

Note

Diversity of the bacteria in the roots of sugarcane used to produce Wasanbon in Kagawa, Japan

Supriadi, Natsuki Shimoura, Masahiro Miyaji, Shigeyuki Tajima, Mika Nomura*

Faculty of Agriculture, Kagawa University, Miki-cho, Kita-gun, Kagawa 761-0795, Japan
*E-mail: nomura@ag.kagawa-u.ac.jp Tel: +81-87-891-3135 Fax: +81-87-891-3021

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Abstract Soil bacteria are an important factor in the cycle of nutrients in soil, while root bacteria in the internal tissues of plants can promote plant growth. The aim of this research was to study the diversity of root bacteria of sugarcane grown in Kagawa, Japan. The sugarcane, derived from *Saccharum sinense* and grown only in this area, is used as a raw material for Wasanbon (a fine-grained Japanese sugar) and is characterized by thin stalks and a low sugar content. To determine its bacterial diversity, bacterial DNA was extracted from the soil and roots, and 16S rRNA genes were sequenced. A total of 1259 operational taxonomic units (OTUs) were detected in the root bacteria and 3894 OTUs in the soil bacteria. The α -diversity between the soil and root bacteria was significantly different. The most abundant class of root and soil bacteria was proteobacteria at more than 50 and 30%, respectively. The endophyte bacteria of rhizobium were also isolated and the *nifH* gene was detected. The relationship between the application of nitrogen-fixing bacteria and the production of Wasanbon should be studied.

Key words: 16S rRNA, endophyte, sugarcane, Wasanbon.

Sugarcane (*Saccharum* sp.) is the main crop in tropical areas for biofuel production and food, and the cane is characterized by thick stalks and a high sugar content. In Brazil, nitrogen is supplied to sugarcane by endophytic bacteria that can fix nitrogen at more than 40 kg N ha⁻¹ year⁻¹ (Urquiaga et al. 2012). The potential nitrogen-fixing bacteria from sugarcane, especially for endophytic diazotrophs, has been widely studied (Boddey et al. 2003). *Acetobacter diazotrophicus* has been isolated from the roots and stems of Brazilian sugarcane (Cavalcante and Dobereiner 1988). Other nitrogen-fixing bacteria, including *Herbaspirillum* sp. (Olivares et al. 1996), *Enterobacter* sp. (Sajjad Mirza et al. 2001), *Azospirillum* sp. (Tejera et al. 2005), *Klebsiella* sp. (Govindarajan et al. 2007), *Burkholderia* sp. (Paungfoo-Lonhienne et al. 2013), and *Bradyrhizobium* sp. (de Matos et al. 2017), have been reported to be isolated from sugarcane roots, stems, and leaves, and most of the bacteria also promote plant growth.

In Japan, sugarcane is grown in a tropic or subtropic areas such as Okinawa and is characterized by thick stalks and a high sugar content (Matsuoka 2006). Kagawa is not temperate area; however, Wasanbon is produced from sugarcane grown only in this area. The varieties cultivated here, originating from *S. sinense*, are different from those in temperate regions and are characterized

by a low sugar content in thin stems. There has been a report on the diversity of endophytes in the roots of four *Saccharum* species, *S. spontaneum*, *S. robustum*, *S. barberi*, and *S. officinarum* (Dong et al. 2018); however, there have been no reports about *S. sinense*.

The aim of this study was to determine the bacterial diversity of the roots and soil using 16S rRNA gene sequencing. The sugarcane was sampled in two different fields: one field was where wheat was cultivated the previous year and the other field was where sugarcane has been grown continuously for 10 years. Samples of sugarcane roots and soil were collected from three separate areas in each field. The sugarcane soil and roots from the field where wheat was cultivated last year were named soil-1 and root-1, respectively, while the soil and sugarcane roots from the field cultivated continuously with sugarcane for 10 years were named soil-10 and root-10, respectively. The roots and soil were collected in August 2018 when the sugarcane was in the elongation stage. The roots were washed three times with sterilized water, washed again with 70% ethanol, and then with 3% sodium hypochlorite. After sterilization, the roots were washed again three times with sterilized water.

Total bacterial genomic DNA was extracted using a PowerSoil DNA isolation kit (Qiagen, Hilden, Germany). A metagenomic amplicon sequencing library

was prepared by using Illumina 16S metagenomics sequencing library preparation (Preparation 16S Ribosomal RNA Gene Amplicons for the Illumina Miseq System, Illumina). In brief, the gene-specific sequences used in this study targeted the V3 and V4 region (Klindworth et al. 2013). The Illumina adapter overhang nucleotide sequences were then added to the gene-specific sequences. The Illumina overhang adapter sequences added to locus-specific sequences were forward overhang: 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-[i7] and reverse overhang: 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-[i5]. Each i7 and i5 is listed in Supplementary Table S1. PCR was performed using a KAPA HiFi Hotstart PCR kit (Sigma-Aldrich, Boston, USA). The amplicons were removed free primers by AMPure XP beads (Beckman Coulter, USA). The quality and concentration of these amplicons were calculated using an Agilent 2100 Bioanalyzer (Agilent, CA, USA). Sequencing was performed using an Illumina Miseq

platform using paired 300 reads (Illumina, San Diego, USA). All data has been registered and the accession numbers are DRA009113 to DRA009123. The plastid and mitochondria data detected in the roots were removed based on the QIIME program (Caporaso et al. 2010). The average reads obtained for Miseq showed that the identified bacterial OTUs of soil-1 and soil-10 were $3,894 \pm 102$ and $3,064 \pm 278$, respectively, and $1,114 \pm 256$ and $1,259 \pm 581$ for root-1 and root-10, respectively. The coverage of all samples was 92 to 97%. Four indices, Chao1 richness index, ACE index, Shannon index, and Simpson index, were also determined to measure the α -diversity of the microbiome (Table 1). Based on the estimation by the Chao1 index, ACE index and Shannon index, the microbial diversity and microbial richness of both soil-1 and soil-10 were significantly higher than that of root-1 and root-10.

The relative abundance of the microbial communities was analyzed (Figure 1). At the phylum level, eight phyla were identified at a rate of over 3% (Figure 1). The

Table 1. Richness and diversity indices for root and soil bacteria of sugarcane.

Sample name	Cleaned reads	Alpha diversity index						
		OTUs number	Total reads	Coverage (%)	Chao1 index	ACE index	Shannon index	Simpson index
Soil-1	27109 \pm 413 ^a	3894 \pm 102 ^a	25930 \pm 363 ^a	92 \pm 0.2 ^a	6723 \pm 105 ^a	6789 \pm 125 ^a	9.8 \pm 0.2 ^a	0.993 \pm 0.001 ^a
Soil-10	27882 \pm 128 ^a	3064 \pm 278 ^a	27135 \pm 228 ^b	94 \pm 0.5 ^a	5603 \pm 296 ^b	5736 \pm 402 ^b	9.2 \pm 0.4 ^b	0.993 \pm 0.003 ^a
Root-1	27218 \pm 106 ^a	1114 \pm 256 ^b	26875 \pm 1083 ^a	97 \pm 0.4 ^b	2135 \pm 423 ^c	2204 \pm 389 ^c	4.2 \pm 0.7 ^c	0.795 \pm 0.045 ^b
Root-10	26383 \pm 978 ^a	1259 \pm 581 ^b	26188 \pm 876 ^{b,a}	97 \pm 0.8 ^b	2233 \pm 856 ^c	2254 \pm 852 ^c	5.3 \pm 2.8 ^c	0.841 \pm 0.1939 ^a

OTU, operational taxonomical unit; ACE, abundance-based coverage estimator. Values are means \pm standard error ($n=3$). Different letters indicate statistically significant differences at the 0.05 probability level according to Fisher's least significant difference (LSD) test.

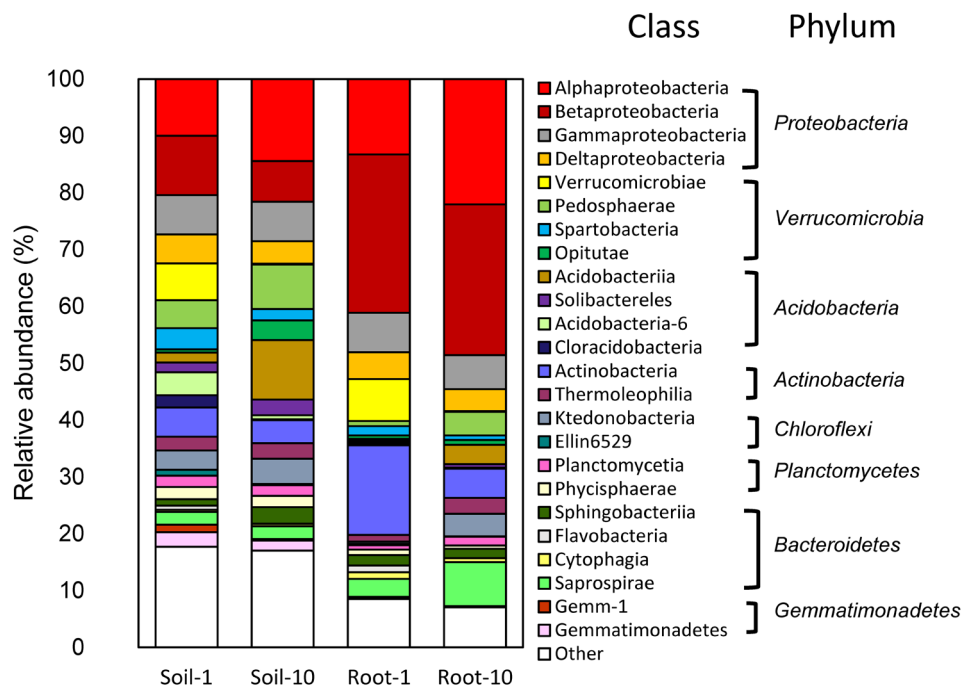


Figure 1. The relative abundance (%) of phylum and class of bacterial community in the soil and roots of sugarcane. The taxa represented accounted for >1% abundance in at least one sample. 'Other' is taxa with a maximum abundance of <1% in each sample.

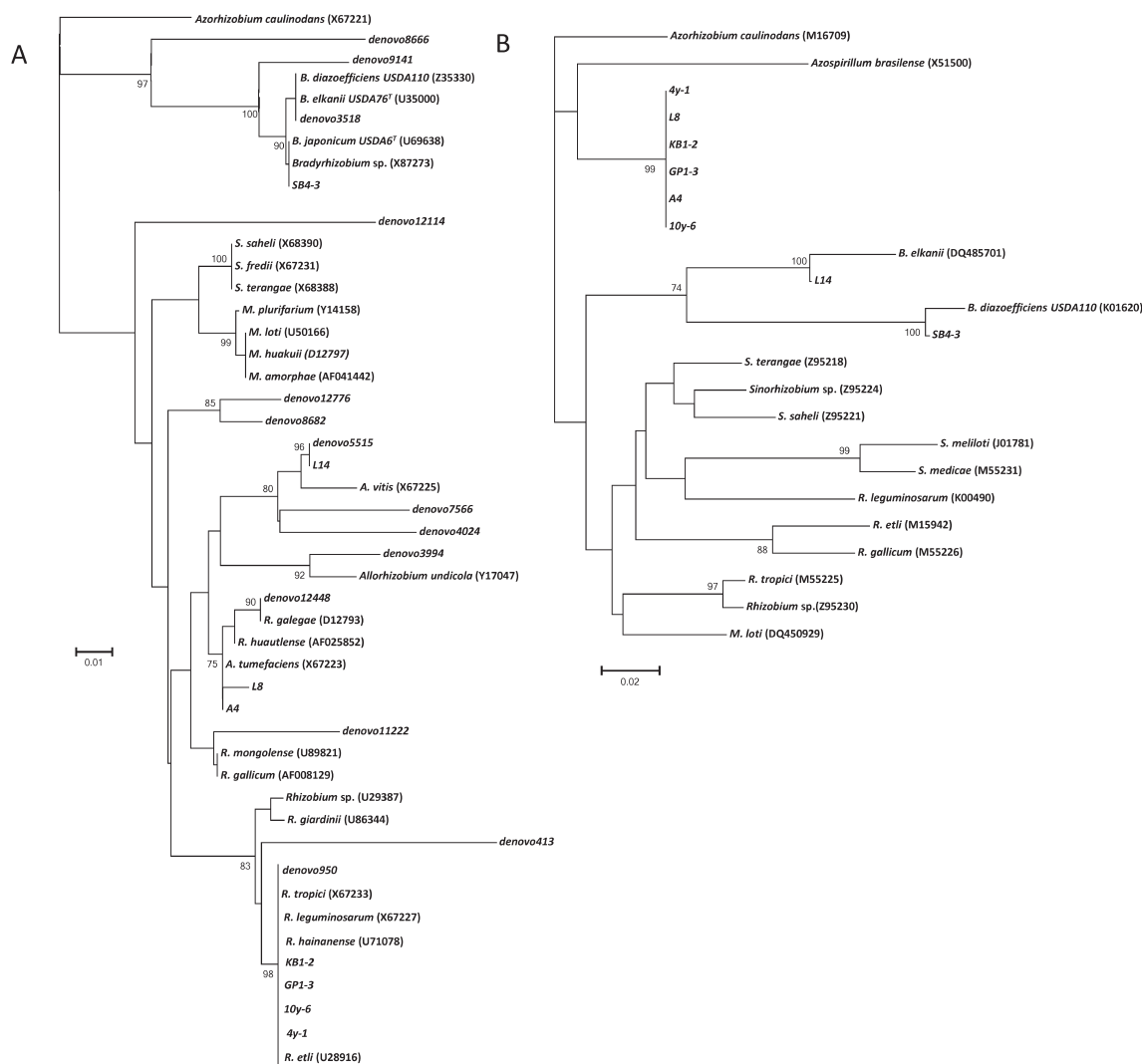


Figure 2. Phylogenetic tree of isolated bacteria from sugarcane based on 16SrRNA (A) and *nifH* (B) sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter. Evolutionary analyses were conducted in MEGA7. The accession number is indicated in parentheses. The denovo number indicates the number of Miseq data isolated in this study. SB4-3, L14, L8, A4, KB1-2, GP1-3, 10y-6, and 4y-1 were subjected to *nifH* sequencing after the bacteria were isolated from the medium. A., *Agrobacterium*; B., *Bradyrhizobium*; M., *Mesorhizobium*; R., *Rhizobium*; S., *Sinorhizobium*.

most abundant phyla in the roots and isolated bacteria from the soil were Proteobacteria, Verrucomicrobia, Acidobacteria, and Actinobacteria, which represented more than 70% of the bacterial taxa in both the soil and roots (Figure 1). The microbial communities in soil-1 and soil-10 and in root-1 and root-10 were similar. The only difference seen was Acidobacteria and Verrucomicrobia (Figure 1). These data indicate that the sugarcane continuous cropping did not change the core microbial community. These core populations are proteobacteria, which is consistent with previous results (Bissett et al. 2016; Lauber et al. 2009). Five classes were more prevalent in the root bacterial populations than in the soil bacterial populations: α -proteobacteria, β -proteobacteria, Cytophagia, Saprospirae, and Actinobacteria (Figure 1). We speculated that these

classes have a higher potential to be isolated as endophyte nitrogen-fixing bacteria. The *Sphingomonas*, *Asticcacaulis*, *Agrobacterium*, and *Rhizobium* species of the α -proteobacteria have been identified as important nitrogen-fixing bacteria (Yeoh et al. 2017). Notably, not only α -proteobacteria but also β -proteobacteria increased as root bacteria. β -proteobacteria were dominant in the roots at 26%, an increase of approximately 2–3 times compared with the 10% in the soil. This result is consistent with a previous result in which β -proteobacteria were present at 5.7% in the roots and predominant compared with 1.4% in the soil (Yeoh et al. 2017). The β -proteobacteria identified in the sugarcane fields in Kagawa (*Achromobacter*, *Burkholderia*, *Ralstonia*, and *Janthinobacterium*) may be endogenous nitrogen-fixing bacteria. However, *Acetobacter*, which

Table 2. Bacterial strains used in this study.

Isolated bacteria	16S rRNA		nifH	
	Best hit spesices	best hit OTU ID	Best hit spesices	Accession number
SB4-3	<i>Bradyrhizobium japonicum</i>	denovo3518	<i>Bradyrhizobium japonicum</i>	LC510362
L14	<i>Rhizobium</i> sp.	denovo5515	<i>Bradyrhizobium elkanii</i>	LC510363
10y-6	<i>Rhizobium</i> sp.	denovo950	Uncultured bacterium	LC510368
4y-1	<i>Rhizobium</i> sp.	denovo950	Uncultured bacterium	LC510369
KB1-2	<i>Rhizobium</i> sp.	denovo950	Uncultured bacterium	LC510366
GP1-3	<i>Rhizobium</i> sp.	denovo950	Uncultured bacterium	LC510367
L8	<i>Agrobacterium</i> sp.	denovo12448	Uncultured bacterium	LC510364
A4	<i>Agrobacterium</i> sp.	denovo12448	Uncultured bacterium	LC510365

has isolated from sugarcane as plant growth promoting bacteria in not only Brazil but also in Okinawa, Japan, was not identified in Kagawa (Asis et al. 2000; Cavalcante and Dobereiner 1988; Lino et al. 2012). We could not find *Acetobacter* from both soils, soil-1 and soil-10, indicating root endophytic bacteria depends on the soil bacterial community. These characters of endophytic bacteria in Kagawa sugarcane might produce unique flavor for Wasanbon sugar.

To know the character of nitrogen-fixing bacteria, bacteria was isolated by two methods; one was used direct isolation by grinding the roots and spread it on HM plates, the sterilization process followed the previous method (Duangkhet et al. 2018). The second methods was trapping by using nodules. The sterilized root of sugarcane was grinded and directly inoculated to soybean, peas and common bean. Then bacteria was isolated from nodules. Through 16S rRNA sequence, the most of isolated bacteria using HM plates were identical to the registrations of denovo950, which belong to *Rhizobium* sp. and some bacteria were denovo5515, denovo 12448, which were classified as *Rhizobium* sp., or *Agrobacterium* sp. (Figure 2A, Table 2). We could only isolate *Bradyrhizobium* sp. from trapping soybean nodule (Table 2). After isolation of bacteria, *nifH* was amplified using previously reported *nifH* primers (Laguerre et al. 2001). Resulting accession number of isolates, SB4-3, L14, L8, A4, KB1-2, GP1-3, 10y-6, and 4y-1, are LC510362-LC510369, respectively (Table 2). The sequencing data showed that not only *Rhizobium* sp. or *Bradyrhizobium* sp. but also *Agrobacterium* sp. contain *nifH* genes. The phylogenetic tree of *nifH* revealed that the isolated bacteria belong to at least two (Figure 2B). The *nifH* gene of SB4-3 strain was derived from *Bradyrhizobium* sp. and another *nifH* gene of isolates which belong to *Rhizobium* sp. and *Agrobacterium* sp. by 16S rRNA were derived from uncultured bacterium (Table 2). This phylogeny is not the strongest evidence for the horizontal-transfer of *nifH* gene. Verification of a horizontal-transfer event is difficult with this limited data. Nevertheless, our data of *nifH* phylogeny showed unexpected features that the gene transfer might exist. More data is needed to prove this hypothesis.

In conclusion, we identified the soil bacteria and endophyte bacteria in the roots of field-grown sugarcane in Kagawa. This report is the first on *S. sinense*, which is used to produce Wasanbon. Diversity was partially different from that in Brazilian and Japanese sugarcane, *S. officinarum*, which might produce unique flavor for Wasanbon sugar. Many *Rhizobium* sp. and *Agrobacterium* sp. with *nifH* were isolated. In the future, the relationship between the application of nitrogen-fixing bacteria and the production of Wasanbon should be studied.

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