinations of *Azospirillum* and *Pseudomonas* strains as reported in this chapter, is a likely logical trend in PGPR technology provided the multispecies nature of the plant root environment. This naturally requires the analysis of interactions between microorganisms and their impact on the plant host for which an application is thought.

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Chapter 22
Co-inoculation of Legumes with *Azospirillum* and Symbiotic Rhizobia

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Abstract Azospirilla are generally regarded as rhizosphere bacteria and colonize the root surface and only a few strains are able to infect plants. The stimulatory effect exerted by *Azospirillum* has been attributed to several mechanisms but under certain environmental and soil conditions, *Azospirillum* can positively influence plant growth, crop yields, and N-content of the legume. Most *Azospirillum*–rhizobacteria co-inoculation studies have focused on the final effects on plant growth and nodulation parameters; in contrast, few data are available on the simultaneous effect of double inoculation and stressful conditions on the early signalling exchange between the symbiotic partners. Co-inoculation of legume with rhizobia plus *Azospirillum* exerted changes on the concentration, content, and/or distribution of several mineral nutrients in roots and/or shoots of plants, change in flavonoids, and can help to tolerate stress. This chapter summarized some effects that occur when legumes are co-inoculated with *Azospirillum*.

22.1 Introduction

Azospirillum (α-subclass of proteobacteria) have been known for many years as plant growth-promoting rhizobacteria (PGPR). They were isolated from the rhizosphere of grasses and cereals all over the world and so far numerous species have been described.

Azospirilla are generally regarded as rhizosphere bacteria, but display strain-specific differences in the way they colonize roots. They predominantly colonize the root surface and only a few strains are able to infect plants. Some *Azospirillum* strains

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have specific mechanisms to interact with roots and colonize even the root interior, while others colonize the mucigel layer or injured root cortical cells. Under certain environmental and soil conditions, *Azospirillum* can positively influence plant growth, crop yields, and N-content of the plant. This plant stimulatory effect exerted by *Azospirillum* has been attributed to several mechanisms. The contribution of biological nitrogen fixation in this plant response, however, has been questioned. One of the most pronounced effects of inoculation with azospirilla on root morphology is the proliferation of root hairs as observed in several grasses, cereals, and legumes under controlled conditions in the greenhouse as well as in the field. The factors, such as the production of plant growth-promoting substances and the increase in the rate of mineral uptake by plant roots have been taken into account to explain the plant yield enhancement and could even be more important in establishing the plant response.

The positive effects of combined inoculation with *Azospirillum* and rhizobacteria have been reported for several legumes and cereals. Most *Azospirillum*–rhizobacteria co-inoculation studies have focused on the final effects on plant growth and nodulation parameters; in contrast, few data are available on the simultaneous effect of double inoculation and stressful conditions on the early signalling exchange between the symbiotic partners. The response of dually inoculated plants and the enhanced growth of legume plants may be attributed to early nodulation, an increase in the number of nodules, and a general improvement in root development. The objective of this chapter was to summarize the effects of co-inoculation with *Azospirillum* and rhizobia on nodulation and growth of legumes.

22.2 Effect of *Azospirillum brasilense* Co-inoculated with Rhizobia on Early Events on Legume Interactions

Leguminous plants have the ability to enter into symbiosis with N_2-fixing bacteria (collectively called rhizobia), which results in the formation of a new plant root organ, the root nodule. The incorporation of atmospheric N_2 into organic material resulting from this *Rhizobium*–legume symbiosis is estimated to account for one-third of the total nitrogen needed for world agriculture. This unique intracellular association contributes significantly to agricultural yields. The formation of nitrogen-fixing nodules results from an interactive process between the legume and the rhizobia, in which signal molecules, play a decisive role. Flavonoids in the root exudate induce the expression of rhizobial nodulation genes (called *nod*, *noe*, and *nol*) that are involved in the biosynthesis and secretion of lipo–chitin oligosaccharides (LCOs) or Nod factors, the bacterial signal molecules (Dardanelli et al. 2010).

They are different methods involved in collection, separation, bioassay, and compound characterization from plant root exudates, especially flavonoids. In this case, studies are much more complicated because the number of compounds is diverse and more selective and sensitive analytical methods are required. The choice of the method depends on the sensitivity required for the purpose at hand, the complexity of

the biological matrix, and pretreatment prior to analysis and include high performance liquid chromatographic (HPLC).

Microbial attachment capacity to plant cells probably acts as a key factor in determining microbe competitiveness to colonize the root. Unfortunately, technical difficulties to purify and identify all possible bacterial surface molecules that could be involved in bacterial attachment have not been completely overcome. In addition, mutations that abolish the production of a particular surface molecule can also influence the production and/or the presence of other bacterial surface components, which makes it difficult to identify which particular surface molecule, is directly responsible for the changes observed in bacterial attachment capacity. In combination with *omics* technologies, the structural determination of bacterial surface molecules and the construction of transgenic plants expressing receptors for bacterial surface components appear as the most promising way to investigate which molecules are actually involved in bacterial attachment, how important they are for successful root colonization under natural conditions, and how they are affected by environmental conditions (Rodríguez-Navarro et al. 2007).

The attachment assays were described by Smit et al. (1986). The rhizobia pellets are washed with phosphate buffer (pH 7.5) and suspended in the same solutions to give a bacterial concentration of 2×10^8 rhizobial cell/mL. In co-inoculation assays, *Azospirillum* should be added to concentrations of 1×10^6 cell/mL. Furthermore, portions of 1 cm roots (five root pieces) are incubated in phosphate buffer with the bacteria for approximately 2 h. Subsequently, the roots are washed, soaked, and the extract obtained is used for dilutions and plate count.

A variety of compounds, such as surface proteins and polysaccharides, have been implicated in adherence of several PGPRs to plant roots. Similar to the *Rhizobium*–legume symbiosis, attachment of *Azospirillum brasilense* cells to wheat (*Triticum aestivum*) roots also can be divided in two different steps (Michiels et al. 1991). The first phase is a weak, reversible, and unspecific binding governed by bacterial surface proteins, CPSs, and flagella. Polar flagella of *A. brasilense* contain an adhesin component that is involved in bacterial attachment to wheat roots. The second attachment phase appears to be irreversible. It occurs 8–16 h after inoculation and is mediated by a bacterial surface polysaccharide. *Azospirillum irakense* cells are mainly associated with rice root hairs, whereas *A. brasilense* cells are mainly located on root surfaces (Zhu et al. 2002). These differences in spatial distribution are the reason why these two species do not compete for root colonization.

The involvement of extracellular fibrils was demonstrated in the irreversible anchoring of *A. brasilense* (Michiels et al. 1991). The nature of this fibrillar material has not been determined yet. Extracellular polysaccharide production also has been related to the process of flocculation of *Azospirillum* cells and might be similar to the fibrillar material produced during root association (Burdman et al. 1998; Skvortsov and Ignatov 1998). The major outer membrane protein of *A. brasilense* appears to be involved in cell aggregation and the first step of attachment. This outer protein exhibits higher affinity to cereal roots than to those of other plants, which may explain why *Azospirillum* is mainly found associated with the rhizosphere of cereals (Rodríguez-Navarro et al. 2007).

However, despite the considerable amount of experimental data concerning *Azospirillum* stimulation of plant development, the mechanisms underlying the association process are still not well understood. This is mainly due to the absence of a clear plant phenotype indicative of a successful interaction, which makes a direct screening of large numbers of mutants not feasible. To get around this difficulty, genes possibly involved in plant interaction have been traced indirectly by characterization of mutants defective in a phenotype that is thought to be involved in plant association, or by screening for conserved structural or functional homology between *Azospirillum* genomic DNA and specific plant interaction genes of other plant-associative bacteria (Steenhoudt and Vanderleyden 2000). Now with the complete sequence of *Azospirillum* and the technological advances (Sozzani et al. 2014), many of the methodological difficulties are to be solved and the contributions of the effect of *Azospirillum* at early stages of interaction are to be studied and analyzed.

Inoculation of *Phaseolus vulgaris* (common bean) with *A. brasilense* Cd promoted root hair formation in seedling roots and significantly increased total and upper nodule numbers at different concentrations of *Rhizobium* inoculum. In experiments carried out in a hydroponic system, *A. brasilense* caused an increase in the secretion of *nod*-gene-inducing flavonoids, as observed by *nod*-gene induction assays of root exudates fractionated by high-performance liquid chromatography (HPLC) (Burdman et al. 1997). When *A. brasilense* Cd is present in salt conditions upon on plant growth, nodulation, flavonoid, and rhizobial lipochitooligosaccharide (LCOs–Nod factor) can change. Reversed-phase (RP) thin-layer chromatography (TLC) is used for the analysis of Nod factors (Spaink et al. 1992). Rhizobia are cultivate, supplemented with 1 mM flavonoids in positive assays, are grown in minimal medium to the end of the exponential phase under both control and test growth conditions. For the radiolabelling of Nod factors, 1 mCi of ^{14}C glucosamine hydrochloride (labelled at C1, specific activity 52 mCi mmol^{-1}, Amersham), yielding a final concentration of 4 mM glucosamine hydrochloride in the medium, is used. TLC plates are exposed to a Kodak® X-Omat R film for 15 days and the film is developed with Kodak® reagents according to the manufacturer's instructions.

Productions LCOs, was sequentially followed after 4, 7, and 14 days during a *Rhizobium–P. vulgaris* cv. Negro Jamapa interaction, in a hydroponics growth system, *A. brasilense* promoted root branching in bean seedling roots and increased secretion of *nod*-gene-inducing flavonoid species, as detected by HPLC. The results also suggested that *A. brasilense* allows a longer, more persistent exudation of flavonoids by bean roots. A general positive effect of *Azospirillum–Rhizobium* co-inoculation on the expression of *nod*-genes by *Rhizobium tropici* CIAT899 and *Rhizobium etli* ISP42, and on nodulation factor patterns, was observed in the presence of root exudates. The negative effects obtained under salt stress on nod-gene expression and on Nod factors' appearance were relieved in co-inoculated plants (Dardanelli et al. 2008).

A. brasilense Sp7 was recently compared with several mutants affected in production of IAA and nitric oxide for their effects on the symbiosis between *Vicia sativa* spp. nigra (vetch) and *Rhizobium leguminosarum* bv. *viciae* (Star et al. 2012). Results from this study confirmed that IAA and nitric oxide produced by *A. brasilense*

also play an important role in co-inoculation of legumes with rhizobia and azospirilla, and confirmed that IAA production by *A. brasilense* is a key component for enhancement of secretion of nod-gene-inducing flavonoids by legume roots (Star et al. 2012).

22.3 Co-inoculation and Strategies to Improve Agronomic Applications

Positive effects of *Azospirillum* inoculation are mainly attributed to improved root development and to the subsequent increase in the rate of water and mineral uptake (Okon and Kapulnik 1986). Biological studies on roots of legumes to observe co-inoculation effects are performed by microscopy test. Seedlings with root lengths of 1.5–2.5 cm are mounted in test tubes on a curled wire with the roots in 25 mL of nutritive solution. Subsequently, the roots are inoculated with *Azospirillum* and roots are observed periodically to microscope to observe root hairs by staining. The roots are shielded from light, and plants are grown at 20 °C for 10 days.

Inoculation with *A. brasilense* clearly increased root branching in Negro Jamapa beans (Dardanelli et al. 2008). At 14 days after inoculation, in the different treatments did not significantly differ in dry weight of shoots and roots, but root dry weight averages were consistently and sometimes significantly higher in the presence of *A. brasilense* Cd, with or without saline stress. In this day, the pH of the plant growth solutions was reduced from pH 6.80 to about 4.59 in controls, 4.78 with *A. brasilense*, 4.47 with *R. tropici* CIAT899 and 4.93 in co-inoculated treatments. Similar growth promotion effects of *A. brasilense* were observed in the presence of *R. etli* ISP42. With 50 mM NaCl, the pH of the solutions was reduced with rhizobia or co-inoculation treatments but not with *A. brasilense* alone (14 days). Acetylene reduction activities were significantly favored in plants co-inoculated with *A. brasilense* and *R. tropici* (140 ± 10 nmol ethylene h^{-1} g root dry $weight^{-1}$) as compared with inoculation with *R. tropici* alone (40 nmol ± 9 ethylene h^{-1} g root dry $weight^{-1}$). The number of nodules did not increase significantly. In these hydroponic systems, the number of nodules formed after 14 days in common bean plants is generally low (about 10 nodules per plant).

Co-inoculation of faba bean with *R. leguminosarum* bv. *viciae* plus *Azospirillum* exerted changes on the concentration, content, and/or distribution of several mineral nutrients in roots and/or shoots of plants, when compared with faba beans inoculated with *Rhizobium* only. The results reported here showed that the qualitative and quantitative effects of mixed inoculation on the mineral composition of *V. faba* varied largely among *Azospirillum* (Rodelas et al. 1999). These data agree with previously reported results on the effects of *Azospirillum* strains on the mineral nutrition of sorghum, wheat, and non-nodulated soybeans (Pacovsky et al. 1985; Bashan et al. 1990).

Dual inoculation with *Rhizobium* and *Azospirillum* can either stimulate or inhibit nodule formation and growth in a given symbiotic system, depending on the

concentrations and timing of inoculation (Plazinski and Rolfe 1985; Yahalom et al. 1991). Inoculation of cereals with very high numbers of *Azospirillum* was found to slightly inhibit root growth (Okon and Kapulnik 1986; Fallik et al. 1988). Both positive and negative effects of *Azospirillum* on nodulation and root development can be mimicked, in some cases, by application of plant growth regulators such as auxins and cytokinins (Volpin and Kapulnik 1994).

This implies that the amount of *Azospirillum* to be used in co-inoculation assays must be previously determined for every crop and combination with the rhizobia. This was shown by Burdman et al. (1997) on common bean. Combined inoculation of potted common bean plants with *Rhizobium* and *Azospirillum* significantly increased both upper and total nodule number and N_2 fixation as compared with inoculation with *Rhizobium* alone. At an *Azospirillum* concentration of 10^8 cfu/mL, the combined inoculation reduced root and shoot dry weight accumulation in comparison with *Rhizobium* alone and with uninoculated controls. However, when the combined inoculation was performed using a lower *Azospirillum* concentration (5×10^6 cfu/mL), positive effects on plant growth were observed, although the enhancement of nodulation and N_2 fixation were not as great as observed at the higher *Azospirillum* concentration.

In 1994, Okon and Labandera summarized *Azospirillum* inoculation experiments according to countries and research groups. Particular emphasis was given, when pertinent, to descriptions of inoculum preparation and inoculation techniques, soil and climatic conditions, experimental layout and, most importantly, yield data expressed as a percentage increase due to inoculation. Later, there were different revisions reporting how legumes were co-inoculated with varying results, depending even on legume genomes. Remans et al. (2008b) worked with two genotypes of common bean, BAT477 and DOR364, and demonstrated contrasting in nodulation response to *A. brasilense* Sp245 when co-inoculated with *Rhizobium*. They observed that *Azospirillum–Rhizobium* co-inoculation as compared to single *Rhizobium* inoculation increased the amount of fixed nitrogen and the yield of DOR364 across all sites. For BAT477, on the contrary, a negative effect of *Azospirillum–Rhizobium* co-inoculation on yield and nitrogen fixation was observed on most of the sites as compared to single *Rhizobium* inoculation. The contrasting response to *Azospirillum–Rhizobium* co-inoculation of BAT477 and DOR364 observed for yield and N_2 fixation is consistent with the difference in nodulation response observed previously in controlled greenhouse experiments (Remans et al. 2008a). The field results indicate that the difference in host responsiveness to *Azospirillum* between bean genotypes can have an important impact on agronomic outputs.

Recently, Hungría et al. (2013) evaluated the combined action of azospirilla and rhizobia inoculants on the yields of soybean and common bean. They demonstrated that the best in-furrow inoculant dose was 2.5×10^5 cells of *A. brasilense*/seed for both crops. Seed co-inoculation with *Azospirillum–Bradyrhizobium* resulted in a mean yield increase of 420 kg/ha (14.1 %) in soybean relative to the non-inoculated control. For common bean, seed inoculation with *R. tropici* increased yield by 98 kg/ha (8.3 %), while co-inoculation with *A. brasilense* in-furrow resulted in the impressive increase of 285 kg/ha (19.6 %).

Summary of commercial field inoculation experiments with inoculants prepared with various *Azospirillum* strains in the world during the last decades were reported for Döbereiner and Pedrosa (1987), Okon and Labandera-González (1994), Bashan et al. (2004), Cassan and Garcia de Salomone (2008), Helman et al. (2011), Hungría et al. (2013), among others. All of them demonstrated that this bacterium is capable of promoting the yield of agriculturally important crops in different soils and climatic regions, using various strains of *A. brasilense*. They concluded that this bacterium is capable of promoting the yield of agriculturally important crops in different soils and climatic regions, if various strains of *A. brasilense* are used. Also, they indicated that there are different aspects of co-inoculation to be studied such as the study of new strains of bacteria combinations with different genotypes of crop plants, and commercial formulations for the development of a mixed commercial product. This is a very critical issue that should be assessed and which demands collaborative efforts from the producing companies, research institutions, and government agencies. Since these products are composed of living organisms, it is very important to confirm the bacterial strain at all stages of the production pipeline and to ensure that high bacterial numbers are maintained until the product reaches farmers (Helman et al. 2011).

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Chapter 23
Interaction of *Azospirillum* and Mycorrhiza

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Abstract The AM fungi interact with *Azospirillum* directly by providing niche and/or habitat or indirectly by modifying host plant morphophysiology. This communication in soil can be beneficial for both the microorganisms and the host plant. The *Azospirillum* inoculation is more successful and more profitable when other microorganisms are co-inoculated with *Azospirillum*. The inoculation consortia apparently work better when VAM fungi are incorporated. So far, observational techniques and morphophysiological analysis techniques have been developed but nonspecifically for this microbial interaction. New co-inoculation methods and polymicrobial formulations with stable, effective, multifunctional, and eco-friendly characteristics are technically demanding.

23.1 Introduction

Mycorrhizal fungi are ubiquitous soil inhabitants forming a symbiotic relationship with roots of most terrestrial plants. Mycorrhiza, as exemplified by Arbuscular Mycorrhizal (AM) fungi, are continuously interactive with plant roots, a wide range of microorganism in the rhizosphere including Plant Growth-Promoting Rhizobacteria (PGPR), Mycorrhizal Helper Bacteria (MHB), and plant pathogens. This collective environment together is referred to as mycorrhizosphere.

Mycorrhiza and bacteria in the mycorrhizosphere often have mutual synergistic interactions (Azcon 2009; Barea et al. 2002, 2005; Finlay 2008). AM fungi interact synergistically with other microorganisms such as nitrogen-fixing bacteria (Brown and Carr 1984; Barea and Azcon-Aguilar 1983), phosphate-solubilizing bacteria

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(Villegas and Fortin 2002; Azcon et al. 1976), and biocontrol agents (Abdel-Fattah and Mohamedin 2000; Barea et al. 1998) to enhance plant growth.

The AM fungi might affect negatively (Cavagnaro et al. 2006), positively (Albertsen et al. 2006), or may have no effect (Olsson et al. 1996) on microbial biomass and growth of specific microbial taxa (Marschner and Timonen 2006). Many studies have shown that some bacterial species respond to the presence of certain AM fungi (Artursson et al. 2006; Andrade 1997), suggesting a high degree of specificity between bacteria associated with AM fungi. Thus, the specific bacteria together with AM fungi may create more indirect synergism for plant growth (Barea 1997), including nutrient acquisition (Barea et al. 2002) and enhancement of root branching (Gamalero et al. 2002). Also, a wide variety of Gram-negative and Gram-positive bacteria stimulates mycorrhizal association with plant root system, and they are usually referred to as MHB (Garbaye 1994).

Anchoring of PGPB (Plant Growth-Promoting Bacteria) to AM fungal structures may have special ecological and biotechnological significance because it may facilitate colonization of new rhizosphere by the bacteria traveling on or moving with fungal hyphae, and may be an essential trait for the development of mixed inocula (Bashan et al. 2004).

23.2 How to Grow Together the Different Microorganisms

AM fungi interact with bacteria directly by providing niche and/or habitat or indirectly by modifying host physiology (Bianciotto et al. 2000; Walley and Germida 1997). Thus, communication between motile bacteria and AM roots in soil can be beneficial for both the microorganisms (Dutta and Podile 2010).

The microsymbionts have distinct habitats in the rhizosphere, such as the nodules (for the *Rhizobium*) and the root interior for the associative diazotrophs and the AM fungi. Both nitrogen-fixers inhabit the interior of the root systems or the mycelial network. Functional interrelations must exist between them and the AM fungi (Bethlenfalvay et al. 1985; Subba Rao 1985; Champawat 1990; Paula et al. 1992).

Azospirillum strains are routinely isolated from agricultural lands and crop plants, including traditional isolations from grasses and cereals (Nath et al. 1997; Weber et al. 1999) from soil, roots, or rhizosphere.

However, *Azospirillum* strains were isolated from the ectomycorrhizal fungi ECM (ectomycorrhiza, sporocarps). Several studies have been reported related to nitrogen-fixing bacteria associated with ectomycorrhizae in conifers such as pine, Douglas fir, and native trees in Southeast Asia belonging to the genera *Azospirillum*, *Bacillus*, *Beijerinckia*, and *Clostridium* mainly (Duponnois and Garbaye 1991; Orozco-Jaramillo and Martinez-Nieto 2009). Also, PGPR such as *Burkholderia*, *Azospirillum*, *Acetobacter*, and *Herbaspirillum* were detected in high numbers on mantle surfaces of ectomycorrhiza of *Fagus sylvatica* by confocal laser scanning

microscopy (Mogge et al. 2000). Three *Azospirillum* strains were isolated from the ectomycorrhizal sporocarps (*Rhizopogon vinicolor*) that colonized Douglas fir trees (Chang and Li 1998).

Some specific studies of *Azospirillum*–AM fungi interaction and its effect on the colonization of both groups of microorganisms have been reported.

Miyauchi et al. (2008) showed that mycorrhizal colonization increased the diazotrophic bacteria colonization in maize roots. The bacteria did not affect root morphological traits and mycorrhizal colonization. This effect suggests a beneficial action of AM fungi in helping the diazotrophic bacteria to penetrate and colonize the plant roots (Raimam et al. 2007).

Mutants of *Azospirillum brasilense* deficient in EPS (exopolysaccharides) were shown to be strongly impaired in their capacity to attach to AM roots and AM fungal structures (Bianciotto et al. 2001). Belimov et al. (1999) found that when barley was inoculated with the AM *Glomus intraradices*, addition of *Azospirillum lipoferum* 137 and the phosphate-solubilizing bacteria, *Agrobacterium radiobacter* and *Flavobacterium* sp., did not affect the intensity of development of mycorrhizal structures in the roots. However, while mycorrhization of the plants was conducive for better establishment of *A. lipoferum* 137 in the rhizoplane, it significantly decreased root colonization of *Flavobacterium* sp.

However, Bauer et al. (2012) did not find interactions between AM fungi and N_2-fixers in communities or *Panicum* monocultures, indicating that short-term effects of these microbial functional groups are additive.

Although some significant studies addressing the impact of *Azospirillum* inoculation on soil microbial communities were made by the Ribosomal Intergenic Spacer Analysis (RISA) or Community-Level Physiological Profiles (CLPPs) techniques, studies of AM fungi or AM fungi/PGPR co-inoculation were made with Denaturing-Gradient Gel Electrophoresis (DGGE) (Trabelsi and Mhamdi 2013; Roesti et al. 2006).

Often, when the specific strains were fluorescently labeled, in situ identification of isolates and localization of *Azospirillum* on roots by confocal laser scanning microscopy or epifluorescence microscopy is possible (Stoffels et al. 2001).

Microorganisms' interactions could be more than just competitive. Bellone and Carrizo de Bellone (2012) described the process of colonization by *Azospirillum brasilense* and *Glomus intraradices* in sugarcane roots. The presence of bacteria in the young roots and their subsequent introduction into the xylem and phloem vessels are favored by the cell endodermal disorganization that occurs during the primal radical growth of the future lateral roots. At the sites of maximal root colonization, the cell wall fungi strongly diminish, what allows better interchanges of metabolites between bacteria and fungi. In the lumen of xylem vessels, the bacteria colonize at random. The presence of intracellular hyphae increases the colonization of the bacteria that occupy the intercellular spaces generated by the fungi. Mycorrhizas are the most common vectors of endophytes of plants. The changes produced in the three symbionts (bacterium, VAM, and plant) facilitate the intimate interaction between them.

23.3 Production of Metabolites

A combination of PGPR with other beneficial microbes such as mycorrhizal fungi induces plants to produce certain metabolites which indirectly make the rhizosphere environment more suitable for their stay (Dutta and Podile 2010).

The colonization generated by both microbial groups stimulates hydrolysis in cell wall hemicelluloses and generates oligosaccharides that will be used in the nutrition process. The enzymes generated during settling are responsible for the morphologic changes that begin in the surface and end up in the inner regions of the cortex. These results suggest that bacteria associated with fungi utilize metabolites produced by plant cells and microsymbionts that interact with them. The increase in the nitrogen-fixing activity (ARA), and in root biomass of both microorganisms, promotes the absorption of nutrients (Bellone and Carrizo de Bellone 2012).

Effects of a mixed inoculant of *Azospirillum* spp. and several AM fungi (*Glomus mosseae*, *G. deserticola*, and natural AM fungi from the test soil) upon mycorrhizal colonization in maize plants were evaluated for enzymatic activities (esterase, phosphatase, trehalase, and chitinase) (Vazquez et al. 2000). The enzymes were indicators for detecting changes in the microbial functional groups in the soil. *Azospirillum* mixed with the AM fungi showed no negative effects on AM fungal establishment. As a result of mycorrhizal colonization and microbial inoculation, modifications of the microbial community structure and ecology were induced.

Bioactive compounds called "Myc factors," similar to "Nod factors" of *Rhizobium*, are suggested to be secreted by mycorrhiza and *Rhizobium* and perceived by host roots for the activation of signal transduction pathway or common symbiosis (SYM) pathway (Kosuta 2003; Roberts et al. 2013). The pathways that prepare plant for both AM and *Rhizobium* infection have some common points. The common SYM pathway prepares the host plant to bring about changes at the molecular and anatomical level with the first contact of fungal hyphae. So far, calcium is supposed to be the hub of secondary messengers via Ca^{2+} spiking in the nuclear region of root hairs (Sieberer et al. 2009).

The plant genes that are expressed by "Myc factors" at the onset of AM-host plant symbiosis and the fungal genes involved in structural and physiological alterations in the host plant have been identified (Akiyama and Hayashi 2006; Dodd and Ruiz-Lozano 2012; Hata et al. 2010). The physiological functions of the host plant need to be modified so that the host plant can provide the fungi with the required organic carbon compounds (through root exudate) in exchange for water and nutrients provided by the fungus.

Juge et al. (2012) showed that soybean plants are under stress when in the presence of the combination of three symbionts (*Bradyrhizobium*, *Azospirillum*, and arbuscular mycorrhizae); they showed that the concentration of coumestrol (Flavonoid) could indicate a common regulating signal between soybean and both *Azospirillum* and mycorrhiza.

23.4 Advantages of Co-inoculation and How to Achieve

Polymicrobial formulations containing a diverse mixture of beneficial rhizosphere microorganisms with multiple functionalities is attractive because combining different classes of soil organisms can take advantage of multiple plant growth-promoting mechanisms and could be applied to multiple crops (Avis et al. 2008; Gravel et al. 2007; Hayat et al. 2010; Malusa et al. 2012; Vestberg et al. 2004). A key concept in constructing effective polymicrobial multifunctional formulations is the selection and use of a right combination of rhizosphere bacteria and fungi that are mutually compatible, have complementary functionalities, effectively colonize the rhizosphere of the crop(s) of interest, and bring about a synergistic promotion of growth and yield of crop(s) (Avis et al. 2008; Azcon-Aguilar 2009; Barea et al. 2005; Hata et al. 2010). It is also important to select a stable and efficacious carrier that would provide a suitable microenvironment for keeping the microbes in the inoculum viable and to develop relatively simple and inexpensive delivery methods. Considering the beneficial effects of: (1) PGPR; (2) AM fungi; and (3) symbiotic N_2-fixers, it is desirable to construct a polymicrobial formulation that contains several microbial strains representing each of these three functional groups and strongly promotes plant growth and yields (Arora et al. 2011; Khalid et al. 2009; Malusa et al. 2012). It is to be expected that a well-designed multifunctional formulations such as the one described would be a welcome addition to the fast-growing inoculant enterprises worldwide. Such an inoculant is also expected to be eco-friendly and suitable for organic farming and other integrated production systems, where synthetic fertilizer inputs are not allowed or restricted by law. However, construction of such complex formulations is technically demanding (Reddy and Saravanan 2013).

The contribution of arbuscular mycorrhizal fungi (AM Fungi) and of PGPR or obligate nitrogen-fixing microsymbionts (*Azospirillum* and *Rhizobium* bacteria) to soil fertility, productivity, and crop yield has been well documented (Subba Rao 1985; Boddey et al. 1991; Jeffries and Dodd 1991; Biró et al. 1993; Bethlenfalvay and Schüepp 1994; Garbaye 1994; Barea et al. 2005).

Synergistic interactions between AM fungi along with a PGPR organism such as *Azospirillum*, *Azotobacter*, *Bacillus*, or *Pseudomonas* species, has also been reported as beneficial for plant growth (Malusa et al. 2012). However, the stimulatory effect of the *Azospirillum* inocula on root growth did not significantly influence the mycorrhization, regardless of the AM fungus involved, either in wheat or in maize plants, in the presence of indigenous AM fungi or when maize plants were artificially inoculated with *G. mosseae* and *Glomus macrocarpum*. *Azospirillum*-AM fungus combination seems suitable for sustainable agriculture practices, since both types of microorganisms are not only compatible with each other but give synergistic benefits to plant productivity (Reddy and Saravanan 2013).

Pacovsky and Fuller (1985) showed that for sorghum inoculated with a vesicular-arbuscular mycorrhizal (VAM) fungus, and a strain of *Azospirillum brasilense*, the presence of *A. brasilense* in the rhizosphere increased VAM colonization and biomass.

Rhizosphere–mycorrhizosphere systems can therefore be tailored to help plants to establish and survive in nutrient-deficient, or degraded habitats or during periods of stress (Smith and Bowen 1979; Sanchez-Diaz et al. 1990).

Positive effects of *A. brasilense* and AM fungal colonization on rice growth and drought resistance have been reported (Ruiz-Sanchez et al. 2011).

Combination of AM fungi and N_2-fixing bacteria helped the legume plants in overcoming drought stress (Aliasgharzad et al. 2006). Effect of *A. brasilense* along with AM can be seen in other crops such as tomato, maize, and cassava (German et al. 2000; Casanovas et al. 2002; Creus et al. 2005). *A. brasilense* and AM combination improved plant tolerance to various abiotic stresses (Joe et al. 2009).

Associative diazotrophs, on the other hand, are not separated from the mycelia of the endomycorrhizal fungi. These nitrogen-fixers can function both inside and outside AM fungi structures. This may result in a competition for nutrients between the two microsymbionts. The success and the failure of the *Azospirillum* and AM fungal co-inoculations may therefore depend on the physiological stage of the host, the time of the infections or on the nutrient demands of the microsymbiont partners (Biró et al. 2000).

For example, increased root colonization by AM fungi was observed when co-inoculated with a range of PGPR including *Azospirillum*, *Azotobacter croococcum*, *Bacillus polymyxa*, and *Pseudomonas stricta* (Artursson et al. 2006; Gamalero et al. 2004; Toro et al. 1997). Barea et al. (1983) similarly showed that maize and ryegrass inoculated with *A. brasilense* and mycorrhizal fungi had comparable N and P contents as to plants grown with fertilizer. Inoculation of plants with *Azospirillum* enhanced mycorrhizal formation and conversely, *Azospirillum* establishment in the rhizosphere was also shown to be improved (Barea et al. 2005). However, increased N acquisition by dual-inoculated plants was attributed to greater N uptake capacity by mycorrhizal infected roots, rather than a direct effect through N_2 fixation. Multilevel interactions between AM fungi, *Azospirillum* and phosphate-solubilizing bacteria PSB have also been reported with indication of synergistic effects when inoculated simultaneously (Muthukumar et al. 2001). However, it is important to also consider that relationships between PGPR and mycorrhizas may not always be positive (Walley and Germida 1997).

AM fungi may inhibit pathogen proliferation through the formation of a bacterial community that limits the pathogen invasion (St-Arnaud and Vujanovic 2007; Li et al. 2007).

There were several observations demonstrating that some pathogen populations were reduced by *Azospirillum* and mycorrhizal fungi inoculation. Maize (*Zea mays*) inoculated with a combination of mycorrhizal fungi, *Glomus fasciculatum*, *Azospirillum* sp., and phosphate-solubilizing bacteria reduced the population of the *Pratylenchus zeae* nematode and simultaneously induced the highest cob yield (Babu et al. 1998).

Inoculation with arbuscular mycorrhizal (AM) fungi and *Azospirillum* sp. suppressed damping-off in chili (*Capsicum* sp.) caused by *Pythium aphanidermatum* (Kavitha et al. 2003).

Addition of *A. brasilense* to the ECM fungi (*Hebeloma crustuliniforme* and *Pisolithus tinctorius*) used as a biocontrol inoculant for pine seedlings against the pine pathogens, *Rhizoctonia solani* and *Fusarium oxysporum*, increased fungal biomass and the shoot/root ratio of the seedlings (Dahm et al. 1998).

Synergistic and antagonistic effects on AM fungi (*G. fasciculatum*) co-inoculated with *A. brasilense* or *Rhizobium meliloti* on the photosynthesis in alfalfa plants might serve as a parameter to measure the effect of the microbial activity and their possible beneficial effects in the field. Beneficial effects of AM on photosynthesis were clearly revealed as enhancement of the electron transport activity per leaf area. When AM was co-inoculated with one of the PGPBs, the antagonistic effect of *A. brasilense* was higher than that of *R. meliloti*, though not strong enough to fully counterbalance the beneficial effect of AM. However, in the case of triple inoculation with both PGPB and AM, electron transport was found to be only slightly lower than in the case of single inoculation by AM, indicating that, in the presence of each other, PGPBs are no longer antagonistic to AM (Tsimilli-Michael et al. 2000).

Similar to AM fungi, ectomycorrhizal fungi also exhibit synergistic interactions with other plant-beneficial organisms such as symbiotic N_2-fixers. For example, ectomycorrhizal symbiosis enhanced the efficiency of inoculation of two *Bradyrhizobium* strains on the growth of legumes (Andre et al. 2005). It is also of interest that similar synergies were seen when AM fungus (*Glomus mosseae*), ECM fungus (*Pisolithus tinctorius*), and *Bradyrhizobium* sp. were used together to inoculate *Acacia nilotica*, enhancement of N_2 fixation, growth, and dry biomass were observed when all three organisms were present (Saravanan and Natarajan 1996, 2000). Moreover, *Bradyrhizobium* sp. when co-inoculated with either the AM fungus or the ECM fungus, gave enhancement of N_2 fixation as compared to the control with *Bradyrhizobium* sp. only.

The co-inoculation of ECM fungi and PGPR may, in some cases, increase fungal colonization (Ouahmane et al. 2009; Domínguez et al. 2012). In other cases, although co-inoculation did not affect the fungal colonization, synergistic effects on the plant growth were observed (Rincon et al. 2005).

However, non-studies of ECM fungi co-inoculated with *Azospirillum* were found.

23.5 Methods of Dual Inoculation

Artificial seed and soil inoculation techniques were, therefore, started over 100 years ago; as a simple application of a mixed nodule extracts (Hiltner 1895).

Many types of formulations and techniques for inoculation of soil microorganisms, especially of PGPB have been tested in recent years (Bashan et al. 2014).

Dodd and Ruiz-Lozano (2012) suggested that combining different classes of soil organisms within one inoculant can take advantage of multiple plant growth mechanisms of such an inoculant to enhance crop productivity. One of the main limitations so far has been the absence of a single polymicrobial inoculant formulation that

would promote the growth of a range of crops including legumes, cereals, and vegetable crops (Reddy and Saravanan 2013).

PGPR strains that are often used (singly or in mixture) include species of genera *Bacillus*, *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Trichoderma*, and *Glomus* (AM) (Reddy and Saravanan 2013).

Often, precultures from Mycorrhizal fungi and PGPR have been performed, separately for each microorganism, previous to the co-inoculations.

Producing ECM fungi is relatively easy and can be produced by fermentation using suitable media; for PGPR + ECM co-inoculations, were often used specific methods of ECM (spore or mycelium) added to PGPR inoculation techniques (Duponnois and Garbaye 1991); in previous studies (Dominguez et al. 2012) co-inoculations of PGPR *P. fluorescens* and black truffle (*Tuber melanosporum*) inocula were performed by simple spore suspensions and *P. fluorescens* cultures.

One of the most common methods of inoculation of AM fungi combined with *Azospirillum* inoculums has been the incorporation of grasses soils, colonized roots fragments, hyphae or spores to the new substrate, or into the seed irrigations.

An example is the study by Bauer et al. (2012); authors test the effects of AM fungi and N_2-fixers (rhizobia and *Azospirillum*) on the productivity, diversity, and species abundances of nutrient-poor grassland microcosms; the AM fungi treatment consisted of a mixture of fungal cultures isolated from prairie soils and cultured with *Sorghum bicolor*, and included various arbuscular mycorrhizal fungi. The soil and coarsely chopped roots from these cultures were used as inocula for this experiment. The N_2-fixer treatment included cysts of wild-type *Azospirillum brasilense*, suspended in KPO_4 buffer (10^7 cfu mL^{-1}) and rhizobia as cultures attached to granular peat (1×10^8 rhizobia g^{-1}).

Other example: In Bellone et al. (2012), sugarcane plants were inoculated with a combination of 1 mL of an *Azospirillum brasilense* culture (1.5×10^7 cfu) and 1 mL of spore suspension of *Glomus intraradices* (100 spores mL^{-1}).

In 2011, Bashan et al. developed two new culture media for mass cultivation of the commonly used plant growth-promoting bacterium *Azospirillum* sp.; based on substitution of glucose in tryptone–yeast extract–glucose medium by Na-gluconate or glycerol.

Separation of the AM fungi and the other usual rhizosphere components (mainly bacteria and other micromycetes) is possible using a sterilization and re-inoculation procedure (Biró et al. 2000). In the microbial treatments, a microbial wash is often included in uninoculated treatments to control for other microorganisms introduced in the AM fungi cultures, but Bauer et al. (2012) did not include this background wash to reduce the potential for contamination with N_2-fixing bacteria. A meta-analysis of AM fungi studies found that the absence of this microbial wash provides a conservative test of the benefits of AM fungi and recommends this approach for factorial manipulation of AM fungi and rhizobia (Hoeksema et al. 2010).

Until now, a few studies have been performed with polymicrobial inoculums including both groups *Azospirillum* and Mycorrhizal fungi at the same time.

A granular clay inoculant containing AM fungus (*Glomus fasciculatum*), *Azospirillum*, and phosphate-solubilizing bacteria (PSB) for easy application in nurseries had a shelf life of up to 60 day (Lilly and Santhanakrishnan 1999).

The technology of macro-alginate beads was used to encapsulate several plant growth-promoting bacteria and mycorrhizae fungi (Bashan et al. 2014). Vassilev et al. (2001) show that inoculation of tomato plants with an AM fungus (*Glomus deserticola*) and a P-solubilizing microorganism (the yeast *Yarrowia lipolytica*) entrapped in alginate can be an efficient technique for plant establishment and growth in nutrient-deficient soils. Several plant growth parameters were equal in treatments inoculated with free and alginate entrapped AM but dual inoculation increased all analyzed variables. Highest rates of the latter were obtained when both fungal microorganisms were entrapped in the carrier. The yeast culture behaved as a "mycorrhiza helper microorganism," enhancing mycorrhization of tomato roots.

Using Plant Growth-Promoting Microorganisms (PGPM) strains that form stable and effective biofilms could be a strategy for producing commercially viable inoculant formulations (Malusa et al. 2012; Seneviratne et al. 2008). A majority of plant-associated bacteria found on roots and in soil are found to form biofilms (Ude et al. 2006). Bacterial, fungal, and bacteria/fungal biofilms were suggested as possible inoculants. This is a novel and interesting idea, but to what extent this approach would be practiced remains to be seen (Reddy and Saravanan 2013).

The most notable phenomenon in *Azospirillum* inoculation, as in the early 1990s (Bashan and Holguin 1997a, b), is that inoculation is more successful and more profitable when other microorganisms are co-inoculated with *Azospirillum*. Inoculation consortia apparently work better when phosphate-solubilizing bacteria, *Azotobacter*, rhizobia, bacilli, and VAM fungi are incorporated, aiding, perhaps, the growth of each other by synergistically providing nutrients, removing inhibitory products, and in the process, enhancing plants' ability to grow better (Bashan et al. 2004).

Anyway, in the *Azospirillum*–Mycorrhiza interaction, the potential changes produced when both groups of microorganisms coexist in the root interior are not well known yet, and the following areas (Bashan et al. 2004) continue to deserve attention for future research: (1) Establish biological markers for the interaction between *Azospirillum* and other microorganisms (especially AM fungi and phosphate-solubilizing bacteria PSB) to select the most compatible combinations for plant inoculation. (2) Use microarrays and proteomics to elucidate the "cross talk" among *Azospirillum*, plants, other rhizosphere bacteria and mycorrhizal fungi.

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Part IV
Agronomic and Industrial Applications Toolbox

Chapter 24
Field Evaluation of Extensive Crops Inoculated with *Azospirillum* sp.

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Abstract The available information about the changes in growth and production of extensive crops due to the inoculation with formulations containing *Azospirillum* sp. is abundant. The crop responses to the presence of *Azospirillum* sp. are generally related with changes in their growth during early stages of development. It leads to variable contributions to the final grain production depending on the differences in the occurrence of abiotic stresses (i.e., water and nutrient uptake). We present, based on recently available articles, the design of field trials and its analysis for describing the value of the inoculation practice in terms of grain production as a tool to support crop management decisions. The ecophysiological responses require the interpretation of the results based on the frequency of their occurrence as well as the discrimination among hierarchical productivity factors (i.e., spatial and temporal variability). Because of the variability in the crop responses to the inoculation with *Azospirillum* sp., a large number of observations are needed to describe significant differences between treatments. In the case of rainfed wheat (*Triticum aestivum* L.) crops from

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the semiarid pampas (Argentina), the range can vary between 60 in seasons with normal rainfall patterns and almost 200 cases in dry seasons. The analysis of distribution response values is a more realistic approach to measure the crop performance to inoculation under the interaction of complex random factors and provides also a probabilistic answer to stakeholders. For example, based on the distribution of maize (*Zea mays* L.) responses to *Azospirillum* sp. from 316 cases located in the pampas region of Argentina it was observed that in more than 81.1 % of the cases the incremental revenue of the practice exceeded its cost and it could be included as a crop management practice under the current production conditions.

24.1 Introduction

The diverse single modes of actions to enhance plant growth and crop production in the presence of *Azospirillum* sp. has been widely studied also under diverse experiences setups. In general, the modes of action that partially explain crop growth and yield responses to the inoculation with *Azospirillum* sp. are both direct and derivate effects enhancing the nutritional status of the plants and mitigating other abiotic stresses. Abundant studies show that *Azospirillum* sp. has the capability, within other attributes, for fixing nitrogen from the air, delivering hormones and enzymes to the rhizosphere that can enhance root growth and promote also the growth of other microbes (Bayan and Levanony 1990; Okon and Labandera-Gonzalez 1994; Dobbelare et al. 2003). Most of the studies that describe several of its modes of action have been performed within pots under controlled conditions in greenhouses (Van Dommelen et al. 2009; Rodrigues et al. 2008) with limitations for the extrapolation to regular crop conditions.

Field trials analyzing the contribution of the inoculation with *Azospirillum* sp. on extensive crops under regular crop production conditions are less abundant. Furthermore, the interpretations of their results are variable and not always conclusive. This could be partially explained because of the complexity in the design of the field studies in response to the diverse mode of interaction of *Azospirillum* sp. and plants under multiplicity in abiotic stress conditions to be mitigated in the presence of the microorganism. The objective of this contribution is to briefly describe several field and data analysis procedures for the evaluation of crop production responses to the inoculation with *Azospirillum* sp.

24.2 Design of Field Experiments

We reviewed 47 articles worldwide published during the last decade showing grain crop production in response to the application of diverse *Azospirillum* sp. inoculants in 12 countries, collecting 347 cases. Most of the studies were performed in cereals' crops (86.7 %), mainly rainfed maize (*Zea mays* L.) and they were located in Latin America, mostly Brazil, and Asia. Approximately, among all the reported crops,

10 % was the mean grain response to the inoculation with *Azospirillum* sp. A greater response was observed in winter cereals (14.0 %) [i.e., wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.)] than in summer cereals (9.5 %) [maize, sorghum (*Sorghum bicolor* L.), rice (*Oryza sativa* L.)] or legumes (6.6 %) [beans (*Phaseolus vulgaris*), soybean (*Glycine max* (L.) Merrill)].

The frequent procedure for the introduction of the *Azospirillum* sp. in field crops is treating the seeds or applying it directly in the sowing furrow with diverse inoculant formulations containing the microorganism. Transplanting seedlings soaked in a solution containing *Azospirillum* sp. was also described in the bibliography to deliver it to the rhizosphere for example of rice crops (Govindarajan et al. 2008; Islam et al. 2012; Khalid et al. 2011). But, because this procedure is not practical and has limited application for extensive production we did not described it in this article. The foliar application of *Azospirillum* sp. was also described in several of the reviewed studies, but with controversial results and not clear recommendations about the moment of application relative to the crop development stage or the environmental conditions for its adequate use (El Habbasha et al. 2013).

The seed treatment with formulations containing *Azospirillum* sp. is preferred to be done without the application in slurry with other products (i.e., micronutrients, fungicides, insecticides, polymers). This is mainly for avoiding potential interactions with the active ingredients as well as with other components of the formulations (i.e., colorants, dispersants, stabilizers). Few studies describe the application of formulations containing *Azospirillum* sp. in the planting furrow during sowing (Hungria et al. 2013). It is a recommended option for reducing the risk of low compatibility treatments applied with the seeds that can reduce the survival of the microbes. In the case of in-furrow treatments, the inoculants were diluted in water-free chlorides in a pH range between 6.0 and 8.0 and were applied at a rate and work pressure that allowed for the delivery of large drops in almost a continues stream below the seedbed (i.e., 30–40 L ha^{-1}, 1.0–2.0 bar and perforated discs as nozzles).

However, several formulations containing *Azospirillum* sp. have been developed for allowing its application on the seeds long term before sowing (Díaz-Zorita et al. 2012), most of the studies described that the inoculated seeds were in the ground within less than 6 h after the application of the biological treatment. Independently of the moment of application of the treatments, it is critical to avoid excessive manipulation of the treated seeds because it can damage the *Azospirillum* sp. cells when exposed to desiccation, high temperatures, ultraviolet light, or toxic compounds. Also, the sowing should be done in a moist seedbed avoiding leaving the seeds exposed to desiccation or direct sun light.

Similar to other inoculation studies, large plots with more than 10 m long are commonly used performing all crop measurements in the central part of them and discharging the borders. This helps to diminish the risk of contamination between treatments as well as for the reduction in the manipulation of the treated seeds because of the use of large seed amounts for sowing. To reduce the potential contamination among different biological seed treatments during the sowing operation, it is recommended the cleaning of the machinery after changing the treatments to be planted as well as to initiate the sowing with the control treatment without inoculation.

Crop management practices that modify the growth of the crops in the presence of abiotic stresses (i.e., nutrient deficiencies, water stress) also affect the performance of the crops treated with *Azospirillum* sp. Thus, it is critical to define crop management practices like irrigation, fertilization, and planting dates and other production conditions (i.e., soil type) in agreement with the objectives of the study. In general, for the quantification of the integral crop performance to *Azospirillum* sp. inoculation, the crop management should reproduce regular production practices avoiding excessive nutrient availability or irrigation practices that limit the possible occurrence of moderate abiotic stresses to be mitigated for the inoculated *Azospirillum* sp. Both, under strong stressful growing conditions (i.e., severe droughts, major nutrients limitations) or in the absence of growth limitations the responses to the biological seed treatment are barely observed (Lana et al. 2012; Mehnaz et al. 2010; Naiman et al. 2009; Naseri et al. 2013). In general, the studied cases were fertilized to rich the crops requirements, mainly NPK, for normal production and only in few situations the crops performed under limited nutrient availability conditions.

The frequent experimental design of these field studies for single locations during few production seasons or few locations during a single season was mostly described in randomized replicated complete blocks. This experimental design has limitations for the analysis of the results and for the interpretation of the contribution of *Azospirillum* sp. to crop production because of the occurrence of random temporal and spatial interactions. Few studies were performed under abundant locations and growing seasons for the contribution of the biological treatment over a range of environmental conditions and regular production practices showing variable results on fixed (i.e., soil type) or random (i.e., production seasons) factors (Díaz-Zorita and Fernández-Canigia 2009). This is the reason why in the following sections of this chapter we will briefly describe two approaches for the interpretation of the crops grain yield responses to the presence of *Azospirillum* sp. taking into account both the spatial (i.e., soil type, crop management practices) and temporal (i.e., production season) variability.

24.3 Soil Type and Temporal Variability Effects on Crop Responses to *Azospirillum* sp. Inoculation

For the quantification of the contribution of wheat seeds inoculation with a formulation containing *Azospirillum brasilense* strain INTA Az39 a field trial was performed during three-consecutive growing seasons in a 50 ha production field located in Bahia Blanca (Buenos Aires Province, Argentina) within the semiarid pampas region. The field lies in a rolling landscape located in the upper valley of the Saladillo Dulce creek containing complex slight to moderate slopes and soils classified as Entic Haplustolls, saline to sodic saline Entic Haplustolls, moderately deep Petrocalcic Paleustols, and shallow and deep Petrocalcic Calciustolls (Soil Survey Staff 1999) always with loam to sandy loam textures in the upper profiles. All the seasons, the wheat crops were managed applying best practices recommended for

high attainable grain production in the semiarid region [i.e., zero tillage practices with chemical weed control, sowing dates during late June and fertilized with 40 kg ha^{-1} of diammonium phosphate (18:46:0)]. The two treatments (control without inoculation and treated seeds with *Azospirillum brasilense*) were yearly placed in paired strips of 8 ha across the described soil types. The shoot dry matter and grain yield measurements were yearly performed in 15 geopositioned sites and then classified into four predominant soil types in pairs of inoculated and control treatments. Descriptive statistics (mean and dispersion parameters) for each of the treatments among spatial (soil type) and temporal (growing seasons) and analysis of variance (ANOVA) based on a factorial split plot design with seasons and the biological seed treatments as main factors and the soil types as split factor were performed. Only the data of shoot dry matter was transformed for the homogenization of the variances (Di Rienzo et al. 2013).

The wheat crops inoculated with *Azospirillum brasilense*, under the studied conditions and averaged among soil types and growing seasons, were observed in 74 % of the sites and trend to show 15 % more shoot dry matter and 17 % grain yield than the control without inoculation (Table 24.1). From the ANOVA, significant effects of the seasons ($P<0.01$) and the biological seed treatments ($P<0.05$) on the grain yields were described with no significant interactions between them ($P<0.21$). The available information was not enough to describe differences among the soil types ($P<0.28$). Sixty-five percent ($P<0.01$) of the wheat grain yield variability was attributed to differences in yearly weather conditions explained mainly for the amount and the distribution of the rainfalls during the growing conditions. The inoculation with *Azospirillum brasilense* explained 4 % ($P<0.05$) of the variability in grain yields, the soil types only 1 % ($P>0.50$), and the remaining 30 % was allocated in the interaction among the studied factors and the experimental error. The greatest response to inoculation, in each of the seasons, was observed in the sites with the highest soil productivity indices corresponding mostly to deep Entic Haplustolls. But, the absence of significant differences between biological treatments within each soil type was attributed to the limited quantity of samples.

The available information from this study showed different deviations of the means among seasons (Table 24.2). Thus, that the sample size for describing differences between the biological treatments with a 95 % of confidence also varied among seasons. For example, in seasons with regular growing conditions 60–75 samples

Table 24.1 Shoot dry matter and grain production of wheat crops grown in a field during three seasons from the semiarid pampas inoculated with *Azospirillum brasilense*

Variable	Treatment	Mean	SD	Min.	Max.	CV (%)
		Mg ha^{-1}				
Shoot dry matter	Control without inoculation	5.8[a]	2.7	2.5	14.2	46.6
	Inoculated	6.7[b]	3.1	2.5	13.6	46.9
Grain yield	Control without inoculation	1.2[a]	0.5	0.5	2.8	39.0
	Inoculated	1.4[b]	0.5	0.6	2.6	36.0

SD standard deviation, *CV* coefficient of variation. In each variable, mean values with different letters show least significant differences ($P<0.05$)

Table 24.2 Maize distribution parameters of control and *Azospirillum brasilense* inoculated maize crops in a database from 316 field experiments performed in Argentina

	Percentile							Mean	SD
	0	5	25	50	75	95	100		
Treatment	Grain yield (kg ha^{-1})								
Control	2,020	4,563	7,169	8,792	10,545	12,855	15,924	8,794	2,512
Inoculated	2,394	5,092	7,583	9,196	11,015	13,095	18,654	9,237	2,562
	Relative yield increase to inoculation (%)								
	−43	−8	1	4	9	24	66	6	11

SD Standard deviation

were needed to describe differences between the means while in the driest and low yielding season more than 200 samples should be required. This large number of observations needed for describing significant differences between the treatments could be diminished limiting the location of samples to spatially homogeneous sites.

24.4 Applying Experimental Data to Evaluate Crop Management Decisions

Experimental data are particularly valuable if they are used to support management decisions. In the previous sections of this chapter, it was shown that *Azospirillum* sp. inoculation is expected to increase crop yields. However, response to *Azospirillum* sp. cannot be predicted with certainty since it is a random variable partially because of its diverse modes of actions mitigation also multiple abiotic stresses during crop growth. In fact, negative, neutral, and positive responses to inoculations with *Azospirillum* sp. were found in any crop species (Araujo et al. 2013; Hungria et al. 2013; Piccinin et al. 2011; Turan and Fikrettin 2013). So, how can the experimental information be used to take decisions? To choose some experiments according to predefined criteria (i.e., soil type or growing season) could be a way and has been discussed in the previous section. But, incorporating particular criteria to select information could be misleading, particularly if it is done by inexperienced persons. In this section, we are analyzing an alternative way to look and explore experimental data to evaluate the convenience of crop inoculations. For this purpose, we used results from 316 field experiments where the grain yields of maize crops under control without inoculation and inoculated with *Azospirillum brasilense* strain INTAAz39 treatments were measured in Argentina. The data were obtained in various locations of the pampas region under regular production practices (i.e., NP fertilization, rotated land, mostly uner zero tillage practices) and during 12 consecutive growing seasons (M. Díaz-Zorita, unpublished data). Because, an ANOVA analysis of the treatment response showed no significant ($P = 0.05$) differences among them, the whole database (316 data set) was used.

Crop management decisions are usually evaluated on the basis of the difference between incremental revenue and incremental costs. If the incremental revenue is higher than the incremental cost, there appears to be an advantage in the technology. In this case, incremental revenue is provided by yield response (yield increment provided by the technology) multiplied by net price (gross price discounted all marketing expenses). As both yield response and price are random variables, incremental income turns out to be a random variable itself. As such, it cannot be described by a static value. Instead, it needs to be described by a distribution function. Using empirical data, the probability distribution of maize yield in the control and inoculated treatments may be described by various percentile values in the range from 0 to 100 (Table 24.2). Moreover, maize yields may be compared statistically in various percentile ranges (Table 24.3). In the database used, the treatments were compared in a wide range of conditions, from crops yielding as low as 2,020 kg ha^{-1} to as high as 18,654 kg ha^{-1} (Table 24.2). Although percentile values differed along the whole distribution, only mean yields of the treatments distribution in the range from 5 to 95 % were significant (Table 24.3). The probability to find differences in both extremes of the data distribution was low; however, in the central 90 % of the distribution, in crops yielding between 6,000 and 12,000 kg ha^{-1}, crops inoculated with *Azospirillum brasilense* had a consistent higher probability of greater yields than control crops (Table 24.3).

Potent tools may be used to extend and analyze the data described. One of this, Monte Carlo simulation, is a technique that allows estimating the probability distribution of an outcome that depends on random variables. Even though it was originally developed with other purposes (i.e., operations research field), it has been widely applied to crop decision analysis under uncertainty (Clow and Flaskerud 2001; Ferreyra et al. 2001; Berger and Pena 2013). In order to use Monte Carlo simulation, probability distributions need to be defined for any random variable involved. From the experimental data of maize yield responses to *Azospirillum brasilense* inoculation in Argentina, a general probability distribution was built showing a range from −43 to 66 % and less than 20 % change of negative responses (Table 24.2). But, as crop response data are not normally distributed, other probability distributions that are

Table 24.3 Mean values of percentile ranges in the empirical distribution of maize grain yields from control and *Azospirillum brasilense* inoculated crops in a database from 316 field experiments performed in Argentina

	Range of the empirical distribution				
	0–5	5–25	25–75	75–95	95–100
Treatment	Grain yield (kg ha^{-1})				
Control	3,691	6,118	8,755	11,541	13,838
Inoculated	3,912	6,566	9,216	11,948	14,448
P	ns	<0.001	<0.001	<0.001	ns
n	16	63	157	64	16
SE	209	92	78	82	316

P statistical significance, *n* number of sites considered in each range, *SE* standard errors of the means, *ns* nonsignificant differences between means

frequently employed, betapert and cumulative, were also tested. The betapert distribution is simple to use in a variety of situations, both when counting with observed data and when working with limited information or also just with only expert opinion. The cumulative distribution can be chosen when large amount of data is available because it implies no data manipulation. These distributions need the subjective estimation of two values to be used as parameters for the range. The values defined as minimum and maximum have zero probability of being sampled in a Monte Carlo simulation process. Therefore, the lowest and the highest values of a sample of real data cannot be used as estimators, which for the exercise performed here were set at −45 and 75 %. In this example, the maize grain yield response to the inoculation with *Azospirillum brasilense* in 316 sites from Argentina was better reproduced when using a cumulative distribution (Table 24.2). The normal and general distribution replicated better the observed data than the betapert distribution but both overestimate the probability of null responses.

To analyze and to make decisions with the results, the maize price has to be described and it is also a random variable. In this analysis, the maize price distribution was defined using a betapert function and the parameters were estimated on the basis of historical prices with inflation adjustment and expert opinion. This is a common procedure when using Monte Carlo simulation in daily decision making. Because of structural changes in commodities markets long time series may not be relevant for producer's decision making in Argentina and we used the last 7 years of maize prices in Argentina at harvest. The resulting betapert distribution for gross price was: 118.7 (minimum), 170.0 (most probable), and 200.0 (maximum) US$ ton^{-1}. For the analysis, the marketing expenses were set at current values with fixed expenses (freight and drying) of 38 US$ ton^{-1} and 3 % of commissions and taxes. Then, the net price was calculated as 97 % of the gross price minus the fixed expenses per produced ton. Using agricultural simulation models and expert opinion, a betapert distribution was defined for maize "base yield" without *Azospirillum brasilense* inoculation. The parameters for this distribution were 5,009 (minimum), 10,630 (most probable), and 12,623 (maximum) kg ha^{-1}, with an expected grain yield of 10,025 kg ha^{-1}. The revenue was calculated as the product between the base yield, the percent of response and the net price. With the distribution shown in the Fig. 24.1, the probability of incremental revenue exceeding the cost of inoculation with *Azospirillum brasilense* can be calculated. If this probability is high enough, there may be no doubt that *Azospirillum brasilense* inoculation will be included as a crop management practice. In this case, based on the distribution of maize responses to *Azospirillum* sp. from 316 cases located in the pampas region of Argentina, it was observed that in more than 81 % of the cases the incremental revenue of the practice exceeded its cost, and it could be included as a crop management practice under the current production conditions.

The approach presented is quite different from the way that usual experimental results are used to help stakeholders. A deterministic result, usually the mean response value, was here replaced by a distribution of values and a probabilistic answer to the stakeholders. This may be considered a more realistic approach to measure the response of the maize crops to inoculation with *Azospirillum brasilense* in Argentina.

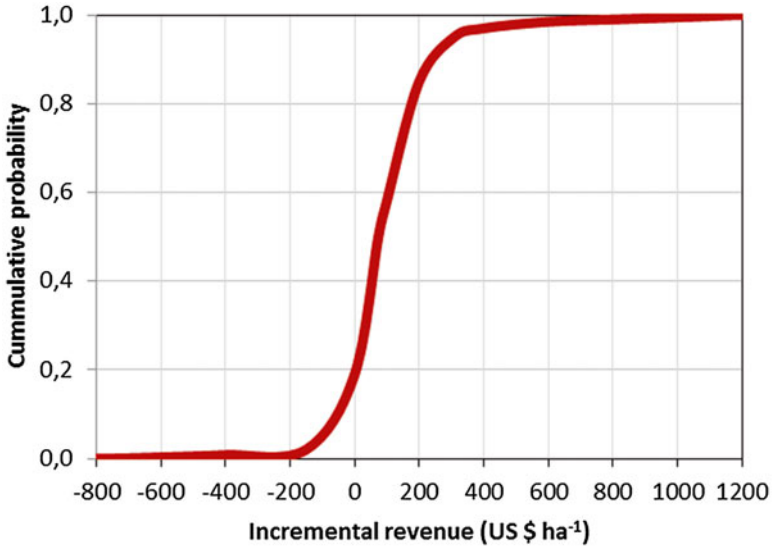

Fig. 24.1 Incremental revenue distribution for *Azospirillum brasilense* inoculation in maize crops from the North of Buenos Aires Province, Argentina

24.5 Conclusions

Abundant field studies, mostly in Latin America and Asia show the increase in grain yields of crops inoculated with *Azospirillum* sp. mainly in cereals. Although this microbe can enhance the nutritional status of the inoculated crops, as well as to reduce minor water or other abiotic stresses, the design of the experiences requires the implementation of normal production practices avoiding major nutrient limitations and extreme water stress conditions.

In general, the delivery of the inoculum to the production system is with the seeds, and it is recommended a careful handling and storage of the treated seeds to facilitate a high survival of the inoculated microbes. The compatibility with other seed treatments and the long-term survival of *Azospirillum* sp. is variable depending on the formulations containing them.

A better interpretation of the results requires the analysis based on the frequency of their occurrence as well as the discrimination among hierarchical productivity factors. And, because of the large variability in the crop responses to the inoculation with *Azospirillum* sp., also large number of observations is needed to describe significant differences between treatments. The analysis of distribution response values is a more realistic approach to measure the crop performance to inoculation under the interaction of complex random factors and provides also a probabilistic answer to stakeholders.

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Chapter 25
Azospirillum spp. and Related PGPRs Inocula Use in Intensive Agriculture

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Abstract From the beginning of plant domestication, extensive farming has been the main strategy adopted by agriculture to produce large amounts of food. However, plant production in a continuously deteriorating environment and an exponentially growing human population are important factors that challenge agriculture nowadays. Moreover, agricultural lands are currently expanded to marginal, arid, or semiarid regions where crops are exposed to abiotic stresses as drought and salinity, this last negative factor aggravated by different anthropogenic actions. In addition, contemporary society requires from agriculture to provide food products with high market and nutritional qualities as fruits and vegetables, which should also be free of agrochemicals. In consequence, it is imperative to develop friendly, non-contaminant, sustainable, and energy-saving plant production strategies. In this regard, vegetable production by intensive farming in controlled environments is continuously expanding. On the other hand, recent reports show that plant inoculation with plant-growth promoting rhizobacteria (PGPR) could improve vegetable quality and yield under abiotic stresses, and to reduce the pressure that current agriculture exerts on the environment. Within this context, our main purpose was to describe a number of techniques aimed to study the plausible beneficial effects of *Azospirillum* and related PGPR inoculation on vegetable growth and nutritional quality, with emphasis on the promotion of antioxidant activity.

25.1 Introduction

With a growing world population that would probably reach 9,000 millions souls in 2050, it will be crucial to redefine agriculture the way we see it today. In order to satisfy the growing demand, food production would need to increase by 70 %

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(Editorial 2010). In addition, this expansion should be attained under sustainability, food safety, and lower economical resources terms (Barrow et al. 2008). This enormous challenge not only demands an optimal use of the actual resources, but also an expansion of the agricultural frontier to arid, semiarid, and/or inhospitable regions of the globe (Lantican et al. 2003; Adesemoye et al. 2008, 2009; Köberl et al. 2011). In these marginal lands, plant growth is usually restricted by different types of abiotic stresses including extreme temperatures, UV radiation, low water and/or nutrients availability, and salinity (Yang et al. 2009). Moreover, it has been predicted that global warming—already in course—will increase drought and salinity thus diminishing crop productivity in different parts of the Earth (Grover et al. 2010; Larson 2013). In addition, the use of pesticides and chemical fertilizers plus soil erosion aggravated the already contaminated agricultural lands, groundwater, and water reservoirs. In consequence, it is imperative to develop friendly, non-contaminant, sustainable, and energy-saving plant production strategies.

On the other hand, a higher demand on fruit and vegetable production is already in course. Worldwide fruit and vegetable production increased 67 % on average from 1990 to 2009 (ISHS 2012). Taking alone, vegetable production during this period increased from 710 to 1,379 million tons, representing 94 % on average (ISHS 2012). This demand has been enhanced by the increasing knowledge on the nutritional value of vegetables, including a high content of vitamins, minerals, dietary fiber, and several compounds that could help to prevent different human pathologies such as cancer, diabetes, and cardiovascular diseases (Le Marchand 2002; Jeremy et al. 2004; Vicente et al. 2014). However, consumers are also aware of the risks associated to the presence of pesticide residues in fruits and vegetables that are usually eaten uncooked. In this context, replacing fertilizers obtained from fossil fuels and chemical syntheses methods by organic fertilizers could help to reduce the pressure that current agriculture exerts on the environment (Barassi et al. 2007).

The above-mentioned obstacles could be partially surmounted by inoculating plants with PGPR (Barassi et al. 2007; Marasco et al. 2012; Berg et al. 2013; Reddy and Saravanan 2013; Turner et al. 2013; Coleman-Derr and Tringe 2014). Indeed, there is data reporting that seed or plant inoculation with some PGPR genus as *Azospirillum*, *Bacillus*, *Enterobacter*, *Klebsiella* and *Pseudomons* can result in more vigorous seedlings, a reduction in the need of fertilizers and/or an increased plant tolerance to abiotic stresses (Kokallis-Burelle et al. 2006; Lamsal et al. 2013; Park et al. 2013). Also, to increase the antioxidant potential and phenolics content in vegetables and fruits, which in turn could help improve their nutritional value (Nautiyal et al. 2008; Kohler et al. 2009; Cappellari et al. 2013; Jain et al. 2014; Ramos-Solano et al. 2014). On the other hand, the increasing knowledge on human microbiome and its contribution to regulate several physiological processes runs parallel to that on plants. In this regard, given the close relationship they maintain with its own microbioma, plants have been considered as meta-organisms (East 2013; Berg et al. 2013; Lebeis 2014). It has been suggested that some of the microorganisms found in the plant microbiome could change the way agriculture is actually performed, thus helping to cope humans with the challenges imposed by a growing global population and progressive weather shifts (Marasco et al. 2012;

Berg et al. 2013; Reddy and Saravanan 2013; Turner et al. 2013; Coleman-Derr and Tringe 2014). In this regard, some organic fertilizers recently introduced into the market are able to stimulate plant growth and development (Malusá et al. 2012). Some of these fertilizers are based on PGPRs, which can help maintain both a healthy plant and soil through different direct and indirect microbial strategies (Bhattacharyya and Jha 2012). These last include a relative protection against plant pathogens, which could be associated in turn to siderophore production, microorganism competition and bacteriocin synthesis (Mitter et al. 2013). A structure improvement as well as bioremediation of soil has also been reported, the last one by means of heavy metal sequestering abilities and/or degradation of toxic xenobiotic compounds as pesticides (Zahir et al. 2004). The PGPRs have also been able to enhance phosphate, organic N, and other nutrients solubility and availability to the plant. The direct plant-growth promotion effect has been associated to different molecular mechanisms of action, such as phytohormone production including auxins, gibberellins, ethylene, and abscisic acid. All the above-mentioned beneficial PGPR effects on plants could help to reduce the amount of dangerous, agroecosystem-disturbing chemical compounds normally used by farmers (Zahir et al. 2004; Singh et al. 2011; Malusá et al. 2012; Reddy and Saravanan 2013; Nadeem et al. 2014).

Even though plant inoculants based on PGPRs have been initially developed for extensive farming, a growing demand to extend their use to intensive agriculture systems such as those used in horticulture, is mounting. Organic, hydroponics, and greenhouse growth are some of the common methods used to grow vegetables. All of them are performed under a controlled environment, condition that in turn could help to obtain more stable PGPR inoculation effects than at the open field (Malusá et al. 2012). In short, seed and/or substrate inoculation with different PGPRs as *Azospirillum*, *Bacillus*, *Enterobacter*, *Klebsiella and Pseudomons* could enhance seedlings vigor, phytohormone production, tolerance to soil pathogens, a higher efficiency in the use of fertilizers, and a better tolerance to environmental stresses (Kokallis-Burelle et al. 2006; Lamsal et al. 2013; Park et al. 2013). Some authors also suggested that PGPR could be able to improve the nutritional value of fruits and vegetables by increasing the antioxidant capacity and phenolics content (Nautiyal et al. 2008; Koheler et al. 2009; Cappellari et al. 2013; Jain et al. 2014; Ramos-Solano et al. 2014).

Amongst PGPRs, *Azospirillum* is one of the most studied genuses. Phytohormone production, nitrogen fixation, and roots proliferation in *Azospirillum*-inoculated plants are all well-known effects that could improve plant growth. However, after an exhaustive literature search, Bashan and de-Bashan (2010) arrived to the conclusion that not a single mechanism of action could explain the promotion of plant growth. Instead, they have proposed a "Multiple Mechanism of Action" where several bacterial effects on plant growth could be acting according to the plant species colonized, *Azospirillum* strain, and the prevailing environmental conditions. An "Additive Hypothesis," where several small mechanisms acting simultaneously or one after the other in order to produce a larger plant growth effect, has been previously presented by Bashan et al. (2004). A cascade or tandem effects where one mechanism could stimulate another one cannot be excluded. In this regard,

phytohormone production, nitric oxide synthesis, higher plant membrane activity, and root proliferation are some of the mechanisms that could be related to each other in order to enhance plant growth (Spaepen et al. 2009; Bashan and de-Bashan 2010).

Despite data on *Azospirillum* genus, its inoculation effects on extensive crops, as well as on its plausible multiple mechanisms of action abound (Spaepen et al. 2009), only recently the research has been focused on its use in intensive horticultural production. Some of these studies have been performed on lettuce growing under normal and salinity growth conditions (Barassi et al. 2006, 2007; Kohler et al. 2009; Fasciglione et al. 2012), tomato (Lamsal et al. 2013; Romero et al. 2014) and pepper (del Amor et al. 2008; Flores et al. 2010).

Within this context, our main purpose was to describe a number of techniques aimed to help study the plausible beneficial effects of *Azospirillum* and related PGPR inoculation on vegetable growth. Also, to evaluate the nutritional value of products obtained, these later methods mainly focused on the promotion of antioxidant activity.

25.2 Seed Inoculation Techniques in Horticultural Species

Successful assays on plant-*Azospirillum* association studies are highly dependent on both effective and adequate root colonization. These requirements imply an optimal bacterial concentration to be applied to seeds, which should be adjusted to each plant species. Sub- or supra-optimal levels could result in either an absence of growth-promotion effects or even in an inhibiting effect on root elongation (Okon and Kapulnik 1986). Inoculation techniques usually involved two steps: (a) inoculant preparation, where a bacterial suspension is mixed with turf, peat moss, culture media, etc. resulting in a solid or liquid formulation (Bashan and de-Bashan 2010; Malusá et al. 2012; Berg et al. 2013), and (b) inoculant application on roots or seeds surfaces.

Particularly, liquid formulations have been adequate to introduce a viable and large amount of *Azospirillum* cells into seeds prior to the germinating process (Creus et al. 1996; Casanovas et al. 2000; Bennet and Wipps 2008). In this regard, *Azospirillum* would be in optimal conditions to colonize roots in the first steps of seed germination and to provide an adequate bacteria/root ratio to exert its beneficial effects. This methodology has proved that a large number of bacterial cells could be introduced into seeds along with the minimum amount of imbibition water germination demands. We have coined the term *intra-semen* to name this inoculation method, also known as biopriming. To determine the amount of water that enters seeds and the optimal inoculation timing, the methodology requires a previous determination of seed imbibition kinetics. Then, a known number of *Azospirillum* cells can be resuspended only in the amount of water seeds intake needed to germinate. Afterwards, seeds with different *Azospirillum* cell concentrations should be available to determine later which one corresponds to the maximum plant-growth promotion effect.

25.2.1 Azospirillum Cultures and Multiplication

Even though each research team has its own protocol to grow and amplify PGPR, we present the method we use in our lab to multiply *Azospirillum*.

Lyophilized *A. brasilense* Sp245 reference strain (donated by J. Döbereiner, CNPBS, Rio de Janeiro, Brazil) is transferred to potato dextrose agar media (USFDA BAM media M127 1998) to obtain stock cultures. Batch cultures are obtained as follows. Bacterial cells are streaked on nutrient agar slant tubes containing Congo Red medium (Rodríguez Cáceres 1982). After 5 days incubation at 32 °C, *Azospirillum* colonies from each tube are suspended in 2 mL phosphate buffer (66 mM, pH 7) by vortex mixing during 15 s at half speed. After that, 4 mL supernatants are transferred to an Erlenmeyer flask containing OAB medium (Okon et al. 1976) and incubated at 32 °C during 17 h with orbital agitation (100 rpm). Bacterial concentration in *Azospirillum* culture is calculated from absorbance determinations at 600 nm. Under the growth conditions described above and according to *A. brasilense* Sp245 growth kinetics described by Molina-Favero et al. (2008), 10^{12} bacterial cells can be obtained at the end of exponential growth phase. Cells were harvested by 15 min centrifugation at $8{,}142 \times g$ in an SS34 Sorvall rotor and resuspended in 66 mM phosphate buffer (pH 7) to the required bacteria·mL^{-1} inoculum concentration.

25.2.2 Seed Imbibitions Kinetics Determination and Inoculation Process

Water intake by seeds follows a three-phase pattern, where a fast initial absorption period (phase I) is followed by an intermission (phase II), and finally by a rise in water intake along with radicle emergence (phase III) (Bewley 1997) (Fig. 25.1). Seeds can withstand desiccation during I and II phases but become susceptible in phase III. A high difference between water potential (matric) of seeds and that of the imbibing solution determines the fast water absorption of phase I. This is however dependent on both seed composition and cortex permeability to water, which differs amongst plant species. With relation to the initial dry weight (DW), water content in different vegetables after phase I was as follows: sweet corn 37–40 %, lettuce 38 %, carrot 34 %, onion 37–39 % (Bennet and Whipps 2008). Taking this into account and considering the ample horticultural multiplicity, it is very important to determine the imbibition curve of seeds on each plant species to be studied.

In short, in the *intra-semen* method seeds need to be immersed in the inoculum during the exponential part of phase I, that is, during the maximum water intake of the imbibition period (Bennet and Wipps 2008). After that, seeds can be superficially blotted or dried up to their original humidity value, which depends on its immediate or late sowing, respectively.

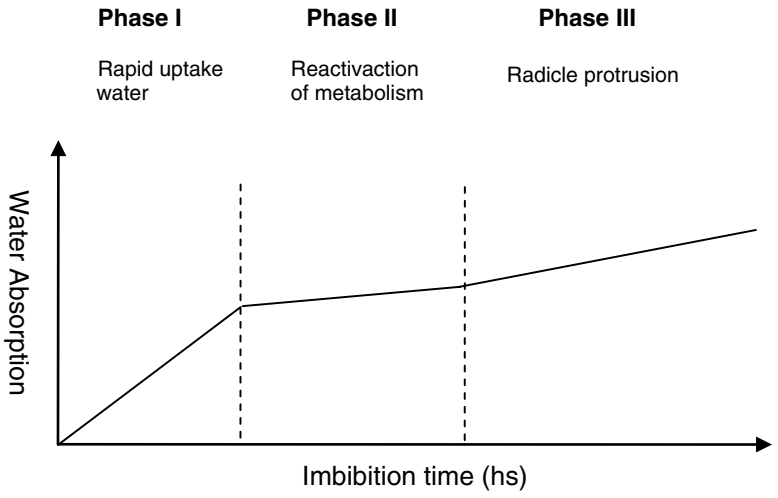

Fig. 25.1 Seed imbibition pattern curve

Equipment and Supplies
Seeds, beakers, analytical balance, kitchen paper rolls, sterile distilled water (SDW), growth chamber.

Procedures
Imbibition curve. Five samples of 50 seeds each are weighed and put in individual beakers, covered by SDW and transferred to a growth chamber, which has been previously set at 25 °C. Every 30 min during the first 5 h and every hour thereafter, water is discarded, seeds blotted between paper layers and weighed. This procedure is repeated until constant weight, which corresponds to the end of phase I.

To determine the imbibition curve, water content data is graphically represented against imbibition time. The time where seeds reach the exponential part of the curve will be the proper one to maintain seeds covered by resuspended *Azospirillum* cells.

Inoculation. No matter which plant species is, seeds need to be disinfected with 1 % NaOCl for 1 min, rinsed three times with SDW and superficially dried on sterile blotting paper. After that, seeds are embedded in the inoculum for the time previously determined for each plant species and according to its imbibition curve. However, some plant species display a lag period of no water absorption before phase I is initiated. During this situation, a presoaking period in SDW is recommended. For example, while *Azospirillum*'s suspensions can be incorporated inside lettuce seeds after 90 min contact with dried seeds, maize imbibition requires 4 h of previous soaking in SDW, followed by an extra 4-h period inoculation.

25.2.3 Determination of Optimum Inoculums Concentration Required to Promote Plant Growth

25.2.3.1 Cool Germination Test

The traditional Germination Test is widely used to determine seed viability (Hampton and TeKrony 1995; ISTA 2014). However, this test does no guarantee an efficient plant performance at the field, where several environmental factors can diminish plant emergence. This observation has conducted to the development of complementing germination tests that tend to evaluate the so-called seed vigor (Hampton and TeKrony 1995). The Cool Germination Test (CGT) is one of these, developed to simulate the adverse conditions related to suboptimal field temperatures operating during germination. This test is widely used in the USA and Europe to evaluate seed vigor in cotton, maize, soy, sorghum, and in several vegetables such as onion, carrot, and beans (Hampton 1992). The CGT has been proved useful in determining fungicide efficacy to select genetic traits related to seed germination on cool and humid soils, to select seed batches devoted to early sowing, to adjust sowing density, and also to evaluate the damaging effects of seed storage on plant physiology (AOSA 1983).

Taking the above into account and the proved *Azospirillum*'s ability to promote plant growth under stressful abiotic conditions (Kohler et al. 2009; Fasciglione et al. 2012; Romero et al. 2014), we have proposed a modified CGT as a complementary tool to determine the optimum bacterial inoculum concentration to be used in *Azospirillum*-vegetable association studies.

Equipment and Supplies
Seeds with a germinating power over 95 %, analytical balance, 20×25-cm filter paper rolls (for small seeds), 20×50-cm filter paper rolls (for large seeds), polyethylene bags, plastic containers to maintain rolls vertically into the germination chamber, germination chamber settled at 25±0.5 °C and a 12-h photoperiod, growth chamber settled at 10±0.5 °C, drying oven.

Procedures
In order to allow humidity and temperature equilibration in all the paper mass, the day before sowing paper towels must be soaked in SDW to saturation, drained of water excess, and stored in a refrigerating chamber at 10 °C.

According to Sect. 25.2.2, seeds are immersed in diluted *Azospirillum* cells suspensions to obtain inocula with different bacterial concentrations, e.g., 0, 10^5, 10^7, 10^9, and 10^{11} cells·seed^{-1}. After superficial drying, a minimum of five lots containing 50 inoculated seeds of each inoculum are sown on paper sheet. The seeds are placed on a doubled layer in two 25-seed rows, 5 and 10 cm from the upper edge of the top of paper and at a distance 2–3 times seeds size (Fig. 25.2a). A second sheet of wet paper is put over the seeds, softly compressed and carefully rolled. To allow a normal plant growth, a firm but not excessively compressed roll must be built, paying special attention to leave ample space at the central part (Fig. 25.2b). Rolls can be maintained as such by means of paper clips or rubber bands.

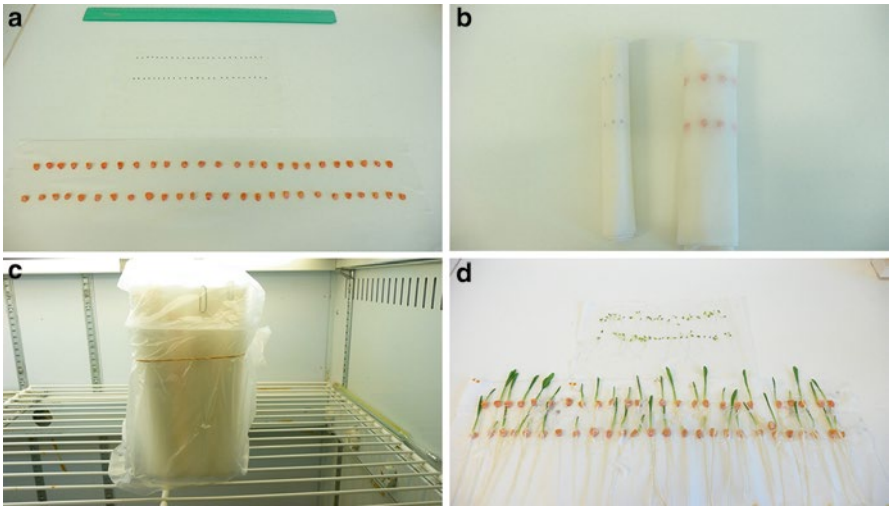

Fig. 25.2 Seed germination in paper rolls. (**a**) *Upper left corner*: 20×25 cm filter paper rectangles sown with 50 lettuce on top and 50 sweet corn on the bottom; (**b**) *upper right corner*: rolled paper after sowing; (**c**) *lower left corner*: sown rolls into polyethylene bags to avoid water evaporation; (**d**) *lower right corner*: seedlings from lettuce and sweet corn distributed as in (**a**)

Sown rolls are accommodated vertically into containers. To prevent water loss by evaporation, containers are introduced in polyethylene bags and properly sealed (Fig. 25.2c). After that, containers are stored during 7 days in the dark, at 10 ± 0.5 °C. This last step gives the name to the test. After the cold period, containers are transferred to a germination chamber settled at 25 ± 0.5 °C and a 12-h photoperiod, where they remain for 7 additional days. Since the maintenance of a high humidity level is required, rolls can be sprayed with SDW if needed. At the end of the 7-day period, rolls are opened and germinated seeds percentage as well as seedlings quality determined (Fig. 25.2d). The criteria used to evaluate seedlings are the same established for each plant species in the standard germination test (ISTA 2014). According to these norms, seedlings can be classified in five groups:

1. Strong, undamaged seedlings.
2. Strong seedlings with a slight development delay, minor damages as short primary and/or secondary roots, sheets with apical scratches, damaged coleoptile but undamaged primary sheet, lightly twisted mesocotile.
3. Subgroup A: small or slightly twisted seedlings, low number of lateral roots.
   Subgroup B: strong seedlings but disproportionately developed.
4. Abnormal seedlings according to ISTA rules and plant species.
5. Dead seeds.

Percentages of each group are calculated. Groups 1 and 2 represent vigorous seeds.

In addition to CGT, plant growth promotion effect of inoculation can be estimated through main root length and aerial height measurements. Complementary information is provided by root DW/aerial DW ratio determination.

25.2.3.2 Salt Germination Test

Both growing soil salinity and freshwater scarcity are environmental factors which could seriously endanger agriculture in a near future. It is of concern that only 2.5 % of the Earth's water is freshwater, and 98.8 % of that water is in ice and groundwater (Gleick 1993). What is more, it is expected that anthropogenic actions will increase both soil and freshwater salinity. As examples, salinity will increase by evaporation in agricultural practices based on flooding and extensive water irrigation; in dam reservoirs and open canal distribution systems; in underground aquifers located near seashore that will drive salt to these reservoirs; etc.

Both germination percentage and rate and also weight are reduced in plants exposed to salt growing conditions (Zhilong 2004; Barassi 2006; Kim 2008). The terminal impact on yield will depend on the plant species and on the degree of stress determined by salt. Traditionally, electrical conductivity of saturated soil paste extract (ECe) has been used to assess soil salinity (Oustan et al. 2007). Using this methodology, Shannon (1997) presented data relating yield decline across a range of salt concentrations. According to these studies, asparagus, red beet, and zucchini squash are salt-tolerant vegetables while lettuce, onion, and carrot are salt-sensitive vegetable species. Broccoli, cabbage, and tomato are moderately tolerant to salt. However, these salt-tolerance differences amongst vegetable species may vary depending on climate, soil conditions, and cultural practices (Shannon 1997).

As already mentioned in the Introduction, plant inoculation with *Azospirillum* can be used as a method to minimize drought and salt stresses (Bashan 2004). Indeed, both germinating power (GP) and aerial biomass increase in carrot and lettuce exposed to low and moderate salt stress has been reported (Barassi 2008). More recently, in order to determine the adequate number of bacteria improving germination under salting conditions, lettuce seeds were inoculated with different numbers of *Azospirillum* cells and exposed to four salt stress levels (Fasciglione et al. 2012). In this regard, plant establishment under salinity depends on successful seed germination (Zhilong 2004; Eraslan 2007). Thus, studies intended to evaluate the effects of PGPR inoculation on plant growth under salt stress conditions should be preceded by salt germination test (SGT) determinations.

Equipment and Supplies

Seeds with germinating power over 95 %, analytical balance, 20-cm diam. filter paper disks, 20-cm diam. petri dishes, polyethylene film, germination chamber settled at 23 ± 0.5 °C and 8-h photoperiod, growth chamber settled at 30 ± 2.0 °C, continuous orbital shaker, conductivity meter, 99 % pure NaCl, SDW.

Procedures

Previously autoclaved filter paper disks are imbibed in SDW or in sterile solutions of different NaCl concentrations. Disinfected seeds are inoculated with different *Azospirillum* cells diluted in 66 mM buffer phosphate, pH 7.0. The volume of buffer used in each case to dilute the cells should be sufficient to completely cover the seeds (Sect. 25.2.2).

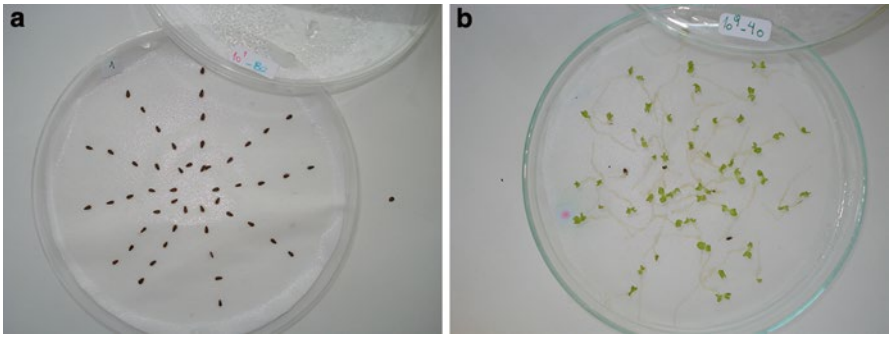

Fig. 25.3 (**a**) *Lactuca sativa* seeds sown on filter paper. (**b**) Seedlings after 10 days growth in a germinating chamber at 23 °C

These solutions are calculated to obtain inocula containing the entire cell per seed levels recommended in the literature, including those below and over these concentrations, e.g., 0, 10^6, 10^7, 10^8, 10^9, 10^{10}, and 10^{11} bacteria·seed^{-1}.

Lots of 50 seeds are immersed in each inoculum and incubated at 30 ± 2.0 °C with orbital shaking (100 rpm). Total incubation time is calculated from the imbibition curve obtained from the selected plant species (Sect. 25.2.2).

Each inoculated seed lot is sown on a petri dish containing two layers of filter paper previously soaked in 10 mL of SDW or in each NaCl solution (Fig. 25.3). To prevent moisture loss in the plate containing the seed, it is recommended to cover them with polyethylene film. Salt concentration to be used in the experiment depends on the plant species (Shannon 1997), being lettuce considered a relatively salt-sensitive vegetable (Fasciglione 2012).

A minimum of five lots containing 50 inoculated seeds of each inoculum is sown on individual petri dishes, wrapped in polyethylene film, and incubated in a germinating chamber at the temperature and photoperiod required by each plant species. Some plant species require keeping seeds refrigerated for about 12–24 h before sowing (ISTA 2014).

Germinated seed percentages are determined 4 and 7 days after sowing (DAS), these values representing germinative energy (GE) and germinating power (GP), respectively. In addition, at the end of a 7-day period seedlings are classified as normal or abnormal, according to the following criteria (ISTA 2014).

I. Normal: strong seedlings with a slight development delay, with minor damages as short primary and/or secondary roots, sheets with apical scratches, damaged coleoptile but undamaged primary sheet, lightly twisted mesocotile. In the case of dicots, it is accepted the presence of one intact cotyledon or three of them.

II. Abnormal: deformed, achromatic, fractured seedlings, twisted or thick roots. In dicots: primary roots emerged after cotyledons or white and yellow cotyledons, glued or necrotic or colorless cotyledons, a twisted or thick or absent hypocotile. Also included in the group: glued cortical seeds, strong but disproportionally developed seedlings, dead seeds.

As mentioned in Sect. 25.2.3.1, plant growth promotion effect of inoculation can be estimated through main root length and aerial height measurements. Complementary information is provided by root DW/aerial DW ratio determination.

Both CGT and SGT provide useful information on the optimal bacterial concentration plant needs to have growth promotion under stressful abiotic conditions. In addition, effective plant colonization regarding inoculum concentration data can be obtained by most probable bacterial number (MPN) or colony forming units (CFU) determination in root seedlings (Postgate 1969; Rodríguez Cáceres 1982).

25.3 Product Quality and Nutritional Value Parameters Determination

25.3.1 Antioxidant Activity

25.3.1.1 β-Carotene Bleaching Test

Beta carotene bleaching test is a spectrophotometric technique used to estimate antioxidant capacity in plants.

Test Principles
Linoleic acid peroxidation induced by heat discolors β-carotene in water. The presence of antioxidants in plant extracts proportionally reduces β-carotene bleaching, which can be quantified by measuring absorbance at 470 nm (A_{470}) (Marco 1968; Miller 1971; Velioglu et al. 1998).

Equipment and Supplies
Analytical balance, spectrophotometer, refrigerated centrifuge, magnetic stirrer, rotary evaporator, boiling flask, centrifuge tubes, test tubes, cuvettes.

Extracting solution: prepare 80 % methanol (v/v) in DW.

β-carotene: dissolve 20 mg β-carotene in 10 mL with chloroform. This stock solution can be preserved up 2 weeks at −20 °C. Working solution (0.2 mg·mL^{-1}) is prepared fresh by diluting 1 mL stock in 10 mL chlorophorm.

Butylated hydroxytoluene (*BHT*): dissolve 0.5 mg BHT in 10 mL with 80 % methanol. This solution (0.05 mg·mL^{-1}) can be preserved up to 2 weeks at −20 °C.

β-carotene-linoleic acid emulsion (*reactant mix*): Prepare fresh. Put 40 μL linoleic acid, 400 μL Tween 20 and 2 mL β-carotene working solution in a 250-mL boiling flask. Remove chlorophorm in rotary evaporator during 10 min at 35 °C. Immediately after, add 100 mL DW and mix vigorously to form an emulsion. Protect from light wrapping boiling flask in aluminum foil.

Procedures
Sample preparation: disrupt 1 g FW plant sample in mortar with pestle and liquid N to obtain a paste. Add 15 mL extracting solution, homogenize, and distribute in

centrifuge tubes. Incubate tubes for 2 h at 70 °C with orbital shaking. *Note*: *in case of processing antocianin-rich tissues*, *temperature must be set at 25* °C *to avoid decomposition of these pigments*. Centrifuge tubes at 13,000 × *g* during 10 min at 4 °C and transfer supernatants to previously conditioned test tubes. If not immediately used, extracted samples can be kept at −20 °C.

Test tube conditioning: put 5 mL β-carotene-linoleic acid emulsion in each test tube. Add 200 μL extract sample to each tube. *Note*: *sample dilution in 80 % methanol is empirically adjusted according to the antioxidant activity plant tissue displays in the assay*. Control is obtained replacing extract sample by 200 μL 80 % methanol. A tube lacking β-carotene emulsion is used as a blank.

A_{470} *determination*: determine A_{470} at *T*0 (initial point). Then, put test tubes in a water bath and incubate at 50 °C in the dark. Determine A_{470} after 30 and 60 min thereafter. Incubation period can be extended up to 120 min, but β-carotene discoloration after 60 min incubation is already evident. All samples are assayed in triplicate.

Antioxidant Capacity Calculation

According to Velioglu et al. (1998), antioxidant capacity can be expressed in four different ways:

1. Antioxidant value (AOX). Plot A_{470} vs. time. The absolute value of slope represents AOX and corresponds to bleaching rates of β-carotene. Determine $AOX_{control}$ and AOX_{sample}, which correspond to reactant mix without antioxidant and with plant extract, respectively. Slope obtained from A_{470} control vs. incubation time plot will be $AOX_{control}$.
2. Antioxidant activity (AA). Calculate percent inhibition relative to control using the equation

$$AA = \left[\left(AOX_{control} - AOX_{sample} \right) / AOX_{control} \right] \times 100$$

3. Oxidation rate ratio (ORR). Determine $AOX_{sample}/AOX_{control}$
4. Antioxidant activity coefficient (AAC). Calculate AAC using the equation

$$AAC = \left[\left(A_{sample} T60 - A_{control} T60 \right) / A_{sample} T0 - A_{control} T0 \right] \times 1{,}000,$$

where:

A *T*60 means A_{470} of the antioxidant mix at 60 min and A *T*0 means A_{470} of the antioxidant mix at 0 min, respectively. *Note*: *T*60 *is the final time of assay*, *which can vary according to the experiment*.

25.3.1.2 DPPH Radical Scavenging Assay

The use of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay provides an easy and rapid way to evaluate plant antioxidants by spectrophotometry.

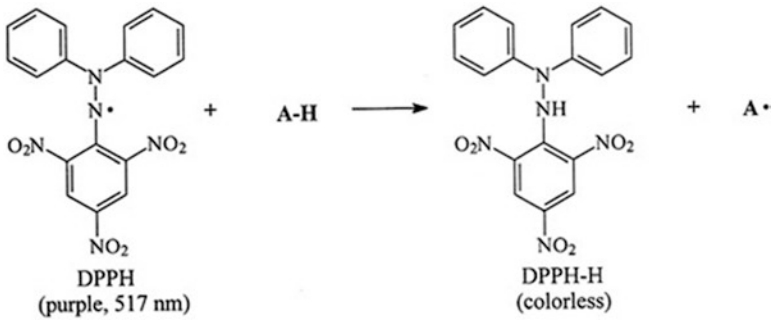

Fig. 25.4 DPPH reduction by an antioxidant compound. *AH* hydrogen atom donor

Test Principles

The deep violet color displayed in methanol by stable-free radical DPPH compound becomes discolored when transformed on its non-radical form by the action of antioxidant H atoms donors (Fig. 25.4). Radical discoloration degree due to the presence of antioxidants can be calculated from optical absorbance data at 517 nm (A_{517}) on control blank and extract samples.

According to Argoti et al. (2011), the radical scavenging activity is expressed in terms of the extract concentration ($\mu g \cdot mL^{-1}$) required to decrease the initial DPPH concentration by 50 % (efficient concentration, EC_{50}). A lower EC_{50} value will mean a higher plant extract capacity to sequester free radicals.

Equipment and Supplies

Analytical balance, spectrophotometer, refrigerated centrifuge, magnetic stirrer, orbital shaker, glass cuvettes.

Extracting solution: prepare 80 % methanol (v/v) in DW.

DPPH solution (*60 μM*; *MW 392.32*): 2.36 mg DPPH are dissolved in 100 mL methanol. To prevent stability decay, the reagent must be prepared the same day to be used and kept refrigerated in the dark.

Stocking ascorbic acid (*AscA*) *solution* (*60 μM*; *MW 176.12*): dissolve 1.06 mg AscA in DW and bring total volume to 100 mL. To prevent antioxidant loss, the reagent must be prepared the same day to be used and kept refrigerated in the dark.

Standard AscA curve: 40–160 μL of 60 μM AscA stocking solution aliquots are taken by duplicate, 1,000 μL 60 μM DPPH added, and total volume completed with DW to a total 3,500 μL on each point. The 40–60 μL rank corresponds to a rank of AscA concentration comprised between 0.12 and 0.49 $\mu g \cdot mL^{-1}$. Let the reaction proceed during 30 min at room temperature and determine A_{517}. Zero spectrophotometer setting is done with 80 % methanol in water.

Procedures

Plant tissue antioxidant extraction: disrupt plant sample in mortar with liquid N. Homogenize 1 g of powder in 10 mL of 80 % methanol and keep refrigerated

with orbital shaking at 100 rpm during 15 min. Centrifuge at 15,000 rpm during 15 min at 3 ± 2 °C. Discard pellet and keep supernatant at −80 °C until use.

Extract analysis: mix 1,000 μL DPPH solution with four different aliquots of extract to obtain samples containing different concentrations of radical scavengers (aliquots are usually between 20 and 100 μL range, which correspond to 2–10 mg FW samples, respectively). Use 80 % methanol to complete volume to a total 3,500 μL. Let the reaction proceed during 30 min at room temperature and determine A_{517}. The same procedure but replacing DPPH by 80 % methanol to complete 3,500 μL volume is used as control blank. Zero spectrophotometer setting is done with 80 % methanol in water. All samples are assayed in triplicate.

DPPH-Scavenging Activity Calculation

1. Calculate DPPH % reduction in each one of the four extract aliquots, according to the equation: DPPH % reduction = $[(A_{517}\ \text{blank} - A_{517}\ \text{sample})/A_{517}\ \text{blank}] \times 100$
2. Graph DPPH % reduction vs. sample concentration ($\mu g \cdot mL^{-1}$). The equation $y = mx + b$ will represent a straight line, where: y = DPPH % reduction; m = slope; x = sample weight; b = y-intercept
3. Replace y by 50 in the above equation and calculate x, that is, $x = (y - b)/m$, where x will represent EC_{50}. Basically, this parameter represents the amount of sample required to discolor 50 % of 60 μM DPPH solution.

As a point of reference to calculate EC_{50}, steps (1)–(3) must also be done following standard AA curve determination.

25.3.2 Determination of Ascorbic Acid Concentration

Ascorbic (AscA, reduced form) and dehydroascorbic (DAscA, oxidized form) acids are important components of plant and animal redox systems. The total sum of both water-soluble compounds is known as AscA in both kingdoms and as vitamin C in man and some animal species unable to synthesize any of them. Both AscA and DAscA are able to quench different ROS species (Allen 1995).

Ascorbic acid abounds in plant tissues, organelles, and extracellular components. It comprises about 90 % of total AscA found in sheets, where its concentration can vary between 20 and 300 mM in cytosol and chloroplast stroma, respectively (Mittler 2002). Ascorbic acid not only counteracts part of the negative oxidative effects generated by photosynthesis but also those produced by ROS under stressful conditions (Mittler 2002). Direct AscA action on ROS negative effects is not restricted to aqueous part of cells, since its role in recycling tocopherols indirectly protects membranes from oxidation (Bruno et al. 2006).

On the other hand, the old story of deadly scurvy disease and its cure exemplifies the important role vitamin C has on maintaining human health. Aside from its antioxidant properties, vitamin C has other important functions. Its role in the metabolism of several amino acids conducting to collagen synthesis in connective tissues is

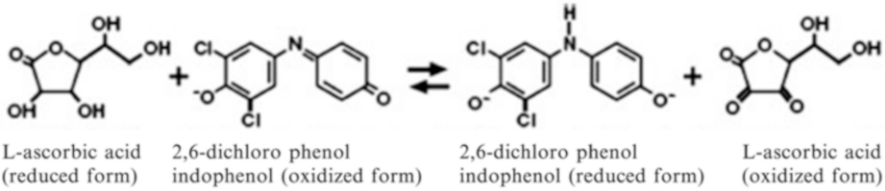

Fig. 25.5 Dichlorophenolindophenol oxidation by ascorbic acid

well known. In addition, vitamin C regulates and participates in enzymatic reactions and transport for neurotransmitters and in hormone biosynthesis. L-ascorbic acid reduces Fe^{3+} to Fe^{2+} from non-heme iron sources and thus enhances iron absorption. L-ascorbic acid can modulate cell growth and differentiation and increase the stability and maximal activity of the hormones oxytocin, vasopressin, cholecystokinin, and α-melanotropin. Epidemiological evidence has also associated vitamin C-containing fruit and vegetable consumption to lower risks of cardiovascular disease (Hacisevki 2009; Drewnowski 2010).

In short, considering its role in protecting plants from abiotic stresses and in preserving human health, AscA and DAscA determination in PGPR-plant association studies should not be excluded.

Test Principles

Dichlorophenolindophenol (DCPIP) reduction by AscA is spectroscopically determined by the method described by Leipner et al. (1997), modified as follows. Blue to pale rose DCPIP discoloration due to AscA activity can be measured at 524 nm (A_{524}) (Fig. 25.5).

Equipment and Supplies

Analytical balance, spectrophotometer, refrigerated centrifuge, centrifuge tubes, disposable plastic cuvettes.

Extracting solution (*2 % metaphosphoric acid*): dissolve 2 g HPO_3 in 100 mL DW.

K2PO4 solution: dissolve 45 g K_2PO_4 in 100 mL DW.

Homocystein solution: dissolve 1 g homocystein in 100 mL DW.

Citrate-phosphate buffer (*2 M*, *pH 2.3*): dissolve 37.6 g citric acid plus 6.16 g Na_2HPO_4 in 100 mL DW.

2.6-dichlorophenolindophenol (*DCPIP*) *solution* (*0.003 %*, *w/v*): immediately before use, dissolve 3 mg DCPIP in 100 mL DW. Keep solution at 4 °C in a dark flask or wrapped in aluminum foil.

Standard AscA 60 mM (*MW 176.12*): immediately before use, dissolve 10.56 mg AscA in 100 mL 2 % metaphosphoric acid. Keep solution at 4 °C in a dark flask or wrapped in aluminum foil.

Procedures

Sample preparation: disrupt 200 mg FW plant sample in mortar with pestle and liquid N to obtain a paste. Add 5 mL of ice-cold extracting solution in the presence of 1 g NaCl, homogenize, and distribute in centrifuge tubes. Centrifuge tubes at 13,000 × *g* during 15 min at 4 °C and transfer supernatants to previously conditioned test tubes. If not immediately used, extracted samples can be kept at −80 °C.

Calibrating curve: 100, 150, 200, and 300 mL aliquots from standard AscA solution were pipetted into plastic cuvettes, that is, covering a 4.2–12.3 $mg \cdot mL^{-1}$ range. Complete total volume to 300 mL with extracting solution.

Cuvette conditioning: Considering that Leipner et al. (1997) method is based on DCPIP reduction by AscA, total AscA (vitamin C) determination in samples will require a previous reduction of DAscA to AscA by homocystein. On the other hand, AscA is obtained from samples where homocystein has been replaced by DW. The following steps describe cuvette conditioning in both assays:

(a) Preparation of cuvettes concerning total AscA determination. Pipet 300 mL sample extract, 200 mL K_2PO_4 solution, and 100 mL homocystein solution into a disposable plastic cuvette.
(b) Preparation of cuvettes concerning AscA determination. Use procedure described in (a), except that homocystein solution is replaced by the same volume of DW.

A_{524} *determination*: Incubate cuvettes during 15 min at 25 °C. Add 1 mL citrate-phosphate buffer 2 M and 1 mL DCPIP solution to each one. Determine A_{524} immediately after. Total AscA content is calculated from the standard AscA curve and the obtained A_{524} sample data. DAscA concentration is calculated according to the equation DAscA = Total AscA (vitamin C) − AscA.

25.3.3 Determination of Total Phenolics Content (Folin–Ciocalteu Assay)

Phenolic compounds comprise a wide range of plant antioxidant substances possessing an aromatic ring with one or more hydroxyl substituents. The Folin–Ciocalteu assay estimates total phenolics content in plant tissues.

Technique Principles

The Folin–Ciocalteu assay relies on the transfer of electrons in alkaline medium from plant phenolic compounds to phosphomolybdic/phosphotungstic acid complexes. Reduced Folin–Ciocalteu reagent forms a blue complex that can be quantified spectroscopically at 724 nm (Singleton and Rossi 1965).

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is used as phenolics standard. Due to the presence of other than GA phenolic compounds in plant tissues, results are expressed in GA equivalents (GAE).

Equipment and Supplies

Analytical balance, spectrophotometer, refrigerated centrifuge, magnetic stirrer, Eppendorf centrifuge tubes, UV/Vis micro cuvettes.

Extracting solution (*ES*): add 0.1 mL of 12,4 M hydrochloric acid to 80 mL methanol and adjust volume to 100 mL with DW.

Gallic acid (*GA*) *solutions* (*2 mM*): dissolve 0.034 g GA (MW 170.12) in 100 mL 80 % methanol in DW to prepare a 2 mM stocking GA solution. Take 0.5, 2.5, 5.0, and 7.5 mL from stocking solution and dilute each one to 10 mL with 80 % methanol to obtain 0.1, 0.5, 1.0, and 1.5 mM GA reference solutions, respectively. Do not dilute stocking solution to obtain the last reference solution point (2 mM).

Folin–Ciocalteu (*FC*) *solution* (*0.25 N*): bring 12.5 mL commercial FC reagent (2 N) to 100 mL with DW. Cover flask with aluminum foil, stir, and let stabilize for 5 min at room temperature.

Sodium carbonate solution (*0.2 M*): weigh 0.572 g $Na_2CO_3 \cdot 10H_2O$ (MW 286.14 g) and dissolve in 10 mL DW.

Procedures

Sample preparation: disrupt 1 g FW plant sample in mortar with pestle and liquid N to obtain a paste. Add 5 mL extracting solution, homogenize, and distribute in centrifuge tubes. Centrifuge tubes at 13,000 × *g* during 10 min at 4 °C and transfer supernatants to test tubes. If not immediately used, extracted samples can be kept at −20 °C.

A_{724} *determination*: From each calibration solution, sample, or blank (ES solution), pipet 50 µL into separate micro cuvettes, and add to each one 475 µL 0.25 N FC. Incubate for 3 min at constant room temperature in the dark. Add 475 µL sodium carbonate solution and incubate 60 min at constant room temperature in the dark. All samples are assayed in triplicate. Determine A_{724} of each solution against the blank and plot absorbance vs. GA concentration. Figure 25.6 shows a typical A_{724} vs. GA concentration plot. Determine curve equation by linear regression. In the example,

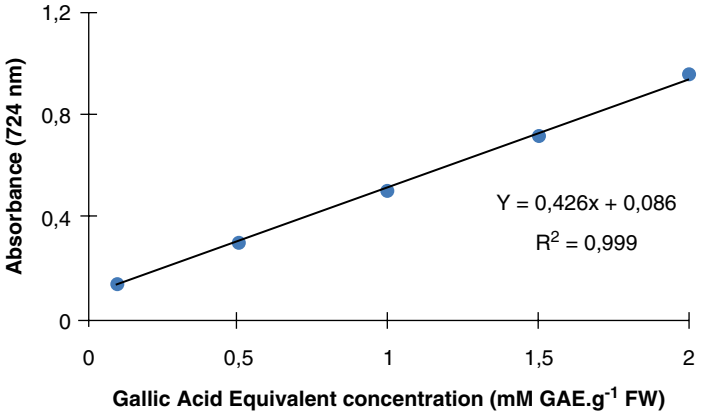

Fig. 25.6 Typical A_{724} vs. gallic acid equivalent concentration plot (Folin–Ciocalteu method)

the equation corresponding to Fig. 25.6 is $y=0.426\ x+0.086$, where $y=A_{724}$ and $x=$mM GA. According to this, sample GA concentration is calculated from $x=(y-0.086)/0.426$ and total phenolics content data expressed as GAE·g^{-1} FW.

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Chapter 26
Inoculant Preparation and Formulations for *Azospirillum* spp.

Yoav Bashan and Luz E. de-Bashan

Abstract In general, shortly after suspensions of *Azospirillum* spp. are inoculated into soil, seed surface, or root surfaces without a proper carrier, the bacteria population declines rapidly. This phenomenon, combined with poor production of bacterial biomass, makes difficult to sustain activity in the rhizosphere, and the physiological state of *Azospirillum* spp. at application time can prevent the buildup of a sufficiently large bacterial population in the rhizosphere. Consequently, a major role of formulation of inoculants is to provide a more suitable microenvironment, combined with physical protection for a prolonged period to prevent a rapid decline of introduced *Azospirillum* spp. Inoculants for field use have to be designed to provide a dependable source of bacteria that survives in the soil and become available to crops, when needed. This chapter provides technical details on production of several formulations proven useful for *Azospirillum* spp. from propagation of the bacterium in culture medium to final formulation for the field, and the industrial considerations involved in the entire process of inoculant production. These include: media for massive propagation, techniques for useful formulation, mode of application out of the laboratory setting, and industrial consideration regarding production of commercial inoculants.

Dedication: This review is dedicated to the memory of the Israeli soil microbiologist Prof. Yigal Henis (1926–2010) of the Faculty of Agriculture, The Hebrew University of Jerusalem in Rehovot, Israel, one of the pioneers of studies of inoculants in Israel.

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26.1 Introduction

The end goal and economic justification of any long-term investigation of the effect of the plant growth-promoting bacteria (PGPB) *Azospirillum* spp. on plant growth and productivity is practical application of inoculants by growers. To that end, inoculants are made of viable cultures with high numbers of *Azospirillum* spp. embedded, suspended, or mixed in a carrier of choice. The later can be either solid or liquid. A fundamental requirement of such technology, in an industrial setting, is the production of cultures of *Azospirillum* spp. of high cell number, usually in fermenters using cheap, yet efficient, culture medium for growth and simple procedures for growing conditions (Bashan 1998).

Azospirillum spp. has been commercially used on a relative large scale in Argentina, Mexico, Europe, South Africa, and India, mainly on cereals, but also on other crops (Díaz-Zorita and Fernández-Canigia 2009; Fuentes-Ramirez and Caballero-Mellado 2005; Hartmann and Bashan 2009). Manufacturers are mostly small- to medium-sized companies and government agencies that are involved in inoculant production (Fuentes-Ramirez and Caballero-Mellado 2005).

This chapter provides technical details on production of several formulations proven useful for *Azospirillum* spp. from propagation of the bacterium in culture medium to final formulation to be used in the field and industrial considerations in the entire process of inoculant production.

26.2 Media for Propagation of *Azospirillum* In Vitro

There are several proven media for successfully growing *Azospirillum* spp. in fermenters. Some are less useful for inoculant production, mainly because of low cell counts and costs.

26.2.1 OAB Medium (a.k.a. Modified NFb)

Comments: This is an improved derivative of the original semisolid, buffer-free, nitrogen-free medium (NFb) that was based on organic acids, mainly malate and succinate, the preferred carbon sources of this bacterium in situ (Döbereiner and Day 1976). The OAB medium is improved by increasing its buffering capacity over the original medium and adding microelements, a limited amount of NH_4Cl to initiate aerobic growth, and a small amount of yeast extract to shorten the lag phase and aid vigorous growth (Okon et al. 1977). While OAB medium is well suited and well recommended for laboratory studies, it is not suitable for mass cultivation in inoculant production at larger scales, larger than laboratory experiments.

OAB medium (with latest minor modifications) is composed of: Solution A [(g/L): DL-malic acid, 5; NaOH, 3; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; NaCl, 0.1; NH_4Cl, 1; yeast extract, 0.1; $FeCl_3$, 0.01; (mg/L): $NaMoO_4 \cdot 2H_2O$, 2; $MnSO_4$, 2.1; H_3BO_3, 2.8; $Cu(NO_3)2 \cdot 3H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$, 0.24; 900 mL distilled water] and Solution B [(g/L): K_2HPO_4, 6; KH_2PO_4, 4; 100 mL distilled water]. After autoclaving and cooling, the two solutions are mixed. The pH of the medium pH is 6.8 (Bashan et al. 1993).

26.2.2 Modified TYG

Comments: TYG medium, based on tryptone, yeast extract and glucose, allows massive cultivation of many PGPB/PGPR. This medium was further developed to enhance the growth of *Azospirillum* by supplementing it with the buffer capacity and micronutrients of the OAB medium that were specially designed for this species (Bashan et al. 2002). Yet, it has a major deficiency. Glucose is not used by some species of *Azospirillum*, such as the most common species used as an inoculant, *A. brasilense*, and glucose is not a preferred carbon source for this genus.

TYG medium contains (g/L): tryptone, 5 (Difco); yeast extract, 5, D-glucose, 5; NaCl, 1.2; $MgSO_4 \cdot 7H_2O$, 0.25; K_2HPO_4 0.13; $CaCl_2$, 0.22; K_2SO_4, 0.17; Na_2SO_4, 2.4; $NaHCO_3$, 0.5; Na_2CO_3, 0.09; Fe(III) EDTA, 0.07; the pH was adjusted to 7.0 after sterilization (Bashan et al. 2002).

26.2.3 BTB-1 and BTB-2

Comments: These media increase mass production of cells more than what is obtained with modified TYG medium and in the shortest period of time (18 h) to a level of ~10^{11} cells/mL. The variant medium using glycerol is much cheaper because industrial grade glycerol from biodiesel production is readily available. Currently, these are the most suitable media for mass production of *Azospirillum* spp.

These media contained the modified TYG medium, where the glucose is replaced by 5 g/L Na-gluconate or 8 mL/L glycerol (Bashan et al. 2011).

26.2.4 General Commercial Microbiological Media (Nutrient Broth Medium, Luria-Bertani Broth, Tryptone Soy Broth, and a Few More of that Type)

Comments: These media produce less than optimal yields of cells for propagation of the bacteria for inoculants compared to the two BTB media. They are still useful for experimental laboratory propagation of *Azospirillum* spp. All these commercial media are prohibitively expensive for industrial-scale inoculant production.

26.3 Justification for Formulation of Inoculant

In general, shortly after suspensions of *Azospirillum* spp. are inoculated into the soil, seed surface, or root surfaces without a proper carrier, the bacteria population declines rapidly. In plantless soil, they disappeared within 15 days (Bashan 1999). This phenomenon, combined with poor production of bacterial biomass, difficulty sustaining activity in the rhizosphere, and the physiological state of *Azospirillum* spp. at application time, prevents the buildup of a sufficiently large *Azospirillum* population in the rhizosphere. A threshold number of cells, ~10^6–10^7 cells·plant^{-1} for *Azospirillum brasilense* is required (Bashan 1986b). The inherent heterogeneity of the soil is the key obstacle, where introduced bacteria sometimes cannot find an empty niche in the soil. These unprotected, inoculated bacteria must compete with the often better-adapted native microflora and withstand predation by soil microfauna. Consequently, a major role of formulation of inoculants is to provide a more suitable microenvironment, combined with physical protection for a prolonged period, to prevent a rapid decline of introduced *Azospirillum* spp. Inoculants for field-scale use have to be designed to provide a dependable source of bacteria that survives in the soil and become available to crops, when needed (Bashan 1998). Many inoculants do not do this; yet, this is the main purpose of inoculant formulation. The three fundamental and essential characteristics for all inoculants are to: (1) support the growth of the intended microorganisms, (2) support the necessary number of *viable* microbial cells in good physiological condition for an acceptable period of time, and (3) deliver enough microorganisms at the time of inoculation to reach a threshold number of bacteria that is usually required to obtain a plant response, i.e., the inoculant must contain enough *viable* bacteria after the formulation process. Formulation is the crucial issue for commercial inoculants. In practice, the formulated inoculant is the sole delivery vehicle of live microorganisms from the factory to the field.

26.3.1 "Primitive" Inoculants: Culture Media with No Additional Formulation

The oldest method of inoculation of seeds and plants with bacterial culture suspension, as has been practice since the pioneering times of plant inoculation decades ago, still prevails today. It is practiced mostly at research facilities. It is a very common practice, especially among highly trained researchers because it is the least laborious.

Obviously, despite the large number of publications demonstrating repeated success of researchers, "no formulation" inoculants are definitely not a practical inoculation technology. This is mainly because the level of expertise needed for proper delivery of the bacteria, under optimal conditions, is many times beyond the skills and the available time of growers. It is also impractical for large-scale application; the large volume of liquids involved, incubation, and refrigeration facilities

needed to maintain culture medium that have no formulation make it unlikely to meet economic and commercial needs. It will serve mainly in the domain of laboratory and greenhouse initial experimentation in research facilities.

26.3.2 Liquid Inoculants

Liquid inoculants are an upgraded derivative of "no-formulation" inoculants to address some of the limitations listed above. Essentially, they are microbial cultures or suspensions amended with substances that may improve stickiness, stabilization, and surfactant and dispersal abilities (for techniques used originally for rhizobia but valid for *Azospirillum* spp., see: Singleton et al. 2002). The main advantage of these inoculants over solid inoculants is that they are easy to handle. Unlike solid carrier-based inoculants, liquid formulations allow the manufacturer to include sufficient amounts of nutrients, cell protectants, and inducers responsible for cell/spore/cyst formation to improve performance. Liquid inoculants containing concentrations of 2×10^9 cells per mL are now common, allowing for lower application rates and increased efficiency in using inoculants (Schulz and Thelen 2008). Further, it is claimed that these inoculants have no contamination and have longer shelf life for some formulations, greater protection against environmental stresses, and increased field efficacy, compared to peat-based inoculants (Singleton et al. 2002). They are compatible with machinery on large farms, such as air seeders and seed augers.

There are no scientific reports concerning the use of liquid formulation for *Azospirillum* spp. Yet, these formulations are sold commercially without specifying their ingredients. The following compounds, used for other PGPB, can be potentially used as additives to create liquid inoculants of *Azospirillum* spp.: sucrose (Cong et al. 2009; Taurian et al. 2010); carboxymethyl cellulose (Jha and Saraf 2012), glycerol, polyvinylpyrrolidone, trehalose, FeEDTA (Albareda et al. 2008; Manikandan et al. 2010; Singleton et al. 2002), gum arabic (acacia gum; Diouf et al. 2003; Gamal-Eldin and Elbanna 2011; Wani et al. 2007).

26.3.3 Peat Inoculants

Peat is the carrier of choice for rhizobia and many PGPB, including *Azospirillum* spp. in North and South America, Europe, and Australia, and the main ingredient of inoculants that are sold in large volumes. Currently, technical details of production of the basic peat-based inoculant, such as grain size, pH, optimal moisture, several amendments, quality of inoculants, quality control standards, and occupational health and safety are common knowledge (Deaker et al. 2011) The physical state of the formulation of peat (solid [powder], pellet, or liquid [slurry]) can make a difference in the success of inoculation in rhizobia, but such studies are not available for *Azospirillum* spp.

There are many small variations of preparing peat inoculant (depending on the desired formulation required) and most are small modification that are based on the following procedure: for preparation of 100 g of inoculant for *Azospirillum* spp., 45 g of ground peat (40 mesh) is thoroughly mixed with 5 g of $CaCO_3$ and 20 mL of tap water (final pH 6.8) stored in polyethylene bags sealed with a plug. The bags are sterilized (gamma-irradiated or tyndelization in an autoclave), and 30 mL of a 24-h-old bacterial culture (approximately 5×10^9 CFU/mL) are aseptically added to each bag, mixed, and incubated for an additional 7 days at 33 ± 2 °C. Every 2 days, the peat is mixed by shaking the bags. The final number of bacteria in the inoculant range from 5×10^7 to 5×10^8 CFU/g of inoculant. The bags are stored for up to several months at 4 ± °C. One day before plant inoculation, the bags are transferred to 30 ± 2 °C for acclimation before inoculation.

Peat is user-friendly to several amendments used for other PGPB but not tested so far for *Azospirillum* spp. Such amendments include: vermiculite (Kokalis-Burelle et al. 2003), chitin, heat-killed *Aspergillus niger* mycelium or spent compost from *Agaricus bisporus* (Manjula and Podile 2001), pyrophyllite (hydrous aluminum silicate) (Meyer et al. 2000, 2001), and charcoal plus an adhesive (Riggs et al. 2001).

Another option to consider for small-scale inoculant production is that some organic wastes and composts can perform equally well or better than peat as a carrier. The main limitation is availability of the raw material for industry. Compost made of cork, bagasse, sawdust, brewery waste, or banana leaves (tested for other PGPB; Bashan et al. 2014) can sustain a small, local inoculant industry where the materials are available. They cannot form a base for a large nationwide industry, especially when the batch raw material is variable.

26.3.4 Synthetic Inoculants

Synthetic formulations based on an assortment of polymers have been continuously evaluated for decades for PGPB because they offer substantial advantages over peat, such as longer shelf life, appropriate survival at the destination field, sufficient cell density, ease of manufacturing, and improved performance in general (Bashan 1998; Bashan et al. 2014; John et al. 2011; Schoebitz et al. 2013). However, the major drawback of polymeric inoculants is that the raw materials for all polymers are relatively expensive compared to peat, soil, and organic waste inoculants and require additional expensive handling by the industry at costs similar to those in the fermentation industry. For agricultural and environmental uses, these polymers include, so far, alginate, agar, λ- and κ carrageenan, pectin, chitosan, bean gum, and proprietary polymers. Several basic requirements for these polymers, which are components of polymeric inoculants, are: (1) nontoxic and free of harmful preservatives that affect bacteria within the inoculant and inoculated plants, (2) slowly degradable in the soil by soil microorganisms, thereby gradually releasing the

bacteria in the needed quantities, usually at the time of seed germination and emergence of seedlings, (3) physical protection for the inoculated bacteria from soil competitors and many environmental stresses (Covarrubias et al. 2012, Schoebitz et al. 2012; Zohar-Perez et al. 2003), (4) sufficient water for survival of the bacteria, and (5) dispersive in water to allow movement of the bacteria from the polymer to the plants. For *Azospirillum* spp. the polymer of choice is alginate (Bashan 1986a; Bashan and Gonzalez 1999; Bashan et al. 2002).

26.3.5 Macro- and Micro-formulations of Alginate

The advantages of alginate formulations are their nontoxic nature, biodegradability, availability at reasonable costs, and slow release into the soil of the entrapped microorganisms, which is controlled by the polymeric structure (Bashan et al. 2002, 2014). Two basic formulations for *Azospirillum* spp. exist: macrobeads (1–4 mm dia.) and microbeads (50–200 μm in dia.) used for several PGPB.

Alginate macrobeads may have solved many of the problems associated with common peat inoculants; yet, their application as agricultural and environmental inoculants have two major disadvantages. First, an additional treatment during sowing is needed even if the inoculant is planted by the seeding machine. In developed countries, the grower who is busy during sowing might be pressed for time and reluctant to incur additional expense and time. In developing countries, the farmer might not inoculate the seeds at all. This happens because of insufficient agricultural education and conservative cultural traditions that make some small-scale farmers suspicious of new technologies, especially those involving live bacteria. Second, the bacteria released from macrobeads need to migrate through the soil toward the plants. Under agricultural practices, when beads are loosely mixed with seeds and sown together by planters, the inoculant beads might fall far from the seeds (up to a few centimeters). Thereafter, the bacteria released from the beads must migrate through the soil, facing competition from the native microflora, sometimes more aggressive and more adapted to the soil than the inoculated strain. Sometimes, the absence of a continuous film of water needed for their movement is an additional limiting factor. These distances, large on a microbial scale, might prove prohibitive for many PGPB, even those with a proven motility in soil, such as *Azospirillum* (Bashan and Levanony 1987).

The microbead concept was conceived to overcome these two fundamental difficulties (Bashan et al. 2002). The underlying hypothesis is that, if the beads are small enough, yet still capable of encapsulating a sufficient number of bacteria, it would be possible to produce a "powder-like" formulation similar to powdered peat inoculants. The seeds are coated with this "bead powder" at the seed-handling facility and sold to the farmer as "improved seeds." Alternatively, the farmer can coat the seeds, as done with peat inoculants.

26.3.5.1 Production of Macrobeads

Basic preparation: A bacterial culture (logarithmic or stationary phase) is aseptically mixed with 2 % (w/v) sodium alginate powder and stirred gently for 1/2–2 h to ensure complete dissolution of all ingredients. The mixture is added dropwise with the aid of a 10-mL sterile syringe into gently stirred, sterilized 0.1 M $CaCl_2$ at room temperature. Beads are immediately formed. The resulting alginate beads (mean diameter, 2 mm) entrap the bacterial cells. The beads are cured in the solution at room temperature for additional 1–3 h. The $CaCl_2$ solution is pumped out, and the beads are washed twice with sterilized tap water. After washings, the beads are incubated in fresh BTB medium (described above) for an additional 24 h for *A. brasilense* in a rotary shaker at 30 ± 2 °C to allow bacteria to multiply further inside the beads (secondary multiplication). Then, the beads are washed twice and collected. The wet beads are kept at 4 ± 1 °C in hermetically sealed flasks under moist conditions for several days. Dry preparation can be achieved by lyophilization or drying at 40 °C in thin layers in trays (Bashan 1986a). After complete dryness, the macrobeads can be stored in hermetically sealed flasks containing silica gel for years (Bashan and Gonzalez 1999).

Based on this principle, equipment were developed to produce macrobeaded inoculants (de-Bashan and Bashan 2010, http://www.bashanfoundation.org/device.html, http://www.bashanfoundation.org/beads/macrobead.html).

26.3.5.2 Production of Microbeads

Production of alginate microbeads is simple and involves low pressure spraying through a small nozzle, resulting in small-diameter droplets of an alginate solution mixed with liquid bacterial culture suspended in a very rich medium (a unit for this purpose is described in Bashan et al. 2002). These droplets, sprayed into a slowly stirred solution of $CaCl_2$, immediately solidify into microbeads at diameters ranging between 100 and 200 μm, which entraps a large number of bacteria (~10^9–10^{11} CFU g^{-1}) (Bashan et al. 2002). Like macrobeads, a description of automated production of microbeads is available (Bashan et al. 2002; de-Bashan and Bashan 2010 http://www.bashanfoundation.org/bead.html). Microbead inoculant of *A. brasilense* Cd is presented in Fig. 26.1.

26.4 Quality Control and Detection of the Strain in the Inoculant

Quality control of inoculants ensures that the declared content of *Azospirillum* spp. is present and that it is alive. Several countries now have guidelines that state how many cells should be in the inoculant. In other countries, this is left to the

Fig. 26.1 (**a**) Dry microbead inoculant of *Azospirillum brasilense* Cd. (**b**) Magnification of single microbeads. *Arrow* indicates a single microbead

manufacturer's discretion. The method for detection of the genus/species/strain varies and depends on the microbiological/molecular analytical capacity of the manufacturer. Generally, traditional, immunological, and molecular methods are employed for *Azospirillum* spp.

26.4.1 Traditional Microbiological Techniques

26.4.1.1 Cultivation of Samples of the Inoculant on Semi-selective Media

The limit of detection depends on the level of contamination by other microorganisms because these methods are based on serial dilutions. Lower limits of detection by these methods are ~10^3–10^5 cfu/g inoculant. Because regulations require that the inoculant will contain at least 10^8 cfu/g, these cultivation methods, despite being almost obsolete in other diagnostic fields, are still useful. These techniques require minimal microbiological infrastructure.

26.4.1.1.1 Semi-selective Media for *Azospirillum*

26.4.1.1.1.1 Congo Red-NFb

This medium is basically the original NFb medium supplemented with 15 mL/L medium of 1:400 aqueous solution of Congo red, autoclaved separately, and added just before using (Rodriguez-Caceres 1982).

Note: This medium permits the recognition of *Azospirillum* colonies on plates and facilitates the isolation of pure cultures since the colonies appear dark red or scarlet with typical colony characteristics, whereas many soil bacteria do not absorb Congo red.

26.4.1.1.1.2 BL and BLCR Media

These two semi-selective media are based on the OAB medium. The BL medium is the OAB medium supplemented with (mg/L) streptomycin sulfate, 200; cycloheximide, 250; sodium deoxycholate, 200; and 2,3,5-triphenyltetrazolium chloride, 15. The BLCR is the BL medium supplemented with an aqueous solution of Congo red (approximately 1 mL of a 1 mg/mL solution per liter) (Bashan and Levanony 1985).

Note: These media are very suitable for the isolation of *Azospirillum* from the rhizosphere since the colonies are easily recognizable, especially on BLCR medium. However, some strains of *A. brasilense* failed to grow on this medium, and the growth of *Azospirillum* on BLCR medium is significantly slower compared to the original OAB medium (about 10 days incubation time). Lower limits of detection are ~10^2–10^3 cfu/g inoculant when the inoculant has a low contamination load. Contaminants are easily recognizable.

26.4.2 Immunological Methods

For immunological detection of *A. brasilense,* only the enzyme-linked immunosorbent assay (ELISA) was developed (Levanony et al. 1987). Although highly specific for the species, lower limit of detection is 10^4 cfu/g inoculant, still useful for inoculant evaluation for the reason explained above for bacteria load in inoculants. When a production of inoculant fails, and the number of *Azospirillum* spp. is below the detection level, but still the manufacturer needs to know the number of *Azospirillum* spp. in the inoculant, this method can be combined with limited enrichment (Bashan et al. 1991). Minor limitations of the immunological method are an access for antibody facility (many commercial facilities are available) and access to plate count reader equipment that reads the ELISA plates.

26.4.3 Molecular Methods

Many molecular methods were developed and are capable of detecting specific *Azospirillum* spp. Yet, only one technique, fluorescent in situ hybridization (FISH) has been shown, so far, to detect this species in inoculants (Bashan et al. 2011) and

later on the roots of inoculated plants (Trejo et al. 2012). Because many FISH procedures are available, the technique described by Stoffels et al. (2001), but with numerous small modifications was used for detection of *A. brasilense* and *A. lipoferum*: Hybridization was performed at 35 % formamide stringency at 46 °C for 2 h. The final concentration of the probe was 3 ng μL^{-1}. Samples were then washed at 48 °C for 5 min with 50 mL of pre-warmed washing buffer. The slides were rinsed for a few seconds with ice cold, deionized water and then air dried. Slides were stored at −20 °C in the dark until observation. Three types of probes were used: an equimolar mixture of probes EUB-338 I (Amann et al. 1990), and II and III (Daims et al. 1999) that cover the domain of bacteria. An *Azospirillum brasilense-specific* probe (Abras 1420, Stoffels et al. 2001) was used for the two *A. brasilense* strains and Alila 1113 (Stoffels et al. 2001) for *A. lipoferum*. The probes Abras 1420 and Alila 1113 were labeled with the fluorochrome FITC (green) and the mix of EUB I, II, and III was labeled with the fluorochrome Cy3 (red). All fluorescent-labeled probes were purchased from Integrated DNA Technologies (Coralville, IA). Before visualization, the slides were mounted in AF1 anti-fading reagent (Citifluor, London, UK). Visualization was done with an epifluorescent microscope (Olympus) with two filters, the Cy3 filter (maximum excitation at 552 nm with maximum emission of light at 565 nm, red fluorescence (Olympus America, Center Valley, PA)) and the FITC filter (maximum excitation at 490 nm with maximum emission of light at 520 nm, green fluorescence (Olympus America)). Positive fluorescent signals that identify the bacteria are a combination of red and green that yields a green-yellow-orange tone, depending on the intensities of the individual color channels. Evaluation of *Azospirillum* spp. in the inoculant can be done either by fluorescent microscopy where images in red and green are superimposed to create a green-yellow-orange tone that provide absolute detection of the species (Fig. 26.2; Bashan et al. 2011) or directly by confocal laser microscopy and quantification by an image analyzer (Trejo et al. 2012).

The main advantage is specific identification of the species. The main limitation of this technique is the skill of the technician and the availability of equipment, which is expensive. Technologies for identifying specific strains of *Azospirillum* spp. in inoculants have not been developed.

26.5 Inoculation Techniques

Azospirillum spp. can be inoculated directly on the seed surface or in the soil. Seed applications greatly outnumber soil applications. This happens because it is easy to use and requires a relatively small amount of inoculant and because *Azospirillum* do not survive well in soils.

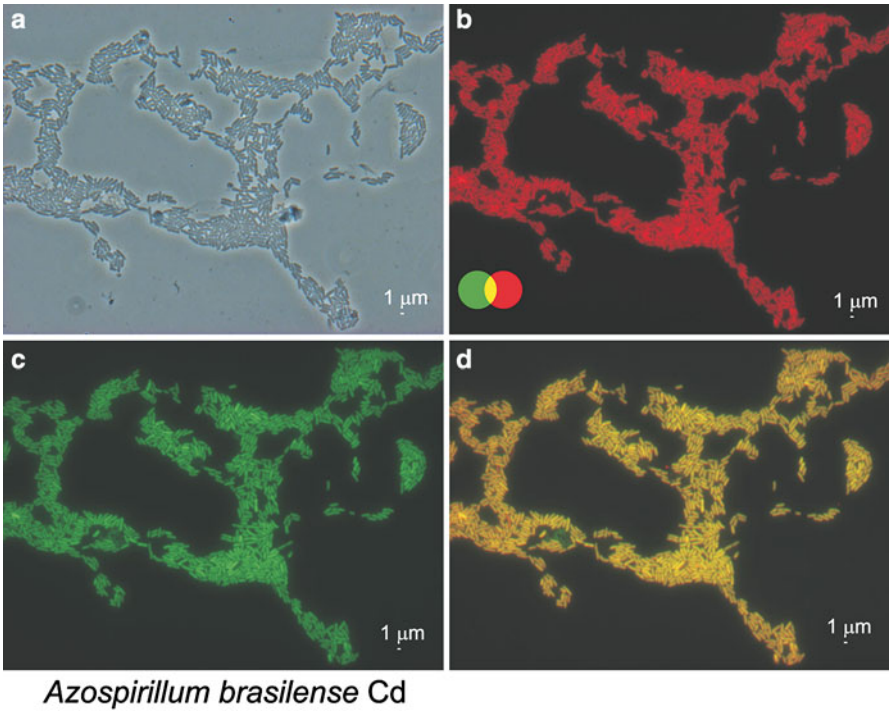

Fig. 26.2 Determination of the quality of the inoculant. Fluorescence in situ hybridization (FISH) of *Azospirillum brasilense* Cd. (**a**–**d**) after formulation and release from microbead alginate inoculant. Bars represent 1 μm. Original figure published in Bashan et al. Biology and Fertility of Soils 47: 963–969 (2011)

26.5.1 Seed Inoculation

There are many small variations of the basic technique. Using a variety of machinery, the basic technology of even seed-coating has not changed for decades. Briefly, prior to sowing, seeds are dusted with peat inoculant, with or without water or adhesive. For small seeds, this is followed by superfine, ground limestone, with or without adhesive, and allowed to dry. Drying can be done in situ or when the coating is applied prior to sowing. The seeds, held in shallow trays, are air-dried or dried by forced air. Coating and drying is also possible by using fluidized beds, where the seeds are floating on a cushion of pressurized air and then sprayed with inoculant. The inoculant is mixed with seeds either by hand, rotating drums that are cheap to operate, large dough or cement mixers, or mechanical tumbling machines. Alternatively, large farm operators use automated seeders fitted with an inoculant

tank, pump, and a mixing chamber commonly used for applying chemical coatings. The latter are not specialized equipment for microbial inoculation, so inoculant may be dislodged from the seeds.

Because every seed needs to be coated with a threshold number of *Azospirillum* spp., adhesives (the same level that were mentioned before for liquid inoculants) are used. A second role of an adhesive is to prevent the inoculant (either dry inoculant as powder or wet inoculant once the moisture evaporates) from dislodging during sowing with the seeding equipment, especially the powdered type, when applied with air seeders. The seeds are then sown with common seeding equipment. While it is commonly agreed that one essential condition to seed coating is adding adhesive materials, there is no agreement on what are the best adhesives. Each manufacturer or experimentalist empirically evaluates which adhesive best fits seeds and inoculants. When seeds are inoculated with liquid inoculant, with or without dissolved adhesive, the inoculant is sprayed directly onto the seeds. After drying, the seeds are sown.

Seed inoculation, despite their popularity in the marketplace, has several significant disadvantages. (1) Each seed, especially small ones, can be coated only with a limited amount of inoculant, which may be a limiting factor because a threshold of *Azospirillum* spp. (10^6–10^7 cfu/g, Bashan 1986b) may be needed for successful inoculation. (2) If an inoculant is not attached well with an adhesive or by pelleting, it may be dislodged by the sowing machinery. (3) In some seedlings, the seed coat is lifted out of the soil during germination, causing desiccation and death of the inoculant bacteria if the inoculant had been applied directly to the seeds. (4) Some species release anti-bacterial compounds from their seeds, which can inhibit the inoculant. (5) Some fungicides and insecticides commonly applied to the seeds may be detrimental to the inoculant (Bashan et al. 2004; Bashan and de-Bashan 2010).

26.5.2 Soil Inoculation

This technique is popular with rhizobia but is far less useful for *Azospirillum* spp. Only a few publications, mostly old, support this application. Therefore, at the state-of-the-art of our knowledge, this technique is not recommended for inoculation of *Azospirillum*.

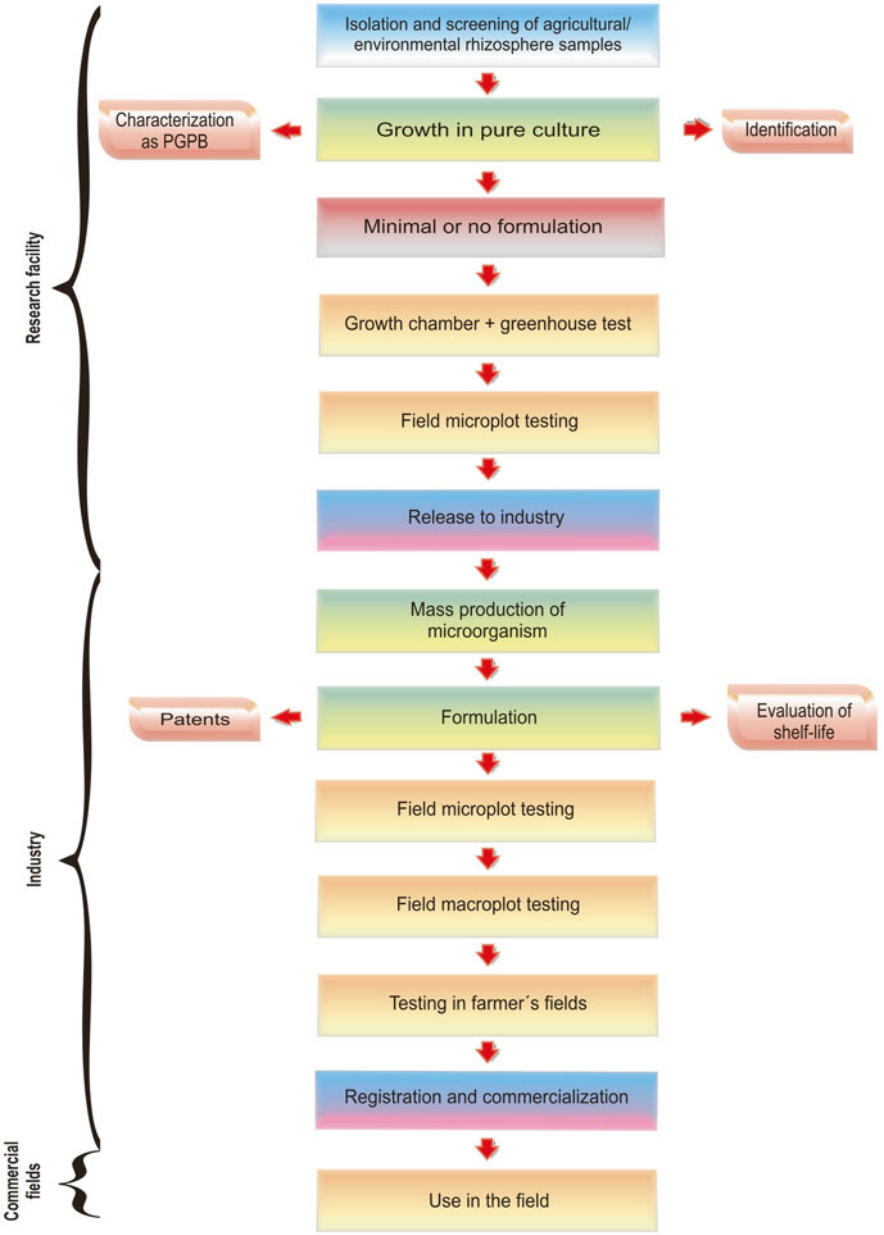

Fig. 26.3 Flow diagram of procedures for developing bacterial inoculants (Original figure published in Bashan et al. Plant and Soil 378: 1–33 (2014))

26.5.3 Industrial Point of View and Industrial Considerations on Inoculants

The industrial point of view of producing *Azospirillum* inoculants is no different from production of any other PGPB inoculant. A flow diagram, showing the procedures for developing microbial inoculants by the industry, is presented in Fig. 26.3. In practical industrial terms, the chosen formulation and method of application determine the potential success of the inoculant more than the specific strain used.

26.6 Conclusions

Inoculation of culture medium as inoculants is a recipe for failure under field condition. Proper formulation of *Azospirillum* is essential and irreplaceable for any successful inoculation beyond the level of test tubes and in vitro studies.

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Chapter 27
Protocol for the Quality Control of *Azospirillum* spp. Inoculants

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Abstract *Azospirillum* has been one of the most studied genera of plant growth promoting rhizobacteria (PGPR) worldwide over the past 50 years. The use of these microorganisms in agriculture practices has been adopted due to their ability to associate in rhizospheric, epiphytic, or endophytic ways with roots and promote whole plant growth or crop productivity. The biological treatment of seeds (inoculation) in more

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than a hundred species of economic or ecological interest has become a common practice in many countries. In Argentina, the Az39 strain of *Azospirillum brasilense*, belonging to the Culture Collection of the Instituto de Microbiología y Zoología Agrícola (IMYZA) of INTA Castelar, was selected in the 1980s after an intensive program to isolate and identify microorganisms for agriculture, according to their agronomic behavior. Since then, its ability to cover the premise for which it was selected has determined that Az39 is largely adopted by inoculant companies in Argentina with the aim of producing biological products for the treatment of several crops. In this chapter, those methods developed and standardized by the network Red de Control de Calidad de Inoculantes (REDCAI) of the Asociación Argentina de Microbiología (AAM) have been adapted as a guide for the quantification of *Azospirillum* spp. and the detection of contaminating microorganisms in biological products, as two of the most basic and important quality control parameters of inoculants.

27.1 Theoretical Framework

In the past 25 years, plant growth promoting diazotrophic rhizobacteria (PGPR) have been widely used in studies of agricultural microbiology worldwide, due to their ability to improve the yield of crops, preserve agro-ecosystems, and reduce the

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environmental impact of the use of mineral fertilizers. Numerous publications have reported the identification and possible manipulation of PGPR, as well as the association of PGPR with higher plants. The most successful associations include those established between nonlegumes or grasses and rhizobacteria of the genera *Pseudomonas*, *Bacillus*, *Azotobacter*, and *Azospirillum*. Among these, *Azospirillum* is one of the most studied due to its ability to significantly improve the growth, development, and yield of numerous species of agricultural interest worldwide (Bashan et al. 2004). The organisms belonging to this genus colonize mainly the elongation zone and root hairs. Some strains of *Azospirillum* sp. can be found inside plants and are thus called facultative endophytes (Figueiredo et al. 2008). Despite the different forms of interaction, when these diazotrophs are associated with grasses, they lead to yield increases of between 5 and 30 % (Baldani et al. 1983; Okon and Labandera 1994). The ability of these organisms to promote plant growth was initially attributed to the process of biological nitrogen fixation, in both rhizospheric and endophytic conditions, but this model had lower agricultural significance than initially expected. Today, one of the main mechanisms proposed to explain the ability of these organisms to promote plant growth is related to their ability to produce or metabolize plant hormone-like compounds, such as indole acetic acid, cytokinins (Tien et al. 1979), gibberellins (Bottini et al. 1989), and ethylene (Strzelczyk et al. 1994), as well as other molecules that regulate plant growth. Many studies have detailed the beneficial effects of the inoculation with PGPR and the important morphophysiological changes that occur in inoculated plants. However, in many cases, the mechanisms or compounds responsible for generating this response have not been identified.

27.1.1 Inoculants of Azospirillum *spp. in Argentina*

When studies of the genus *Azospirillum* began in Argentina with the aim to introduce them as rhizobacteria of agricultural use, one of the main drawbacks was that there were no local isolates of this microorganism. Following the guidelines proposed by Dr. Johanna Döbereiner from the laboratory of Agrobiology of EMBRAPA, Brazil (Döbereiner and Day 1976), and Dr. Yaacov Okon from the Hebrew University of Jerusalem, Israel (Okon et al. 1977), a considerable number of presumptive isolates of this genus were obtained, but due to the lack of a simple and accurate descriptive method or confirmatory molecular techniques they were only considered as presumptive. With this premise, subsequent studies aimed to isolate bacterial strains of *Azospirillum* from the main crops of Argentina, mainly maize and wheat.

After extensive research on the physiological properties of the presumptive isolates, such as the use of different carbon and nitrogen sources, a defined culture medium, highly useful for recognition and isolation of strains obtained in natural

conditions, was developed. The main features to identify the microorganisms in this medium were scarlet red staining of the colonies and rough and flattened structure on their surface. These allowed easy identification and selection even in the presence of other rhizosphere microorganisms. Thus, this medium, proposed by Enrique Rodríguez Cáceres in 1982, was named RC (Fig. 27.2). After that, this new tool allowed isolating a large number of strains from different crops and obtaining a collection of 64 strains, which were lyophilized for conservation at the Instituto de Microbiología y Zoología Agrícola (IMYZA) of the Instituto Nacional de Tecnología Agropecuaria (INTA) Castelar (Buenos Aires, Argentina).

27.1.2 Azospirillum brasilense *Az39*

An intense program was then held at the IMYZA from 1981 until 1995, to select and identify strains of *Azospirillum* sp. potentially applicable in agriculture and to assess their ability to promote growth in cultivable species such as wheat and corn in experimental fields of the province of Buenos Aires (Rodríguez Cáceres et al. 1995). The information obtained allowed confirming the positive effect of inoculation with *A. brasilense* in both crops and selecting the Az39 strain (obtained from the roots of wheat grown in soils of Marcos Juárez, Córdoba, Argentina) and the Cd strain (collection strain) as the ones with best performance within the group, due to their ability to increase yields of both crops from 13 to 33 %. Based on the information generated with this program, and in agreement with the inoculant companies of our country, the Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA) postulated the *A. brasilense* Az39 native strain as that recommended for the production of inoculants initially intended for crops of maize and wheat in Argentina.

27.2 Protocol for the Evaluation of Inoculants Containing *Azospirillum* spp.

27.2.1 Conservation of Samples

In the case that the samples are transported to the laboratory in their original container, during transportation and storage, these must be kept under the conditions recommended by the manufacturer. Rapid temperature changes can cause changes in moisture in the solid products by condensation or induce physiological changes in the microorganisms. Control samples must be kept at room temperature and away from direct sunlight.

27.2.2 Sampling Procedures

Depending on the availability and sample sizes, these can be divided according to the following recommendations:

27.2.2.1 Aqueous Liquid Inoculants

- Sanitize containers with alcohol 70°.
- Homogenize by manual agitation.
- Remove 10 mL of the product under flame or laminar flow, with syringe and needle or sterile pipette.

27.2.3 Enumeration of Viable Cells of **Azospirillum** *spp. Using the Plate Count Technique by Spreading on the Surface in Red Congo (RC) Medium*

27.2.3.1 Preparation of the Homogenate

- Shake the container vigorously for 30 s.
- Remove 10 mL of the product under flame or laminar flow, with syringe and needle or sterile pipette.
- Dilute with 90 mL of physiological solution, with the addition of Tween 80 to a final concentration of 0.01 % (0.360 mL of Tween 80 stock solution. Methodological Annex 3) per 90 mL of physiological solution. This is considered the 10^{-1} dilution.
- Shake for 15–20 min in any of the following conditions:
  - Linear stirrer with a 250-mL Erlenmeyer flask (point 3 or similar).
  - Orbital shaker with a 500-mL Erlenmeyer flask at 150 rpm (2.5 of eccentricity).
  - Magnetic stirrer with 250-mL Erlenmeyer flask at 300–350 rpm with a magnetic bar (40 × 7 mm).

27.2.3.2 Preparation of Working Dilutions

- Remove the Erlenmeyer flask from the agitator (the 10^{-1} dilution must be homogeneous, avoiding precipitation or phase separation).
- Remove 1 mL of the 10^{-1} dilution, preferably with automatic pipette, and place it in a test tube containing 9.0 mL of sterile saline.
- This dilution is called 10^{-2}. Vortex for 20 s to mix thoroughly.
- Repeat the previous step until dilution 10^{-7}, as shown in Fig. 27.1.

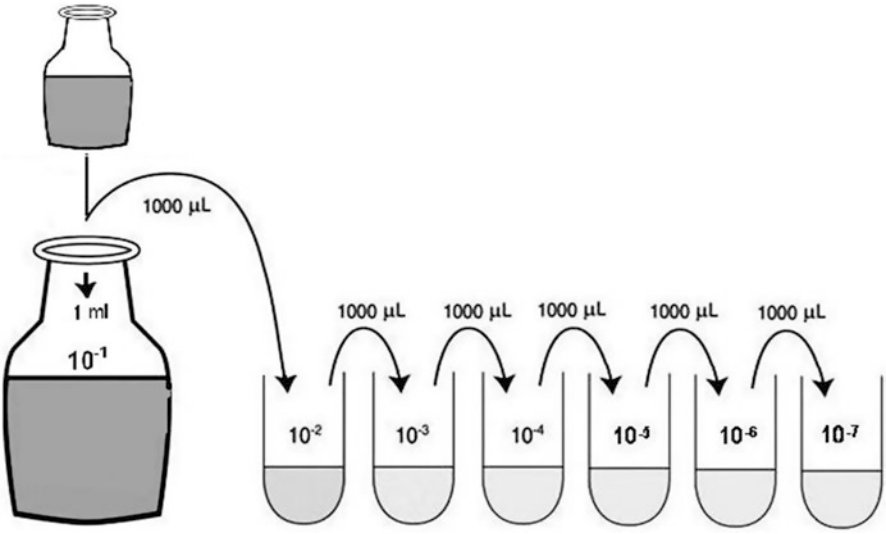

Fig. 27.1 Scheme of the tenfold dilution procedure to estimate viable cell enumeration

27.2.3.3 Dilution Plating

- Perform plate dilutions 10^{-4}, 10^{-5}, and 10^{-6} in triplicate, by spreading of 0.1 mL of the adequate dilution onto the surface of Petri dishes containing RC medium (15–20 mL) according to Rodríguez Cáceres (1982), with a Drigalsky spatula. The spatula to be used in spreading can be built with a glass rod (preferably 4 mm in diameter), a conditioned platinum spatula, a welding electrode, etc.
- Dry the plates in an incubator prior to their use.
- Begin with the highest dilution. Place 0.1 mL in the middle of the plate and, with the Drigalsky spatula previously sterilized, spread the liquid on the surface.
- Leave for 15 min with the agar side down until the liquid is completely absorbed.

27.2.3.4 Incubation

- Incubate the plates upside down in an incubation chamber between 28 and 30 °C. If space is limited and it is necessary to stack plates, take care not to exceed six plates.

27.2.3.5 Reading

- Reading is carried out 4 days after seeding and verified 2 days later.
- Plates showing between 30 and 300 colonies are counted to verify the proportionality between dilutions. If two successive dilutions present plates within these values, the corresponding calculation for each dilution is made according

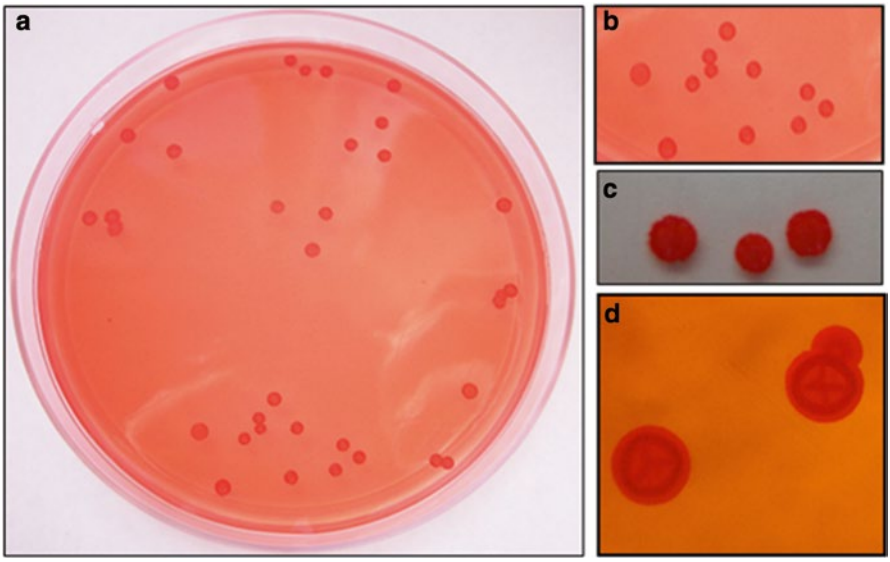

Fig. 27.2 Scarlet red colonies of *Azospirillum* spp. in Petri dishes containing RC culture medium, according to Rodríguez Cáceres (1982). Photographic credit: (**a**, **b**) Mariana Puente, (**c**) Luciana Di Salvo, and (**d**) Lina Lett

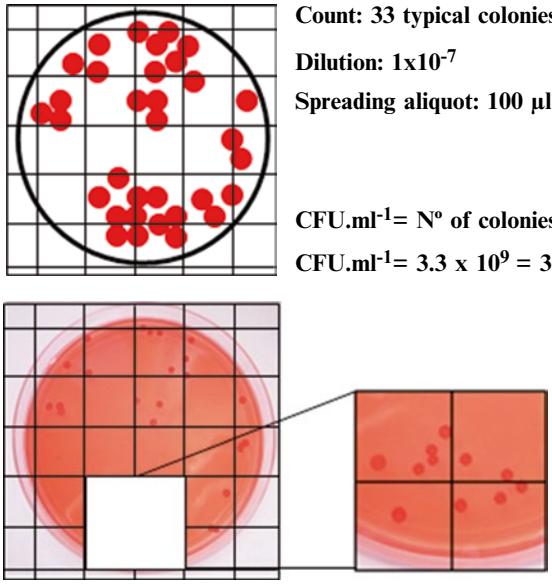

Fig. 27.3 Schematic counting of the colonies in plate. Photographic credit: Mariana Puente

to the formula described below and then the result is averaged. Typical colonies of *Azospirillum* spp. are usually scarlet red, circular, convex, 1–3 mm in diameter, and raised edges, as shown in Fig. 27.2. Count example is shown in Fig. 27.3.

Formula

The result is expressed as colony forming units per milliliter ($CFU \cdot mL^{-1}$) and is calculated as follows:

$$CFU \cdot mL^{-1} = \text{no of colonies counted} \times 10 \times \text{dilution factor}$$

where number of colonies is the average number of colonies present in the three reading plates, aliquot factor is 10 if 0.1 mL is used for spreading on the plates, and dilution factor is the inverse of the dilution in which the colonies are counted to obtain the result.

27.2.4 Reference Procedure for the Detection of Contaminating Microorganisms

To detect contaminating microorganisms, we suggest direct spreading from the container with spatula and by depletion in plates with trypticase soy agar (TSA) for bacteria in general or with Sabouraud agar for saprophytic fungi. In addition, we suggest carrying out Gram staining and observing a direct sample under a microscope.

27.2.4.1 Spreading in Culture Medium for the Detection or Quantification of Contaminants

- Spread on the surface a loaded spatula obtained directly from the sample without burning.
- Incubate the TSA plates in an incubation chamber at 28–30 °C for 48–72 h and the Sabouraud agar plates at 24 °C for 72 h.

27.2.4.2 Observation of Slides Stained with Gram Stain Modified by Hucker

Preparation of Smears

- On a clean slide, place a drop of the material to be analyzed.
- Spread with a spatula and fix by cutting several times the oxidant flame of the burner.
- Proceed to staining.

Staining Technique

(a) Reagents

Preparation of reagents and solutions. See Methodological Annex 4. Follow the procedure of the manufacturer if you work with a commercial kit.

(b) Smear staining

- Wash with the crystal violet working solution for 1 min.
- Rinse and wash with water.
- Wash with Lugol's iodine solution and leave for 1 min. Rinse and wash with water.
- Discolor with alcohol until total removal of the stain.
- Rinse and wash with water.
- Wash with safranin solution for 2–3 min. Rinse and wash with water.
- Dry and observe under a microscope.

Recording

Seek for the presence of microorganisms with staining and morphological features not compatible with *Azospirillum* sp., i.e., Gram (+) or Gram (−) rods of great size, cocci, and vibrios. Additionally, the cell morphology of *Azospirillum* sp. should be determined due to its cell dimorphism.

27.2.4.3 Observation of a Direct Slide (Fresh)

- On a clean slide, place an aliquot of the material to be analyzed (a drop of the formula if the inoculant is liquid or a drop of dilution 10^{-1} if the inoculant is solid, allowing the particulate to sediment).
- Cover with a coverslip.
- Observe under the microscope.

Recording

Bacteria of the genus *Azospirillum* sp. are mobile Gram (−) rods, but their shape and mobility depend on the physiological state in which the sample is preserved. You should seek for the presence of microorganisms with morphological features compatible to those mentioned and high mobility in spiral form.

27.2.5 Evaluation of pH

Aqueous Liquid Inoculant

pH is measured directly from the product.

Fig. 27.4 Growth of *Azospirillum brasilense* Az39 in NFb semisolid medium and evaluation of the diazotrophic ability in microaerophilic conditions, according to Döbereiner et al. (1995). Photographic credit: Carolina Castaño

27.2.6 Procedure to Evaluate the Microaerophilic and Diazotrophic Capacity of Presumptive Bacterial Colonies

Since it is common to observe dimorphism of colonies in RC medium plates inoculated with *Azospirillum* sp., our strategy to assume the presence of the microorganism is based on the evaluation of the bacterial ability to fix atmospheric nitrogen under microaerophilic conditions, by means of a procedure modified from Döbereiner et al. (1995).

27.2.6.1 Preparation of the Medium

- Prepare semisolid NFb medium as mentioned in point 2 of the Methodological Annex and as it is presented in Fig. 27.4.
- Load 10 mL sterile flasks with 5 mL of freshly sterilized medium.

27.2.6.2 Inoculation of NFb Medium

- Inoculation is carried out from the presumptive bacterial colonies obtained in the RC medium.
- Select a colony with an inoculating loop and seed at depth on the semisolid medium.
- Incubate between 4 and 6 days in an oven at 28–30 °C.
- Presumptive growth is considered in those cases in which the presence of a cloud or halo of growth is observed below the surface of the culture medium (Fig. 27.4).

Methodological Appendix

Agar-RC Medium (Agar–Congo Red)

Component	Amount
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
$FeCl_3 \cdot 6H_2O$	0.015 g
DL-malic acid	5.0 g
KOH	4.8 g
Congo red solution[a]	15.0 mL
Agar	20.0 g
H_2O	1,000 mL

Adjust to pH 7 with 0.1 N of KOH
[a]Congo red solution

Component	Amount
Congo red	2.5 g
H_2O	1,000 mL

NFb Semisolid Medium to Evaluate the Microaerophilic Diazotrophic Activity

Component	Amount
D-malic acid	5.0 g
K_2PO_4H	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.1 g
$Ca_2Cl \cdot 2H_2O$	0.02 g
Bromothymol blue solution[b]	2.0 mL
Micronutrient solution[c]	2.0 mL
$FeCl_3 \cdot 6H_2O$	0.015 g
KOH	4.5 g
Agar	1.8 g
Distilled water	1,000 mL

Adjust to pH 6.8 with 0.1 N of KOH
[b]Bromothymol blue basic solution

Component	Amount
Bromothymol blue	0.5 g
KOH 0.2 N	100 mL

Component	Amount
H_3BO_3	0.286 g
$MnSO_4 \cdot H_2O$	0.235 g
$ZnSO_4 \cdot 7H_2O$	0.024 g
$CuSO_4 \cdot 5H_2O$	0.008 g
$Na_2MoO_4 \cdot 2H_2O$	0.2 g
H_2O	200.0 mL

[c]Micronutrient solution

Tween 80 Stock Solution at 2.5 % (w/v)

Component	Amount
Tween 80	5 g
Distilled water	200 mL

Gram Staining Technique Modified by Hucker

Crystal Violet Reaction

Crystal violet mother solution (*A*)	
Crystal violet	5 g
Alcohol 95°	25 mL
Ammonium oxalate mother solution (*B*)	
Ammonium oxalate	2 g
Distilled water	200 mL

Prepare solutions A and B separately and use a *working solution* prepared with 4 mL of solution A, 36 mL of distilled water, and 160 mL of solution B.

Lugol's Iodine Solution

Iodine	1 g
Potassium iodide	2 g
Distilled water	300 mL

Safranin Reagent

Safranin	2.5 g
Alcohol 95°	100 mL

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