J. Phycol. **47,** 1350–1359 (2011)

DOI: 10.1111/j.1529-8817.2011.01062.x

CELL-CELL INTERACTION IN THE EUKARYOTE-PROKARYOTE MODEL OF THE MICROALGAE *CHLORELLA VULGARIS* AND THE BACTERIUM *AZOSPIRILLUM BRASILENSE* IMMOBILIZED IN POLYMER BEADS[1]

Luz E. de-Bashan

Environmental Microbiology Group, Northwestern Center for Biological Research (CIBNOR), Mar Bermejo 195, Col. Playa Palo de Santa Rita, La Paz, B.C.S. 23090, Mexico

The Bashan Foundation, 3740 NW Harrison Blvd., Corvallis, Oregon 97330, USA

Michael Schmid, Michael Rothballer, Anton Hartmann

Helmholtz Zentrum München, German Research Centre for Environmental Health, Department of Microbe-Plant Interactions, Ingolstaedter Landstrasse 1, D-85764 Neuherberg, Germany

and Yoav Bashan[2]

Environmental Microbiology Group, Northwestern Center for Biological Research (CIBNOR), Mar Bermejo 195, Col. Playa Palo de Santa Rita, La Paz, B.C.S. 23090, Mexico

The Bashan Foundation, 3740 NW Harrison Blvd., Corvallis, Oregon 97330, USA

Cell-cell interaction in the eukaryote-prokaryote model of the unicellular, freshwater microalga *Chlorella vulgaris* Beij. and the plant growth-promoting bacterium *Azospirillum brasilense*, when jointly immobilized in small polymer alginate beads, was evaluated by quantitative fluorescence in situ hybridization (FISH) combined with SEM. This step revealed significant changes, with an increase in the populations of both partners, cluster (mixed colonies) mode of colonization of the bead by the two microorganisms, increase in the size of microalgae-bacterial clusters, movement of the motile bacteria cells toward the immotile microalgae cells within solid matrix, and formation of firm structures among the bacteria, microalgae cells, and the inert matrix that creates a biofilm. This biofilm was sufficiently strong to keep the two species attached to each other, even after eliminating the alginate support. This study showed that the common structural phenotypic interaction of *Azospirillum* with roots of higher plants, via fibrils and sheath material, is also formed and maintained during the interaction of this bacterium with the surface of rootless single-cell microalgae.

***Key index words:* *Azospirillum*; biofilm; *Chlorella*; microalgae; plant growth-promoting bacteria**

***Abbreviations:* CLSM, confocal laser scanning microscopy; DB, dissolved beads; FISH, fluorescence in situ hybridization; PGPB, plant growth-promoting bacteria; RGB, red-green-blue image; SB, sliced beads; SGM, synthetic growth medium**

In many cases, studies of plant-microorganism interactions involve a host plant with a small genome, such as *Arabidopsis thaliana* or rice; yet, most studies involving plant growth-promoting bacteria (PGPB) employed plants with far larger genomes, in which these PGPB commonly interact, such as maize (2.5 Gb), oat (11.4 Gb), or wheat (16 Gb). A simpler model for eukaryote-prokaryote interaction, using the green microalga *C. vulgaris* (having the smallest plant genome, ~45–49 Mb (Eckardt 2010) and the PGPB *A. brasilense* jointly trapped in alginate beads was proposed (de-Bashan and Bashan 2008).

Every unit in this model, technically a single polymeric bead, includes microalgae, bacteria, and a polymer matrix holding the microorganisms together. *Chlorella* spp. (Chlorophyceae) are simple, immotile, unicellular, aquatic green microalgae. *Chlorella* has been used in studies of photosynthesis, synthesis of carbohydrates in microalgae, and respiration (Ilangovan et al. 1998). *Chlorella* spp. and land plants, such as soybean (*Glycine max*) and maize (*Zea mays*), form a monophyletic lineage, the chlorophytes (fig. 3 in Bhattacharya and Medlin 1998). From a biotechnological standpoint, attention has been drawn to the potential of mass cultivation of this microalga for the production of high-value, low-volume compounds, such as pigments in the food industry, including the health-food market in industrialized countries (Lebeau and Robert 2006), application in wastewater treatment (Oswald 1992), and as biofuel (Mata et al. 2010).

Except for symbiotic rhizobia, *Azospirillum* is the most studied agricultural PGPB (Bashan and de-Bashan 2005). It is a highly competent, rhizo-

[1]Received 24 November 2010. Accepted 31 March 2011.
[2]Author for correspondence: e-mail bashan@cals.arizona.edu.

sphere-dwelling diazotroph that is also very versatile in its nitrogen transformations and carbon consumption and acts as a general PGPB for numerous plant species (Bashan et al. 2004), including *Chlorella* (Gonzalez and Bashan 2000), and uses a multitude of growth promotion mechanisms (Bashan and de-Bashan 2010). Alginate is the most commonly used polymer for microbial cell encapsulation, also called immobilization, where microorganisms are encapsulated in small cavities within the polymeric matrix (Smidsrød and Skjåk-Bræk 1990). Immobilization of microorganisms in alginate beads is a widely used technique when viable microbial cells are required in biotechnological processes (Prasad and Kadokawa 2009).

Several biochemical, biophysical, and metabolic aspects of this model have been studied (de-Bashan and Bashan 2008 and references therein). Yet, the intimate interaction between the cells of the microorganisms within the bead was initially studied solely by TEM a decade ago (Lebsky et al. 2001, de-Bashan et al. 2002). The working hypothesis of our study was that, similar to the interaction of *Azospirillum* cells with roots of crops, where anchoring material is produced to maintain the bacteria in its favorite location on the root, the interaction with the surface of rootless single-cell microalgae will produce some physical interactions between the two microorganisms leading to a creation of a strong attachment between the two partners. The general purpose of this study was to explore, specifically and quantitatively, the structural interaction between the cells of the prokaryote and eukaryote partners of this model.

To understand the details of such interactions, we used a combination of visual quantification techniques: (i) FISH evaluated by confocal laser scanning microscopy (CLSM) and epifluorescence microscopy (Axioplan, Zeiss, Oberkochen, Germany), (ii) comparing these images with images obtained by SEM of beads from the same batch of the model, and (iii) image-analysis quantification software. FISH has been widely used to investigate cultivation-independent bacterial communities in a range of ecosystems (Daims et al. 2001, Bertaux et al. 2007, Dazzo et al. 2007). This technique combines molecular identification, enumeration, and localization of physiologically active bacteria. FISH detects nucleic acid sequences by a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the fixed cells (Moter and Göbel 2000). Specifically, it has been used in the PGPB field of research to assay colonization of wheat roots by *A. brasilense* (Assmus et al. 1995) and *A. amazonense* and other diazotrophic PGPB in sugarcane plantlets (Oliveira et al. 2009).

These combined techniques allowed us to specifically detect *A. brasilense* inside the beads, to quantify the number of each microorganism in the interaction and quantitatively determine the developing dynamics, formation of single and dual species clusters, movements of bacteria within a solid bead, and detailed structural formations between the microalgae and bacteria.

MATERIALS AND METHODS

Microorganisms and growth conditions. The unicellular microalga *C. vulgaris* (UTEX 2714, University of Texas, Austin, TX, USA) was used. Before immobilization in alginate beads, the microalgae were cultured in sterile mineral medium (C30) for 5 d (Gonzalez et al. 1997), and *A. brasilense* Cd (DMS 1843, Braunscheweg, Germany) was grown in nutrient broth (Sigma) at 35 ± 2°C for 18 h in a rotary shaker (New Brunswick Scientific, Series 25, Edison, NJ, USA) at 120 rpm.

Immobilization of microorganisms in alginate beads. Microorganisms were immobilized according to de-Bashan et al. (2004). Briefly, 20 mL of axenically grown cultures of *C. vulgaris*, containing 6.0×10^6 cells $\cdot$ mL^{-1}, was harvested by centrifugation (Hemle Z 200A, Wehingen, Germany) at 2,000*g* and washed twice with sterile saline solution (0.85% NaCl). The cells were then mixed with 80 mL sterile, 6000-cP 2% alginate solution (a solution made of alginate mixed at 14,000 and 3500 cP) and stirred for 15 min. Beads (2–3 mm in diam.) were automatically produced in a 2% $CaCl_2$ solidification solution (de-Bashan and Bashan 2010). The beads were left for 1 h at 22 ± 2°C for curing and then washed in sterile saline solution. As controls, cultures of *A. brasilense* ($\sim 10^9$ CFU $\cdot$ mL^{-1}), and cultures of *C. vulgaris* (6.0×10^6 cells $\cdot$ mL^{-1}) were immobilized similarly. Because immobilization normally reduces the number of *Azospirillum* in the beads, a second incubation step was necessary for cultures of *A. brasilense* after the initial curing and washing, a process that recovered the population of bacteria in the mix. The second incubation lasted overnight in diluted nutrient broth (1:10). Where jointly immobilized cultures of *A. brasilense* and *C. vulgaris* were used (tested model), the same concentration of each microorganism, as used in pure cultures, was mixed prior to incorporation with alginate beads, but the volume of each microbial culture was reduced to 10 mL before adding alginate.

Culture conditions. Microorganisms immobilized alone or jointly were grown under batch conditions for 10 d. Synthetic growth medium (SGM) was prepared with the following (in mg $\cdot$ L^{-1}): 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 (89). The cultures were incubated in 250 mL unbaffled Erlenmeyer flasks (100 mL medium with 4 g of beads) at 28 ± 2°C, agitated at 120 rpm, with constant light at a density of 60 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} photon flux. At days 1, 3, 7, and 10, 25 beads were sampled for analysis.

Fixation and preparation of samples for FISH. For dissolved beads (DB), the alginate matrix of 10 beads was dissolved in 1 mL 4% sodium bicarbonate (Sigma) for 30 min to evaluate the strength of attachment between the two partners of the model. One mL of DB was centrifuged (14,000*g*); the pellet was washed twice in 1 X PBS (15% v/v 200 mM sodium phosphate buffer/130 mM NaCl at pH 7.4) and fixed with 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO, USA) for 1 h at 4°C. After fixation, the pellet was 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 each sample was added to a gelatin-coated (0.1% w/v, 0.01% w/v chromium potassium sulfate) microscope slide, air-dried, and dehydrated by successive 50, 80, and 96% ethanol washes (3 min each). Samples were air-dried again (Daims et al. 2005).

For sliced beads (SB), five beads were sliced with a sterile scalpel; slices were mounted on gelatin-coated (0.1% w/v, 0.01% w/v chromium potassium sulfate) microscope slides,

attached to the slide by adding 1 drop of warm low-melt agarose solution (0.25% w/v, Sigma), and dried at 37°C for 45 min. The samples were then fixed with 50 μL 4% paraformaldehyde and incubated at 4°C for 1 h. Afterward, the paraformaldehyde was removed by pipetting; the samples were 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 was based on the technique described by Assmus et al. (1995), with numerous small modifications. 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 prewarmed 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 visualization. An equimolar mixture of probes was used: EUB-338 I (Amann et al. 1990), II, and III (Daims et al. 1999), all detected the domain *Bacteria.* These three probes, when combined, detected almost all bacteria. For *A. brasilense*, we used the specific probe Abras 1420 (Stoffels et al. 2001). The EUB-338 I, II, and III probes were labeled with the fluorochrome fluorescein isothiocyanate (FITC), and *Abras* 1420 was labeled with the fluorochrome Cy3. All fluorescent-labeled probes purchased from Thermo Electron, Ulm, Germany. Before visualization, the slides were mounted in AF1 antifading reagent (Citifluor, Electron Microscopy Sciences, Hatfield, PA, USA).

Visualization. For CLSM, an LSM 510 META system with an Axiovert 100 M inverted microscope (Zeiss) was used (Schmid et al. 2009). A helium neon laser provided the excitation wavelength of 543 nm (Cy3), and an argon ion laser provided the excitation wavelength of 488 nm (FITC). To distinguish between the fluorescence from Cy3 and FITC-labeled oligonucleotide probes, the specific signals were depicted in red and green, respectively. The third color channel (helium laser, 633 nm singular wavelengths) was used to visualize autofluorescence of the microalgae and was assigned a blue color. The three signals were combined and depicted as a red-green-blue (RGB) image. An Apochromat 63 X/1.2 water immersion lens was used for all analyses and acquisitions of images. Analyses of images used a software LSM 510 v4.2 (Zeiss).

For epifluorescence microscopy, an Axioplan 2 (Zeiss), equipped with a mercury lamp (HXP120, Osram, Munich, Germany) and Zeiss filter sets for FITC/GFP (Emitter BP 525/50, Beamsplitter FT 495, Exciter BP 470/40), Cy3 (Emitter BP 605/70, Beamsplitter FT 570, Exciter BP 545/25), and Cy5 (Emitter BP 690/50, Beamsplitter FT 660, Exciter BP 640/30) excitation were used. An Apochromat 63 X/1.2 water immersion lens (Zeiss) was used for all observations. Images were recorded with the CCD camera AxioCam MRm controlled by the software AxioVision Rel. v4.6 (Zeiss) and further processed with Adobe Photoshop v8.0 (Adobe Systems, Mountain View, CA, USA).

A major technical difficulty of observing microalgae-bacteria interaction 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 was adopted, where the ultrabrilliant microalgae were obscured by a black circle, allowing visualization 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 but not blue. Additionally, to enhance clarity of the images, time of exposure was 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* varied in their fluorescence color from yellow-green to orange, arising from different intensities of the separately recorded red and green channels. Similarly, the microalgae show slightly different tones, ranging from magenta to a light cyan. The major difference, however, was the presence of the blue color fraction, which is absent in *A. brasilense* signals.

Quantification. Cell counting and measuring populations, cluster size of the microalgae and of the bacteria, and the distance between microalgal clusters and bacterial clusters in all FISH images obtained from the confocal laser scanning and epifluorescence microscopies were quantified using image analyzer software (Image Pro-Plus 4.1, Media Cybernetics, Silver Spring, MD, USA).

Scanning electronic microscope. Five beads were fixed with glutaraldehyde following the method of Bashan et al. (1986), with modifications. Briefly, beads were fixed for 5 h in a 5% (v/v) glutaraldehyde solution in 1 M 4-(-2 hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer at pH 7.2. After fixation, the beads were washed twice in 1 M HEPES and then dehydrated with increasing ethanol concentrations, 25% for 10 min, 50% for 30 min, 70% for 10 h, and 100% for 60 min, at 4°C. Samples were frozen in liquid nitrogen, sliced to half, and dried with CO_2 in a critical point dryer (Samdri-PVT-3B, Tousimis Research, Rockville, MD, USA). The slices were mounted on a stub, submitted to osmium vapor for 4 d, and coated with palladium foil for 35 min at 40 mA in a sputter coater (Vaccum Desk II, Denton, Scotia, NY, USA).

Another set of five beads was fixed with Davidson's fixative (Howard and Smith 1983) and embedded in paraffin for histological sectioning with a microtome (Leica RM2025, Nussloch, Germany). Sections were mounted on a precoated slide and stored at room temperature until used. Before processing for visualization, the paraffin was removed from the slides by immersing them in xylene for 10 min, followed by immersion in fresh xylene for another 10 min. The samples were rinsed for 5 min in sterile deionized water and air-dried. Slides were submitted to osmium vapor for 4 d and coated with palladium, as described earlier.

Visualization was done in a scanning electron microscope (Hitachi S-3000N, Tokyo, Japan) at 15 kV, using a 45° angle of the slide to the electron beams. The photomicrographs were obtained with software (Quartz PCI v5.5, Quartz Imaging, Vancouver, Canada).

Experimental design and statistical analysis. Cultures were prepared in triplicate, where a single flask served as one replicate. Each experiment was repeated three times. FISH analysis was performed on five replicates. A total of 130 photomicrographs were analyzed for quantifying microorganisms, measuring microalga and bacteria cluster size, and measuring distance between microalgae and bacteria clusters. A total of 83 SEM photomicrographs were analyzed. Data were analyzed first by one-way ANOVA and then by Tukey's post hoc analysis or by Student's *t*-test, with confidence set at $P \leq 0.05$. All statistical analyses were performed with computer software (Stastica v6.0, StatSoft, Tulsa, OK, USA).

RESULTS

Populations of C. vulgaris *and* A. brasilense *immobilized alone or jointly within one bead.* Population dynamics of the two microorganisms within a single bead showed that populations of *C. vulgaris* immobilized alone or jointly with *A. brasilense* increased similarly for 3 d. Later, joint immobilization yielded larger populations for up to 10 d (Fig. 1a, capital

letter analysis). At that time, the population of *C. vulgaris* growing alone declined. The population of *A. brasilense* jointly immobilized with *C. vulgaris* was significantly higher 7 d after inoculation (Fig. 1b, capital letter analysis). After 3 d of incubation, populations of *A. brasilense* grown alone declined. When *A. brasilense* was jointly immobilized with the microalgae, the decline was delayed until 10 d after inoculation (Fig. 1b, lowercase letter analysis).

Effect of joint immobilization on the size of microalgae clusters within the bead. Two types of beads (intact and those where the alginate was dissolved and removed) were evaluated. In beads containing only *C. vulgaris*, the size of the clusters changed only slightly, usually by insignificant amounts (Fig. 2, a and b, lowercase letter analysis). When immobilized with *A. brasilense*, clusters grew continuously, reaching significantly larger sizes after 7 d (DB, Fig. 2b) and 10 d (intact beads, Fig. 2a) after immobilization. When the beads were dissolved, the microorganisms maintained their colonies in the same formation as in intact beads; the shape of the colony was preserved even after the alginate was removed.

Cell-cell interaction of A. brasilense *and* C. vulgaris. The FISH technique allowed identification of *A. brasilense* interacting with microalgae because the bacteria were specifically labeled by the fluorescent probes. Positive fluorescence signals from *A. brasilense* varied in their color from yellow-green to orange, where the microalgae show slightly different tones, ranging from magenta to a light cyan. The microalgae show autofluorescence in all channels used for detection; there was no need to label these cells with specific probes. In addition to their fluorescence characteristics, the size of cells of the two species was markedly different and served as another differentiating criterion.

Colonization of polymer beads and interaction of the two species occurred in the numerous cavities within the beads (Fig. 3). At the time of inoculation

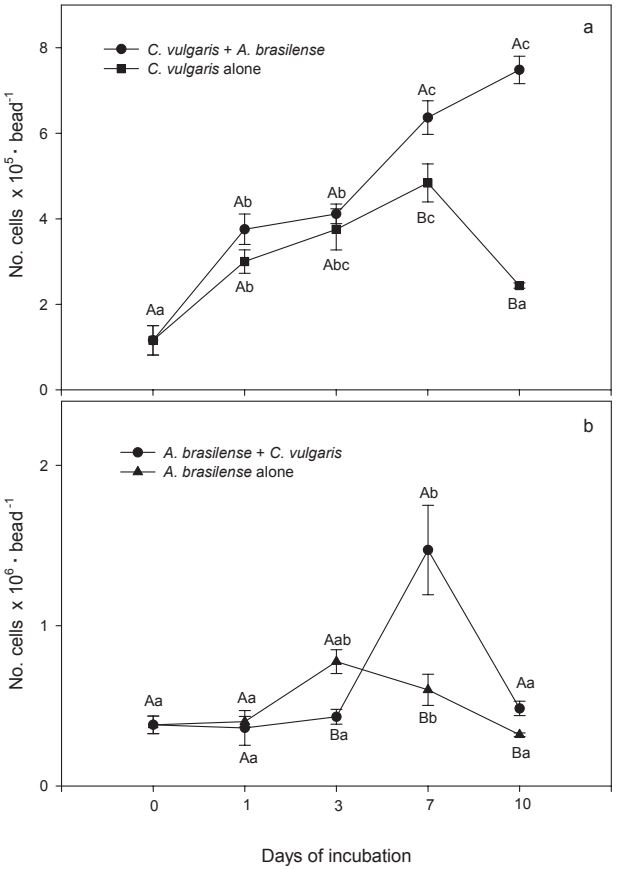

FIG. 1. Multiplication of *Chlorella vulgaris* and *Azospirilllum brasilense* growing immobilized alone and jointly in alginate beads. (a) Populations of *C. vulgaris*. (b) Populations of *A. brasilense*. Points on each curve denoted by a different lowercase letter differ significantly at $P < 0.05$ in one-way analysis of variance, according to Tukey's post hoc analysis. Points denoted by a different capital letters at each day of incubation differ significantly at $P < 0.05$ in Student's *t*-test. Bars represent the SE.

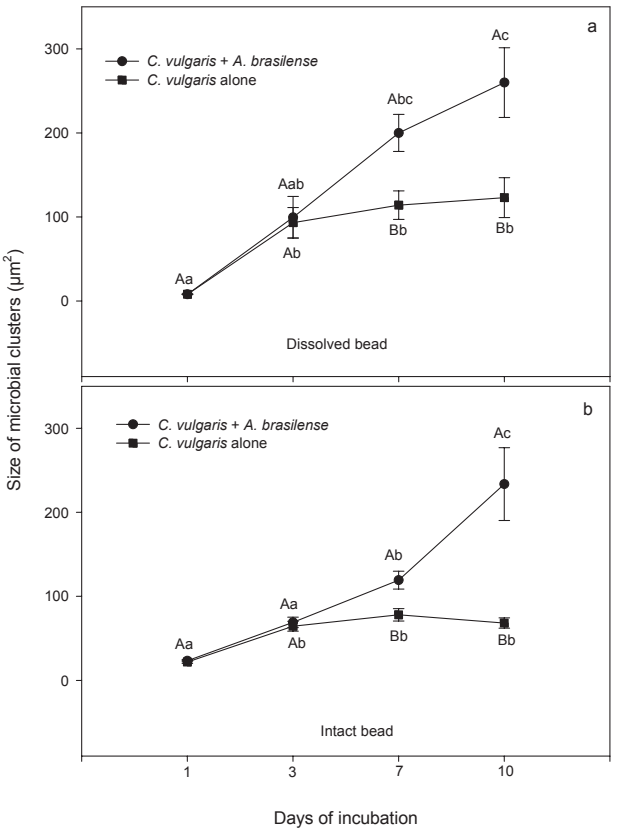

FIG. 2. Size of microbial clusters of *Chlorella vulgaris* and *Azospirillum brasilense* growing immobilized alone and jointly in alginate bead under different conditions in the bead. (a) Clusters measured in bead where the alginate matrix was dissolved before analysis. (b) Clusters measured in bead that was sliced open and the counting was performed in the presence of the alginate matrix. Points on each curve denoted by a different lowercase letter differ significantly at $P < 0.05$ in one-way analysis of variance, according to Tukey's post hoc analysis. Points denoted by a different capital letter at each day of incubation differ significantly at $P < 0.05$ in Student's *t*-test. Bars represent the SE; its absence indicates negligible SE.

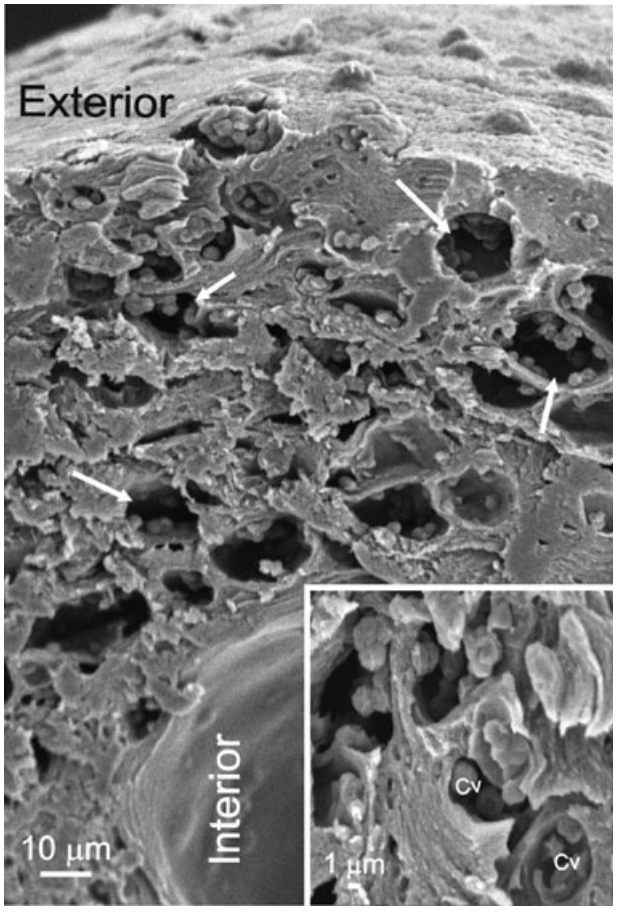

FIG. 3. SEM of cross-section of the bead model of the eukaryote-prokaryote model shows polymeric structure made of cavities (arrows) where microorganisms are interacting and the limited space available for the interaction. Cv, *Chlorella vulgaris*.

and up to 1 d after joint immobilization, there are no clusters composed of both microorganisms. Only individual cells and cell clusters of the same species were observed (Fig. 5, a–c). Less commonly, cells of *A. brasilense* were attached to individual microalga (Fig. 7a). Within the beads, distance between clusters of each kind of microorganism decreased with time, starting at day 3 (Fig. 4) and continuing thereafter. Clusters composed of both microorganisms started to form at day 3 (Fig. 5d). At day 7 and day 10 after immobilization, the distance between the two species shrunk to ~1 μm, which suggests physical attachment (Fig. 4). The FISH observations indicate that clusters of the two microorganisms were mainly formed at day 7 through day 10 (Fig. 5, g and j). When each microorganism was immobilized separately, they formed single-species aggregates (Fig. 5, e, f, h, i, and l), as is common for aging colonies of *Azospirillum* spp. or cells under stress, as would be the case if oxygen was low (Hartmann and Hurek 1988, Burdman et al. 2001). Possibly, this formation was forced on proliferating microalgae cells because there is limited space for growth within the cavities.

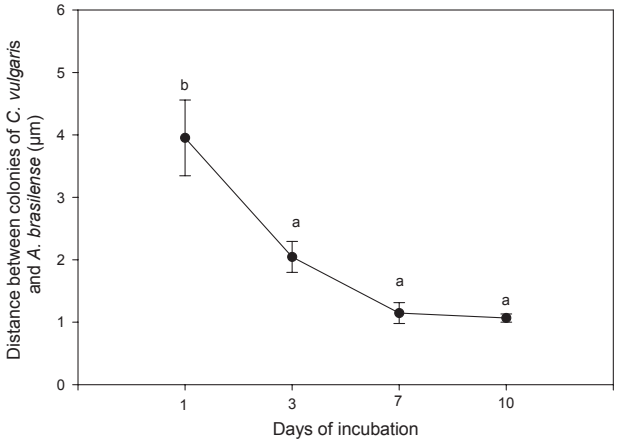

FIG. 4. Distance between clusters of *Chlorella vulgaris* and *Azospirillum brasilense* that were jointly immobilized in alginate beads. Points on each curve denoted by a different lowercase letter differ significantly at $P < 0.05$ in one-way analysis of variance, according to Tukey's post hoc analysis. Bars represent the SE.

With time, and most prominently at day 7 after joint immobilization, *A. brasilense* and *C. vulgaris* produced the cluster-type interaction, where small connections between cells of the two species, made of fibril and sheath materials, were commonly observed (Fig. 6). The makeup of these materials is unknown. Both microorganisms apparently were connected to the inert alginate and to each other by these connections, forming solid structures (Fig. 6, arrows). This type of attachment was strong from the onset of the interaction. When the alginate matrix was dissolved, the two microorganisms stayed attached (Fig. 7).

DISCUSSION

Much knowledge about interactions of the rhizosphere-dwelling *Azospirillum* spp. with plant cells comes from studies of attachment to roots of many plant species, but only a very small number of studies describe its interaction with single cells, mostly microalgae (Steenhoudt and Vanderleyden 2000, Bashan et al. 2004). Attachment of *Azospirillum* is fundamental for a long-term association with the roots of the host plant for three reasons. (i) Without a secure attachment, bacteria may detach from the root as it grows through the soil, and water may wash bacteria from the rhizoplane; then it may perish in the surrounding, nutrient-deficient soil. *Azospirillum* is known to survive poorly in many soils without host plants (Bashan 1999). (ii) If the bacteria are not attached to root epidermal cells, substances excreted by the plant and the bacteria diffuse into the rhizosphere and are consumed by nutritionally versatile microorganisms before reaching *Azospirillum* or the plant. When bacteria attach to roots, part of the excreted substances diffuses into the intercellular spaces of the root cortex.

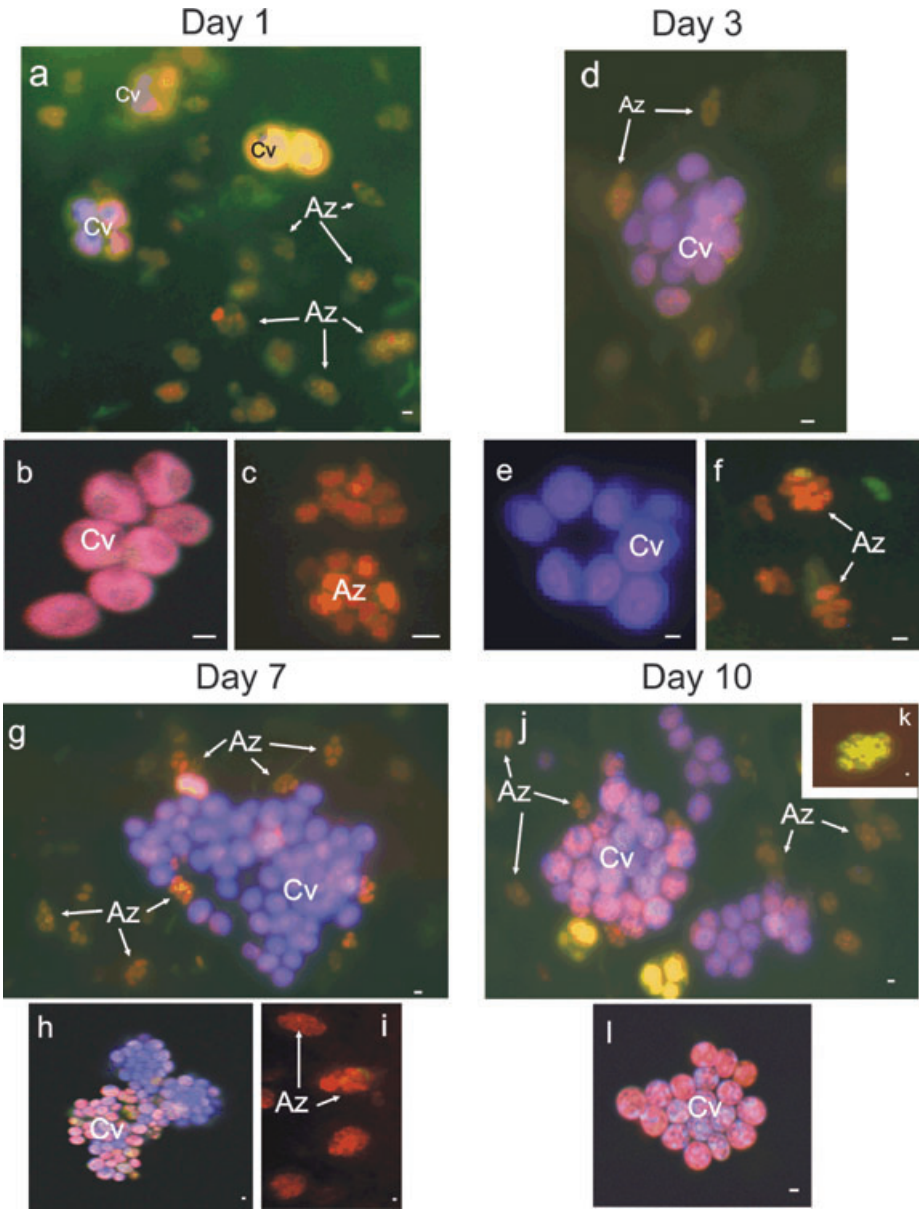

FIG. 5. Patterns of interaction between *Chlorella vulgaris* and *Azospirillum brasilense* by fluorescence in situ hybridization (FISH) photomicrographs (a, d, g, j) with time. (b, c, e, f, h, i, k, l) Microorganisms immobilized alone (controls). All photomicrographs were taken by the epifluorescence microscopy Axioplan 2, except b and h, which were taken with CSLM. *C. vulgaris* was not labeled while cells of *A. brasilense* were labeled with the probes Abras 1420 Cy3 (red color) and EUB338Mix FITC (green) via FISH. Positive fluorescent signals identifying *A. brasilense* are therefore a combination of red and green without any blue, resulting in a green-yellow to orange tone, depending on the intensities of the individual color channels. Autofluorescence of the microalgae in the different images may vary from magenta to light cyan based on the selected channel exposure time but is characterized by the presence of a blue signal fraction in addition to red and green. Cv, *C. vulgaris;* Az, *A. brasilense.* Scale bars, 1 μm.

(iii) Sites on roots without attached *Azospirillum* are vulnerable to aggressive, possibly unbeneficial colonizers.

In large and small water bodies, interaction of phytoplankton (mainly made of microalgae) and bacteria is well known (Makk et al. 2003, Sapp et al. 2007). These associations also occur in artificial biofilms created in treatment of hazardous materials (Muñoz and Guieysse 2006, Tang et al. 2010). Similarly, expansion of bacterial aggregates to a biofilm-sized layer under wet conditions by epiphytes has been documented (Lindow and Brandl 2003). Yet, the detailed nature of these latter interactions has not been studied. Only a handful of studies show the detailed interaction between microalgae and bacteria in artificial biofilms and constructed models, as follows: (i) Coculturing of the bacterium *Brevundimonas* sp. with unicellular microalgae *Chlorella ellipsoidea* showed direct adhesion of bacterial cells to the surface of the microalgae cells

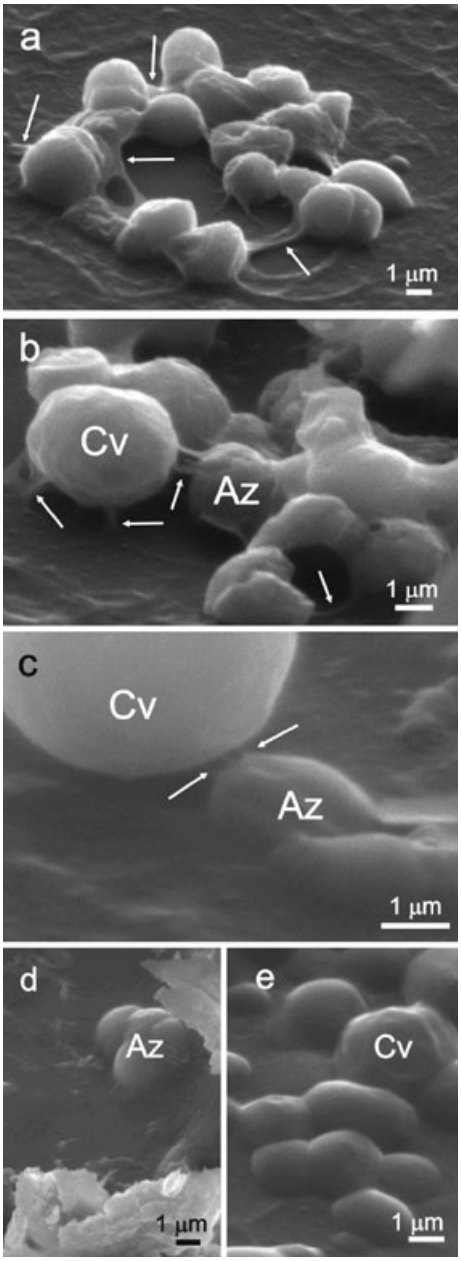

FIG. 6. SEM of inside the cavities (shown in Fig. 3) of the bead eukaryote-prokaryote model illustrates interaction between cells of *Chlorella vulgaris* and *Azospirillum brasilense* 7 d after being jointly immobilized. (a) A cluster made of the two microorganisms connecting to each other and to the alginate matrix by a network of fibril material and sheaths (arrows). (b) Enlargement of fibril material connection among the microorganisms and to the alginate matrix (arrows). (c) Very high magnification of cell–cell interaction between a microalgae cell and a bacterium via fibril connections (arrows). (d, e). Controls of immobilized microorganisms growing separately. There are no connections between cells or to the surface. Cv, *C. vulgaris*; Az, *A. brasilense*.

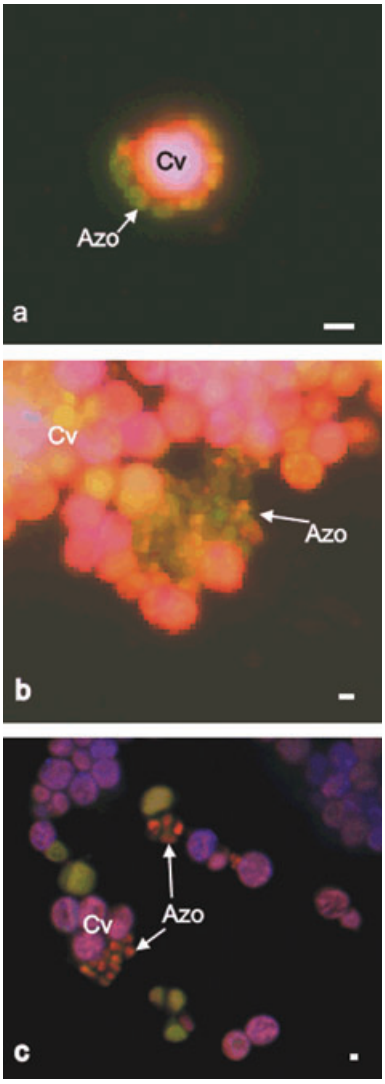

FIG. 7. Epifluorescence microscopy (a, b) and confocal scanning laser microscopy (c) of the interaction between *Chlorella vulgaris* and *Azospirillum brasilense* after the alginate matrix was dissolved, but maintaining the cells of the two attached microorganisms after 1 d (a), 7 d (b), and 10 d (c). *C. vulgaris* was not labeled where cells of *A. brasilense* were labeled using fluorescence in situ hybridization (FISH) for visualization. The variation in the colors is explained in the legends to Figure 5. Cv, *C. vulgaris;* Azo, *A. brasilense*. Scale bars, 1 µm.

as single cells or clusters. This interaction forms abundant crinkles on the surface *C. ellipsoidea* cells. No evidence of a specific sheath excreted from *C. ellipsoidea* cells was observed, nor was any explanation why crinkles were formed provided (Park et al. 2008). (ii) In a trial where *C. sorokiniana* and one of its symbiont (*Microbacterium* sp.) were coflocculated, the microalgae produced sheath; however, no details on the structural nature of the interaction are known (Imase et al. 2008). (iii) During immobilization of *A. brasilense* with *C. vulgaris* in alginate beads, the following events were qualitatively observed by TEM over the period of observation: After random immobilization within the confinement of one bead, where most cells of each species occupy their own cavities, motile populations of *A. brasilense* presumably moved toward the immobile

C. vulgaris cells. The two populations eventually merged into a mixed cluster, and some electronally dense unknown material was present in shared cavities. The report concluded that joint immobilization enhanced the vitality of the microalga *C. vulgaris* and delayed senescence (Lebsky et al. 2001).

Our current study expanded upon these initial observations by using a quantitative approach, precise molecular detection techniques, and three types of microscopy. An apparent similarity between the interactions of *Azospirillum* with microalgae and plant roots was demonstrated, namely, formation of connecting fibril and sheath material, a biofilm, that bind both microorganisms together and to the solid structure of the alginate matrix. Even when the alginate bead matrix was dissolved, the attachments in the cell clusters remained, indicating strong structural connections between the cells. These structures may be made of (i) several high-molecular-weight carbohydrates containing lipopolysaccharide-protein and polysaccharide-lipid complexes that *Azospirillum* is known to produce in large quantities, even though their precise nature is unknown (Del Gallo and Haegi 1990, Skvortsov and Ignatov 1998, Puente et al. 1999, Konnova et al. 2001); (ii) the sheath produced by *Chlorella* species (Imase et al. 2008); or (iii) a mix of all of these. The rigidness of the bacteria-microalgae attachment, even after completely dissolving the alginate support with sodium bicarbonate, can be explained by the complexity of the biofilm produced by the interacting *Azospirillum*-microalgae community, which cannot be broken as easily as the alginate polymer, where only a single substitution of Ca with Na is needed for dissolving the polymer.

The preferred sites for *Azospirillum* attachment and for root surface colonization, in most plant species studied, are the elongation and root hair zones, where the bacteria mainly cluster in an aggregate form of colonization supported by massive fibril material that connects the bacteria to the epidermal surface. Aggregation is one of the most basic phenomena of *Azospirillum* colonization of roots (Bashan et al. 2004). Physical attachment of *Azospirillum* spp. to single-cell plants, such as microalgae, has not been previously reported. Our study showed that the typical clustering (aggregation) by *A. brasilense* colonization of roots was similarly maintained when interacting with clusters of microalgal cells. Although clusters of microalgae contain numerous individual cells, they lack any apparent differentiation, unlike tissues of higher plants, which are the common hosts of *Azospirillum*.

Azospirillum spp. has two different phases of attachment to roots. The primary adsorption phase is fast, weak, and governed probably by bacterial proteins and mediated by lectins, hydrophobicity, pH, and charge (Bashan and Levanony 1989, Castellanos et al. 1997, 1998, Antonyuk et al. 1999, Egorenkova et al. 2000, Burdman et al. 2001, Yegorenkova et al. 2001, Pinheiro et al. 2002). The second phase (called anchoring) is stronger, takes several hours to develop, is irreversible, and is based on bacterial extracellular surface polysaccharides involving a network of fibril material and a large amount of mucigel-like substances that permanently connects the bacteria to the root surface and prevents removal from the site (Del Gallo and Haegi 1990, Bashan et al. 1991a,b, Michiels et al. 1991, Bashan and Holguin 1993, Skvortsov and Ignatov 1998, Bianciotto et al. 2001, Fedonenko et al. 2002). The polar flagellum of *A. brasilense*, which is primarily used for swimming, was also implicated in the attachment process of the bacteria to roots (Croes et al. 1993, Grilli Caiola et al. 2004). These connecting structures were observed during interaction of *Azospirillum* with several plant species and can be considered a phenotype of its interaction phase with roots. This study expands this view, demonstrating that the anchoring mode of attachment of *Azospirillum* also includes single-cell microalgae.

The phenotypic characteristic of this microalgae-PGPB model is enhanced multiplication of the microalgae and eventually some decline in the *Azospirillum* population (Gonzalez and Bashan 2000, de-Bashan and Bashan 2008). In this study, using different microscopic-molecular tools instead of traditional microbial counting, the general behavior of the *Chlorella-Azospirillum* model was confirmed. Because each microalgal cell can be precisely located and counted by our techniques, it demonstrated that, even within the confines of small bead cavities, the size of the microalgal clusters significantly increased.

Azospirilla are highly motile bacteria with developed chemotaxis (Zhulin and Armitage 1992) and aerotaxis (Reiner and Okon 1986), both in vitro (Zhulin and Armitage 1993) and in situ (Bashan 1986), and are capable of seeking and swimming toward the roots of the host plant (Bashan and Levanony 1987, Bashan and Holguin 1994, 1995). When immobilized within an alginate bead, the bacteria migrate toward the host microalgae. This phenomenon is likely a result of the bacteria partly dissolving the alginate polymer with the organic acids they produced. All Azospirilla abundantly produce the hormone indole-3 acetic acid (IAA; Steenhoudt and Vanderleyden 2000) that also acts as a weak acid. Additionally, *A. brasilense* produces gluconic acid (Rodriguez et al. 2004). IAA produced in this model is known to enhance the growth of these microalgae (de-Bashan et al. 2008).

In summary, this study demonstrated that the phenotypic structural interaction of *Azospirillum* with roots, composed of fibril and sheath material, a biofilm, also formed and was maintained during the interaction of this bacterium with the surfaces of single-cell microalgae. The microalgae-bacteria structures are apparently strong enough to allow firm

connections between the prokaryotic and eukaryotic partners. The interaction was favorable for the microalgae. These findings enhance the validity of this prokaryotic-eukaryotic model that is useful for basic studies of the physiology and molecular biology of plant-bacterium interactions.

We thank Maria del Carmen Rodríguez, Eulalia Meza, and Ariel Cruz of CIBNOR for assisting in histological sectioning and SEM; and Noga Bashan of The Technion, Israel Institute of Technology, Haifa, Israel, for technical assistance. Ira Fogel of CIBNOR provided editorial suggestions. This study was supported by Sistema Nacional de Investigadores-Consejo Nacional de Ciencia y Tecnología (SNI-CONACYT contract 90089; CONACYT contract 23917), Secretaria de Medio Ambiente y Recursos Naturales (SEMARNAT contract 23510), and time for writing by the Bashan Foundation, USA. Y. B. received a short-term research fellowship in 2009 from the German Academic Exchange Service (DAAD), Bonn, Germany.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. & Stahl, D. A. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* 56:1919–25.

Antonyuk, L. P., Sumaroka, M. V. & Ignatov, V. V. 1999. Effect of wheat lectin on the membrane phospholipid content of *Azospirillum brasilense*. *Appl. Biochem. Microbiol.* 35:53–5.

Assmus, B., Hutzler, P., Kirchhof, G., Amann, R. I., Lawrence, J. R. & Hartmann, A. 1995. *In situ* localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescence labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. *Appl. Environ. Microbiol.* 61:1013–9.

Bashan, Y. 1986. Migration of the rhizosphere bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* towards wheat roots in the soil. *J. Gen. Microbiol.* 132:3407–14.

Bashan, Y. 1999. Interactions of *Azospirillum* spp. in soils: a review. *Biol. Fertil. Soils* 29:246–56.

Bashan, Y. & de-Bashan, L. E. 2005. Bacteria/plant growth-promotion. *In* Hillel, D. [Ed.] *Encyclopedia of Soils in the Environment*, vol. 1. Elsevier, Oxford, UK, pp. 103–15.

Bashan, Y. & de-Bashan, L. E. 2010. How the plant growth-promoting bacterium *Azospirillum* promotes plant growth-a critical assessment. *Adv. Agron.* 108:77–136.

Bashan, Y. & Holguin, G. 1993. Anchoring of *Azospirillum brasilense* to hydrophobic polystyrene and wheat roots. *J. Gen. Microbiol.* 139:379–85.

Bashan, Y. & Holguin, G. 1994. Root-to-root travel of the beneficial bacterium *Azospirillum brasilense*. *Appl. Environ. Microbiol.* 60:2120–31.

Bashan, Y. & Holguin, G. 1995. Inter-root movement of *Azospirillum brasilense* and subsequent root colonization of crop and weed seedlings growing in soil. *Microb. Ecol.* 29:269–81.

Bashan, Y., Holguin, G. & de-Bashan, L. E. 2004. *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003). *Can. J. Microbiol.* 50:521–77.

Bashan, Y. & Levanony, H. 1987. Horizontal and vertical movement of *Azospirillum brasilense* Cd in the soil and along the rhizosphere of wheat and weeds in controlled and field environments. *J. Gen. Microbiol.* 133:3473–80.

Bashan, Y. & Levanony, H. 1989. Factors affecting adsorption of *Azospirillum brasilense* Cd to root hairs as compared with root surface of wheat. *Can. J. Microbiol.* 35:936–44.

Bashan, Y., Levanony, H. & Klein, E. 1986. Evidence for a weak active external adsorption of *Azospirillum brasilense* Cd on wheat roots. *J. Gen. Microbiol.* 132:3069–73.

Bashan, Y., Levanony, H. & Whitmoyer, R. E. 1991a. Root surface colonization of non-cereal crop plants by pleomorphic *Azospirillum brasilense* Cd. *J. Gen. Microbiol.* 137:187–96.

Bashan, Y., Mitiku, G., Whitmoyer, R. E. & Levanony, H. 1991b. Evidence that fibrillar anchoring is essential for *Azospirillum brasilense* Cd attachment to sand. *Plant Soil* 132:73–83.

Bertaux, J., Gloger, U., Schmid, M., Hartmann, A. & Scheu, S. 2007. Routine fluorescence *in situ* hybridization in soil. *J. Microbiol. Methods* 69:451–60.

Bhattacharya, D. & Medlin, L. 1998. Algal phylogeny and the origin of land plants. *Plant Physiol.* 116:9–15.

Bianciotto, V., Andreotti, S., Balestrini, R., Bonfante, P. & Perotto, S. 2001. Extracellular polysaccharides are involved in the attachment of *Azospirillum brasilense* and *Rhizobium leguminosarum* to arbuscular mycorrhizal structures. *Eur. J. Histochem.* 45:39–49.

Burdman, S., Dulguerova, G., Okon, Y. & Jurkevitch, E. 2001. Purification of the major outer membrane protein of *Azospirillum brasilense*, its affinity to plant roots, and its involvement in cell aggregation. *Mol. Plant-Microbe Interact.* 14:555–61.

Castellanos, T., Ascencio, F. & Bashan, Y. 1997. Cell-surface hydrophobicity and cell-surface charge of *Azospirillum* spp. *FEMS Microbiol. Ecol.* 24:159–72.

Castellanos, T., Ascencio, F. & Bashan, Y. 1998. Cell-surface lectins of *Azospirillum* spp. *Curr. Microbiol.* 36:241–4.

Croes, C. L., Moens, S., van Bastelaere, E., Vanderleyden, J. & Michiels, K. W. 1993. The polar flagellum mediates *Azospirillum brasilense* adsorption to wheat roots. *J. Gen. Microbiol.* 139:2261–9.

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H. & Wagner, M. 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Syst. Appl. Microbiol.* 22:434–44.

Daims, H., Ramsing, N. B., Shleifer, K.-H. & Wagner, M. 2001. Cultivation-independent, semiautomatic determination of absolute bacterial cell numbers in environmental samples by fluorescence in situ hybridization. *Appl. Environ. Microbiol.* 67:5810–8.

Daims, H., Stoecker, K. & Wagner, M. 2005. Fluorescence in situ hybridization for the detection of prokaryotes. *In* Osborn, A. M. & Smith, C. J. [Eds.] *Molecular Microbial Ecology*. Taylor and Francis Group, New York, pp. 223–7.

Dazzo, F. B., Schmid, M. & Hartmann, A. 2007. Immunofluorescence microscopy and fluorescence *in situ* hybridization combined with CMEIAS and other image analysis tools for soil- and plant-associated microbial autecology. *In* Hurst, C. J., Crawford, R. L., Garland, J. L., Lipson, D. A., Mills, A. L. & Stetzenbach, L. D. [Eds.] *Manual of Environmental Microbiology*, 3rd ed. American Society for Microbiology, Washington, D.C., pp. 712–33.

de-Bashan, L. E., Antoun, H. & Bashan, Y. 2008. Involvement of indole-3-acetic-acid produced by the growth-promoting bacterium *Azospirillum* spp. in promoting growth of *Chlorella vulgaris*. *J. Phycol.* 44:938–47.

de-Bashan, L. E. & Bashan, Y. 2008. Joint Immobilization of plant growth-promoting bacteria and green microalgae in alginate beads as an experimental model for studying plant-bacterium interactions. *Appl. Environ. Microbiol.* 74:6797–802.

de-Bashan, L. E. & Bashan, Y. 2010. Immobilized microalgae for removing pollutants: review of practical aspects. *Bioresour. Technol.* 101:1611–27.

de-Bashan, L. E., Bashan, Y., Moreno, M., Lebsky, V. K. & Bustillos, J. J. 2002. Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilense*. *Can. J. Microbiol.* 48:514–21.

de-Bashan, L. E., Hernandez, J. P., Morey, T. & Bashan, Y. 2004. Microalgae growth-promoting bacteria as ''helpers'' for microalgae: a novel approach for removing ammonium and phosphorus from municipal wastewater. *Water Res.* 38:466–74.

Del Gallo, M. & Haegi, A. 1990. Characterization and quantification of exocellular polysaccharides in *Azospirillum brasilense* and *Azospirillum lipoferum*. *Symbiosis* 9:155–61.

Eckardt, N. A. 2010. The *Chlorella* genome: big surprises from a small package. *Plant Cell* 22:2924.

Egorenkova, I. V., Konnova, S. A., Skvortsov, I. M. & Ignatov, V. V. 2000. Investigation of the initial stages of interaction of the bacterium *Azospirillum brasilense* with wheat seedling roots: adsorption and root hair deformation. *Microbiology (Moscow)* 69:103–8.

Fedonenko, Y. P., Zatonsky, G. V., Konnova, S. A., Zdorovenko, E. L. & Ignatov, V. V. 2002. Structure of the O-specific polysaccharide of the lipopolysaccharide of *Azospirillum brasilense* Sp245. *Carbohydr. Res.* 337:869–72.

Gonzalez, L. E. & Bashan, Y. 2000. Growth promotion of the microalga *Chlorella vulgaris* when coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium *Azospirillum brasilense*. *Appl. Environ. Microbiol.* 66:1527–31.

Gonzalez, L. E., Cañizares, R. O. & Baena, S. 1997. Efficiency of ammonia and phosphorus removal from a Colombian agroindustrial wastewater by the microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus*. *Bioresour. Technol.* 60:259–62.

Grilli Caiola, M., Canini, A., Botta, A. L. & Del Gallo, M. 2004. Localization of *Azospirillum brasilense* Cd in inoculated tomato (*Lycopersicon esculentum* Mill.) roots. *Ann. Microbiol.* 54:365–80.

Hartmann, A. & Hurek, T. 1988. Effect of carotenoid overproduction on oxygen tolerance of nitrogen fixation in *Azospirillum brasilense* Sp7. *J. Gen. Microbiol.* 134:2449–55.

Howard, D. W. & Smith, C. S. 1983. *Histological Techniques for Marine Bivalve Mollusks*. NOOA Technical Memorandum NMFS-F/NEC-25. U.S. Department of Commerce, Woodshole, Massachusetts, 102 pp.

Ilangovan, K., Cañizares-Villanueva, R. O., González-Moreno, S. & Voltolina, D. 1998. Effect of cadmium and zinc on respiration and photosynthesis in suspended and immobilized cultures of *Chlorella vulgaris* and *Scenedesmus acutus*. *Bull. Environ. Contam. Toxicol.* 60:936–43.

Imase, M., Watanabe, K., Aoyagi, H. & Tanaka, H. 2008. Construction of an artificial symbiotic community using a *Chlorella*-symbiont association as a model. *FEMS Microbiol. Ecol.* 63:273–82.

Konnova, S. A., Brykova, O. S., Sachkova, O. A., Egorenkova, I. V. & Ignatov, V. V. 2001. Protective role of the polysaccharide containing capsular components of *Azospirillum brasilense*. *Microbiology (Moscow)* 70:436–40.

Lebeau, T. & Robert, J. M. 2006. Biotechnology of immobilized micro algae: a culture technique for the future? *In* Rao, S. [Ed.] *Algal Cultures, Analogues of Blooms and Applications*. Science Publishers, Enfield, New Hampshire, pp. 801–37.

Lebsky, V. K., Gonzalez-Bashan, L. E. & Bashan, Y. 2001. Ultrastructure of coimmobilization of the microalga *Chlorella vulgaris* with the plant growth-promoting bacterium *Azospirillum brasilense* and with its natural associative bacterium *Phyllobacterium myrsinacearum* in alginate beads. *Can. J. Microbiol.* 47:1–8.

Lindow, S. E. & Brandl, M. T. 2003. Microbiology of the phyllosphere. *Appl. Environ. Microbiol.* 69:1875–83.

Makk, J., Beszteri, B., Ács, É., Márialigeti, K. & Szabó, K. 2003. Investigation on diatom-associated bacterial communities colonizing an artificial substratum in the river Danube. *Large Rivers* 14:249–65. (Arch. Hydrobiol. Suppl. 147).

Mata, T. M., Martins, A. A. & Caetano, N. S. 2010. Microalgae for biodiesel production and other applications: a review. *Renew. Sust. Energ. Rev.* 14:217–32.

Michiels, K. W., Croes, C. L. & Vanderleyden, J. 1991. Two different modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots. *J. Gen. Microbiol.* 137:2241–6.

Moter, A. & Göbel, U. B. 2000. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *J. Microbiol. Methods* 41:85–112.

Muñoz, R. & Guieysse, B. 2006. Algal–bacterial processes for the treatment of hazardous contaminants: a review. *Water Res.* 40:2799–815.

Oliveira, A. L. M., Stoffels, M., Schmid, M., Reis, V. M., Baldani, J. I. & Hartmann, A. 2009. Colonization of sugarcane plantlets by mixed inoculations with diazotrophic bacteria. *Eur. J. Soil Biol.* 45:106–13.

Oswald, W. J. 1992. Microalgae and wastewater treatment. *In* Borowitzka, M. A. & Borowitzka, L. J. [Eds.] *Microalgal Biotechnology*. Cambridge University Press, Cambridge, UK, pp. 305–28.

Park, Y., Je, K.-W., Lee, K., Jung, S.-E. & Choi, T.-J. 2008. Growth promotion of *Chlorella ellipsoidea* by co-inoculation with *Brevundimonas* sp. isolated from the microalga. *Hydrobiologia* 598:219–28.

Pinheiro, R. D., Boddey, L. H., James, E. K., Sprent, J. I. & Boddey, R. M. 2002. Adsorption and anchoring of *Azospirillum* strains to roots of wheat seedlings. *Plant Soil* 246:151–66.

Prasad, K. & Kadokawa, J.-I. 2009. Alginate-based blends and nano/microbeads. *Microbiol. Monographs* 13:175–210.

Puente, M. E., Holguin, G., Glick, B. R. & Bashan, Y. 1999. Root surface colonization of black mangrove seedlings by *Azospirillum halofraeference* and *Azospirillum brasilense* in seawater. *FEMS Microbiol. Ecol.* 29:283–92.

Reiner, O. & Okon, Y. 1986. Oxygen recognition in aerotactic behavior of *Azospirillum brasilense* Cd. *Can. J. Microbiol.* 32:829–34.

Rodriguez, H., Gonzalez, T., Goire, I. & Bashan, Y. 2004. Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp. *Naturwissenschaften* 91:552–5.

Sapp, M., Schwaderer, A. S., Wiltshire, K. H., Hoppe, H.-G., Gerdts, G. & Wichels, A. 2007. Species-specific bacterial communities in the phycosphere of microalgae? *Microb. Ecol.* 53:683–99.

Schmid, M., Iversen, C., Gontia, I., Stephan, R., Hofmann, A., Hartmann, A., Jha, B., Eberl, L., Riedel, K. & Lehner, A. 2009. Evidence for a plant-associated natural habitat for *Cronobacter* spp. *Res. Microbiol.* 60:608–14.

Skvortsov, I. M. & Ignatov, V. V. 1998. Extracellular polysaccharides and polysaccharide-containing biopolymers from *Azospirillum* species: properties and the possible role in interaction with plant roots. *FEMS Microbiol. Lett.* 165:223–9.

Smidsrød, O. & Skjåk-Bræk, G. 1990. Alginate as immobilization matrix for cells. *Trends Biotechnol.* 8:71–8.

Steenhoudt, O. & Vanderleyden, J. 2000. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiol. Rev.* 24:487–506.

Stoffels, M., Castellanos, T. & Hartmann, A. 2001. Design and application of new 16S rRNA-targeted oligonucleotide probes for the *Azospirillum-Skermanella-Rhodocista*-cluster. *Syst. Appl. Microbiol.* 24:83–97.

Tang, X., He, L. Y., Tao, X. Q., Dang, Z., Guo, C. L., Lu, G. N. & Yi, X. Y. 2010. Construction of an artificial microalgal-bacterial consortium that efficiently degrades crude oil. *J. Hazard. Mater.* 181:1158–62.

Yegorenkova, I. V., Konnova, S. A., Sachuk, V. N. & Ignatov, V. V. 2001. *Azospirillum brasilense* colonization of wheat roots and the role of lectin-carbohydrate interactions in bacterial adsorption and root-hair deformation. *Plant Soil* 231:275–82.

Zhulin, I. B. & Armitage, J. P. 1992. The role of taxis in the ecology of *Azospirillum*. *Symbiosis* 13:199–206.

Zhulin, I. B. & Armitage, J. P. 1993. Motility, chemokinesis, and methylation-independent chemotaxis in *Azospirillum brasilense*. *J. Bacteriol.* 175:952–8.