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Root Colonization and Systemic Spreading of *Azoarcus* sp. Strain BH72 in Grasses

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The invasive properties of *Azoarcus* sp. strain BH72, an endorhizospheric isolate of Kallar grass, on gnotobiotically grown seedlings of *Oryza sativa* IR36 and *Leptochloa fusca* (L.) Kunth were studied. Additionally, *Azoarcus* spp. were localized in roots of field-grown Kallar grass. To facilitate localization and to assure identity of bacteria, genetically engineered microorganisms expressing β-glucuronidase were also used as inocula. β-Glucuronidase staining indicated that the apical region of the root behind the meristem was the most intensively colonized. Light and electron microscopy showed that strain BH72 penetrated the rhizoplane preferentially in the zones of elongation and differentiation and colonized the root interior inter- and intracellularly. In addition to the root cortex, stelar tissue was also colonized; bacteria were found in the xylem. No evidence was obtained that *Azoarcus* spp. could reside in living plant cells; rather, plant cells were apparently destroyed after bacteria had penetrated the cell wall. A common pathogenicity test on tobacco leaves provided no evidence that representative strains of *Azoarcus* spp. are phytopathogenic. Compared with the control, inoculation with strain BH72 significantly promoted growth of rice seedlings. This effect was reversed when the plant medium was supplemented with malate (0.2 g/liter). N_2 fixation was apparently not involved, because the same response was obtained with a *nifK* mutant of strain BH72, which has a Nif$^-$ phenotype. Also, Western blot (immunoblot) analysis of protein extracts from rice seedlings gave no indication that nitrogenase was present. PCR and Western immunoblotting, using primers specific for eubacteria and antibodies recognizing type-specific antigens, respectively, indicated that strain BH72 could colonize rice plants systemically, probably mediated by longitudinal spreading through vessels.

Various microorganisms can live under diverse circumstances in higher and lower plants endophytically (43). The relationship can even be obligate for the microbial partner as for arbuscular mycorrhizal fungi (15), which cannot be grown successfully in the absence of a host plant. The endophyte may be able to reside inside the living cell like arbuscular mycorrhizal fungi (15) or intercellularly like the fungus *Sphacelia typhina* (Fr.) Tul. in tall fescue grass (18). The plant-microbe interaction may be beneficial to the host as in the well-known endosymbiosis of legumes with rhizobia, or the ecological impact may be not so obvious as in endophytic bacteria occupying the storage vacuole in cells of sugar beet roots (25). According to Quispel (43), microorganisms that rapidly kill the host cells or damage and ecologically threaten the plant should not be called endophytes, nor should this term be used for microbes which spend only a negligible part of their life cycle inside the plant.

Recently Döbereiner et al. (11) suggested that the term "endophytic diazotroph" be used for microaerobically nitrogen-fixing bacteria like *Acetobacter diazotrophicus* and *Herbaspirillum* spp. which, in addition to other sources, were isolated from seeds of rice, roots, stems, and leaves of sugar cane, but not from soil. Several other nonpathogenic, microaerobically nitrogen-fixing soil and root isolates belonging to the genera *Azospirillum* (32, 51) and *Alcaligenes* (59) also have the potential to colonize and reside in roots of grasses. While beneficial effects on plants induced by *Azospirillum* species are well documented (reviewed by Okon [39]), data about the biological impact of other grass-associated diazotrophs are limited.

Another group of microaerobically N_2-fixing root isolates belongs to *Azoarcus* spp. (50). Up to now, bacteria assigned to this genus were obtained mainly from the rhizosphere of *Leptochloa fusca* (L.) Kunth, commonly called Kallar grass (48, 50), an undomesticated C_4 plant highly tolerant of soil salinity, alkalinity, and waterlogged conditions. This grass has been known for long to be associated with nitrogen-fixing bacteria (35). Various diazotrophs described as *Beijerinckia*, *Klebsiella*, (60) and *Zoogloea* (5) species have been isolated from roots. In 1984, two unidentified diazotrophic rod-shaped bacterial strains named BH72 and H6a2, which were presumed to be predominant in the root interior of field-grown Kallar grass (48) and were not antigenically related (46), were isolated. To confirm their presence in roots visually and to detect colonization sites, immunofluorescence studies were carried out. By using a mixture of specific antibodies directed against strains BH72 and H6a2, bacteria isolated from the root interior could be localized in the intercellular gas spaces (aerenchyma) in the roots of these plants (46). Using a mixed inoculum of both strains, immunogold-silver staining confirmed that they were able to colonize aerenchyma of roots of Kallar grass when grown in gnotobiotic culture with nondiseased plants (44). Bacteria entered the root through wounds, where lateral roots emerge, and between epidermal and cortical cells. They also

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appeared to occasionally invade outer cells of the not yet differentiated root tissue or epidermal cells by passing through the outer tangential longitudinal wall. Our recent taxonomic studies showed that the two endorhizospheric isolates are not related to each other. Strain H6a2 was assigned to the gamma subclass of the domain *Proteobacteria*, whereas strain BH72 belongs to the beta subclass and forms a new genus *Azoarcus* with other isolates from Kallar grass (50). Because the bacteria of this genus, in contrast to bacterial strain H6a2, were repeatedly isolated by us from the Kallar grass field under study, we were interested in a detailed analysis of colonization capacities of *Azoarcus* spp.

This report deals with the localization of *Azoarcus* spp. in Kallar grass and rice and the biological impact of these bacteria on the plant. A polyphasic approach was used on field-grown and gnotobiotically grown plants, including immunogold localizations at light and electron microscopic levels, visualization of genetically engineered microorganisms, Western (immunoblot) blot analysis, PCR, yield estimations of rice seedlings, and a pathogenicity test. While no evidence was obtained that these bacteria could establish endosymbiosis with Kallar grass and rice, endorhizospheric isolate *Azoarcus* sp. strain BH72 (i) invaded roots inter- and intracellularly, (ii) entered the xylem, which probably allowed symptomless systemic spreading in rice, and (iii) increased yield of rice by processes other than N_2 fixation, indicating that this bacterium may live in Kallar grass and probably also in other plants as an endophyte.

(A preliminary account of this study was presented previously [22].)

MATERIALS AND METHODS

Bacterial strains and plants. The following bacterial strains were obtained from the culture collection Laboratorium voot Microbiologie, Ghent, Belgium: *Alcaligenes piechaudii* NCMB 1051^T, *Comamonas acidovorans* Stanier 14^T, *Comamonas terrigena* NCIB 8193^T, *Azorhizobium caulinodans* ORS571^T, *Azoarcus communis* S2, and *Herbaspirillum seropedicae* Z78. *Azoarcus* sp. strain BHNK4 is a *nifK* mutant of strain BH72, obtained after marker exchange mutagenesis (23). This bacterial strain has a Nif$^-$ phenotype in pure culture. All other bacteria mentioned in the text were isolated by B. Reinhold from the rhizosphere of Kallar grass (47, 48, 50). Seeds of *Oryza sativa* IR36 were a kind gift from A. Kaplan, University of Idaho, Moscow. Seeds from Kallar grass were obtained from our own harvests. Potted plants of *Nicotiana tabacum* petit Havana SR1 were kindly provided from M. van den Bulke, University of Gent, Ghent, Belgium.

Construction of Tn*5-gus* insertions. Strain D3 is a mutant strain of *Azoarcus* sp. strain BH72, selected for constitutive expression of *Escherichia coli* β-glucuronidase (GUS) on complex medium, as well as on synthetic medium with KNO_3 or N_2 as a nitrogen source. Mutants were obtained by transposon mutagenesis after surface mating using plasmid pSB165, which carries a transcriptional operon fusion transposon (Tn*5-gusA1* [53]).

Cultivation of plants. Surface sterilization and germination of Kallar grass seeds were carried out as described previously (28). Seeds of *O. sativa* IR36 were surface sterilized by vigorous shaking at 25°C for 40 min in a solution containing 30 ml of commercial sodium hypochlorite, 1 g of Na_2CO_3, 30 g of NaCl, and 1.5 g of NaOH per liter of distilled water. After two washes for 10 min in sterile distilled water, seeds were transferred aseptically on agar plates containing medium of the following composition (per liter): yeast extract, 1 g; peptone, 3 g; glucose, 1 g; agar, 15 g. For germination, plates were incubated for 12 h at 37°C and 4 days at 25°C in a 12-h day–12-h night cycle. Seedlings not contaminated by microorganisms were aseptically transferred into glass tubes containing the following medium (per liter) if not indicated otherwise: DL-malic acid, 0.2 g titrated with KOH to pH 6.8; L-proline, 0.58 g; KH_2PO_4, 1.5 g; K_2HPO_4, 1.0 g; ferric citrate, 13 mg; KCl, 0.15 g; $CaCl_2 \cdot 2H_2O$, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; Na_2MoO_4, 2 mg; H_3BO_3, 3 mg; $MnSO_4 \cdot H_2O$, 2 mg; $ZnSO_4 \cdot 7H_2O$, 0.2 mg; $CuSO_4 \cdot 5H_2O$, 0.1 mg; agar, 4 g; pH 6.8. The tubes were subsequently inoculated with washed 10^7 cells per ml of medium of a mid-logarithmic culture grown on SM medium (45). If not indicated otherwise, plants were grown for 3 weeks in a 12-h day–12-h night cycle at 32 ± 0.5°C in a water bath, keeping roots in the dark by adding black ink to the water. For estimation of dry weight, plants were rinsed in tap water to remove residues of agar and subsequently lyophilized to complete dryness. Protein from rice seedlings was estimated after extraction from plants in 33% (wt/vol) NaOH at 37°C for 15 min.

Polyclonal antibodies. To enhance specificity, antiserum to strain BH72 elicited in rabbits (46) was adsorbed with cross-reactive antigens of strain S7a1, a nondiazotrophic member of the beta subclass of *Proteobacteria* also isolated from Kallar grass (50), by affinity chromatography (58), using 7 mg of whole-cell protein coupled to 1 ml of cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions. Prior to coupling, sodium dodecyl sulfate (SDS)-soluble cell extracts were dialyzed against $NaHCO_3$ buffer (0.1 M, pH 8.3) for 4 days to remove SDS. The antiserum raised against purified *Rhodospirillum rubrum* Fe protein of the nitrogenase (dinitrogenase reductase) was kindly provided by P. W. Ludden, Madison, Wis., and used as 1.5 mg of the freeze-dried serum dissolved in 100 μl of sterile distilled water. For Western blot analysis and immunolabelling of sections, both preparations of polyclonal antibodies were adsorbed with roots of axenically grown Kallar grass (45) as outlined previously (46).

Extraction of protein. SDS-soluble bacterial components were prepared from whole cells as outlined by Kiredjian et al. (26). Bacteria were grown for 40 h at 28°C on combined nitrogen in Roux flasks or on N_2 in rubber-stopper-closed 10-liter flasks containing 1 liter of nitrogen-free SM medium (21) and a headspace consisting of 99% N_2 and 1% O_2. Composition of medium in Roux flasks per liter was as follows: DL-malic acid, 2.5 g titrated with KOH to pH 6.8; KH_2PO_4, 1.5 g; K_2HPO_4, 1.0 g; NaCl, 1 g; NH_4Cl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; $MnSO_4 \cdot H_2O$, 0.01 g; $Na_2MoO_4 \cdot 2H_2O$, 0.002 g, FeEDTA (0.66 [wt/vol] in water), 10 ml; yeast extract, 1 g; LabLemco powder, 3 g; agar, 15 g; pH 6.8. One-milliliter SDS extracts were prepared from 150 mg (bacteria [wet weight]) each. Proteins from pools of 10 plants were extracted by homogenizing lyophilized plant fractions in extraction buffer according to VandenBosch et al. (55) with a mortar and pestle. Before spinning, bacteria were subjected to four freeze-thaw cycles.

Extraction of DNA. Lyophilized plant fractions were frozen at −70°C, and plants (three plants in each pool) were broken with a mortar and pestle. Subsequently, homogenate was extracted for 30 min at 37°C in buffer containing 200 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA, 0.5% (wt/vol) SDS, and 3 mg of RNase A per ml. For a 10-mg sample, 0.12 ml of buffer was used. The samples were then digested with proteinase K at 0.1 mg/ml for 30 min at 37°C, and the DNA was extracted by standard procedures (3). DNA of

bacteria was prepared by the method of Marmur (36), with modifications as outlined previously (47).

PCR conditions. Primers TH1 and TH2 (20) correspond to regions 20 to 43 and 552 to 557 in the *E. coli* 16S rRNA sequence (6), respectively, which are rather conserved among eubacteria. PCRs were carried out according to Hurek et al. (20) at condition A with an annealing temperature of 70°C and 38 cycles.

Western blots. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using Laemmli's system (29). Components subjected to electrophoresis were electroeluted (54) onto nitrocellulose sheets (BA85; Schleicher und Schüll, Dassel, Germany) for 90 min at 24 V and 100 mA, with a semidry electroblotter (JKA-Biotech, Dassel, Denmark), using a buffer containing (per liter of distilled water) 14.4 g of glycine, 3 g of Tris, 200 ml of methanol, and 1.5 mM SDS. Nitrocellulose matrices were blocked with bovine serum albumin (BSA) in Tris-buffered saline (TBS) (54) treated for 2 h with primary antibody diluted 10,000-fold in Tris-buffered saline containing 0.5% BSA and for 2 h with swine anti-rabbit peroxidase conjugate (Dako Patts, Glostrup, Denmark), diluted 1,000-fold in the same buffer. Blots were developed with 4-chloro-1-naphthol as the substrate (16).

Histochemical detection of β-D-glucuronidase. Roots of rice plants were rinsed several times in 25 mM phosphate buffer (pH 6.8) (25°C) until agar was removed from roots. Subsequently, the intact root system was incubated for 3 h at 45°C in 50 mM sodium cacodylate buffer with 0.5 mg of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) per ml. Roots were inspected immediately with a Wild M 3 compound microscope. Photomicrographs were recorded on Kodak Ektachrome 50 DX films.

Treatment of field-grown plants. Roots of Kallar grass for sectioning were sampled in November 1988 in the Punjab region of Pakistan from the same field which had been used for previous studies in 1984 (46–48, 50). Plants were carefully dug up with the center of the crown of roots enclosed in a ball of soil about 30 cm in diameter. Plants were processed within 3 h of collection. Root bales were shaken to remove loosely adhering soil and then washed with tap water. As judged by the development of a yellowish to brownish color, roots of different ages were chosen. Roots from the center at 2- to 10-cm soil depth were excised from plants and divided into two representative fractions. One was used for microscopy and treated immediately. The other one was used within 96 h for isolation of diazotrophs (50). Lower parts of stem bases containing root nodes from the center of the crown of roots were sampled and also processed immediately.

Microscopy and immunocytochemistry on resin-embedded material. Samples from in situ-grown plants were embedded in LR White acrylic resin, soft grade (Agar Scientific Ltd., Essex, United Kingdom), and heat cured at 54°C as described previously (44). Before infiltration, representative evacuated root segments (each 2 to 3 mm long; fraction I) from 2-, 5-, and 9-mm soil depth and blocks of stem bases (border length, approximately 1 mm) were fixed in glutaraldehyde (1% [wt/vol] in 50 mM sodium cacodylate buffer [pH 7.2]) and dehydrated in ethanol. Samples from gnotobiotically grown plants, including tissue stained positive for GUS and bacteria enclosed in 1.5% agar, were embedded into the same resin but UV polymerized at −20°C as described previously (24). Semithin and ultrathin sections were obtained on an LKB Ultratome III (LKB, Bromma, Sweden), using glass knifes made with the LKB Knivemaker 7800.

Ultrathin sections were mounted onto Parlodion-protected copper grids fitted with a carbon-coated Formvar film. For immunolabelling, grids were preadsorbed on a drop of 2% BSA in phosphate-buffered saline (PBS) (9) and then transferred to the primary antibodies diluted 250-fold in 2% BSA in PBS for 1 h. After washing in PBS, grids were placed on a drop of goat anti-rabbit gold conjugate (15-nm-diameter particles; Auroprobe EM GAR G15 [Janssen Life Sciences Products, Nettetal, Germany]) diluted 40-fold in 2% BSA in PBS. After 1 h, grids were rinsed in PBS and in distilled water and poststained with 4% (wt/vol) aqueous uranyl acetate and lead hydroxide (37). The preparations were examined with a Zeiss EM 109 electron microscope operated at 80 kV, and photographs were recorded on Agfa HDU1P film.

Semithin sections were mounted in groups of three to four on glass slides treated with poly-L-lysine (34) and immunolabelled by using reagents and incubation conditions as applied for electron microscopy, with a slight modification: primary and secondary antibodies were diluted in solutions containing 0.1% (vol/vol) Tween 20 and 0.1% (vol/vol) Triton X-100. The photochemical silver reaction for signal amplification was done as described previously (44). Specimens were examined with an Olympus Vanox-S or a Leitz Diaplan microscope. Pictures were recorded on Kodak Ektachrome 50 DX or 160 DX film.

RESULTS

Pathogenicity test. To obtain an indication whether *Azoarcus* spp. could cause defense reactions in the plant, a commonly used pathogenicity test (27) was done. For infection, *Azoarcus* sp. strain BH72, *Azoarcus indigens* VB32^T, *A. communis* S2, and *Pseudomonas syringae* pv. syringae as a control were infiltrated into the intercellular spaces of an old leaf of an intact plant of *N. tabacum* petit Havana SR1 at a dose of 10^7 CFU per leaf area. Whereas *Azoarcus* spp. did not elicit a hypersensitivity response leading to visible necrosis in the leaf within 14 days, parts of the leaf treated with the pathogen *P. syringae* pv. syringae developed this phenotype within 2 days (not shown).

Screening for putative colonization sites by GUS assay. For a systematic evaluation of colonization sites in roots of grasses, we inoculated rice with an *Azoarcus* sp. strain BH72 mutant positive for constitutive GUS expression and screened roots after 1 to 3 weeks by a histochemical assay for the presence of this enzyme. We observed a patchy staining profile of the fibrous root system of rice with intensive, sometimes alternate staining of the apical (Fig. 1a) regions of the root behind the meristem. In most cases, the first 1 to 2 mm immediately proximal to the root apex was heavily surface colonized (Fig. 1b). Stained regions below the root hair zone often corresponded to colonization of the root interior (Fig. 1c). Uninoculated plants never stained positive for GUS when subjected to the assay (not shown). Inspection of the roots with higher magnification revealed that intensity of staining always coincided with intensity of surface colonization by bacteria expressing GUS at the terminal part of the roots (Fig. 1a and b). However, at regions more distant to the root apex, we sometimes observed zones stained positive but not colonized at all (not shown), stressing that microscopic controls for presence of bacteria are indispensable. Unspecific GUS staining was probably caused by diffusion of indoxyls before dimerization to an insoluble precipitate (10). The same colonization pattern was observed with Kallar grass seedlings (not shown).

Effect of inoculation on growth of rice. To assess the nitrogen fixation potential of *Azoarcus* spp. in our gnotobiotic system and to rule out the effect of the bacteria on plant growth, inoculation studies were carried out. When rice was inoculated with *Azoarcus* sp. strain BH72 or *Azoarcus* sp. strain

FIG. 1. *Azoarcus* sp. strain D3 cells colonizing roots of rice seedlings, rendered visible by staining for the presence of GUS with X-Gluc. (a) Primary root of a rice seedling, showing alternate staining of the root tip in the zones of cell elongation and differentiation. The arrow marks the region shown in panel b. Bar, 1 mm. (b) Higher magnification of the stained region proximal to the root apex from panel a, showing cells of strain D3 heavily colonizing the surface of the root. Bar, 10 μm. (c) Interference-contrast image of a transverse thick section (5 μm) of the stained region distal to the root apex from section a, showing the volume of a plant cell occupied by *Azoarcus* sp. strain D3. Bar, 10 μm.

BHNK4, which has a Nif$^-$ phenotype, there were significant increases of plant biomass and total protein over those of aseptically grown control plants when malate was not added to the growth medium (Table 1). Both inocula caused essentially the same growth responses, strongly suggesting that N_2 fixation was not involved.

Colonization of roots of Kallar grass and rice by *Azoarcus* sp. strains BH72 and D3 was not accompanied by inhibition of root growth or other macroscopically visible disease symptoms, whether or not K-malate was added to the growth medium of the plants. However, with malate supplement, dry weight or total protein of rice plants significantly decreased upon inoculation with strain BH72 (Table 1). Because addition of this carbon source promoted the colonization of roots by strain D3, we usually supplemented plant growth medium with malate to facilitate localization of bacteria in root tissue.

Specificity of the antibodies. To characterize the polyclonal antibodies, Western blot analysis of whole-cell SDS extracts of *Azoarcus* spp. and reference bacteria were carried out. One-dimensional SDS-PAGE, electroblotting, and immunostaining of SDS-extracted bacterial components (Fig. 2) revealed that the preparation of the antiserum raised against *Azoarcus* sp. strain BH72 was specific for the genus. Representative strains of four different species of *Azoarcus* had one or more antigens in common not shared with reference bacteria belonging to other genera. The antiserum recognized a significantly smaller number of components in whole-cell SDS extracts of isolates other than strain BH72. Western blot analysis detected only one band or even failed to detect antigens in whole-cell extracts of *Azoarcus* sp. strain S5b2 or *Azoarcus* sp. strain 6a3, respectively, both of which are less related to other members of the genus *Azoarcus* (20, 50). No cross-reaction was found with nitrogen-fixing *H. seropedicae* Z78 (lane 10). No additional bands appeared when preparations of N_2-grown (lane 2) instead of combined-nitrogen-grown (lane 1) strain BH72 were used, indicating that our polyclonal antibodies were not detecting proteins specifically expressed during nitrogen fixation.

Specificity of the staining reaction. To validate the immunocytochemical staining reaction on resin-embedded material,

TABLE 1. Effects of *Azoarcus* sp. strain BH72 and the Nif$^-$ mutant strain BHNK4 on growth of rice seedlings

Treatment[a]	Inoculum	Dry wt[b] (mg)	Total protein[c] (mg)
Without malate		14.5 ± 1.9 (a)	0.55 ± 0.06 (a)
With malate		14.7 ± 2.1 (a)	0.54 ± 0.04 (a)
Without malate	*Azoarcus* sp. strain BH72	16.8 ± 1.5 (b)	0.79 ± 0.07 (b)
	Azoarcus sp. strain BHNK4	16.5 ± 1.3 (b)	0.80 ± 0.07 (b)
With malate	*Azoarcus* sp. strain BH72	10.9 ± 3.2 (c)	0.46 ± 0.05 (c)

[a] Either 0.2 g of K-DL-malate per liter was added to the plant growth medium, or this supplement was omitted.

[b] Values represent means of 16 to 19 plants each. Results followed by different letters are significantly different from each other at the 5% level according to the Mann-Whitney test.

[c] Values represent means of six pools of three plants (each) measured three times each. Statistical analysis is indicated as in footnote *b*.

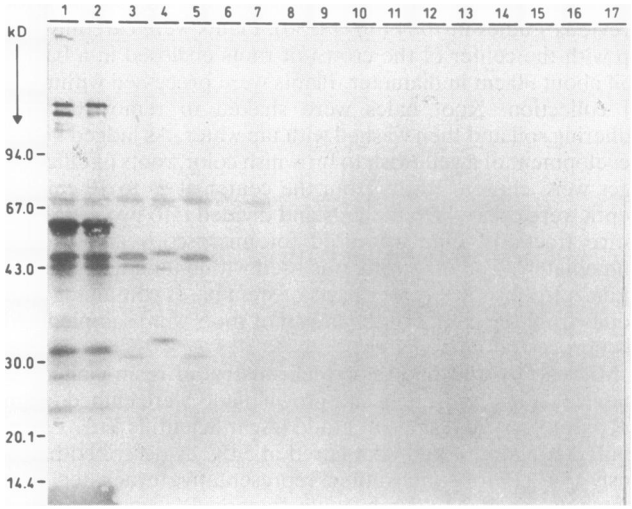

FIG. 2. Western blot of whole-cell SDS extracts of *Azoarcus* spp. (lanes 1 to 7) and reference bacteria (lanes 8 to 17), incubated with antiserum to cells of *Azoarcus* sp. strain BH72. Lanes: 1, *Azoarcus* sp. strain BH72; 2, *Azoarcus* sp. strain BH72 grown on N_2; 3, *A. communis* SWuB3^T; 4, *A. indigens* VB32^T; 5, *A. communis* S2; 6, *Azoarcus* sp. strain 6a3; 7, *Azoarcus* sp. strain S5b2; 8, *Azoarcus* sp. strain S7a1; 9, *H. seropedicae* Z78; 10, *H. seropedicae* Z78 grown on N_2; 11, isolate H6a2; 12, *Azospirillum halopraeferens* Au4^T; 13, *Azospirillum lipoferum* Rp5; 14, *Alcaligenes piechaudii* NCMB 1051; 15, *Azorhizobium caulinodans* ORS571^T; 16, *C. acidovorans* Stanier 14^T; 17, *C. terrigena* NCIB 8193^T. Bacteria were grown on combined nitrogen if not indicated otherwise. Proteins applied in equal amounts were separated by SDS-PAGE, using an 8 to 15% linear gradient.

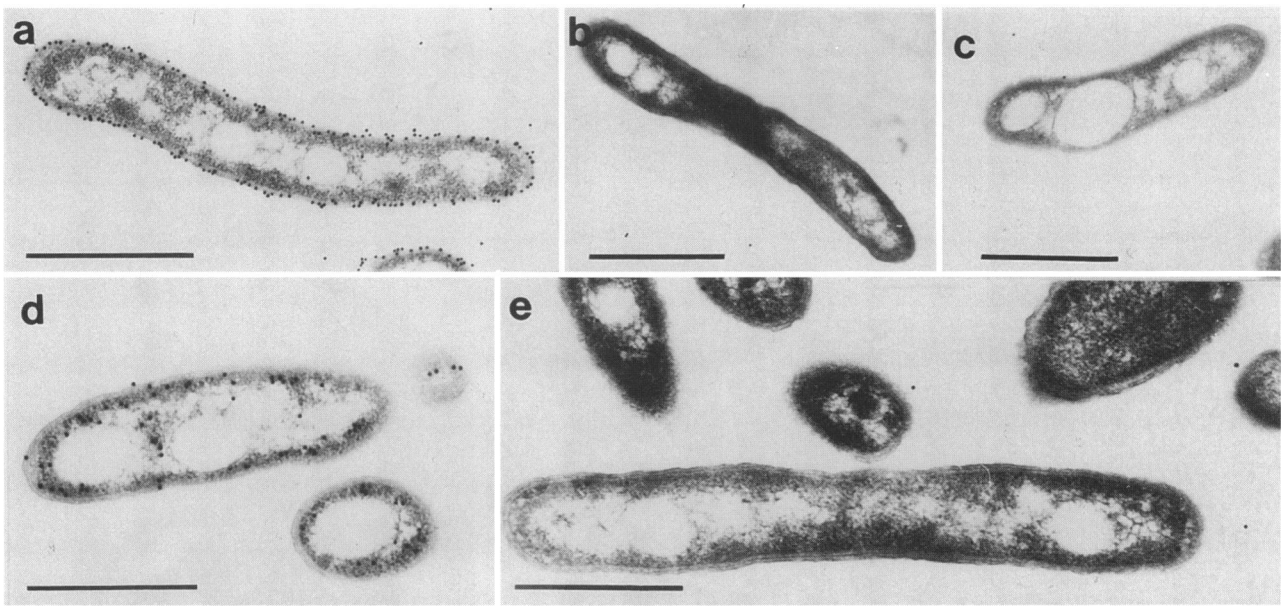

FIG. 3. TEMs of immunolabelled *Azoarcus* sp. strain BH72 after exposure of sections to antiserum directed against strain BH72 (a) and antiserum to dinitrogenase reductase (d and e). Bacteria were grown on combined nitrogen (a to c and e) or on N_2 (d). Views b and c show sections subjected to the same staining procedure, but the primary antibodies were either omitted (b) or replaced by preimmune serum of genus-specific antibodies (c). Bars, 1 μm.

control incubations with pure cultures were run. They showed that immunogold labelling of the embedded specimen was specific: (i) the cell surface of *Azoarcus* sp. strain BH72 was labelled with the antiserum raised against *Azoarcus* sp. strain BH72 (Fig. 3a); (ii) no significant labelling occurred when GAR G15 was used alone (Fig. 3b) or (iii) the primary antibodies were replaced by preimmune serum (Fig. 3c); (iv) no significant labelling was detected on reference strains *Azospirillum halopraeferens* Au4^T and S7a2 (not shown), both isolates from Kallar grass roots; and (v) the cytoplasm of N_2-grown (Fig. 3d) but not of NH_4-grown *Azoarcus* sp. strain BH72 (Fig. 3e) was labelled with antiserum to dinitrogenase reductase.

Detection of *Azoarcus* sp. in the root cortex. Light and electron microscopic examination of resin-embedded roots of Kallar grass and rice seedlings grown axenically with *Azoarcus* sp. strain BH72 or D3 confirmed the potential of these bacteria to colonize the cortex of roots. Colonization of the tissue occasionally resulted in inter- and intracellular multiplication of bacteria in the root (Fig. 1c and 4a to h). Identity of the inoculum inside cells of roots was confirmed by histochemical detection of GUS (Fig. 1c) followed by immunocytochemical localization of bacteria on subsequent sections by transmission electron microscopy (Fig. 4d to g). There and also in intercellular spaces (not shown), *Azoarcus* spp., like other root-colonizing bacteria whether phytopathogenic (52) or not (55), were often embedded in a matrix (Fig. 4d to g) which was also labelled (Fig. 4e), indicating that bacteria released antigenic material which was part of the matrix. In contrast to the ultrastructural appearance of unstained specimens (transmission electron micrographs [TEMs] of unstained specimens are shown in Fig. 4c and h to k), the ultrastructure of tissue subjected to histochemical GUS staining prior to fixation (TEMs of stained specimens are shown in Fig. 4a, b, and d to g) always appeared to be distorted, probably because of heat treatment at 45°C for 3 h prior to fixation. While cells of the root cap and apical meristem were never found to be penetrated, their surfaces were commonly colonized by these bacteria, albeit less intensely than the subsequent zones of active cell division, elongation, and differentiation (not shown). Preferentially in the zone of cell expansion, *Azoarcus* sp. penetrated the root (Fig. 4i to k). When *Azoarcus* sp. strain BH72 invaded highly vacuolate cells in the zone of elongation in roots of rice (Fig. 4i) or Kallar grass, bacteria were never surrounded by additional membranes (Fig. 4h and j) and never found inside the vacuole or cytoplasm of a healthy-looking cell. Rather, plant cells appeared to degenerate when bacteria had penetrated the cell wall (Fig. 4j and k). The same applies to plants which were grown in a medium not supplemented with malate.

Field studies are always required to evaluate laboratory experiments. Therefore, we attempted to localize *Azoarcus* spp. also in field-grown Kallar grass, where we observed three major types of mature roots: type a, diameter approximately 2 mm, almost no lateral roots; type b (the predominant type), diameter approximately 0.7 to 2 mm, with medium frequency of laterals; and type c, diameter ≤0.5 mm, with very high frequency of lateral roots. No rhizodermal cells which were apparently still alive were seen. In none of the roots did we obtain convincing evidence for frequent colonization of the cortex by *Azoarcus* spp., but we could detect the bacteria in stelar tissue of the transition zone between shoot and roots (see below).

Detection of *Azoarcus* sp. in the vascular system. *Azoarcus* sp. strain BH72 was able to invade the stele of roots (Fig. 5). Bacteria were able to proliferate in the intercellular spaces of Kallar grass, move between cells of stelar tissue, and finally enter parenchyma cells or fibers (Fig. 5a). Xylem vessels and adjacent fibers might be colonized via penetration through pits (Fig. 5b). Besides in gnotobiotically grown Kallar grass (not shown) and rice plants (Fig. 5c), immunogold labelling for dinitrogenase reductase (Fig. 5d) or for *Azoarcus* sp. (Fig. 5c and e) revealed that bacteria including *Azoarcus* spp. colonized stelar tissue in situ. However, besides in the root base, in

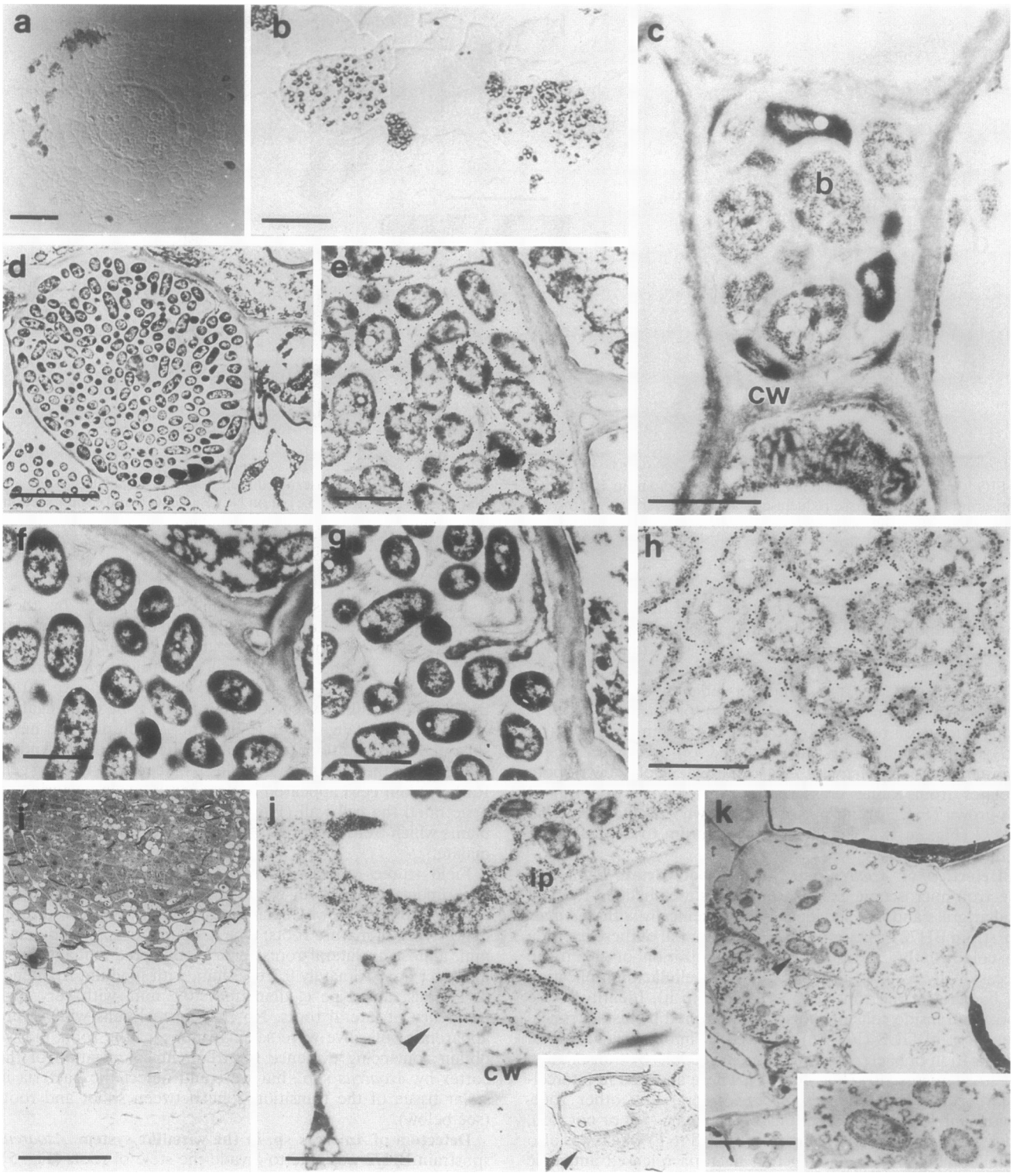

FIG. 4. Series of micrographs illustrating intra- and intercellular colonization of root cortex of rice and Kallar grass seedlings by *Azoarcus* sp. strains D3 (a, b, d, e, f, and g) and BH72 (c, h, i, j, and k). Some tissue had been subjected to GUS staining before embedding (a, b, d, e, f, and g). (a) General view of a transverse section through a differentiated root of a rice seedling showing a large proliferation of bacteria deep in the cortex. Light micrograph with interference-contrast illumination, immunogold-silver stained for *Azoarcus* spp. with whole-cell antiserum raised against *Azoarcus* sp. strain BH72. Bar, 50 μm. (b) Higher magnification of part of section a, depicting intra- and intercellular enlarged populations of *Azoarcus* sp. Bar, 10 μm. (c) TEM of a transverse section through the subapical region of a root of Kallar grass without aerenchyma invaded by *Azoarcus* sp. strain BH72, showing complete occupation of the intercellular space by bacteria (b). cw, plant cell wall. Bar, 1 μm. (d) TEM of a subsequent section of Fig. 1c, which showed a plant cell in a root of rice occupied by *Azoarcus* sp. strain D3. Bar, 5 μm. (e) Higher magnification of a subsequent section of panel d, right side, supporting taxonomic affiliation of bacteria in a plant cell by immunogold labelling using whole-cell

field-grown plants, colonization of stelar tissue was found only in roots of type b.

Detection of *Azoarcus* sp. in rice plants by Western blot analysis and PCR. To determine whether *Azoarcus* sp. spreads in the whole vegetative body of these plants, protein as well as DNA extracted from shoots, root bases (transition zone), and roots of rice inoculated with strain BH72 were screened for bacteria by Western blot analysis and PCR. For specific detection of *Azoarcus* sp. strain BH72, polyclonal antibodies raised against whole cells of this bacterium were affinity purified as described by Fleischmann et al. (12) for an extracellular antigen (19) migrating in SDS-PAGE with an apparent molecular mass of 110 kDa. While in control blots, where the primary antibodies were replaced by preimmune serum, no bands were detected (Fig. 6A), the purified antibodies specifically detected type-specific antigens with molecular masses ranging from 110 to 160 kDa, which are apparently restricted to strain BH72 (Fig. 6B; see also Fig. 2). With these antibodies, bands in the expected range of apparent molecular weight were detected in protein preparations of all fractions of inoculated but not uninoculated rice plants within 3 weeks after inoculation. However, when growth medium was not supplemented with malate, antigens in shoots could not be detected by Western blot analysis (not shown), suggesting that colonization was significantly reduced. Furthermore, we obtained no evidence that *Azoarcus* sp. strain BH72 expressed detectable amounts of dinitrogenase reductase in axenic culture with rice seedlings after 3 weeks of incubation with (Fig. 6c) or without malate supplement (not shown).

PCR amplification of eubacterial 16S ribosomal DNA (rDNA) confirmed that bacteria were present in root, shoot, and root base of inoculated but not uninoculated rice, even when malate was not added to the plant growth medium (Fig. 7). An amplification product of the appropriate size (525 bp) was obtained for pure cultures of *Azoarcus* sp. strain BH72, as well as roots or shoots of inoculated but not of aseptically grown rice seedlings. The smaller product found in shoots but not in roots is probably due to amplification of rice chloroplast 16S rDNA (EMBL accession number X15901), which should give a 480-bp product and served as an internal standard.

DISCUSSION

By immunocytochemical localization by light and electron microscopy in combination with histochemical localization of reporter gene expression, we confirmed previous results that endorhizospheric bacteria originating from Kallar grass were able to invade roots of gnotobiotically grown Kallar grass seedlings inter- and intracellularly (44) and extended this observation to rice. Besides occasional entry into cells of the not yet differentiated root tissue, *Azoarcus* sp. strain BH72 also colonized cortical and stelar cells. It was highly unlikely that obligately endophytic eubacteria in surface-sterilized seeds of rice, which did not grow on germination plates but which colonized tissues of seedlings, had caused the infections observed, because we histochemically detected a mutant with a Gus^+ phenotype inside roots and found no evidence for colonization of aseptically grown plants by PCR targeted for eubacterial 16S rDNA.

Among root-colonizing nonpathogenic bacteria, a capacity to penetrate into cells seems to be quite common and not restricted to *Azoarcus* sp. strain BH72. Bacteria belonging to several genera have been found inside cells of roots of healthy, disease-free sugar beet plants (25). Rhizobia have also been shown to occasionally penetrate root cells (31). From light microscopic studies employing fluorescent antibody staining techniques, Schank et al. (51) obtained evidence that besides the cortex, neither the vascular system nor the endodermis of several field-grown grasses was colonized by members of *Azospirillum brasilense*. It was stated that azospirilla occurred in cortical cells (51). Using immunogold electron microscopy, Levanony et al. (32) concluded that *A. brasilense* Cd probably penetrated the root via the middle lamella of the epidermal layer, forming an intercellular association with cortical cells of wheat roots. Bacteria did not colonize endodermis and vascular system; however, young live root cells were occasionally invaded too (4). Intracellular ingress has also been claimed to accompany colonization of rice roots by *Alcaligenes faecalis* A15 (59). The sporadic occurrence of the diazotrophic *Enterobacter agglomerans* C1 in the stele of gnotobiotically grown wheat seedlings was accompanied by an infection intensity close to a pathogenic relationship (33).

The stele has been considered to be invariably colonized by pathogens (8), where saprophytes may also occur (14). Despite previous reports suggesting vascular tissue to be an important colonization site for diazotrophs (30, 41), information about specific diazotrophic bacteria in the stele is still scarce. Colonization of vascular tissue by *Azoarcus* sp. strain BH72 is in accord with a systemic occurrence of these bacteria in rice. Similar results were presented recently for the diazotrophic plant pathogen *Herbaspirillum rubrisubalbicans* (11). The stele may be important for longitudinal and lateral spreading of nonpathogenic diazotrophic bacteria as well, allowing colonization of the shoot and the fibrous root system without repeated ingress through the rhizoplane. Recently it was shown that cellulolytic enzymes are present in this bacterium (49), suggesting that an active process of penetration might be involved in the infection process of *Azoarcus* sp. strain BH72 in vivo.

Whether Kallar grass was grown in quartz sand (44) or in situ, endorhizospheric bacteria affiliated with the genus *Azoarcus* were shown to be able to reside in apparently disease-free plants. Also, failure to elicit a hypersensitivity reaction in tobacco provided evidence that representative members of this group are not phytopathogenic. Furthermore, similar to results obtained in inoculation studies with members of the genus

antiserum raised against *Azoarcus* sp. strain BH72. Bacteria are embedded in a matrix which appears to be at least partially of bacterial origin. Bar, 1 μm. (f) Subsequent section of panel e subjected to the same staining procedure, with primary antibodies omitted. Bar, 1 μm. (g) Subsequent section of panel f subjected to the same staining procedure, with the primary antibodies replaced by preimmune serum. Bar, 1 μm. (h) TEM of *Azoarcus* sp. strain BH72 tightly packed in a cell of a root of Kallar grass and apparently not enclosed by additional membranes. Immunogold labelling as in panel e. Bar, 1 μm. (i) TEM of a transverse section through the infection zone with highly vacuolated plant cells either disrupted or with local degradation of cytoplasm in an undifferentiated primary root of rice proximal to the root apex. Bar, 50 μm. (j) Higher magnification of the infection region presented in panel i showing *Azoarcus* sp. strain BH72 in a plant cell between the apparently intact protoplast (ip) and the plant cell wall (cw); immunogold labelling as in panel e. Bar, 1 μm. Overview of the plant cell from the same section is shown in the lower right corner; bacterium is marked by arrowhead. (k) Higher magnification of the infection region presented in panel i showing *Azoarcus* sp. strain BH72 cells inside a plant cell in the root tissue with highly disorganized cytoplasm; immunogold labelling as in panel e. Closeup of the same section is in the lower right corner, showing genus-specific labelling of bacterial cells. Bar, 5 μm.

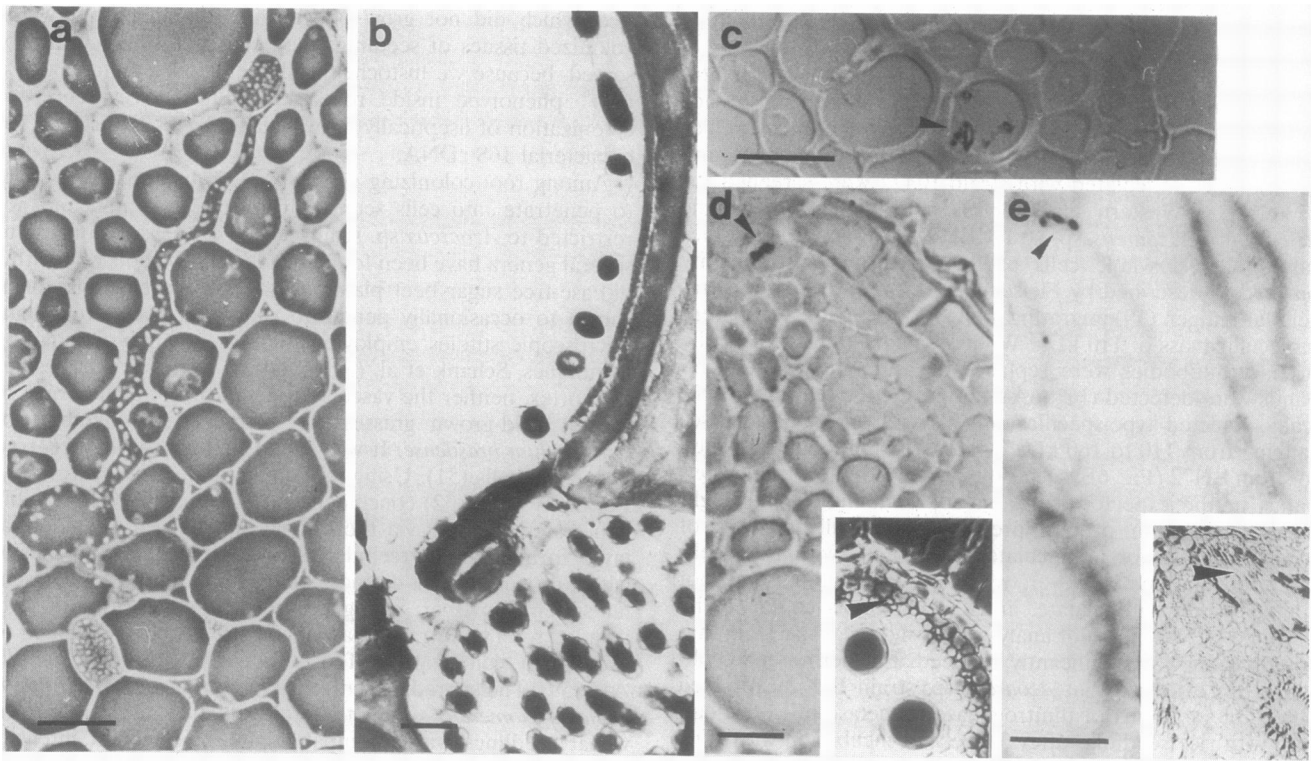

FIG. 5. Diazotrophic bacteria colonizing vascular tissue of rice and Kallar grass. (a) General view of the stele in the mature region of a root of Kallar grass grown for 7 weeks in gnotobiotic culture with *Azoarcus* sp. strain BH72 without malate supplement. Bacteria penetrating between parenchyma cells and fibers in the stele, accompanied by massive inter- and intracellular multiplication, are seen. Light micrograph with inverse phase-contrast illumination. Bar, 10 μm. (b) Higher magnification TEM of part of a subsequent section corresponding to that shown in panel a, showing profuse multiplication of bacteria in the fiber adjacent to the xylem vessel which is also colonized, *Azoarcus* sp. strain BH72 probably moving through a pit. Bar, 1 μm. (c) *Azoarcus* sp. strain BH72 colonizing xylem in a root of 3-week-old rice plant. Light micrograph with interference-contrast illumination, immunogold-silver stained for *Azoarcus* spp. with whole-cell antiserum raised against *Azoarcus* sp. strain BH72. Bar, 10 μm. (d) Diazotrophic bacteria colonizing a stelar cell (probably pericycle) in a root (type b) of Kallar grass grown on a field in the Punjab of Pakistan. Light micrograph with bright-field illumination, immunogold-silver stained for dinitrogenase reductase. Bar, 10 μm. An overview of the section is presented in phase-contrast illumination in the lower right corner. (e) Colony of *Azoarcus* spp. in a stelar cell proximal to a vessel in the transition region of roots and stems of field-grown Kallar grass in the Punjab of Pakistan. Light micrograph with bright-field illumination, immunogold-silver stained for *Azoarcus* spp. as in panel c. Bar, 10 μm. An overview of the section is presented in interference-contrast illumination in the lower right corner.

Azospirillum (38), we also found that *Azoarcus* inoculation could benefit plant growth. Results of Western blot analysis and inoculation experiments with a Nif$^-$ mutant of *Azoarcus* sp. strain BH72 indicated that N_2 fixation was apparently not involved in promotion of plant growth by strain BH72. Effects might have been caused by, for example, enhancing plant mineral uptake and improving plant water relationships (reviewed by Okon [39]). However, addition of malate to the plant growth medium caused a significant decrease in plant dry weight and protein content and promoted colonization of roots by the bacterial strains. This observation indicated that depending on the experimental conditions applied, the usually neutral or beneficial relationship may become detrimental. Variations in plant response have also been obtained for plant growth-promoting members of the genus *Azospirillum*, where inoculation may decrease yield as well (42). In our case, decreases of plant weight (dry weight) in the presence of malate were probably caused by a random albeit massive inter- and intracellular invasion and proliferation of *Azoarcus* sp. strain BH72 in the apical region of roots behind the meristem, resulting in premature cell death.

In mature roots of Kallar grass and rice, most plant cells outside the endodermis are dead. Death of rhizodermal cells may be attributed to a phenomenon termed root cortex death, which is known to occur in roots of monocots (17) and which has been suggested to be under genetic control of the plant (13). Death of cortical cells in roots of rice (1, 56) and also in Kallar grass (46) is related to the formation of an aerenchyma. These intercellular gas spaces originate lysigenously by collapse and lysis of cells in roots of rice (1, 56) and probably also in Kallar grass. Usually aerenchyma is already obvious just behind the meristem (2) and becomes well developed in nodal roots of maize within the first 3 to 4 cm behind the root tip, where cell expansion is complete (7). When *Azoarcus* sp. strain BH72 massively invaded the elongation and differentiation zones, plant cells were apparently destroyed after bacteria had penetrated the cell wall.

Independent of supplementation with this carbon source, invasion of tissue, which is determined to die anyway, was not followed by persistent residence of bacteria inside living cells. Comparable results were obtained for *A. brasilense*: electron microscopic studies revealed that upon inoculation of wheat, *A. brasilense* Cd did not apparently reside in living plant cells as well (4, 32).

Premature cell death of invaded plant cells may represent a strictly localized hypersensitivity reaction. Changes in the

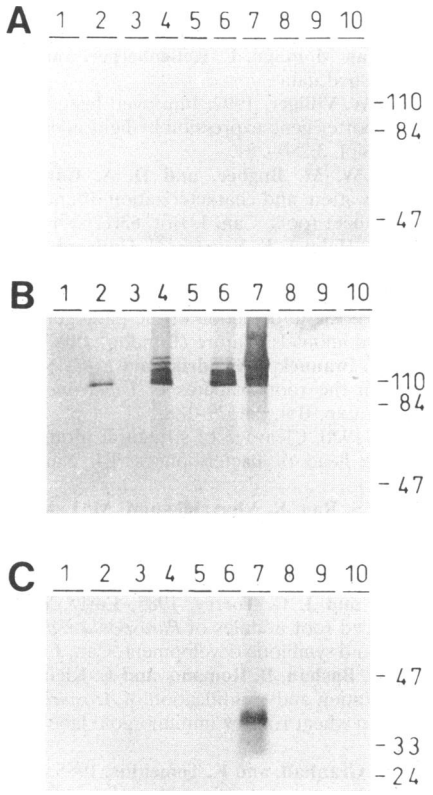

FIG. 6. Detection of *Azoarcus* sp. strain BH72 in rice seedlings by Western blot analysis after 3 weeks of incubation. Proteins applied in amounts equivalent to equal amounts of proteins were separated on a SDS–9% polyacrylamide gel. Western blots were incubated with preimmune serum (A), affinity-purified polyclonal antibodies for type-specific antigens of *Azoarcus* sp. strain BH72 (B), or anti-dinitrogenase reductase antibodies (C). Lanes: 1, 3, and 5, total protein preparations of shoots, root bases, and roots of aseptically grown rice, respectively; 2, 4, and 6, total protein preparations of shoots, root bases, and roots of rice inoculated with *Azoarcus* sp. strain BH72, respectively; 7 to 10, whole-cell SDS extracts of *Azoarcus* sp. strain BH72 grown on N_2 (lane 7), *A. communis* SWuB3^T (lane 8), *A. indigens* VB32^T (lane 9), and *Azoarcus* sp. strain S5b2 (lane 10). Positions of molecular weight markers (in thousands) are indicated on the right.

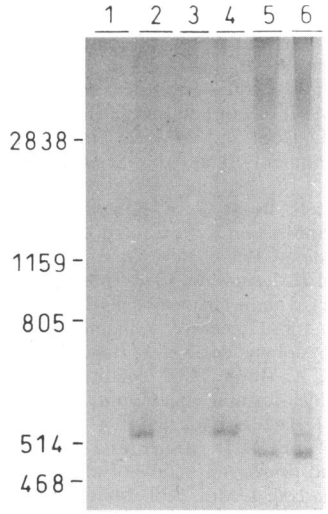

FIG. 7. Detection of *Azoarcus* sp. strain BH72 in rice seedlings by PCR with 16S rRNA-targeted primers specific for eubacteria. Rice plants were grown for 3 weeks without addition of carbon source to the medium. PCR products amplified from total genomic DNA were visualized on a 6% nondenaturing polyacrylamide gel stained with Amersham's silver stain kit (code RPN.17; Amersham International, Amersham, United Kingdom) for DNA. Sizes are given in base pairs. Lanes: 1, water (control); 2, 0.5 ng of DNA of *Azoarcus* sp. strain BH72; 3, 5 ng of DNA of roots from aseptically grown rice; 4, 5 ng of DNA of roots from rice inoculated with *Azoarcus* sp. strain BH72; 5, 5 ng of DNA of shoots from aseptically grown rice; 6, 5 ng of DNA of shoots from rice inoculated with *Azoarcus* sp. strain BH72.

protein composition of rice roots or shoots could not be detected in one-dimensional SDS-PAGE in the range of 15 to 100 kDa (not shown), indicating that pathogenesis-related proteins were not expressed. Parasitic or pathogenic-type interactions have been observed frequently and seem to accompany establishment of an overall ecologically beneficial relationship (43) even in symbiotic mycorrhizal (15) and nodule systems (31, 57). For example, during nodule morphogenesis, rhizobia have been reported to cause localized deleterious effects on host tissue, apparently terminating in death of a single invaded cell caused by a hypersensitivity reaction response (57) or by entry of masses of bacteria (31), or even transient growth depressions have been observed in arbuscular mycorrhizal plants (reviewed by Harley [15]).

In conclusion, members of the genus *Azoarcus* are likely to belong to the indigenous nonpathogenic microflora of *L. fusca*, preferably using cortex and vascular tissue of roots for residence and vessels for spreading through the vegetative body. After active penetration of the rhizoplane mostly in the subapical region of the root axis, bacteria which most likely did not fix a considerable amount of nitrogen were able to reside in healthy young plants. In contrast to interaction of rhizobia with plants, long-term survival of cortical cells was apparently not required for *Azoarcus* spp. to reside in the disease-free host. Observation of a positive growth response of and a similar colonization pattern in rice is of particular interest because it extends the host range of *Azoarcus* spp. According to the terminology of Quispel (43), the diazotroph *Azoarcus* sp. strain BH72 may be properly called an endophyte. We obtained no evidence that *Azoarcus* spp. could establish an endosymbiosis with rice or Kallar grass, although strain BH72 was apparently able to penetrate plant cells. Therefore, we suggest not classifying grass-associated bacteria of the genus *Azoarcus* as endophytic diazotrophs (40), because this term has been used for *Acetobacter diazotrophicus* and *Herbaspirillum* spp. with reference to Schanderl's symbiosis theory, implicating a symbiotic bacteroid-like relationship of these microorganisms inside the cytoplasm of living plant cells (11). Clearly, more work on the biology of *Azoarcus* spp. and other nonpathogenic microorganisms, which may live endophytically in plant tissue, is needed to understand the key aspects of their relationship to their plant host and their role in the plant-root-soil continuum.

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