九州大学学術情報リポジトリ
Kyushu University Institutional Repository

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

Matsuyama, Nobuaki
Faculty of Agriculture, Tokyo University of Agriculture | Laboratory of Plant Pathology, Division of Plant Pathology and Pesticide Science, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University

Daikohara, Makiko
Faculty of Agriculture, Tokyo University of Agriculture

Yoshimura, Keiko
Faculty of Agriculture, Tokyo University of Agriculture

Manabe, Kayo
Faculty of Agriculture, Tokyo University of Agriculture

他

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：

J. Fac. Agr., Kyushu Univ., **54** (1), 1–11 (2009)

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

Nobuaki MATSUYAMA[1,2*], Makiko DAIKOHARA[1], Keiko YOSHIMURA[1], Kayo MANABE[1], Md. Abu Ashraf KHAN[2], Maria Salete de MELO[2], Hiromitsu NEGISHI[1], Kazuo SUYAMA[1], Kenichi TSUCHIYA[2] and Naruto FURUYA[2]

Laboratory of Plant Pathology, Division of Plant Pathology and Pesticide Science, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University, Fukuoka 812–8581, Japan
(*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% $CaCl_2 \cdot 2H_2O$ (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-

[1] Faculty of Agriculture, Tokyo University of Agriculture, Atsugi 243–0034, Japan
[2] Laboratory of Plant Pathology, Division of Plant Pathology and Pesticide Science, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University, Fukuoka 812–8581, Japan
* Corresponding author (E–mail: nobuaki.matsuyama@jcom.home.ne.jp)

Table 1. List of bacteria tested

Genus & Species	Strains
(Gram positive bacteria)	
Bacillus subtilis	NBRC 13719^{T}, AKU–AB89, AKU–AN83530, NIAES1702,AKU558
Clavibacter michiganensis subsp. *michiganensis*	NBRC 12471, NBRC 13762, NIAS–N6206, NIAS–N6601 (t)
C. michiganensis subsp. *sepedonicus*	ATCC 33113^{T}, NBRC 13763, NBRC 13764 (po)
C. michiganensis subsp. *nebraskens*	ATCC 27794^{T} (co)
(Gram negative bacteria)	
Acidovorax avenae subsp. *avenae*	ATCC 19860^{T}, HNAES–H8206, HNAES –H8210, HNAES –H8505 (r)
**Agrobacterium* biovar 1 (=*A. tumefaciens*)	ATCC 23308^{T} (Ti), NIAS–Ku7415 (ro.,Ti), MAFF 301001(*Prunus* 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.)
Agrobacterium biovar 2 (=*A.rhizogenes*)	ATCC 11325^{T}(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)
Agrobacterium biovar 3 (=*A. vitis*)	NBRC 15140^{T}, MAFF 302147, MAFF 302148, MAFF 302149, MAFF 302150, MAFF 302297, MAFF 302652 (g.v.,Ti)
A. rubi	NBRC 13261^{T}, NBRC 13260 (*Rubus* 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)
Bradyrhizobium japonicum	NBRC 15001 (root nodule, soy) (*R. japonicum*)
Mesorhizobium huakuii	NBRC 15243^{T} (root nodule,m.vetch.) (*R. huakuii*)
Rhizobium etli	NBRC 15573^{T} (root nodule,kid.b.)
R. galegae	NBRC 14965^{T} (root nodule, *Galega orientalis*)
R. leguminosarum	NBRC 14778^{T}, NBRC 13337 (root nodule,w.clov.)
R. phaseoli	NBRC 14168, NBRC 14994 (root nodule,kid.b)
R. tropici	NBRC 15247^{T} (root nodule,kid.b.)
Sinorhizobium meliloti	NBRC 14782^{T} (root nodule,med.)(*R.meliloti*)
Burkholderia andropogonis	JCM 10487^{T}, MAFF 301006 (su.g), MAFF 301129 (tu)
B. caryophylli	ATCC 25418^{T}, NIAS1192, NIAS 1406, MAFF 301060, MAFF 302555 (car), AKU–Yame1 (r.p.g.)
B. cepacia complex	ATCC 25416^{T} (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]
B. gladioli pv. *alliicola*	ATCC 19302^{T} (o)
B. gladioli pv. *gladioli*	ATCC 10248^{T} (gla), MAFF 302515 (tu), MAFF 302537 (o), MAFF 302544 (r), MAFF 301580(den), NIAS1064, NIAS 1065 (fr)
B. gladioli	MAFF 302409 (a.b.), MAFF 302418 (g.g), MAFF 302424 (cy), AKU–H–1, AKU–H–2 (r)
B. glumae	MAFF 301169^{T}, 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)
B. plantarii	JCM 5492^{T}, MAFF 302387, MAFF 302392, MAFF 302412, MAFF 302467, MAFF 302470, MAFF 302475, MAFF 302481, MAFF 302484, MAFF 302485, AZ8201(r)
B. vandii	JCM 7957^{T}(va)
Comamonas acidovorans	ATCC 15668^{T} (soil)
Erwinia carotovora subsp. *atroseptica*	ATCC 33260^{T} (po)
E. carotovora subsp. *carotovora*	ATCC 15713^{T} (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)
E. chrysanthemi pv. *chrysanthemi*	SU E8301 (ep), AKU–Ku8601 L1 (pear), TUA –Ichihara 1–1 (pear), SU–Ech44
E. chrysanthemi pv. *dianthicola*	Dianthi 1e, 2n (car)
E. chrysanthemi pv. *zeae*	NCPPB 377^{T}, R–7, R–8, Corn801 (co), ALE829p
E.herbicola pv. *milletiae*	NIAS 1 (Japanese wisteria)
Herbaspirillum rubrisubalbicans	ATCC 19308^{T}, MAFF 301626, MAFF 301628(s.c)
Pseudomonas aeruginosa	ATCC 7700T
P. fluorescens	ATCC 13525^{T}, ATCC 520049
P. syringae pv. *lachrymans*	NIAS1319, NIAS1321 (cu)
P. syringae pv. *pisi*	MAFF 301211, MAFF 301213(pea)
P. syringae pv. *tabaci*	KTES–PA28, NIAS–P41, AKU–Ku8102
P. syringae pv. *syringae*	ATCC 19310^{T}, NIAS I
Ralstonia solanacearum	ATCC 11696^{T}, 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
R. pickettii	ATCC 27511^T (H)
Xanthomonas campestris pv. *campestris*	ATCC 33913^T (b.s), NIAS I
X. campestris pv. *citri*	N6113–1, N6829–1–3, N6831–1–1, Kawa (ci) (*X. axonopodis* pv. *citri*)
X. campestris pv. *cucurbitae*	Ishikawa I
X. campestris pv. *pisi*	NIAS II
X. campestris pv. *pruni*	NIAS 1–10–1
X. campestris pv. *vitians*	NIAS I
X. oryzae pv. *oryzae*	ATCC 35933^T, 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 (tumorigenetic); 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 (Kersters 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 3days on King's B (KB; peptone 20 g, K_2HPO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 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, K_2HPO_4 0.7 g, KH_2PO_4 0.1 g, $MgSO_4 \cdot 7H_2O$ 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 μl (70 μl 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% $CaCl_2 \cdot 2H_2O$ (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, chlofoform–methanol–0.2% $CaCl_2 \cdot 2H_2O$ (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*

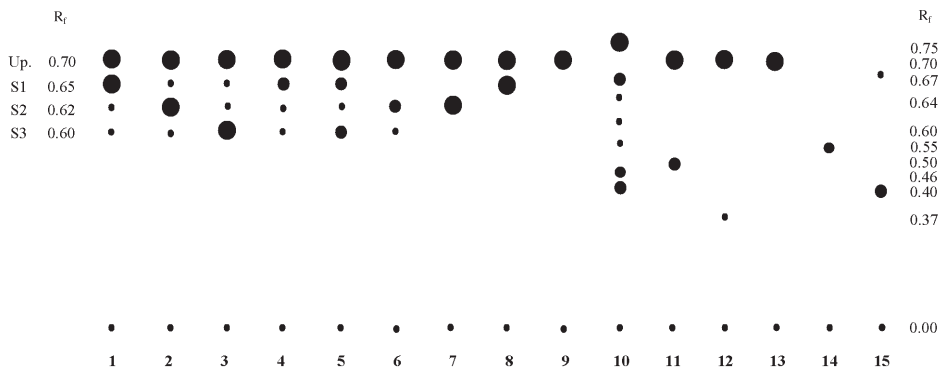

Fig. 1. Diagrammatic representations of TLC profiles of gram negative and positive bacteria.
1. *Burkholderia cepacia*, 2. *B. caryophylli*, 3. *B. plantarii & B. vandii*, 4. *B. gladioli*, 5. *B. glumae*, 6. *B. andropogonis*, 7. *Ralstonia solanacearum*, 8. *Erwinia carotovora*, 9. *E. chrysanthemi*, 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.

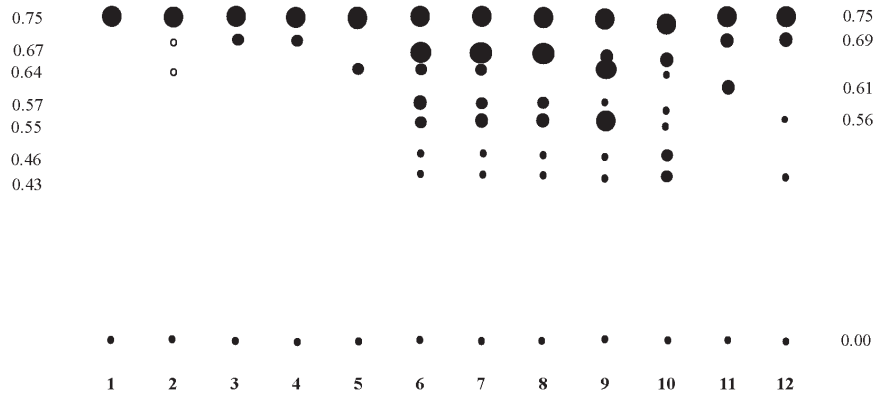

Fig. 2. Diagrammatic representations of TLC profiles of *Bradyrhizobium, Sinorhizobium, Rhizobium, Mesorhizobium* species and *Agrobacterium* species.
1. *Bradyrhizobium japonicum*, 2. *Sinorhizobium meliloti*, 3. *Rhizobium leguminosarum*, 4. *Rhizobium galegae*, 5. *Mesorhizobium huakuii*, 6. *R. tropici*, 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.

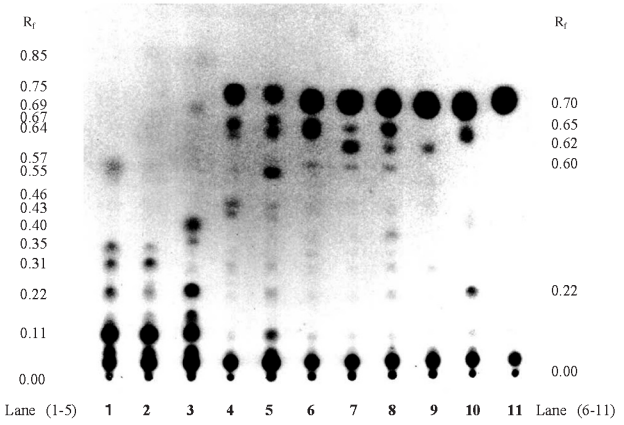

Fig. 3. Chromatograms of aminolipids isolated from phytopathogenic and non–pathogenic bacteria.
1. *Clavibacter michiganensis* subsp. *michiganensis* NBRC 12471, 2. *C. michiganensis* subsp. *sepedonicus* NBRC 13764^T, 3. *Bacillus subtilis* NBRC 13719^T, 4. *Agrobacterium* biovar 1 Ku7415 (Ti), 5. *A.* 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.

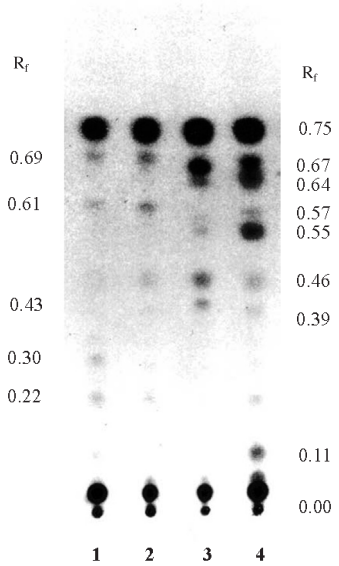

Fig. 4. Chromatograms of aminolipids isolated from various *Agrobacterium* species.
1. *Agrobacterium* biovar 3 MAFF 302150 (Ti), 2. *A.* biovar 3 MAFF 302297 (Ti), 3. *A.* biovar 1 Ku7415 (Ti), 4. *A.* biovar 2 Ku7411 (Ri).

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. *alliicola*. 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 (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

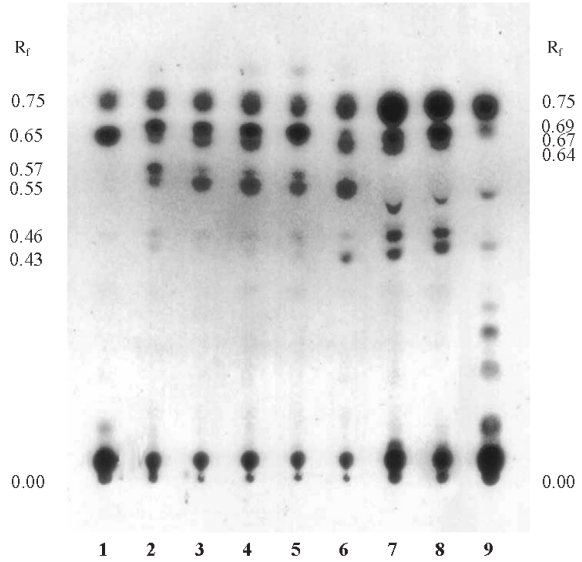

Fig. 5. Chromatograms of aminolipids isolated from *Mesorhizobium, Rhizobium* and *Agrobacterium* species.
1. *Mesorhizobium huakuii* NBRC 15243^T, 2. *Rhizobium tropici* NBRC 15247^T, 3. *Agrobacterium* sp. Sa–4 (cherry, Ti), 4. *Agrobacterium* sp. NBRC 15292 (cherry, Ti), 5. *Agrobacterium* sp. NBRC 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.

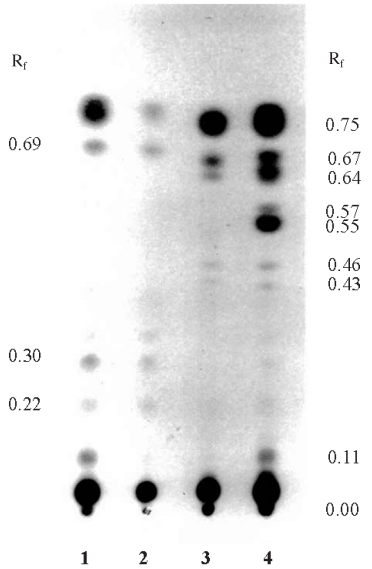

Fig. 6. Chromatograms of aminolipids isolated from *Rhizobium* and *Agrobacterium* species.
1. *Rhizobium leguminosarum* NBRC 13337, 2. *R. leguminosarum* NBRC 14778^T, 3. *Agrobacterium* biovar 1 Ku7415 (Ti), 4. *A.* biovar 2 Ku7411(Ri).

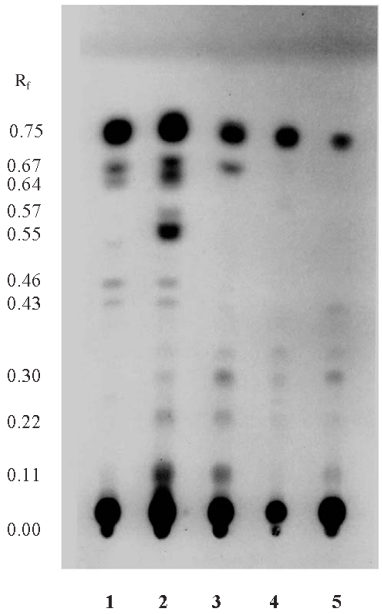

Fig. 7. Chromatograms of aminolipids isolated from *Agrobacterium* and *Rhizobium* species.
1. *Agrobacterium* biovar 1 Ku7415(Ti), 2. *A.* biovar 2 Ku7411(Ri), 3. *Rhizobium phaseoli* NBRC 14994, 4. *Sinorhizobium meliloti* NBRC 14782, 5. *Bradyrhizobium japonicum* NBRC 15001.

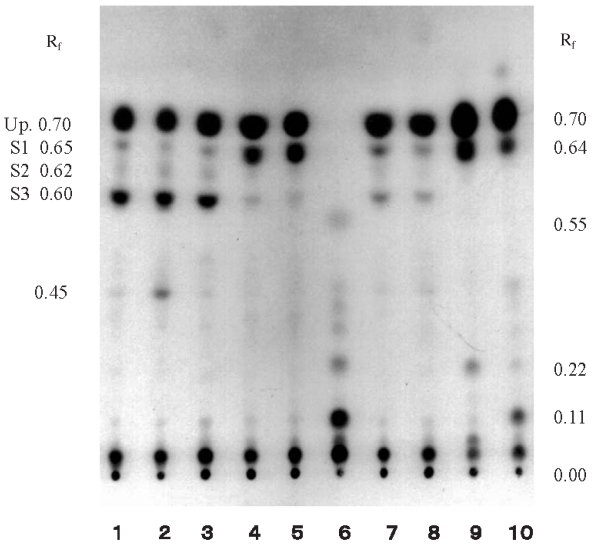

Fig. 8. Chromatograms of aminolipids isolated from various phytopathogenic bacteria.
1. *Burkholderia vandii* JCM 7957^T, 2. *B. plantarii* MAFF 302484, 3. *B. plantarii* MAFF 302475, 4. *B. cepacia* 356–5, 5. *B. cepacia* ATCC 25416^T, 6. *Clavibacter michiganensis* subsp. *michiganensis* N6206, 7. *B. glumae* Kyu82–34–2, 8. *B. glumae* Ku8111, 9. *Erwinia carotovora* subsp. *carotovora* 486–4, 10. *E. carotovora* subsp. *carotovora* N7129.

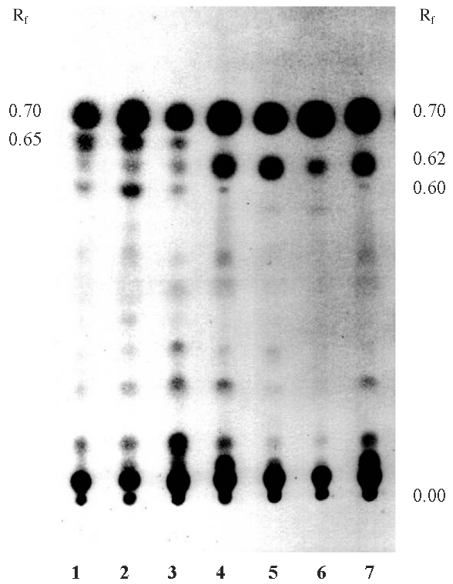

Fig. 9. Chromatograms of aminolipids isolated from *Burkholderia* species and *Ralstonia solanacearum*.
1. *Burkholderia gladioli* pv. *alliicola* ATCC 19302^T, 2. *B. glumae* MAFF 301169^T, 3. *B. gladioli* H–1, 4. *B. andropogonis* MAFF 301006, 5. *R. solanacearum* C319, 6. *R. solanacearum* ATCC 11676^T, 7. *B. andropogonis* MAFF 301006.

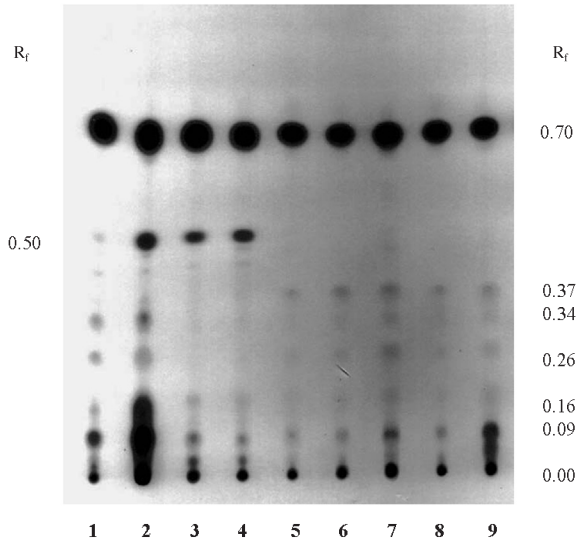

Fig. 10. Chromatograms of aminolipids isolated from *Xanthomonas* species.
1. *Xanthomonas campestris* pv. *citri* N6113–1, 2. *X. campestris* pv. *citri* N6829–1–9, 3. *X. campestris* pv. *citri* N6831–1–1, 4. *X. campestris* pv. *citri* Kawa, 5. *X. oryzae* pv. *oryzae* Q75114, 6. *X. oryzae* pv. *oryzae* 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.

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 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 *nebraskens*. 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. lichenformis* 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 (Mahenthiralingam 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.

REFERENCES

Ballard, R. W., N. J. Palleroni, M. Doudoroff, R. Y. Stanier, and M. Mandel 1970 Taxonomy of aerobic pseudomonads: *Pseudomonas cepacia, P. marginata, P. alliicola* and *P. caryophylli*. *J. Gen. Microbiol.*, **60**: 199–214

Bevivino, A., S. Tabacchioni, L. Chiarini, M. V. Del Carusi, M. Gallo and P. Visca 1994 Phenotypic comparison between rhizosphere and clinical isolates of *Burkholderia cepacia*. *Microbiology*, **140**: 1069–1077

Bouzar, H., J. B. Jones and N. C. Hodge 1993 Differential characterization of *Agrobacterium* species, using carbon–source utilization patterns and fatty acid profile. *Physiology and Biochemistry*, **83**: 733–737

Broughton, W. J. 2003 Roses by other names: Taxonomy of the *Rhizobiaceae*. *J. Bacteriol.*, **185**: 2975–2979

Buddenhagen, I., L. Sequeira and A. Kelman 1962 Designation of races in *Pseudomonas solanacearum*. *Phytopathology*, **52**: 726 (abstract)

Chase, R. C., R. E. Stall, N. C. Hodge and J. B. Jones 1992 Characterization of *Xanthomonas campestris* strains from Aroids, using physiological, pathological and fatty acid analyses. *Phytopathology*, **82**: 754–760

Coenye, T., B. Holmes, K. Kersters, J. R. W. Govan and P. Vandamme 1999 *Burkholderia cocovenenans* (van Damme *et al.*, 1960) Gills *et al.* 1995 and *Burkholderia vandii* Urakami *et al.*, 1994 are junior synonyms of *Burkholderia gladioli* (Severini 1913) Yabuuchi *et al.* 1993 and *Burkholderia plantarii* (Azegami *et al.* 1987) Urakami *et al.* 1994, respectively. *Int. J. Syst. Bacteriol.*, **49**: 37–42

Colwell, R. R. 1970 Polyphasic taxonomy of bacteria. Iizuka, H., Hasegawa, T. (ed.) *Proc. First Int. Conf. Culture Collections*, University of Tokyo Press, Tokyo, pp. 421–436

Cook, D., E. Barlow and L. Sequeira 1989 Genetic diversity of *Pseudomonas solanacearum*: detection of restriction fragment length polymorphisms with DNA probes that specify virulence and hypersensitivity response. *Mol. Plant–Microbe Interact.*, **2**: 113–121

Counsell, T. J. and R. G. E. Murray 1986 Polar lipid profiles of the genus *Deinococcus*. *Int. J. Syst. Bacteriol.*, **36**: 202–206

Daikohara, M., K. Manabe, K. Yoshimura and N. Matsuyama 2003 Trials for simplified differentiation of phytopathogenic bacteria by a modified rapid extraction–TLC method. *Jpn. J. Phytopathol.*, **69**: 309 (abstract in Japanese)

De Boer, S. H. and M. Sasser 1986 Differentiation of *Erwinia carotovora* sp. *carotovora* and sp. *atroseptica* on the basis of cellular fatty acid composition. *Can. J. Microbiology*, **32**: 796–800

De Vos, P. and J. De Loy 1983 Intra– and intergeneric similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. *Int. J. Syst. Bacteriol.*, **33**: 487–509

De Vos, P., M. Goor, M. Gills and J. De Ley 1985 Ribosomal ribonucleic acid cistron similarities of phytopathogenic *Pseudomonas* species. *Int. J. Syst. Bacteriol.*, **35**: 169–184

Dittmer, J. C. and R. L. Lester 1964 A simple specific spray for the detection of phospholipids on thin–layer chromatography. *J. Lipid Res.*, **5**: 126–127

Dristig, M. C. G. and J. C. Dianese 1990 Characterization of *Pseudomonas solanacearum* biovars based on membrane protein patterns. *Phytopathology*, **80**: 641–646

Eardly, B. D., S. M. Nour, P. B. van Berkum and R. K. Selander 2005 Rhizobial 16S rRNA and *dnaK* genes: mosaicism and the uncertain phylogenetic placement of *Rhizobium galegae*. *Appl. Environ. Microbiol.*, **71**(3): 1328–1335

Farrand, S. K., P. B. van Berkum and P. Oger, P. 2003 *Agrobacterium* is a definable genus of the family Rhizobiaceae. *Int. J. Syst. Evol. Microbiol.*, **53**: 1681–1687

Furuya, N., T. Masunaga, A. A. Khan, K. Iiyama, M. Matsumoto and N. Matsuyama 2000 Bacterial wilt of russell prairie gentian caused by *Burkholderia caryophylli*. *J. Gen. Plant Pathol.*, **66**: 316–322

Furuya, N., F. Shimokusuzono, Y. Nakamura, K. Nishimura, M. Takeshita, N. Matsuyama, K. Manabe and Y. Takanami 2004 Crown gall of tobacco caused by *Agrobacterium tumefaciens* biovar 1 in tobacco field. *J. Gen. Plant Pathol.*, **70**: 39–44

Galbraith, L. and S. G. Wilkinson 1991 Polar lipids and fatty acids of *Pseudomonas caryophylli, Pseudomonas gladioli* and *Pseudomonas pickettii*. *Jour. Gen. Microbiol.*, **137**: 197–202

Gillis, M., Van V. Tran, R. Bardin, M. Goor, P. Hebbar, A. Willems, P. Segers, K. Kersters, T. Heulin, and M. P. Fernandez 1995 Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N_2–fixing isolates from rice in Vietnam. *Int. J. Syst. Bacteriol.*, **45**: 274–289

Hasegawa, T., M. P. Lechevalier and H. A. Lechevalier 1979

Phospholipid composition of motile actinomycetes. *J. Gen. Appl. Microbiol.*, **25**: 209–213

Hirakawa, Y., H. Ura, N. Furuya and N. Matsuyama 1999 Taxonomic reexamination of *Burkholderia plantarii* and *B. vandii*. *Kyushu Pl. Prot. Res.*, **45**: 1–4

Hirakawa, Y. N. Furuya, K. Iiyama, H. Ura, K. Matsumoto, M. Takeshita, N. Matsuyama and Y. Takanami, Y. 2001 Comparative studies of *Burkholderia plantarii* and *B. vandii* based on chemotaxonomic markers. *Kyushu Pl. Prot. Res.*, **47**: 33–38

Jarvis, B. D. W., S. Sivakumaran, S. W. Tighe and M. Gills 1996 Identification of *Agrobacterium* and *Rhizobium* species based on cellular fatty acid composition. *Plant Soil*, **184**: 43–158

Kawaguchi, A., H. Sawada, K. Inoue and H. Nasu 2005 Multiplex PCR for the identification of *Agrobacterium* biovar 3 strains. *J. Gen Plant Pathol.*, **71**: 54–59

Kersters, K., and J. De Ley 1984 Genus Ⅲ. *Agrobacterium* Conn 1942. *In* Bergey's manual of systematic bacteriology, ed. by Krieg, N. R. and Holt, J. G., The Williams & Wilkins Co., Baltimore., **Vol. 1** pp. 244–254

Khan A. A., and N. Matsuyama 1998 A rapid extraction TLC method for differentiation of *Burkholderia* spp., *Ralstonia solanacearum, Herbaspirillum rubrisubalbicans* and *Pseudomonas syringae* pathovars. *J. Fac. Agr., Kyushu Univ.*, **44**: 49–58

Kori, Y., N. Furuya, K. Tsuno and N. Matsuyama 1992 Differentiation of *Erwinia chrysanthemi* and *Erwinia carotovora* subsp. *carotovora* by the cellular fatty acid analysis. *J. Fac. Agr., Kyushu Univ.*, **37**: 173–178

Komura, I., K. Yamada, S. Otsuka and K. Komagata 1975 Taxonomic significance of phospholipids in coryneform and nocardioform bacteria. *J. Gen. Appl. Microbiol.*, **21**: 251–265

Lechevalier, M. P., C. De Bievre and H. Lechevalier 1977 Chemotaxonomy of aerobic actinomycetes: phospholipids composition. *Biochem. Syst. Ecol.*, **5**: 249–260

Lessie, T. G., W. Hendrickson, B. D. Manning and R. Devereux 1996 Genomic complexity and plasticity of *Burkholderia cepacia*. *FEMS Microbiol Lett.*, **144**: 117–128

Li, X. and A. C. Hayward 1994 Bacterial whole cell protein profiles of the rRNA group Ⅱ pseudomonads. *J. Appl. Bact.*, **77**: 308–318

Mahenthiralingam, E. and P. Vandamme 2005 Taxonomy and pathogenesis of the *Burkholderia cepacia* complex. *Chron. Respir. Dis.*, **2**(4): 209–217

Margaret, A. R. 1988 Use of fatty acid for the identification of phytopathogenic bacteria. *Plant Disease*, **72**: 460–463

Matsuyama, N., I. H. Mian, A. M. Akanda and N. Furuya 1993a On a rapid identification of phytopathogenic bacteria by the direct colony thin–layer chromatography (1) *Proc.Assoc. Pl. Prot. Kyushu*, **39**: 60–63

Matsuyama, N., I. H. Mian, A. M. Akanda and N. Furuya 1993b Application of the direct colony TLC method for identification of phytopathogenic bacteria. *J. Fac. Agr., Kyushu Univ.*, **37**: 283–287

Matsuyama, N., I. H. Main, A. M. Akanda and N. Furuya 1993c Comparative studies on thin–layer chromatograms of lipids from various phytopathogenic bacteria. *Ann. Phytopath. Soc. Japan*, **59**: 528–534

Matsuyama, N. 1993d Rapid identification of phytopathogenic bacteria by direct colony thin–layer chromatography. *Proc. 6th. international congress of plant pathology* (ICPP1993) Montreal, Canada pp. 44

Matsuyama, N. 1995a Trials for rapid identification of phytopathogenic bacteria by HPLC and the direct colony TLC. *J. Fac. Agr., Kyushu Univ.*, **40**(1, 2): 87–91

Matsuyama, N. 1995b Application of the direct colony TLC for identification of phytopathogenic bacteria (Ⅲ). Distinction of the pseudomonads in the rRNA–homology group Ⅱ (*Burkholderia* spp.). *J. Fac. Agr., Kyushu Univ.*, **40**(1, 2): 189–196

Matsuyama, N., Y. Ueda, K. Iiyama, N. Furuya, H. Ura, A. A. Khan and M. Matsumoto 1998 Rapid Extraction–HPLC as a tool for presumptive identification of *Burkholderia gladioli, B. glumae* and *B. plantarii* causal agents of various rice disease. *J. Fac. Agr., Kyushu Univ.*, **42**: 265–272

Matsuyama, N. 1998 Presumptive identification of several phytopathogenic bacteria by novel diagnostic tests. *J. Fac. Agr., Kyushu Univ.*, **43**(3, 4): 337–343

Matsuyama, N., A. A. Khan, K. Yoshimura, N. Furuya, K. Manabe, M. Daikohara, K. Suyama and H. Negishi, H. 2003a Rapid identification of phytopathogenic bacteria by an improved extraction–TLC method. *Proc. 8 th. international congress of plant pathology* (ICPP2003), Christchurch, New Zealand. pp. 96

Matsuyama, N., A. A. Khan, K. Yoshimura, N. Furuya, K. Manabe, M. Daikohara, K. Suyama and H. Negishi 2003b Simplified differentiation of various plant pathogenic bacteria by an improved rapid extraction–TLC method. *Jpn. J. Phytopathol.*, **69**: 309 (abstract in Japanese)

Matsuyama, N., M. Daikohara, K. Manabe, N. Furuya, K. Suyama, H. Negishi and B. Wong 2004 Comparison of *Agrobacterium* and *Rhizobium* species with the lipid chromatograms obtained by the rapid extraction–TLC method. *Jpn. J. Phytopathol.*, **70**: 284 (abstract in Japanese)

Matsuyama, N., M. Daikohara, K. Manabe, N. Furuya, K. Suyama, H. Negishi and B. Wong 2005 Similarity for TLC chromatograms of amino lipids from *Rhizobium tropici* and some *Agrobacterium* species. *Jpn. J. Phytopathol.*, **71**: 291 (abstract in Japanese)

Matsuyama, T., M. Sogaya, K. Kaneda and T. Yano 1986 Rapid detection of bacterial lipids by direct colony thin–layer chromatography. *Proc. 23rd. international symposium of advances in chromatography*, Chiba, Japan. pp. 127–128

Matsuyama, T., M. Sogaya and T. Yano 1987 Direct colony thin–layer chromatography and rapid characterization of *Serratia marcescens* mutants defective in production of wetting agents. *Applied and Environmental Microbiology*, **53**: 1186–1187

Narayanasamy, P. 2003 Plant pathogen detection and disease diagnosis. (2nd ed.) 155–159, Marcel Dekker, Inc., New York

Oyaizu, H., and K. Komagata 1983 Grouping of *Pseudomonas* species on the basis of cellular fatty acid composition and the quinone system with special reference to the existence of 3–hydroxy fatty acids. *J. Gen. Appl. Microbiol.*, **29**: 17–40

Palleroni, N. J., R. Kunisawa, R. Contopoulou and M. Doudoroff 1973 Nucleic acid homologies in the genus *Pseudomonas*. *Int. J. Syst. Bacteriology*, **23**: 333–339

Palleroni, N. J. 2005 *In* Bergey's Mannual Syst. Bacteriol. Second edition **Vol. 2** Part C: 575–600

Richard, C., H. Monteil, F. Megraud, R. Chatelain and B. Laurent 1981 Phenotype characteristics of 100 strains of *Pseudomonas cepacia* proposition of a biovars classification. *Ann Biol Clin.*, **39**: 9–15

Sawada, H., Y. Takikawa and H. Ieki 1992 Fatty acid methyl ester profiles of the genus *Agrobacterium*. *Ann. Phytopath. Soc. Japan*, **58**: 46–51

Sawada, H. 1994 Studies on differentiation and taxonomy of the genus *Agrobacterium*. *Bull. Fruit Tree Res. Stn.* Extra **No. 5**: 1–110

Sawada, H., H. Ieki and I. Matsuda 1995 PCR Detection of Ti and Ri plasmids from phytopathogenic *Agrobacterium* strains. *Appl. and Environ. Microbiol.*, **61**(2): 828–831

Schleifer, K. H. and O. Kandler 1972 Peptidoglycan types of bacterial cell–walls and their taxonomic implications. *Bacteriological Reviews*, **34**: 407–477

Sotokawa, N., and Y. Takikawa 2003 Diversity of *Burkholderia cepacia* Genomovar I. *Jpn. J. Phytopathol.*, **69**(3): 302 (abstract in Japanese)

Staneck, J. L. and G. D. Roberts 1974 Simplified approach to identification of aerobic actinomycetes by thin–layer chromatography. *Appl. Microbiol.*, **28**: 226–231

Stanier, R. Y., N. J. Palleroni and M. Doudorof 1966 The aerobic

pseudomonads: a taxonomic study. *J. Gen Microbiol.*, **43**: 159–271

Starr, M. P., C. L. Jenkins, L. B. Bussey and A. G. Andrews 1997 Chemotaxonomic significance of the xanthomonadins, novel brominated aryl–polyene pigments produced by bacteria of the genus *Xanthomonas. Arch. Microbio*l., **113**: 1–9

Stead, D. E. 1992 Grouping of plant–pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profile. *Int. J. Syst. Bacteriol.*, **42**: 281–295

Suzuki, K., M. Goodfellow and A. G. O'Donnell 1993 Cell Envelopes and Classification *In* Handbook of New Systematics (eds. Goodfellow, M., O'Donnell, A. G.), Academic Press Inc., San Diego, CA 92101, USA pp. 195–250

Tabacchioni, S., P. Visca, L. Chiarini, A. Bevivino, C. Di Serio, S. Fancelli and R. Fani 1995 Molecular characterization of rhizosphere and clinical isolates of *Burkholderia cepacia. Res Microbiol.*, **146**: 531–542

Tighe, S. W., P. de Lajudie, K. Dipietro, K. Lindstrom, G. Nick and B. D. Jarvis 2000 Analysis of fatty acids and phenotypic relationships of *Agrobacterium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium* species using the Sherlock Microbial Identification System. *Int. J. Syst. Evol. Microbiol.*, **50**(2): 787–801

Ura, H., M. Matsumoto, K. Iiyama, N. Furuya and N. Matsuyama 1998 PCR–RFLP analysis for distinction of *Burkholderia* species, causal agents of various rice diseases. *Kyushu Pl. Prot. Res.*, **44**: 5–8

Urakami, T., C. Ito–Yoshida, H. Araki, T. Kijima, K. Suzuki and K. Komagata 1994 Transfer of *Pseudomonas plantarii* and *Pseudomonas glumae* to *Burkholderia* as *Burkholderia* spp. and description of *Burkholderia vandii* sp.nov. *Int. J. Syst. Bacteriol.*, **44**: 235–245

Vandamme, P., B. Pot, M. Gillis, P. De Vos, K. Kesters and J. Swing 1996 Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol. Rev.*, **60**: 407–438

Viallard, V., I. Poirier, B. Cournoyer, J. Haurat, S. Wiebkin, K. Ophel–Keller and J. Balandreau 1998 *Burkholderia graminis* sp. nov., a rhizospheric *Burkholderia* species, and reassessment of *[Pseudomonas] phenazinium, [Pseudomonas] pyrrocinia and [Pseudomonas] glathei as Burkholderia. Int. J. Syst. Bacteriol.*, **48**: 549–563

Villa, J. E., K. Tsuchiya, M. Horita, M. Natural, N. Opina and M. Hyakumachi 2005 Phylogenetic relationships of *Ralstonia solanacearum* species complex strains from Asia and other continents based on 16S rDNA, endoglucanase, and *hrpB* gene sequences. *J. Gen Plant Pathol.*, **71**: 39–46

Wells, J. M., J. E. Butterfield and L. G. Revear 1993 Identification of bacteria associated with postharvest diseases of fruits and vegetables by fatty acid composition. *Postharvest Pathology and Mycotoxins*, **83**: 445–447

Xiang, Li, M. Dorsch, T. DelDot, L. Sly, I. Stackebrandt and A. C. Hayward 1993 Phylogenetic studies of the rRNA group II pseudomonads based on 16S rRNA gene sequences. *J. Appl. Bacteriol.*, **74**: 324–329

Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki and M. Arakawa 1992 Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group Ⅱ to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb.nov. *Microbiol Immunol.*, **36**: 1251–1275

Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta and Y. Nishiuchi 1995 Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: Proposal of *Ralstonia picketti* (Ralston, Palleloni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. *Microbiol. Immunol.*, **39**: 897–904

Yamamoto, S., P. J. M. Bouvet and S. Harayama 1999 Phylogenetic structures of the genus *Acnetobacter* based on *gyrB* sequences : comparison with grouping by DNA–DNA hybridization. *Int. J. Syst. Bacteriol.*, **49**: 87–95

Yohalem, D. S. and J. W. Lorbeer 1994 Intraspecific metabolic diversity among strains of *Burkholderia cepacia* isolated from decayed onions, soils, and the clinical environment. *Antonie Leeuwenhock*, **65**: 111–131

Young, J. M., Y. Takikawa, L. Gardan and D. E. Stead 1992 Changing concepts in taxonomy of plant pathogenic bacteria. *Annual Review of Phytopathology*, **30**: 67–105

Young, J. M., L. D. Kuykendall, E. Martinez–Romero, A. Kerr and H. Sawada 2001 A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie *et al.*, 1998 as new combination: *Rhizobium radiobacter*, *R. rhizogenes, R. rubi, R. undicola and R. vitis. Int. J. Syst. Evol Microbiol.*, **51**: 89–103

Young, J. M., L. D. Kuykendall, E. Martinez–Romero, A. Kerr and H. Sawada 2003 Classification and nomenclature of *Agrobacterium* and *Rhizobium* – a reply to Farrand *et al.* (2003). *Int. J. Syst. Evol Microbiol.*, **53**: 1689–1695