

Presumptive Differentiation of Phytopathogenic and Non-pathogenic Bacteria by Improved Rapid-Extraction TLC Method

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<https://doi.org/10.5109/14029>

出版情報：九州大学大学院農学研究院紀要. 54 (1), pp.1-11, 2009-02-27. Faculty of Agriculture, Kyushu University

バージョン：

権利関係：

Presumptive Differentiation of Phytopathogenic and Non-pathogenic Bacteria by Improved Rapid-Extraction TLC Method

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(Received October 23, 2008 and accepted December 5, 2008)

TLC profiles of aminolipids extracted from phytopathogenic and non-pathogenic bacteria with chloroform–methanol–0.3%NaCl (2:1:0.2, v/v/v) or 2–propanol are useful for discrimination of bacteria. For many bacteria, each TLC profile is genus or species specific and highly reproducible. For most gram–negative bacteria, the uppermost spot (Up) appeared at ca. R_f 0.7 on the chromatograms developed with chloroform–methanol–0.2% CaCl₂·2H₂O (55:35:8, v/v/v). This spot was absent on the chromatograms of gram–positive bacteria, *Clavibacter michiganensis*. The profiles of *Agrobacterium* spp. and *Rhizobium* spp. were different from other gram–negative bacteria with the uppermost spots at ca. R_f 0.75. For the case of *Agrobacterium* spp., the chromatograms of the strains belonging to the same biovar were identical. Distinct differences were found among the profiles of *Agrobacterium* biovar 1, *A.* biovar 2, *A.* biovar 3 and *A. rubi*. The profiles of *Rhizobium* spp., except for *R. tropici*, and their relatives such as *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium* spp. were quite simple and different from those of *Agrobacterium* spp. For the case of *Burkholderia* species, except for *B. andropogonis*, three spots (designated as S1, S2, S3) appeared under the uppermost spot (Up) and their profiles were species specific for several species such as *B. plantarii* and *B. caryophylli*. On the chromatogram of *B. andropogonis*, the S1 spot (non–phosphorous) was absent and the S3 spot was faint. The profiles of 96 *Ralstonia solanacearum* strains from various sources were identical. For the case of *Erwinia carotovora* an intensive benchmark spot appeared at R_f 0.64 but this spot was absent on the chromatograms of pathovars of *E. chrysanthemi* and *E. herbicola*. Clear diversity in profiles was observed between *Xanthomonas campestris* and *X. oryzae*. The profile of pathovars of *Pseudomonas syringae* were identical and simple. Substitution of chloroform solvent systems with less hazardous organic solvents was tested. 2–propanol for the lipid extraction and 1–butanol–acetic acid–water (3:1:1 and 5:3:1, v/v/v) for the developing solvents were usable, though development with the butanol systems was highly time–consuming.

INTRODUCTION

A number of scientists have stated that the phylogenetic classification should be based on the concept of polyphasic taxonomy (Colwell, 1970; Gills *et al.*, 1995; Vandamme *et al.*, 1996). The identification of microorganisms should also be practiced using the same polyphasic concepts. However, the presumptive differentiation of microorganisms requires the establishment of one or several benchmarks that are easy to detect, highly reproducible and consistent with the phylogenetic classification. Much effort has been applied to ascertain the suitability of various cell wall and/or membrane components such as peptidoglycans, proteins, and fatty acids as markers for preliminary differentiation of bacteria (Suzuki *et al.*, 1993; Schleifer and Kandler, 1972; Dristig and Dianese, 1990; Li and Hayward, 1994; Chase *et al.*, 1992; De Boer and Sasser, 1986; Bouzar *et al.*, 1993; Stead,

1992; Wells *et al.*, 1993; Margaret, 1988; Galbraith and Wilkinson, 1991; Sawada *et al.*, 1992; Kori *et al.*, 1992; Jarvis *et al.*, 1996; Young *et al.*, 1992). Meanwhile in the clinical field, simplified identification of aerobic and infectious actinomycetes such as genus *Nocardia* with diaminopimelic acid (DAP) isomers was performed successfully by using thin–layer chromatography (Staneck and Roberts, 1974). This procedure is now well established and used as a routine test for the identification of the actinomycetes.

In 1993, the application of the direct colony thin–layer chromatography (invented by Matsuyama *et al.*, 1986) for the presumptive differentiation of phytopathogenic bacteria was firstly performed (Matsuyama *et al.*, 1993a, b, c, d). This method was then simplified and developed into a rapid extraction TLC method (Khan and Matsuyama, 1998). This novel method was improved well (Matsuyama *et al.*, 2003a, b; Furuya *et al.*, 2004) and has been used for obtaining the fingerprint profiles of phytopathogenic bacteria and some of non–pathogenic bacteria. The details will be presented in this report.

MATERIALS AND METHODS

Bacterial isolates tested

As can be seen in Table 1, a total of 315 strains, pre-

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Table 1. List of bacteria tested

Genus & Species	Strains
(Gram positive bacteria)	
<i>Bacillus subtilis</i>	NBRC 13719 [†] , AKU-AB89, AKU-AN83530, NIAES1702, AKU558
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	NBRC 12471, NBRC 13762, NIAS-N6206, NIAS-N6601 (t)
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	ATCC 33113 [†] , NBRC 13763, NBRC 13764 (po)
<i>C. michiganensis</i> subsp. <i>nebraskens</i>	ATCC 27794 [†] (co)
(Gram negative bacteria)	
<i>Acidovorax avenae</i> subsp. <i>avenae</i>	ATCC 19860 [†] , HNAES-H8206, HNAES-H8210, HNAES-H8505 (r)
* <i>Agrobacterium</i> biovar 1 (= <i>A. tumefaciens</i>)	ATCC 23308 [†] (Ti), NIAS-Ku7415 (ro.,Ti), MAFF 301001 (<i>Prunus</i> sp.,Ti), MAFF 301222, MAFF 301223 (mar.,Ti), MAFF 301224 (ro.,Ti), MAFF 301276, MAFF 301277, MAFF 301278 (chry.,Ti), MAFF 106577, MAFF 106578, MAFF 106579, MAFF 106580, MAFF 106581, MAFF 106582, MAFF 106583, MAFF 106584, MAFF 106585, MAFF 106586 (mel.,Ri), NBRC 14555, NBRC 15188, NBRC 15191 (mel.,Ri), MAFF 520014, MAFF 520019, MAFF 520020 (non-pathogenic, cu.rhiz.)
<i>Agrobacterium</i> biovar 2 (= <i>A. rhizogenes</i>)	ATCC 11325 [†] (app.,Ri), NIAS-Ku7411 (ro.,Ri), NBRC 14793, NBRC 15196, NBRC 15198, NBRC 15201 (ro.,Ti), MAFF 301279, MAFF 301280, MAFF 301539 (ro.,Ti), Kerr84 (non-pathogenic, soil)
<i>Agrobacterium</i> biovar 3 (= <i>A. vitis</i>)	NBRC 15140 [†] , MAFF 302147, MAFF 302148, MAFF 302149, MAFF 302150, MAFF 302297, MAFF 302652 (g.v.,Ti)
<i>A. rubi</i>	NBRC 13261 [†] , NBRC 13260 (<i>Rubus</i> sp.,Ti)
Unclassified agrobacteria (cherry)	NBRC 15292, NBRC 15293, NBRC 15294, NBRC 19295 (cher.,Ti), Sa-4 (cher.,Ti)
Unclassified agrobacteria (kiwi)	NBRC 15296, NBRC 15297 (kw.,Ti)
<i>Bradyrhizobium japonicum</i>	NBRC 15001 (root nodule, soy) (<i>R. japonicum</i>)
<i>Mesorhizobium huakuii</i>	NBRC 15243 [†] (root nodule, m.vetch.) (<i>R. huakuii</i>)
<i>Rhizobium etli</i>	NBRC 15573 [†] (root nodule, kid.b.)
<i>R. galegae</i>	NBRC 14965 [†] (root nodule, <i>Galega orientalis</i>)
<i>R. leguminosarum</i>	NBRC 14778 [†] , NBRC 13337 (root nodule, w.clov.)
<i>R. phaseoli</i>	NBRC 14168, NBRC 14994 (root nodule, kid.b.)
<i>R. tropici</i>	NBRC 15247 [†] (root nodule, kid.b.)
<i>Sinorhizobium meliloti</i>	NBRC 14782 [†] (root nodule, med.) (<i>R. meliloti</i>)
<i>Burkholderia andropogonis</i>	JCM 10487 [†] , MAFF 301006 (su.g), MAFF 301129 (tu)
<i>B. caryophylli</i>	ATCC 25418 [†] , NIAS1192, NIAS 1406, MAFF 301060, MAFF 302555 (car), AKU-Yame1 (r.p.g.)
<i>B. cepacia</i> complex	ATCC 25416 [†] (o), TARC 243-4, TARC 356-3, TARC 356-5 (o), Pc4 (le.rhiz.), Pc6W (c.ca.rhiz.), Pc13, Pc14, Pc16, Pc17M-1, Pc17M-2 (w.o.rhiz.), Pc20, Pc22w, Pc23, Pc24 (soy.rhiz.), Pc28, Pc29, Pc30, Pc33SW, Pc34 (bar.rhiz.), Pc35, Pc36, Pc39, Pc40, Pc40M, Pc41, Pc42, Pc43 (to.rhiz.), Pc518 (f.soil), Pc685(H), Pc2046 (H), Pc2423 (unknown) [Genomvar 1], Pc639(H, 3-B), Pc1211 (H, 3-B), Pc1751(H, 3-A), Pc2046 (H, 3-A), Pc3018 (H, 3-B), MAFF 302528 (r. rhiz, 3-B) [Genomvar 3]
<i>B. gladioli</i> pv. <i>alliicola</i>	ATCC 19302 [†] (o)
<i>B. gladioli</i> pv. <i>gladioli</i>	ATCC 10248 [†] (gla), MAFF 302515 (tu), MAFF 302537 (o), MAFF 302544 (r), MAFF 301580 (den), NIAS1064, NIAS 1065 (fr)
<i>B. gladioli</i>	MAFF 302409 (a.b.), MAFF 302418 (g.g), MAFF 302424 (cy), AKU-H-1, AKU-H-2 (r)
<i>B. glumae</i>	MAFF 301169 [†] , AKU-Ku8104, AKU-Ku8112, AKU-Ku8114, NIAES-N7503, NIAES-N7504, KNAES 2, KNAES 8012, KNAES 8015, KNAES 8020, Kyu82-34-2, AZ8224, AZ84448, AKU-T-2, AKU-T-7 (r)
<i>B. plantarii</i>	JCM 5492 [†] , MAFF 302387, MAFF 302392, MAFF 302412, MAFF 302467, MAFF 302470, MAFF 302475, MAFF 302481, MAFF 302484, MAFF 302485, AZ8201(r)
<i>B. vandii</i>	JCM 7957 [†] (va)
<i>Comamonas acidovorans</i>	ATCC 15668 [†] (soil)
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>	ATCC 33260 [†] (po)
<i>E. carotovora</i> subsp. <i>carotovora</i>	ATCC 15713 [†] (po), NIAS-EH8519 (cu), NIAS-N7101 (s.pe), NIAS-N7129 (rad), LSPPM 473-1 (c.ca), LSPPM 489-4 (ca), LSPPM 493-1 (po), IU 645ar (c.ca)
<i>E. chrysanthemi</i> pv. <i>chrysanthemi</i>	SU E8301 (ep), AKU-Ku8601 L1 (pear), TUA-Ichihara 1-1 (pear), SU-Ech44
<i>E. chrysanthemi</i> pv. <i>dianthicola</i>	Dianthi 1e, 2n (car)
<i>E. chrysanthemi</i> pv. <i>zeae</i>	NCPPB 377 [†] , R-7, R-8, Corn801 (co), ALE829p
<i>E. herbicola</i> pv. <i>milletiae</i>	NIAS 1 (Japanese wisteria)
<i>Herbaspirillum rubrisubalbicans</i>	ATCC 19308 [†] , MAFF 301626, MAFF 301628 (s.c)
<i>Pseudomonas aeruginosa</i>	ATCC 7700 [†]
<i>P. fluorescens</i>	ATCC 13525 [†] , ATCC 520049
<i>P. syringae</i> pv. <i>lachrymans</i>	NIAS1319, NIAS1321 (cu)
<i>P. syringae</i> pv. <i>pisi</i>	MAFF 301211, MAFF 301213 (pea)
<i>P. syringae</i> pv. <i>tabaci</i>	KTES-PA28, NIAS-P41, AKU-Ku8102
<i>P. syringae</i> pv. <i>syringae</i>	ATCC 19310 [†] , NIAS I
<i>Ralstonia solanacearum</i>	ATCC 11696 [†] , AKU 6211 (t), AKU 6224 (t), AKU 6227 (t), AKU 6237 (ma), AKU 6257 (ep), AKU 6277 (po), AKU 6303 (ep), AKU 6509 (to), AKU 6511 (ep), AKU 6513 (ep), AKU 6515 (t), AKU 7501-1 (ep), MAFF 301559 (po), NESVOT8109, NESVOT8202 (s.p), NESVOT8224, NESVOT7601-1 (ep), NESVOT7602-1 (t), KTES-C319-SR, BY-4 (to), Brasil; 13, 72, 92, 128, 788, 800, 578, 613, 799, 933, 61, 113, 131, 66, 67, 98, 106, 964, 982, 1005 (po), 7, 127, 162, 468, 582, 20, 629 (pe), 129, 656 (cu), 19, 630, 633, 31, 33, 35, 855, 798, 1033, 62, 76, 985, 140, 290, 535, 49, 1102, 1103, 1104 (t), 555 (to), 51, 56, 71, 79, 87, 795, 796, 804, 858, 534 (ep), 574, 575, 576, 577, 579, 603 (eu), 47 (S.g), 73, 401, 655, 661, 821 (ba), Bangladesh; C1 (chi), E1 (ep), P1 (po), T1 (t)

Table 1. Continued

Genus & Species	Strains
<i>R. pickettii</i>	ATCC 27511 ^r (H)
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	ATCC 33913 ^r (b.s), NIAS I
<i>X. campestris</i> pv. <i>citri</i>	N6113-1, N6829-1-3, N6831-1-1, Kawa (ci) (<i>X. axonopodis</i> pv. <i>citri</i>)
<i>X. campestris</i> pv. <i>cucurbitae</i>	Ishikawa I
<i>X. campestris</i> pv. <i>pisi</i>	NIAS II
<i>X. campestris</i> pv. <i>pruni</i>	NIAS 1-10-1
<i>X. campestris</i> pv. <i>vitians</i>	NIAS I
<i>X. oryzae</i> pv. <i>oryzae</i>	ATCC 35933 ^r , Q75114, Q7781, Q7602, Q7660, T7144 (r)

Note: Alphabetical abbreviation in the parentheses mean as follows;

a.b, adzuki bean; ba, bananas; bar.rhiz., barley rhizosphere; b.s, brussels sprout; ca:cabbage; car., carnation; c.ca, chinese cabbage; c.ca.rhiz., chinese cabbage rhizosphere; cher, cherry; ci, citrus; chi, chili; chry, chrysanthemum; co, corn; cu, cucumber; cy, cymbidium; den, dendrobium; ep, egg plant; eu, eucalyptus; f.soil, forest soil; fr, freesia; g.g, green gram; gla, gladiolus; g.v, grape vine; kid.b., kidney bean; le, lettuce; le.rhiz., lettuce rhizosphere; ma, marguerite; med, medicago; mel, melon; m.vetch, milky vetch; pe, pepper; po, potato; o, onion; ra, radish; r, rice; r.rhiz., rice rhizosphere; ro, rose; r.p.g., russell prairie gentian; s.c, sugarcane; S.g, *Solanum gilo*; s.g, sudan grass; s.p, sweet pepper; soy, soy bean; t, tomato; to, tobacco; to.rhiz., tobacco rhizosphere; tu, tulip; va, vanda; w.o, welsh onion; w.o.rhiz., welsh onion rhizosphere; w.clov., white clover; H, human (clinical); Ti, harboring Ti plasmid (tumorigenic); Ri, harboring Ri plasmid (rhizogenetic).

Abbreviation of institutions and culture collections:

AKU and Ku, Faculty of Agriculture, Kyushu University, Fukuoka, Japan; ATCC, American Type Culture Collection, USA; AZ, Dr. K. Azegami; HNAES, Hokuriku Agricultural Experiment Station, Jyoetsu, Japan; JCM, Japan Collection of Microorganisms, Saitama, Japan; KNAES and Kyu, Kyushu National Agricultural Experiment Station, Nishigoushi, Kumamoto, Japan; KTES, Kagoshima Tobacco Experimental Station, Japan; LSPPM, Ministry of Agriculture, Thailand; MAFF, Ministry of Agriculture, Forestry and Fishery, Japan; NBRC, Biological Resource Center, Chiba, Japan (IFO); NIAES, National Institute of Agro-environmental Sciences, Ibaraki, Japan; NIAS, National Institute of Agricultural Sciences, Tokyo, Japan (NIAES); NRSVOT, National Research Station of Vegetable Ornamental and Tea, Iwate, Japan; SU, Shizuoka University, Shizuoka, Japan; TUA, Tokyo University of Agriculture, Atsugi, Japan.

* The taxon of the genus *Agrobacterium* is indicated according to the nomenclature, biovar system (Kerstens and De Ley, 1984; Sawada *et al.*, 1995).

viously identified by standard tests, were used in this study.

Extraction of bacterial aminolipids and thin layer chromatography

The procedures for extraction of lipids from bacterial whole-cells and the development with the thin-layer chromatography (TLC) are as follows. Each bacterial isolate was cultured at 25 °C for 3 days on King's B (KB; peptone 20 g, K₂HPO₄ 1.5 g, MgSO₄·7H₂O 1.5 g, agar 15 g in 1 litre of 1% glycerol solution, pH 7.2, Eiken Co.) slant medium. For the *Xanthomonas* species, nutrient broth agar (NBA; beef extract 5 g, peptone 5 g, NaCl 5 g, agar 15 g in 1 litre of distilled water, pH 7.0) slant medium was also used. Since King's B medium was not suitable for culturing *Rhizobium etli*, the medium No. 805 (yeast extract 1 g, mannitol 5 g, K₂HPO₄ 0.7 g, KH₂PO₄ 0.1 g, MgSO₄·7H₂O 1 g, agar 15 g in 1 litre of distilled water, pH 7.0-7.2) was used.

Four loopfuls of bacterial cells were pasted onto the bottom of a small glass-vial (1 cm in diameter, 5 cm in length) and 1 ml of a chloroform-methanol-0.3% NaCl solution (2:1:0.2, v/v/v) was added. Bacterial cells were suspended roughly in the solution by stirring gently with the flame-sterilized loop. The vial was capped tightly and allowed to stand undisturbed for 30 min at room temperature. Forty μ l (70 μ l for the cases *Agrobacterium* and *Rhizobium* spp.) of the transparent lower chloroform-layer which contains lipids was taken with a micropipette and spotted onto the origin spots of a pre-coated silica gel TLC plate (Merck Co., Si 60, 0.25 mm in thickness). The origin spots were placed at 2.5 cm from the

bottom edge of the silica gel plate and separated 1.5 cm each other. To avoid the edge effects during TLC development, the spots at the extreme left and right sides were placed 2.5 cm from the respective edges of the plate. Development of the TLC plates was conducted in a rectangular glass tank (25 × 25 × 12 cm) sheeted inside with filter-papers moistened well with the developing solvents. One hundred ml of chloroform-methanol-0.2% CaCl₂·2H₂O (55:35:8, v/v/v) was used for development. The development was conducted at 25 °C for 90 min. Renewal of the solvent at each development and keeping vapor-saturation in the tank are preferable for reproducible results. After development, the TLC plate was dried for ca.10 min in a draft chamber. Aminolipids on TLC plate were detected by spraying of ninhydrin (Ninhydrin spray, Wako Chem. Co.) followed by heating at ca. 100 °C for 5 min in a small oven. The chromatograms obtained were recorded by a personal computer using a scanner and the resulting images treated with Adobe Photoshop 5.0 LE to adjust contrast and brightness for optimal visualization of spots.

Substitution of chloroform solvent systems with less hazardous organic solvents was tested (Daikohara *et al.*, 2003). Instead of chloroform-methanol-0.3% NaCl (2:1:0.2, v/v/v) solution for extraction of lipids, acetonitrile, methanol, ethylacetate and 2-propanol were individually tested. For the developing solvent, 14 solvent systems were tested. The constituents were as follows: hexane-ether-acetic acid (90:10:1, 90:20:1, v/v/v), 1-butanol-acetic acid-water (3:1:1, 5:3:1, v/v/v), heptanes-isopropylether-acetic acid (60:40:4, v/v/v), diisobutylketone-acetic acid-water (40:25:4, v/v/v), cyclohexane-

dioxane (7:3, v/v), ethylacetate–carbontetrachloride (6:4, v/v), 2–butanol–acetic acid–water (3:1:1, v/v/v), 2–Methyl–2–propanol (*tert*–Butyl alcohol)–acetic acid–water (5:3:1, v/v/v), phenol–water (8:2, v/v), 2–propanol–12% ammonia water (4:1, v/v), petroleum ether–diethyl ether–acetic acid (90:10:1, 10:20:1, v/v/v).

RESULTS

Comparison of the thin–layer chromatograms of aminolipids extracted from 315 phytopathogenic and non–pathogenic bacteria (Table 1) was conducted. TLC profiles were characteristic at the genus or species level and highly reproducible. This method is quite easy to practice and suitable for preliminary differentiation of various phytopathogenic bacteria.

Extraction of lipids and their development on silica gel plate

Chloroform–methanol–0.3% NaCl (2:1:0.2, v/v/v) and 2–propanol were suitable for the extraction of lipids from whole–cells of bacteria. For the development of the extracts on silica gel TLC plates, chloroform–methanol–0.2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (55:35:8, v/v/v) was optimal. Though butanol–acetic acid–water (3:1:1 and 5:3:1, v/v/v) is less hazardous, development with this solvent system gave different profiles with the chloroform solvent system, naturally, and required 3.5 hrs.

Reproducibility of chromatograms

The chromatographic profiles were highly reproducible when culture age and chromatographic conditions were uniformly maintained.

Chromatograms of the gram–positive and negative bacteria

As can be seen in Figs. 1, 2, 3, 8, striking differences

between the TLC profiles of gram–positive and negative bacteria were observed. For the chromatograms of gram–negative bacteria such as *Burkholderia*, *Ralstonia*, *Erwinia*, *Pseudomonas* and *Xanthomonas*, the uppermost spot (Up) appeared at ca. R_f 0.7. For genera *Agrobacterium* and *Rhizobium*, the uppermost spot appeared at ca. R_f 0.75. Below the uppermost spots various benchmark spots appeared. Their profiles were genus or species specific (Figs. 1–10).

For gram–positive bacteria such as *Clavibacter michiganensis* and *Bacillus subtilis*, spots at R_f 0.70–0.75 were absent. Though a spot appeared near R_f 0.7 for the case of *B. subtilis*, it was quite faint (Fig. 3).

Chromatograms of *Agrobacterium* and *Rhizobium* spp.

For the cases of *Agrobacterium* and *Rhizobium* species, the uppermost spots (Up) appeared at ca. R_f 0.75. Distinct diversities in chromatographic profiles were found among *A. biovar* 1, *A. biovar* 2, *A. biovar* 3 and *A. rubi* (Figs. 2–7). The profile corresponded clearly with each biovar and not with the symptoms generated by Ti or Ri plasmid. The profiles of the agrobacteria isolated from cherry and kiwi fruit (these strains were originally isolated by Sawada (1994) and have been reported as novel *Agrobacterium* spp.) resembled with the profiles of biovar 2 and *R. tropici*, though minor differences were found (Fig. 5). For example, the profiles of the strains from cherry and kiwi fruit were different each other with respect to the presence of R_f 0.65 spot (Fig. 5). The profile of *R. tropici* was almost the same as that of the cherry type. With the exception of the profile of *R. tropici*, the profiles of *R. leguminosarum* (Type species), *R. phaseoli*, *R. galegae*, *R. etli* and their relatives such as *Bradyrhizobium japonicum*, *Mesorhizobium huakuii*, *Sinorhizobium meliloti* were quite simple and obviously different from those of *Agrobacterium*

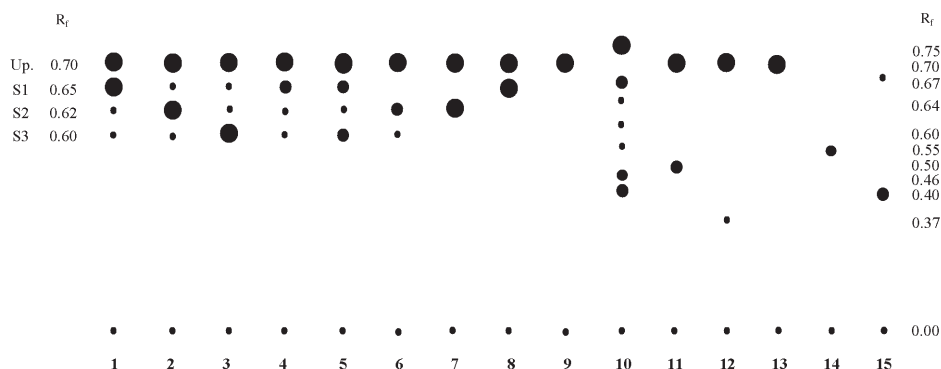


Fig. 1. Diagrammatic representations of TLC profiles of gram negative and positive bacteria.

1. *Burkholderia cepacia*, 2. *B. caryophylli*, 3. *B. plantarii* & *B. vandii*, 4. *B. gladioli*, 5. *B. glumae*, 6. *B. andropogonis*, 7. *Ralstonia solanacearum*, 8. *Erwinia carotovora*, 9. *E. chrysanthemi*, 10. *Agrobacterium* biovar 1, 11. *Xanthomonas campestris* pathovars, 12. *X. oryzae*, 13. *Pseudomonas syringae* pathovars, 14. *Clavibacter michiganensis* pathovars, 15. *Bacillus subtilis*.

Up: Uppermost spot appearing at R_f 0.70, S1, S2, S3: Benchmark spots appear on the TLC chromatograms of *Burkholderia* species. Diagrammatic representation was performed with the result for each type strain.

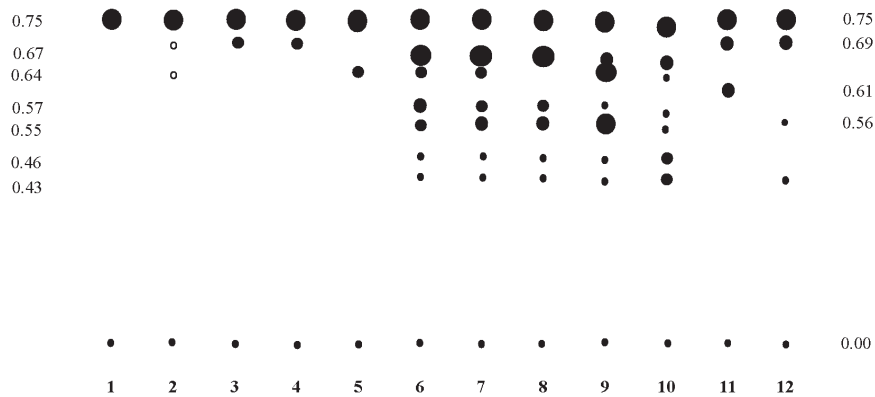


Fig. 2. Diagrammatic representations of TLC profiles of *Bradyrhizobium*, *Sinorhizobium*, *Rhizobium*, *Mesorhizobium* species and *Agrobacterium* species.

1. *Bradyrhizobium japonicum*, 2. *Sinorhizobium meliloti*, 3. *Rhizobium leguminosarum*, 4. *Rhizobium galegae*, 5. *Mesorhizobium huakuii*, 6. *R. tropici*, 7. *Agrobacterium* sp. (cherry), 8. *Agrobacterium* sp. (kiwi), 9. *Agrobacterium* biovar 2, 10. *Agrobacterium* biovar 1, 11. *Agrobacterium* biovar 3, 12. *Agrobacterium rubi*
Diagrammatic representation was performed with the result for each type strain.

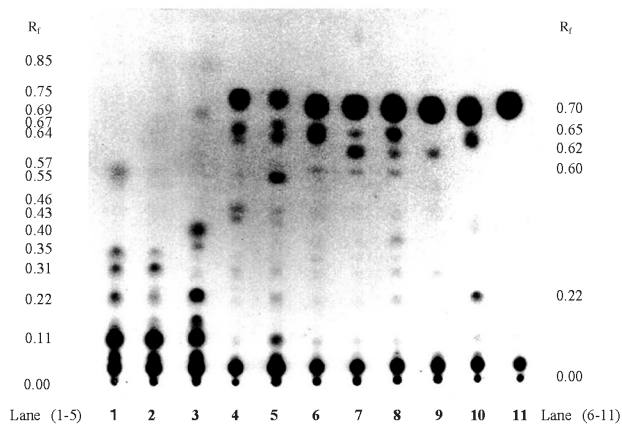


Fig. 3. Chromatograms of aminolipids isolated from phytopathogenic and non-pathogenic bacteria.

1. *Clavibacter michiganensis* subsp. *michiganensis* NBRC 12471, 2. *C. michiganensis* subsp. *sepedonicus* NBRC 13764^T, 3. *Bacillus subtilis* NBRC 13719^T, 4. *Agrobacterium* biovar 1 Ku7415 (Ti), 5. *A.* biovar 2 Ku7411 (Ri), 6. *Burkholderia cepacia* ATCC 25416^T, 7. *B. caryophylli* MAFF 301192, 8. *B. gladioli* H-1, 9. *Ralstonia solanacearum* MAFF 301559, 10. *Erwinia carotovora* subsp. *carotovora* 486-4, 11. *Pseudomonas syringae* pv. *syringae* I.

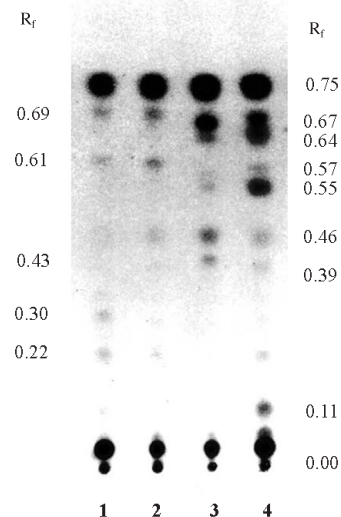


Fig. 4. Chromatograms of aminolipids isolated from various *Agrobacterium* species.

1. *Agrobacterium* biovar 3 MAFF 302150 (Ti), 2. *A.* biovar 3 MAFF 302297 (Ti), 3. *A.* biovar 1 Ku7415 (Ti), 4. *A.* biovar 2 Ku7411 (Ri).

spp. (Fig. 2, 5–7) in so far as the strains tested.

Chromatograms of *Burkholderia* species

Under the uppermost spot (Up, R_f 0.70), several spots designated as S1 (R_f 0.65), S2 (R_f 0.62) and S3 (R_f 0.60) appeared (Figs. 1, 3, 8, 9). The profiles of S1, S2, S3 spots were characteristic for some *Burkholderia* species. An intensive S3 spot was always observed on the chromatograms of *B. plantarii* and *B. vandii* (Figs. 1, 8). The profile of *B. caryophylli* was quite unique and its S2 spot always appeared intensively (Figs. 1, 3). An intensive S1 spot was characteristic for the profiles of the type strains of *B. gladioli* pv. *gladioli* and pv. *allii-cola*. However, this profile was not common for other

strains. No distinct differences between *B. gladioli* and *B. glumae* were observed, though the profiles of both type strains were different. The profiles of the *B. cepacia* complex were not uniform. The profile of the type strain (ATCC 25416^T) was highly reproducible and an intensive S1 spot always appeared (Figs. 1, 3, 8). This profile of the type strain, however, was inconsistent with the some profiles of clinical and environmental strains. The chromatogram of *B. andropogonis* was clearly different from those of other *Burkholderia* spp. In particular, the profile of *B. andropogonis* lacked an S1 spot (Fig. 9). By spraying of Dittmer's reagent (Dittmer and Lester, 1964), used for the detection of phosphate, we clarified that the Up, S2 and S3 spots are phosphatidyl aminolip-

ids, while S1 is not a phospholipid.

Chromatograms of *Ralstonia* species

A distinct benchmark spot at R_f 0.62 appeared without exception for 96 strains from various plant sources

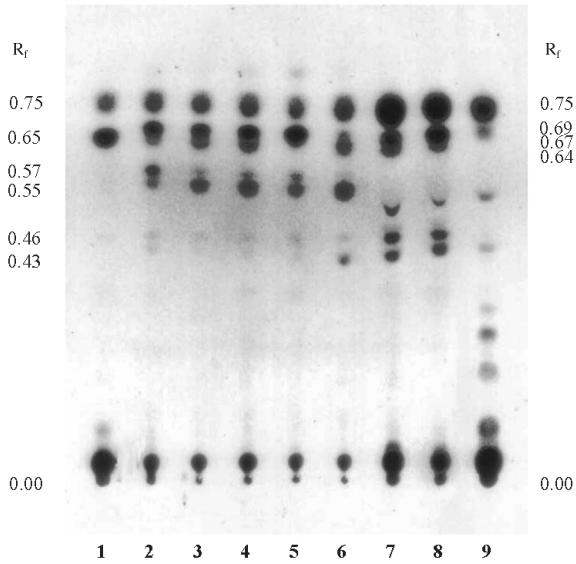


Fig. 5. Chromatograms of aminolipids isolated from *Mesorhizobium*, *Rhizobium* and *Agrobacterium* species. 1. *Mesorhizobium huakuii* NBRC 15243^T, 2. *Rhizobium tropici* NBRC 15247^T, 3. *Agrobacterium* sp. Sa-4 (cherry, Ti), 4. *Agrobacterium* sp. NBRC 15292 (cherry, Ti), 5. *Agrobacterium* sp. NBRC 15297 (kiwi, Ti), 6. *A.* biovar 2 ATCC 11325^T (Ri), 7. *A.* biovar 1 ATCC 23308^T (Ti), 8. *A.* biovar 1 Ku7415 (Ti), 9. *A. rubi* NBRC 13260.

(Figs. 1, 3, 9). No major differences in the profiles were observed between *R. solanacearum* and *R. pickettii*.

Chromatograms of *Erwinia* species

The benchmark spot of *E. carotovora* appeared at

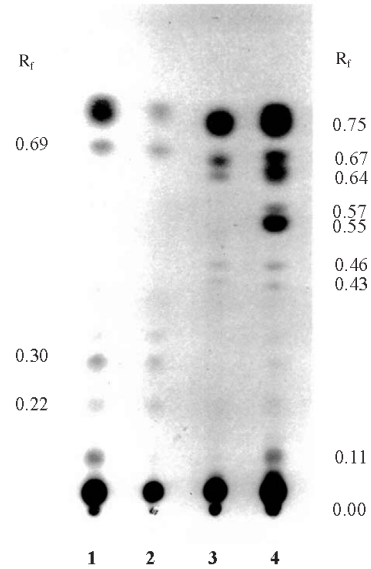


Fig. 6. Chromatograms of aminolipids isolated from *Rhizobium* and *Agrobacterium* species.

1. *Rhizobium leguminosarum* NBRC 13337, 2. *R. leguminosarum* NBRC 14778^T, 3. *Agrobacterium* biovar 1 Ku7415 (Ti), 4. *A.* biovar 2 Ku7411 (Ri).

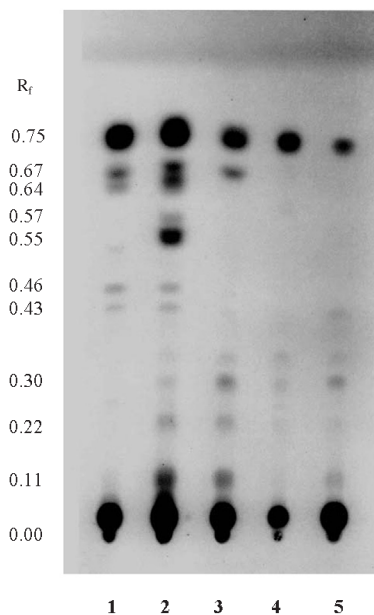


Fig. 7. Chromatograms of aminolipids isolated from *Agrobacterium* and *Rhizobium* species.

1. *Agrobacterium* biovar 1 Ku7415 (Ti), 2. *A.* biovar 2 Ku7411 (Ri), 3. *Rhizobium phaseoli* NBRC 14994, 4. *Sinorhizobium meliloti* NBRC 14782, 5. *Bradyrhizobium japonicum* NBRC 15001.

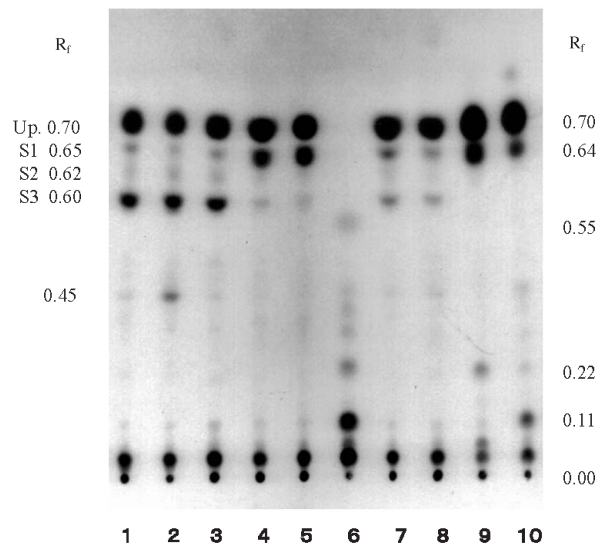


Fig. 8. Chromatograms of aminolipids isolated from various phytopathogenic bacteria.

1. *Burkholderia vandii* JCM 7957^T, 2. *B. plantarii* MAFF 302484, 3. *B. plantarii* MAFF 302475, 4. *B. cepacia* 356-5, 5. *B. cepacia* ATCC 25416^T, 6. *Clavibacter michiganensis* subsp. *michiganensis* N6206, 7. *B. glumae* Kyu82-34-2, 8. *B. glumae* Ku8111, 9. *Erwinia carotovora* subsp. *carotovora* 486-4, 10. *E. carotovora* subsp. *carotovora* N7129.

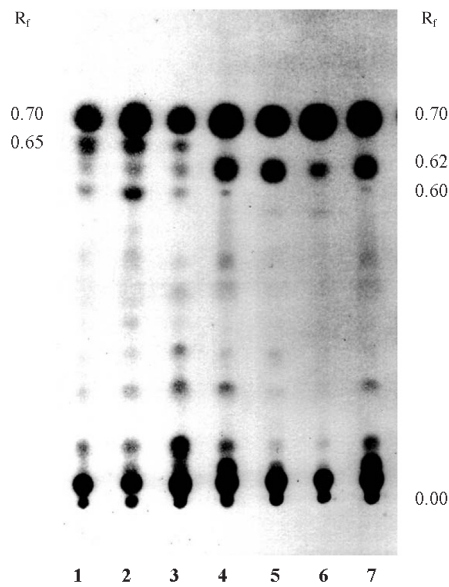


Fig. 9. Chromatograms of aminolipids isolated from *Burkholderia* species and *Ralstonia solanacearum*.

1. *Burkholderia gladioli* pv. *alliicola* ATCC 19302^T, 2. *B. glumae* MAFF 301169^T, 3. *B. gladioli* H-1, 4. *B. andropogonis* MAFF 301006, 5. *R. solanacearum* C319, 6. *R. solanacearum* ATCC 11676^T, 7. *B. andropogonis* MAFF 301006.

R_f 0.64 (Figs. 1, 3, 8) and this spot was absent on the chromatograms of *E. chrysanthemi* and *E. herbicola*.

Chromatograms of *Xanthomonas* species

As can be seen in Figs. 1 and 10, the profiles of *X. campestris* pv. *citri* (*X. axonopodis* pv. *citri*) and *X. oryzae* pv. *oryzae* were clearly different. The profiles of *X. campestris* pathovars were identical and the benchmark spot appeared at R_f 0.50. This spot was absent on the chromatogram of *X. oryzae* pv. *oryzae*. The differences between both species appeared more striking when cultured on NBA slant medium instead of King's B medium. Before the spraying of the ninhydrin, several yellowish spots were seen near the uppermost spot at R_f 0.70. Several differences in the profiles of these yellow spots were observed between *X. campestris* and *X. oryzae* (data not shown).

Chromatograms of *Acidovorax*, *Comamonas*, *Herbaspirillum* and *Pseudomonas* spp.

The profiles of *A. avenae*, *C. acidovorans*, *H. rubrisubalbicans*, *P. fluorescens*, *P. aeruginosa* and *P. syringae* pathovars were simple and only an uppermost spot appeared at R_f 0.70. For the chromatograms of *H. rubrisubalbicans* and *P. aeruginosa*, characteristic spots that could be pigments appeared above the uppermost spot.

DISCUSSION

Since morphological characteristics are not always determinative keys in bacterial identification, routine

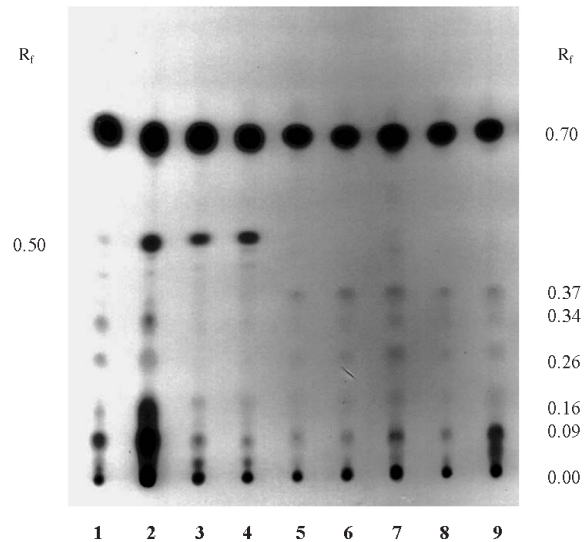


Fig. 10. Chromatograms of aminolipids isolated from *Xanthomonas* species.

1. *Xanthomonas campestris* pv. *citri* N6113-1, 2. *X. campestris* pv. *citri* N6829-1-9, 3. *X. campestris* pv. *citri* N6831-1-1, 4. *X. campestris* pv. *citri* Kawa, 5. *X. oryzae* pv. *oryzae* Q75114, 6. *X. oryzae* pv. *oryzae* Q7781, 7. *X. oryzae* pv. *oryzae* Q7602, 8. *X. oryzae* pv. *oryzae* Q7660, 9. *X. oryzae* pv. *oryzae* T7144. NBA medium was used for culture.

characterization of 40–50 biochemical and physiological properties, along with pathogenicity tests have been used to identify phytopathogenic bacteria. However, these experiments are highly laborious, time-consuming and require technical skills. Recently, DNA and/or RNA analyses have been introduced into the identification process. The analysis of the complete base sequences of bacterial genomes is the most reliable procedure for the identifying bacteria. However, it is difficult and expensive to practice, and so alternative methods such as DNA-DNA/DNA-rRNA hybridization, RFLP and 16S/23S rRNA gene analyses and others have been proposed (Palleroni *et al.*, 1973; De Vos and De Ley, 1983; De Vos *et al.*, 1985; Xiang *et al.*, 1993; Yamamoto *et al.*, 1999). Fatty acid analysis of bacterial membranes by GLC and the protein analysis by electrophoresis also have been used and are recognized to be reliable methods (Oyaizu and Komagata, 1983; De Boer and Sasser, 1986; Galbraith and Wilkinson, 1991; Chase *et al.*, 1992; Sawada *et al.*, 1992; Kori *et al.*, 1992; Stead, 1992; Bouzar *et al.*, 1993; Jarvis *et al.*, 1996; Tighe *et al.*, 2000; Khan *et al.*, 2002; Dristig and Dianese, 1990; Li and Hayward, 1994; Schleifer and Kandler, 1972).

For systematic classification of actinomycetes and some bacteria such as genus *Deinococcus*, the TLC profiles of polar lipids have been used effectively (Komura *et al.*, 1975; Lechevalier *et al.*, 1977; Hasegawa *et al.*, 1979; Counsell and Murray, 1986). This technique was simplified and the direct colony TLC method was invented (Matsuyama *et al.*, 1986, 1987). In 1993, one of the authors tried to apply this method for presumptive differentiation of various phytopathogenic bacteria

(Matsuyama *et al.*, 1993a, b, c, d). The chromatographic profiles of some phytopathogenic bacteria were genus or species specific and thus useful for differentiation (Matsuyama, 1995a, b, 1998; Furuya *et al.*, 2000, 2004; Khan *et al.*, 2000; Narayanasamy, 2003; Palleroni, 2005). The chromatographic profiles were stable if culture conditions were uniformly maintained. The benchmark spot or spots appeared clearly on the chromatogram for cultures up to seven days old. The appearance of the benchmark spot or spots was likewise unaffected in such cultures (Matsuyama *et al.*, 1993a, 2003a, b). In the case of the direct colony TLC method, pasting the bacterial colonies on the origin spot of the silica gel plate, drying the samples, and developing the plate twice in the same direction with two different kinds of chloroform solvent systems were laborious and time-consuming. Therefore, simplifying the original method became a high priority and led to invention of the rapid extraction-TLC method (Khan and Matsuyama, 1998). Since its original invention, this method has been considerably improved (Matsuyama *et al.*, 2003a, b). This method now is quite easy to practice and the results obtained are highly reproducible.

Striking differences in TLC profiles were observed between *C. michiganensis*, gram-positive bacteria, and negative bacteria (Figs. 1, 3, 8). The uppermost spot at R_f 0.70 (Up), which was commonly observed on the chromatograms of gram-negative bacteria, was absent on the chromatograms of three subspecies of *C. michiganensis* such as subsp. *michiganensis*, *sepedonicus* and *nebraskensis*. A similar profile was also observed for *Micrococcus luteus* (NBRC 16250, datum was not shown). At first, the absence of this Up spot was assumed to be a common characteristic of gram-positive bacteria. However, it was not universal and the spots with similar R_f value of R_f 0.70 were also observed for *Bacillus* spp., gram-positive bacteria, such as *B. cereus*, *B. licheniformis* and others (data were not shown). For *B. subtilis*, a spot at R_f 0.69 was faintly observed (Fig. 3). Based on its reaction to the Dittmer's reagent and comparison with the authentic samples, the Up spot was assumed to be phosphatidyl ethanolamine or its relatives. The uppermost spots of *Agrobacterium* spp. and *Rhizobium* spp. appeared at ca. R_f 0.75 and were clearly different from those of other gram-negative bacteria.

The chromatograms of *A. biovar* 1, *A. biovar* 2, *A. biovar* 3 and *A. rubi* were distinctly different from each another (Figs. 2–7). The chromatograms of the strains belong to the same biovar were identical without exception. The difference between *A. biovar* 3 and other *Agrobacterium* species was obvious (Figs. 2, 4). Though the identification of *A. biovar* 3 with PCR techniques was newly reported (Kawaguchi *et al.*, 2005), reliable *A. biovar* 3 discrimination with this TLC method can also be performed (Matsuyama *et al.*, 2004).

Lately, the amalgamation of two genera, *Agrobacterium* and *Rhizobium*, into the single genus *Rhizobium* and corresponding striking changes of the genus and species names such as *R. radiobacter*, *R. rhizogenes* and *R. vitis* were proposed (Young *et al.*,

2001; Young *et al.*, 2003). However, vigorous opposition to this proposal has also been expressed (Farrand *et al.*, 2003; Broughton, 2003). In our present experiments, we note that the chromatograms of *R. etli*, *R. galegae*, *R. leguminosarum* (the type species of genus *Rhizobium*), *R. phaseoli* and their relatives such as *B. japonicum*, *M. huakuii* and *S. meliloti* were quite different from those of *A. biovar* 1 and *A. biovar* 2 (Figs. 2, 5–7).

The one exception, however, was the chromatogram of *R. tropici*, which resembled the chromatograms of *A. biovar* 2 and *Agrobacterium* species which were isolated originally from cherry and kiwi fruit by Sawada (1994) (Matsuyama *et al.*, 2005). In particular, the profile of *R. tropici* was identical with that of the cherry isolates (Fig. 5). These TLC results agreed partly with 16S rDNA analyses and fatty acid analyses (Sawada, 1994; Tighe *et al.*, 2000). Although the authors could not decide if both genera should be amalgamated as genus *Rhizobium*, we point out that *R. tropici* is unique in the genus *Rhizobium* and will be involved in the same chromosomal group with *A. biovar* 2 (Fig. 5). Similar data has been reported by other groups (Sawada *et al.*, 1992; Sawada, 1994; Tigh *et al.*, 2000; Eardly *et al.*, 2005).

The profiles of *Burkholderia* spp. are characteristic and three spots designated as S1, S2, S3 appeared under the uppermost spot (Up) of ca. R_f 0.70 on the chromatogram. The profiles of these three spots were species specific for the cases of *B. plantarii*, *B. caryophylli*, and *B. andropogonis*. Though *B. vandii* was reported to be a novel species of genus *Burkholderia* (Urakami *et al.*, 1994), objections have been made that *B. vandii* will turn out to be a synonym or junior synonym of *B. plantarii* (Ura *et al.*, 1998; Hirakawa *et al.*, 1999; Coenye *et al.*, 1999; Hirakawa *et al.*, 2001). As shown in Fig. 8, the chromatograms of *B. vandii* and *B. plantarii* were identical (Matsuyama *et al.*, 2003a, b). As reported before, a distinction between *B. gladioli* and *B. glumae* was not observed by the TLC, but was observed by HPLC (Matsuyama *et al.*, 1998).

The profiles of the *B. cepacia* complex were varied, though S1, S2 and S3 spots always appeared. For strains from onion (isolated in Thailand), the chromatograms agreed well with that of the type strain (ATCC 25416^T, onion). However, some profiles, especially of clinical or environmental strains, were not always identical with that of type strain. Thus, *B. cepacia* is a complex of strains (Stanier *et al.*, 1966; Ballard *et al.*, 1977; Richard *et al.*, 1981; Bebivino *et al.*, 1994; Tabacchioni *et al.*, 1995; Yohalem and Lorbeer, 1994; Lessie *et al.*, 1996; Sotokawa and Takikawa, 2003) and nine new species names have now been proposed. The difficulties in identifying *B. cepacia* complex strains have been elucidated (Mahenthalingam and Vanddamme, 2005).

The profile of *B. andropogonis* lacks S1 spot and is quite different from the profiles of other *Burkholderia* species (Figs. 1, 9). The 16SrDNA sequences data (Viallard *et al.*, 1998) support this result. At first sight the profile rather resembled with that of *R. solanacearum*.

It has been well documented that races, genetic

groups and phylotypes exist in *R. solanacearum* species complex (Buddenhagen *et al.*, 1962; Cook *et al.*, 1989; Villa *et al.*, 2005). However, no differences were observed for the TLC chromatogram of 96 strains of *R. solanacearum* isolated from various plants and various places. The benchmark spot distinctly appeared at R_f 0.62 (Figs. 1, 3, 9). The profile of *R. pickettii* was almost identical with that of *R. solanacearum*. In 1992, *R. solanacearum* initially was placed in the genus *Burkholderia* (Yabuuchi *et al.*, 1992) and transferred afterwards to the genus *Ralstonia*. The TLC profile of *R. solanacearum* is obviously different from those of *Burkholderia* spp. (Figs. 1, 3, 8, 9).

The differences between *E. carotovora* and other *Erwinia* spp. such as *E. chrysanthemi* and *E. herbicola* were obvious. The benchmark spot appeared at R_f 0.64 on the chromatograms of subspecies *carotovora* and *atroseptica* of *E. carotovora* (Figs. 1, 3, 8).

The distinct benchmark spot appeared at R_f 0.5 on the chromatogram of *X. campestris*. This spot was absent on the chromatogram of *X. oryzae* (Fig. 10). The benchmark spot was detected more clearly when NBA medium was used. It has been documented that the chromatographic profiles of yellow pigments in xanthomonads are species specific (Starr *et al.*, 1997). In our experiments obvious differences between the chromatograms of *X. campestris* pv. *citri* and *X. oryzae* pv. *oryzae* for yellow pigments were also observed (data not shown).

The identification of the causal agent of devastating plant diseases, especially the identification of phytopathogenic bacteria, is currently highly laborious and time-consuming. For rapid identification of these bacteria various chemotaxonomic means have been used. The usefulness of the cellular fatty acid methylester (FAME) analyses by gas-liquid chromatography (GLC) has been well documented and reliable procedures have been established. Lately, the analyses of the polar lipids extracted from whole-cells of microbes have also been stressed. In 1993, the authors have first reported the usefulness of TLC profiles of aminolipids extracted from whole-cells of bacteria as an important tool for the rapid and presumptive differentiation of phytopathogenic bacteria. Since then, these procedures have been simplified and well established. This procedure is quite easy to implement and the profiles obtained are highly reproducible.

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