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Detection and identification of bacteria intimately associated with fungi of the order *Sebacinales*

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Summary

Because of their beneficial impact on plants, the highly diverse mycorrhizal fungi grouped in the order *Sebacinales* lay claim to high ecological and agricultural significance. Here, we describe for the first time associations of Sebacinoid members with bacteria. Using quantitative PCR, denaturating gradient gel electrophoresis and fluorescence *in situ* hybridization, we detected an intimate association between *Piriformospora indica* and *Rhizobium radiobacter*, an α-Proteobacterium. The stability of the association, vertical transmission of the bacteria during asexual fungal reproduction and fungal plant colonization was monitored using *R. radiobacter*-specific primers. Treatment of mycelium or fungal protoplasts with antibiotics highly efficient against the free bacteria failed to cure the fungus. Barley seedlings dip-inoculated with *R. radiobacter* showed growth promotion and systemic resistance to the powdery mildew fungus *Blumeria graminis* comparable to *P. indica* inoculation. By screening additional isolates of the *Sebacina vermifera* complex, three species-specific associations with bacteria from the genera *Paenibacillus*, *Acinetobacter* and *Rhodococcus* were found. These findings suggest that *Sebacinales* species regularly undergo complex interactions involving host plants and bacteria reminiscent of other ectomycorrhizal and endomycorrhizal associations.

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Introduction

In natural ecosystems, plants experience complex interactions with microorganisms on physical, metabolic and functional levels, and hardly a single plant family has been recognized, which is not living in symbiosis with microorganisms (Smith and Read, 1997; Frey-Klett *et al.*, 2007). Plant symbiotic microbes can grow, propagate and interact not only in form of individual cells but also as multitrophic communities. In many cases this might be the key for the widespread success of host–microbe symbioses because of the immense biochemical and physiological diversity among microbial species and the ability of microbial cells and microbial communities to precisely sense and properly respond to changing environments.

Here we describe symbiosis of barley with species of the fungal order *Sebacinales* with emphasis on fungus-associated bacteria. *Sebacinales*, the most basal Basidiomycota group with known mycorrhizal members, are ubiquitously distributed and are found on all continents in temperate and subtropical climates associated with orchids, liverwort thalli and Ericaceae as ectomycorrhizal and endomycorrhizal fungi (Selosse *et al.*, 2007; Schäfer and Kogel, 2008). Recent studies indicate that they also form a novel type of mutualistic symbiosis with a broad spectrum of monocotyledonous and dicotyledonous plants (Weiss *et al.*, 2004; Matheny *et al.*, 2007), including crop plants such as barley, maize, tomato and – in contrast to arbuscular mycorrhiza fungi – *Brassicaceae* (Varma *et al.*, 1999; Peskan-Berghoefer *et al.*, 2004, Deshmukh *et al.*, 2006). *Sebacinales* are divided into two clades (Selosse *et al.*, 2007). Clade A consists of ectomycorrhizae and ectendomycorrhizae species whereas Clade B includes ericoid along with cultivable orchid root colonizing mycorrhiza species of the complex *Sebacina vermifera* and *Piriformospora indica* (Varma *et al.*, 1998; Weiss *et al.*, 2004). Plants colonized by these

species display improved growth and fitness. Barley plants colonized by *P. indica* show higher grain yield and superior resistance against various root and leaf pathogens (Waller *et al*., 2005; Deshmukh *et al*., 2006). Because of its remarkable beneficial activity the *Sebacinales* have attracted considerable interest as potential biocontrol agents, especially because these fungi are not obligate biotrophs and thus axenically cultivable.

Many recent reports on mycorrhizal interactions suggest that biological activities brought about by the symbiosis ought to be considered under the premise of a more complex tripartite interplay of the host plant with the mycorrhiza fungus and fungus-associated bacteria. These bacteria can associate with fungal spores (Walley and Germida, 1997), hyphae (Nurmiaho-Lassila *et al*., 1997; Sbrana *et al*., 2000), or are present as endobacteria inside fungal cells (Bertaux *et al*., 2005; Lumini *et al*., 2006). In many cases these associations show a certain type of specificity (Artursson *et al*., 2006). In 1994, Garbaye (1994) introduced the term mycorrhization helper bacteria for bacteria associated with mycorrhizal fungi which consistently promote mycorrhizal development. There is a wide variety of beneficial effects mediated by these bacteria to the mycorrhizal partner which include inhibition or promotion of germination and alterations to foraging behaviour, hyphal branching (fungal architecture), growth, survival, reproduction, exudate composition and production of antibacterial metabolites (Rainey *et al*., 1990; Frey-Klett and Garbaye, 2005; Aspray *et al*., 2006; Riedlinger *et al*., 2006; Frey-Klett *et al*., 2007). Although most studies of bacterial interaction have been conducted in ectomycorrhizal systems, the interaction between bacteria and arbuscular mycorrhizal (AM) symbioses has also been shown (Bianciotto *et al*., 1996; Duponnois and Plenchette, 2003). Bacteria have been shown to increase the germination and growth of AM fungi thus helping in the symbiosis (Artursson *et al*., 2006). These beneficial mycorrhizal associates belong to diverse bacterial groups, including Gram-negative Proteobacteria, e.g. *Agrobacterium*, *Azospirillum*, *Azotobacter, Burkholderia*, *Bradyrhizobium*, *Enterobacter*, *Pseudomonas, Klebsiella* and *Rhizobium*, Gram-positive Firmicutes like *Bacillus*, *Brevibacillus* and *Paenibacillus*, as well as Gram-positive *Actinobacteria* (*Rhodococcus*, *Streptomyces* and *Arthrobacter*). The contribution of the bacterial partners on plant's physiology and – on another scale – on natural ecosystems is not understood.

We demonstrate here stabile associations of fungal species of the order *Sebacinales* with bacteria known to interact with ectomycorrhiza and endomycorrhiza. We show that *P. indica* is associated with a strain of the species *Rhizobium radiobacter* and that this bacterium exhibits a considerable biological activity to the host plant.

Results

Piriformospora indica *is associated with* R. radiobacter

Under standard culture conditions as used for fungal propagation, *P. indica* develops spherical fungal colonies within 3–4 weeks in the transparent medium. However, crushing the mycelium with a fine blender and subsequent microscopic examination of the supernatant upon bacterial live–dead staining indicated the presence of rod-shaped bacteria of 1–1.5 μm in length. This initial observation suggested that there is a tight association of *P. indica* with bacteria. To check this notion, we traced the presence of specific bacteria in the original *P. indica* isolate *P. indica*-DSM11827 deposited in 1997 immediately after the discovery of the fungus in the Indian Thar desert (Table 1). Using universal primers, almost full-length fragments of the entire bacterial 16S-rRNA gene were amplified from the fungal metagenomic DNA of *P. indica*-DSM11827 as well as in other fungal cultures

Table 1. *Sebacinales* analysed for bacterial presence.

Isolate	Host	Associated bacteria
Piriformospora indica-DSM11827[a]	*Prosopis julifora* and *Zizyphus nummularia*[b]	*Rhizobium radiobacter* (GenBank Accession No. EU669179)
S. vermifera-MAFF305838[c]	*Caladenia tesselata*	*Paenibacillus* sp. (GenBank Accession No. EU669180)
S. vermifera-MAFF305828[c]	*Eriochilus cucullatus*	*Acinetobacter* sp. (GenBank Accession No. EU669181)
S. vermifera-MAFF305835[c]	*Caladenia catenata*	*Rhodococcus* sp. (GenBank Accession No. EU669182)
S. vermifera-MAFF305837[c]	*Caladenia dilatata*	P[d]
S. vermifera-MAFF305830[c]	*Crytostylis reniformis*	P[d]
S. vermifera-MAFF305842[c]	*Microtis uniflora*	P[d]
Multinucleate *Rhizoctonia*-DAR29830[e]		P[d]

a. Type species *Piriformospora indica* was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.
b. Fungus was originally isolated from rhizosphere of these plants.
c. Culture collection numbers: Isolates of *Sebacina vermifera* were obtained from the National Institute of Agrobiological Sciences, Tsukuba, Japan.
d. Present (P) but bacterial identity not yet determined.
e. The isolate DAR29830 was kindly provided by Karl-Heinz Rexer, University of Marburg, Germany.

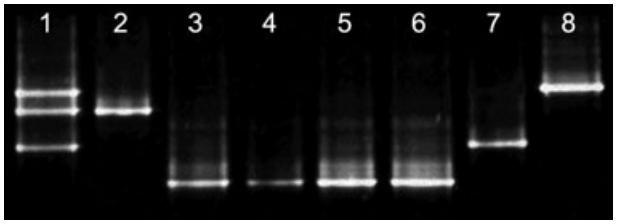

Fig. 1. Detection of *P. indica*-associated bacteria by DGGE. The 16S-rRNA gene fragments of 500 bp were obtained from the metagenome of bacterial communities associated with different isolates of *P. indica*. Lane 1, reference pattern composed with three known bacterial strains (*E. coli*, *H. frisingense* Mb11, *Acinetobacter* sp.); lane 2, *Acinetobacter* sp.; lane 3, *P. indica*-DSM11827; lane 4, *P. indica*-JE1; lane 5, *R. radiobacter* PABac-DSM isolated from *P. indica*-DSM11827; lane 6, *R. radiobacter* PABac-JE isolated from *P. indica*-JE1; lane 7, *E. coli*; lane 8, *Herbaspirillum frisingense Mb11*. Sequence data from the bands in lanes 3 and 4 showed 100% identity with the 16S-rRNA gene sequences of the bacterial isolates PABac-DSM and PABac-JE in lanes 5 and 6.

including *P. indica*-JE1, *P. indica*-HA and *P. indica*-ND (Fig. S1). Fragments showed identical 16S-rRNA gene-coding sequences, designating the bacterium as α-Proteobacterium of the genus *Rhizobium* (GenBank Accession No. EU669179) with the highest similarity to the species *R. radiobacter* (synonym *Agrobacterium radiobacter or Agrobacterium tumefaciens*) (Young *et al.*, 2001). These data suggested that *P. indica* contains a single bacterial strain. To confirm this finding, we employed denaturing gradient gel electrophoresis (DGGE) of the seminested PCR products of the 16S-rRNA-coding gene. DGGE revealed the presence of a single high intensity band in *P. indica*-DSM11827 and *P. indica*-JE1 (Fig. 1). Direct PCR amplification also showed the same pattern of bands (not shown). DNA sequences of the PCR product were identical to a 500 bp region of the 16S-rRNA gene obtained from metagenomic DNA of *P. indica*-DSM11827.

For further characterization we attempted to isolate bacteria from the fungal mycelium of *P. indica*-DSM11827 and *P. indica*-JE1. Growth of isolated bacteria – named PABac-DSM and PABac-JE respectively – was observed 3 days after inoculation of bacteria released from mechanically sheared fungal hyphae in Luria–Bertani (LB) medium supplemented with 0.8% sucrose. Microscopic analysis revealed the presence of a pure culture of rod-shaped bacteria of 1–1.5 μm in length (Fig. S2). By using the primer pair 27f and 1495r to amplify the 16S-rRNA gene, a PCR product of the expected size was obtained (Fig. S1). Cloning, sequencing and comparative 16S-rRNA gene sequence analysis showed that all obtained sequences were identical to each other and were also identical to those isolated from metagenomic DNA of different *P. indica* isolates. After BLAST search and phylogenetic analysis with ARB software (Fig. 2) and EzTaxon server (Chun *et al.*, 2007), the sequence was identified as belonging to an α-Proteobacterium of the genus *Rhizobium* (GenBank Accession No. EU669179) with the highest similarity and 100% homologue to the species *R. radiobacter*-AJ389909. The phenotypic characters (data not shown) were congruent with those reported for *R. radiobacter* (Young *et al.*, 2001 and references therein).

Denaturing gradient gel electrophoresis profiles of a direct and seminested PCR assay using 16S-rRNA gene

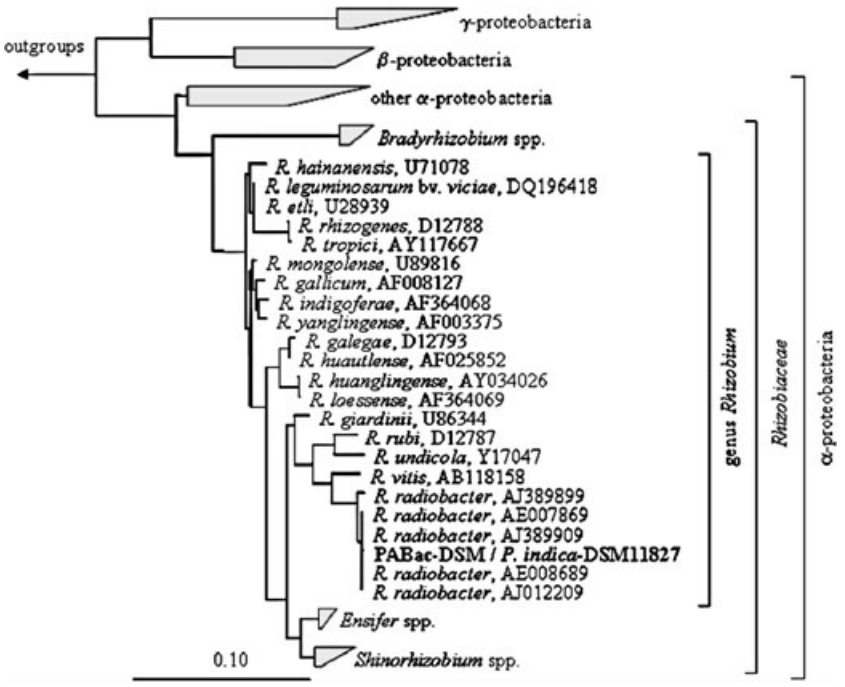

Fig. 2. Phylogenetic classification of *P. indica*-associated bacteria. Phylogenetic tree based on comparative sequence analysis of 16S-rRNA-coding genes of *P. indica*-associated bacteria and representatives of related *Rhizobium radiobacter*/*Agrobacterium tumefaciens* group. The bar indicates 10% sequence divergence.

Table 2. Efficacy of antibiotics against *R. radiobacter*.

Antibiotic	OD_{600} after 48 h
Spectinomycin (300 μg ml^{-1})	0.000
Spectinomycin (200 μg ml^{-1})	0.010
Ciprofloxacin (200 μg ml^{-1})	0.013
Ciprofloxacin (100 μg ml^{-1})	0.017
Cefatoxime (300 μg ml^{-1}) + Ticarcillin (150 μg ml^{-1})	0.019
Oxytertacycline (100 μg ml^{-1})	0.030
Rifampicin (100 μg ml^{-1})	0.032
Cefatoxime (500 μg ml^{-1})	0.050
Oxytertacycline (200 μg ml^{-1})	0.070
Cefatoxime (250 μg ml^{-1})	0.091
Ticarcillin (150 μg ml^{-1})	0.501
Lincomycin (100 μg ml^{-1})	2.160
Carbenicillin (100 μg ml^{-1})	2.243
Gentamicin (100 μg ml^{-1})	2.510
Gentamicin (50 μg ml^{-1})	2.450
Control	2.499

Bacteria (PABac-DSM) were grown in LB medium containing various antibiotics at 25°C. OD600 was measured 48 h after inoculation.

primers corroborated presence of a single high intensity band in PABac-DSM and PABac-JE identical in electrophoretic mobility and proved to have the same sequence as obtained from the metagenomic DNA of *P. indica*-DSM11827 and *P. indica*-JE1 (Fig. 1). The quantification of the ratio of bacterial to fungal DNA in different fungal cultures by real-time quantitative PCR showed an average of 0.035 ng of *R. radiobacter* DNA per 100 ng of *P. indica* DNA.

Treatments for potential curing P. indica *from* R. radiobacter

With the aim of eliminating *R. radiobacter* from *P. indica*-DSM11827, bacteria (*R. radiobacter* PABac-DSM) were first cultivated in LB medium in the presence of various antibiotics while the efficacy of the chemicals was proved by OD_{600} measurement after 48 h (Table 2). Among other antibiotics spectinomycin and ciprofloxacin were found to be highly efficient in completely inhibiting the growth of *R. radiobacter* PABac-DSM *in vitro*. However, after axenic culturing of *P. indica*-DSM11827 for 2 months at 25°C in the presence of ciprofloxacin (200 μg ml^{-1}) and/or spectinomycin (300 μg ml^{-1}) bacteria were neither eliminated from the hyphae nor eliminated from chlamydospores as evidenced by PCR analysis. In analogy to the strategy followed in arbuscular mycorrhiza (Lumini *et al*., 2007), *P. indica* was intended to be cured from bacteria by producing successive vegetative generations starting from single chlamydospores. Five generations of single spores (G1, G2, G3, G4, G5) were grown on agar plates containing 300 μg ml^{-1} spectinomycin and 10 colonies from each generation were tested for bacterial presence by conventional PCR using universal eubacterial primers. However, bacteria were detected in all generations (data not shown). In a complementary approach, young growing hyphae were transferred to freshly prepared plates containing antibiotics every fourth day for five times. In all seven tested *P. indica* colonies, conventional PCR with universal primes as well as real-time quantitative PCR with primers specific for the *Rhizobium/Agrobacterium* intergenic transcribed spacer resulted in PCR products of the appropriate sizes, confirming the presence of *R. radiobacter*. In another attempt, hyphal protoplasts were isolated and subsequently regenerated on plates containing *Aspergillus* minimal medium with 0.3 M sucrose, spectinomycin (300 μg ml^{-1}) and ciprofloxacin (300 μg ml^{-1}) at 30°C and growing mycelium was transferred every second day for eight consecutive days, to fresh medium plates containing antibiotics. All seven independent regenerated *P. indica* colonies gave positive signals after real-time quantitative PCR analysis for the presence of bacterial 16S-23S rRNA intergenic transcribed spacer (ITS) sequences.

Piriformospora indica *is intimately associated with* R. radiobacter

In order to locate bacteria associated with *P. indica*, fungal preparations from axenic cultures of *P. indica*-DSM11827 as well as cells of a pure culture of strain PABac-DSM were stained by fluorescence *in situ* hybridization (FISH). The presence of bacterial rRNA in the fungal mycelium was proven by application of a probe specific for eubacteria (EUB-338-mix) (Table 3). Concomitantly, a colocalized signal was detected by using the Rh-1247 probe, which is specific for rRNA of bacteria belonging to the *Rhizobium* group (Fig. 3A-C). In order to exclude the detection of unspecific hybridizations EUK-516 probes were introduced that are specific for 18S-rRNA of eukaryotes. These analyses further implicated association of bacteria with mycelium and chlamydospores of *P. indica* and further confirmed the low number of associated bacteria as was already indicated by real-time PCR-based quantification.

Rhizobium radiobacter *induces growth promotion and disease resistance in barley*

Most substantial biological activities of *P. indica* and related *S. vermifera* species in various host plants are growth promotion and systemic induced resistance to fungal pathogens (Deshmukh *et al*., 2006). To assess the biological activity of isolated bacteria, roots of 3-day-old barley seedlings were dip-inoculated with PABac-DSM (OD_{600} 1.6). Upon 3 weeks, treated plants showed an increase in shoot fresh weight of 17% over control plants demonstrating the growth promoting activity of the bacterium (Table 4). Moreover, the same plants were more

Table 3. Phylogenetic oligonucleotide probes used for FISH analyses.

Probe[a]	Target[a]	Position[a,b]	Sequence (5′-3′)[a]	FA (%)[a,c]	Specificity[a]
EUK-516	18S-rRNA	502–517	ACCAGACTTGCCCTCC	0–50	*Eukarya*
EUB-338	16S-rRNA	338–355	GCTGCCTCCCGTAGGAGT	0–50	Most bacteria
EUB-338 II	16S-rRNA	338–355	GCAGCCACCCGTAGGTGT	0–50	*Planctomycetales*
EUB-338 III	16S-rRNA	338–355	GCTGCCACCCGTAGGTGT	0–50	*Verrucomicrobiales*
LGC-354-a	16S-rRNA	354–371	TGGAAGATTCCCTACTGC	35	Firmicutes (Gram-positive bacteria with low DNA G + C content)
LGC-354-b	16S-rRNA	354–371	CGGAAGATTCCCTACTGC	35	Firmicutes (Gram-positive bacteria with low DNA G + C content)
LGC-354-c	16S-rRNA	354–371	CCGAAGATTCCCTACTGC	35	Firmicutes (Gram-positive bacteria with low DNA G + C content)
Rh-1247	16S-rRNA	1247–1252	TCGCTGCCCACTGTG	35	*Rhizobium* sp., *Agrobacterium* sp., *Ochrobactrum* sp., some *Azospirilla* sp., few *Sphingomonas* sp.

a. Data taken from probe base (http://www.microbial-ecology.net/probebase) (Loy *et al*., 2003; 2007).
b. Position according to Brosius *et al*. (1981).
c. Formamide in the hybridization buffer.

resistant to the biotrophic fungal leaf pathogen *Blumeria graminis* f.sp. *hordei*. Barley leaves showed a decrease in the frequency of powdery mildew pustules of 64% over control (plants treated only with LB medium; Table 4). To compare the bacteria-mediated activity with that from the fungus, we inoculated roots with *P. indica*. We found an increase in fresh shoot biomass (27%) and a decrease in the number of powdery mildew pustules (54%) as compared with non-colonized plants (Table 4).

In order to further characterize the bacterium, we grew *R. radiobacter* PABac-DSM in mineral salt medium supplemented with 0.5% glucose and 500 μg ml^{-1} of tryptophan. Under this cultivation conditions, the bacterium exhibited a substantial production of indole-3-acetic acid (IAA) up to 40 μg ml^{-1} after 24 h at 25°C in the presence of tryptophan. In the absence of this amino acid, however, there was no production of IAA demonstrating tryptophan-dependent IAA synthesis by *R. radiobacter* PABac-DSM.

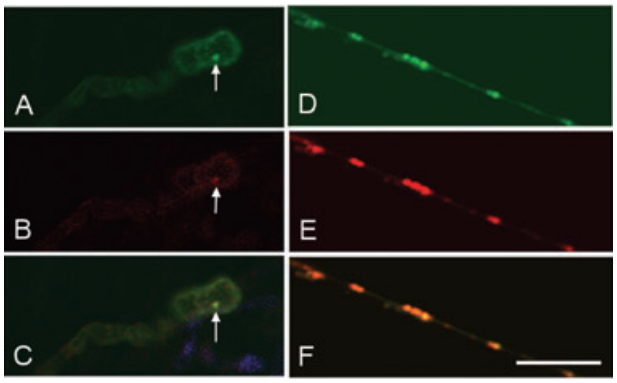

Fig. 3. Detection of fungus-associated bacteria by fluorescence *in situ* hybridization (FISH). Fixed fungal mycelia of *P. indica*-DSM11827 (A–C) and *S. vermifera*-MAFF305838 (D–F) were used for FISH analysis. FISH was performed with EUB-338-mix-FITC (green), specific for the domain *Bacteria* (A and D) and with either Rh-1247-Cy3 probe (red), specific for the *Rhizobium* group (B–C) or LGC-mix-Cy3 (red), specific for the Firmicutes group to which *Paenibacillus* sp. belongs (E–F). While Fig. 3A and D shows the green channel, Fig. 3B and E shows the red channel and Fig. 3C and F shows the superimposed images. In Fig. 3C and F, the composed rgb-images result in a yellow colour for the bacteria, indicating colabelling by EUB-338-mix-FITC and Rh-1247-Cy3 (C) and colabelling by EUB-338-mix-FITC and LGC-354-Cy3 (F). In A– C, arrows indicate the bacterial signals. Scale bar = 10 μm.

Bacterial associations are common in Sebacinales

The hypothesis was followed that other species of the order *Sebacinales* commonly contain endocellular bacteria. Therefore, seven *S. vermifera* isolates were selected, which were originally collected from different autotrophic orchids in Australia (Warcup, 1988). All the strains were previously proven to exhibit beneficial biological activity in barley (Deshmukh *et al*., 2006). After PCR and sequencing analyses using bacterial universal primers we provided evidence that all *S. vermifera* isolates contained bacteria although from different genera (Table 1). We studied the bacterial association of *S. vermifera*-MAFF305838 in more detail. Using FISH and confocal laser scanning microscopy on axenically grown fungal cultures, we detected an intracytoplasmic localization of bacterial cells within hyphae (Fig. 3D–F). Spherical bacteria of 0.5–1 μm size were detected with the probe EUB-338-mix, indicative for almost all bacteria, and colocalized with probe LGC-354-mix,

Table 4. Biological activity conferred by *R. radiobacter* in barley.

Treatment	Increase in shoot weight over control (%)	Decrease in powdery mildew pustules over control (%)
R. radiobacter	17.07**	63.90**
P. indica	27.35**	53.56**

Asterisks denote statistically significant differences between the respective values of endophyte-colonized and non-colonized plants (**Student's *t*-test $P < 0.001$).

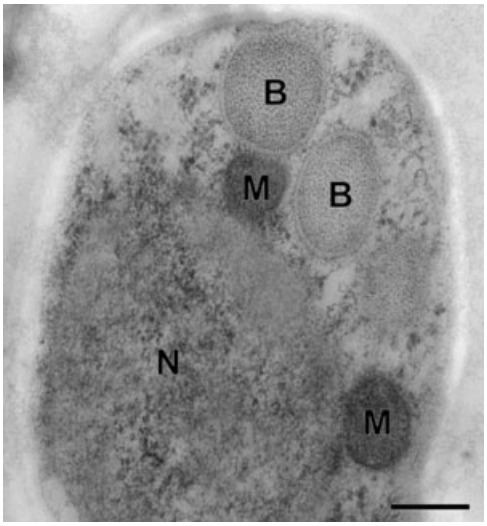

Fig. 4. Transmission electron micrograph of fungal mycelium of *S. vermifera*-MAFF305838 containing endocellular bacteria (B), a nucleus (N) and mitochondria (M) (Scale bar = 200 nm).

specific for the Firmicutes group to which *Paenibacillus* belongs (Table 3). The number of bacteria varied from one to three per hyphal cell. To exclude unspecific probe labelling, the samples were hybridized with EUK-516 probe (specific for 18S-rRNA of eukaryotes). To further substantiate the presence of endobacteria, transmission electron microscopy of *S. vermifera*-MAFF305838 was done. The mycelium from liquid culture was frozen at high pressure and cryosubstituted in glutaraldehyde and uranyl acetate. Examination of the samples confirmed the presence of endobacteria within the cytosol of intact cells of *S. vermifera*-MAFF305838 (Fig. 4).

Discussion

We demonstrate here that species of the *Sebacinales* regularly undergo complex symbioses involving plants and bacteria of different genera. Sebacinoid fungi were formerly shown to possess a broad plant host spectrum (Varma *et al*., 1999; Weiss *et al*., 2004) and, so far investigated, exhibit beneficial biological activities to their hosts (Deshmukh *et al*., 2006). Our finding that the Sebacinoid symbiosis involves bacteria leads to a more complex picture and requires reconsideration of the role played by the fungus in its symbiotic interaction with the plant. However, not only complexity but also intimacy apparently constitutes a crucial trait in bacteria–Sebacinoid associations. First, associations of bacteria with Sebacinoid fungi are rather diverse. We found bacterial species belonging to three different genera, namely *Paenibacillus*, *Acinetobacter* and *Rhodococcus* in three phylogenetically and morphologically distinct members of the *S. vermifera* species complex and *R. radiobacter* closely associated with *P. indica*. Second, isolation and subsequent axenic cultivation of fungus-associated bacteria were successful, so far, only with *R. radiobacter* from *P. indica*. Third, bacteria-free Sebacinoid fungi could not be obtained so far. These tight relational characteristic impedes investigations on the importance of each partner for mediating biological activity and for plant colonization. Intriguingly, these close associations of Sebacinoid fungi with bacteria resemble those previously detected in other mycorrhizal associations (Frey-Klett *et al*., 2007).

Bianciotto *et al*. (2004) recently demonstrated for the AM fungus *Gigaspora margarita* a continuous vertical transmission of its endobacteria from one generation to another guaranteeing the enduring nature of the association. Comparably, *R. radiobacter* was found in developing *P. indica* chlamydospores and in fungal colonies regenerated from single spores or from hyphal tip cells. Moreover, bacteria were still traceable in the fungal cultures after five fungal root passages (data not shown).

Different strategies to obtain bacteria-free fungus failed, which further argues against a rather temporal or loose association. Neither cultivation of hyphae in axenic culture under high antibiotic concentrations nor successive *in vitro* single-spore isolation steps nor the exposure of fungal protoplast to antibiotics in the regenerating medium resulted in bacteria-free *P. indica.* In presence of antibiotics, the fungus grew slowly that may indicate an adverse antibiotic effect on bacteria or on the fungus itself. However, after removing the antibiotics, the amount of bacterium was in range as found in untreated *P. indica*. These findings suggest that either the bacterium is protected inside the fungus or its absence would reduce fungal fitness as reported for *G. margarita* cured from its endocellular bacteria (*Candidatus* Glomeribacter gigasporarum) (Lumini *et al*., 2007). However, *R. radiobacter* could be isolated from fungal mycelium and multiplied in liquid cultures demonstrating that the bacterium is not entirely dependent on the fungus. That the association bases on a critical balance between bacterium and fungus is suggested by an experiment in which *R. radiobacter* was added in abundance to fungal suspension cultures. Here, the bacteria overgrew and entangled the hyphae and the fungus eventually died (data not shown). Interestingly, incubation of crushed hyphae, containing hyphae and bacteria released from mechanical shearing, resulted in clearance of the medium after 2 days. This finding suggests a certain affinity between fungal hyphae and bacteria. Further analysis will show how specific this absorption is.

Using various molecular strategies (16S-rRNA gene sequences analysis, DGGE analysis and real-time quantitative PCR analysis), the intimate association between *P. indica* and *R. radiobacter* was further characterized. We could unequivocally prove the presence of the identical

bacterial strain in the original isolate *P. indica*-DSM11827 deposited in 1997 by A. Varma and in all fungal cultures derived thereof, i.e. *P. indica*-JE1, *P. indica*-HA and *P. indica*-ND. All bacterial sequences from *P. indica* isolates and the bacterial cultures isolated from *P. indica* were identical within the amplified region of the bacterial 16S-rRNA gene. Consistently, DGGE analysis of single-step and seminested PCR assay using 16S-rRNA gene primers revealed a single dominant band for *P. indica*-DSM11827 and *P. indica*-JE1 as well as for PABac-DSM and PABac-JE, further emphasizing that only one distinct bacterial species was associated with *P. indica*.

A cytohistochemical approach using FISH in combination with the *Rhizobium*-specific probe Rhi-1247 showed an association of *R. radiobacter* with spores and hyphae. In general the number of bacteria per hypha was low, which is consistent with the low number of endobacteria (2–20 per cell) present in the ectomycorrhizal fungus *Laccaria bicolor* (Bertaux *et al*., 2003; 2005). Quantitative PCR analysis further supported the microscopic data revealing a ratio of 0.02–0.035 ng of bacterial DNA per 100 ng of *P. indica* DNA. The low number of bacteria within hyphae may explain why we failed to detect *R. radiobacter* by electron microscopy. In contrast, a combined strategy that included electron microscopy and FISH detected the endobacterial nature of *Paenibacillus* sp. in *S. vermifera*-MAFF305838 with roughly 1–3 bacteria per fungal cell. Different to other fungus–endobacterial associations (Partida-Martinez *et al*., 2007), there was no evidence for a focal accumulation of *R. radiobacter* or *Paenibacillus* in fungal hyphae.

It is certainly of interest to clarify the impact of each Sebacinoid symbiosis partner on beneficial effects in colonized plants. A first step has been done by examining the biological potential of *R. radiobacter* (PABac-DSM) in barley. The bacterium qualitatively and quantitatively induced symbiosis phenotypes comparable to those induced by *P. indica*, e.g. growth augmentation and systemic resistance to powdery mildew. Consistently, the potential of specific strains of *R. radiobacter* for improvement of plant performance in integrated production systems has been reported earlier. *R. radiobacter* strain 204 increased barley root and shoot length as well as improved crop yield in barley and wheat leading to its commercial distribution as biofertilizer in Russia (Humphry *et al*., 2007).

Rhizobium radiobacter (syn. *A. tumefaciens*) is well known as a soil-borne bacterium that infects dicotyledonous plants and often causes crown gall disease characterized by the neoplastic growth of infected plant tissue. Tumour induction is dependent on the integration of a bacterial virulence plasmid (Ti) into the plant genome, which contains genes encoding the phytohormones auxin and cytokinin. In our experiments, PABac-DSM did not trigger any harmful effect on barley. The *virD2* gene was detected in PABac-DSM by PCR analyses indicating that the Ti plasmid is present in this strain. However, the *isopentenyltransferase* (*ipt*) gene, which is associated with cytokinin biosynthesis, could not be detected (S. Wagner, unpublished). Hence, the lack of the *ipt* gene may explain the non-pathogenic nature of this bacterial strain. Concordantly, a non-pathogenic strain of *A. radiobacter* containing *virD2* but not *ipt* was previously described by Haas *et al*. (1995). These authors speculated that the strain arose from a pathogenic progenitor through a deletion in the T-DNA. Moreover, coexistence of symbiosis- and pathogenicity-determining genes has been shown to occur in strains of *Rhizobium rhizogenes* enabling this bacterium to induce nodules or tumours in plants (Velazquez *et al*., 2005).

A recent report by Sirrenberg *et al*. (2007) demonstrated production of IAA in liquid culture of *P. indica*, which might induce growth promotion. Because bacteria-free fungus is not available, it remains vague whether the fungus itself, the bacterium or even both partners produced the hormone. We found that *R. radiobacter* (PABac-DSM) produces IAA in the presence of tryptophan. Despite the fact that Salkowski reagent also detects indole pyruvic acid and indole acetamide in addition to IAA (Glickmann and Dessaux, 1995), the method is fairly accurate because IAA is usually known as the main excreted microbial auxin. IAA is best known for its role in plant signal transduction (Quint and Gray, 2006). However, this hormone can act as a signal molecule in bacteria and fungi (Leveau and Preston, 2008) and induce adhesion and filamentation of *Saccharomyces cerevisiae* (Prusty *et al*., 2004). Importantly, IAA has been implicated in plant–microbe compatibility (Robert-Seilaniantz *et al*., 2007). This could be accomplished by suppression of defence reactions otherwise elicited by fungal Microbe-Associated Molecular Patterns (Navarro *et al*., 2006; Wang *et al*., 2007). Notably, in barley and *Arabidopsis*, root colonization by *P. indica* leads to suppression of pathogenesis-related (PR) genes (Deshmukh and Kogel, 2007). Thus, it is tempting to speculate that bacteria-derived auxin contributes to successful root colonization by Sebacinoid fungi. Further research is required to demonstrate that the bacterium is the only source of auxin and that auxin would mimic interaction phenotypes mediated by host plant colonization.

Experimental procedures

Fungal material

Piriformospora indica isolates were obtained from the following sources: *P. indica*-DSM11827 from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany;

P. indica-JE1 from Dr Ralph Ölmüller, Jena, Germany; *P. indica*-HA from Dr Holger Deising, Halle, Germany; *P. indica*-ND from Dr Ajit Varma, New Delhi (Table 1). All isolates stem from one original sample collected in the Thar desert, India in 1997 (Varma *et al*., 1998). They were propagated in liquid *Aspergillus* minimal medium at room temperature (Pham *et al*., 2004). *S. vermifera* strains (Table 1) were propagated in Malt-Yeast-Extract-Pepton medium (aqueous solution of 7 g l^{-1} malt extract, 1 g l^{-1} peptone, 0.5 g l^{-1}, yeast extract).

DNA isolation, PCR and sequencing

High chromosomal weight DNA from 2-week-old *P. indica* cultures was isolated using the NucleoSpin® Tissue Kit (Macherey-Nagel, Germany) according to the instruction manual. 16S-rRNA gene was amplified by using the bacterial universal primer pair 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1495r (5′-CTAC GGCTACCTTGTTACGA-3′). A conventional PCR amplification was performed in a Gene Amp® PCR System 9700 PE Applied Biosystem thermo cycler in a total volume of 25 μl containing 2× PCR Master Mix (Fermantas, Life Sciences, St Leon-Rot, Germany), 75–100 ng of DNA and 1 μM of each primer. After an initial denaturation step at 95°C for 5 min, 34 cycles with denaturation at 95°C for 1 min, primer annealing at 59°C for 1 min, elongation at 72°C for 1.45 min and a final extension at 72°C for 10 min were performed. The obtained PCR products were purified using a Gel Extraction Kit (Promega, Mannheim, Germany) and cloned into the pGEM-T vector (Promega) following the manufacturer's instructions. DNA from 25 plasmids was extracted with Wizard® Plus SV Minipreps (Promega) and submitted for sequencing to AGOWA GmBH, Berlin, Germany. Direct sequencing of PCR products was also performed with the primer pair 27f and 1495r. Sequences were assembled with the Sequencher 3.1.1 software (Gene Codes Corporation) and analysed with the ARB software package (http://www.arb-home.de) (Ludwig *et al*., 2004).

Phylogenetic analysis

The 16S-rRNA-coding gene sequences obtained from the sequenced plasmids and from direct sequencing were added to an existing database of well-aligned small-subunit rRNA gene sequences by using the fast alignment tool implemented in the ARB software package (http://www.arb-home.de) (Ludwig *et al*., 2004). Sequences were proof read according to the chromatograms and wrong positions in the alignments were manually corrected if needed. Phylogenetic analyses were performed by applying maximum likelihood, maximum parsimony and neighbour-joining methods by use of respective tools in the ARB software package.

Isolation of bacteria

Mycelia of 14-day-old *P. indica*-DSM11827 and *P. indica*-JE1 cultures were crushed in Gamborg B5 medium (Duchefa Biochemie, the Netherlands) supplemented with 0.45 M mannitol using a fine blender. Homogenate was filtered through a miracloth (22–25 μm) filter and centrifuged at 100 *g* for 7 min. The supernatant was collected and subsequently centrifuged at 3200 *g* for 10 min. The bacterial cell pellet was resuspended in LB medium containing 0.8% sucrose and inoculated in the same medium at 22°C for 2 days under gentle shaking. The bacterial culture was streaked on LB medium plate and incubated for 2 days at 25°C. Thirty bacterial colonies were randomly picked from the plates and identified by sequencing the 16S-rRNA gene using a universal primer pair 27f and 1495r as described above. Phenotypic characterization and identification at the species level were performed using fatty acid analysis of whole cell extract as described by Kämpfer and Kroppenstedt (1996) and physiological characterization using 90 physiological biochemical tests was performed by the method described by Kämpfer *et al*. (1991).

Denaturing gradient gel electrophoresis

DNA extraction for DGGE analysis was performed using the FastDNA® Spin Kit for soil (MP Biomedicals, LLC., Illkirch, France) according to the manufacturer's protocol. A seminested PCR was performed to amplify a 500 bp region of the bacterial 16S-rRNA-coding gene. First, almost the entire 16S-rRNA gene was amplified using the above mentioned bacterial primer pair. The cycle conditions differ in 25 cycles of amplification. Subsequently, next PCR was performed using 27f (with a 42 bp GC clamp on the 5′ end) and reverse R518 (5′-ATTACCGCGGCTGCTGG-3′) universal primers (Vanhoutte *et al*., 2005). The PCR mix contained (final concentrations) 1× Thermophilic DNA Polymerase Buffer (Promega), 2.5 mM $MgCl_2$ (Promega), 0.025 mM of each dNTP (Fermantas, Life Sciences), 0.25 μM of each primer and 0.05 U μl^{-1} *Taq* DNA polymerase (Promega). One microlitre of first PCR product was used as template in a total volume of 50 μl reaction. Thermal cycling conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 25 amplification cycles with heat denaturation at 94°C for 1 min, primer annealing at 59°C for 45 s and extension at 72°C for 40 s. A final elongation step at 72°C for 10 min completed the reaction. The PCR products were analysed with standard horizontal agarose gel electrophoresis on a 1% agarose gel.

Additionally a single-step PCR amplification of a 500 bp region of the 16S-rRNA was performed using the forward primer 27f or F357 (5′-TACGGGAGGCAGCAG-3′) (Vanhoutte *et al*., 2005), with a 42 bp GC clamp on the 5′ end, in combination with the reverse primer R518. PCR was performed under the above mentioned conditions. The cycle conditions differ only in 32 cycles of amplification.

Denaturing gradient gel electrophoresis analyses were performed using an 8% (wt/vol) acrylamide-bisacrylamide gel (Liqui-Gel™ 37.5:1; MP Biomedicals) with a 35–75% linear urea-formamide (Fluka, Seelze, Germany) denaturing gradient (100% denaturant corresponds to 40% formamide plus 7 M urea). After adding the loading buffer (0.05% bromophenol blue and 0.05% xylene cyanol in 70% glycerol), 20 μl of each sample was loaded on the DGGE gel and submitted to electrophoresis in 1× TAE buffer at 60°C with a constant voltage of 50 V for 20 h using a Bio-Rad DCode™ Universal Mutation Detection System. The gels were stained in the dark for 20 min in ethidium bromide and subsequently washed with 1× TAE buffer. The following bacterial strains were used as reference: *Escherichia coli, Herbaspirillum frisingense* strain Mb11 and *Acinetobacter* sp. The obtained DGGE bands for *P. indica*-DSM11827, *P. indica*-JE1, PABac-DSM and PABac-JE isolate were excised; the DNA fragments were purified using Wizard® Plus SV Minipreps (Promega) and submitted for sequencing to AGOWA GmBH, Berlin, Germany.

Real-time PCR quantification

Genomic DNA of PABac-DSM was used as template to amplify the 16S–23S-rRNA intergenic transcribed spacer region, using primer ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITS_Reub (5′-GCCAAGGCATCCACC-3′) (Cardinale *et al*., 2004). A PCR product of the expected size (1.4 kb) was cloned and sequenced as described earlier. The sequences obtained were used to design the *Rhizobium/Agrobacterium*-specific primer pair ITS_Rhf (5′-TCAGCACATAACCACACCAATCGCG-3′) and ITS_Rhr (5′-TG CTTTGTACGCTCGGTAAGAAGGG-3′). These primers were used in real-time PCR to quantify the amount of bacterium in *P. indica* cultures. Amplifications were performed in 25 μl SYBR® Advantage® qPCR Premix (Clontech Laboratories, CA, USA) according to manufacturer's instructions with 200 nM oligonucleotides, 100–300 ng of fungal genomic DNA, and carried out with a Stratagene-Mx3000P® QPCR SystemMx3000P (Stratagene Research, La Jolla, CA, USA). After an initial activation step at 95°C for 1 min, 45 cycles (95°C for 5 s and 65°C for 25 s) were performed and a single fluorescent reading was obtained at 65°C of each cycle step. A melting curve was determined at the end of cycling to ensure the amplification of a single PCR product. Cycle threshold values were determined with the Mx3000P V2 software supplied with the instrument. A standard curve using different dilutions of bacterial DNA was prepared and was used to calculate the amount of bacterial DNA in fungal samples.

Treatment of P. indica *with antibiotics*

Hyphae were cultured for 5 generations in the presence of either spectinomycin (300 μg ml^{-1}) or ciprofloxacin (200 and 500 μg ml^{-1}) and in combination. Both antibiotics were effective against PABac-DSM *in vitro* (Table 2). Additionally, single-spore culturing was performed in the presence of these antibiotics. Chlamydospores were harvested from 4-week-old plates using a 0.05% Tween-20 solution, and purified three times by centrifugation at 100 g for 7 min. The pellet was resuspended before each centrifugation step in 0.05% Tween-20 containing 300 μg ml^{-1} spectinomycin. Spores were finally treated with spectinomycin (300 μg ml^{-1}) for 3 h and subsequently plated on *Aspergillus* minimal medium plates containing spectinomycin (300 μg ml^{-1}). A single germinating spore was picked using a stereomicroscope (MZ16F, Leica, Germany) and used as inoculum on antibiotic containing agar plates. This was termed generation 1 (G1) of the single-spore culture. Spores were harvested after 3 weeks from these plates and plated to produce further generations of single-spore cultures (G2–G5) in the same way. After every generation, fungal samples were taken and bacterial detection was performed by conventional PCR using universal eubacterial primers and with real-time PCR using specific primers as described above.

Additionally, young growing mycelium from *P. indica* was picked with the help of a stereomicroscope and transferred to new plates containing *Aspergillus* minimal medium with antibiotics (spectinomycin 300 μg ml^{-1} and ciprofloxacin 300 μg ml^{-1}) and incubated at 24°C. Every fourth day the growing mycelium was transferred alternatively to fresh plates or to liquid *Aspergillus* minimal medium containing antibiotics for a total of five times. Finally young mycelium was transferred on *Aspergillus* minimal medium plates and liquid medium without antibiotics, grown for 3 weeks and used for DNA isolation. Seven independent treated colonies were checked for bacterial presence as described above.

Piriformospora indica *protoplast isolation and treatment with antibiotics*

Fresh mycelia from *P. indica*-DSM11827 were crushed and filtered through miracloth. The filtrate was collected by centrifugation and resuspended in liquid *Aspergillus* minimal medium. After 3 days the young mycelium was collected using a miracloth filter, washed twice with 0.9% NaCl and resuspended in SMC buffer (1.33 M sorbitol, 50 mM $CaCl_2$, 20 mM MES buffer pH 5.8) containing 2.5% lysing enzymes from *Trichoderma harzianum* (L1412 Sigma). The suspension was incubated for 1 h at 37°C. The activity of the lysing enzymes was stopped by adding STC buffer (1.33 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris-HCl; pH 7.5). Protoplasts were filtered through miracloth and collected by centrifugation. The pellet was washed three times in STC buffer containing spectinomycin (300 μg ml^{-1}) and ciprofloxacin (300 μg ml^{-1}) and diluted to a final concentration of 1×10^8 cells per ml. The preparation was checked under a phase-contrast microscope to ensure the absence of any mycelial fragments or spores. Liquid *Aspergillus* minimal medium containing 0.3 M sucrose, spectinomycin (300 μg ml^{-1}) and ciprofloxacin (300 μg ml^{-1}) was used for regeneration of protoplasts. Regeneration was observed after 72 h incubation at 30°C. Young growing mycelium from seven colonies were picked with the help of a stereomicroscope and transferred to new plates containing *Aspergillus* minimal medium with antibiotics. Every second day for eight consecutive days the growing mycelium was transferred to fresh *Aspergillus* minimal medium plates containing antibiotics. Finally young mycelium was transferred on medium plates without antibiotics, grown for 3 weeks and used for DNA isolation and checked for bacterial presence as described above.

Fluorescence in situ *hybridization*

Two to four-week-old fungal cultures or overnight grown bacterial cultures were fixed by adding 50% ethanol and incubation at 4°C for 3–4 h. Thereafter, cultures were washed three times in 1× PBS and finally resuspended in a 1:1 mixture of 1× PBS and $Ethanol_{absolute}$. Samples were stored at –20°C. Fixed fungal material was dehydrated in an increasing ethanol series (50%, 80% and 96% ethanol, 3 min each). FISH was performed as described by Manz *et al*. (1996). Hybridization was carried out for 90 min at 46°C, followed by a stringent washing step at 48°C for 10–15 min. All steps of FISH with fungal material were carried out in eppendorf tubes (humid chamber). After the washing step, the material was spread onto glass slides. Two microlitres of the fixed bacterial suspension were immobilized on hydrophobic Teflon-coated slides in 8 mm hybridization wells (Roth GmbH, Karlsruhe) and hybridization was carried out as described above. Before observation, the slides were mounted in AF1 antifading reagent (Citifluor, London, UK).

The fluorescent tagged oligonucleotide probes used in this study were purchased from Thermo Electron Corporation GmbH, Ulm, Germany. These were EUB-338-mix [an equimolar mixture of EUB-338 (Amann *et al.* 1990), EUB-338-II and

EUB-338-III (Daims *et al*., 1999)], LGC-354-mix [an equimolar mixture of LGC-354-a, LGC-354-b and LGC-354-c (Meier *et al*., 1999)], Rhi-1247 (Ludwig *et al*., 1998) and EUK-516 (Amann *et al*., 1990) (Table 3). All of them were labelled either with FITC, Cy3 or Cy5.

Microscopic analysis

Hybridized samples were analysed with a confocal laser scanning microscope (CLSM 510 Axiovert 100 M; Zeiss, Jena, Germany) equipped with an argon laser (laserline 488 nm) and two helium–neon lasers (laserlines 543 and 633 nm), for the excitation of FITC, Cy3 and Cy5 respectively. Plan-Neofluar 100 X/1.3 oil and Apochromat 63 X/1.2 water immersion lenses were used for all analysis and image acquisitions. Monochrome images were taken sequentially at each wavelength to optimize scan conditions and laser settings. Artificial colours were assigned to the fluorescent images resulting from each excitation wavelength: green for 488 nm, red for 543 nm and blue for 633 nm. Superimpositions were processed with the Zeiss software package LSM 510, version 3.5.

Electron microscopy

For ultrastructural studies, cells were fixed and micrographs were taken as described by Straube *et al*. (2006).

Colorimetric assay for indole-3-acetic acid determination

Production of IAA by *R. radiobacter* was determined according to Tsavkelova *et al*. (2007). *H. frisingense* strain Mb11 (aux^+ reference strain) and *Herbaspirillum hiltneri* strain N3 (aux^- reference strain) were included in the analyses as positive and negative control respectively.

Plant materials and growth conditions

Kernels of barley cv. Golden Promise were sterilized with 3% sodium hypochloride for 2 h, rinsed in water and germinated for 3 days. Subsequently, roots of seedlings were inoculated in a homogenized mycelial solution (1 g ml^{-1}) or bacterial suspension in LB medium (OD_{600} 1.6) for 1.5 h. Inoculated seedlings were transferred to pots containing a 2:1 mixture of expanded clay (Seramis®, Masterfoods, Verden, Germany) and Oil-Dri® (Damolin, Mettmann, Germany). Plants were grown in a growth chamber at 22°C/18°C day/night cycle, 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density). Plants were fertilized once after 2 weeks with 20 ml of a 0.5% Wuxal top N solution (Schering, N/P/K: 12/4/6) per pot containing three plants.

Biological activity of PABac-DSM

Golden Promise plants were harvested 3 weeks after root inoculation with bacteria or *P. indica*, and shoot fresh weight was measured. For the assessment of systemic resistance induction, the youngest leaves were harvested for a detached leaf-segment test with *B. graminis* f.sp. *hordei* (Waller *et al*., 2005).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Detection of bacterial 16S-rRNA gene by agarose gel electrophoresis. Ethidium bromide-stained agarose gel showing PCR products of 16S-rRNA-coding regions amplified with universal bacterial primers 27f and 1495r from *P. indica*'s metagenomic DNA or from bacteria isolated from fungal samples. Lane M, 1 kb plus marker; lane 1, *P. indica*-DSM11827; lane 2, *P. indica*-JE1; lane 3, *P. indica*-HA; lane 4, *P. indica*-ND; lane 5, PABac-DSM isolated *P. indica*-DSM11827; lane 6, PABac-JE isolated from *P. indica*-JE1; lane 7, non-template control.
Fig. S2. Light microscopy of the pure culture of *R. radiobacter* (PABac-DSM). Rod shaped bacteria were observed using epifluorecent microscope (Axioplan 2, Zeiss). (Scale bar = 3 μm).

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