Chapter 10
Azospirillum Cell Aggregation, Attachment, and Plant Interaction

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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.

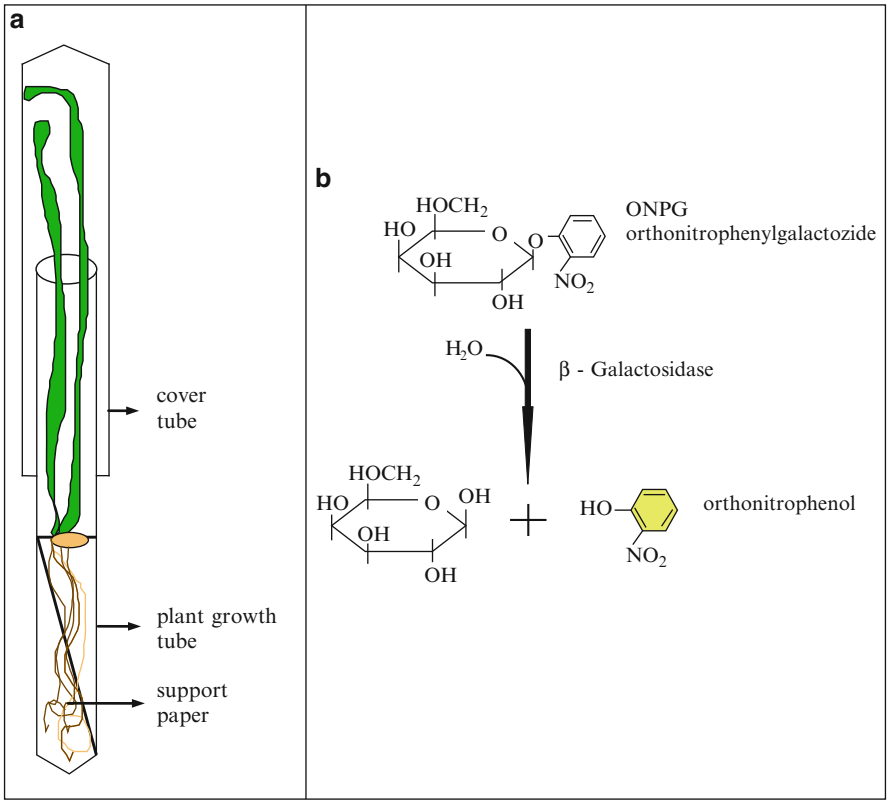

Fig. 10.1 (**a**) Plant growth system. (**b**) β-Galactosidase activity

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.

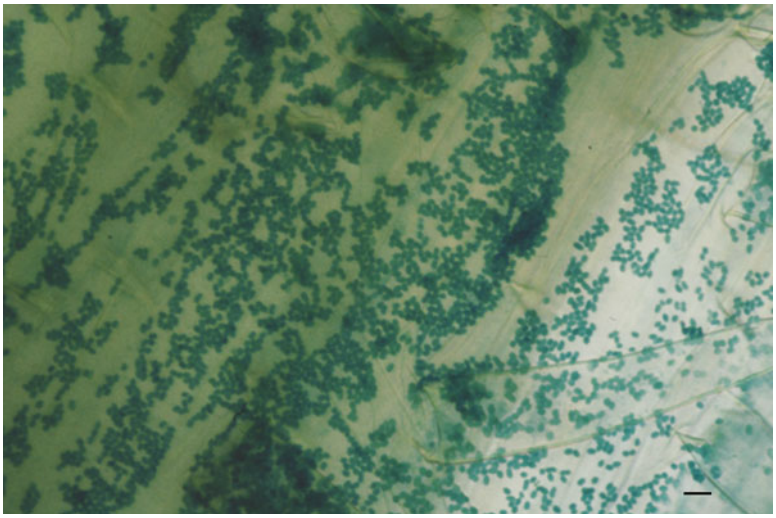

Fig. 10.2 Light micrograph of *A. brasilense* Sp7 [pLA-*lacZ*] cells on the surface of wheat roots. The *Azospirillum* cells contain pLA-*lac*Z, a constitutive *lac*Z fusion, which is expressed in all physiological conditions. They appear in shades of green to blue following X-Gal staining. Bar size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