FEMS Microbiology Ecology 25 (1998) 117–128

In situ localization of two methanotrophic strains in the rhizosphere of rice plants

Bettina Gilbert [a], Bernhard Aßmus [b], Anton Hartmann [b], Peter Frenzel [a,*]

[a] *Max-Planck-Institut für Terrestrische Mikrobiologie, Karl-von-Frisch Straße, D-35043 Marburg, Germany*
[b] *GSF - National Research Center for Environment and Health, Institute of Soil Ecology, Ingolstaedter Landstr. 1, D-85764 Neuherberg, Germany*

Received 3 July 1997; revised 23 October 1997; accepted 23 October 1997

Abstract

Methanotrophic bacteria play an important role in regulating the methane flux from rice fields to the atmosphere. The abundance of methane-oxidizing bacteria is very heterogeneous along the rice root as revealed by most probable number (MPN) counts and by scanning confocal laser microscopy (SCLM). Two methanotrophic strains, Rp1 and Rp2, were isolated from high dilutions of MPN counts from the rhizoplane of rice roots grown in natural rice field soil. We used monoxenically grown rice plants to relocalize these strains on and in rice roots by means of polyclonal antisera and 16S rRNA probes. Strain Rp1 was even detected in xylem vessels. The two isolates also were able to recolonize roots grown in natural soil under competitive conditions. We found large colonies in deep grooves on the root surface between iron oxide precipitates. Some cells also were observed in the root cortex. The distribution of methane-oxidizing bacteria probably reflects the changing availability of methane and oxygen in the rhizosphere. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: Methanotrophic bacteria; Colonization of rice roots; SCLM; Fluorescently labeled antibody; 16S rRNA probe

---

1. Introduction

The atmospheric trace gas methane is radiatively and chemically active and plays an important role in tropospheric and stratospheric chemistry [1]. Atmospheric methane mixing ratios have increased considerably over the last decades resulting in concerns about its contribution to a global climate change. While methanogenic archaea produce methane in anoxic environments, methanotrophic bacteria limit the amount of methane actually emitted. Methanotrophs can be divided into two phylogenetically and physiologically different groups. Type I methanotrophs cluster in the gamma subdivision, whereas type II methanotrophs cluster in the alpha subdivision of the *Proteobacteria*.

On a global scale, methanotrophic bacteria oxidize more than half of the methane produced [2]. Rice fields account for approx. 20% of global methane emissions, estimations ranging from 10 to 25%. Field measurements indicate that 10 to 50% of the methane produced in rice fields is not emitted due to its reoxidation in the rhizosphere and at the soil surface

---

* Corresponding author. Tel.: +49 (6421) 178 820; Fax: +49 (6421) 178 809; E-mail: frenzel@mailer.uni-marburg.de

0168-6496/98/$19.00 © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.
PII S0168-6496(97)00088-3

[3,4]. Methanotrophs are strictly aerobic because their key enzyme, methane monooxygenase, requires molecular oxygen. They occur at oxic-anoxic interfaces where both methane and oxygen are available. In rice fields, the rhizosphere is such an environment because rice roots are supplied with atmospheric oxygen through the aerenchyma. Oxygen diffuses into the soil, creating an oxygenated zone around the roots [5–7]. On the other hand, the aerenchyma serves as a conduit for methane from methanogenic bulk soil to the atmosphere. Both the rhizoplane and the rhizosphere are therefore suspected to house methane-oxidizing bacteria (MOB).

The association of MOB with plants has been studied with both classical and molecular techniques. Our own MPN counts revealed a significant enrichment of MOB in the rice rhizosphere [8]. Sediment-free roots of many aquatic macrophytes oxidized methane [9] and so did rice roots, too [10]. Recently, Watanabe et al. [11] reported on the oxidation of propylene to propylene oxide by excised roots and a basal portion of the stem indicating the presence and activity of methane monooxygenase. Phylogenetic signature probes applied to RNAs extracted from plant roots revealed that type II methanotrophs dominated in this environment [9]. Finally, there is indirect evidence from MPN counts of surface-sterilized roots that MOB are present within rice roots [12]. However, a direct observation of endophytically occurring methanotrophs is still lacking.

Scanning confocal laser microscopy (SCLM) in combination with fluorescently labeled oligonucleotide probes or antibodies is a powerful tool for studying the spatial structure of biofilms, activated sludge or the rhizosphere [13–15]. The specimen is scanned by a focused laser beam and only the fluorescent signals emerging from the focused plane are detected by a photomultiplier (for reviews see [16,17]). Optical thin-sectioning without destroying the specimen becomes possible and problems resulting from autofluorescence of root material and soil particles are diminished.

The goal of our study was to describe the association of two methanotrophic isolates with rice roots. Monoxenic and soil-grown rice plants were studied using immunological methods and in situ hybridization techniques in combination with confocal laser scanning microscopy.

2. Materials and methods

2.1. Bacterial strains and media

All bacterial strains used in this study are listed in Table 1. The methanotrophic strains Rp1 and Rp2 were isolated from the rhizoplane of rice roots grown in natural soil. The original abundance was 10^8 and 10^{12} bacteria per g dw of root biomass, respectively. Rp1 and Rp2 are both type II methanotrophs that have to the best of our knowledge not been described before. Further characterization is in progress and will be published elsewhere. Using anti-Rp1 antiserum, it could be shown that five other methanotrophic isolates were identical to Rp1. To avoid the isolation of fast-growing organisms that are not abundant in the original environment, high dilutions (10^{-7} and 10^{-12} g^{-1} of dry root material for Rp1 and Rp2, respectively) of MPN counts served as inocula for enrichment cultures. Thus, the original abundance of the isolates in situ was known. The MPN technique for rhizoplane specimens was described previously [8]. Methanotrophs were grown in nitrate mineral salts medium containing (in grams per liter) KNO_3, 1.0; KH_2PO_4, 0.54; $Mg_2SO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2\ H_2O$, 0.015 (Mg_2SO_4 and $CaCl_2$ were added after autoclaving). The pH was adjusted to 6.8. A trace element solution was added after autoclaving [8]. The cells were shaken at 200 rpm under a methane-air headspace (approx. 20% methane).

2.2. Monoxenic culture of rice plants

Seeds of *Oryza sativa* var. Roma type japonica were shaken in 30% sterilization solution (100% solution contains per liter: 105 ml sodium hypochlorite solution, 3 g Na_2CO_3, 100 g NaCl, 5 g NaOH) for 30 min at 50 rpm. The seeds were washed in sterile tap water three times for 30 min each. They were germinated on yeast extract agar plates (containing in grams per liter: yeast extract 2, peptone 3, agar 17) in order to check for contamination. When the seedlings reached a height of 2–3 cm, they were aseptically transferred to glass tubes with cotton stoppers, which permit gas exchange but prevent bacterial contamination. The tubes had a diameter of 3.5 cm and a length of 35 cm, and contained 120 g of glass beads (0.5–1 cm in diameter) and 50 ml of Hoagland's medium (as described in [18] but without malic acid and proline). The medium was mixed with a pre-culture of either isolate Rp1 or Rp2 to give an optical density of approx. $OD_{578nm} = 0.05$. The tubes were incubated in jars under an atmosphere of methane, synthetic air (79.5% N_2/20.5% O_2) and CO_2 (25%/70%/5%) that was renewed every week. Seeds and plants were incubated in a temperature-controlled room at 25°C in a light/dark cycle of 12/12 h at a radiation intensity of approx. 80 μE m^{-2} s^{-1}.

2.3. Production and purification of polyclonal antisera

The anti-Rp1 and anti-Rp2 sera were prepared by Eurogentec, Seraing, Belgium, by injecting a suspension of washed, pasteurized bacteria (approx. 1×10^9 cells (200 μl)$^{-1}$) intramuscularly into rabbits according to their standard immunization protocol. The pre-immunsera showed a positive reaction with *Escherichia coli*. After an initial purification using a protein A-purification kit (Sigma, Deisenhofen, Germany), antibodies against *E. coli* were removed by affinity adsorption with *E. coli*. The antisera were tested for cross-reactivity by ELISA. The ELISA test was described in principle by Schloter et al. [19]. It is based on a colorimetric test in PVC-microtiter plates (Costar, 205 Broadway, Cambridge, MA 02139, USA) with an anti-rabbit IgG conjugated to horseradish peroxidase. The evaluation was made by an ELISA-plate photometer.

2.4. Culture of rice plants in soil

The rice field soil [8] was filled into 600 ml glass beakers and kept under flooded conditions at 25°C. For inoculation experiments, approx. $1 \cdot 10^{11}$ cells of strain Rp1 or Rp2 were mixed with 700 g dry weight (dw) of water-saturated soil. After one week of preincubation, MPN counts of these soil samples revealed 1000 times more methanotrophs than in non-inoculated rice field soil ($5 \cdot 10^8$ vs. $2.3 \cdot 10^5$ MOB (g dw)$^{-1}$, respectively) and about ten times more than in natural rhizospheric soil ($3.7 \cdot 10^6$ MOB (g dw)$^{-1}$). The beakers were planted with 3 or 4 rice seedlings each. They were incubated in a greenhouse and illuminated in a light/dark cycle of 16/8 h at a light intensity of 500 μE m^{-2} s^{-1}.

2.5. Preparation of root pieces for MPN counts of MOB

Rice plants were grown one by one in plastic tubs (15·4·2 cm^3, 80 g dry weight of natural soil) that were incubated in a temperature-controlled room at 25°C. They were illuminated in a light/dark cycle of 12/12 h at a light intensity of approx. 50 μE m^{-2} s^{-1}. The roots were gently washed with tap water until all soil particles were removed. The roots were cut to pieces of 0.5–3 cm in length. Length and diameter of the root pieces were measured using a binocular. Length, diameter and number of lateral roots were estimated. Root pieces were crushed with pestle and mortar. Only a very few organisms adhered to the residual root fibers after this treatment as verified by fluorescein isothiocyanate (FITC) staining and epifluorescence microscopy [8]. The crushed roots were suspended in 1–2 ml of nitrate mineral salts medium and served as inoculum for MPN dilutions (described in [8]). After 3 weeks of incubation, positive wells had a cloudy appearance. Most probable numbers were obtained using Rowe's tables [20].

2.6. Sample preparation and multiple staining of rhizosphere bacteria for SCLM

Roots were fixed in 1% formaldehyde, 60 mM ethylene glycol-bis(β-aminoethylether), 10% dimethyl sulfoxide according to Bauwens et al. [21]. Further sample processing also followed [21] except for the RNAse A treatment which was omitted. During the first fixation step a mild vacuum was sometimes applied to the root pieces to infiltrate the aerenchyma and to ensure that the aerenchymatous tissues also were fixed. Optical cross-sections of root pieces fixed with or without low pressure did not show any morphological differences.

The procedure for combined staining with antibodies and rRNA-directed probes was performed as described previously [22]: After blocking the roots for 20 min with blocking solution (3% bovine serum albumin in sterile PBS; PBS: phosphate buffered saline; pH 7.2), they were incubated for 30 min with the primary antibody, rinsed with washing solution (0.3% bovine serum albumin in 1/10 PBS), and incubated for 40 min with the secondary FITC-labeled antibody (MoBiTec, Göttingen, Germany). All steps were performed at 37°C. Root segments were washed for 5 min with PBT (0.1% Tween-20 (v/v) in PBS) and then fixed with methanol/acetone (1:1) for 15 min at 25°C. After rinsing with 50% methanol and with hybridization solution, the hybridization procedure was followed according to Manz et al. [23]. The root pieces were finally stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min at 25°C [24], air-dried and mounted in Citifluor antifading solution (Citifluor Ltd, Canterbury, Kent, UK). If no hybridization with 16S rRNA probes was desired, the counterstaining with DAPI was performed immediately after the immunofluorescence staining.

The following oligonucleotide probes were used: (i) Eub338, complementary to a region of the 16S rRNA specific for the domain *Bacteria* [25], (ii) 9α, complementary to a region of the 16S rRNA specific for serine pathway methylotrophs [26,27], (iii) EubM, a mixture of probes complementary to regions of the 16S rRNA specific for *Bacteria*: Eub338 [25], Eub927 [28], Eub785, Eub1055 and Eub1088 [29]. To control hybridization stringency, 10% NaCl but no formamid was used for all three probes. The probes were purchased already coupled with tetramethylrhodamine-5-isothiocyanate (TRITC) and purified by high-pressure liquid chromatography (MWG Biotech, Ebersberg, Germany). They were 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.

We investigated roots from monoxenic plants aged 2 and 4 weeks inoculated with Rp1 (5 plants) and Rp2 (2 plants) and from monoxenic plants aged 8 weeks inoculated with Rp1 (10 plants). In addition, we investigated plants that had been grown for 9 weeks in Rp1- or Rp2-enriched soil (4 plants

Table 1

Bacterial strains used in this study, their source and phylogenetic position and positive (+) or negative (−) reaction with the polyclonal antisera anti-Rp1 and anti-Rp2, respectively

Strain	Phylogenetic position	anti-Rp1	anti-Rp2	source
Rp1	α-proteobacteria	+	−	own isolate
Rp2	α-proteobacteria	−	+	own isolate
Methylocystis parvus	α-proteobacteria	−	−	NCIMB11132[a]
Methylosinus trichosporium strain OB3b	α-proteobacteria	−	−	C. Murrell[b]
Methylosinus sporium	α-proteobacteria	−	−	C. Murrell[b]
Bradyrhizobium japonicum strain USDA	α-proteobacteria	−	−	D. Werner[c]
Rhizobium tropici CIAT 899	α-proteobacteria	−	−	D. Werner[c]
Rhizobium trifolii DSM 30141	α-proteobacteria	−	−	D. Werner[c]
Agrobacterium rhizogenes A4	α-proteobacteria	−	−	D. Werner[c]
Agrobacterium tumefaciens C 58	α-proteobacteria	−	−	D. Werner[c]
Nitrobacter whittenburyi	α-proteobacteria	−	−	W. Tappe[d]
Nitrosomonas europaea	β-proteobacteria	−	−	W. Tappe[d]
Azoarcus communis	β-proteobacteria	−	−	B. Reinhold-Hurek[e]
Methylobacter agile	γ-proteobacteria	−	−	C. Murrell[b]
Methylobacter whittenburyi	γ-proteobacteria	−	−	C. Murrell[b]
Methylomonas methanica	γ-proteobacteria	−	−	NCIMB11130[a]
Methylococcus capsulatus	γ-proteobacteria	−	−	NCIMB 11132[a]
Eschericchia coli DH5α	γ-proteobacteria	−	−	B. Reinhold-Hurek[e]
Pseudomonas putida	γ-proteobacteria		−	R. Conrad[e]
Pseudomonas Ps88	γ-proteobacteria	−	−	R. Conrad[e]
Clostridium strain XB 90	Gram+, high GC	−	−	P. Janssen[e]
Bacillus subtilis	Gram+, low GC	−	−	M. Marahiel[c]
Methanobacterium bryantii	euryarchaeota	−	−	P. Janssen[e]

The selected bacteria are phylogenetically related to isolates Rp1 and Rp2 or may occur in rice field soil.

[a] National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom.

[b] University of Warwick, United Kingdom.

[c] Philipps University, Marburg, Germany.

[d] KFA, Jülich, Germany.

[e] MPI für Terrestrische Mikrobiologie, Marburg, Germany.

each). Representative parts of the roots were selected according to their morphological appearance (colour, thickness) and to the root region (root tip, root hair zone, root with lateral roots).

2.7. *Scanning confocal laser microscopy (SCLM)*

An inverted scanning confocal laser microscope (LSM 410, Zeiss, Jena, Germany) equipped with excitation laser lines at 364, 488, and 543 nm was used as described in [13].

3. Results

3.1. *Numbers of MOB on single root pieces and on selected root regions*

By MPN counts we found large variations in numbers of MOB on single roots. The highest numbers were found on dark-brown roots (with iron oxide deposition, [30]). Values ranged from $<0.9\times10^3$ to $>6.3\times10^5$ g^{-1} fresh weight. That was equivalent to <0.1 MOB mm^{-2} and >123 MOB mm^{-2} of root surface, respectively. In order to get a larger sample size and thus a lower detection limit, root tips and lateral root regions of several roots of one plant were pooled. Numbers of MOB were generally lower on root tips than in the region with lateral roots (Table 2).

3.2. *Specificity of antisera*

Antisera were produced against the two methanotrophic strains Rp1 and Rp2 isolated from the rhizoplane of rice roots. Specificity of antisera was tested against several methanotrophs, other proteobacteria of the α, β and γ subgroup, and a few other strains that may occur in rice field soil. Purified anti-Rp1 and anti-Rp2 sera showed no cross-reactivity against the tested strains (Table 1). Consequently, the antisera were regarded as strain-specific and used not only with monoxenic but also with soil-grown plants.

Non-specific binding of the secondary FITC-labeled antibody was not observed with monoxenic plants. With soil-grown roots, however, we observed some non-specific binding to soil particles, probably caused by antigenic determinants that are known to be part of the organic fraction of soils [31]. Those areas exhibiting fluorescent amorphous particles of varying sizes and shapes were clearly distinguishable from bacteria with well defined cell boundaries.

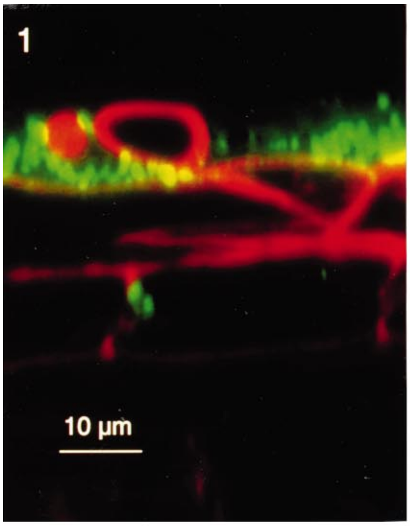
3.3. *Colonization of monoxenic plants with Rp1 and Rp2*

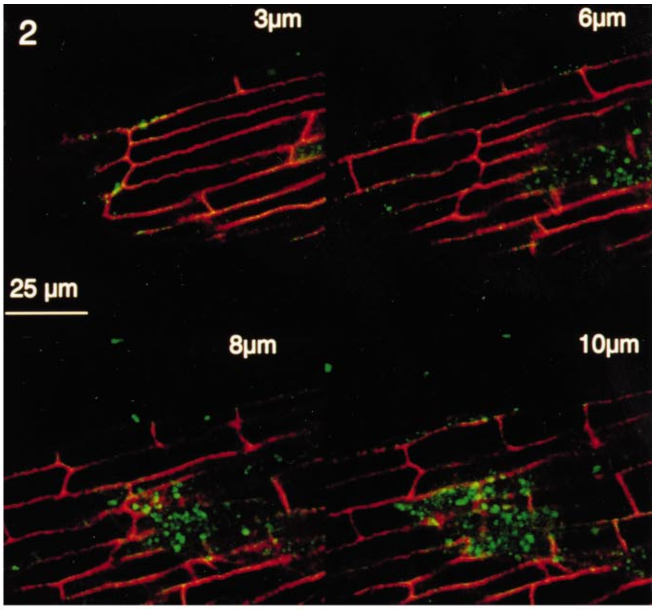
The colonization of rice roots was very heterogeneous. Some roots were not colonized at all and these could not be distinguished by any macroscopic feature. On other roots, colonization ranged from single MOB cells through microcolonies and clumps of bacteria to thick bacterial layers. These bacteria were all stained by DAPI and by the FITC-labeled strain-specific antibody. A few epidermal plant cells were densely packed with bacteria. The nuclei of these cells were either not visible or visible only as a shadow, apparently being degraded, while nuclei of the neighbouring epidermal and cortex cells were usually intact. The bacteria were stained by DAPI, and very few cells at the surface were labeled by antibody. This may suggest that the roots were contaminated by unidentified bacteria. However, we

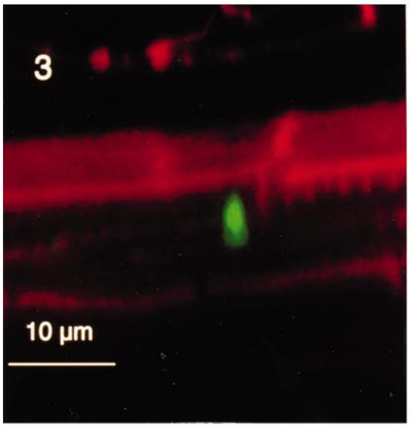
Table 2
Numbers of MOB on different regions of rice roots

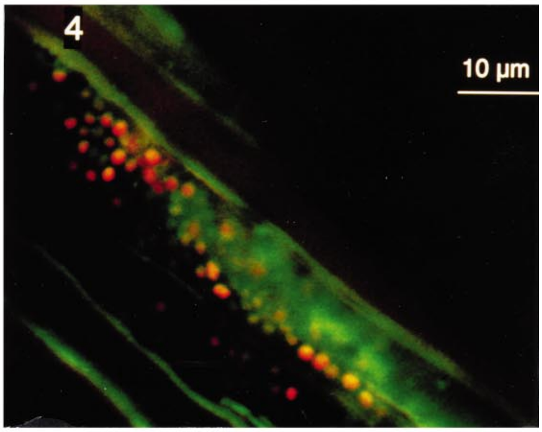
Age of rice plant	Root tip		Root region with lateral roots	
Weeks	MPN 10^5 MOB (g fw)$^{-1}$	mg fw	MPN 10^5 MOB (g fw)$^{-1}$	mg fw
3	<0.1	7.4	6.0 (3.7; 9.8)	16.5
6	0.5 (0.3; 0.8)	2.1	5.0 (3.1; 8.2)	16.0
6	<0.4	1.3	1.0 (0.7; 1.6)	157.0

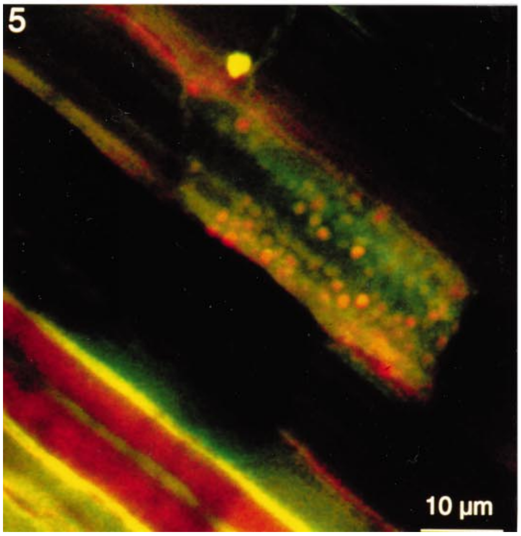
Root tips (the first 0.5 cm of the root) and lateral root regions of several roots (of one plant) were combined in order to get an appropriate sample size. The sample size is indicated in mg fresh weight (fw). Asymmetric 95% confidence limits calculated according to Cochran [49] are indicated in brackets. The detection limit was approx. 10^4 MOB (g fw)$^{-1}$.

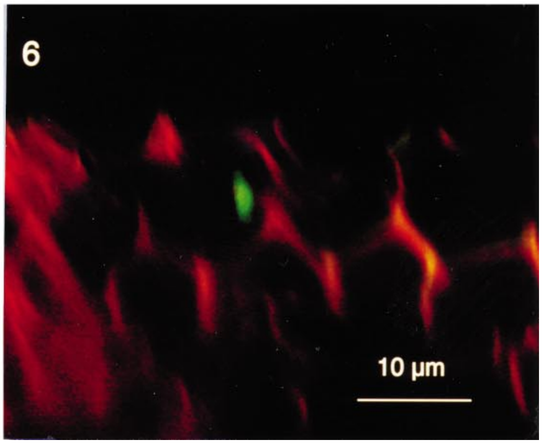

1
10 µm


2
3µm
6µm
25 µm
8µm
10µm


3
10 µm


4
10 µm


5
10 µm


6
10 µm

Figs. 1–6. Confocal microscopy of rice roots. The methanotrophic isolates Rp1 and Rp2 were detected by strain-specific polyclonal antisera and a FITC-labeled secondary antibody. In some cases, a TRITC-labeled 16S rRNA probe complementary to a region specific for the domain *Bacteria* (EubM) was used (4, 5). The excitation wavelength was 488 nm for the fluorochrome FITC and 543 nm for the plant cell wall fluorescence and for the fluorochrome TRITC. 1–3: Roots of monoxenic plants grown in liquid culture with glass beads. 4–6: Roots of soil-grown plants. The natural soil was inoculated with isolate Rp1 and contained 1000 times more methanotrophs than non-inoculated natural soil ($5 \cdot 10^8$ MOB (g dry weight)$^{-1}$ vs. $2.3 \cdot 10^5$ MOB (g dry weight)$^{-1}$, respectively). 1: Dense bacterial cell layer of isolate Rp1 on the root surface and two bacterial cells in the root cortex. Sagittal optical section ('z-scan'). 2: Colony of isolate Rp1 in the root cortex. The cells are located in the 2nd and 3rd cortical cell layer. Individual optical sections were collected at intervals of 1 μm (z-series). Four of them are shown as examples. 3: One bacterial cell in the xylem tissue. Sagittal optical section ('z-scan'). 4: Rp1 colony in a deep groove between the iron oxide coating on a dark-brown root. About one-third of the antibody-labeled cells were also stained by 16S rRNA probe EubM. The iron oxide appears black to dark-brown. 5: Rp1 colony in an epidermal cell. The plant cells surrounding the colonized cell appear black or dark-brown because of iron oxide precipitates. 6: Rp2 cell in the outer cortical layer. The cell surface appears black because of iron oxide precipitates. Vertical optical section ('z-scan').

←

think that the plants were still gnotobiotic but that diffusion of the antibody was hampered by the dense matrix of bacteria. This interpretation is supported by the finding that we always found part of the antibody-labeled cells on the root surface additionally stained by probe Eub338 or 9α, but never found cells in the endorhizosphere that bound to one of the 16S rRNA probes.

The autofluorescence of root cortex and epidermal cell walls was relatively high. Therefore, it was easy to define cell boundaries and to localize bacteria exactly. When bacteria situated in deeper layers of a root specimen are viewed with a laser microscope, however, the autofluorescence is likely to cause shadowing effects [32]. Indeed, it was often difficult to localize MOB in relation to the plant cell walls in the fully differentiated aerenchymatous region and in the stelar cylinder.

We observed that some colonization characteristics depended on the plant's age. In young monoxenic rice plants (2 and 4 weeks old), MOB were found mainly at the sites of emergence of lateral roots, on root tips and in the area about 50 μm behind the root cap. Apart from these hot spots, strain Rp1 was rarely found on young roots and if so, only single cells occurred. In contrast, the surface of lateral roots was sometimes covered with strain Rp2, and the distribution of bacterial cells followed the plant cell wall pattern. With roots of 4 week-old plants, a few single antibody-labeled Rp1 cells were found in the outer cortical layers.

While observing many roots of young plants (2 and 4 weeks old) that were devoid of any MOB or colonized only by a few single cells, we observed a certain correlation: if nuclei were present in root cortex cells (stained by DAPI), MOB could usually be found on the rhizoplane, whereas if no nuclei were visible, there usually were no bacterial cells on the root surface.

Older plant's roots (8 to 10 weeks old) were colonized to a greater extent by strain Rp1 as compared to younger monoxenic plants. Here again, the distribution pattern of bacteria sometimes followed the plant cell wall pattern. MOB on the root surface preferentially colonized grooves between two epidermal cells on the root surface. In some cases, we observed a bacterial layer that was up to 10 μm thick (Fig. 1.1). Strain Rp1 also colonized the interior of rice roots (Fig. 1.1). Single cells and microcolonies were found in the root cortex, e.g. in the 2nd and 3rd cortical layer (Fig. 1.2). Whether the bacteria were located intra- or intercellularly could not be determined with certainty. Very few cells also were observed in xylem vessels of the central cylinder (Fig. 1.3). In the neighbouring area of these 'truly endophytic' cells no lesions could be found.

3.4. Colonization of rice roots grown in natural soil

Rice roots are known to oxidize the surrounding soil and reduced iron species in particular, resulting in brown iron oxide coatings. We distinguished three morphological stages of iron oxide precipitation. (i) With white or light-brown roots, iron oxide precipitates followed the plant cell wall pattern. At the sites of emergence of lateral roots, a ring of brown precipitate had often been formed. (ii) Some cells in a given area were covered with iron oxide while others remained free giving the root a chequered pattern. Macroscopically, the roots were brown. (iii) Dark-brown roots were covered all over with iron oxide. It did not fluoresce and appeared black in the confocal image.

The colonization pattern of the two isolates did not differ on roots from 9 week-old plants grown in inoculated soil. Root tips did not have a large cap and were not colonized. White and light-brown roots were scarcely colonized by single cells that often were found at the emergence sites of lateral roots and in grooves between epidermal cells. Some were labeled only by antibody, indicating that these were Rp1 or Rp2 cells that were not active. Some were labeled by one of the 16S rRNA probes (EubM and 9α were used alternatively), indicating that these were active bacteria but not strain Rp1 or strain Rp2. Some were labeled by an antibody and a 16S rRNA probe, indicating that these Rp1 or Rp2 cells were active.

On dark-brown roots, we often found deep grooves densely packed with antibody-labeled bacteria. In one case, about one third of the Rp1 cells also were labeled by probe EubM (Fig. 1.4) and the intensity of the label varied suggesting varying ribosome contents. Usually, though, much less MOB were labeled by probe EubM or 9α than by antibody. Another colony of Rp1 cells was observed in an epidermal cell (Fig. 1.5). We also saw some Rp1 and Rp2 cells in the outer cortical cell layer (Fig. 1.6). However, it often was impossible to check whether the root surface was intact in this area because the iron oxide-covered surface appeared black in the vertical optical section (z-scan).

4. Discussion

Here, we present for the first time direct evidence that methanotrophic bacteria (MOB) live in very close association with wetland rice plants and may even colonize the endorhizosphere. Our isolates that were obtained from high MPN dilutions of the rice rhizoplane successfully recolonized roots of monoxenic plants. They formed dense layers and clumps on the root surface, colonized epidermal cells, they invaded the roots and were detected in the cortex and even in xylem vessels. The ability of MOB to penetrate rice roots had been suggested from findings of MOB in surface-sterilized roots [12]. Our isolates were also detected on plants that were grown in inoculated soil which means that they survived and established themselves under competitive conditions.

The SCLM is a non-invasive method and represents an alternative to conventional thin-sectioning techniques. In particular, it allows to study large samples helping to localize hot spots of bacterial colonization. In our study we applied a combination of polyclonal antisera and 16S rRNA oligonucleotide probes. The polyclonal antisera were strain-specific after affinity purification with *E. coli*. We assume that the antigenic determinants were quite stable under adverse environmental conditions because we detected bacterial cells that apparently were not physiologically active as they were not labeled by a 16S rRNA probe. Especially in soils, bacteria frequently enter a dormant state in which they are viable yet non-culturable [33]. This would not necessarily affect (polyclonal) antibody binding, since surface structures might be preserved and there are several antigenic determinants on the cell surface. It would, however, lower the ribosome content [34] and result in a weak or undetectable signal when using 16S rRNA probes [22]. On the other hand, if cells are labeled with antibody and with 16S rRNA probe, we may assume that these cells probably were metabolically active (e.g. Fig. 1.5).

We could not find any endorhizospheric cells to which one of the 16S rRNA probes had bound. Either these cells were physiologically inactive or the fluorescence signal intensity of the probes was too low. The signal of the antibodies was amplified by the label of the secondary antibody, whereas each 16S rRNA probe had only one fluorochrome molecule.

Bacterial colonization of rice roots was very heterogeneous in its extent. This was proposed after our preliminary studies of roots with a scanning electron microscope (data not shown). Some roots were devoid of any bacteria, others were scarcely colonized (e.g. in the root hair zone), and again others exhibited hot spots of colonization with thick clumps of bacteria in some areas. Kimura et al. [35] found that rice roots were scarcely colonized except for regions with root hairs and with lateral root emergence. The heterogeneity was confirmed for MOB by MPN counting of single root pieces and of pooled pieces from several roots (Table 2) and it was even more obvious when examining root pieces with the SCLM. On some pieces we could hardly find any bacteria (MOB or heterotrophs) despite of their general abundance in this environment.

Bacterial colonization of MOB can be expected where oxygen is available. Indeed, it seems to begin at the sites of lateral root emergence and in the elongation zone behind the root tip as these are hot spots of bacterial colonization on roots of young rice plants. The base of lateral roots may be a favorable site because the root aerenchyma may be in direct contact with the environment there [36]. With soil-grown roots, we observed a ring of brown iron oxide precipitate around the emerging lateral root indicating oxygen leakage. The elongation zone may be a favorable site as well. It has been suggested to be another site of oxygen release because aerenchyma formation begins there [37] and the root epidermis is not yet differentiated. However, the metabolic oxygen demand of the root is also high in this region.

With monoxenic rice plants, we often observed that the pattern of MOB cells followed the plant cell wall pattern. This is a well known colonization pattern for other bacteria (e.g. [38,39]) and is usually ascribed to exudation of organic substrates at these sites [40]. However, it was rather unexpected for MOB as they cannot utilize complex organic substrates for growth and are even inhibited by them [41,42]. They do utilize some amino acids as N-sources, though, and these are also among the root exudates of rice. In addition and perhaps more important, plant cell walls may be sites of oxygen leakage [37]. This hypothesis was supported by our finding that iron oxide precipitates also followed the plant cell wall pattern in an early stage. Also, with soil-grown plants, the deep grooves that occurred along the cell walls of epidermal cells were often extensively colonized (confer also [43]). In one case, one-third of the antibody-labeled cells in such a groove were additionally stained by probe EubM in various intensities (Fig. 1.4), indicating that these cells were metabolically active. This suggests that these grooves were not artifacts due to the fixation process but represented niches where oxygen may be available and MOB may be actively oxidizing methane.

The root tips of monoxenic plants but not of soil-grown plants also were colonized by MOB, sometimes intensively. In natural soil, root cap cells usually are sloughed off as the tip grows although it was shown that bacteria may colonize root tips of soil-grown wheat [44]. In monoxenic plants, there was less abrasion, and root caps were very large and densely colonized by microbes [18,45].

In young monoxenic rice plants (2 to 4 weeks old), we seldom found endophytic MOB but in old plants (8 to 10 weeks old), we regularly found antibody-labeled bacterial cells in the root cortex and sometimes even in xylem vessels. In soil-grown roots, we found some bacteria in the root cortex, but iron oxide precipitations made it difficult to find and localize the bacteria precisely. MPN counts of surface-sterilized soil-grown roots indicated that approx. 1% of the total root MOB (after homogenization) occurred in the endorhizosphere. The ability to invade a rice root may have great advantages for MOB because the oxygen partial pressure will be higher inside the root than on the root surface or in the rhizosphere where competition for oxygen is high. The question of how and where the strains Rp1 and Rp2 penetrate the root remains unsolved. We presume that MOB enter the roots through natural lesions, e.g. at wounds, or at sites where lateral roots emerge. These sites were also hot spots of colonization. However, in order to get into the xylem, the bacteria must penetrate the endodermis, possibly through pits as shown for *Azoarcus* sp. [18]. Because of shadowing effects the maximum depth for microscopical examination was often limited to about 30 μm which is about the thickness of the root cortex [36]. Therefore, it is possible that colonization of the central cylinder may be more intense than we found.

Why is the colonization of rice roots by MOB so heterogeneous? We have to keep in mind that it depends on the availability of oxygen and methane. It is useful to consider the difference between white and dark-brown roots. There are several different situations where roots may be white or light-brown. It may be that no oxygen leaks into the soil, no iron oxide is formed, and thus aerobic bacteria cannot colonize the root. It may be that the rhizosphere is highly oxygenated and iron oxide precipitates are formed primarily in the surrounding soil. Then, roots are only scarcely colonized by MOB because enough oxygen is available in the surrounding soil and rhizoplane MOB are severely methane-limited. On the other hand, dark-brown roots are covered with iron oxide and only very little oxygen is likely to overcome this barrier. Therefore, bacteria in need of oxygen have to cling to dark-brown roots as much as possible. However, the question of a possible correlation between the formation of iron oxides and extensive colonization of the root interior could not be addressed because of the shadowing effects of iron oxides.

Various studies with classical and molecular techniques [9,12] have revealed that methanotrophs may be associated with wetland plants and rice roots in particular. All plant roots are associated with numerous species of microorganisms, beneficial as well as pathogenic. In particular, diazotrophic bacteria have been reported to invade rice roots [18,46]. Many rhizobacteria are known for their plant growth-promoting capacities [47]. They are more or less closely associated to roots and enhance growth by nitrogen fixation, phosphate solubilization, phytohormone production etc. Whether or not MOB are plant growth-promoting, remains to be elucidated. MOB have been found in a variety of different environments and cannot be regarded as explicit rhizobacteria [48]. However, type II organisms may be useful to the plant with respect to their nitrogen fixing ability. Indeed, our isolates belonged to MOB type II and signature probe hybridizations to bacterial RNA preparations from plant roots of aquatic macrophytes also revealed that type II methanotrophes dominated in this environment [49]. More research is needed to fully understand the interaction between MOB and rice plants.

Acknowledgments

We thank those people who kindly provided strains, P. Hutzler (GSF-National Research Center for Environment and Health, Institute of Pathology, D-85764 Neuherberg) for enabling studies with the confocal laser microscope, W. Liesack for the phylogenetic classification of the isolates Rp1 and Rp2, and R. Conrad for discussion. This study was financially supported by the Deutsche Forschungsgemeinschaft (Fr 1054/1) and the European Commission (Contract No EV5V-CT94-0499 and BIO4-CT96-0419).

References

[1] Lelieveld, J., Crutzen, P.J. and Brühl, C. (1993) Climate effects of atmospheric methane. Chemosphere 26, 739–768.

[2] Reeburgh, W.S., Whalen, S.C. and Alperin, M.J. (1993) The role of methylotrophy in the global methane budget. In: Microbial Growth on C1 Compounds (Murrell, J.C. and Kelly, D.P., Eds.), pp. 1–14. Intercept, Andover.

[3] Epp, M.A. and Chanton, J.P. (1993) Rhizospheric methane oxidation determined via the methyl fluoride inhibition technique. J. Geophys. Res. 98, 18413–18418.

[4] Denier van der Gon, H.A.C. and Neue, H.-U. (1996) Oxidation of methane in the rhizosphere of rice plants. Biol. Fertil. Soils 22, 395–366.

[5] Armstrong, W. (1971) Radial oxygen losses from intact rice roots as affected by distance from the apex, respiration and waterlogging. Physiol. Plant. 25, 192–197.

[6] Trolldenier, G. (1988) Visualization of oxidizing power of rice roots and of possible participation of bacteria in iron deposition. Z. Pflanzenernähr. Bodenk. 151, 117–122.

[7] Frenzel, P., Rothfuss, F. and Conrad, R. (1992) Oxygen profiles and methane turnover in a flooded rice microcosm. Biol. Fertil. Soils 14, 84–89.

[8] Gilbert, B. and Frenzel, P. (1995) Methanotrophic bacteria in the rhizosphere of rice microcosms and their effect on porewater methane concentration and methane emission. Biol. Fertil. Soils 20, 93–100.

[9] King, G.M. (1994) Associations of methanotrophs with the roots and rhizomes of aquatic vegetation. Appl. Environ. Microbiol. 60, 3220–3227.

[10] Frenzel, P. and Bosse, U. (1996) Methyl fluoride, an inhibitor of methane oxidation and methane production. FEMS Microbiol. Ecol. 21, 25–36.

[11] Watanabe, I., Hashimoto, T. and Shimoyama, A. (1997) Methane-oxidizing activities and methanotrophic populations associated with wetland rice plants. Biol. Fertil. Soils 24, 261–265.

[12] Bosse, U. and Frenzel, P. (1997) Activity and distribution of methane-oxidizing bacteria in flooded rice soil microcosms and in rice plants (*Oryza sativa*). Appl. Environ. Microbiol. 63, 1199–1207.

[13] Aßmus, B., Hutzler, P., Kirchhof, G., Amann, R., Lawrence, J.R. and Hartmann, A. (1995) In situ localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. Appl. Environ. Microbiol. 61, 1013–1019.

[14] Wagner, M., Amann, R., Kämpfer, P., Aßmus, B., Hartmann, A., Hutzler, P., Springer, N. and Schleifer, K.-H. (1994) Identification and in situ detection of gram-negative filamentous bacteria in activated sludge. Syst. Appl. Microbiol. 17, 405–417.

[15] Wolfaardt, G.M., Lawrence, J.R., Robarts, R.D., Caldwell, S.J. and Caldwell, D.E. (1994) Multicellular organization in a degradative biofilm community. Appl. Environ. Microbiol. 60, 434–446.

[16] Laurent, M., Johannin, G., Gilbert, N., Lucas, L., Cassio, D., Petit, P.X. and Fleury, A. (1994) Power and limits of laser scanning confocal microscopy. Biol. Cell 80, 229–240.

[17] Shotton, D. and White, N. (1989) Confocal scanning microscopy: three-dimensional biological imaging. Trends Biochem. Sci. 14, 435–439.

[18] Hurek, T., Reinhold-Hurek, B., van Montagu, M. and Kellenberger, E. (1994) Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. J. Bacteriol. 176, 1913–1923.

[19] Schloter, M., Bode, W., Hartmann, A. and Beese, F. (1992) Sensitive chemiluminescence-based immunological quantification of bacteria in soil extracts with monoclonal antibodies. Soil Biol. Biochem. 24, 399–403.

[20] Rowe, R., Todd, R. and Waide, J. (1977) Microtechnique for Most-Probable-Number Analysis. Appl. Environ. Microbiol. 33, 675–680.

[21] Bauwens, S., Katsanis, K., van Montagu, M., van Oostveldt, P. and Engler, G. (1994) Procedure for whole mount fluorescence in situ hybridization of interphase nuclei on *Arabidopsis thaliana*. Plant J. 6, 123–131.

[22] Aßmus, B., Schloter, M., Kirchhof, G., Hutzler, P. and Hartmann, A. (1997) Improved in situ tracking of rhizosphere bacteria using dual staining with fluorescence-labeled antibodies and rRNA-targeted oligonucleotides. Microb. Ecol. 33, 32–40.

[23] Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.-H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst. Appl. Microbiol. 15, 593–600.

[24] Porter, K.G. and Feig, Y.S. (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 25, 943–948.

[25] Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.

[26] Tsien, H.C., Bratine, B.J., Tsuji, K. and Hanson, R.S. (1990) Use of oligodeoxynucleotide signature probes for identification of physiological groups of methylotrophic bacteria. Appl. Environ. Microbiol. 56, 2858–2865.

[27] Brusseau, G.A., Bulygina, E.S. and Hanson, R.S. (1994) Phylogenetic analysis and development of probes for differentiating methylotrophic bacteria. Appl. Environ. Microbiol. 60, 626–636.

[28] Giovannoni, S.J., DeLong, E.F., Olsen, G.J. and Pace, N.R. (1988) Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. 170, 720–726.

[29] Lee, S., Malone, C. and Kemp, P.F. (1993) Use of multiple 16S rRNA-targeted fluorescent probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. Mar. Ecol. Prog. Ser. 101, 193–201.

[30] Chen, C.C., Dixon, J.B. and Turner, F.T. (1980) Iron coatings on rice roots: Mineralogy and quantity influencing factors. Soil Sci. Soc. Am. J. 44, 635–638.

[31] Schloter, M., Aßmus, B. and Hartmann, A. (1995) The use of immunological methods to detect and identify bacteria in the environment. Biotechnol. Adv. 13, 75–90.

[32] Czymmek, K.J., Whallon, J.H. and Klomparens, K.L. (1994) Confocal microscopy in mycological research. Exp. Mycol. 18, 275–293.

[33] Roszak, D.B. and Colwell, R.R. (1987) Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51, 365–379.

[34] Schaechter, M.O., Maaloe, O. and Kjelgaard, N.O. (1958) Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. 19, 592–606.

[35] Kimura, M., Murakami, H. and Wada, H. (1989) Microbial colonization and decomposition processes in rice rhizoplane I. Microbial colonization associated with lateral root emergence. Soil Sci. Plant Nutr. 35, 63–70.

[36] Butterbach-Bahl, K. (1993) Mechanismen der Produktion und Emission von Methan in Reisfeldern. IFU-Schriftenreihe 14-93, ISBN 3-927548-53-7, Wiss.-Verl. Maraun, Frankfurt/M.

[37] Ando, T., Yoshida, S. and Nishiyama, I. (1983) Nature of oxidizing power of rice roots. Plant Soil 72, 57–71.

[38] Bennett, R.A. and Lynch, J.M. (1981) Bacterial growth and development in the rhizosphere of gnotobiotic cereal plants. J. Gen. Microbiol. 125, 95–102.

[39] Chin-A-Woeng, T.F.C., de Priester, W., van der Bij, A.J. and Lugtenberg, B.J.J. (1997) Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365, using scanning electron microscopy. MPMI 10, 79–86.

[40] Bowen, G.D. (1979) Integrated and experimental approaches to the study of growth of organisms around roots. In: Soil-Borne Plant Pathogens (Schippers, B. and Gams, W., Eds.), pp. 207–227. Academic Press, London.

[41] Eccleston, M. and Kelly, D.P. (1973) Assimilation and toxicity of some exogenous C_1 compounds, alcohols, sugars and acetate in the methane-oxidizing bacterium *Methylococcus capsulatus*. J. Gen. Microbiol. 75, 211–221.

[42] Gilbert, B. (1997) Methanotrophe Bakterien in der Reisrhizosphäre. Dissertation Universität Marburg. Tectum Verlag, Marburg.

[43] Foster, R.C., Rovira, A.D. and Cock, T.W. (1983) Ultrastructure of the Root-Soil Interface. The American Phytopathological Society, St. Paul, MN, USA.

[44] Campbell, R. and Greaves, M.P. (1990) Anatomy and community structure of the rhizosphere. In: The Rhizosphere (Lynch, J.M., Ed.), pp. 11–34. John Wiley and Sons, Chichester, New York.

[45] Wiehe, W., Hecht-Buchholz, C. and Höflich, G. (1994) Electron microscopic investigations on root colonization of *Lupinus albus* and *Pisum sativum* with two associative plant growth

promoting rhizobacteria, *Pseudomonas fluorescens* and *Rhizobium leguminosarum* bv. trifolii. Symbiosis 17, 15–31.

[46] Reding, H.K., Hartel, P.G. and Wiegel, J. (1991) Effect of *Xanthobacter*, isolated and characterized from rice roots, on growth of wetland rice. Plant Soil 138, 221–230.

[47] Glick, G.B. (1995) The enhancement of plant growth by free-living bacteria. Can. J. Microbiol. 41, 109–117.

[48] Hanson, R.S. and Hanson, T.E. (1996) Methanotrophic bacteria. Microbiol. Rev. 60, 439–471.

[49] Cochran, W.G. (1950) Estimation of bacterial densities by means of the "Most Probable Number". Biometrics 6, 105–116.