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Communication

Burkholderia vietnamiensis Isolated from Root Tissues of Nipa Palm (*Nypa fruticans*) in Sarawak, Malaysia, Proved to Be Its Major Endophytic Nitrogen-Fixing Bacterium

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Root-associating bacteria of the nipa palm (*Nypa fruticans*), preferring brackish-water affected mud in Sarawak, Malaysia, were investigated. In a comparison of rhizobacterial microbiota between the nipa and the sago (*Metroxylon sagu*) palm, it was found that the nipa palm possessed a group of *Burkholderia vietnamiensis* as its main active nitrogen-fixing endophytic bacterium. Acetylene reduction by the various isolates of *B. vietnamiensis* was constant (44 to 68 nmol h^{-1} in ethylene production rate) in soft gel medium containing 0.2% sucrose as sole carbon source, and the bacterium also showed motility and biofilm-forming capacity. This is the first report of endophytic nitrogen-fixing bacteria from nipa palm.

Key words: *Nypa fruticans*; *Burkholderia vietnamiensis*; endophytic bacterium; nitrogen fixation

The nipa palm (*Nypa fruticans*, family Arecaceae) is a gregariously growing monoecious tropical mangrove palm, adapted to river courses affected by brackish water.[1)] The genus *Nypa* consists of only one species, *N. fruticans*, and this palm has a subterranean stout stem that grows beneath the ground with only large maximum 15 m compound leaves emerging from stout stems upwards above the water table.[2)] According to *Asahi Encyclopedia*, *World of Plants*,[3)] the compound leaves of nipa in Sarawak, Malaysia, are one of the world's largest fronds. Since nipa sap contains more than 16% w/v sugars,[4)] it is estimated that grown nipa palm can produce 6,480–15,600 liters of ethanol ha^{-1} y^{-1} as compared to 3,350–6,700 liters ha^{-1} y^{-1} for sugarcane.[5)] Although the nipa palm is thus a large biomass-producing wild plant in mangrove areas, agricultural studies on this palm are limited because of its uncommon living environment. Soil analysis in the nipa habitat has revealed poor availability of nitrogen and phosphorus along with soil salinity,[6)] but sustainably high productivity is observed for this palm. Hence it has been speculated that the productivity of the nipa is supported by unknown mechanisms for its fertility maintenance, in which symbiotic microbes are involved.[7,8)]

Hence we investigated the endophytic nitrogen-fixing bacteria of the nipa palm, and also the sago palm (*Metroxylon sagu*) for comparison. Palm roots of nipa and sago were sampled from Mukah District, Sarawak (N 02.900, E 112.288 and N 02.912, E 112.146 respectively) during the wet season in mid-February 2008. Primary and secondary roots were randomly sampled from five and four specimens of nipa and sago palm respectively. The roots were carefully dug out from the soil, and the rhizosphere soil was gently removed by spraying with clean water. Fresh root samples were kept in sterile zip-lock polyethylene bags, and stored in a cooling box at 4 °C during transportation of less than 1 d.

In the laboratory, root samples were first washed with 70% ethanol for 30 s, and then surface-sterilized with diluted commercial bleach containing 0.5% sodium hypochlorite (Chlorox®, Rawang, Malaysia) for 5 min, followed by rinsing three times with sterilized water for 10 min each time. In view of the relatively high concentration of sucrose in the sap of the nipa palm,[4)] surface-sterilized root cut vertically in 10 mm long was inoculated into gellan gum soft gel medium[9)] that contained 0.2% w/v sucrose as sole carbon source.

The soft gel medium was incubated at 25 °C in the dark, until a subsurface bacterial pellicle emerged (14 d). Using a micropipette, 10 µl of cultured medium containing the subsurface pellicle was spread over a modified Winogradsky's agar (MWA) plate[9)] containing 0.2% sucrose for purification of microbial colonies. The bacteria thus obtained as pure isolates were kept in 10% glycerol stock at −20 °C. By determining the sequence of the entire 16S rRNA gene, as described previously,[10)] all the isolates were tentatively identified at the levels of genera and species.

An acetylene reduction assay was done as described previously,[10)] at 7-d-incubation at 25 °C, with *Klebsiella pneumoniaea* ATCC 8724 as positive control. Although the root tissues were carefully cut out from fresh, non-damaged parts and surface-sterilized as described above, some saprophytes might have moved into the root tissues from the cut part and spread quickly over the inner tissues. Therefore, it was necessary to screen for endophytic nitrogen-fixers and any additional factors. Using several soil bacteria and rhizobacteria, it has been found that sucrose used as a carbon source tends to suppress acetylene reduction in many soil and rhizos-

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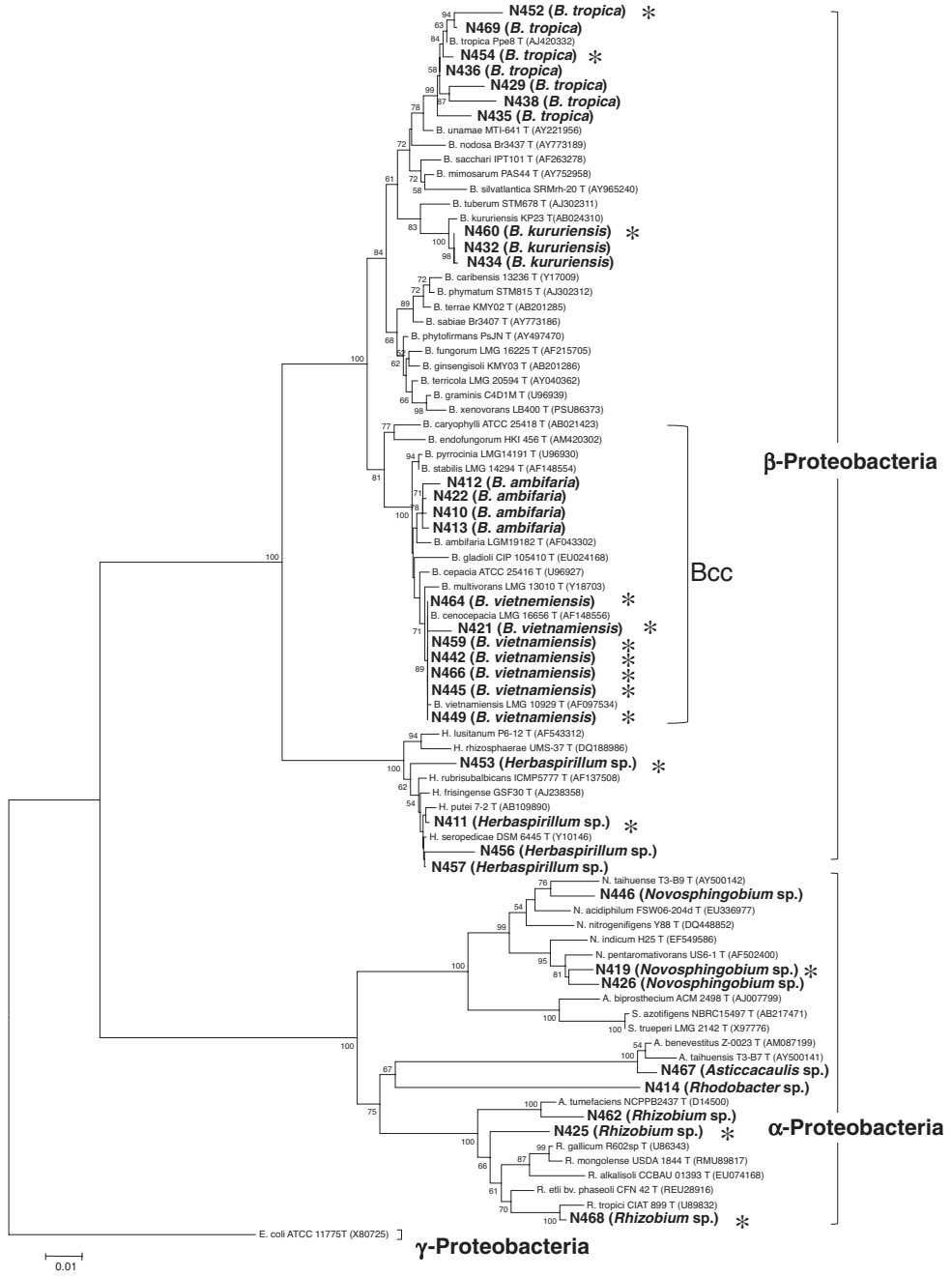

Fig. 1. Phylogenetic Position of *Burkholderia vietnamiensis* and Some Other Important Proteobacteria Isolated from Roots of Nipa Palm.

The phylogenetic relations among *Burkholderia*, *Herbaspirillum*, and some important diazotrophic α-proteobacteria from the root of the nipa were computed based on 1.5 kb 16S rRNA gene sequences using the Maximum Composite Likelihood Method, and are in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (the complete deletion option). A phylogenetic tree showing the relations of *B. vietnamiensis* to all the type strains of *Burkholderia* spp. and some environmentally important diazotrophic and non-diazotrophic bacteria subclasses of α- and β-Proteobacteria isolated from the nipa root (some *Herbasirillum* spp. of β-Proteobacteria, some *Novosphingobium* spp., and *Rhizobium* spp. of α-Proteobacteria). The numbers at the nodes indicate the levels of the bootstrap generated by a neighbor-joining analysis[11] of 1,000 replicates. Bootstrap values below 50% are not indicated. The scale bar represents 0.01 substitutions per nucleotide site. The DNA database accession number for each reference type strains is shown in parentheses. Phylogenetic analyses were conducted in MEGA version 4[12] and the sequence of *Escherichia coli* ATCC 11775^T was used as outgroup. Acetylene-reducing isolates are marked with an asterisk.

pherous bacteria, although active reduction is observed in the same bacteria cultured in soft gel medium containing D-mannitol or mixture of D-glucose and DL-malic acid (Hara, Tang, and Hashidoko, unpublished data). Considering this less responsive sucrose as the most dominant sugar in nipa sap, it is reasonable that the sucrose-containing assay medium for acetylene reduction is an appropriate medium for searching for endophytic nitrogen-fixers in nipa.

The bacteria obtained from the surface-sterilized roots of the nipa palm (5 specimens) by trapping culture consisted of 58 isolates, of which 21 isolates were identified as members of the genus *Burkholderia*. One diagnostic character of the bacterial isolates was that 11 isolates among the 21 isolates of *Burkholderia* were the members of the *B. cepacia* complex (Bcc), as shown in the phylogenetic tree of the genus *Burkholderia* (Fig. 1).[11–14] In our screening and characterization of

root-associated bacteria of sago palm, 20 out of a 61 isolates were identified as the genus *Burkholderia*, but no Bcc member was found in these *Burkholderia* spp. from the sago roots.

Of the total, 11 root-associated Bcc isolates from nipa, acetylene reduction (32 to 66 nmol h^{-1} in the sucrose medium) was observed in seven isolates (N421, N442, N445, N449, N459, N464, and N466, accession nos. AB568116, AB568117, AB568118, AB568311, AB568119, AB568312, and AB568313 in DDBJ), all of which were identical as the same species close to *B. cenocepacia*[15] and *B. vietnamiensis*.[16] In the database search for the 16S rRNA gene sequence, the bacterium showing the best match to those of the N_2-fixing isolates was *B. vietnamiensis* G4 (accession nos. CP000614 and CP000615), with 99–100% homology. *B. cenocepacia* and *B. vietnamiensis* are almost indistinguishable by sequence homologies on their 16S rRNA genes. However, *B. cenocepacia* (genomovar III) is highly human pathogenic,[17,18] while *B. vietnamiensis* (genomovar V) is non-pathogenic to humans.[17–19] The pathogenicity of *B. cenocepacia* is genetically detectable by *cblA* (coding giant cable pilus protein) and *esmR* (epidemic strain marker of *B. cepacia*).[17,20] *B. vietnamiensis*[16] and other plant-associating nitrogen-fixing *Burkholderia* spp. isolated from sugarcane and maize[21] are free from these transmissibility factors. Therefore, these seven isolates of the nipa palm were tentatively identified as *B. vietnamiensis* (Fig. 2), a plant-origin, non-pathogenic Bcc member.[18] The marker genes for the human pathogenic *Burkholderia* will be tested for root endophytic *B. vietnamiensis* in the near future.

These seven isolates of *B. vietnamiensis* were obtained from four different sources of nipa specimens, and all were highly active nitrogen-fixers, and moderate motility and tolerance to 1.75% NaCl in nitrogen-poor medium were observed for *B. vietnamiensis* N421, N442, and N445 among the bacteria tested (Table 1). Furthermore, none of the other nipa root-associated Bcc bacteria that were identical as *B. ambifaria* reduced acetylene. On the other hand, two *B. tropica* and a *B. kururiensis* of total 10 isolates of the non-Bcc group from the nipa were acetylene reducing, but the other seven isolates did not reduce acetylene (Fig. 1). Considering all these factors together, it is reasonable to regard this species as an endophytic nitrogen-fixing *B. vietnamiensis* of the nipa palm. Here we tentatively identified these nitrogen-fixing *B. vietnamiensis* as endophytic nitrogen-fixing bacteria. Similar endophytic bacteria, many *Burkholderia* spp., such as *B. vietnamiensis* LMG10929 isolated from rice roots in Vietnam,[22] have also been reported as bio-fertilizing agents,[23] but as for the endosymbiont-like nitrogen fixer from nipa, this is the first report.

On the other hand, sago root for comparison with nipa provided 20 isolates of *Burkholderia* out of 61 rhizobacteria, but none of them were members of Bcc despite the same isolation process. Non-Bcc *Burkholderia* from sago palm consisted of *B. unamae*, *B. phenazinium*, *B. mimosarum*, *B. nodosa*, *B. gladioli*, *B. tropica*, *B. phymatum*, *B. tropicalis*, and *B. fungorum* as isolates, but none of these reduced acetylene in the 0.2% sucrose medium. Shrestha *et al.* have reported an investigation of nitrogen-fixers in the root of sago palm in Philippines,[24] in which study *B. tropicalis* of a non-Bcc member was isolated as the only bacterium of the genus *Burkhlderia*, while two isolates of *Azospirillum amazonense* from

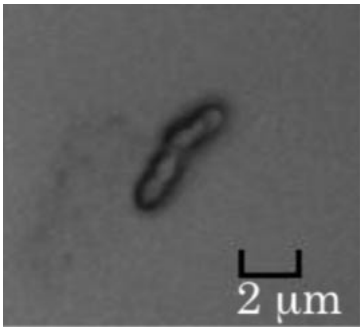

Fig. 2. Observation of *B. vietnamiensis* (N445) under Laser Scanning Electron Microscopy.

B. vietnamiensis (isolate N445) was inoculated into potato-dextrose broth and incubated for 4 d at 28 °C in the dark without agitation. A cell suspension (20 μl) was spread over glass slide plates and air-dried. The bacterial cell morphology was observed under a laser scanning electron microscope (Violet Laser Color 3D Profile Microscope, Keyence, Osaka, Japan), with a 150 × objective lens and a 10 × ocular lens.

Table 1. Properties of Some *B. vietnamiensis* and Other Palm Isolates

Code	Bacterium	Source	C_2H_2 reduction (nmol h^{-1})	Motility	Tolerance to 1.75% NaCl ($OD_{665\,nm}$)	Biofilm formation
N421	*B. vietnamiensis*	nipa 2	44	+	0.20	+
N442	*B. vietnamiensis*	nipa 5	55	+	0.28	+
N445	*B. vietnamiensis*	nipa 5	68	+	0.38	+
N459	*B. vietnamiensis*	nipa 4	64	±	0	±
N460	*B. kururiensis*	nipa 4	82	+	0	+
S39	*A. amazonense*	sago 1	40	−	0	±
S81	*A. amazonense*	sago 1	65	−	0	−
S70	*B. unamae*	sago 2	0	+	0.10	±
ATCC8724	*K. pneumoniae*	−	2	−	0.44	−

One hundred μl of diluted cell suspension ($OD_{665\,nm}$ 0.006–0.007) was added to 10 ml of MW medium supplemented with 0.2% w/v sucrose (pH 6.2) and solidified with 0.3% gellan gum in a 18-cm test tube. For the motility test, the soft gel medium was voltexed for 10 s for uniform inoculation, and the culture tube was incubated for 48 h at 28 °C in the dark. Of the bacterial cells in the gelled medium, the growth patterns were compared to a reference culture, non-motile *K. pneumoniae*.[9] For salt tolerance, 100 μl of bacterial cell suspension ($OD_{665\,nm}$ 0.6–0.7 in water) was inoculated into 10 ml of NaCl-containing liquid MW medium (0.2% sucrose and 1.75% NaCl) in the test tube, shake-cultured for 48 h at 28 °C at 100 rpm, and then cell growth was monitored at 665 nm. C_2H_2 reduction was done for only a single trial, but reduction by S81 and *K. pneumonia* ATCC 8724 in this assay system showed high reproducibility (n = 3–5). Motility test: +, horizontally dispersed; ±, blurred; −, formed aggregate or particle. Biofilm formation (24 h, on glass wall of culture test tube): +, formed; ±, formed but ambiguous; −, not formed.

the root tissues of sago palm exhibited relatively high acetylene reduction in the 0.2% sucrose medium (Table 1), in contrast with a previous report on sago nitrogen-fixers, mostly γ-Proteobacteria and their consortia.[24] As the genus *Azosprillum* is widely known as a common endophytic nitrogen-fixer throughout the family Poaceae,[25] our screening system is probably functional in searching for endophytic nitrogen-fixing bacteria. Member of *B. vietnamiensis* or another Bcc member were not found in the sago palm. In a preliminary screening, neither the coconut palm nor the oil palm possessed nitrogen-fixing *B. vietnamiensis* (Tang, Melling, Goh, and Hashidoko, unpublished data).

As described above, the nipa grows efficiently in nutrient-deficient, brackish swamp areas, probably due to the contribution of such highly effective endophytic nitrogen-fixing bacteria. The high motility and biofilm-forming property of some *B. vietnamiensis* N421, N442, and N445 support the speculation that these *B. vietnamiensis* isolates from the nipa root inhabit the intercellular spaces in aerenchyma. The high motility of the endophytes probably assists its rapid spread through the root tissues along the cortical gas-filled space, while the biofilm formation of the bacteria leads to long, stable colonization in the inner tissues of the host plants.[26] Although this stage of the investigation cannot determine the contribution of nitrogen by these root-associating diazotrophs to the host plants, it is strongly suggested that *B. vietnamiensis* is a candidate nitrogen-fixing endophyte. As palm plants in tropical and sub-tropical zones are prospective bio-resources for bio-fuel production,[5] the discovery of *B. vietnamiensis* as a potent candidate for endophytic nitrogen-fixer in the nipa palm may encourage further systematic investigation of its functionality as a bio-fertilizing agent, including its capabilities of phytohormone production as Cocking *et al.* have suggested.[27] It may be applicable as a bio-fertilizing agent for economically important palms, particularly the sago palm, a fresh-water swamp-adapted palm used as a plantation crop in Sarawak-Malaysia for starch production.

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