RESEARCH ARTICLE

Molecular and culture-dependent analyses revealed similarities in the endophytic bacterial community composition of leaves from three rice (*Oryza sativa*) varieties

Lucía Ferrando, Jimena Fernández Mañay & Ana Fernández Scavino

Cátedra de Microbiología, Departamento de Biociencias, Facultad de Química, Universidad de la República, Montevideo, Uruguay

Correspondence: Lucía Ferrando, Cátedra de Microbiología, Departamento de Biociencias, Facultad de Química, Universidad de la República, Gral. Flores 2124, Casilla de Correo 1157, Código Postal 11800, Montevideo, Uruguay. Tel.:+598 29244209; fax: +598 29241906; e-mail: lucia.ferrando1@gmail.com

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Abstract

The endophytic bacterial communities of the three most important rice varieties cultivated in Uruguay were compared by a multiphasic approach. Leaves of mature plants grown in field experiments for two consecutive crop seasons were studied. No significant differences were found in the heterotrophic bacterial density for the three varieties. *Pantoea ananatis* and *Pseudomonas syringae* constituted 51% of the total of the isolates. These species were always present regardless of the variety or the season. Molecular analysis based on the 16S rRNA gene was performed by terminal restriction fragment length polymorphism (T-RFLP) and cloning. T-RFLP analysis revealed that bacterial communities grouped according to the variety, although the three varieties presented communities that showed 74% or higher similarities. *Brevundimonas*, the dominant genus in the clone library (18% of the clones), which might be present in all varieties according to T-RFLP profiles, was not recovered by cultivation. Conversely, bacteria from the genus *Pseudomonas* were not detected in the clone library. These results indicate that communities established in leaves of physiologically different rice varieties were highly similar and composed by a reduced group of strongly associated and persistent bacteria that were partially recovered by cultivation.

Introduction

Rice (*Oryza sativa*), the most important cereal crop, feeds more than 50% of the world's population (Mano & Morisaki, 2008). The development of a sustainable production system is a major challenge for researchers and rice producers. Endophytes, as defined by Hallmann *et al.* (1997), are bacteria detected in surface-sterilized plants or extracted from inside plant tissues that do not have harmful effects on the host. Endophytic bacteria have been isolated from a diverse and broad spectrum of plant species and tissues (Rosenblueth & Martínez-Romero, 2006).

Most studies on rice endophytes have been focused on diazotrophic bacteria (Pains Rodrigues *et al.*, 2008) because of the benefit that plants can obtain from them. However, nitrogen-fixing bacteria represent a small fraction of the total endophytic population cultivated from stems and roots of rice in different crop stages (Barraquio *et al.*, 1997; Stoltzfus *et al.*, 1997). Diversity of culturable endophytes inhabiting cultivated rice has been successfully assessed by different authors which have isolated bacteria from seeds, roots, stems, and leaves of the plant (Okunishi *et al.*, 2005; Mano *et al.*, 2006, 2007; Tian *et al.*, 2007; Yang *et al.*, 2008; Loaces *et al.*, 2011).

Endophytic bacterial diversity in rice plants has been partially assessed using 16S rRNA gene-based techniques (Sun *et al.*, 2008; Prakamhang *et al.*, 2009) or targeting specific taxa or functional genes (Tian *et al.*, 2007; Wu *et al.*, 2009). Chelius & Triplett (2001) designed primers targeting the 16S rRNA gene to overcome the undesirable interference of chloroplast and mitochondrial DNA from the host plant. However, the results obtained by applying this strategy to different plants were not always entirely successful (Idris *et al.*, 2004; Rasche *et al.*, 2006; Becker *et al.*, 2008; Sun *et al.*, 2008). Alternative strategies, like

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enrichment of bacterial cells from plant tissues before further culture-independent analyses, seem to be promising (Ikeda *et al.*, 2009).

Several authors have shown that cultivar type may influence the associated bacterial communities in different plants. Sessitsch *et al.* (2002) reported the detection of plant tissue and variety specific endophytes in three potato varieties. Also, Rasche *et al.* (2006) showed that the diversity of bacterial endophytes colonizing sweet pepper was affected by chilling and cultivar type. Furthermore, it was observed that the rice variety influenced the ammonia-oxidizing community composition (Briones *et al.*, 2002) and the expression of diazotrophic endophytic bacteria (Knauth *et al.*, 2005).

The three Uruguayan most important rice varieties are distinguished by their different physiological properties which enable them to face successfully adverse environmental conditions (i.e. low temperatures, presence of pathogens like *Rhizoctonia oryzae*) or to obtain higher crop yields (Blanco *et al.*, 2003). We hypothesize that these characteristics could select different endophytic bacterial communities. Thus, the aim of this work was to elucidate and to compare the community composition of the endophytic bacteria inhabiting leaves of three Uruguayan rice varieties. T-RFLP analysis and 16S rRNA gene cloning were combined with enumeration and identification of the predominant isolates to elucidate and to compare the community composition of these rice varieties.

Materials and methods

Field site and sampling

The irrigated rice system studied in this work consists of two annual crop seasons followed by 4 years of pastures for livestock feeding (unusual for temperate rice production), which is a typical management employed in Uruguay.

The experimental site was located at the National Agricultural Research Institute (Instituto Nacional de Investigación Agropecuaria, Unidad Paso de la Laguna) in Treinta y Tres, southeast Uruguay (32°55′S and 54°50′ W). Soil characteristics were the following: silty loam in texture, pH 5.2, N-NH_4^+ 0.3 mM, 3.0% organic C and 5.0% organic matter.

Three *O. sativa* varieties were studied: *O. sativa*, type Japonica, variety INIA Tacuarí (IT); *O. sativa*, type Indica, varieties INIA Olimar (IO) and El Paso 144 (EP).

The three rice varieties were obtained from local crossing (inbred varieties) and selected according to their different characteristics. These varieties, released in 1985 (EP), 1992 (IT), and 2002 (IO), represent 95% of cropped rice in Uruguay. The selection allowed to differentiate the varieties with high yields (IO), high cold tolerance (IT) or resistance to pathogens, specially *R. oryzae* (EP) (Blanco *et al.*, 2003). The experiment was set out as a completely randomized design with three replicated plots (approximately 12 m^2 each one) per treatment.

Standard conditions were used for rice cultivation. IT and EP were fertilized with 22, 30, and 30 kg N Ha^{-1} at seeding, tillering, and booting, respectively. For IO, nitrogen fertilization consisted of 14, 35, and 21 kg N Ha^{-1} at seeding, tillering, and internode elongation, respectively. A mixture of herbicides Quinclorac, Clomazone and Propanil was applied at 1.4, 0.8, and 4.0 L Ha^{-1}, respectively, at the postemergence stage.

Samples were collected near harvest in 2005 and 2006, between 120 and 140 days after seeding. Three replicated plots were analyzed separately for each treatment in both sampling years. Sampling was differently performed in the two successive years.

In 2005, one rice plant was collected from five different points randomly distributed in each replicated plot and then pooled. Then, the pools of leaves obtained from each replicated plot were disinfected and used for counts and molecular analyses.

In 2006, three different points randomly distributed within each replicated plot were sampled (triplicates) to study the intra plot count deviation. Five plants were collected from each of the three sampling points and mixed separately. The three pools of rice leaves obtained for each replicated plot were disinfected separately and then used for counts.

Surface disinfection of rice leaves

Collected rice plants were kept at 4 °C until further processing. Pooled leaves from the different treatments obtained as was explained previously were disinfected. Leaves were separated (5 cm upper from roots) and cut into fragments of 10–15 cm length. Fragmented leaf pieces were brushed and washed with tap water and detergent. Distilled water was used for the last rinse. Two 100 mL successive washes were made with 5% sodium hypochlorite for 5 min with agitation, followed by three washes with sterile distilled water (Loaces *et al.*, 2011). To verify surface disinfection efficiency, leaves were rolled on Nutrient Agar and R2A Agar and the plates were incubated at 30 °C for 48 h. No growth was observed.

Enumeration and isolation of endophytic bacteria

Disinfected plant material (5 g) was macerated in a sterilized mortar with a 10 mL sterile saline solution under aseptic conditions.

In the 2005 sampling, dilutions on sterile 0.9% NaCl solution prepared from macerates were plated out on R2A Agar (Difco) (incorporated seeding) and Nutrient Agar (Difco) (surface seeding) and incubated at 30 °C for 72 h. At least three colonies per observed morphotype (approximately 20 isolates) were randomly selected and picked from the highest dilutions and then purified and identified.

In the 2006 sampling, heterotrophic endophytic bacteria were counted on R2A as was described previously, except that the triplicates of each replicated plot were analyzed separately. Additionally, heterotrophic siderophore-producing bacteria (SPB) were enumerated in R2A-CAS medium (Schwyn & Neilands, 1987) with PIPES final concentration reduced to 50 mM, for SPB. Plates were incubated at 30 °C for 72 h. Siderophore production was confirmed by the formation of an orange halo in R2A-CAS medium. Diazotrophic bacteria were enumerated in vials by most probable number (MPN) using RMR media (Elbeltagy *et al.*, 2001) and confirmed by the acetylene reduction assay (ARA) (Paerl, 1998). Siderophore-producing bacteria and diazotrophic bacteria were counted pooling triplicate macerates from each replicated plot. Isolates were obtained from higher dilution plates or vials of different media utilized. At least three colonies were randomly picked from each morphotype. Redundant isolates, within each replicate, with similar colony morphology and amplified ribosomal DNA restriction analysis (ARDRA) profile were discarded.

Dry weight was determined for plant material by drying 10.0 g of each sample at 100 °C until constant weight.

Statistical analysis

Statistical analysis of data was performed using the free software PAST 1.42 (http://folk.uio.no/ohamer/past/). Bacterial counts were submitted to analysis of variance (ANOVA one way) and the Tukey test ($P < 0.05$) was used for comparison of means.

DNA extraction and 16S rRNA gene amplification

Total DNA was extracted from rice material. The surface disinfected frozen samples were pulverized in sterilized precooled mortars with liquid nitrogen and transferred into sterilized 1.5-mL tubes immediately. Disinfected pooled rice leaves (10 g) were extracted according to Doyle & Doyle, 1987. Briefly, pulverized plant material was treated with CTAB buffer (100 mM Tris.HCl pH 8.0; 20 mM EDTA; 1.4 M NaCl, 2% CTAB, 0.2% mercaptoethanol) and then incubated at 65 °C for 45 min. After chloroform/isoamylic alcohol (24 : 1 v/v) extraction, DNA was precipitated by isopropanol addition. DNA was centrifuged for 10 min at 9600 ***g***, resuspended in TE buffer and subjected to RNAase treatment (10 μg mL^{-1} final concentration).

Pure cultures (1.5 mL) were centrifuged (10 min at 15 000 ***g***, 4 °C), and the pellets were extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI).

Extracted DNA from leaf samples and isolates was used as templates for 16S rRNA gene amplification with primers pair 8f and 1492r. All PCRs were carried out in 25 μL (total volume) mixtures containing approx. 100 ng of total DNA, 0.4 mM of each primer, 1.5 mM $MgCl_2$, Taq buffer, 0.17 mM of each dNTP, and 1.2 U of Taq DNA polymerase (Invitrogen, San Diego, CA). The reactions were performed in a Perkin-Elmer, GeneAmpPCR System 2400 thermocycler using the following program: initial denaturation step at 94 °C for 30 s, followed by 30 cycles at 92 °C for 60 s, 60 °C for 60 s, and 72 °C for 60 s, with a final extension step at 72 °C for 5 min

Screening of endophytic diversity by 16S rRNA gene T-RFLP

T-RFLP analysis of the endophytic bacterial community present in rice leaves of each one of the three replicated plots per treatment sampled in 2005 was performed. To avoid the amplification of the chloroplast or mitochondrial DNA, two different PCR approaches were used. Firstly, direct PCR amplification using primers 799f (position 781–799 according to *Escherichia coli* number) and 1492r (position 1492–1510 according to *E. coli* number) was performed. Primer 799f was 5′ labelled with 6-carboxyfluorescein (Sessitsch *et al.*, 2002). PCRs were carried out in 50 μL (total volume) mixtures using the PCR conditions described previously except for primers concentration (0.1 mM of each primer). Technical triplicates were performed and pooled after amplification. Secondly, a nested PCR approach was assayed. First a PCR of 15 cycles with primers 8F and 23Sr FGPL132′-38R (position 110–140 according to *E. coli* number) (Normand *et al.*, 1996) was carried out in 25 μL (total volume) mixtures containing 0.5 μM final primer concentration (the other conditions remained the same as those described earlier). Then, a 25 cycles PCR using 1 μL of the first PCR product as template was performed (including controls without DNA from the first PCR). Technical duplicates and triplicates were performed for the first and second PCR step, respectively. These replicates were pooled after PCR amplification, and labelled PCR products were concentrated and desalted with MICROCON®100 columns (Amicon Inc., MA). Approximately 300 ng of labelled PCR products were digested with AluI or HhaI (Fermentas).

After enzyme inactivation by heating at 65 °C, 15 min, aliquots (4 μL) of the digest were mixed with a master mix (16 μL) containing deionized formamide, loading buffer [Applied Biosystems Instruments (ABI), Foster City, CA], and 0.5 μL of a DNA fragment length standard (ROX 500; ABI). After denaturing of the DNA at 94 °C for 5 min and immediate chilling on ice, they were loaded onto a capillary automated DNA sequencer (3100 Genetic Analyzer). After electrophoresis, the length of fluorescently labelled terminal restriction fragments (T-RFs) was analyzed by the GeneScan 3.1 software (ABI).

Terminal restriction fragment (T-RF) sizes between 40 and 445 bp with peak heights larger than 50 fluorescence units were considered for the analysis to obtain reproducible T-RF profiles (Osborn *et al.*, 2000). Strategies used to prevent plant plastids interference were quite successful because not more than 30% of the total fluorescence signal gave T-RF sizes coincident with mitochondrial or chloroplast rice DNA.

The four data sets (amplicons retrieved from primers 8f or 799f restricted with HhaI or AluI) obtained for each replicated plot were run by duplicate. Total intensity of fluorescence present in each electropherogram was compared within each data set, and T-RFLP data were standardized to the lowest quantity as was previously described (Dunbar *et al.*, 2001). All profiles were aligned considering identical the T-RFs that differed by < 3 bp. T-RF composite profiles were constructed by calculating the mean of peak heights from duplicate runs obtained from each replicated plot. Peaks that were absent in one duplicate run were omitted unless they were detected in both replicates of the remaining two plots from the same treatment. Data of relative abundance of each fragment were utilized to perform UPGMA clustering analysis with the Morisita similarity index using the Past 1.42 software (http://folk.uio.no/ohammer/past/). This analysis included the four data sets (accounting for 86 different T-RFs) for each replicated plot by treatment.

Cloning and phylogenetic analysis

The rice variety El Paso 144 sampled in 2005 was further selected for cloning because of its high diversity revealed in the T-RFLP analysis. Fingerprinting of plots B and C contained all peaks present in the three rice varieties studied, thus pooled DNA from these two replicated plots was utilized. The PCR product obtained as explained previously (nested PCR approach) was cloned using a TOPO TA cloning kit (Invitrogen Corp.) following the protocol of the manufacturer. Clones were subjected to tooth pick PCR (115 clones), using primers T3–T7 provided with the cloning kit. The amplified products were digested with MspI–RsaI and grouped into 19 operational taxonomic units (OTUs) based on their restriction patterns. At least two clones representative from each OTU group were sequenced with vector-specific primers T3 and T7. All sequencing reactions were carried out at Macrogen Sequencing Service, Korea, using an ABI PRISM 3730XL capillary sequencer (Applied Biosystems, Foster City, CA). About 60% of retrieved clone sequences corresponded to bacterial gene, and the remaining sequences corresponded to mitochondria and chloroplast inserts.

Rarefaction analysis of the library was performed taking into account only bacterial sequences using the free software Analytic Rarefaction 1.3 (http://www.uga.edu/~strata/software/), developed by Steven Holland.

The 16S rRNA gene was partially sequenced for isolates obtained using the primer 8F described previously. Nucleotide sequence data reported in this paper have been deposited in the EMBL GenBank, and accession numbers corresponding to clones and isolates are shown in Fig. 2.

At least one representative of each clone and isolate per OTU was used to construct the phylogeny. For 16S rRNA gene phylogenetic analysis, alignments and phylogenetic distance dendrograms were constructed using the neighbor-joining algorithm (Saitou & Nei, 1987) as implemented in MEGA4 (Tamura *et al.*, 2007). Evolutionary distances were calculated using Jukes–Cantor method (Jukes & Cantor, 1969).

Results

Abundance and distribution of culturable endophytic bacteria in the three rice varieties

Heterotrophic counts, corresponding to the 2006 rice crop, were performed in triplicates taken from each replicated plot. The abundance of heterotrophic bacteria was not significantly different between the three rice varieties (Table 1). Similar results were obtained on the pooled leaves from each replicated plot of the 2005 harvest, being

Table 1. Enumeration of heterotrophic endophytic bacteria in leaves of three rice varieties (2006 crop season)

	Log_{10} N of CFU* (g fresh weight)$^{-1}$		
Plot	EP	IT	IO
A†	3.53 ± 0.31	nd	4.24 ± 0.40
B†	3.95 ± 0.21	3.21 ± 0.17	3.62 ± 0.65
C†	4.10 ± 0.44	3.60 ± 0.17	4.60 ± 0.97
Mean	3.86 ± 0.30	3.40 ± 0.28	4.15 ± 0.50

nd, not determined.

*CFU: colony forming units. *Oryza sativa* variety: EP, El Paso 144; IT, INIA Tacuarí; IO, INIA Olimar.

†The values represented the mean of triplicate samples taken from each of three replicated plots (A, B and C, except for IT for which plants from only two replicated plots were analyzed).

the heterotrophic numbers in the same range for the two media utilized (data not shown). Heterotrophic siderophore-producing bacteria (HSPB) counts (expressed as Log N bacteria (g fresh weight)$^{-1}$) were 2.42 ± 0.31, 3.49 ± 0.67 and 3.72 ± 0.16 for IT, IO, and EP, respectively. HSPB densities did not show significant differences between the rice varieties. Although diazotrophic bacterial numbers were low, < 3.33 expressed as Log N bacteria (g fresh weight)$^{-1}$, a few isolates were obtained and identified (isolates D1, D34 and D43; Fig. 2a).

The culturable heterotrophic endophytes (79 isolates) detected in each rice variety in two successive crops is shown in Table 2. Representative isolates of each OTU were successfully identified because partial sequence of the 16S rRNA gene showed 97% or higher similarity with NCBI database sequences. Sequences corresponding to the same OTU were grouped, and the lowest similarity percentage is shown in Table 2.

Leaves of the three rice varieties had in common a few genera of bacteria. Predominant isolates recovered from the highest dilution counts were affiliated mainly to the *Gammaproteobacteria* (*Pantoea* spp. and *Pseudomonas* spp.) and to the *Actinobacteria* (*Curtobacterium* spp. and *Microbacterium* spp.). The third common phylogenetic group was the *Alphaproteobacteria*, with *Sphingomonas* as the main genus retrieved.

Pantoea ananatis and *Pseudomonas syringae* pv. *actinidiae* were always present, accounting for 51% of the heterotrophic bacteria isolated (Table 2). The less frequent OTUs (Pg1, Mb1 y Cb1), associated with the species *Pantoea agglomerans*, *Microbacterium testaceum*, and *Curtobacterium flaccumfaciens* pv. *beticola*, respectively, represented 25% of

Table 2. Dominant heterotrophic endophytic bacteria isolated from leaves of three rice varieties in two successive crop seasons

					Isolates from each sample‡					
					EP		IT		IO	
OTU*	Representative isolates	Closest relative (accession number)	% Identity†	Total number of isolates	2005	2006	2005	2006	2005	2006
Pa1	Pt17, pt31, pt29, pt2	*Pantoea ananatis 3Pe76* (EF178449)	≥ 98	27	4	8	3	2	1	9
Pg1	Ps24, pt6	*Pantoea agglomerans HK 14-1* (AY335552)	≥ 99	8		4		1	1	2
P1	Pt59, Mb16	*Pseudomonas syringae* pv. *actinidiae* (EU906856)	≥ 99	13	3	3	1	2	2	2
P2	Pa1, Pt15	*Pseudomonas* sp. *AHL 2* (AY379974)	≥ 99	4		4				
P3	Ps19	*Pseudomonas graminis KF701* (AB109886)	99	1					1	
P4	Ps22	*Pseudomonas syringae* pv. *phaseolicola* 1448A (CP000058)	99	1			1			
P5	Ps21	*Xanthomonas axonopodis* pv. *dieffenbachiae* 05-220 (EU203153)	100	1			1			
P6	S6	*Xanthomonas* sp. *Aed03* (EU740995)	100	1			1			
Sp1	S4	*Sphingomonas yabuuchiae* (AB071955)	100	1		1				
Sp2	S11	*Sphingomonas azotifigens* (AB217473)	98	1						1
Sp3	S2	*Sphingomonas yabuuchiae* (AB071955)	99	1				1		
Sp4	J52	*Sphingomonas yunnanensis* (EU730917)	99	1	1					
Mb1	Mb2, Mb3, Mb21	*Microbacterium testaceum SE017* (AF474325)	≥ 98	6	1		2	2		1
Cb1	S7, JT, Mb23	*Curtobacterium flaccumfaciens* pv. *beticola* (AY273208)	≥ 98	6	1	1	3			1
Cb2	JS14, Mb18, IS14	*Curtobacterium citreum Z10zhy* (AM411064)	≥ 98	3	1	1				1
St	J210	Staphylococcus cohnii JL812 (EF512729)	99	1						1
Sz	J237	Aurantimonas ureilytica 5715S-12 (DQ883810)	97	1						1
Mt	JB	*Methylobacterium* sp. *Pd-S-(s)-l-D-4(3)* (AB242971)	97	1				1		
B1	152	*Bacillus megaterium HDYM-24* (EF428248)	99	1					1	
		Total isolates		**79**	**11**	**22**	**12**	**9**	**6**	**19**

*Operational Taxonomic Units defined by ARDRA profiles.

†Several isolates were sequenced for each ARDRA profile. The lowest values of identity among the several isolates sequenced are shown.

‡*Oryza sativa* variety: EP, El Paso 144; IT, INIA Tacuarí; IO, INIA Olimar.

the isolates and were detected at least once in each rice variety. Similarly, the genus *Sphingomonas*, distributed in four OTUs, was recovered from the three varieties. The ten remaining profiles that grouped 19% of the isolates were randomly distributed in the rice varieties or crop seasons except for OTU P2 (*Pseudomonas* sp. AHL2), which was found exclusively in the variety El Paso in 2006.

These results indicate that the culturable endophytic communities were similar in abundance and were dominated by two species (*P. ananatis* and *P. syringae*) persistently associated with the three rice varieties.

Molecular fingerprinting of the endophytic bacterial community in the three rice varieties

T-RFLP profiles of endophytic communities from replicated plots of the EP, IO, and IT rice varieties were compared. The primer 799f allowed the amplification of the nine samples but it did not discriminate among them because all the samples showed 92% or higher similarity (data not shown). The nested amplification allowed a better discrimination, although the Plot B of the variety IT did not show good restriction profiles and it was excluded from this analysis. Thus, four data sets (two PCR strategies and two restriction enzymes) were combined, and each plot (except by plot B of the variety IT) was represented by between 27 and 49 T-RFs.

The clustering analysis showed that endophytic communities from different plots of the same variety were highly similar (more than 85%) (Fig. 1). The two varieties of Indica origin (EP and IO) sustained more similar (80%) bacterial communities.

On the other hand, the lowest similarity (74%) was observed when varieties EP and IO were compared with IT. These differences were not explained by any dominant T-RF in the four data sets (data not shown).

Phylogenetic analysis of the endophytic bacterial community

Although DNA from only one rice variety was cloned, this clone library was useful to compare with the isolated bacteria and to infer the identity of the main T-RFs because no significant differences were found between varieties according to the T-RFLP clustering analysis.

Although 30% and 10% of the analyzed clones corresponded to rice mitochondria and chloroplast, respectively, the analysis of endophytic community composition was performed quite successfully. Confirmation of endophytic diversity coverage was conducted by constructing rarefaction curves for the 16S rRNA gene sequences cloned.

The 16S rRNA gene library showed a community composed of six main phylogenetic groups. Most of the

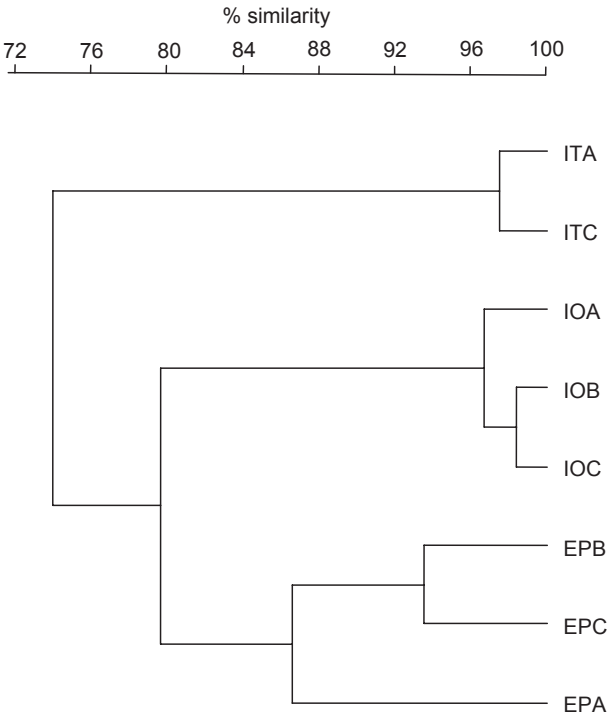

Fig. 1. UPGMA dendrogram showing clustering analysis and similarity percentage among T-RFLP profiles corresponding to the three treatments studied. The four data sets obtained were included (amplicons retrieved from primers 8f or 799f restricted with HhaI or AluI). Rice varieties are indicated as IT (INIA Tacuarí), IO (INIA Olimar), and EP (El Paso 144). Replicates of each treatment are indicated as A, B, and C.

sequences clustered within the *Alphaproteobacteria* (46%) including *Brevundimonas* (18%), *Erythrobacter–Porphyribacter* (13%), and *Sphingomonas* (11%) as the dominant genera. *Actinomycetales* was the second numerically important phylogroup (16%) with *Patulibacter* as the main genus (5%). *Firmicutes* were represented by the genus *Bacillus* (5%) and by the species *Aerococcus viridans* (7%). *CFB* phylogroup (*Cytophaga–Flexibacter–Bacteroides*) was represented only by the genus *Chryseobacterium* (11%) and the *Gammaproteobacteria* (11%) mostly by the genus *Pantoea* (9%). The sixth group belonged to the order *Rubrobacterales* (5%) with *Brachybacterium* as the main genus.

Phylogenetic relationships between 16S rRNA gene sequences of the clones and isolates were compared with sequences retrieved from the NCBI database (Fig. 2). Most of the clone sequences were closely related (more than 98% sequence identity) to isolated bacteria previously described. Four of the six phylogenetic groups observed in this work were represented by isolates and clones, and only two phylogroups (*CFB* and *Rubrobacterales*) were detected exclusively by molecular analysis.

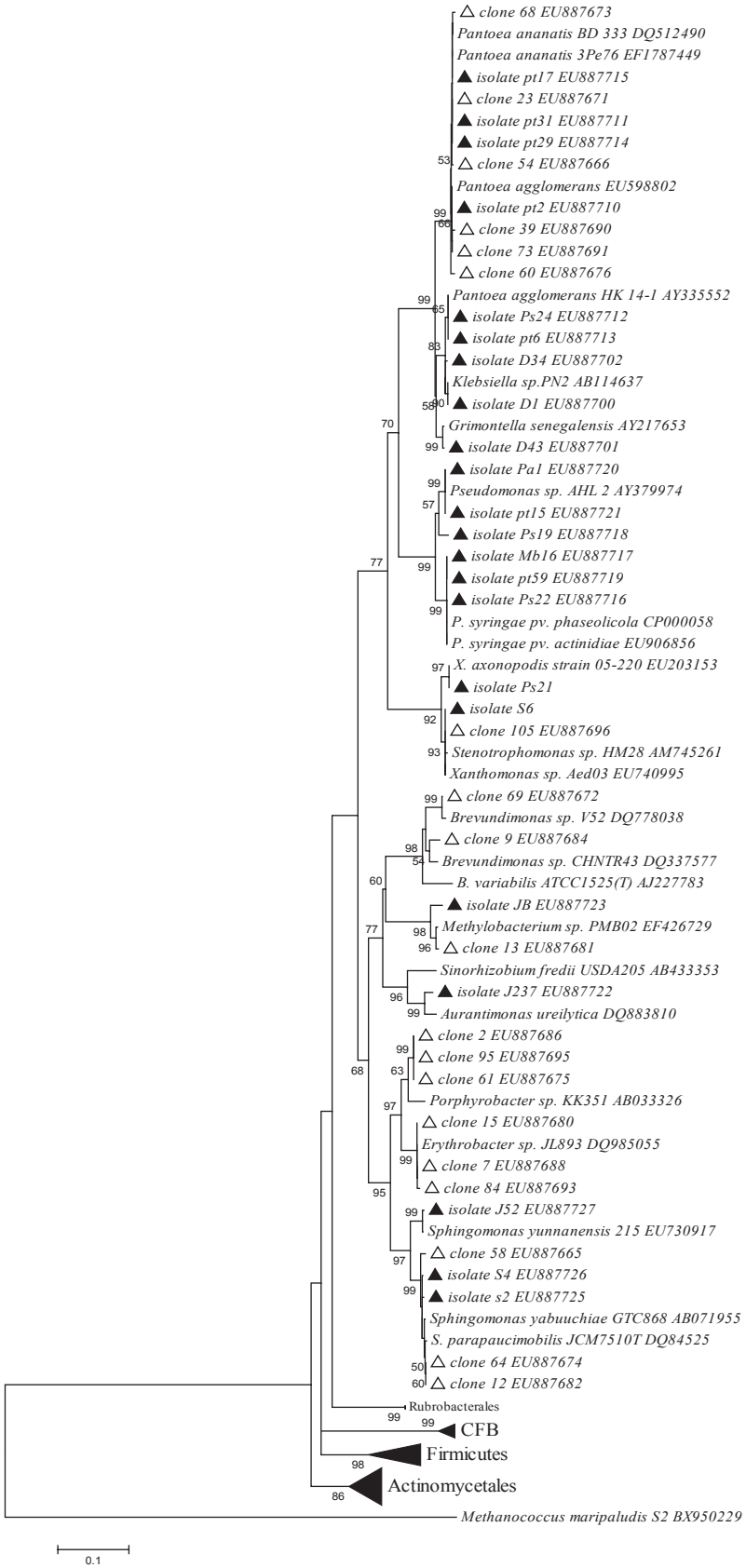

Fig. 2. Continued.

These results suggest that culture and molecular approaches allowed to reach a wide range of the bacterial diversity at this level of taxonomic resolution.

However, certain genera were detected only by one approach. Thus, 47% of the sequences in the clone library had no close relatives among the isolates within the same

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences corresponding to clones (empty triangles) and isolates (filled triangles) retrieved from endophytic communities inhabiting rice leaves. (a) *Gammaproteobacteria*, *Alphaproteobacteria*. (b) *CFB*, *Firmicutes*, *Rubrobacterales* and *Actinomycetales*. The reference sequences are shown by species or clone name followed by the GenBank accession number. Bootstrap values > 50% (100 data re-samplings) are given. The bar represents 10% divergence.

genus. The two main genera (*Brevundimonas* and *Porphyrobacter*/*Erythrobacter*) of the *Alphaproteobacteria* were only retrieved by culture-independent analysis despite that *Brevundimonas* was the dominant genus in the clone library. On the other hand, some genera within the *Gammaproteobacteria*, like *Pseudomonas* (filled triangles in Fig. 2), were represented exclusively by isolates. This class also included diazotrophic endophytes related to the genera *Klebsiella* (isolates D1 and D34) and *Enterobacter* (isolate D43) that were unlikely to be detected in the clone library because they were recovered in low dilutions of RMR medium.

Phylogenetic assignment of the T-RFs

To elucidate the community structure in the EP variety, the sequences retrieved from the 16S rRNA gene clone library and from the isolates were used to predict *in silico* the identity of the peaks in the T-RFLP fingerprints (Table 3). The phylogenetic affiliation was performed using the data set comprised of the 8F primer and both restriction enzymes, but only HhaI results are shown because these profiles were the most diverse and no additional information was provided with the AluI data set. Some of the T-RF sizes were expressed as a base pair range to account for the different possible assignments. Table 3 summarizes the T-RFs present in the profiles of plots B and C of the EP variety. The different approaches used, i.e. isolation, cloning, and T-RFLP, were correlated in this table.

Some isolates belonging to the genus *Curtobacterium*, which was also detected by cloning, were not listed in Table 3 because their T-RF sizes were larger than standard marker coverage. For the same reason, the T-RFs representing clones of *Aerococcus viridians* are not in this Table.

Consistent results were obtained by both molecular methods, except for some clones of *Actinobacteria* (i.e. *Brachybacterium* sp., clones 29 and 123, Fig. 2) which accounted for about 10% of the clone library, and were not found in the T-RFLP profiles.

Most of the T-RFs could be explained by the *in silico* fragments inferred from clones or isolates obtained (Table 3). Only three T-RFs (58–63, 136–138, and 357–361 bp), present in both samples (EPB and EPC), could not be assigned.

Table 3. Phylogenetic assignment of T-RF retrieved from endophytic communities of the EP rice variety. Predicted T-RFs correspond to *in silico* HhaI restriction analysis of clones and isolates sequences obtained from plots B and C

Community T-RF size (bp)	Isolates	Clones	Closest relative (accession number)	16S rRNA % identity	Affiliation
81–84	J52		*Sphingomonas yunnanensis strain 215* (EU730917)	99	
		C2	Uncultured alpha proteobacterium (AJ318120)	97	
		C58	*Sphingomonas yabuuchiae* (AB071955)	98	*Alphaproteobacteria*
		C95	*Porphyrobacter* sp. *KK351* (AB033326)	97	
		C84	*Erythrobacter* sp. *JL893* (DQ985055)	99	
		C12, C64	*Sphingomonas yabuuchiae* (AB071955)	> 98	
		C61	*Patulibacter minatonensis* (AB193261)	99	*Actinobacteria*
94		C11	*Chryseobacterium* sp. *TM3_8* (DQ279360)	> 99	*Bacteroidetes*
141–145*	Mb5		*Microbacterium testaceum* strain *SE017* (AF474325)	99	*Actinobacteria*
213–215	Pt59		*Pseudomonas syringae* pv. *coryli*, strain *NCPPB 4273* (AJ889841)	99	*Gammaproteobacteria*
		C105	*Xanthomonas albilineans GPEPC73* (FP565176)	98	
226		C8	*Patulibacter minatomensis* isolate *CP177-2* (AB193261)	99	*Actinobacteria*
		C3, C17, C47, C94	*Chryseobacterium* sp. *BBTR48* (DQ337589)	> 97	*Bacteroidetes*
239	J210		*Staphylococcus cohnii* strain *JL812* (EF512729)	100	*Firmicutes*
		C127	*Bacillus pumilus* strain *BSH-4* (EF488975)	99	
333		C69	*Brevundimonas* sp. Tibet-IBa1 (DQ108394)	99	*Alphaproteobacteria*
		C9†	*Brevundimonas* sp. *CHNTR43* (DQ337577)	98	
373–377		C37	*Salinibacterium* sp. *7320* (AM111058)	97	*Actinobacteria*
	Ps24		*Pantoea agglomerans* strain *WAB1913* (AM184254)	100	
		C60	*Pantoea ananatis* strain *BD 333* (DQ512490)	99	*Gammaproteobacteria*
	Pt2		*Pantoea ananatis* strain *JA04* (DQ365569)	99	
	Pt31		*Pantoea ananatis* strain *3Pe76* (EF178449)	> 99	
		C54†	*Brevundimonas* sp. *CHNTR43* (DQ337577)	98	*Alphaproteobacteria*

*Rice Mitochondria presents a restriction fragment of 140–142 bp. Rice Chloroplast does not present restriction site.
†Clones closely related to the same *Brevundimonas* sp. but presenting different T-RFs.

This analysis would indicate that the T-RF of 333 bp, present in all samples with fluorescence intensities ranged between 2% and 18%, could be assigned to the genus *Brevundimonas*, dominant in the clone library analyzed from the EP variety. Similarly, the second most widespread T-RF (226 bp), detected in two plots of IO and one of EP, which was not represented by isolated bacteria, could be assigned to the *Actinobacteria* or to *CFB* (Table 3).

Discussion

Mature leaf tissues of rice are a selective environment for the establishment of a bacterial endophytic community. These bacteria should be able to resist and adapt to physiological changes experienced by the rice plant as the crop cycle evolves. During the mature plant stage, the accumulated carbohydrates in leaf and culm translocate into the grains (Yoshida, 1981). Thus, the nutrient concentration decreases in mature leaves being less available for bacteria. These conditions may select for bacteria that might have a strong interaction with the rice plant and become adapted to this particular niche. Furthermore, this interaction may be influenced by the particular physiology of the rice variety.

The present work describes and compares the endophytic bacterial community structure from leaves of three cultivated rice varieties by molecular and cultivation approaches. Information from three replicated plots per variety and from two successive crop seasons was taken into account for the comparison.

Densities of the viable endophytes determined in this work were in the same range as those reported by other authors (Mano *et al.*, 2007; Prakamhang *et al.*, 2009). In a previous work, it has been shown that the density of heterotrophic endophytes in rice leaves did not increase significantly along the crop cycle (Loaces *et al.*, 2011). The results obtained in the present work suggest that endophytic heterotrophic bacteria present at the mature stage in rice leaves have similar densities regardless of the rice variety or the crop season.

We also found that there was no apparent preferential association of species among the different varieties. A reduced group of species was strongly and persistently associated with rice leaf in any variety. *Pantoea* was the predominant genus accounting for 44% of the isolates, and *P. ananatis* represented more than 15% of the isolates recovered in any of the samples. This species has been detected in all tissues of the variety INIA Olimar throughout the whole cycle of the rice crop (Loaces *et al.*, 2011). The same species was also retrieved from rice seeds in Japan (Mano *et al.*, 2006), and *P. agglomerans* has been isolated from rice roots as a nitrogen-fixing bacterium in Thailand (Prakamhang *et al.*, 2009). Verma *et al.* (2004) observed that *Pantoea* sp. was a more aggressive colonizer than *Ochrobactrum* sp. when both strains were co-inoculated in rice seeds. This suggests that bacteria of the genus *Pantoea* may have the ability to colonize and grow inside the rice tissues in different agroecosystems.

The genera *Curtobacterium* and *Microbacterium*, which summarized 19% of the isolates, were alternatively present in the three varieties. Only 12% of the isolates, which belonged to the genera *Sphingomonas*, *Xanthomonas*, *Methylobacterium*, *Staphylococcus*, *Bacillus*, and *Aerobacter*, were randomly distributed among the samples. Thus, most of the isolates were found in two successive rice crop seasons, suggesting that their presence is not fortuitous and that a strong association between these bacteria and the plant may be involved in such selection. Several authors reported that at least a portion of the endophytes found in leaves are seed-borne (McInroy & Kloepper, 1995; Kaga *et al.*, 2009; Loaces *et al.*, 2011). Our results suggest that the bacteria isolated remained associated with the plant irrespective of the variety and may be transferred through the seeds.

T-RFLP and cloning were a suitable methodological combination to compare the endophytic communities from the three different rice varieties and inferring the identity of their members. The nested PCR strategy, used to avoid the chloroplast and mitochondrial interference, was partially successful and enough amounts of data were obtained to perform the community analysis.

Endophytic bacterial communities corresponding to the three rice varieties presented highly similar T-RFLP profiles (Fig. 1). In addition, the culture-dependent analysis showed that the main heterotrophic endophytic bacteria were common to the three varieties.

Most of the phylotypes observed, detected by cloning and isolation, were distributed in five main phylogroups: *Alphaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, *Firmicutes*, and *CFB* (Fig. 2). In contrast to the dominance of *Betaproteobacteria* observed in rice roots by 16S rRNA gene cloning with primers 799f-1492r (Sun *et al.*, 2008), we found that *Alphaproteobacteria* predominated in the clone library constructed from rice leaves of the EP variety using the nested PCR strategy.

Many phylotypes of the clone library were represented by the isolates, but some exceptions should be pointed out. *Brevundimonas*, affiliated to the *Alphaproteobacteria*, represented the main genus in the clone library and was not recovered by isolation in spite of the use of two different media. Bacteria from this genus have been isolated from rice as endophytic nitrogen-fixing bacteria (Prakamhang *et al.*, 2009) and detected in low proportion in a 16S rRNA gene clone library from rice roots (Sun *et al.*,

2008). Besides diazotrophy, *Brevundimonas* can be beneficial to the plant because of its suppressive effect against pathogenic fungi (De Boer *et al.*, 2007) or its nematicidal ability (Zheng *et al.*, 2008). *Brevundimonas* has also been retrieved as endophyte from diverse plant tissues like cotton roots (Hallmann *et al.*, 1999), banana shoots (Thomas *et al.*, 2008), carrots (Surette *et al.*, 2003), and tomato leaves (Enya *et al.*, 2007). In addition, the T-RF of 333 bp, which might be assigned to the genus *Brevundimonas*, was present in all the samples. However, the failure to recover it in any of the samples may be attributed to the limitations of the cultivation method. Lower incubation temperatures (20–25 °C) than that employed in this work could be more appropriate to recover bacteria of this genus.

Similarly, the genus *Chryseobacterium* (11% of the clone library) was only detected by molecular analysis in this work. However, species of *Chryseobacterium* have been isolated from rice roots (Mano *et al.*, 2007) and leaves of sugar beet (Shi *et al.*, 2009). Also, the genera *Porphyrobacter*/*Erythrobacter* (13% of the clone library) had no close relatives among the isolates. These genera were detected in aquatic ecosystems (Hiraishi & Imhoff, 2005; Shiba & Imhoff, 2005), although not reported as endophytes. Because the respective clones of these genera were not represented by an exclusive T-RF (Table 3), the importance and distribution of these bacteria in the three varieties could not be established.

In contrast to the above described genera, *Pseudomonas* was widely detected by isolation but not recovered by cloning. This genus was found as a dominant group of siderophore-producing bacteria that can be isolated from all tissues throughout the rice crop cycle (Loaces *et al.*, 2011). The T-RF 213–215 bp, which could correspond to the isolates of *P. syringae* recovered in this work (Table 3), was only detected in one or two of three replicated plots of each rice variety representing less than 7% of the total intensity of fluorescence in any sample. Interestingly, many *Pseudomonas* spp. have been considered as potential biological inoculants because of their properties as pathogen antagonists or plant growth–promoting bacteria. However, if *Pseudomonas* depends on their capacity to colonize plant leaves, our results suggest that their true ability to be effectively promoting plant growth should be further studied.

In conclusion, the structure of endophytic bacterial communities in mature leaves of the rice varieties INIA Tacuarí, INIA Olimar and El Paso 144, was highly similar. Although molecular and culture-dependent analyses revealed differences in the dominant species, similarities in community composition of the three varieties were maintained. While *Pantoea* and *Pseudomonas* predominated among the isolated bacteria, *Brevundimonas* seems to be the most abundant and widespread genus detected by molecular analysis.

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