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A Rapid Extraction – TLC Method for Differentiation of *Burkholderia* spp., *Ralstonia solanacearum*, *Herbaspirillum rubrisubalbicans* and *Pseudomonas syringae* Pathovars

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8

A rapid extraction –TLC method was conducted for differentiation of *Burkholderia* spp., *Ralstonia solanacearum*, *Herbaspirillum rubrisubalbicans* and *Pseudomonas syringae* pathovars. Lipids were extracted from bacterial cells in a small glass–vial using 0.2 ml of chloroform – methanol – 0.3% NaCl solution (2 : 1 : 0.4, v/v/v). The development of TLC plate was performed with chloroform – methanol – 0.2% $CaCl_2 \cdot 2H_2O$ solution (55 : 35 : 8, v/v/v) for 1 h at 25 °C and the amino–lipid spots were visualized by spraying ninhydrin and successive heating at 100 °C for 10 min. The chromatograms were characteristic at both the generic and species level. This method will be practical for rapid differentiation as well as identification of *Burkholderia* spp., *R. solanacearum*, *H. rubrisubalbicans* and *P. syringae* pathovars.

INTRODUCTION

Routine identification of phytopathogenic bacteria is generally conducted by a combination of physiolosical and biochemical tests. However, these tests are time consuming and require extensive experience to analyze the results. Therefore, the development of rapid methods has been emphasized. Both nucleic acid–based methodologies (Pecknold and Grogan, 1973; Bereswill *et al.*, 1994) and chemotaxonomic methods (Ikemoto *et al.*, 1978; De Boer and Sasser, 1986; Roy, 1988; Van Zyl and Steyn, 1990; Chase *et al.*, 1992; Kori *et al.*, 1992; Li and Hayward, 1994) have been used in bacterial taxonomy. However, these methods require expensive facilities. Though the lipid profiles have not been used widely, extensive research works show the potentiality of the method for the identification of bacteria. Matsuyama *et al.* (1986) developed the direct colony TLC (thin layer chromatography) method for the rapid detection and identification of bacterial lipids. Successful identification of phytopathogenic bacteria by this direct colony TLC method has been reported (Matsuyama *et al.*, 1993a; Matsuyama and Furuya, 1993b; Matsuyama, 1995). However, the direct colony TLC method needs drying the bacterial colony on TLC plate before development. Furthermore, duplicated developments with two kinds of developing solvents are necessary. Recently, we modified the original method and then used the modified method for the identification of phytopathogenic bacteria (Khan and Matsuyama, 1998). We have continued to improve the method for lipid extraction process and the results are presented in this report.

MATERIALS AND METHODS

Bacterial strains

Altogether 79 strains of *Burkholderia* spp., *Ralstonia solanacearum*, *Herbaspirillum rubrisubalbicans* and *Pseudomonas syringae* pathovars (all were formerly member of pseudomonads) were included in this experiment. The details of the strains are given in Table 1.

Table 1. List of *Burkholderia* spp., *Herbaspirillum rubrisubalbicans*, *Ralstonia solanacearum* and *Pseudomonas syringae* pathovars used in this experiment.

Bacteria	Isolate	Source[a)]
B. caryophylli	ATCC 25418^T	ATCC
	NIAS 1192	NIAS
	NIAS 1406	〃
	MAFF 301060	NIAR
	MAFF 302555	〃
B. cepacia	ATCC 25416^T	ATCC
	343–4	NIAS
	356–3	〃
	356–5	〃
B. gladioli pv. *gladioli*	ATCC 10248^T	ATCC
	NIAS 1065	NIAS
	E–14	AKU
	MAFF 302515	NIAR
	MAFF 301064	〃
	MAFF 301580	〃
	MAFF 302385	〃
	MAFF 302537	〃
	MAFF 302544	〃
B. gladioli pv. unidentified	MAFF 302418	〃
	MAFF 302409	〃
	MAFF 302424	〃
B. glumae	MAFF 301169^T	〃
	MAFF 302423	〃
	2	KNAES
	Kyu 82–34–2	〃
	8015	〃
	8020	〃
	N 7401	NIAS
	N 7501	〃
	N 7504	〃
	N 7505	〃
	Ku 8104	AKU
	Ku 8105	〃
	Ku 8112	〃
B. plantarii	MAFF 301723^T	NIAR
	MAFF 302387	〃
	MAFF 302484	〃
	MAFF 302412	〃
	MAFF 302467	〃
	MAFF 302470	〃

Continued Table 1

Bacteria	Isolate	Source[a)]
	MAFF 302475	NIAR
	MAFF 302481	〃
	MAFF 302485	〃
	MAFF 302392	〃
B. vandii	JCM 7957[T]	JCM
B. andropogonis	MAFF 301006	NIAR
	MAFF 301129	〃
H. rubrisubalbicans	MAFF 301626	〃
	MAFF 301628	〃
R. solanacearum	ATCC 11696[T]	ATCC
	C 319	KTES
	8107	NIVOT
	8224	〃
	6511	NIAS
	Ku 7502–1	AKU
	855	UNB
	Z 9	IPSA
	Z 10	〃
P. syringae pv. *syringae*	ATCC 19310[T]	ATCC
pv. *oryzae*	MAFF 301538	NIAR
pv. *tabaci*	PA 28	KTES
pv. *tabaci*	P 41	NIAS
pv. *tabaci*	Ku 8102	AKU
pv. *lachrymans*	NIAS 1319	NIAS
pv. *lachrymans*	NIAS 1321	〃
pv. *mori*	P 23	〃
pv. *pisi*	MAFF 301211	NIAR
pv. *pisi*	MAFF 301213	〃
pv. *theae*	MAFF 750001	〃
pv. *coronafaciens*	MAFF 301314	〃
pv. *phaseolicola*	MAFF 301616	〃
pv. *phaseolicola*	MAFF 301716	〃
pv. *japonica*	MAFF 301163	〃
pv. *atropurpurea*	MAFF 301307	〃
pv. *morsprunorum*	MAFF 301444	〃
pv. *myricae*	MAFF 301464	〃
pv. *tomato*	MAFF 301593	〃
pv. *glycinea*	KN 28	NIAS
pv. *striafaciens*	P 71	〃

[a)]ATCC: American Type Culture Collection, USA.
NIAS : National Institute of Agricultural Sciences, Tokyo, Japan.
NIAR: National Institute of Agrobiological Resources, Tsukuba, Japan.
AKU: Faculty of Agriculture, Kyushu University, Fukuoka, Japan.
KNAES: Kyushu National Agricultural Experiment Station, Kumamoto, Japan.
JCM: Japan Collection of Microorganisms, Saitama, Japan.
KTES: Kagoshima Tobacco Experiment Station, Japan.
NIVOT: National Research Institute of Vegetables, Ornamental Plants and Tea, Morioka, Japan.
UNB: University of Brasilia, Brasilia, Brazil.
IPSA : Institute of Post Graduate Studies in Agriculture, Gazipur, Bangladesh.
Type culture: Small T at the shoulder of isolate number indicates type culture.

Growth conditions

All bacterial strains were grown on slants of King's B agar medium (Eiken Chem. Co.). The medium consists of 20.0 g peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, 15.0 g agar and 1 litre of 1% glycerol solution, pH 7.2. Slant cultures were grown at 30 °C for 3 days.

Lipid extraction

One loopful bacterial cells was collected in a small glass vial from the slant culture and 0.2 ml of chloroform – methanol – 0.3% NaCl solution (2 : 1 : 0.4, v/v/v) was added and the vial was capped tightly. Bacterial cells were suspended gently and kept at least for 15 min at room temperature.

Thin layer chromatography

Lipid extract (about 10 μl) was spotted on the origin of pre-coated silica gel TLC plate (Merck Co., Si 60, 0.25 mm in thickness) maintaining at a distance of 1.0 cm from the bottom edge of the plate and 1.5 cm from each other sample spots. The development of TLC plate was conducted in a rectangular glass tank with chloroform – methanol – 0.2% $CaCl_2 \cdot 2H_2O$ solution (55 : 35 : 8, v/v/v) solvent system for 1 h at 25 °C. After development the TLC plate was dried well.

Detection of lipid spots

Lipid spots on TLC plate were detected by spraying ninhydrin (Ninhydrin spray, Tokyo Kasei Chem. Co.) followed by heating in an oven at 100 °C for 10 min. The chromatograms were recorded by photocopy, photograph and/or computer (Adobe photoshop 3.0 J).

RESULTS

Figures 1, 2 and 3 show the chromatographic profiles of lipids from *Burkholderia* spp., *R. solanacearum*, *H. rubrisubalbicans* and *P. syringae* pathovars. Lipid spots observed on TLC plate discriminate the strains at species level. The presence or absence and relative size of three benchmark spots designated as S_1, S_2, and S_3 (Figs. 1, 2 and 3) under the common spot (Rf 0.76) were species specific. The spot S_2 was larger than spots S_1 and S_3 in *B. caryophylli*, whereas spot S_1 was larger than S_2 and S_3 in case of *B. cepacia*. The chromatograms of *B. gladioli*, *B. glumae*, *B. plantarii* and *B. vandii* resembled each other. However, spot S_3 was found larger than S_1 and S_2 in *B. plantarii* and *B. vandii* (Figs. 1, 2 and 3).

The chromatograms of *B. andropogonis* and *R. solanacearum* were roughly similar at Rf 0.62–0.76 area and spot S_1 was absent. Spot S_3 was absent or faint in *R. solanacearum* and different from *B. andropogonis*. The chromatograms of *H. rubrisubalbicans* and *P. syringae* pathovars were very special type, where spots S_1, S_2 and S_3 were not detected but a spot at Rf 0.83 appeared in *H. rubrisubalbicans* along with the common spot at Rf 0.76 (Figs. 2 and 3).

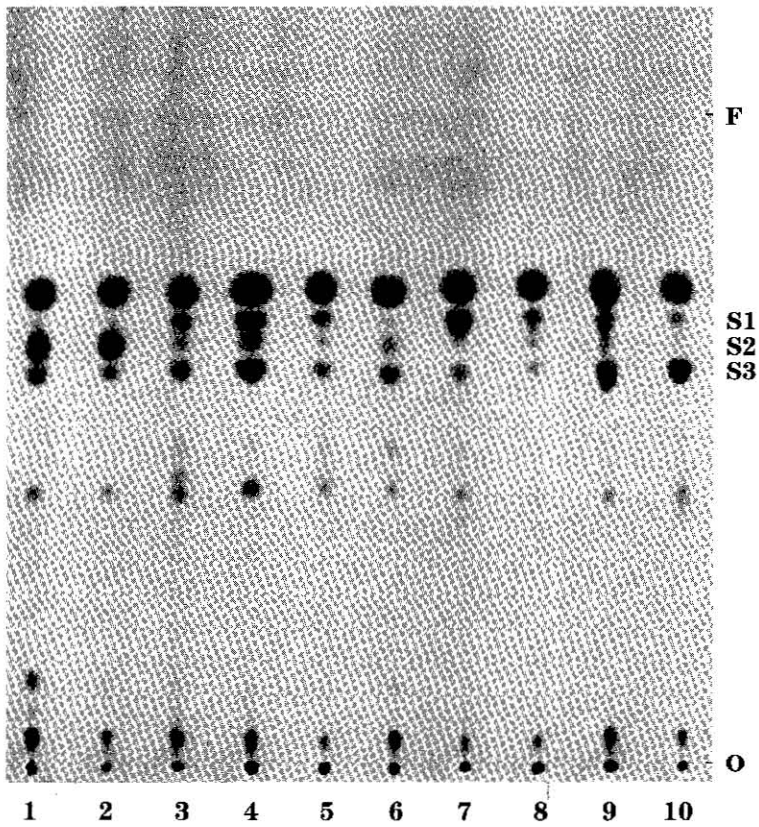

Fig. 1. TLC chromatograms of lipids from *Burkholderia* spp.
Lanes 1, *B. caryophylli* ATCC 25418^T; 2, *B. caryophylli* NIAS 1406; 3, *B. gladioli* pv. *gladioli* ATCC 10248^T; 4, *B. gladioli* pv. *gladioli* MAFF 302515; 5, *B. glumae* MAFF 301169^T; 6, *B. glumae* N 7501; 7, *B. cepacia* ATCC 25416^T; 8, *B. cepacia* 356–5; 9, *B. plantarii* MAFF 301723^T; 10, *B. vandii* JCM 7957^T.
F: Solvent front, O: Origin; S_1, S_2 and S_3 indicate the benchmark spots.

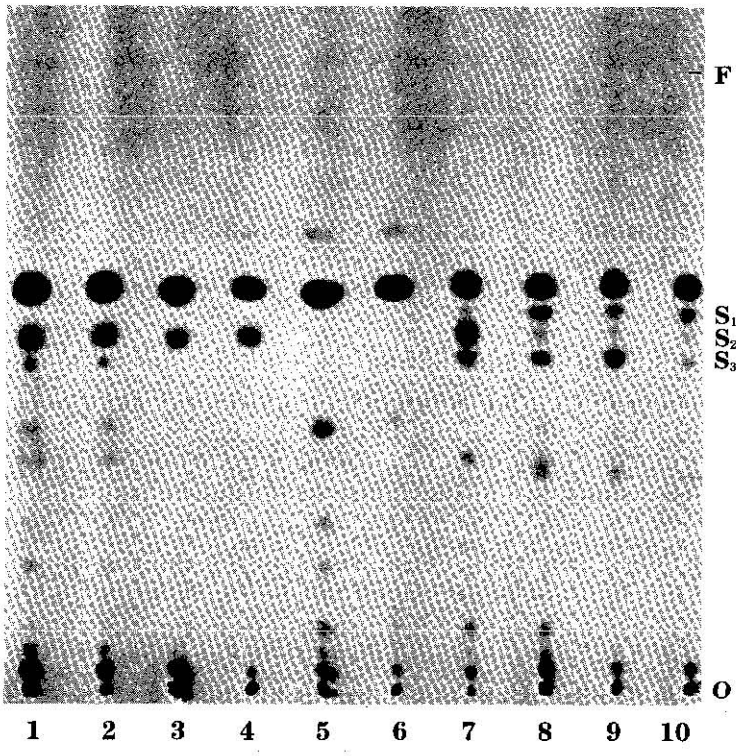

Fig. 2. TLC chromatograms of lipids from *Burkholderia* spp., *R. solanacearum* and *H. rubrisubalbicans*.
Lanes 1, *B. andropogonis* MAFF 301006; 2, *B. andropogonis* MAFF 301129; 3, *R. solanacearum* ATCC 11696^T; 4, *R. solanacearum* Ku 7502–1; 5, *H. rubrisubalbicans* MAFF 301626; 6, *H. rubrisubalbicans* MAFF 301628; 7, *B. caryophylli* ATCC 25418^T; 8, *B . gladioli* pv. *gladioli* ATCC 10248^T; 9, *B. plantarii* MAFF 301723^T; 10, *B. cepacia* ATCC 25416^T.
F: Solvent front, O: Origin; S_1, S_2 and S_3 indicate the benchmark spots.

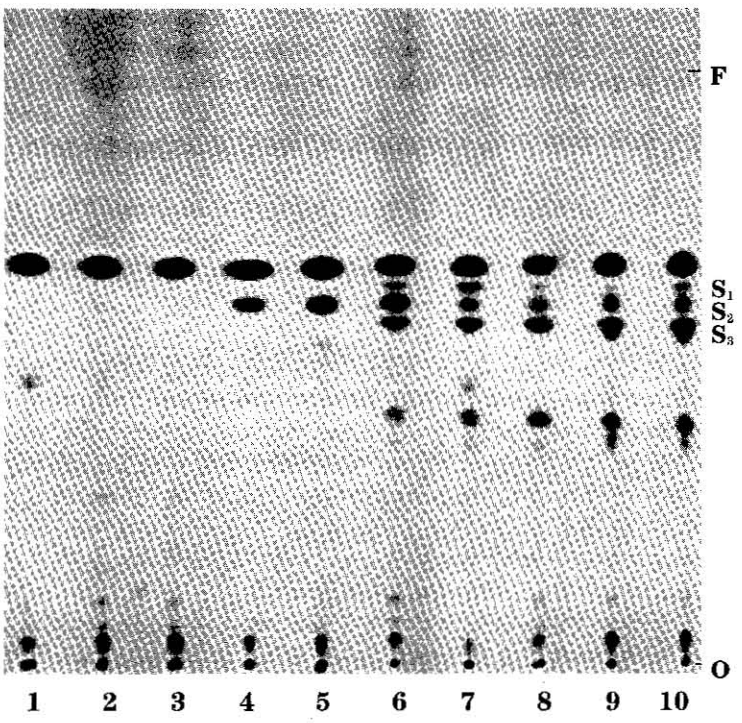

Fig. 3. TLC chromatograms of lipids from *P. syringae*, *R. solanacearum* and *Burkholderia* spp.
Lanes 1, *P. syringae* pv. *syringae* ATCC 19310^T; 2, *P. syringae* pv. *phaseolicola* MAFF 301616; 3, *P. syringae* pv. *tomato* MAFF 301593; 4, *R. solanacearum* ATCC 11696^T; 5, *R. solanacearum* C 319; 6, *B. caryophylli* MAFF 301060; 7, *B. gladioli* pv. *gladioli* MAFF 301580; 8, *B. glumae* Ku 8112; 9, *B. plantarii* MAFF 302412; 10, *B. plantarii* MAFF 302470.
F: Solvent front, O: Origin; S_1, S_2 and S_3 indicate the benehmark spots.

DISCUSSION

Rapid and easy identification methods for phytopathogenic bacteria have been sought for some time. PCR–based molecular methods (Jensen *et al.*, 1993; Bereswill *et al.*, 1994; Manceau and Horvais, 1997) and chemotaxonomic markers like fatty acid profiles (Ikemoto *et al.*, 1978; De Boer and Sasser, 1986; Roy, 1988; Chase *et al.*, 1992; Kori *et al.*, 1992; Stead, 1992) and protein profiles (Van Zyl and Steyn, 1990; Li and Hayward, 1994) have been used for the rapid identification of phytopathogenic bacteria. A stable marker is needed for identification and classification of different groups of bacteria. Though the lipid profiles have not been used routinely in bacterial identification, extensive studies have shown their potential as taxonomic markers (Counsell and Murray, 1986; Matsuyama *et al.*, 1987). The direct colony TLC method for rapid detection and identification of bacterial lipids was developed and used for the identification of *Serratia* spp. (Matsuyama *et al.*, 1986, 1987). The rapid identification of phytopathogenic bacteria by using this direct colony TLC has also been reported (Matsuyama *et al.*, 1993a; Matsuyama and Furuya, 1993b; Matsuyama, 1995). Recently, we used the simple TLC method for the identification of phytopathogenic bacteria (Khan and Matsuyama, 1998). However, both the direct colony TLC and the simple TLC methods need 1 – 2 h for drying the bacterial colony/lipid spot on the TLC plate before developments. This constraint is possible to overcome by our present method. Hence this method is less time consuming and easy to perform.

The taxonomic utility of lipid profiles requires reasonable uniformity within the group. The lipid profiles of strains of each species used in this experiment showed obviously uniformity within the species and distinguishable from the member of other species on the basis of three benchmark spots (S_1, S_2 and S_3) appeared on TLC plate under the common spot at Rf 0.76 (Figs. 1, 2 and 3). The results of our present method agreed well with the former results by the direct colony TLC and simple TLC (Matsuyama and Furuya, 1993b; Matsuyama, 1995; Khan and Matsuyama, 1998). Lipid spots appeared at Rf 0.42–0.52 were also important benchmarks for some strains and these spots were not detectable by the original direct colony TLC method (Matsuyama and Furuya, 1993b; Matsuyama, 1995). The chromatograms of the most members of *Burkholderia* such as *B. caryophylli, B. cepacia, B. gladioli, B. glumae, B. plantarii* and *B. vandii* resembled each other by lipid profiles but were clearly distinct from those of other strains which were also members of rRNA homology group–II pseudomonads; which justified the proposal of shifting *P. caryophylli, P. cepacia, P. gladioli, P. glumae* and *P. plantarii* into the new genus *Burkholderia* (Yabuuchi *et al.*, 1992; Urakami *et al.*, 1994). The chromatograms of *R. solanacearum* (*P. solanacearum*) were roughly similar to those of *B. andropogonis* but quite different from other *Burkholderia* spp. The lipid profile of *H. rubrisubalbicans* (*P. rubrisubalbicans*) was quite unique. In case of *P. syringae* pathovars, only the common spot at Rf 0.76 was observed. Fatty acid profiles of *P. syringae* pathovars also differentiated them from rRNA homology group II pseudomonads. Again *H. rubrisubalbicans* and *R. solanacearum* were also discriminated from other members of rRNA homology group II pseudomonads on the basis of hydroxy fatty acid composition (Stead, 1992; Young *et al.*, 1992). These results were also supported by the findings of our TLC methods. The substances visualized by ninhydrin

spray perhaps aminolipids, characterization of lipids is subject for others.

This rapid extraction–TLC method will be practical and convenient for rapid differentiation as well as identification of *Burkholderia* spp., *R. solanacearum, H. rubrisubalbicans* and *P. syringae* pathovars.

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