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Design of Sphingomonad-Detecting Probes for a DNA Array, and Its Application to Investigate the Behavior, Distribution, and Source of Rhizospherous *Sphingomonas* and Other Sphingomonads Inhabiting an Acid Sulfate Soil Paddock in Kalimantan, Indonesia

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Throughout Central and South Kalimantan, Indonesia, strongly acidic soil (pH 2.1–3.7) is widely distributed, and the local acidic soil-tolerant plants, including local rice varieties, often possess sphingomonads in their rhizosphere and rhizoplane. To investigate the behavior of sphingomonads inhabiting the rhizosphere of such acid-tolerant plants, we designed 13 different DNA array probes (each of 72 mer) specific to a group of sphingomonads, using a hypervariable V6 region of the 16S rRNA gene. This DNA array system was used preliminarily for an analysis of microfloral dynamisms, particularly of sphingomonads, in acidic paddock ecosystems, and the results suggest that the acid-tolerant local rice shares rhizospherous sphingomonads with wild *Juncus* sp., a predominant weed that thrives in acidic paddocks during the off-season for rice farming. This tentative conclusion supports the bio-rationality of the traditional rice farming system with respect to functional rhizobacteria.

Key words: acid sulfate soil paddock; *Juncus* sp.; source of rhizospherous sphingomonads; *Sphingomonas*-detecting DNA array probes

Throughout Central and South Kalimantan, Indonesia, the acidic land allows the growth and regeneration only of limited acid-tolerant plants, including local rice varieties for traditional rice farming.[1,2] In an investigation of the microfloral composers in the rhizosphere of such acid-tolerant plants, we frequently isolated *Sphingomonas* spp. and other sphingomonads of α-proteobacteria. Sphingomonads are Gram-negative rods of the family Sphingomonadaceae, α-subclass proteobacteria, and possession of an outer membrane composed mainly of sphingolipids is a common characteristic of them.[3–5] Sphingomonads, including the genus *Sphingomonas*, are often oligotrophiles and sometimes diazotrophiles, and are commonly isolated from soil. Polyaromatic-degrading and polychlorophenol-degrading sphingomonads have often been isolated from sewage sludge or polluted soils.[6,7] Some sphingomonads also have the ability to digest naturally occurring polysaccharides that are normally highly resistant to degradation. For example, *S. adhaesiva* decomposes agarose,[5] while *Sphingomonas* sp. strain A1 utilizes alginate as a carbon source.[8] Many sphingomonads behave as biofilm-forming bacteria to tolerate highly starving conditions.[8,9] Sphingomonads are also isolated from fresh water[10] and the phytoplane.[11,12] Previous investigations of the population size of sphingomonads in Gramineae, including the rice plant (*Oryza sativa*), confirmed dense colonization on floral buds and ears.[11,13] These phylloplane-attaching sphingomonads are likely to be tolerant of UV irradiation, and this adaptation is also closely related to their ability to form biofilms.[14,15] Due to this property of the phytoepiphytes, it is reasonable to hypothesize that phyllospherous sphingomonads are seed-borne and disperse from the seed coat over aerial parts of the

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seedlings. In fact, we have also isolated some sphingomonads as minor seed-epiphytes from rice seeds, not only from the surface of palea but also from surface-sterilized rice seeds, but they were relatively minor composers (unpublished data).

During our investigation of microfloral components in the rhizoplane of acid-tolerant plants inhabiting acid-sulfate soil in Central and South Kalimantan, we also frequently isolated several sphingomonads with a high population density.[1] Some of these bring a certain benefit to the host plants due to their abilities to fix nitrogen, neutralize their inhabiting micro-environment, accumulate polysaccharides in the rhizosphere, and their promotion of a synergistic bio-consortium to acquire a stable niche.[16,17] After this discovery, queries then arose as to the original source of such rhizospherous sphingomonads of the local rice varieties. As a means of identifying the original source of the rhizospherous sphingomonads, we applied a DNA array technique[18–20] to pursue and detect certain members of the genus *Sphingomonas*. Although some applications of the DNA array technique to specific bacterial detection and identification have been attempted,[21,22] bacterial identification at the species level among microfloral composers is still insufficient. Wang *et al.* have designed 40 mer probes from three different regions of the 16S rRNA gene for one bacterial species, and prepared a set of probes for 40 fecal bacteria. Species-specific detection of the bacterial composers in human feces was unsuccessful, and bacterial composition and detection were sufficient only at the limited genus level used in the probe design. In this paper, we show the results of our DNA array analysis to suggest the distribution, behavior and sources of sphingomonads in the acidic soils in South Kalimantan.

Materials and Methods

Rhizoplane and/or soil bacteria and rhizosphere bacterial communities used as DNA sample sources for hybridization. The isolated and stocked strains of rhizoplane bacteria used in this study were *Sphingomonas rosa* EC-K013 (accession no. AB121233) and *Burkholderia cepacia* EC-K014 (AB121232). Both of these strains were isolated from the rhizoplane of *Melastoma malabathricum* growing on a path between acidic paddocks (pH 3.1–3.2) at Jelapatbaru, a village northwest of Banjarmasin, South Kalimantan. The root fragments of local rice variety and *Juncus* sp. used for DNA extraction for rhizospherous microfloral communities were obtained from acid-sulfate soil paddocks at Gambut, South Kalimantan, in March of 2004.

Design of sphingomonad-detecting DNA array probe. We were interested in utilizing the DNA array technique for specific detection of sphingomonads using a hypervariable region V6 in the 16S rRNA gene for the array probes.[23] This was a realistic possibility due to the massive bacterial 16S rRNA gene sequence information available for new strains and species within various world-wide gene bank databases. In the DDBJ database,[24] the species-specific regions around positions 940–1090 in *E. coli* were collected from 43 different *Sphingomonas* species as a means to cover almost all of the groups on the phylogenetic tree of the *Sphingomonas* genus.[25,26] These sequences were compared to one another in order identify the most divergent regions of DNA sequences within the 16S rRNA gene. Positions 986–1055 for *Escherichia coli*, involving the V6 region, was accordingly chosen as a variable region among some species of the group *Sphingomonas*.[23] From the 43 sphingomonads, 13 representative species in the region were selected as target sequences for probe design (Fig. 1). Although some genera of Sphingomonadaceae (*e.g.*, *Caulobacter* sp. and *Blastobacter* sp.) are indistinguishable from genus *Sphingomonas* by PCR detection using *Sphingomonas*-specific primers,[11] these Sphingomonadaceae bacteria possessed genus-specific DNA sequences in this region distinguishable from all of the other sphingomonads.

A total of 13 types of probes were designed for sphingomonads. Specifically, 10 kinds of probes were created for the detection of *Sphingomonas* (*S. rosa* IAM14222, *S. ursincola*, *S. echinoids* DSM1805-T, *S. capsulata* GIFU11526, *S. adhaesiva* GIFU11458T, *S. aurantiaca* MA306a, *S. pituitos* EDIV, *S. paucimobilis* 12576T, *S. aromaticivorans* IFO16084, and *S. stygialis* SMCCB0712) and two additional probes were designed for the detection of *Sphingobium* spp. (*S. chlorophenolicum* RA2 and *S. yanoikuyae* Q1). In addition to these 13 sphingomonad-specific probes, we also designed five independent probes for non-sphingomonad Sphingomonadaceae (*Porphyrobacter tepidorius* DSM10594, *Caulobacter leidyi* ATCC15260, *C. henricii* ATCC15253, *Brevundimonas vesicularis* LMG2350, and *Blastobacter* sp. SMCCB0477). Probes for α-proteobacteria (*Rhizobium etli* TAL182, U28939), β-proteobacteria (*Burkholderia cepacia* ST-200, AB051408, *Herbaspirillum putei* 7-2, AB109890), and a common region for class proteobacteria (positions 1041–1112 for *E. coli*) were also designed. All probes were created as synthetic oligonucleotides and were purchased from Sigma Genosys (Ishikari, Japan).

DNA array. Micro Spotting Solution (TeleChem International, Fullerton, CA) was added to an oligonucleotide solution, and a 20 μM probe solution was prepared. This solution was stamped onto Carbostation-U (Nisshinbo, Chiba, Japan), a type of glass slide which is coated with polycarbodiimide with a Micro Spotter (Teramecs, Kyoto, Japan), and is used for microarray analysis. After the spotting, rehydration (exposing to vapor for 2–3 s) was carried out, and the glass slide was subsequently dried on a 80 °C hot-plate for 2–3 s. Probes were immobilized by exposing them to 600 mJ of UV irradiation. Prior to use, the background

Consensus sequence (References)	acaggtgctgcatggctgtcgtcagctcgtgtcgtgagatgttgggttaagtcccgcaacgagcgcaaccct
Sphingomonas yanoikuyae Q1	aacgtttgacatccctatcgcggatcgtggagacactttccttcagttcggctggataggtgacaggtgctg
Sphingobium chlorophenolicum RA2	aacgtttgacatccctagtgtggattgtggagacacattccttcagttcggctggctaggtgacaggtgctg
Sphingomonas rosa IAM 14222	agcgtttgacatcctcatcgcgatttccagagatggatttcttcagttcggctggatgagtgacaggtgctg
Sphingomonas ursincola	agcgtttgacatgtctagtatgtttcccagagatgggttacttcagttcggctggctagaacacaggtgctg
Sphingomonas echinoides DSM 1805-T	agcgtttgacatgtccggacgatttcgggagaccgatctcttcccttcggggactggaacacaggtgctgca
Sphingomonas adhaesiva 11458T	agcgtttgacatgtccggacgacttccagagatggatttccttccttcggggactggaacacaggtgctgca
Sphingomonas aurantiaca MA306a	agcgtttgacatggcaggacgacttccggagacggatttcttcccttcggggacctgcacacaggtgctgca
Sphingomonas aerolata NW12	agcgtttgacatggcaggacgacttctggagacagatttcttcccttcggggacctgcacacaggtgctgca
Sphingomonas pituitos EDIV	agcgtttgacatggtaggacgacttccagagatggatttcttcccttcggggacctacacacaggtgctgca
Sphingomonas paucimobilis 12576T	agcgtttgacatggtaggacgacttccagagatggatttcttccttcggggacctacacacaggtgctgcat
Sphingomonas capsulata 11526	agcctttgacatcccgcgctatatcgagagatcgatagttcctttcggggacgcggtgacaggtgctgcatg
Sphingomonas aromaticivorans IFO 16084	agcgtttgacatcccgcgctacttccagagatggaaggttcccttcggggacgcggtgacaggtgctgcatg
Sphingomonas stygialis SMCCB 0712	agcgtttgacatcccgcgctaacccgagagatcgggtgttcccttcggggacgcggtgacaggtgctgcatg
Porphyrobacter tepidarius DSM10594	agcctttgacatcctaggacgacttctggagacagatttcttcccttcggggacctagtgacaggtgctgca
Caulobacter leidyi ATCC15260	agcgtttgacatgtccggacgatttccagagatggatctcttcccttcggggactggaacacaggtgctgca
Caulobacter henricii ATCC15253	acctttgacatgcccggaccgccacagagatgtggttttcccttcggggactgggacacaggtgctgcatg
Brevundimonas vesicularis LMG 2350	acctttgacatgcctggaccgccagagagatctggctttcccttcggggactaggacacaggtgctgcatg
Blastobacter sp. SMCC B0477	agcgtttgacatgtctagtatgtttcccagagatgggttacttcagttcggctggctagaacacaggtgctg
Burkholderia cepacia ST-200	tacccttgacatggtcggaatcccgctgagaggtgggagtgctcgaaagagaaccggcgcacaggtgctgca
Herbaspirillum putei 7-2	tacccttgacatggatggaatcccgaagagatttgggagtgctcgaaagagaaccatcacacaggtgctgca
Rhizobium etli TAL 182	agcccttgacatgcccggctacttgcagagatgcaaggttcccttcggggaccgggacacaggtgctgcatg

Fig. 1. Probe Design for *Sphingomonas*-Detecting DNA Array.

Sequence information for DNA probes of 72 mer were collected from the DNA database. The bacterial strains used in the probe design are listed here, and each accession no. is described in the text.

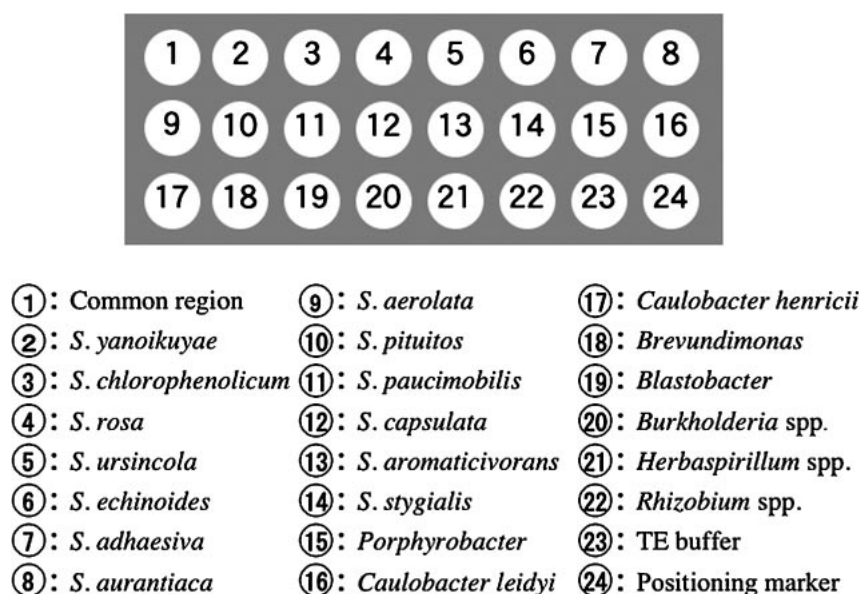

Fig. 2. DNA Array and Probe Location.

DNA probes designed as listed in Fig. 1 were stamped according to the locations shown here. Usually, the stamping was triplicated on a slide glass, so hybridization was also triplication for one DNA sample. At spot no. 1, 72 mer of a consensus sequence for most eubacteria was stamped as a reliable positive control for proteobacteria, whereas spot no. 23 is a positioning marker of a 20 mer oligonucleotide, the 5′ end of which was labeled with rhodamine.

area of the arrays was blocked with BlockAce (Snow Brand Milk Products, Tokyo), and the arrays were washed with TE buffer twice and then dried. All probes were stamped with at least two or three replications. The DNA array layout is illustrated in Fig. 2.

Labeling and hybridization. Approximately 2 kb of the rRNA gene region was amplified from a 100 ng template (*e.g.*, environmental DNA (eDNA)) with 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and LDBact-132aA18 (5′-CCGGGTTTCCCCATTCGG-3′) primers.[27] To obtain labeled rRNA gene fragments, 1 µl of 1 mM Cy5-dCTP (a fluorescence-labeled cytocin-dinucreotide, PE Applied Biosystems, Boston, MA) was added in a PCR reaction; it contained 4 µl of 1 mM dCTP and 5 µl of 2 mM dATP, dGTP and dTTP mixture solution. PCR amplification was performed with the KOD dash enzyme (Toyobo, Tokyo) in a final volume of 50 µl. After the first denaturation step at 94 °C for 4 min, thermal cycling was repeated 40 times under the following conditions: 94 °C for 30 s, 60 °C for 30 s, 68 °C for 2 min, and a final extension at 68 °C for 7 min. A 72 mer oligonucleotide designed from a common region in the 16S rRNA gene of proteobacteria (positions 1041–1112) was used as a positive control.[28] A 20 mer oligonucleotide in which the 5′ end was labeled with rhodamine was also stamped as a positioning marker. Approximately 1 µg of Cy5-dCTP-labelled

DNA fragments eliminating unincorporated dNTPs was dissolved in a 20-μl hybridization buffer (final conc. $0.3 \times$ SSC, 20% formamide, 0.3% SDS), then denatured at 97 °C, and was immediately snap-cooled on ice. The DNA fragments were subsequently dropped onto an array, covered with a thin cover slip, and sealed using HybChamber™ (Genemachines, Fullerton, CA). The Tm in the $0.3 \times$ SSC and 20% formamide conditions was calculated for each of our 72 mer probes (OLIGO™, Takara, Ohtsu, Japan), and the lowest hybridization temperature was found to be 51.2 °C. After 5 h of hybridization, the cover slip was removed with $0.2 \times$ SSC, 0.1% SDS and the array was washed twice in the same buffer for 5 min with shaking. The slides were then washed three times with $0.2 \times$ SSC for 3 min with shaking. The slides were then rinsed once with $0.05 \times$ SSC and centrifuged ($1{,}000 \times g$ for 1 min) for drying.

Imaging and data analysis. In order to evaluate the intensity of the fluorescence of each probe, hybridized DNA arrays were scanned by Scan Array 4000 (GSI Lunomics, Billeria, MA). The acquired image was then analyzed with Gene Pix 4000 analysis software (Inter Medical, Nagoya, Japan). Signal intensities were normalized by the intensity of a positive control (spot no. 1).

Specific hybridization of a sphingomonad from pure and mixed culture. Sphingomonas rosa EC-K013 and *Burkholderia cepacia* EC-K014 were subcultured on slants of modified Winogradsky's medium (mineral mixture, 0.5% sucrose, 0.005% yeast extract, and 1.5% agar) from frozen 10% glycerol stock solutions. Each cell suspension (containing approximately 1×10^4 cells/100 μl) was uniformly inoculated onto an N-free soft gel medium.[29] This soft gel medium was solidified with 0.3% gellan gum (pH 6.2 or 4.0), and contained Winogradsky's mineral mixture with 1% sucrose as the sole carbon source. Inoculated samples were incubated at 20 °C under dark conditions.

For DNA extraction, approximately 50 μl of the bacterial mat taken directly by micropipette from the soft gel medium was subjected to DNA extraction with the Isoplant II kit. It is important to note that it is known that gellan gum does not interfere with the efficiency of PCR reactions.[30] The resulting DNA sample was applied to DNA array analysis.

DNA extraction from acidic paddock soil and soil bacterial culture. To prepare environmental DNA (e-DNA) samples, we first attempted direct extraction[31,32] of paddock soil in South Kalimantan. First, approximately 1 g of soil was suspended into 20 ml of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) containing 100 units of lysozyme (4 units mg^{-1}, ICN Biomedicals, CA, USA). This soil-solution mixture was maintained at 37 °C for 1 h and then centrifuged at a low speed ($1{,}000 \times g$, for 5 min) to separate the heavy soil particles. The resulting supernatant was collected and transferred to sterile 50 ml-Falcon tubes, mixed with an equivalent volume of *n*-propanol, and then centrifuged at $5{,}500 \times g$ for 15 min. We utilized the Isoplant II DNA extraction kit according to the supplier's instructions (Wako Pure Chemical Industries, Osaka, Japan) combined with lysozyme-treatment and/or sonication with glass beads to extract bacterial DNA from a portion of the resulting residue. However, due to the large amount of humic substances, we obtained a pellet that was deep brown in color. To minimize contaminated polyphenols in the sample, we also attempted to wash out the humic substances with TE buffer several times before the DNA extraction process as described above.

DNA preparation from soil bacterial culture was also attempted. Into a 50-ml Falcon tube, 25 ml of an autoclaved $1 \times$ Hoagland's No. 2 solution-base cultured medium containing 0.2% sucrose (pH 5.2) was poured, and 100 μl of a supernatant of the paddy soil suspension (20 mg soil/0.5 ml sterilized water in a 1.5-ml Eppendorf tube) was inoculated. The medium was incubated at 28 °C for 72 h in the dark, at which point the medium became slightly turbid, indicative of oligotrophic bacterial growth. The medium was centrifuged at $5{,}500 \times g$, and the resulting supernatant was gently decanted. The resulting bacterial pellet was subjected to DNA extraction with the Isoplant II kit according to the supplier's instructions.

DNA extraction from rhizoplane washings and/or plant residues in the paddock. DNA extraction from rhizoplane microfloral components of local rice varieties and *Juncus* sp. was performed as follows: ten living roots of local paddy plants were collected from five different specimens grown for approximately one week after transplanting them in an acid-sulfate soil paddock. The samples, including root tips, were dug up with the paddock soil under mid-acidic (pH 3.7–4.1) water. The soil surrounding the roots was first physically removed by rough manual agitation. It was then completely washed out from the rhizoplane with clean water emitted from a spray bottle. Scissors were surface-sterilized and washed with sterile water, and then used to cut the soil-less roots at 3–5 cm from the tip. Ten root fragments were placed into a polyethylene bag filled with 3 ml of TE buffer (pH 8.0). The roots were then gently scraped over a polyethylene bag to obtain root surface tissues together with rhizoplane bacteria. The resulting tissue suspension was then collected in a sterilized 1.5-ml Eppendorf tube and centrifuged to obtain a precipitate. DNA extraction from the resulting pellet was subsequently performed with the Isoplant II DNA extraction kit.

As a reference sample of sphingomonad, rhizosphere bacteria of a sapling of *Melastoma malabathricum*, an acid-sulfate soil-adapting plant, were also treated by the same procedures as described above. First, non-sterilized seeds of *M. malabathricum* was sown on a sterilized vermiculite bed to wet them with 1/4 Hoagland's No. 2

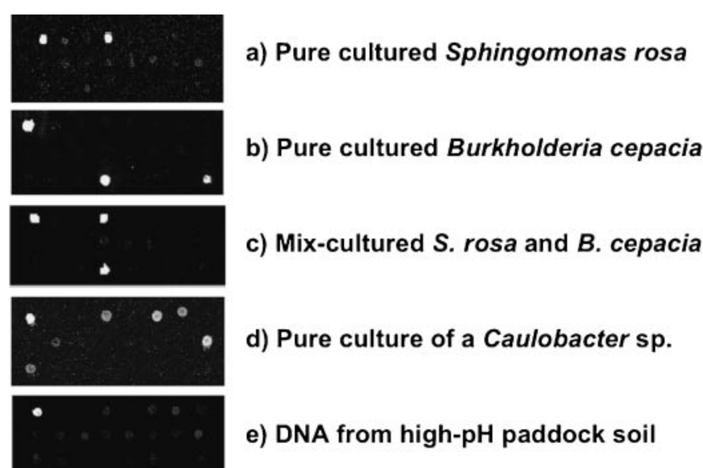

Fig. 3. DNA Array Patterns of Pure and Mixed Cultures of Reference Bacteria and Trial Samples.
(a) *Sphingomonas rosa* EC-K013, the DNA of which was directly extracted from colony pure-cultured in gellan gum-base soft gel medium at 23 °C for one week in the dark. (b) *Burkholderia cepacia* EC-K014, and (c) mix-cultured *S. rosa* and *B. cepacia*, both pure- and mix-cultured under the same conditions as (a). (d) *Caulobacter* sp. isolated from *Plantago lanceolata*,[29] and (e) DNA from wet soil that possessed relatively high pH but in which rice productivity was low. The soil contained dead roots and a relatively large mass of organic matter.

adjusted at pH 3.0, and left for germination and seedling growth in a plant growth chamber at 25 °C. After the 3-month seedlings were harvested, their root washings were inoculated to Hoagland's No. 2 plus 0.2% sucrose liquid medium (pH 5.2), and left for 72 h of incubation at 28 °C in the dark.

Results

Distinguishable detection of sphingomonads in pure and mixed cultures with different soil bacteria

Single cultures of *Sphingomonas rosa* EC-K013 incubated in N-free Winogradsky's mineral mixture-base soft gel medium (pH 6.2) were directly extracted from the layered colony with the gel matrix in the medium and tested for the DNA array (Fig. 3a). The array analysis showed specific hybridization to the array probe, which was designed for *S. rosa* (spot no. 4). In the case of *Burkholderia cepacia* EC-K014, it also showed a single hybridization at *B. cepacia* (spot no. 20 in Fig. 3b). Similar specificity was also observed on a *Herbaspirillum* sp. isolated from stem marrow of *Scleria sumatrensis* and another isolate of *Sphingomonas* sp. from rhizosphere of Kalimantan local rice. These *Herbaspirillum* sp. and *Sphingomonas* sp. selectively hybridized with spots no. 4 and no. 21 respectively, although the hybridization intensity was less than 50% of the reference spot.

Thus the array probes (72 mer) along with sequence variation, as shown in Fig. 1, were sufficient to distinguish different subclasses of bacteria. Also, our DNA array clearly showed a sufficient performance for distinction of *S. rosa* EC-K013 from other species of *Sphingomonas* and other genera of Sphingomonadaceae. Hence, we concluded that our DNA array showed sufficient performance for the distinction of sphingomonads and other genera of Sphingomonadaceae. DNA from the mixed cultured medium of *S. rosa* EC-K013 and *B. cepacia* EC-K014 showed selective hybridization on the spots (nos. 4 and 20) with almost equivalent intensity of fluorescence (Fig. 3c).

This DNA array method is certainly unable to distinguish sphingomonads at the species level because some probes showed cross-hybridization on some probes. For instance, *Caulobacter* sp. (AB086019) isolated from the rhizoplane of a *Plantago lanceolata* specimen sampled in a sandy coastal area of Ishikari, Hokkaido, Japan,[29] showed a distinguishable hybridization to the probe of *Caulobacter leidyi* (spot no. 16) with 49% intensity to spot no. 1, but it also hybridized with several *Sphingomonas* (spots nos. 4, 6, 7, 10, and 17, with hybridization intensities of 3–31%). Despite the non-specific hybridization to some *Sphingomonas* spp. (Fig. 3d), significant hybridization of the *Caulobacter* sp. to spot no. 16 suggests the availability of the DNA array probe for detection of sphingomonads. In a paddock soil showing a relatively high soil pH (6.1–6.2) due to water-logged conditions through the dry seasons, DNA successfully extracted from the sampled soil gave a sole fluorescence at spot no. 1 for the common region of proteobacteria (Fig. 3e).

Failure to detect sphingomonads in bulky, acidic paddock soils in South Kalimantan

We failed to extract bacterial DNA directly from acidic paddock soil by the several procedures described above, but we were successful in extracting eDNA from bulky paddock soil that contained fragments of dead rice roots, enabling us to obtain PCR products for the 16S rRNA gene of proteobacteria. In the DNA array analysis, a high intensity of fluorescence was observed only at spot no. 1 for a common sequence of proteobacteria. Although we have observed many *Burkholderia* spp. and sphingomonads as the most representative bacterial

Table 1. DNA Array Patterns of the Rhizospherous Microflora on an Acid-Tolerant Local Rice Variety

	a) Pot-grown *Melastoma* (cultured root washings)	b) Local rice root (physically scraped)	c) Local rice root (treated with lysozyme)	d) Rice stem residues (brown-colored)	e) Slime in stagnant water (red-colored slime)	f) Black matters in soil surface (*Juncus* root residues)	g) *Juncus* sp. root (physically scraped)	h) *Juncus* sp. root (treated with lysozyme)
Consensus sequence	100	100	100	100	100	100	100	100
S. yanoikuyae	0.1	0	0.2	0	0.1	0.3	0.8	0.2
S. chlorophenolicum	0	0	0	0	0	0	0	0
S. rosa	20.1	5.8	2.4	3.5	0.6	13.5	4.4	2.4
S. ursincola	0	0	0	0	0	0	0	0
S. echinoides	0	0	0	0.1	0	0.1	0.3	0
S. adhaesiva	1.4	0	0	0.1	0	0	0.1	0
S. aurantiaca	4.9	0	0	0	0	0	0	0
S. aerolata	2.3	0	0	0	0	0.1	0.1	0
S. pituitos	68.7	0.1	0	0	0	0.1	0.3	0
S. paucimobilis	45.3	0	0	0	0	0.1	0.1	0
S. capsulata	0	1.5	3.9	0.9	0.2	21.2	7.2	3.9
S. aromaticivorans	0	6.0	9.0	2.2	2.2	26.1	17.4	9.0
S. stygialis	0.8	4.9	10.9	1.8	2.0	30.7	18.3	10.9
Porphyrobacter	0	0	0	0	0	0.1	0	0
Caulobacter leidyi	0	0.1	0.2	0.2	0.1	0.2	0.5	0.2
Caulobacter henricii	0	0.6	1.0	1.1	0.4	0.5	5.0	1.0
Brevundimonas	0	0.2	0.1	0.2	0.2	0.2	1.4	0.1
Blastobacter	0.2	0	0	0	0	0	0.2	0
Burkholderia spp.	0	9.8	8.4	11.4	3.2	6.7	11.4	8.4
Herbaspirillum spp.	0	16.1	21.8	21.4	11.7	7.3	20.0	21.8
Rhizobium spp.	0	0.1	0.1	0.3	0.2	0	0.3	0.1

DNA extraction was attempted in several samples to be subjected to PCR amplification for labeling. The DNA sources were as follows: (a) Root washings of a pot-grown *Melastoma malabathricum* sapling under acidified, nitrogen-starving conditions (vermiculite bed wet with N-free 1/4 Hoagland no. 2 of pH 3.0) were shaken in Hoagland no. 2 plus 0.2% sucrose at 28 °C for 3 days and subjected to DNA extraction as described. (b) Rice roots soaked in sterilized water in a small polyethylene bag were mechanically damaged on the surfaces over polyethylene film of the plastic bag to yield tiny, floating substances, and the resulting water phase with floating substances was immediately collected by centrifugation and subjected to DNA extraction, while (c) rice roots were treated with lysozyme at 40 °C for 30 min in a polyethylene bag, and then physically scraped as described above. (d) Rice straw after harvest, plant residues at the base of which possessed a brown-colored slime inside and outside; the residues was directly extracted for DNA preparation. (e) Slime in the acidic, stagnant water in the paddock were collected from utricularia (a bladderwort) leaves floating in the paddock water, since a large mass of slime often aggregates on aquatic plants. (f) Black-colored organic matter is distributed throughout the shallow part of paddock soil. Probably, this organic matter is the residue of the *Juncus* root mat, but its original source has not yet been identified. In the direct DNA extraction method, a DNA sample applicable for DNA array was effectively obtained. (g) Physical scraping of *Juncus* sp. root soaked in sterilized water in a small polyethylene bag was done with as rice roots in (b), and (h) *Juncus* sp. roots in a polyethylene bag were treated with lysozyme at 40 °C for 30 min, as in (c). The relative fluorescent intensity of each spot is shown by a calculated percentage toward reference spot no. 1 for the common sequence of proteobacteria. Significant hybridization (10% or more of the reference spot) is indicated by screen tone on the back, and framed bacteria in the Table are likely to be close to major bacterial composers among the investigated habitats in the acidic paddock.

genera in the rhizosphere of local rice plants grown in acid-sulfate paddocks in several Kalimantan regions,[1,16] neither the paddock soil nor the paddock soil with dead rice root residues showed any evidence that sphingomonads inhabit paddock soil.

Bacterial components inhabiting plain acidic paddock soil were therefore cultured in 1 × Hoagland's No. 2 plus 0.2% sucrose (pH 5.2) and incubated for 3 d to recover cultured soil bacteria. This liquid medium is an effective one which enables selective culturing of oligotrophic bacteria that originally inhabited acidic paddock soil. Almost all of the sphingomonads isolated from the rhizoplane of local rice plants grown in acid-sulfate soil paddocks in South Kalimantan were oligotrophic bacteria and showed high growth performance in the Hoagland's No. 2 medium. In contrast, many of the β- and γ-proteobacteria that were found in the rhizoplane as auxotrophs grew slowly in the medium. Hence it was speculated that incubation of the paddock soils under oligotrophic conditions is favorable for good cell growth of sphingomonads possibly inhabiting the acidic soil. In consequence, however, the DNA sample that was extracted from the cultured oligotrophic bacteria did not show any hybridization toward probes for sphingomonads. The intense fluorescence observed clearly indicated that this DNA sample hybridized with probes specific to a common sequence of proteobacteria (spot no. 1) and *Burkholderia* spp. only (spot no. 20) (Fig. 2).

By the same process, we also tested rhizospherous microflora of *Melastoma malabathricum* seedlings grown in an acidic nursery bed. In contrast to the bacterial culture from the soil, this was more specific to sphingomonads. Based on DNA array analysis, a sphingomonad close to *Sphingomonas pituitos* was a major microfloral component in this bacterial culture and rhizospherous microflora of the *M. malabathricum* seedlings (Table 1, a).

Detection of sphingomonads as predominant microfloral components in the rhizosphere of local rice varieties grown in the acidic paddock in South Kalimantan

The DNA array technique indicated that sphingomonads are one of the common microfloral components in the rhizosphere of a local rice variety, Siam Unus, that is cultivated in acid-sulfate paddocks in the Gambut area of South Kalimantan. The microfloral components from local rice rhizosphere showed the best performance in hybridization to the DNA array probe of *Herbasprillum* spp. (spot no. 21). Furthermore, those of *Sphingomonas rosa* (spot no. 4), *S. aromaticivorans* (spot no. 13), *S. capsulata* (spot no. 12), *S. stygialis* (spot no. 14), and *Burkholderia* spp. (spot no. 20) also showed significant hybridizing intensities for major microfloral composers (Table 1, b and c). This DNA array pattern was reproducible in DNA obtained from the surfaces of

root fragments that were either treated with lysozyme or physically scraped.

As shown in Table 1 (d and e), samples obtained from slime and rice stem residues that were floating on stagnant water did produce significant hybridizing spots on probes for sphingomonads. In contrast, the blackish organic-matter-like mat that is often found in shallow parts of the bulky soil (less than 5 cm deep) in the *Juncus* sp.-emerging paddock definitely possessed several sphingomonads. Specifically, DNA from this sample hybridized with probes for *S. rosa*, *S. capsulata*, *S. stygialis*, and *S. aromaticivorans* as spots nos. 4, 12, 13, and 14 respectively (Table 1, f).

Rhizosphere of a wild Juncus *sp. grown in the acidic paddock in South Kalimantan, a habitat of sphingomonads*

The acidic paddocks in Gambut often overflood and are therefore not suitable for rice transplanting due to an excessively high water table. Hence, from September until mid March, farmers do not cultivate rice in the flooded paddocks.[2] During the rainy season, the stagnant water in the paddocks usually showed a pH range of 3.7–4.1, a level that is relatively moderate but still acidic. In the rainy season, a *Juncus* sp. (family Juncaceae) predominantly emerges as spontaneous vegetation in the paddock, and it grows as an aquatic emergent plant which covers the paddock almost completely within a 1-month time frame. Rhizoplane-attaching bacterial communities inhabiting the root surface of the wild *Juncus* sp. were obtained with lysozyme-treatment and/or mechanical scraping from 10 root fragments. Including the root tip, samples were 2–4 cm in total length. Rhizobacterial DNAs were extracted directly from the components using a DNA extraction kit. The template was applied to labeling with PCR and successive DNA array analysis for specific detection of sphingomonads. DNA array analysis resulted in a pattern similar to those of the rhizoplane microflora of local rice varieties and the blackish mat described above. The rhizoplane microflora of *Juncus* sp. obtained by the physical scraping method showed relatively high hybridization to probes that were specific for *Herbaspirillum* spp. (spot no. 21), *S. stygialis* (spot no. 13), and *S. aromaticivorans* (spot no. 14), as shown in Table 1 (g and h).

Discussion

Previous studies have led researchers to conclude that many sphingomonads are soil bacteria.[5,7,9,33] However, our investigations of sphingomonads obtained from the root surface of South Kalimantan rice varieties have led us to speculate that the root-attaching sphingomonads did not come from the soil. In fact, sphingomonads that are characteristically found in acidic soil regions in Central and South Kalimantan are always isolated from the rhizosphere or in specified plant surfaces growing in the acidic soil.[1,16] DNA directly extracted from the soil of local paddocks that possessed higher soil pH (6.1–6.2) hybridized only with spot no. 1 for the common region of proteobacteria. This suggests that the neutral paddock soil contains different groups of soil bacteria (*e.g.*, γ- and δ-proteobacteria) as major microfloral composers rather than Sphingomonadaceae and β-proteobacteria common in acidic soil (*Burkholderia* spp. and *Herbaspirillum* spp.).

The DNA array technique, which facilitates detection of sphingomonads, was found to be a powerful tool applicable in the present study as a method of characterizing the ecological behaviors of rhizobacteria in the acid-tolerant local rice varieties. The aforementioned results indicate that these sphingomonads cannot survive in highly acidic, bulky soil without the presence of living plant roots, unlike *Burkholderia* spp. This was suggestive, because we have often isolated *Burkholderia* spp., such as *B. cepacia* and *B. tropicalis*, from the acidic paddock soil (pH 3.0–3.3), while sphingomonads were from the rhizosphere and rhizoplane of local acidic soil-adapting plants in bacterial screening on Winogradsky's agar plate (unpublished data). Hence it is reasonable to conclude that the surfaces of the living roots in the acidic soil are a source of plant-associating sphingomonads and some other rhizobacteria, as well as of certain plant residues there.

At the investigation site, the local multiple-transplanting system in the rice cultivation process is inherited from former generations.[1] This nursery system is advantageous for a stepwise adaptation of the nursery plants to low pH.[34] During this off-season of rice cultivation (November to March), water-emergent *Juncus* grows densely and finally occupies the acidic paddocks to form a pure community and a thick root mat there. At the beginning of the rice transplanting season, local farmers cut and clean off the aboveground part of the *Juncus* community with large sickles, but the farmers never turn over the paddock soil. Considering that this rice cultivation process is uniquely developed and maintained in the local, acidic soil regions, transplanted rice in the rainy season has chance enough to receive functional rhizobacteria from the predominant weeds, and their roots reside in the paddocks. In the case of sphingomonads in South Kalimantan paddocks, this horizontal transmission-like spreading of rhizospherous sphingomonads is not a spontaneous event, but rather the outcome of human activity in the local farming system.

Juncus spp. are known to be a representative acidic soil indicator,[35] and this genus can adapt to acidic soil conditions with several acid-tolerant strategies, including bacterial plaque on the rhizoplane.[36] In a similar manner, functional rhizobacteria might be involved in the surviving strategy of the wild *Juncus* community in the acidic soil paddocks of South Kalimantan. In the *Juncus* sp.-emerging paddock, blackish organic matter often accumulates in a shallow part of the bulk soil, and our DNA array analysis showed that this organic

matter possesses high populations of sphingomonads (Table 1, f). In the paddocks, the aboveground portion of the *Juncus* plants is eliminated before rice transplanting in March, at which point most of the current-year root mat of the *Juncus* community has already turned into a condensed, organic mass, while DNA array analysis revealed that regenerated *Juncus* communities possess a high population of sphingomonads in the rhizosphere (Table 1, g and h). *Via* this organic matter in soil and/or directly from the rhizosphere of the living *Juncus* community, a major part of the sphingomonads are probably supplied to the rhizosphere of transplanted local rice varieties.

Hasegawa *et al.* have reported that rice productivity is positively correlated to soil acidity in the local paddocks in the Gambut area of South Kalimantan.[1] In addition, many of the highly productive local rice varieties in acidic soils also possess sphingomonads as one of their major rhizospherous microfloral composers. These are tolerant to acidic conditions.[1,16] Taken together, certain contributions of the rhizospheous sphingomonads to rice production were tentatively inferred.[16,37] In fact, in ammonia-assimilating *Melastoma malabathricum*, it has been found that *S. rosa* EC-K013 promotes the growth of saplings under nitrogen-starving and strongly acidic (pH 3.0) nursery conditions.[38]

Our sphingomonad-detecting DNA array probes made it possible to investigate and pursue microfloral succession in the rhizosphere of several acid-tolerant plants, since the rhizospherous sphingomonads are most likely to be a mediator in the rhizosphere of acid-tolerant plants in South and Central Kalimantan.[16] We were thus able effectively to monitor microfloral succession, which was affected by the growth stage of the host plant and/or the external environment. Among the group of sphingomonads, the hypervariable region V6 in the 16S rRNA gene varies widely at the species level, and this sequence variation, distinguishable among many of the sphingomonads, was most beneficial for designing sphingomonad-detecting DNA array probes. Although precise data collection for each bacterial strain used for probe design is necessary along with several improvements in the array probes, including shortening the probe size to delete 20–30 bases from both 5′- and 3′-terminals in the V6 region, the DNA array technique is useful tool to investigate the ecological behavior of sphingomonads. Using these DNA array probes, answers to questions about the ecological roles of rhizospherous sphingomonads in acid-tolerant plants in Kalimantan and other areas should be obtained in the near future.

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