

# Endophytic Bacterial Diversity in Rice (*Oryza sativa* L.) Roots Estimated by 16S rDNA Sequence Analysis

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Received: 5 January 2007 / Accepted: 9 June 2007 / Published online: 10 August 2007  
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**Abstract** The endophytic bacterial diversity in the roots of rice (*Oryza sativa* L.) growing in the agricultural experimental station in Hebei Province, China was analyzed by 16S rDNA cloning, amplified ribosomal DNA restriction analysis (ARDRA), and sequence homology comparison. To effectively exclude the interference of chloroplast DNA and mitochondrial DNA of rice, a pair of bacterial PCR primers (799f–1492r) was selected to specifically amplify bacterial 16S rDNA sequences directly from rice root tissues. Among 192 positive clones in the 16S rDNA library of endophytes, 52 OTUs (Operational Taxonomic Units) were identified based on the similarity of the ARDRA banding profiles. Sequence analysis revealed diverse phyla of bacteria in the 16S rDNA library, which consisted of alpha, beta, gamma, delta, and epsilon subclasses of the Proteobacteria, *Cytophaga/Flexibacter/Bacteroides* (CFB) phylum, low G+C gram-positive bacteria, *Deinococcus-Thermus*, Acidobacteria, and archaea. The dominant group was Betaproteobacteria (27.08% of the total clones), and the most dominant genus was *Stenotrophomonas*. More than 14.58% of the total

clones showed high similarity to uncultured bacteria, suggesting that nonculturable bacteria were detected in rice endophytic bacterial community. To our knowledge, this is the first report that archaea has been identified as endophytes associated with rice by the culture-independent approach. The results suggest that the diversity of endophytic bacteria is abundant in rice roots.

## Introduction

Endophytic bacteria are defined as those bacteria that can be isolated from surface-disinfected plant tissues or extracted from within the plants and that are not observed to harm the host plants [18]. Endophytic bacteria ubiquitously inhabit most plant species, and have been isolated from a variety of plants [31]. Recently, it has been reported that endophytic bacteria may promote plant growth and suppress plant diseases probably by means similar to plant growth-promoting rhizobacteria (PGPR) [14]. Therefore, a better understanding of endophytic bacteria may help to elucidate their function and potential role more effectively in developing sustainable systems of crop production.

So far, most information on endophytic bacterial diversity has been obtained by using culture-dependent approaches. However, due to the unknown conditions for growth requirements of many bacteria and the presence of cells which are in a viable but noncultivable state [44], the portion of microbial diversity which has been obtained by conventional cultivation techniques is less than 1% of the bacterial species present [1]. Therefore, culture-independent molecular approaches based on 16S rRNA gene analysis such as PCR amplification of 16S rDNAs, amplified ribosomal DNA restriction analysis (ARDRA), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment

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length polymorphism (T-RFLP) have been successfully used for bacterial community analysis in a great variety of environments, including soil ecosystems [8], marine [6], rhizosphere [41], food [5], and human intestine [25] to overcome the limitations of culture-dependent approaches.

However, these culture-independent approaches used on endophytic bacteria have met with limited success due to disturbances from chloroplast 16S rDNA and mitochondrial 18S rDNA. Chelius and Triplett [4] have successfully designed a pair of bacterial primers to amplify bacterial sequences directly from maize roots by PCR and excluded the disturbances of organelle DNA.

Rice is a graminaceous phreatophyte and one of the world's most important grain food crops. The aims of this paper were to explore the feasibility of identifying rice endophytic bacteria using culture-independent methods and especially to obtain a better understanding of bacterial community structure and diversity using the methods of amplified ribosomal DNA restriction analysis (ARDRA) and sequencing of 16S rDNA clones.

## Materials and Methods

### Sampling of Plants and Field Site Description

Plant samples (*Oryza sativa* L., variety 90-3) were obtained from the agricultural experimental base in Luannan County, Hebei province, China. The physico-chemical characteristics of the rice field soil were as follows: pH 7.68, 36% clay, 1.86% organic matter, and 0.144% total nitrogen. Rice had been grown in this field for 3 years. Samples of rice plants at the tillering stage were collected from the wetland rice field in June 2004 and immediately transported to the laboratory.

### Surface Sterilization of Rice Roots

Rice roots were washed with tap water to remove attached clay. Subsequently, the roots were immersed in 70% ethanol for 3 min, washed with fresh sodium hypochlorite solution (2.5% available Cl<sup>-</sup>) for 5 min, rinsed with 70% ethanol for 30 s, and finally washed five times with sterile distilled water. To confirm that the sterilization process was successful, the aliquots of the sterile distilled water used in the final rinse were set on tryptic soy agar (TSA) medium plates. The plates were examined for bacterial growth after incubation at 28 °C for 3 days. Rice root samples that were not contaminated as detected by culture-dependent sterility test were used for further analysis.

### Total DNA Extraction

The hot CTAB procedure [48] was used with some modifications. About 1 g of the surface-sterilized rice roots

was frozen with liquid nitrogen and ground to a fine powder in a sterilized and precooled mortar. The fine powder was suspended in preheated 9 ml CTAB extraction buffer [2% (W/V) cetyltrimethylammonium bromide, CTAB; 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 1.5% polyvinyl-pyrrolidone, PVP; 0.5% 2-mercaptoethanol] and mixed by inverting the tube several times, followed by incubation in a 60 °C water bath for 45–60 min. DNA was then extracted twice with chloroform-isoamylalcohol (24:1 v/v), followed by precipitation with 0.6 volumes of isopropanol for 2 h at –20 °C. DNA was centrifuged at 12000×g for 10 min at 4 °C, washed with 70% ethanol, and then air-dried. Finally, the DNA was resuspended in 50 µl of sterile Milli-Q water.

### Amplification of the Bacterial 16S rRNA Genes

After comparing the eight prokaryotic universal primers previously published (8f [10], 338f [38], 799f [4], 968f [20], 518r [38], 926r [30], 1378r [20], and 1492r [29]) with rice mitochondrial 18S rDNA (GenBank accession number: AB076666) and chloroplast 16S rDNA (GenBank accession number: X15901) sequences, the pair of primers 799f (position 781 through 799 according to *E. coli* number) and 1492r (position 1492 through 1510 according to *E. coli* number) were selected to amplify the DNA of rice endophytic bacteria. The pair of primers 799f and 1492r would not amplify rice chloroplast DNA. PCR amplification with 799f and 1492r could give a bacterial product approximately as 735 bp and a rice mitochondrial product as 841 bp. The 50 µl PCR reaction mixture contained 100 ng of DNA extract, 1×Taq reaction buffer, 20 pmol of each primer, 200 µM each dNTP, and 1.5 U of Taq DNA polymerase (Promega). After initial denaturation at 94 °C for 5 min, each thermal cycling was as follows: denaturation at 94 °C for 1 min, annealing at 52 °C for 45 s, and elongation at 72 °C for 1 min. At the end of 30 cycles, the final extension step was at 72 °C for 8 min.

Products of six parallel PCRs were combined and electrophoretically separated. The band of size approximately 735 bp in the electrophoresis pattern was excised from an agarose gel and purified by the Wizard SV Gel and PCR Clean-up System (Promega) as described by the manufacturer.

### Construction of 16S rDNA Clone Library

The purified PCR products were ligated into the pGEM-T-easy vector according to the protocol supplied by the manufacturer (Promega). *E. coli* DH5α competent cells (Tiangen Co., China) were transformed with the ligation products and spread onto Luria-Bertani agar plates with ampicillin (100 µg ml<sup>-1</sup>) and X-gal/IPTG on the surface for

standard blue and white screening. Colonies randomly picked were screened directly for inserts by performing colony PCR with primers for the vector (primers T7 and Sp6). All clones containing inserts of the correct size were stored in Luria-Bertani medium containing 20% (v/v) glycerol at  $-70^{\circ}\text{C}$ .

#### ARDRA Procedure

ARDRA was performed to analyze the diversity of positive clones. The PCR reactions (25  $\mu\text{l}$ ) contained  $1\times$  Taq reaction buffer, 200  $\mu\text{M}$  dNTP, 10 pmol each T7 and SP6 PCR primers, 1.5  $\mu\text{l}$  *E. coli* containing cloned insert DNA, and 0.75 U of Taq DNA polymerase (Promega). Thermal cycling conditions were as follows: an initial denaturation at  $94^{\circ}\text{C}$  for 5 min followed by 30 cycles of  $94^{\circ}\text{C}$  for 1 min,  $51^{\circ}\text{C}$  for 1 min,  $72^{\circ}\text{C}$  for 1 min, with a final extension at  $72^{\circ}\text{C}$  for 8 min. Digestion of the PCR product (5  $\mu\text{l}$ ) was done at  $37^{\circ}\text{C}$  for 4 h using the restriction enzymes *Hae*III and *Rsa*I. The restriction fragments were separated on a 2.5% agarose gel running in  $1\times$  TAE buffer at 100 V for approximately 1 h. Fragments shorter than 80 bp were not taken into consideration because they were very close to the detection threshold. According to ARDRA patterns, clones were grouped into OTUs as described by Sessitsch et al. [40].

#### Estimation of the Size of Clone Library

Two approaches were used to estimate extent of the sample size (number of clones screened by ARDRA) and yielded a certain fraction of the species (sequence types) present. To estimate the representation of the phylotypes, the clone coverage was calculated with the following equation:  $C = (1 - n1/N) \times 100\%$ , where  $n1$  is the number of single clones, and  $N$  is the total number of clones in the clone library [15]. Diversity of the clone library was investigated by rarefaction analysis. Rarefaction curves were calculated using the freeware program aRarefactWin [21].

#### Sequencing and Phylogenetic Analysis

Clones representing each distinct ARDRA pattern were chosen for sequencing. Partial sequences of cloned 16S rRNA genes were sequenced by Invitrogen Co. (Shanghai, China) with the ABI 377 automated DNA sequencer. The presence of possible chimeric sequences was investigated by using the CHIMERA\_CHECK program of the Ribosomal Database Project II (RDP II) [34]. All sequences obtained were compared with sequences in the GenBank/EMBL/DDBJ database by using the BLASTN search program. The most similar sequences were further aligned by CLUSTAL X (version 1.81) [45]. Phylogenetic trees

were inferred by the neighbor-joining method [39] with the two-parameter model of Kimura [26] of the Treecon software (version 1.3 b). Statistical significance levels of interior nodes were determined by bootstrap analysis (1,000 data resamplings) [13], and values above 50% are reported.

#### Nucleotide Sequence Accession Numbers

The nucleotide sequence data reported in this paper had been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession no. DQ340871–DQ340923.

## Results

#### DNA Extraction and PCR Amplification

Total DNA was extracted from rice roots using the CTAB approach. Root tissue is rich in phenolic compounds, complex carbohydrates such as lignin, and other plant secondary metabolites, all of which may interfere with DNA amplification by PCR. In order to remove the possible inhibitors of PCR, 1/10 dilution of template DNA was used.

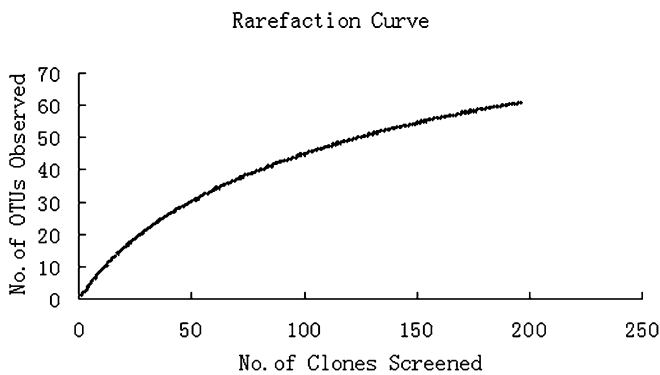
After electrophoresis, two bands of PCR products were displayed on the agarose gel. One band between 700 bp and 800 bp could be the bacterial 16S rDNA fragments, and the other band between 800 bp and 900 bp could be the rice mitochondrial 18S rDNA fragments. The purified PCR products between 700 bp and 800 bp were used for constructing a 16S rDNA clone library of rice endophytic bacteria.

#### ARDRA and Statistical Analysis of the Clone Library

In order to identify the diversity of endophytic bacteria from rice roots, the 16S rDNA clone library was constructed. One hundred and ninety-six clones containing the inserts of correct size were verified by colony PCR. All of the positive clones were screened by ARDRA. The ARDRA patterns obtained with *Hae*III digestion had more bands than that with *Rsa*I digestion. An OTU was defined as a group of clones that had identical banding patterns obtained from the two independent digestions. According to ARDRA patterns, 196 clones were grouped into 54 OTUs. The result of statistical analysis showed that coverage of the clone library was 90.6%, and the calculated rarefaction curve is shown in Fig. 1.

#### Phylogenetic Analysis of 16S rDNA Library Established from the Endophytic Bacterial Community of Rice Roots

According to ARDRA results, representative clones of all OTUs were selected for 16S rDNA sequence analysis. In



**Figure 1** Rarefaction curve of endophytic bacterial 16S rDNA clone library of rice roots

order to confirm the ARDRA validity in this study, 2–3 clones of five randomly selected OTUs were sequenced, which showed that sequences of different clones from the same OTU were uniform. Then one clone selected randomly from each OTU of the rest was sequenced. Using the CHIMERA\_CHECK program, two sequences representing two OTUs were identified as chimeric sequences and discarded, so this clone library included 52 OTUs comprising 192 clones.

The phylogenetic analysis of the 192 clones revealed that the majority of clones were affiliated with Proteobacteria (59.26%). Other clones belonged to low G+C gram-positive bacteria phylum (6.76%), CFB division (4.17%), *Deinococcus-Thermus* (1.56%) and Acidobacteria (0.52%). Furthermore, 3.13% of the clones belonged to archaea. Nearly 15% (14.58%) of the sequences showed high similarity with uncultured bacterial sequences. Details of all OTUs in the clone library are listed in Table 1.

The sequences related to Proteobacteria made up the largest fraction of the clone library, which included alpha, beta, gamma, delta, and epsilon subclasses. The Betaproteobacteria comprising 15 OTUs were the most dominant subclass of Proteobacteria (Fig. 2a). Among them, two predominant groups were related to *Gallionella* sp. and *Burkholderia* spp. The sequences related to Gammaproteobacteria, the second most abundant fraction, comprised 9 OTUs (Fig. 2b). In the Gammaproteobacteria, 2 OTUs including 20 clones were identified as *Stenotrophomonas* spp., which was the most dominant genus in the clone library. Two other dominant OTUs in this subclass were clustered with *Enterobacter* sp. and *Pantoea* sp. The Alphaproteobacteria were the third abundant subclass (Fig. 2c). Most dominant OTU in the subclass had the highest similarity to *Brevundimonas* sp. The phylogenetic diversity of Delta- and Epsilonproteobacteria was much less, only 2 OTUs belonged to Delta-proteobacteria (Fig. 2c), and 1 OTU was affiliated with Epsilonproteobacteria (Fig. 2b).

Except for Proteobacteria, the remaining OTUs belonged to low G+C gram-positive bacteria (Fig. 2d), CFB division

(Fig. 2b), *Deinococcus-Thermus* (Fig. 2c), Acidobacteria (Fig. 2b), uncultured bacteria (Fig. 2d), and archaea. Within these OTUs, there were four predominant OTUs including more than five clones. Two out of the four OTUs were related to uncultured bacteria. The third OTU clustered with *Flavobacterium* sp. of CFB phylum, but identity of 16S rDNA gene partial sequence was only 95%. The fourth OTU had high identity with *Methanospirillum* sp., a member of archaea.

## Discussion

It is essential to design highly specific primers for investigation of endophytic bacterial diversity with culture-independent approaches, since plant chloroplast 16S rDNA and mitochondrial 18S rDNA have high homology to bacterial 16S rDNAs. The results of this study suggested that primers of 799f and 1492r could effectively exclude the interference of rice chloroplast DNA and mitochondrial DNA and could be fit for study on rice endophytic bacteria by culture-independent methods. Chelius and Triplett [4] applied these primers (799f–1492r) to successfully study bacterial diversity in association with the roots of *Zea mays* L. by using culture-independent clonal assessment. They retrieved six bacterial divisions, but no single mitochondrial, chloroplast, or eukaryotic sequences in the bacterial clone library. The 799f primer was highly specific for bacterial 16S rRNA gene sequences except for those of Verrucomicrobia, Cyanobacteria, Spirochetes, and all Obsidian Pool (OP) candidate divisions [4]. On the other hand, some euryarchaeal sequences could also match primer 799f [4]. Upon alignment with the small subunit rRNA genes of 41 Viridiplantae chloroplast and 16 Viridiplantae mitochondria known in the GenBank database, it was found that the pair of primers (799f and 1492r) would not amplify chloroplast 16S rDNA of 41 plants and mitochondrial 18S rDNA of six Chlorophyta plants, and the products of amplifying mitochondrial 18S rDNA of nine Streptophyta except *Chaetosphaeridium globosum* were larger than those of bacteria. Therefore, it is deduced that this pair of primers may be employed as universal primers in studies on endophytic bacteria in the Viridiplantae by culture-independent approaches.

The coverage result of the clone library suggested that the unique sequence types sampled from this library approached the total number of unique sequences within the library. In addition, the rarefaction curve also tended to plateau, indicating that this library was large enough to reflect the endophytic bacterial diversity of rice roots.

The most dominant group in our clone library was related to Proteobacteria, which was consistent with other studies [4, 22, 24]. However, the proportion of subclasses

**Table 1** Distribution of 16S rDNA clones detected from endophytes in rice roots

Group	No. of OTUs	No. of clones	%Total clones	Closest NCBI match	% Identity
Alphaproteobacteria	7	24	12.49		
		11	5.73	<i>Brevundimonas diminuta</i> LMG 2337 (AJ227779)	99
		2	1.04	<i>Methylobacterium</i> sp.P1 (AF148859)	99
		2	1.04	<i>Sinorhizobium teranga</i> LMG 7834 (X68388)	99
		5	2.60	<i>Novosphingobium tardaugens</i> ARI-1 (AB070237)	97
		1	0.52	<i>Caulobacter</i> sp. A1 (AF361188)	97
		2	1.04	<i>Kaistina koreensis</i> (AB127972)	98
		1	0.52	<i>Kaistina koreensis</i> (AB127972)	99
		Betaproteobacteria	15	52	27.08
9	4.69			<i>Achromobacter xylosoxidans</i> (AF531768)	99
6	3.13			<i>Burkholderia</i> sp. 56 (AB114270)	99
6	3.13			<i>Burkholderia fungorum</i> (AF215705)	99
1	0.52			<i>Acidovorax facilis</i> CCUG 2113 (AF078765)	99
2	1.04			<i>Comamonas testosterone</i> SB2 (AY050494)	99
2	1.04			<i>Curvibacter gracilis</i> 7-1(AB109889)	99
1	0.52			<i>Delftia acidovorans</i> WDL34 (AF538930)	98
1	0.52			<i>Delftia tsuruhatensis</i> HR4 (AY302438)	100
3	1.56			<i>Herbaspirillum frisingense</i> Mb11 (AJ238359)	99
1	0.52			<i>Hydrogenophaga taeniospiralis</i> (AF078768)	99
1	0.52			<i>Variovorax</i> sp. WDL1(AF538929)	99
2	1.04			<i>Duganella violaceinigra</i> (AJ871470)	97
2	1.04			<i>Methyloversatilis universalis</i> (DQ923115)	99
13	6.77			<i>Gallionella ferruginea</i> (L07897)	94
2	1.04	<i>Sterolibacterium denitrificans</i> (AJ306683)	94		
Gammaproteobacteria	9	48	25.00		
		16	8.33	<i>Stenotrophomonas maltophilia</i> 15 (AY169434)	99
		12	6.25	<i>Enterobacter</i> sp. B509 (AB049108)	99
		10	5.21	<i>Pantoea</i> sp.(AJ002811)	99
		4	2.08	<i>Stenotrophomonas</i> sp. B50 (AF128871)	99
		1	0.52	<i>Acinetobacter baumannii</i> DSM30008 (X81667)	99
		2	1.04	<i>Alkanindiges illinoisensis</i> (AF513979)	97
		1	0.52	<i>Methylophaga marina</i> ATCC 35842 (X87338)	97
		1	0.52	<i>Plesiomonas shigelloides</i> NCIMB9242 (X60418)	94
		1	0.52	<i>Pseudomonas stutzeri</i> (Y18006)	100
Deltaproteobacteria	2	3	1.56		
		2	1.04	<i>Geobacter</i> sp. CdA-3 (Y19191)	98
		1	0.52	<i>Bdellovibrio bacteriovorus</i> BRP4 (AF148939)	97
Epsilonproteobacteria	1	6	3.13		
		6	3.13	<i>Sulfurospirillum multivorans</i> K (X82931)	98
CFB	3	8	4.17		
		5	2.60	<i>Flavobacterium psychrophilum</i> IFO15942 (AB078060)	95
		2	1.04	<i>Sphingobacterium</i> sp. p11E (AJ496037)	98
Low G+C gram Positive bacteria	5	1	0.52	<i>Flavobacterium frigidis</i> LMG 21922 (AJ557887)	96
		13	6.77		
		2	1.04	<i>Lachnospiraceae bacterium</i> 19gly4 (AF550610)	98
		1	0.52	<i>Clostridium</i> sp XB 90 (AJ229234)	98
		4	2.08	<i>Planomicrobium okeanokoites</i> 120804KB1 (AY730790)	100
Deinococcus-Thermus	1	3	1.56	<i>Planomicrobium mcmeekinii</i> S23F2 (AF041791)	98
		3	1.56	<i>Acidaminobacter hydrogenoformans glu65</i> (AF016691)	94
		3	1.56		
Acidobacteria	1	3	1.56	<i>Deinococcus indicus</i> Wt/1a (AJ549111)	96
		1	0.52		
		1	0.52	<i>Holophaga foetida</i> DSM6591T (X77215)	95

**Table 1** (continued)

Group	No. of OTUs	No. of clones	%Total clones	Closest NCBI match	% Identity
Uncultured bacterium	6	28	14.58		
		10	5.21	Uncultured bacterium (AM157348)	99
		11	5.73	Uncultured bacterium 734 (AF513103)	96
		1	0.52	Uncultured bacterium (AY945880)	97
		3	1.56	Uncultured Bacteroidetes bacterium (DQ330488)	90
		1	0.52	Uncultured bacterium (AB161300)	98
		2	1.04	Bacterium Ellin514 (AY960777)	95
Archaea	2	6	3.13		
		5	2.60	<i>Methanospirillum</i> sp. A2 (AJ133792)	97
		1	0.52	<i>Candidatus Methanoregula boonei</i> (DQ282124)	96

of Proteobacteria was different in endophyte clone libraries of various plants. In some studies on the endophytes by culture-independent approaches, *Delta*- and *Epsilonproteobacteria* were rarely reported, but they were found in our clone library. It is indicated that the diversity of endophytic bacteria was abundant in rice roots, and there might be some extent of specificity between the endophytes and their host plants.

Three predominant OTUs comprising more than 12 clones were found in the clone library, of which two OTUs belonged to the Gammaproteobacteria. The first OTU showed the highest similarity to *Stenotrophomonas maltophilia* including 16 clones. By the culture-dependent method, *S. maltophilia* was isolated as endophytes from cotton roots and stems [35]. *S. maltophilia* plays important roles in agricultural production as a plant growth-promoting bacterium, which could suppress disease development by secretion of antibiotics [23], production of extracellular enzymes such as protease and chitinase [9, 28], and potential root colonization [9]. The second OTU included 12 clones which had highest similarity to *Enterobacter* sp. B509, a nitrogen-fixing bacterium isolated from wild rice [11]. As one of the most universal of endophyte genera, some strains of *Enterobacter* had nitrogen-fixing ability or antagonistic activity to phytopathogens [17, 46]. The third OTU was related to *Gallionella ferruginea* with 94% identity of 16S rDNA partial sequence, which was the predominant OTU of Betaproteobacteria. The result indicates that this bacterium is likely to be a potential novel species.

Another two dominant groups were observed in this clone library. One group clustered with *Burkholderia*, a genus widely distributed in natural environments such as plant interior and rhizosphere [2, 43]. In addition, *Burkholderia* sp. has shown a variety of biological functions including crop yield enhancement [43], plant growth promotion [3], plant pathogens suppression [3], as well as diverse pesticides degradation [7]. The other group had a high similarity to *Pantoea* sp. A previous study found that some strains of this

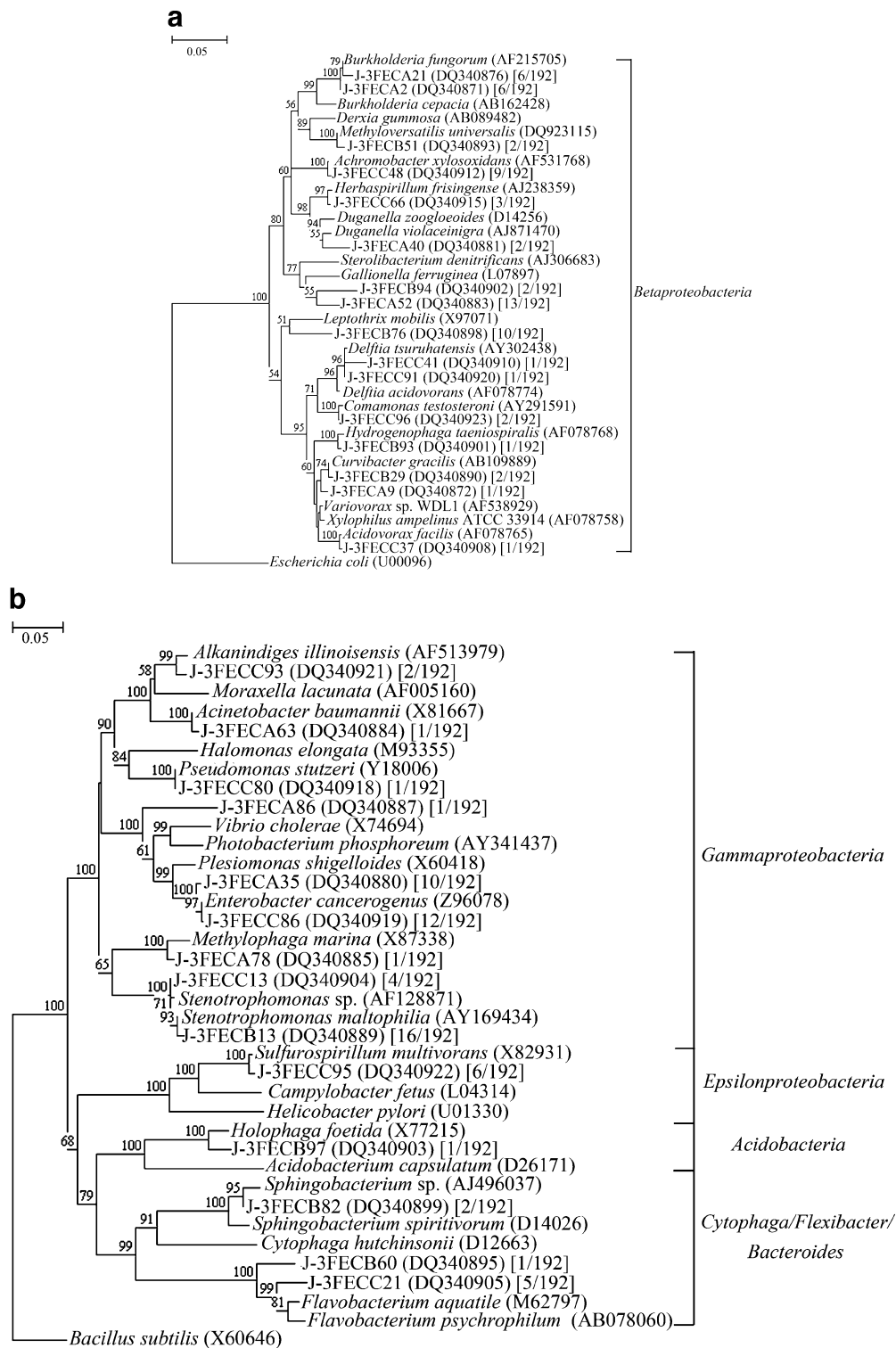
genus isolated as endophyte from rice seeds could promote plant growth [47]. In addition, some sequences of which phylotypes related to nitrogen-fixing *Herbaspirillum frisingense* Mb11 isolated from the C4-fiber plant *Miscanthus sacchariflorus* [27] and *Delftia tsuruhatensis* HR4 isolated from rice rhizoplane [19] were also found in this clone library. *D. tsuruhatensis* HR4 showed strong antagonistic activity in vitro to a variety of fungal plant pathogens and high nitrogen-fixing activity [19].

Anaerobic clostridia have been rarely isolated from plants by culture-dependent approaches, probably due to the oxygen requirement for aerobic culture conditions. However, Minamisawa et al. [36] reported that ubiquitous endophytic clostridia in gramineous plants exhibited nitrogen-fixing ability in association with nondiazotrophic endophytes. Clustering analysis indicated that some sequences in our clone library were related to clostridia.

In this clone library, some sequences had high identity with *Brevundimonas diminuta*, *Kaistina koreensi*, and *Methyloversatilis universalis*. To our knowledge, these strains have not been observed previously as an endophytic bacterium. In addition, sequences of some clones showed low identity with the cultured bacterial genera but high identity with the uncultured bacteria, revealing the presence of some uncultured bacteria in the rice endophytic bacterial community.

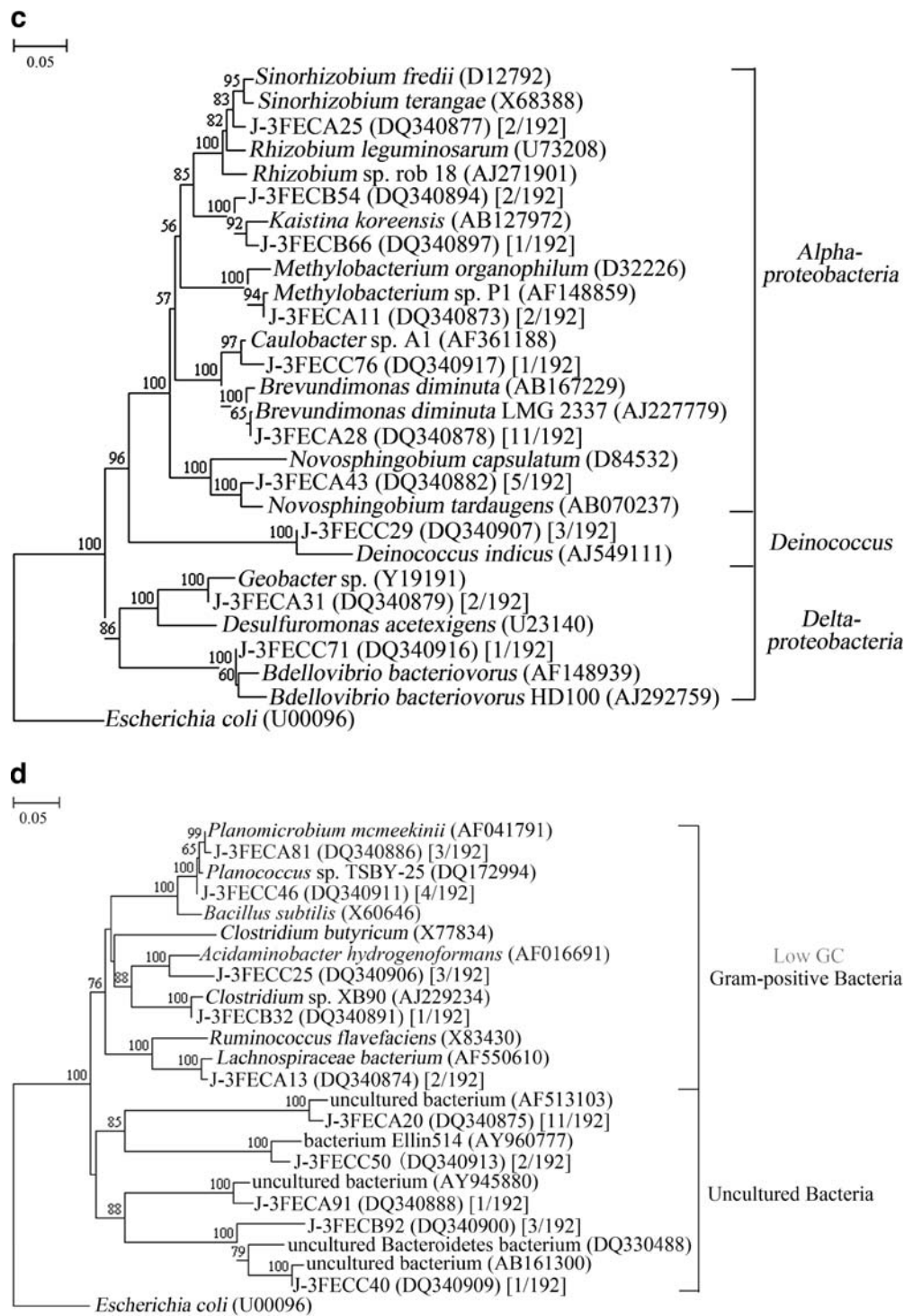
Rice paddy soil is one of the dominant sites for methane release, which suggests there are abundant methanogens in this environment. Methanogens are the largest group of archaea and previous studies have confirmed that an abundant diversity of archaea occurs on rice roots [33], rhizosphere [32], and in the surrounding paddy soil [16]. To our knowledge, this is the first report of endophytic archaea inhabiting rice roots as demonstrated by 16S rDNA cloning and sequencing.

Nevertheless, studies on endophytic bacterial diversity by 16S rDNA cloning and sequencing have some limitations. The biases in genomic DNA extraction, PCR



**Figure 2** 16S rDNA-based dendrogram showing the phylogenetic relationship of clones from rice roots. Phylogenies were inferred using the neighbor-joining analysis and trees were generated using Treecon 1.3 b. As outgroup reference, sequences of *E. coli* are related to Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, *Deinococcus*, gram-positive bacteria, and uncultured bacteria; sequences of *Bacillus subtilis* are related to Gammaproteobacteria, Epsilonproteobacteria, CFB, and Acidobacteria. Numbers in *parentheses* represent the sequence

accession numbers in DDBJ/EMBL/GenBank. Numbers in *square brackets* indicate the clone number out of the total clones. Numbers at *branch points* indicate bootstrap values at or above a 50% threshold. The scale bar represents a 5% estimated difference in nucleotide sequence. **a** Betaproteobacteria. **b** Gammaproteobacteria, Epsilonproteobacteria, CFB, and Acidobacteria. **c** Alphaproteobacteria, Deltaproteobacteria and *Deinococcus*. **d** Gram-positive bacteria and uncultured bacteria



**Figure 2** (continued)

amplification, and cellular rDNA copy number were well known limitations for all molecular approaches relying on PCR amplification of rRNA genes from genomic DNA [12, 37, 42]. In addition, the cloning sequences usually could not provide any information on the function of the individual community members. Also, this method could

only analyze species structure, but not the dominant populations. Therefore, combining the above approach with quantitative techniques, such as FISH or RT-PCR of functional genes, may explore the dominant populations more effectively. Although the approach of library construction may distort the structure of a bacterial community

to some extent, it is a practicable method to provide better knowledge about the endophytic bacterial diversity and a preview for obtaining the endophytic cultures. It was also found that the rice endophytic community was very complex and a lot of uncultured rice endophytes could be interesting subjects for further exploration.

**Acknowledgements** This work was supported by the National Natural Science Foundation of China (Project No. 30370032 and 30170035), the Education Ministry Foundation of China (Project No. 20060028001), and the Natural Science Foundation of Hebei (Project No.2004000106).

## References

- Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 59:143–169
- Araújo WL, Marcon J, Maccheroni W, van Elsas JD Jr, van Vuurde JW, Azevedo JL (2002) Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* citrus plants. *Appl Environ Microbiol* 68:4906–4914
- Bevivino A, Sarrocco S, Dalmastri C, Tabacchioni S, Cantale C, Chiarini L (1998) Characterization of a free-living maize-rhizosphere population of *Burkholderia cepacia*: effect of seed treatment on disease suppression and growth promotion of maize. *FEMS Microbiol Ecol* 27:225–237
- Chelius MK, Triplett EW (2001) The diversity of archaea and bacteria in association with the roots of *Zea mays* L. *Microb Ecol* 41:252–263
- Cocolin L, Rantsiou K, Iacumin L, Cantoni C, Comi G (2002) Direct identification in food samples of *Listeria* spp. and *Listeria monocytogenes* by molecular methods. *Appl Environ Microbiol* 68:6273–6282
- Cottrell MT, Kirchman DL (2000) Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. *Appl Environ Microbiol* 66:5116–5122
- Daubaras DL, Danganan CE, Hübner A, Ye RW, Hendrickson W, Chakrabarty AM (1996) Biodegradation of 2,4,5-trichlorophenoxyacetic acid by *Burkholderia cepacia* strain AC1100: evolutionary insight. *Gene* 179:1–8
- Dunbar J, Takala S, Barns SM, Davis JA, Kuske CR (1999) Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Appl Environ Microbiol* 65:1662–1669
- Dunne C, Crowley JJ, Moenne-Loccoz Y, Dowling DN, de Bruijn FJ, O’Gara F (1997) Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* W81 is mediated by an extracellular proteolytic activity. *Microbiology* 143:3921–3931
- Edwards U, Rogall T, Blacker H, Emde M, Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes: characterization of a gene coding for 16S ribosomal RNA. *Nucl Acids Res* 17:7843–7853
- Elbeltagy A, Nishioka K, Sato T, Suzuki H, Ye B, Hamada T, Isawa T, Mitsui H, Minamisawa K (2001) Endophytic colonization and *in planta* nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. *Appl Environ Microbiol* 67:5285–5293
- Farrelly V, Rainey FA, Stackebrandt E (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl Environ Microbiol* 61:2798–2801
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791
- Feng Y, Shen D, Song W (2006) Rice endophyte *Pantoea agglomerans* YS19 promotes host plant growth and affects allocations of host photosynthates. *J Appl Microbiol* 100:938–945
- Good IL (1953) The population frequencies of species and the estimation of population parameters. *Biometrika* 40:237–264
- Großkopf R, Stubner S, Liesack W (1998) Novel euryarchaeotal lineages detected on rice roots and in the anoxic bulk soil of flooded rice microcosms. *Appl Environ Microbiol* 64:4983–4989
- Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. *Can J Microbiol* 43:895–914
- Hallmann J, Quadt-Hallmann A, Rodriguez-Kabana R, Kloepper JW (1998) Interactions between *Meloidogyne incognita* and endophytic bacteria in cotton and cucumber. *Soil Biol Biochem* 30:925–937
- Han J, Sun L, Dong X, Cai Z, Sun X, Yang H, Wang Y, Song W (2005) Characterization of a novel plant growth-promoting bacteria strain *Delftia tsuruhatensis* HR4 both as a diazotroph and a potential biocontrol agent against various plant pathogens. *Syst Appl Microbiol* 28:66–76
- Heuer H, Smalla K (1997) Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. In: van Elsas JD, Trevors JT, Wellington EMH (eds) *Modern soil microbiology*. Marcel Dekker, New York, pp 353–373
- Holland S (2004) Analytic rarefaction. <http://www.uga.edu/~strata/software/>. Accessed 09 July 2007
- Idris R, Trifonova R, Puschenreiter M, Wenzel WW, Sessitsch A (2004) Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. *Appl Environ Microbiol* 70:2667–2677
- Jakobi M, Winkelmann G, Kaiser D, Kempler C, Jung G, Berg G, Bahl H (1996) Maltophilin: a new antifungal compound produced by *Stenotrophomonas maltophilia* R3089. *J Antibiot* 49:1101–1104
- Kaiser O, Pühler A, Selbitschka W (2001) Phylogenetic analysis of microbial diversity in the rhizosphere of oilseed rape (*Brassica napus* cv. Westar) employing cultivation-dependent and cultivation-independent approaches. *Microb Ecol* 42:136–149
- Kibe R, Sakamoto M, Yokota H, Ishikawa H, Aiba Y, Koga Y, Benno Y (2005) Movement and fixation of intestinal microbiota after administration of human feces to germfree mice. *Appl Environ Microbiol* 71:3171–3178
- Kimura M (1980) A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120
- Kirchhoff G, Eckert B, Stoffels M, Baldani JJ, Reis VM, Hartmann A (2001) *Herbaspirillum frisingense* sp. nov., a new nitrogen-fixing bacterial species that occurs in C4-fibre plants. *Int J Syst Environ Microbiol* 51:157–168
- Kobayashi DY, Reedy RM, Bick JA, Oudemans PV (2002) Characterization of a chitinase gene from *Stenotrophomonas maltophilia* strain 34S1 and its involvement in biological control. *Appl Environ Microbiol* 68:1047–1054
- Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) *Nucleic acid techniques in bacterial systematics*. Wiley, New York, pp 115–175
- Liu WT, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol* 63:4516–4522
- Lodewyck C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgey M, van der Lelie D (2002) Endophytic bacteria and their potential application. *Crit Rev Plant Sci* 86(6):583–606

32. Lu Y, Conrad R (2005) In situ stable isotope probing of methanogenic archaea in the rice rhizosphere. *Science* 309:1088–1090
33. Lu Y, Lueders T, Friedrich MW, Conrad R (2005) Detecting active methanogenic populations on rice roots using stable isotope probing. *Environ Microbiol* 7:326–336
34. Maidak BL, Cole JR, Parker CT, Garrity GM Jr, Larsen N, Li B, Lilburn TG, McCaughey MJ, Olsen GJ, Overbeek R, Pramanik S, Schmidt TM, Tiedje JM, Woese CR (1999) A new version of the RDP (Ribosomal Database Project). *Nucl Acids Res* 27:171–173
35. McInroy JA, Kloepper JW (1995) Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant Soil* 173:337–342
36. Minamisawa K, Nishioka K, Miyaki T, Ye B, Miyamoto T, You M, Saito A, Saito M, Barraquio W, Teaumroong N, Sein T, Tadashi T (2004) Anaerobic nitrogen-fixing consortia consisting of clostridia isolated from gramineous plants. *Appl Environ Microbiol* 70:3096–3102
37. More MI, Herrick JB, Silva MC, Ghiorse WC, Madsen EL (1994) Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. *Appl Environ Microbiol* 60:1572–1580
38. Øverås L, Forney L, Daae FL, Torsvik V (1997) Distribution of bacterioplankton in menomictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl Environ Microbiol* 63:3367–3373
39. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
40. Sessitsch A, Reiter B, Pfeifer U, Wilhelm E (2002) Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and *Actinomycetes*-specific PCR of 16S rDNA genes. *FEMS Microbiol Ecol* 39:23–32
41. Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Appl Environ Microbiol* 67:4742–4751
42. Suzuki MT, Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* 62:625–630
43. Tabacchioni S, Bevivno A, Chiarini L, Visca P, Del Gallo M (1993) Characteristics of two rhizosphere isolates of *Pseudomonas cepacia* and their potential plant-promoting activity. *Microb Rel* 2:161–168
44. Tholozan JL, Cappelier JM, Tissier JP, Delattre G, Federighi M (1999) Physiological characterization of viable-but-nonculturable *Campylobacter jejuni* cell. *Appl Environ Microbiol* 65:1110–1116
45. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl Acids Res* 25:4876–4882
46. Tsuda K, Kosaka Y, Tsuge S, Kubo Y, Horino O (2001) Evaluation of the endophyte *Enterobacter cloacae* SM10 isolated from spinach roots for biological control against Fusarium wilt of spinach. *J Gen Plant Pathol* 67:78–84
47. Verma SC, Ladha JK, Tripathi AK (2001) Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. *J Biotech* 91:127–141
48. Xie Zh-W, Ge S, Hong D-Y (1999) Preparation of DNA from silica gel dried mini-amount of leaves of *Oryza rufipogon* for RAPD study and total DNA bank construction. *Acta Botanica Sinica* 41:802–807