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Original article

Isolation, partial identification and application of diazotrophic rhizobacteria from traditional Indian rice cultivars

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ABSTRACT

A diversity of N₂-fixing (diazotrophic) bacteria was isolated from two traditional rice cultivars, Sataria and Kartiki, from the rice growing area of Mithila region of North Bihar, India, where low levels of nitrogen fertilizers are applied. Nitrogen-free semisolid media NFB, JMV and LGI with different carbon sources and pH-values were used for enrichment and isolation of root-associated diazotrophs. The colonization density of roots by diazotrophs, as estimated from positive pellicle formation at highest dilution in nitrogen-free enrichment media, was 10⁶–10⁸ diazotrophic bacteria per g fresh root weight. Roots of the cultivar Kartiki were found to be more densely colonized endophytically by diazotrophs as detected after chloramine T (1%) surface disinfection. To ascertain the phylogenetic affiliation of the isolates, phylogenetic oligonucleotide probes and the Fluorescent *in situ* Hybridization (FISH) technique were applied. Using group-specific rRNA directed oligonucleotide probes, the majority of the isolates could be identified as alpha-, beta-, or gamma-proteobacteria. Using 16S and 23S rRNA-directed genus- or species-specific probes, *Herbaspirillum seropedicae*, *Azospirillum amazonense*, *Burkholderia cepacia/vietnamiensis*, *Rhizobia* and *Pseudomonas* spp. were found to be the most prominent root associated culturable diazotrophs. Diazotrophic *Gluconacetobacter* spp. were also demonstrated as colonizers of rice roots. *Burkholderia cenocepacia*, *Pseudomonas* sp. and three diazotrophic PGPR reference strains were used for the inoculation of axenically grown rice seedlings to determine the plant growth promoting potential. Significant increases in the shoot length (up to 60%), shoot dry weight (up to 33%) and the grain yield (up to 26%) per plant were observed in non-axenic pot and field trials. Using semisolid enrichment media after surface sterilization of field grown inoculated rice roots and oligonucleotide probing of the diazotrophic enrichment cultures, a sustainable colonization with the inoculated bacteria could be demonstrated.

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1. Introduction

The presence of plant growth promoting N₂-fixing bacteria and the possibility of a significant increase in plant performance and yield under nutrient limiting conditions by root-associated bacteria have been discussed for many years. Especially rice as important food source for billions of people is in the centre of interest. The estimation for biological nitrogen fixation in rice is quite divergent [4] and it has been clearly demonstrated that the contribution of nitrogen fixation is dependent on the rice variety. We tested two traditional rice varieties, “Sataria” and “Kartiki” from the rice growing area of Mithila region of North Bihar (N 26° 21'; E 86° 04'), India, where yields of about 3.0–3.5 ton per hectare are sustainable even without use of nitrogen fertilizers.

The interest in plant associated diazotrophic bacteria was much stimulated by the discovery of endophytic diazotrophic bacteria in graminaceous crop plants, such as sugar cane, maize, wheat and rice [2,24,33]. Some of these bacteria colonize the interior of roots in the intercellular spaces and also the central cylinder, such as *Herbaspirillum seropedica* and *Herbaspirillum rubrisubalbicans* as well as *Gluconacetobacter diazotrophicus* in sugar cane [15,27]. These and other diazotrophic bacteria, such as *Azoarcus* sp. occur endophytically in graminaceous plants [14,30]. In addition, Rhizobia have been observed as endophytic diazotrophs in rice plants, when rice is grown in rotation with legumes [42]. Diazotrophic endophytic bacteria colonize the roots in quite high numbers and often occur systematically in the plant without causing negative impact to the plant. On the contrary, this endophytic colonization was shown to have the potential to improve the nutrition, growth and health of the plants. In contrast, members of the species *Azospirillum brasilense* are mostly rhizoplane colonizers with only some potential to enter the rhizodermis, as in the case of *A. brasilense* Sp245 [32,38].

In rice, β -proteobacteria of the genus *Burkholderia*, e.g. *Burkholderia brasilensis*, and *Burkholderia vietnamensis* [11] and *Burkholderia* spp. [25] have been reported in high numbers. These bacteria colonize the rice plants systemically, although the highest numbers were observed in the roots. In addition, β -proteobacterium *Azoarcus* was demonstrated to colonize rice systemically [13]. In the interaction with a fungus and under microaerobic conditions, these bacteria change their physiology, forming so-called diazosomes [30]. More recently, Kirchof et al. [18] found a new diazotrophic *Herbaspirillum* species (*Herbaspirillum frisigense*) to colonize the roots of the C4 grasses *Miscanthus sinensis* and *Pennisetum purpureum* and characterized *Herbaspirillum hiltneri* colonizing wheat and barley endophytically. It is an attractive hypothesis, that some endophytic bacteria enter a symbiotic state and interact with the plant host in a way, not yet understood. While in the conventional identification of bacteria, time consuming series of physiological and biochemical tests are necessary, the identification of isolated bacteria using fluorescence *in situ* hybridization (FISH) and molecular phylogenetic probes targeting 16S- or 23S-rRNA is rapid [1,19,26]. Using recently developed probes, whole cell hybridization with fluorescently labeled probes is possible for all members of the genus *Azospirillum* as well as *Herbaspirillum* [18,33,40].

In this communication, we present a large number of diazotrophic bacteria isolated from rice roots with three time levels of surface sterilization (0, 2, 10 min) using chloramine T. The isolates were identified using 16S- and 23S-rRNA directed group, genus and species-specific oligonucleotide probes and fluorescence *in situ* hybridization (FISH) analysis. 16S-sequence analysis was performed with selected isolates to prove the FISH-result. Finally, selected bacterial isolates and type strains were used in inoculation experiments to demonstrate the potential to improve the growth performance and grain yield of the inoculated rice plant.

2. Materials and methods

2.1. Rice cultivars

Rice plants of two traditional cultivars Sataria (normal harvest variety) and Kartiki (early harvest variety) were collected from unfertilized fields from Madhubani district (Mithila region) of North Bihar (N 26° 21'; E 86° 04'), India and transferred aseptically to the lab in sterile boxes. Bacteria were isolated from root parts as described below.

2.2. Isolation of diazotrophic bacteria from rice roots

Roots were washed free of adhering soil particles for 15 min in 0.5× PBS solution and were treated afterwards with freshly prepared 1% chloramine T (C₇H₇ClNO₂Sn – Sigma-Aldrich, USA) for 0, 2 or 10 min. After thorough washing, the roots (0.5 g fresh weight) were homogenized with a sterile mortar in 9.5 ml 4% sucrose solution. Aliquots of 0.1 ml of serial dilutions (up to 10⁻⁸) were inoculated into vials containing 5 ml of the respective semisolid medium.

For the enrichment and isolation of diazotrophic bacteria, three different semisolid nitrogen free media (JMV, Nfb and LGI) [9] were applied with the following composition:

- (A) JMV: mannitol as carbon source 5 g, K₂HPO₄ 0.6 g, KH₂PO₄ 1.8 g, MgSO₄ × 7H₂O 0.2 g, NaCl 0.1 g, CaCl₂ × 2H₂O 0.2 g, bromothymol blue (0.5% [w/V] solution in 0.2 M KOH) 2 ml, trace element solution (ZnSO₄ 100 mg/l, MnCl₂ × 4H₂O 30 mg/l, H₃BO₃ 300 mg/l, CoCl₂ × 6H₂O 200 mg/l, CuCl₂ × 2H₂O 10 mg/l, NiCl₂ × H₂O 20 mg/l, Na₂MoO₄ × 2H₂O 30 mg/l) 2 ml, Fe-EDTA solution (1.6% [w/v]) 4 ml, KOH 4.5 g, vitamin solution (riboflavine 10 mg/l, thiamin-HCL × 2H₂O 50 mg/l, nicotinic acid 50 mg/l, pyridoxin-HCL 50 mg/l, Ca-pantothenate 50 mg/l, biotin 100 mg/l, folic acid 200 mg/l, vitamin B₁₂ 200 mg/l), pH 4.2–4.5, volume was complemented to 1000 ml, 2.1 g agar was added for semisolid medium.
- (B) Nfb: the medium had the same composition cited above for JMV medium with the exception that malic acid (5 g/l) as carbon source and 1.8 g agar for the preparation of semi-solid medium were used, pH was adjusted to 6.5 with 10 N KOH solution.
- (C) LGI: this medium had the same composition as it is cited for the Nfb medium with the exception that sucrose is used as carbon source, pH was adjusted to 6.0–6.2 with 10 M H₂SO₄.

After incubation for four to five days at 30 °C, diffuse subsurface pellicles appeared. Bacteria from the highest dilution vial showing a pellicle formation (see Table 3) were transferred to new sterile semi-solid medium for a second incubation. After new pellicle formation, cells were plated on nitrogen free solid medium with trace amount of yeast extract. Single, separated colonies growing on these plates were re-inoculated into new semi-solid medium. Bacteria from growth pellicles in these vials were finally transferred to nutrient broth agar plates. For long term storage at –20 °C, pure cultures were centrifuged and re-suspended in sterile PBS/glycerin (4:1).

2.3. Fixation of cells and fluorescent in situ hybridization with phylogenetic probes

Cell cultures were fixed in 4% paraformaldehyde solution for at least 2 h at 4 °C. To screen all isolates for phylogenetic affiliation, samples were hybridized with group, genus and species specific phylogenetic, fluorescently labeled probes (listed in Table 1).

Fluorescent in situ Hybridization (FISH) with fluorochrome (FITC, Cy3 and Cy5) labeled oligonucleotide probes was performed according to previously described protocols [1,21]. All rRNA-targeted oligonucleotide probes used in this study, were synthesized and fluorescently labeled by ThermoHybaid Division Interactiva (Ulm, Germany).

Hybridizations were performed on Teflon coated glass slides with 6 or 8 wells (Marienfeld, Bad Mergentheim, Germany) for independent positioning of the samples. Aliquots of reference cells were spotted on single wells, dried at 46 °C and dehydrated using an ascending Ethanol series (5 min each 50, 80 and 100% ethanol). Hybridizations were carried out for at least 1.5 h at 46 °C in 10 µl hybridization buffer containing 0.9 M sodium-chloride (pH 8.0), 0.01% (w/v) SDS, 10 mM Tris/HCl (pH 8.0), various amounts of deionized

formamide, and 30 ng of Cy3/Cy5 labeled and/or 50 ng of FITC labeled probes. After hybridization in a humid chamber, slides were rinsed with pre-warmed washing buffer and washed for 20 min at 48 °C in a water bath. The washing buffer contained 20 mM Tris/HCl (pH 8.0), 0.01% (w/v) SDS, from 0.056 M to 0.9 M sodium-chloride, depending on the stringency according to the hybridization buffer, and 0.25 mM EDTA (pH 8.0). To remove salts, slides were rinsed with deionized water. After air drying of the slides, samples were embedded and mounted in Citifluor AF-1 (Citifluor Ltd., London, UK) to circumvent bleaching effects and sealed with a cover slip.

For epifluorescence microscopic analysis a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany) equipped with a mercury lamp HBO50 (Osram, Munich, Germany) and high quality (HQ) filter sets for FITC (Emitter HQ 535/50, Beamsplitter Q 505 LP, Exciter HQ 480/40), Cy3 (Emitter HQ 610/75, Beamsplitter Q 570 LP, Exciter HQ 545/30) and Cy5 (Emitter HQ 700/75, Beamsplitter Q 660 LP, Exciter HQ 620/60) excitation were used. All filter sets were obtained from AHF Analy-sentechnik (Tübingen, Germany). A Plan-Neofluar 100×/1.3 oil immersion objective (Zeiss) served for all observations.

2.4. Extraction of genomic DNA and PCR amplification of *nifH* gene

For DNA extraction, colonies from bacterial isolates were cultured in 3 ml of liquid 1/2 DYGS medium overnight at 30 °C. The cells were centrifuged and further used for DNA extraction. Genomic DNA was extracted and purified by use of the FastDNA spin kit (Qbiogene Inc., CA, USA) according to the manufacturer's protocol. Amplification of the *nifH* gene from the extracted DNA was performed using the primers Pol F (5' TGCGAYCCSAARGCBGACTC-3') and Pol R (5'-ATSGCCATCATYTCRCCGGA-3'). Amplification was performed in 50 µL final volume containing 1 µL genomic DNA

Table 1 – 16S- and 23S-rRNA directed oligonucleotide probes used

Probes	Specificity	rRNA-target site	% Formamide	Reference
Eub338	Bacteria	16S	0	[1]
Alf1b	Alpha proteobacteria	16S	20	[22]
Bet42a	Beta proteobacteria	23S	35	[22]
Gam42a	Gamma proteobacteria	23S	35	[22]
Ps56a	<i>Pseudomonas</i>	23S	0	[36]
SUBU 1237	<i>Burkholderia</i>	16S	60	[41]
Bcv13b	<i>B. cepacia</i> , <i>B. vietnamiensis</i>	23S	25	[41]
HERB 1432	<i>Herbaspirillum</i>	16S	35	[37]
HERB68	<i>Herbaspirillum</i>	16S	35	[37]
Hsero445	<i>H. seropedica</i>	16S	35	[37]
Hrubri445	<i>H. rubrisubalbicans</i>	16S	60	[37]
Hfris445	<i>H. frisingense</i>	16S	50	[37]
Rhi 1247	<i>Rhizobia</i>	16S	35	[21]
Glac 1424	<i>Gluconobacter/Acetobacter</i>	16S	30	[27]
Azo 440a	<i>Azospirillum-Skermanella-Rhodocysta</i> -cluster	16S	50	[42]
AZO1-665	<i>Azospirillum</i> subcluster	16S	50	[42]
Aama1250	<i>A. amazonense</i>	16S	50	[42]
CF 319a + b	<i>Cytophaga-flavobacterium-bacterioides</i>	16S	35	[23]
HGC69a	High G + C Gram-Positive bacteria	23S	30	[31]
LGCabc	Low G + C Gram-Positive bacteria	23S	20	[24]

(50 ng), 20 pmol each of forward and reverse primer, PolF and PolR, a 200 μM concentration of each of dNTPs (Sigma, USA), 10 \times *Taq* polymerase buffer and 2.5 U of *Taq* polymerase (Sigma, USA). PCR conditions consisted of initial denaturation step at 94 °C for 4 min, 30 amplification cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and primer extension at 72 °C for 2 min; followed by a final extension at 72 °C for 5 min with MyCycler™ PCR System (BioRad, USA). Aliquots of the PCR products were analyzed in 1.5% (wt/vol) agarose gels (Sigma, USA) by horizontal gel electrophoresis. DNAs were visualized by UV excitation after staining with ethidium bromide (0.5 mg l⁻¹). PCR products were eluted from agarose gels, purified and sequenced.

2.5. 16S-rDNA sequencing, phylogenetic analysis and tree reconstruction

Almost full length 16S-rRNA coding gene fragments were amplified from 100 ng of the respective isolated and purified DNA using the flanking primer pair 616-F 5'-AGA-GTT-TGA-TYM-TGG-CTC-AG-3' and 630-R 5'-CAK-AAA-GGA-GGT-GAT-CC-3' [17]. The reaction mixture with a total volume of 50 μl contains 50 pmol of each primer, 5 μl 10 \times *Taq* reaction buffer (Promega, Madison, Wisconsin), 200 μM of each dNTP's and 2.5 U of *Taq* Polymerase (Promega, Madison, Wisconsin). The amplification was carried out in a programmable Thermal Cycler (Primus-96, MWG-Biotech, Ebersberg, Germany). Cycle program starts with an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing 50 °C for 45 s and elongation at 72 °C for 1 min. Cycling was completed by a final elongation step at 72 °C for 5 min. Successful amplification results in PCR products of about 1500 bp length sizes of the respective PCR products were determined by fluorescent imaging by standard horizontal agarose gel electrophoresis with ethidium bromide stained DNA [35]. The obtained PCR products were purified with the commercially available Nucleo-Spin® Extract Kit (Macherey & Nagel, Düren, Germany) according to manufactures protocol. The purified DNA was sequenced directly using an ABI-Prism-377 automated Sequencer (Applied Biosystems, Foster City, Germany) and the Big-Dye-Terminator sequencing Kit (Applied Biosystems). We use in addition to the amplification primer mentioned above the internal primers 608-F (5'-CCG-CAC-AAG-CGG-TG G-3'), binding position 931–945 and 612-RII, (5'-GTA-AGG-TTY-TNC-GCG-T), binding position 969–984 according to Brosius et al. [5]. Phylogenetic analyses of the obtained 16S-rRNA sequences were performed with the software package ARB (<http://www.arb-home.de>) [20]. All sequences were aligned automatically with the implemented tool FAST_Aligner according to homologous positions of an existing alignment of about 160.000 SSU-rRNA sequences in the database (SILVA-94, <http://www.arb-silva.de>) [29]. Wrong alignment positions and ambiguities were corrected manually with the help of the sequencing chromatograms and secondary structure data. Phylogenetic trees were calculated by applying "Maximum Parsimony" (ARB, PHYLIP) [10], "Maximum Likelihood (fast DNAML program), [28] and "Neighbor-Joining" methods [34].

2.6. Inoculation of the rice cultivar "Kartiki" with diazotrophic bacteria

Surface sterilization of rice seeds: rice seeds were surface disinfected by using 1% streptomycin plus a few drops of Tween 80 for 20 min followed by 2 \times washing with sterile water. Thereafter, seeds were treated with 0.1% HgCl₂ for 10 min (with constant agitation) and washed thrice with sterile water. Seeds were transferred to NB plates for germination in the dark at room temperature and to monitor residual infections. Germinated seeds completely free from infection were chosen for further inoculation experiments.

Axenic rice plant inoculation system: two isolates, JZ4 (*Burkholderia cenocepacia*) and NZ5 (*Pseudomonas* sp.) from this work and three diazotrophic PGPR reference strains were used for the inoculation to know their potential for promoting plant growth. JZ4 and NZ5 were predominant isolates (from the highest dilution) of nitrogen-free enrichment cultures of the rice cultivar "Kartiki" after surface treatment for 10 min with 1% chloramine T, arguing for a tight physical association with the root or even for an endophytic localization. These isolates also showed the presence of *nifH* gene as evidenced by amplification of a characteristic 360 bp gene fragment. Hoaglands medium [12] with all salts and micronutrients but without nitrogen was prepared and 60 ml of this medium was used per test tube (100 ml capacity) and solidified with 0.6% agar. Before solidification, 10⁸ cells of an overnight grown bacterial culture were added per tube. As un-inoculated control, tubes with 1 ml sterile water added were prepared. After solidification, germinated rice seeds were transferred to the test tube and incubated in a growth chamber at 28 °C and 10 h light (250 $\mu\text{mol}/\text{m}^2/\text{s}$) per day for 2–3 weeks.

Pot and field experiments: thereafter, five seedlings of each rice cultivar were transplanted into pots containing soil without any added nitrogen for further growth. Thirty replicates for each treatment were maintained. Pots were constantly watered for proper growth. After 60 days some of the plants from pots were replanted in the experimental plots (2.5 \times 2.0 m) for yield analysis at the harvest (at day 120). Remaining pots were left for a further period of 30 days and thereafter all growth data such as shoot and root length and dry weight were determined from these pot grown plants. The grain yield per plant data was obtained from the experimental field grown plants at harvest time. The data were analyzed using one-way ANOVA. The soil used in the pot experiments were taken from the experimental plots itself and it was analyzed for different physical and chemical parameters (see Table 2). Finally, root samples were taken

Table 2 – Physicochemical properties of the soil used in pot culture experiment

Parameters	Content	Nature of the soil
pH	8.3	Basic
Electrical conductivity	0.28 dsnl	Average
Organic C	0.73%	Medium
Phosphorus	26.92 kg ha ⁻¹	Medium
Potassium	268.50 kg ha ⁻¹	Medium
Nitrogen	0.065%	Low

from some of the inoculated plants, washed thoroughly, treated with 1% chloramine T for 10 min and homogenized as described above. The aliquots were transferred to nitrogen-free semisolid medium to test for the presence of nitrogen fixing bacteria.

3. Results and discussion

3.1. Numbers of diazotrophic bacteria associated with the roots

In the roots of the rice cultivar Sataria taken from rice field in the Mithila region, North Bihar, 10^7 bacteria per gram fresh root weight were obtained in Nfb medium without application of chloramine T. A reduced amount of viable bacteria (10^5 and 10^3 bacteria per gram fresh root weight) were found in root homogenate after 2 and 10 min surface disinfection with 1% chloramine T solution, respectively (Table 3). In JMV and LGI medium also 10^7 bacteria per gram root fresh weight were estimated. After 2 and 10 min surface disinfection, 10^4 and 10^2 bacteria were determined in JMV medium, while no pellicle formation was observed in LGI medium.

In the roots of cultivar Kartiki, 10^8 bacteria per gram fresh root weight were determined using Nfb medium in untreated roots, while 10^6 and 10^4 bacteria were estimated in root homogenates after 2 and 10 min chloramine T treatment, respectively. In JMV medium, positive pellicle formation gave an estimate of 10^8 , 10^7 and 10^5 diazotrophic bacteria in root homogenates after 0, 2 and 10 min surface disinfection, respectively (Table 3). In LGI medium 10^7 , 10^6 , and 10^3 diazotrophic bacteria were estimated after 0, 2 and 10 min of surface disinfection.

These results suggest differences in the bacterial colonization of roots of these two rice cultivars. In the cultivar Sataria a lower extent of potentially endophytic colonization of the roots occurred, since no bacteria could be isolated from surface disinfected roots in LGI medium and three orders of magnitude less diazotrophic bacteria were detected in JMV after 2 min surface disinfection. In contrast, a higher number

of diazotrophic bacteria colonized the roots in the early harvest variety Kartiki. Even 10^6 and 10^7 bacteria per gram root fresh weight were found after 2 min treatment with chloramine T and 10^3 – 10^5 bacteria per gram root fresh weight were recorded after 10 min treatment with chloramine T in malate-containing Nfb and JMV media. This observation suggests a high degree of endophytic colonization of cultivar Kartiki. To test this further, the cultivar showing the higher degree of endophytic colonization (Kartiki) was chosen for inoculation studies (see below).

3.2. Identification of the bacterial isolates

A total number of 170 isolates were obtained from the enrichment cultures and tested with 16S- and 23S-rRNA directed phylogenetic oligonucleotide probes (Table 1) for the characterization of their phylogenetic affiliation in a top to bottom approach. All these isolates gave positive hybridization with the probe Eub-338. Using group-specific probes for the alpha-, beta- and gamma-proteobacteria (Gram-negative bacteria) 156 bacterial isolates gave positive hybridization signals. The remaining 14 isolates were tested with the oligonucleotide probes for the *Cytophaga-Flavobacterium-Bacteroides* group (CF319 a, b), the low GC Gram-positive (LGC a, b, c) and high-GC Gram-positive (HGC-69a), but only one isolate could be identified as high-GC Gram-positive bacterium. Within each proteobacterial group, oligonucleotide probes with increased specificity down to the genus and species level (Table 4) were applied to identify the bacteria.

Alpha-proteobacteria: from the 51 isolates (35 isolated from the cultivar Sataria and 16 from Kartiki) only four isolates gave positive hybridization with the probe Azo-440a, specific for the *Azospirillum-Skermanella-Rhodocysta*-cluster (Tables 1 and 4). The probes AzoI-665 specific for the *Azospirillum* subcluster, containing *A. brasilense*, *Azospirillum lipoferum*, *Azospirillum doebereineriae*, *Azospirillum largomobile*, and *Azospirillum halopraeferens* [40] was applied to narrow down the identification which gave negative results in all four cases (data not shown). Three of these isolates finally hybridized with the species-specific probe Aama-1250 and could therefore be identified as *A. amazonense*, while the fourth isolate failed to hybridize with any of the available probes (Table 4). It may have a mutation in the probe binding site and thus failed to be identified using this approach or may represent a yet not identified *Azospirillum* species.

Using the probe Glac-1424 for the *Gluconobacter-Acetobacter* cluster, nine isolates gave positive hybridization. Diazotrophic *Gluconacetobacter* isolates had been reported from sugar cane, coffee and pineapple [2]. Association of *G. diazotrophicus* with Korean wetland rice variety has been reported recently [25].

Interestingly, 26 isolates belonging to the alpha-proteobacteria could be grouped as *Rhizobiaceae* using the probe Rhi-1247 (Tables 1 and 4). The full length 16S-rDNA sequence of one selected isolate (NY11) was determined which clearly confirmed the identification as *Rhizobium* sp. with the highest similarity of 99.8% to the next nearer classified *Rhizobium* sp. 1003, AB054953 and 98.5% similarity to the two *Rhizobium tropici* strains D12798 and X67233 (Fig. 1). Further testing with species-specific probes and sequencing of the 16S rDNA of the other isolates, especially which failed to hybridize with

Table 3 – Cell numbers (MPN/pellicle formation) of diazotrophs in two rice cultivars

Treatments		Cultivars	
		Sataria	Kartiki
Nfb	0 min chloramin T	10^7	10^8
	2 min chloramin T	10^5	10^6
	10 min chloramin T	10^3	10^4
JMV	0 min chloramin T	10^7	10^8
	2 min chloramin T	10^4	10^7
	10 min chloramin T	10^2	10^5
LGI	0 min chloramin T	10^7	10^7
	2 min chloramin T	0	10^6
	10 min chloramin T	0	10^3

Table 4 – Isolates belonging to α -, β - and γ -subgroups of proteobacteria

Isolates belonging to α -subgroup of proteobacteria						Isolates belonging to β -subgroup of proteobacteria									Isolates belonging to γ -subgroup of proteobacteria		
Isolates	Probes					Isolates	Probes							Isolates	Probes		
	Alf 1b	AZO 440a	Aama 1250	Rhi 1247	Glac 1424		Beta 42a	SUBU 1237	Bcv 13b	HERB 1432	HERB 68	Hfris 445	Hrubri 445		Hsero 445	Gam 42a	Ps 56a
NA-1	+			-	+	NA-4	+	-		+	+	-	-	+	NA-6.3	+	+
NA-1.2	+	-		-	+	JA-3	+	+	-	-					NA-6.3.2	+	+
NA-5	+	-		-	-	JA-6	+	+	+						NA-10	+	+
NA-6	+	-		-	+	JA-7	+	+	+						NA-12.1	+	+
NA-8	+	-		+	-	JA-8	+	+	+						NA-13	+	+
NA-11.2	+	-		+	-	JA-9	+	+	+						JA-2	+	+
NA-15	+	-		-	+	JA-14	+	+	+						JA-4	+	+
NB-1	+	-		+	-	JA-15	+	+	-						JA-10	+	-
NB-2	+	-		+	-	LA-1	+	+	+						JA-16	+	-
NB-3	+	-		+	-	LA-2	+	+	+						LA-4	+	-
NB-4	+	-		+	-	LA-3	+	+	+						LA-11	+	-
NB-5	+	-		-	+	LA-5	+	+	+						LA-12	+	+
NB-6	+	-		+	-	LA-6	+	-		+	+	-	-	+	LA-2.2	+	+
NB-9	+	-		-	-	LA-7	+	+	+						NX-3	+	+
NB-12	+	-		+	-	LA-8	+	+	+						NX-3.2	+	+
NB-13	+	-		-	-	LA-10	+	+	+						NX-5	+	-
NB-15	+	-		+	-	NX-8	+	-		+	+	-	-	+	NX-6	+	+
NC-1	+	-		+	-	JX-1.3	+	+	+						NX-7	+	+
NC-3	+	-		+	-	JX-2.1	+	+	+						NY-3.2.1	+	+
NC-4	+	-		+	-	JX-2.2	+	-		+	+	-	-	+	NY-8.1	+	+
NC-5	+	-		+	-	JX-4	+	+	+						NY-9	+	+
NC-6	+	-		+	-	JY-1	+	+	+						NZ-2	+	+
NC-8	+	-		-	-	JY-2									NZ-3	+	+
NC-9	+	-		-	-	JY-2.1	+	+	+						NZ-5	+	+
NC-10	+	-		+	-	JY-3.1	+	+	+						NZ-6	+	+
NC-13	+	-		+	-	JY-3.2	+	+	+						NZ-7	+	+
JA-1	+	-		-	-	JY-3.3	+	+	+						NZ-8	+	+
JA-5 ^a	+	+	-			JY-4	+	+	-						JX-1.1	+	+
JA-12 ^a	+	+	+			JY-5	+	+	-						JX-1.2.1	+	+
JB-9	+	-		-	-	JY-6	+	+	+						JY-2.2	+	+
JB-9.2	+	-				JZ-1	+	+	+						JZ-3.1.1	+	+
JB-10	+	-		-	-	JZ-2	+	+	-						LX-2	+	+
JB-12	+	-		+	-	JZ-3	+	+	+						LX-3	+	-
LA-9 ^a	+	+	+			JZ-3.1	+	+	+						LX-4	+	+
LA-13	+	-		-	-	JZ-4	+	+	+						LX-4.2	+	+
NX-1	+	-				JZ-4.1	+	+	+						LX-4.3	+	-
NY-3	+	-		+	-	LX-5	+	-		+	+	-	-	+	LX-5.2	+	+
NY-3.2	+	-		+	-	LX-6.1	+	-		+	+	-	-	+	LY-3.1	+	-
NY-4	+	-		+	-	LX-6.2	+	-		+	+	-	-	+	LY-4	+	+
NY-6.1	+	-		+	-	LY-1	+	+	+						LY-5	+	+
NY-7	+	-		-	+	LY-2	+	+	+						LY-6	+	+
NY-8	+	-		+	-	LY-3.1.1	+	-		+	+	-	-	+	LY-8	+	+
NY-10	+	-		-	-	LY-7	+	-		+	+	-	-	+	LZ-1	+	-
NY-11	+	-		+	-	LY-7.1	+	+	-						LZ-3	+	+

(continued on next page)

Table 4 (continued)

Isolates belonging to α -subgroup of proteobacteria				Isolates belonging to β -subgroup of proteobacteria				Isolates belonging to γ -subgroup of proteobacteria						
Isolates		Probes		Isolates		Probes		Isolates		Probes				
Alf 1b	AZO 440a	Aama 1250	Rhi 1247	Glac 1424	Beta 42a	SUBU 1237	Bcv 13b	HERB 1432	HERB 68	Hfris 445	Hrubri 445	Hsero 445	Gam 42a	Ps 56a
+	-	-	+	-	+	+	-	+	+	-	-	+	+	-
NZ-1					LY-9								LZ-5	
NZ-4					LY-9.1									
NZ-5.1					LY-10									
NZ-9					LY-11									
LX-1					LY-12									
LX-1.2					LZ-1.2									
LY-3 ^a					LZ-2									
					LZ-4									
					LZ-6									
					LZ-7									
Total: 51 isolates				Total: 54 isolates				Total: 45 isolates						

Isolates nomenclature: N, J and L denote the medium used – Nfb, JMV and LGI, respectively. A, B and C denote the chloramine T treatment for 0, 2 and 10 min, respectively, for the rice cultivar Sataria. X, Y and Z denote the chloramine T treatment for 0, 2 and 10 min, respectively, for the rice cultivar Kartiki. The numerals 1, 2, 3, etc. denote the number of isolates from same source; (+) and (-) sign denote positive and negative hybridization signals with the respective probe.

^a These isolates gave negative hybridization signals with the probe AZO1 665.

Rhi 1247, has to be performed to identify these isolates further. It was already reported by Yanni et al. [42] and Schloter et al. [37] and confirmed recently by Chi et al. [6] that Rhizobia can colonize various graminaceous plants including rice endophytically. A PGPR-effect was demonstrated for *Rhizobium leguminosarum* bv. trifolii inoculated to rice [6,42]. Twelve alpha-proteobacteria could not be characterized to the genus and species level using this approach.

Beta-proteobacteria: out of 54 isolates identified as beta-proteobacteria (16 from cultivar Sataria and 38 from Kartiki) 11 gave positive hybridization with the probes HERB-68 and HERB-1432, specific for the genus *Herbaspirillum*. They hybridized additionally with the species-specific probe Hsero-445 for *H. seropedica* and were negative in the FISH-analysis using the probes Hfris-445 and Hrubri-445 (Table 4). This confirms the earlier finding [4] that *H. seropedica* colonizes rice roots. Colonization of vascular tissue in leaves of Sorghum by *Herbaspirillum* has also been reported earlier [16]. Using the hybridization probe SUBU-1237 for the *Burkholderia-Sutarella* genus cluster, 43 isolates could be grouped into this cluster. Further hybridization of these isolates with the probe Bcv-13b specific for *Burkholderia vietnamiensis* and *Burkholderia cepacia* [39] revealed that 30 isolates represent bacteria of the *B. cepacia* complex, from which some are known as diazotrophs associated with rice plants [4,9,25]. Analysis of the 16S-rDNA sequence affiliates isolate JZ4 clearly to the *B. cepacia* complex with the highest similarity of 99.9% to the strains *Burkholderia* sp., AM747631 and AB212227, *B. cepacia*, AY741330 and AY741341 and *Burkholderia anthina*, AJ420880 (Fig. 1). Thirteen isolates apparently belong to the *Burkholderia* genus, which are not linked closely to the *B. cepacia* complex. Further characterization of these isolates by 16S-rDNA sequencing is necessary to determine the exact phylogenetic affiliation.

Gamma-proteobacteria: from the 45 isolates (12 from cultivar Sataria and 33 from Kartiki), 35 gave positive hybridization with the probe Ps-56a and could therefore be identified as *Pseudomonas* sp. (Table 4). You and Zhou [43] had already reported about the isolation of a nitrogen-fixing endophytic diazotroph from rice plants, which they classified at that time as *Alcaligenes faecalis* A15. This strain was also shown to fix nitrogen in rice callus cultures using the ¹⁵N tracer technique [43]. Later the strain A15 was reclassified to the species *Pseudomonas stutzeri* [41]. The association of *Pseudomonas* spp. with rice has also been reported earlier [25]. To address the plant growth promoting potential, one *Pseudomonas* sp. isolate (NZ5) was included in the rice inoculation experiment. The exact affiliation of the remaining 10 isolates determined as gamma-proteobacteria has to be further analyzed by extensive 16S-rDNA sequencing.

Amplification of *nifH* gene: to confirm the potential for nitrogen fixation, the presence of the structural gene for nitrogenase reductase (*nifH*) was determined by *nifH* gene amplification with genomic DNA extracted from seven representative isolates belonging to α -, β - and γ -subgroups of proteobacteria (shown in Fig. 2). Amplification with the gene specific primers yielded the expected 360-bp size product on agarose gel. In case of one isolate (NY7) there was an additional band below the main band which was removed during elution and purification. The DNA sequence data matched with the predicted *nifH* sequence (data not shown).

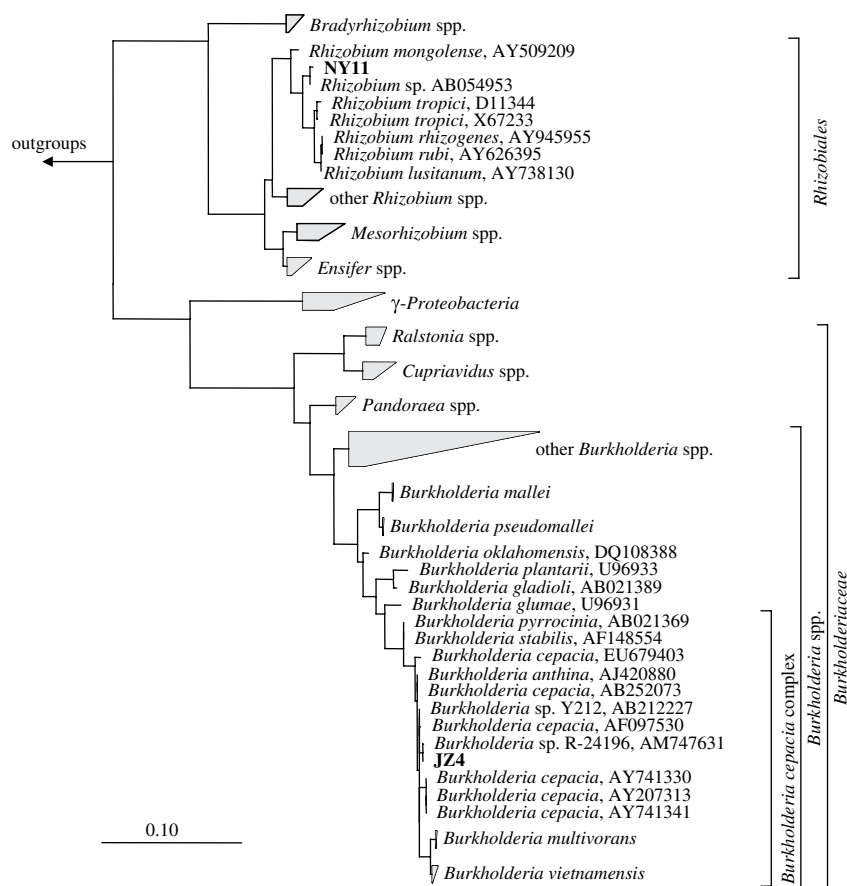


Fig. 1 – Phylogenetic tree showing the relationships among 16S-rDNA sequences of isolates NY11 and JZ4 and the most similar sequences retrieved from databases.

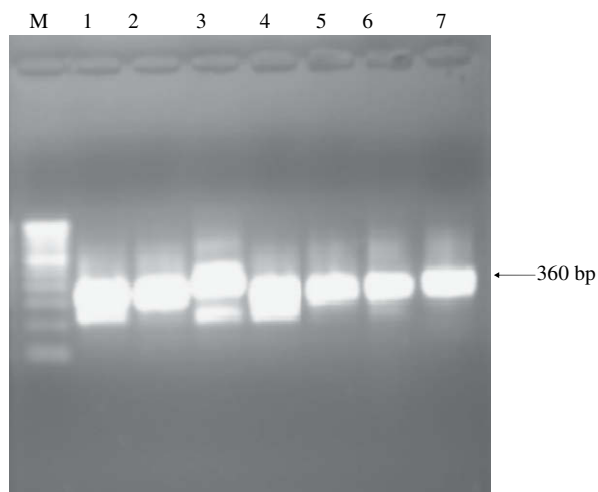


Fig. 2 – Agarose gel electrophoresis of PCR products obtained by amplifying *NifH* gene from the genomic DNA of bacterial isolates -1: NB-3, 2: JA-12, 3: NY-7 (alpha-subgroup); 4: JZ-4, 5: LX-5 (beta-subgroup) and 6: NZ-5, 7: LX-4 (gamma-subgroup). M, Marker 100 bp DNA ladder.

3.3. Impact of inoculated diazotrophic bacteria on growth and yield of rice

Selected diazotrophic bacteria, including identified isolates (*B. cepacia* complex strain JZ4 and *Pseudomonas* strain NZ5) from the present study and the standard reference cultures (*H. seropedicae* Z67^T, *G. diazotrophicus* Pal5^T, and *A. brasilense* Sp7^T), were used for inoculation of Kartiki rice seedlings to determine the plant growth data, like shoot and root length and dry weight [3] as well as yields. The effects of the inoculation on growth and yield in pot and field experiments are summarized in Table 5. A significant increase in shoot length, shoot dry weight and grain yield per plant were observed in pot and field experiments. Maximum increase in shoot length was observed in the case of *B. cepacia* and *H. seropedicae* (>75%). About 50% increase in the shoot length was observed with *G. diazotrophicus* and *Pseudomonas* sp.; *A. brasilense* gave an increase of 36% in shoot length. Over all more than 50% increase in shoot length was observed after inoculation of rice with these diazotrophic bacteria. A similar trend in the increase of shoot dry weight was observed. The increase in average shoot dry weight varied from 21% to 48%, in case of *A. brasilense* and *B. cepacia*, respectively. Generally about 4–13% increase in root length and root dry weight was noticed in the cultivar Kartiki due to bacterial inoculation. Another

Table 5 – Inoculation experiment with rice cultivar Kartiki by selected diazotrophic bacteria

Parameters	Control		<i>H. seropedicae</i> Z67 ^T		<i>G. diazotrophicus</i> PAL5 ^T		<i>A. brasilense</i> Sp7 ^T		<i>B. cenocepacia</i> JZ4		<i>Pseudomonas</i> sp NZ5	
	Mean ± SD	% Increase	Mean ± SD	% Increase	Mean ± SD	% Increase	Mean ± SD	% Increase	Mean ± SD	% Increase	Mean ± SD	% Increase
Shoot length plant ⁻¹ (cm)	43.60 ± 1.40 ^e	75	76.50 ± 3.00 ^b	54	67.20 ± 5.60 ^c	36	59.40 ± 2.40 ^d	89	82.50 ± 2.90 ^a	64.00 ± 2.80 ^{c,d}	47	
Root length plant ⁻¹ (cm)	9.00 ± 0.40 ^c	8	9.70 ± 0.70 ^{a,b}	4	9.40 ± 0.50 ^{b,c}	1	9.10 ± 0.20 ^{b,c}	13	10.20 ± 0.50 ^a	9.34 ± 0.30 ^{b,c}	4	
Shoot dry weight plant ⁻¹ (g)	7.20 ± 0.15 ^d	36	9.80 ± 0.39 ^b	32	9.50 ± 0.52 ^b	21	8.70 ± 0.36 ^c	48	10.70 ± 0.81 ^a	9.30 ± 0.57 ^{b,c}	29	
Root dry weight plant ⁻¹ (g)	0.78 ± 0.04 ^b	7	0.85 ± 0.05 ^a	5	0.83 ± 0.04 ^{a,b}	3	0.81 ± 0.03 ^{a,b}	8	0.86 ± 0.03 ^a	0.84 ± 0.04 ^a	6	
Grain weight plant ⁻¹ (g)	1.24 ± 0.03 ^d	28	1.59 ± 0.10 ^{a,b}	25	1.55 ± 0.05 ^{a,b}	22	1.51 ± 0.06 ^{b,c}	31	1.63 ± 0.08 ^a	1.53 ± 0.30 ^c	23	

Shoot/root length and dry weight data were obtained after 90 days of transplantation of plants to pot. The grains yield data was obtained using experimental plot grown plants at the harvest (120 days). Mean average value ± SD obtained from 20 individual plants (four replicates, each replicates having five plants) are shown. Values in the rows denoted by a different letter indicate significant difference at P ≤ 0.05 in one-way ANOVA.

interesting observation was a marked difference in the rooting pattern, such as increased branching of the roots, in all inoculated plants as compared to un-inoculated control plants. This indicates the possible involvement of hormonal effects, possibly by the auxin indole-3-acetic acid and related compounds as has been exemplified in detail for *Azospirillum brasilense* [7,8]. An average of 25% increase in grain yield at harvest was observed in all inoculated plants. These results clearly demonstrate the plant growth promotion in rice, both in terms of growth and yield, by all five diazotrophic bacteria. However, it cannot be concluded from these results whether this growth stimulation is due to hormonal effects on the root system and concomitant improved nutrient uptake or through a contribution through nitrogen fixation. Finally, it could be demonstrated using root samples of the plants after harvest that after surface sterilization with 1% chloramine T, pellicle formation up to 10⁻⁵ dilutions in semi-solid media occurred. Applying the FISH-technique and species-specific probes the inoculated diazotrophic species could be confirmed in all cases in the pellicles (not shown). This makes it highly probable, that the inoculated bacteria colonized the rice plants had efficiently colonized the roots (probably even in the endorhizosphere) and stayed there in considerable numbers until harvest time.

4. Conclusion and outlook

Using the nested application of phylogenetic oligonucleotide probes, bacterial isolates from enrichment cultures with nitrogen-free semisolid NFB, JMV and LGI media could be identified without applying other biochemical testing. This approach directly allowed the rapid and partial identification of a high diversity of diazotrophic bacterial strains in two Indian rice cultivars, Sataria and Kartiki. Well known diazotrophic species (like *Azospirillum amazonense*, *H. seropedica*, *B. vietnamiensis*, *Rhizobium* sp. and *Pseudomonas* sp.) as well as bacteria with until now not clarified phylogenetic affiliation were found. The localization of the diazotrophs in or on the surface of roots could only preliminarily be demonstrated by the use of different treatments with chloramine T. Further detailed analysis using FISH and confocal laser scanning microscopy is necessary to describe their exact endophytic localization within the plant. The inoculation of rice seedling with selected bacteria in axenic experiments and its further extension to fields proved the plant growth promoting potential of these diazotrophic bacteria in rice. The possible involvement of nitrogen fixation by the inoculated bacteria especially in the traditional early harvest variety Kartiki needs to be proven by ¹⁵N-dilution analysis and N-balance studies.

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