APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1995, p. 1013–1019 Vol. 61, No. 3
0099-2240/95/$04.00+0

In Situ Localization of *Azospirillum brasilense* in the Rhizosphere of Wheat with Fluorescently Labeled, rRNA-Targeted Oligonucleotide Probes and Scanning Confocal Laser Microscopy

BERNHARD ASSMUS,[1] PETER HUTZLER,[2] GUDRUN KIRCHHOF,[1] RUDOLF AMANN,[3] JOHN R. LAWRENCE,[4] AND ANTON HARTMANN[1]*

Institut für Bodenökologie[1] and Institut für Pathologie, biomedizinische Bildanalyse,[2] GSF-Forschungszentrum für Umwelt und Gesundheit, D-85764 Oberschleißheim, and Lehrstuhl für Mikrobiologie, Technische Universität München, D-80290 Munich,[3] Germany, and National Hydrology Research Institute, Environment Canada, Saskatoon, Saskatchewan, Canada S7N 3H5[4]

Received 22 August 1994/Accepted 13 October 1994

The colonization of wheat roots by *Azospirillum brasilense* was used as a model system to evaluate the utility of whole-cell hybridization with fluorescently labeled, rRNA-targeted oligonucleotide probes for the in situ monitoring of rhizosphere microbial communities. Root samples of agar- or soil-grown 10- and 30-day-old wheat seedlings inoculated with different strains of *A. brasilense* were hybridized with a species-specific probe for *A. brasilense*, a probe hybridizing to alpha subclass proteobacteria, and a probe specific for the domain *Bacteria* to identify and localize the target bacteria. After hybridization, about 10 to 25% of the rhizosphere bacteria as visualized with 4′,6-diamidino-2-phenylindole (DAPI) gave sufficient fluorescence signals to be detected with rRNA-targeted probes. Scanning confocal laser microscopy was used to overcome disturbing effects arising from autofluorescence of the object or narrow depth of focus in thick specimens. This technique also allowed high-resolution analysis of the spatial distribution of bacteria in the rhizosphere. Occurrence of cells of *A. brasilense* Sp7 and Wa3 was restricted to the rhizosphere soil, mainly to the root hair zone. C-forms of *A. brasilense* were demonstrated to be physiologically active forms in the rhizosphere. Strain Sp245 also was found repeatedly at high density in the interior of root hair cells. In general, the combination of fluorescently labeled oligonucleotide probes and scanning confocal laser microscopy provided a very suitable strategy for detailed studies of rhizosphere microbial ecology.

The rhizosphere is a habitat of extraordinary significance for biocoenosis in soils. Root exudates provide large amounts of easily degradable organic carbon sources which attract and support an abundant and highly active rhizosphere microflora (11). The rhizosphere exhibits plant-microorganism symbioses of crucial importance, e.g., the nodules induced by rhizobia in leguminous plants (56) and the mycorrhizae (1, 9). It plays a key role in the nutrition and health of plants. Rhizosphere populations and their physiological activities therefore contribute considerably to primary production in terrestrial ecosystems.

In addition to the true symbiotic plant-microorganism systems, a large variety of plant-associated soil bacteria with less intimate associations are able to enhance the growth of a wide range of economically important crops. This taxonomically diverse group is called plant growth-promoting rhizobacteria (24). *Azospirillum* is a genus of versatile plant growth-promoting rhizobacteria which colonizes the rhizosphere mainly of cereals in tropical and subtropical regions (34, 37). Their potentially plant-beneficial abilities include nitrogen fixation (48), nitrate reduction (19), and phytohormone production (18, 29). These plant growth-promoting capabilities led to growth stimulation in field experiments when proper inoculation techniques were used (35). However, failures and a lack of reproducibility have been reported (7). Therefore, more basic biological knowledge is needed to understand the rhizosphere interaction and colonization by *Azospirillum* species before a selection of strains that perform well under field conditions can be obtained (25).

For such a purpose, traditional microbial methods do not provide appropriate tools. Isolation of microorganisms from natural samples and cultivation do not allow an exact localization and often detect only a minor portion of naturally occurring microorganisms (50, 55). Traditional methods selectively alter the apparent contribution of one particular group to the whole community (53). Microscopic examination with classical stains, e.g., 4′,6-diamidino-2-phenylindole (DAPI) (38), or the use of enzymatic color-producing reactions, e.g., the reduction of 2,3,5-triphenyltetrazolium chloride (36), reveals the organisms in their natural habitat. However, the taxonomic affiliation of the observed bacterial cells is often uncertain. More recently, fluorescence-labeled probes such as antibodies (8) and rRNA-targeted oligonucleotides (3) have been introduced as tools in microbial ecology. These probes allow an in situ identification of bacteria, even if the bacteria had not been cultured before (4). Hybridization with fluorescently labeled rRNA-targeted probes has been performed to investigate microorganisms in aquatic ecosystems (20, 32), biofilms (5, 39, 40), and sediments (13, 47). However, experience with this technique in the soil habitat is limited at the moment (16). One reason for this is the low physiological activity of many soil microorganisms. Cells may have entered a quiescent state (31) which results in a low ribosome content (43). The amount of

* Corresponding author. Mailing address: Institut für Bodenökologie, GSF-Forschungszentrum für Umwelt und Gesundheit, Ingolstädter Landstr. 1, D-85764 Oberschleißheim, Germany. Phone: 49 (89) 3187-3415. Fax: 49 (89) 3187-3376.

rRNA is directly correlated to the fluorescence conferred by rRNA-targeted probes (12), and hence a low physiological activity may result in a weak or even undetectable hybridization signal. The second challenge when investigating soil- or plant-associated microorganisms with fluorescent probes is the severe autofluorescence of plant material and mineral particles (16, 17). Furthermore, an exact localization of the bacteria in thick samples remains difficult because of problems with limited depth of focus.

Scanning confocal laser microscopy (SCLM) should circumvent the last two problems. In this technique, which has been reviewed recently (46, 49), the specimen is scanned with a focused laser beam and the fluorescent signals are detected by a photomultiplier. A confocal pinhole allows only signals arising from the focused plane to be detected. With SCLM, nondestructive optical sections of a sample are obtained and disturbing effects from out-of-focus fluorescence are reduced (57). SCLM has already been used in microbial ecology to study the spatial structure of microbial biofilms (10, 28, 58) and recently was combined with the use of fluorescent antibodies (44) or oligonucleotide probes (14, 52, 54). In this work, we used the root colonization of monoxenic and soil-grown wheat seedlings by *Azospirillum brasilense* as a model system for the in situ monitoring of rhizosphere bacteria with fluorescently labeled rRNA probes and SCLM.

MATERIALS AND METHODS

Bacterial strains and media. *A. brasilense* Sp7 was obtained from the German Collection of Microorganisms, Brunswick, Germany. Strains Sp245 and Wa3 were kindly provided by J. Döbereiner, EMBRAPA, Rio de Janeiro, Brazil, and C. Christiansen-Weniger, Wageningen, The Netherlands, respectively. The bacteria were grown at 30°C in minimal medium containing (in grams per liter) KH_2PO_4, 0.15; K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.25; $CaCl_2 \cdot 2H_2O$, 0.15; and sodium succinate, 5.0. For solid medium, 0.5 g of NH_4Cl per liter and 12 g of agar per liter were added. Trace element and ferric iron solutions were prepared by the method used for Nfb medium (27), and 1 ml was added to the medium after autoclaving. The final pH was adjusted to 6.8.

Inoculation and growth of wheat seedlings. Seeds of wheat cultivar PF839197 provided by J. I. Baldani, EMBRAPA, were germinated for 2 days on moistened paper at room temperature and then transferred to tubes filled with approximately 40 g of unsterilized soil (sandy loam; 44% sand, 38% silt, 18% clay, 1.12% total organic carbon). The tubes were drained to ensure unsaturated conditions. At the time of planting, inoculation was performed: *A. brasilense* cells were harvested at mid-logarithmic growth phase and washed in sterile phosphate-buffered saline (PBS; pH 7.2), and approximately 10^8 cells were added to each tube. The seedlings were grown in a greenhouse at 28°C and moistened every 3 days with 5 ml of distilled water.

Monoxenic cultures were grown in agar tubes containing medium as described above, except that succinate and NH_4^+ were omitted and only 8 g of agar per liter was used. The medium was autoclaved in the tubes and kept at 42°C. Approximately 10^8 washed cells from an overnight culture of the desired strain were added. The tube was gently shaken and put on ice. After the agar had solidified, a sterilized germinated wheat seed was placed on the surface with sterile forceps. The tubes were sealed with sterile cotton. The grains were sterilized as follows (6): seeds were treated with 95% ethanol for 1 min and then with acidified hypochlorite (solution A [5% NaOCl in water], 20 ml; solution B [1 M KH_2PO_4, 160 ml; concentrated HCl, 40 ml; sterile water, 50 ml], 4 ml; solution C [Tween 80, 1%, vol/vol], 2 ml; sterile water, 174 ml) for 5 min. The seeds were washed four times for 1 min in sterile water and then immersed in sterile water for 4 h. Treatment with acidified hypochlorite and the washing steps were repeated, and the seeds were kept in 30% hydrogen peroxide for 5 min, washed again four times with sterile water, and immersed for a further 1 h in sterile water. Then the seeds were germinated on Luria broth agar plates in the dark at room temperature for 2 days. Only seeds from plates on which no contaminating microorganisms were observed were chosen. This procedure prevented growth of contaminants in most cases. However, it could not always prevent the occurrence of yeasts and slender spore-forming rods.

Monoxenic cultures were also performed in tubes containing sterile quartz sand (average grain size, 0.8 mm) and liquid medium as described above but without addition of succinate.

Preparation of samples and slides. After 10 and 30 days, seedlings were carefully removed from the tubes and the roots were washed by shaking in sterile PBS. Standard fixation and dehydration procedures (3) were slightly modified, as follows. Root pieces 15 to 25 mm in length from all parts of the root system were transferred to fixation buffer (4% paraformaldehyde in PBS) and fixed for 2 h at 28°C. Samples were washed and dehydrated in 50, 80, and 96% ethanol (5 min each) and then immobilized on gelatin-coated slides (3) with a droplet of 0.25% agarose on both ends. After being dried, slides could be stored at room temperature for several months.

Probes. The following oligonucleotide probes were used: (i) EUB338, complementary to a region of the 16S rRNA specific for the domain *Bacteria* (2); EUK1379, complementary to a region of the 18S rRNA of the domain *Eucarya* (20); (iii) ALF1b, complementary to a region in the 16S rRNA conserved in the alpha subclass of *Proteobacteria* and a few other bacteria (33); and (iv) AB, complementary to a region of the 23S rRNA of *A. brasilense* (23).

Oligonucleotide probes were synthesized with a C6-TFA amino-linker [6-(trifluoroacetylamino)hexyl(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite] at the 5′ end (MWG Biotech, Ebersberg, Germany), labeled with tetramethylrhodamine-5-isothiocyanate (TRITC; Molecular Probes, Eugene, Oreg.), and purified as described by Amann et al. (3) or purchased already coupled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester and purified by high-pressure liquid chromatography (FLUOS; MWG Biotech). In the latter case, the purification procedure (3) started with the separation of labeled and unlabeled oligonucleotides in a polyacrylamide gel. The probes were finally dissolved in TE buffer (10 mM Tris hydrochloride [pH 7.2], 1 mM EDTA) to a final concentration of 50 μg ml^{-1} and stored at −20°C.

The DNA-specific dye DAPI (Sigma) was stored in a 0.35-mg ml^{-1} aqueous stock solution at 4°C.

In situ hybridization. The hybridization procedure followed in principle the method published by Manz et al. (33). The root pieces were immersed in 15 μl of hybridization buffer (0.9 M NaCl, 20 mM Tris hydrochloride [pH 7.2], 0.01% sodium dodecyl sulfate [SDS], 5 mM EDTA). The buffer contained 20% formamide if probe ALF1b was used. Then 2 μl of probe solution was added. The slides were incubated for 2 h at 46°C in an equilibrated humidity chamber. The probes were removed with 5 ml of washing solution (20 mM Tris, 0.01% SDS, 5 mM EDTA, 0.9 M NaCl [180 mM NaCl when probe ALF1b was used]), and the slides were immersed in 50 ml of washing solution at 48°C for 20 min. The slides were then rinsed with distilled water and allowed to air dry. If counterstaining with DAPI was desired (38), the stock solution was diluted 500-fold in distilled water and 20 μl of the working solution was applied to each root piece. After incubation for 10 min at room temperature, the slides were rinsed with distilled water, air dried, and mounted in antifading solution (22).

Epifluorescence microscopy was performed with an Axioplan microscope (Zeiss, Oberkochen, Germany) equipped with filter sets 01, 09, and 15. Color micrographs were taken on Kodak Ektachrome P1600 color reversal film. Exposure times were 0.03 s for phase-contrast micrographs and 4 to 45 s for epifluorescence micrographs.

SCLM. An LSM 410 inverted scanning confocal laser microscope (Zeiss) equipped with three lasers (Ar ion, UV; Ar ion, visible; and HeNe, supplying excitation wavelengths at 365, 488, and 543 nm, respectively) was used to record optical sections. A 100× oil immersion lens (NA 1.3) was used. Monochrome sequences of images were taken along the optical axis (*z* axis) with increments between 0.7 and 1.2 μm. For sagittal images (*z* scans), *z* increments of 0.15 μm were chosen. Artificial-color images were rearranged from sequentially recorded monochrome images or projections of *z* sequences. Red-green anaglyphs were calculated from monochrome sequences. Colored three-dimensional reconstructions from polyfluorescent image sequences were obtained by first calculating monochrome stereo pairs and then combining the results within a true-color red-green-blue display (rgb display). All image combining, processing, and analysis was performed with the standard software package provided by Zeiss.

Preliminary experiments were made with an MRC 600 scanning confocal laser microscope (Bio-Rad Microscience, Toronto, Ontario, Canada) equipped with an Ar ion laser (excitation wavelength, 488 nm).

RESULTS

General remarks. The fluorescent signal of the hybridized bacteria allowed detection of azospirilla on root samples from agar-, quartz sand-, or soil-grown wheat seedlings. Unstained bacterial cells in control experiments exhibited only a very weak autofluorescence within the excitation wavelengths used. This background was not increased by hybridization experiments with the eucaryotic probe EUK1379. This assay ensured that no nonspecific adsorption of oligonucleotides, e.g., on cell wall components, occurred.

Staining *A. brasilense* cells with the species-specific AB probe resulted in only a mediocre signal. The fluorescence intensity was high enough for detection in pure cultures and on root samples from monoxenic agar-grown seedlings. However, when the probe was applied to azospirilla colonizing roots of plants grown in soil microcosms or monoxenically in quartz

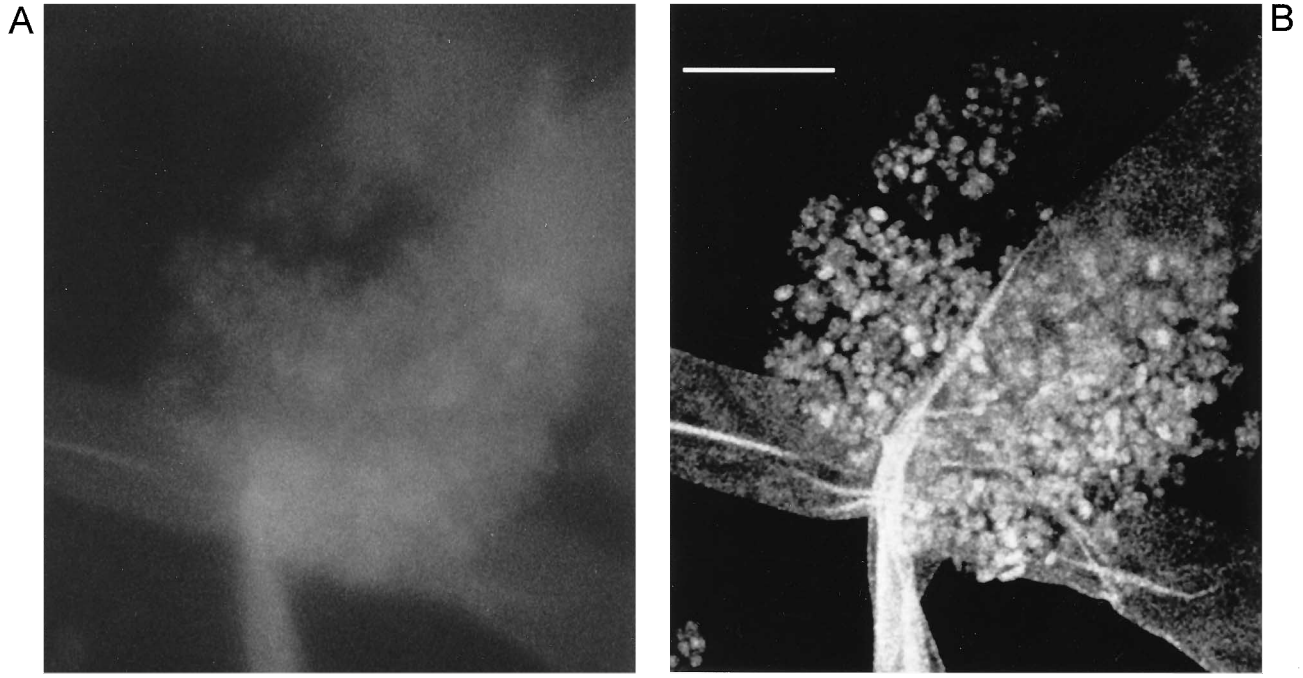


FIG. 1. Cell clump of *A. brasilense* Sp7 in the root hair zone of an agar-grown wheat seedling. Bacteria were hybridized with TRITC-labeled probe AB. (A) Epifluorescence micrograph. (B) Confocal image of the same site, showing a projection of a *z* sequence (7 μm). The excitation wavelength was 543 nm. Bar, 15 μm.

sand-filled tubes, the hybridization signal was too low for documentation, although the same cells bound readily detectable amounts of probes ALF1b or EUB338. Signal quantification by SCLM on pure cultures of *A. brasilense* Sp7 and Sp245 revealed that the signal intensity of probe AB was approximately 40% of that of probe EUB338 and 50% of that of probe ALF1b.

Comparison between epifluorescence and scanning laser microscopy. The root material exhibited strong autofluorescence regardless of the excitation wavelengths used. This background was further increased during the fixation and dehydration procedure. Also, clay and organic particles which became suspended in the mounting solution severely hampered conventional epifluorescence microscopy. Therefore, studies were restricted to a minor part of the root hair zone. Bacteria colonizing the surface of the main root could rarely be detected. Furthermore, the viewing and the documentation of thick hybridized samples (usual thickness range, 0.5 to 1 mm) were severely limited by "out-of-focus blur," and the exact localization of bacteria was doubtful in many cases.

In contrast, these problems could be largely overcome if SCLM was used. The images obtained included only signals from focused planes. Projections of *z* sequences resulted in reconstructions of the whole scanned part of the specimen in focus. The significantly decreased background signal and further contrast enhancement procedures lead to a considerable improvement in image quality (Fig. 1 and 2). In addition, colors could be assigned to the original images collected at various excitation wavelengths, and when these images were combined in an rgb display, the results of double or even triple labeling could be viewed in a single image (see Fig. 3).

The spatial arrangement of a scanned sample was confirmed by *z* scans (see Fig. 3C), which, for example, could distinguish between bacteria colonizing the interior of a root hair cell and those enveloped only by the crumpled cell wall of a collapsed root hair. For direct visualization of the three-dimensional information, anaglyphs from monochrome *z* sequences which could be viewed with red-green glasses or colored stereo pairs from double- or triple-stained samples were calculated (see Fig. 4).

Colonization patterns and occurrence of C-forms. *A. brasilense* cells appeared throughout the root samples of wheat seedlings grown monoxenically in agar tubes. The bacteria colonized root hairs as well as primary and secondary root surfaces (Fig. 2) but were most abundant in the root hair zone. In roots from 10-day-old seedlings, they appeared mainly as microcolonies, whereas in samples from 30-day-old plants, they formed dense layers and clumps (Fig. 1). The same colonization patterns occurred on monoxenic plants in quartz sand and in nonsterile soil microcosms (Fig. 2), where the azospirilla were stained with the group-specific probe ALF1b. Root samples from soil were also colonized by various other bacteria and occasionally fungal hyphae. About 10 to 25% of the DAPI-stained cells also gave a signal with the bacterial consensus probe. Within the latter, azospirilla were recognized by hybridization with probe ALF1b in combination with typical morphology and growth in cell clumps. Even after inoculation, azospirilla constituted only a small proportion of the rhizosphere bacterial community.

In the rhizosphere of plants grown in quartz sand and occasionally also of those grown in agar, cell clumps were observed which consisted to a remarkable degree of enlarged cyst-like cells specifically hybridizing with probes ALF1b or AB, respectively (Fig. 3A). Interestingly, these cells exhibited the same signal strength as the normal ovoid to rod-shaped bacteria, which indicates high physiological activity. When wheat seedlings had been inoculated with *A. brasilense* Sp245, specifically stained cells were repeatedly found at high density in the interiors of noncollapsed root hairs (Fig. 3B and C). The cell walls of these root hairs were not obviously disrupted. This phenomenon was not observed in every sample, but when it was detected, up to one-third of the root hairs were affected.

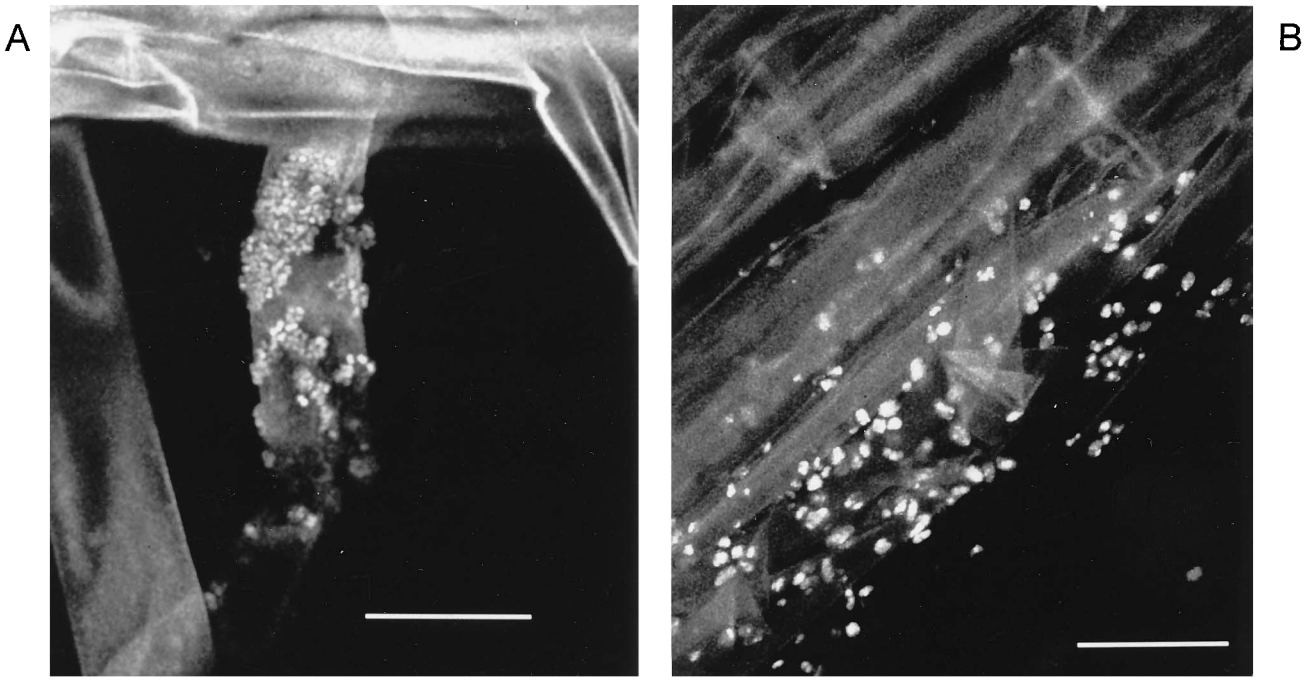


FIG. 2. Root hair of a soil-grown wheat seedling (A) and root surface of a quartz sand-grown wheat seedling (B) colonized by *A. brasilense* Sp245. Bacteria were hybridized with TRITC-labeled probe ALF1b. The excitation wavelength was 543 nm. Bars, 20 μm (A) and 10 μm (B).

The highest densities of bacteria were always in the tip of the invaded root hairs. In root samples from the soil microcosms, bacteria not hybridizing to probe ALF1b also were found in root hairs. ALF1b-stained bacteria showed up inside root hair cells only if the seedling had been inoculated with strain Sp245. No bacteria could be detected inside cortex or xylem cells of the root cylinder. Cells of strains Sp245 and Wa3, however, could be seen below the outer cortex cells in intercellular spaces of inoculated plants (data not shown).

DISCUSSION

SCLM enabled us to achieve greater insight into the spatial distribution of introduced and naturally occurring bacteria in the rhizosphere of wheat seedlings. The image quality was significantly improved by reducing background fluorescence via single-spot excitation. In addition, optical sections completely in focus are produced and can be combined into a multicolor extended-focus image. With SCLM and the commonly included software, three-dimensional information is obtained and can be visualized in a convenient way, e.g. by red-green anaglyphs or colored stereo image pairs. SCLM is therefore an important tool, especially in combination with specific molecular and serological probes, for in situ studies in microbial ecology.

A prerequisite for the successful use of rRNA-targeted probes for the detection of microorganisms is the presence of a sufficiently large number of ribosomes in the target cells. The results obtained clearly demonstrated that the ribosome content of a significant part of the rhizosphere microorganisms was high enough to render them detectable by in situ hybridization with fluorescent oligonucleotide probes. This is in contrast to the situation in bulk soil, where without nutrient addition only a very small fraction of the DAPI-stained cells could be detected (16). It also became evident that the enlarged cyst-like forms of *A. brasilense* that have been repeatedly reported to occur in the rhizosphere of colonized plant roots (37) contain ample ribosomes and consequently are physiologically active cells and not dormant forms. Previously, this was shown only in liquid cultures, where cyst-like forms of *A. brasilense*, which can be obtained by specific cultivation conditions (42), exhibited some nitrate reductase activity (51). Cyst-like forms appeared mainly in the rhizosphere of sand-grown plants. The large pore volume in sand-filled microcosms may have allowed increased oxygen access to the bacteria compared with the situation in agar or soil. Thus, oxygen protection of nitrogenase might be the primary function of the cell envelope of these cyst-like forms.

The less intense fluorescence conferred by the *A. brasilense*-specific probe AB compared with probes EUB338 and ALF1b may be due to a higher-order structure in the fixed ribosomes partially covering the target site of the 23S rRNA in situ. The search for other suitable target sequences which may be more readily accessible should resolve this problem. With the group-specific probe ALF1b, the colonization behavior of azospirilla could be monitored successfully. We confirmed that strain Sp245 enters the interior of root hair cells which had apparently intact cell walls. The ability of this strain to penetrate root cell walls had been suggested for many years because of the original isolation from surface-sterilized roots (37), and it was recently shown by detection with monoclonal antibodies in ultrathin sections of the root cylinder (45). In this study, we could not detect specifically stained cells of *A. brasilense* Sp245 within cells of the root cylinder. This could be due to a limited accessibility of the cell lumen of the vascular system by the probes rather than to a lack of invasive potential of this strain. To demonstrate internal colonization in the xylem, an improvement of sample processing, e.g., by enzymatic plant cell wall digestion, will be necessary.

It is not known whether the invasion of root hair cells by *A. brasilense* Sp245 is the initial step in the colonization of root cortex and xylem cells analogous to root nodulation by rhizobia

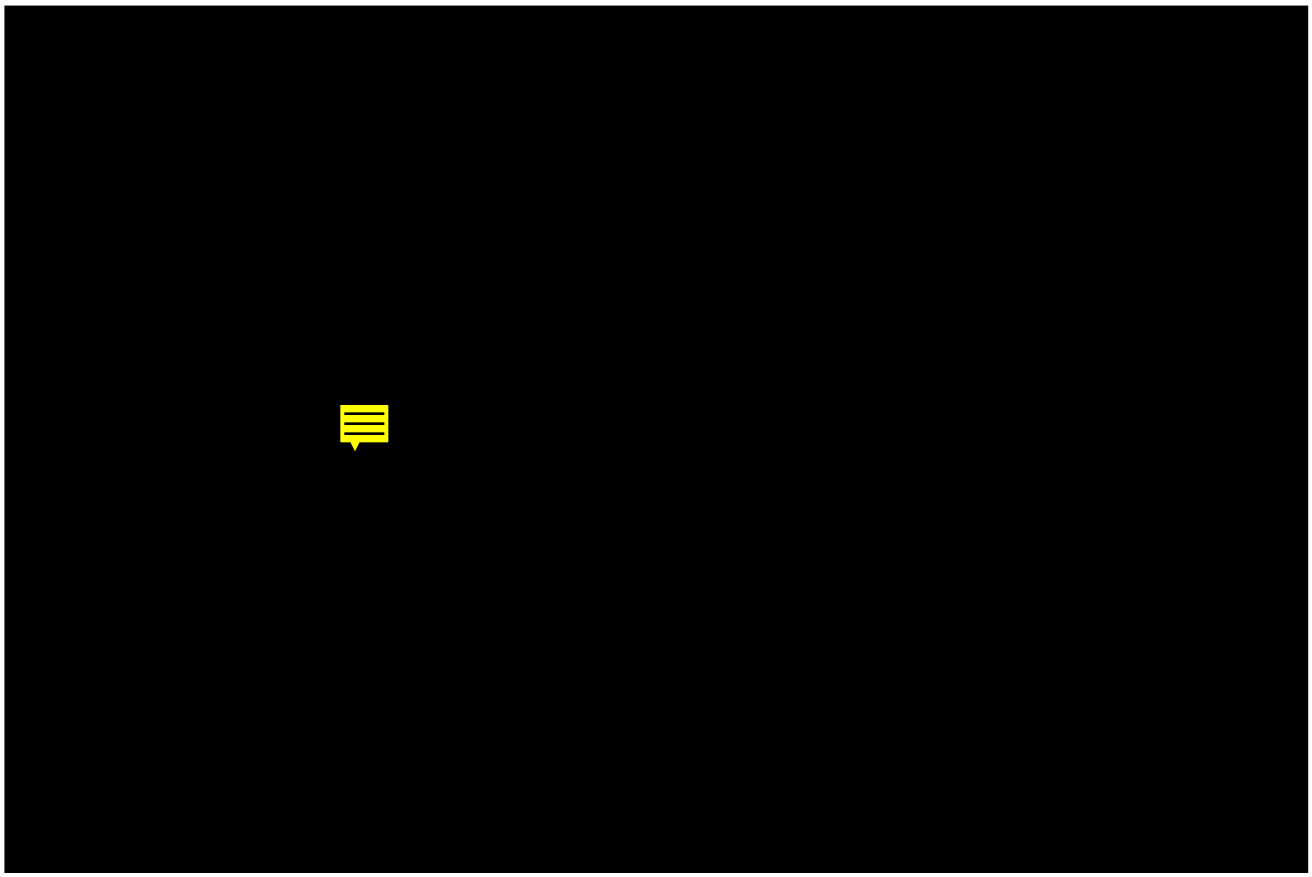


FIG. 3. (A) Tight cell clump of *A. brasilense* Sp245 adhering to a bundle of root hairs of a quartz sand-grown wheat seedling. Staining was carried out with TRITC-labeled probe ALF1b. Excitation wavelengths were 543 and 488 nm for red and green fluorescence, respectively. Projection of *z* sequences (28 μm) is displayed as an rgb image. Bar, 15 μm. Note the enlarged cyst-like cell forms (arrow). (B and C) Root hair zone of a soil-grown wheat seedling, colonized by various bacteria including *A. brasilense* Sp 245 growing internally in a root hair (arrow). Staining was carried out with TRITC-labeled probe ALF1b, FLUOS-labeled probe EUB338, and DAPI. Excitation wavelengths were 543, 488, and 365 nm for red, green, and blue fluorescence, respectively. Signals are displayed as rgb images. Bar, 15 μm (bar applies to panels B and C). (B) *xy* scan. (C) *z*-scan; depth, 15 μm. The yellow line in panel B indicates the approximate orientation of the *z* scan.

FIG. 4. Root hair zone of a soil-grown wheat seedling including a colony of *A. brasilense* Sp245. Staining and excitation were as described for Fig. 3B and C. A three-dimensional reconstruction of a projection of *z* sequences (12 μm) is displayed as a colored stereo pair. Bars, 15 μm.

or whether *A. brasilense* organisms enter the vascular system through lesions. Probably, the invasive potential of a plant growth-promoting rhizobacterium is of key importance because the organism escapes competition among rhizosphere bacteria and achieves close contact with the host. Colonization of the root interior has been reported for a number of diazotrophic plant growth-promoting rhizobacterium genera, including *Azospirillum*, *Herbaspirillum*, *Acetobacter* (15), *Azoarcus* (41), and *Alcaligenes* (59). Colonization of the endorhizosphere was previously monitored by using either polyclonal antisera (30, 41) or activity of reporter genes in genetically engineered derivatives of the investigated strain (21, 26), mostly in monoxenic plant cultures and on thin sections, which may have damaged the viewed plant cells. There are few examples of monitoring a naturally grown endophytic microorganism via whole-cell hybridization with rRNA-directed probes; one involves squashed nodules of *Alnus* spp. colonized by *Frankia* spp. (17). Our study demonstrates that the combination of rRNA-directed oligonucleotide probes and SCLM now enables in situ investigations of specifically stained rhizobacteria from soil-grown plants by using relatively undisturbed samples. This strategy was introduced very recently for the specific localization of bacteria in marine bivalves (14) and in activated-sludge flocs (52, 54) and has now been extended to the rhizosphere habitat. Investigations of bacteria in their specific microniches in a wide range of habitats should be possible by this technique.

ACKNOWLEDGMENTS

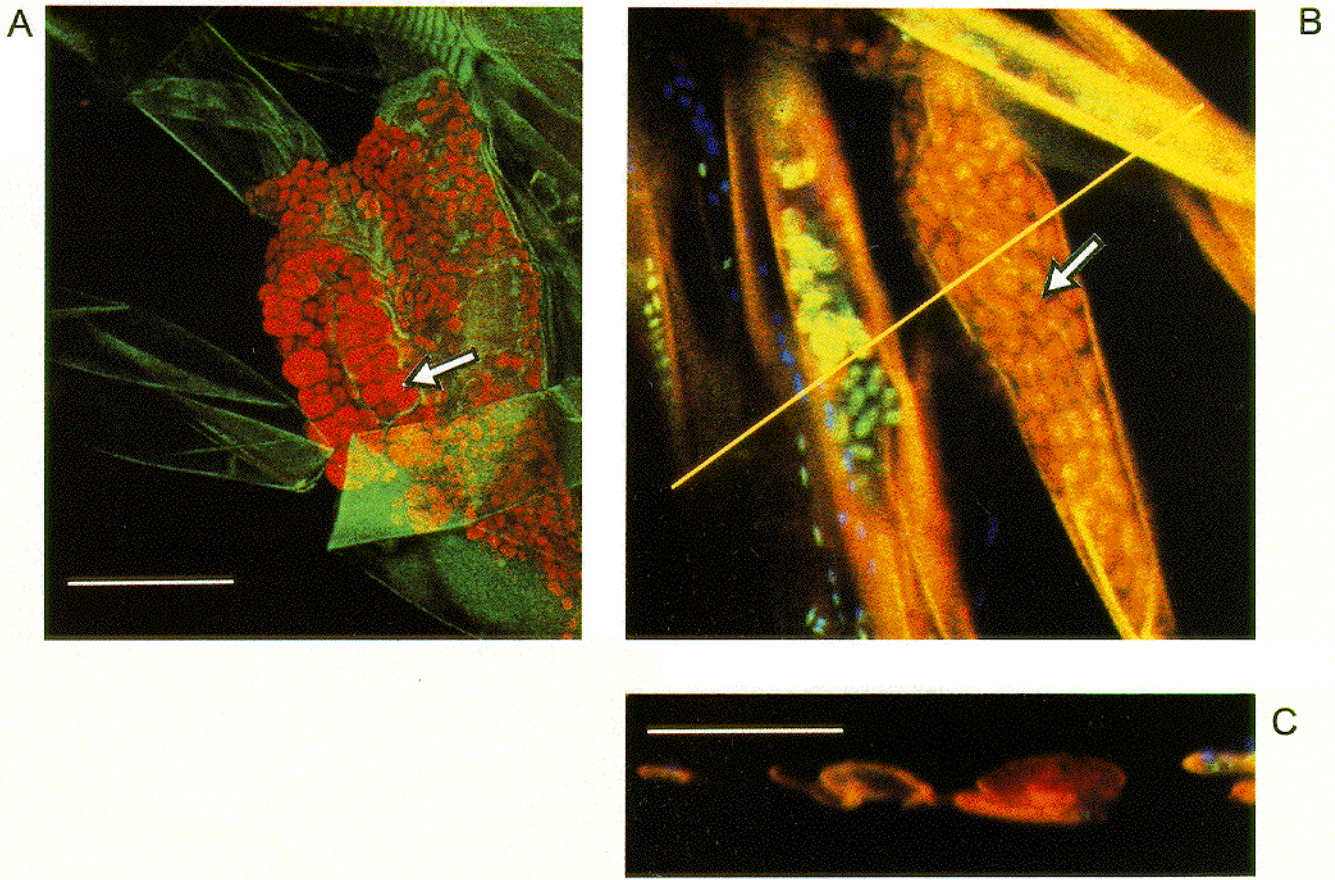
This work was supported by the Deutsche Forschungsgemeinschaft and the Canadian-German scientific and technological research cooperation (CAN ENV 27).

We thank A. Voss, GSF, Oberschleißheim, Germany, and G. Swerhone, NHRI, Saskatoon, Canada, for their excellent technical support. We also appreciate support and helpful suggestions by J. Döbereiner, EMBRAPA, Rio de Janeiro, Brazil.

REFERENCES

1. **Abbott, L. K., and C. Gazey.** 1994. An ecological view of the formation of VA mycorrhizas. Plant Soil **159:**69–78.
2. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
3. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
4. **Amann, R. I., N. Springer, W. Ludwig, H.-D. Görtz, and K.-H. Schleifer.** 1991. Identification *in situ* and phylogeny of uncultured bacterial endosymbionts. Nature (London) **351:**161–164.
5. **Amann, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl.** 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl. Environ. Microbiol. **58:**614–623.
6. **Baldani, J. I. (EMBRAPA, Rio de Janeiro, Brazil).** 1992. Personal communication.
7. **Bashan, Y., and H. Levanony.** 1990. Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture. Can. J. Microbiol. **36:**591–608.
8. **Bohlool, B. B., and E. L. Schmidt.** 1980. The immunofluorescence approach in microbial ecology. Adv. Microb. Ecol. **4:**203–241.
9. **Bowen, G. D.** 1994. The ecology of ectomycorrhiza formation and functioning. Plant Soil **159:**61–67.
10. **Caldwell, D. E., D. R. Korber, and J. R. Lawrence.** 1992. Confocal laser microscopy and digital image analysis in microbial ecology. Adv. Microb. Ecol. **12:**1–67.
11. **Campbell, R., and M. P. Greaves.** 1990. Anatomy and community structure of the rhizosphere, p. 11–34. *In* J. Lynch (ed.), The rhizosphere. John Wiley & Sons, Inc., New York.
12. **DeLong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243:**1360–1363.
13. **DiChristina, T. J., and E. F. DeLong.** 1993. Design and application of rRNA-targeted oligonucleotide probes for the dissimilatory iron- and manganese-reducing bacterium *Shewanella putrefaciens*. Appl. Environ. Microbiol. **59:**4152–4160.
14. **Distel, D. L., and C. M. Cavanaugh.** 1994. Independent phylogenetic origins of methanotrophic and chemolithoautotrophic bacterial endosymbioses in marine bivalves. J. Bacteriol. **176:**1932–1938.
15. **Döbereiner, J.** 1992. History and new perspectives of diazotrophs in association with non-leguminous plants. Symbiosis **13:**1–13.
16. **Hahn, D., R. I. Amann, W. Ludwig, A. D. L. Akkermans, and K.-H. Schleifer.** 1992. Detection of micro-organisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. J. Gen. Microbiol. **138:**879–887.
17. **Hahn, D., R. I. Amann, and J. Zeyer.** 1993. Whole-cell hybridization of *Frankia* strains with fluorescence- or digoxigenin-labeled, 16S rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. **59:**1709–1716.
18. **Hartmann, A., M. Singh, and W. Klingmüller.** 1983. Isolation and characterization of *Azospirillum* mutants excreting high amounts of indolacetic acid. Can. J. Microbiol. **29:**916–923.
19. **Hartmann, A., and W. Zimmer.** 1994. Physiology of *Azospirillum*, p. 15–41. *In* Y. Okon (ed.), *Azospirillum*/plant associations. CRC Press, Inc., Boca Raton, Fla.
20. **Hicks, R. E., R. I. Amann, and D. A. Stahl.** 1992. Dual staining of natural bacterioplankton with 4′,6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. **58:**2158–2163.
21. **Hurek, T., B. Reinhold-Hurek, M. van Montgau, and E. Kellenberger.** 1994. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. J. Bacteriol. **176:**1913–1923.
22. **Johnson, G. D., and G. M. de C. Nogueira Araujo.** 1981. A simple method of reducing the fading of immunofluorescence during microscopy. J. Immunol. Methods **43:**349–350.
23. **Kirchhof, G., and A. Hartmann.** 1992. Development of gene-probes for *Azospirillum* based on 23S-rRNA sequences. Symbiosis **13:**27–35.
24. **Kloepper, J. W.** 1994. Plant growth-promoting rhizobacteria (other systems), p. 137–166. *In* Y. Okon (ed.), *Azospirillum*/plant associations. CRC Press, Inc., Boca Raton, Fla.
25. **Kloepper, J. W., and C. J. Beauchamp.** 1992. A review of issues related to measuring colonization of plant roots by bacteria. Can. J. Microbiol. **38:** 1219–1232.
26. **Kluepfel, D. A.** 1993. The behavior and tracking of bacteria in the rhizosphere. Annu. Rev. Phytopathol. **31:**441–472.
27. **Krieg, N. R., and J. Döbereiner.** 1984. Genus *Azospirillum*, p. 94–104. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
28. **Lawrence, J. R., D. R. Korber, B. D. Hoyle, J. W. Costerton, and D. E. Caldwell.** 1991. Optical sectioning of microbial biofilms. J. Bacteriol. **173:** 6558–6567.
29. **Lebuhn, M., and A. Hartmann.** 1994. Production of auxin and L-tryptophan related indolic and phenolic compounds by *Azospirillum brasilense* and *Azospirillum lipoferum*, p. 145–147. *In* M. H. Ryder, P. M. Stephens, and G. D. Bowen (ed.), Improving plant productivity with rhizosphere bacteria. Proceedings of the 3rd International Workshop on Plant Growth-Promoting Rhizobacteria. Commonwealth Scientific and Industrial Research Organisation, Adelaide, Australia.
30. **Levanony, H., Y. Bashan, B. Romano, and E. Klein.** 1989. Ultrastructural localization and identification of *Azospirillum brasilense* Cd on and within wheat root by immuno-gold labeling. Plant Soil **117:**207–218.
31. **Lewis, D. L., and D. K. Gattie.** 1991. The ecology of quiescent microbes. ASM News **57:**27–32.
32. **Lim, E. L., L. A. Amaral, D. A. Caron, and E. F. DeLong.** 1993. Application of rRNA-based probes for observing marine nanoplanktonic protists. Appl. Environ. Microbiol. **59:**1647–1655.
33. **Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer.** 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst. Appl. Microbiol. **15:**593–600.
34. **Michiels, K., J. Vanderleyden, and A. Van Gool.** 1989. *Azospirillum*-plant root associations: a review. Biol. Fertil. Soils **8:**356–368.
35. **Okon, Y., and C. A. Labandera-Gonzalez.** 1994. Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biol. Biochem. **26:**1591–1601.
36. **Patriquin, D. G., and J. Döbereiner.** 1978. Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. Can. J. Microbiol. **24:**734–742.
37. **Patriquin, D. G., J. Döbereiner, and D. K. Jain.** 1983. Sites and processes of associations between diazotrophs and grasses. Can. J. Microbiol. **29:**900–915.
38. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:**943–948.
39. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:**1354–1360.
40. **Ramsing, N. B., M. Kühl, and B. B. Jörgensen.** 1993. Distribution of sulfate-reducing bacteria, O_2 and H_2S in photosynthetic biofilms determined by

oligonucleotide probes and microelectrodes. Appl. Environ. Microbiol. **59:** 3840–3849.
41. **Reinhold, B., T. Hurek, and I. Fendrik.** 1987. Cross-reaction of predominant nitrogen-fixing bacteria with enveloped, round bodies in the root interior of Kallar grass. Appl. Environ. Microbiol. **53:**889–891.
42. **Sadasivan, L., and C. A. Neyra.** 1985. Flocculation in *Azospirillum brasilense* and *A. lipoferum*: exopolysaccharides and cyst formation. J. Bacteriol. **163:** 716–723.
43. **Schaechter, M. O., O. Maaloe, and N. O. Kjeldgaard.** 1958. Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. **19:**592–606.
44. **Schloter, M., R. Borlinghaus, W. Bode, and A. Hartmann.** 1993. Direct identification, and localization of *Azospirillum* in the rhizosphere of wheat using fluorescence-labelled monoclonal antibodies and confocal scanning laser microscopy. J. Microsc. **171:**173–177.
45. **Schloter, M., G. Kirchhof, U. Heinzmann, J. Döbereiner, and A. Hartmann.** 1994. Immunological studies of the wheat-root-colonization by the *Azospirillum brasilense* strains Sp7 and Sp245 using strain-specific monoclonal antibodies, p. 291–297. *In* N. A. Hegazi, M. Fayez, and M. Monib (ed.), Nitrogen fixation with non-legumes. American University of Cairo Press, Cairo, Egypt.
46. **Shotton, D., and N. White.** 1989. Confocal scanning microscopy: three-dimensional biological imaging. Trends Biochem. Sci. **14:**435–439.
47. **Spring, S., R. Amann, W. Ludwig, K.-H. Schleifer, H. van Gemerden, and N. Petersen.** 1993. Dominating role of an unusual magnetotactic bacterium in the microaerobic zone of a freshwater sediment. Appl. Environ. Microbiol. **59:**2397–2403.
48. **Tarrand, J. J., N. R. Krieg, and J. Döbereiner.** 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov., and two species, *Azospirillum lipoferum* (Beijerink) comb. nov. and *Azospirillum brasilense* sp. nov. Can. J. Microbiol. **24:**967–980.
49. **Tekola, P., Q. Zhu, and J. P. A. Baak.** 1994. Confocal laser microscopy and image processing for three-dimensional microscopy. Hum. Pathol. **25:**12–21.
50. **Torsvik, V., J. Goksöyr, and F. L. Daae.** 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56:**782–787.
51. **Ückert, J., J. Döbereiner, I. Fendrik, and E.-G. Niemann.** 1991. Nitrate reductase activity of *Azospirillum brasilense* Sp7 and Sp245 and C-forms in continuous culture, p. 249–254. *In* M. Polsinelli, R. Materassi, and M. Vincenzini (ed.), Nitrogen fixation. Kluwer Academic Publishers, Dordrecht, The Netherlands.
52. **Wagner, M., R. Amann, P. Kämpfer, B. Aßmus, A. Hartmann, P. Hutzler, N. Springer, and K.-H. Schleifer.** 1994. Identification and *in situ* detection of gram-negative filamentous bacteria in activated sludge. Syst. Appl. Microbiol. **17:**405–417.
53. **Wagner, M., R. Amann, H. Lemmer, and K.-H. Schleifer.** 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. **59:**1520–1525.
54. **Wagner, M., B. Aßmus, R. Amann, P. Hutzler, and A. Hartmann.** *In situ* analysis of microbial consortia in activated sludge using fluorescently labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. J. Microsc., in press.
55. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature (London) **345:**63–65.
56. **Werner, D.** 1992. Physiology of nitrogen-fixing legume nodules: compartments and functions, p. 399–431. *In* G. Stacey, R. H. Burris, and H. J. Evans (ed.), Biological nitrogen fixation. Chapman & Hall, New York.
57. **White, J. G., W. B. Amos, and M. Fordham.** 1987. An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. J. Cell Biol. **105:**41–48.
58. **Wolfaardt, G. M., J. R. Lawrence, R. D. Robarts, S. J. Caldwell, and D. E. Caldwell.** 1994. Multicellular organization in a degradative biofilm community. Appl. Environ. Microbiol. **60:**434–446.
59. **You, C., and F. Zhou.** 1989. Non-nodular endorhizospheric nitrogen fixation in wetland rice. Can. J. Microbiol. **35:**403–408.


A
B
C