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Rapid Identification of Phytopathogenic Bacteria by TLC

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A rapid identification by TLC was performed for phytopathogenic bacteria (*Burkholderia* spp., *Ralstonia solanacearum* and *Herbaspirillum rubrisubalbicans*). One loopful bacterial cell was suspended in 0.2 ml of chloroform-methanol (2 : 1, v/v) in a small glass-vial and kept for 15 min in room temperature. About 10 μl of lipid extract was spotted on the origin of silica gel TLC plate and dried well. The plate was developed with chloroform-methanol-0.2% calcium chloride (55:35:8, v/v/v) for 1 hr at 25 °C. After drying, the spots were visualized by spraying ninhydrin and successive heating at 100 °C for 10 min. The chromatograph was recorded by a photograph and/or photocopy. The lipid spots appeared on TLC plate at Rf 0.42–0.83 area were reliable benchmarks for differentiation of rRNA homology group II pseudomonads. The chromatograms of *Burkholderia caryophylli, B. cepacia, B. gladioli, B. glumae, B. plantarii* and *B. vandii* resembled each other but were distinct at species level and clearly different from *R. solanacearum, B. andropogonis* and *H. rubrisubalbicans*. The chromatographs of *B. andropogonis* and *R. solanacearum* were found roughly similar, but characteristic spots at Rf 0.42–0.52 region were absent in *R. solanacearum*. On the other hand, the profile of *H. rubrisubalbicans* was quite unique. This TLC method will be practical for rapid identification of phytopathogenic bacteria.

INTRODUCTION

The identification of phytopathogenic bacteria usually requires a series of physiological and biochemical tests under a determinative scheme and a host pathogenicity tests. These all tests are time consuming and in some cases difficult to interpret the results. Recently, chemotaxonomy has been introduced in systematic taxonomy and identification of bacteria by cellular fatty acid analyses has been well established (Chase *et al.*, 1992; De Boer and Sasser, 1986; Ikemoto *et al.*, 1978; Kori *et al.*, 1992; Roy, 1988). However, this method needs facilities and preparation of samples are time consuming.

The direct colony TLC for lipid identification was firstly invented by Matsuyama *et al.* (1986) and applied successfully for rapid identification of phytopathogenic bacteria (Matsuyama *et al.*, 1993a, b, c; Matsuyama and Furuya, 1993; Matsuyama, 1995a,b). This method, however, needs about 1–2 hr for drying the bacterial colony on TLC plate before development and requires two times developments in two different solvent systems. The modification of original direct colony TLC method was conducted and results were presented in this report.

MATERIALS AND METHODS

Bacterial strains

Thirty one isolates of *Burkholderia* spp., *Ralstonia solanacearum* and *Herbaspirillum rubrisubalbicans* (all were formerly rRNA homology group II pseudomonads) were tested in this experiment. The details of the isolates were given in Table 1.

Table 1. List of bacterial isolates used in this study.

Phytopathogenic bacteria	Isolate	Source
Burkholderia caryophylli	NIAS 1192	NIAS
	NIAS 1406	NIAS
B. cepacia	ATCC 25416^T	ATCC
	343-4	NIAS
	356-3	NIAS
	356-5	NIAS
B. gladioli pv. *gladioli*	ATCC 10248^T	ATCC
	NIAS 1065	NIAS
	E-14	AKU
	MAFF 302515	NIAR
B. gladioli pv. unidentified	MAFF 302418	NIAR
B. glumae	MAFF 301169^T	NIAR
	2	KNAES
	N 7504	NIAS
	N 7501	NIAS
	Kyu 82-34-2	KNAES
B. plantarii	MAFF 301723^T	NIAR
	MAFF 302387	NIAR
	MAFF 302484	NIAR
B. vandii	JCM 7957^T	JCM
B. andropogonis	MAFF 301006	NIAR
	MAFF 301129	NIAR
Herbaspirillum rubrisubalbicans	MAFF 301626	NIAR
	MAFF 301628	NIAR
Ralstonia solanacearum	ATCC 11696^T	ATCC
	C 319	KTES
	8107	NRIV
	8224	NRIV
	6511	NIAS
	Ku 7502-1	AKU
	855	UNB

NIAS: National Institute of Agricultural Sciences, Tokyo, Japan.
ATCC: American Type Culture Collection.
AKU: Faculty of Agriculture, Kyushu University, Fukuoka, Japan.
NIAR: National Institute of Agricultural Resources, Tsukuba, Japan.
KNAES: Kyushu National Agricultural Experiment Station, Kumamoto, Japan.
JCM: Japan Collection of Microorganisms, Saitama, Japan.
KTES: Kagoshima Tobacco Experiment Station, Japan.
NRIV: National Reasearch Institule of Vegetable, Ornamental Plant and Tea Morioka Branch, Japan.
UNB: University of Brasilia, Brasilia, Brazil.
Type culture: Small T at the shoulder of isolate number indicated type culture.

Growth conditions

Each culture was grown on a slant of King's B agar medium (20.0 g peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, 15.0 g agar, 1 liter of 1% glycerol solution, pH7.2, Eiken Chem. Co.) at 30 °C for 3 days.

Lipid extraction

One loopful bacterial cell was placed in a small glass vial and 0.2 ml of chloroform-methanol solution (2:1, v/v) was added and the vial was capped tightly. Bacterial cells were mixed gently and kept for 15 min at room temperature.

Thin-layer chromatography

About 10 μl of lipid extract was spotted on the origin of pre-coated silica gel TLC plate (Merck Co. Si 60, 0.25 mm in thickness) and completely dried by a hair drier. Sample spots were placed aparting 1.5 cm each other at a distance of 1.0 cm from the bottom edge of the plate. The plate was developed with chloroform-methanol-0.2% calcium chloride (55:35:8, v/v/v) solvent system for 1 hr and dried well. The development was conducted in an incubator at 25 °C.

Detection of spots

Detection of the spots on TLC plate was performed by spraying ninhydrin (Ninhydrin spray, Tokyo Kasei Chem. Co.) followed by heating in an oven at 100 °C for 10 min. The spraying of sulfuric acid-dichromate (0.6% $K_2Cr_2O_7$ in 50% H_2SO_4) followed by heating at 130 °C for 15 min was also conducted for visualizing of the spots of some pseudomonads. The chromatograms were recorded by photograph and/or photocopy.

RESULTS

The chromatographic profiles of *Burkholderia* spp., *Ralstonia solanacearum* and *Herbaspirillum rubrisubalbicans* were shown in Figs. 1 and 2. Distinct differences were found among the chromatographic profiles at species level. There were three benchmark spots, S_1, S_2 and S_3 (Fig. 1, 2) under the common spot (Rf 0.76) represented well the characteristics of each species. The existence and relative size of the spots were species specific. In *B. caryophylli*, spot S_2 was larger than spots S_1 and S_3, whereas spot S_1 was larger than S_2 and S_3 in the case of *B. cepacia*. The chromatograms of *B. gladioli*, *B. glumae*, *B. plantarii* and *B. vandii* resembled each other. However, spots S_1 and S_3 were larger than S_2 in *B. gladioli* and *B. glumae*. Again, spot S_2 was slightly prominent in *B. gladioli* than that of *B. glumae*. *B. plantarii* and *B. vandii* were not distinguishable, where spot S_3 was larger than spots S_1 and S_2 (Fig. 1, 2).

The chromatograms of *B. andropogonis* and *R. solanacearum* were similar at Rf 0.62–0.76 area but differences were observed at Rf 0.42–0.52. Characteristic spots at Rf 0.42–0.52 area were absent in *R. solanacearum*. A spot at Rf 0.90 was also detected in *B. andropogonis* by spraying with sulfuric acid-dichromate but was not detected in *R. solanacearum*.

The profile of chromatograms of *H. rubrisubalbicans* was very special, where spots S_1, S_2 and S_3 were absent but a spot at Rf 0.83 appeared along with the common spot at Rf 0.76 (Fig. 2).

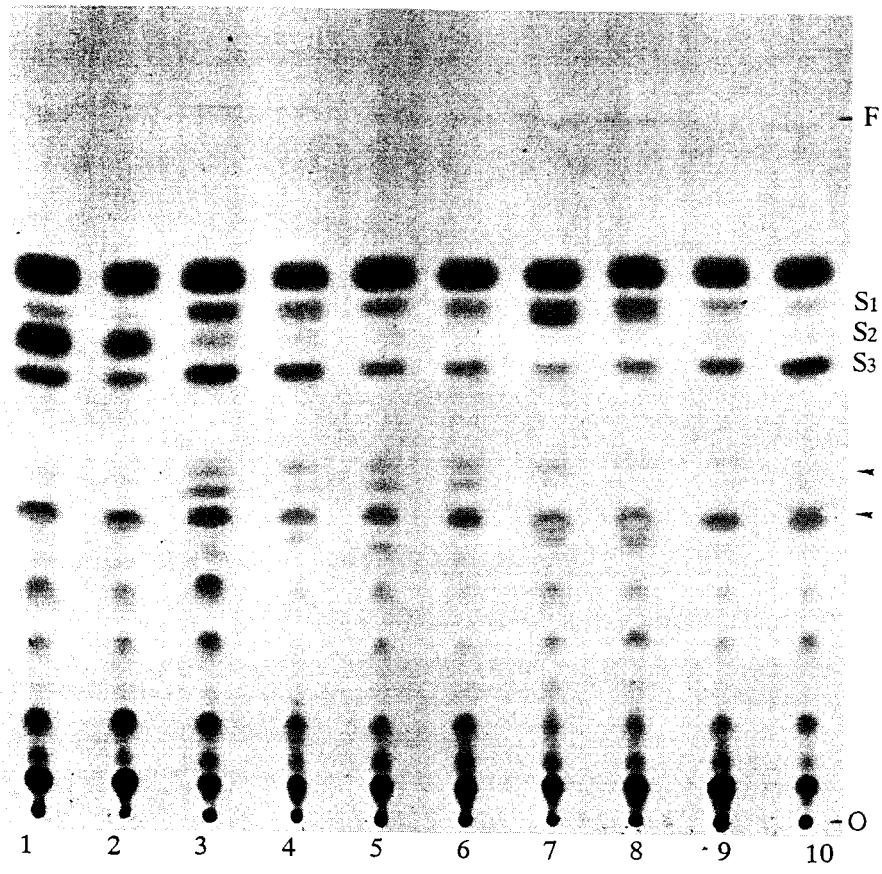

Fig. 1. TLC chromatograms of lipids from phytopathogenic bacteria.
1. *B. caryophylli* NIAS 1192
2. *B. caryophylli* NIAS 1406
3. *B. gladioli* pv. *gladioli* ATCC10248^T
4. *B. gladioli* pv. unidentified MAFF 302418
5. *B. glumae* MAFF 301169^T
6. *B. glumae* Kyu 82-34-2
7. *B. cepacia* ATCC 25416^T
8. *B. cepacia* 356-5
9. *B. plantarii* MAFF301723^T
10. *B. plantarii* MAFF302387

F: Solvent front, O: Origin
Double arrow heads indicate Rf 0.42–0.52

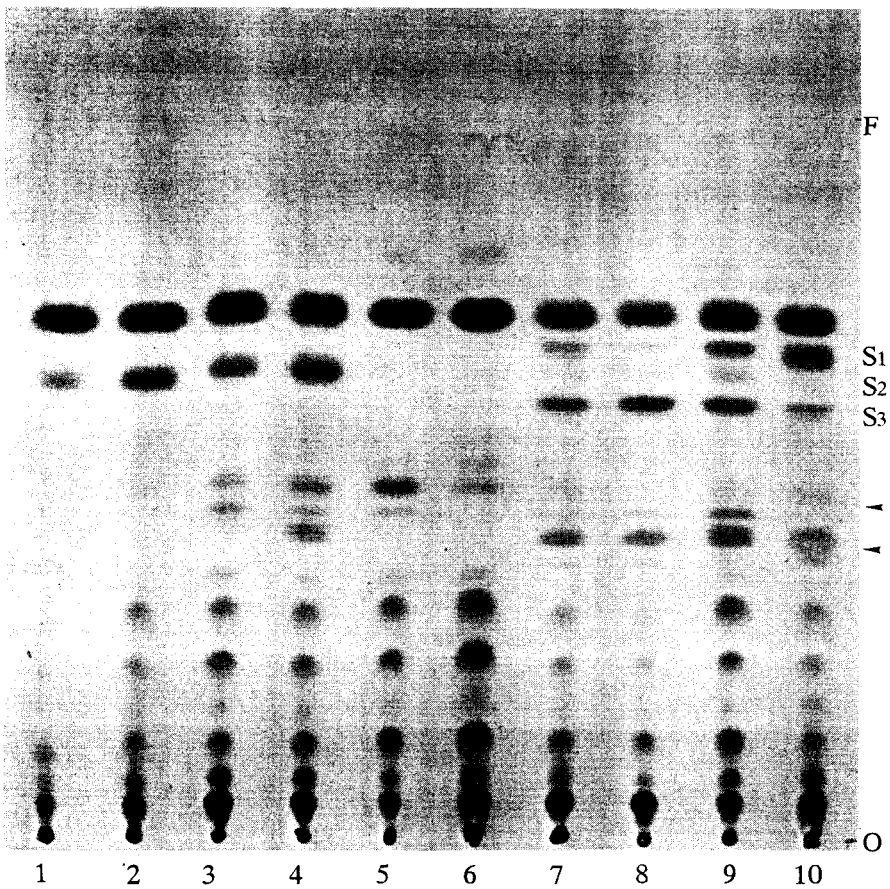

Fig. 2. TLC chromatograms of lipids from phytopathogenic bacteria.
1. *R. solanacearum* ATCC11696^T
2. *R. solanacearum* C 319
3. *B. andropogonis* MAFF 301006
4. *B. andropogonis* MAFF 301129
5. *H. rubrisubalbicans* MAFF 301626
6. *H. rubrisubalbicans* MAFF 301628
7. *B. plantarii* MAFF301723^T
8. *B. vandii* JCM 7957^T
9. *B. gladioli* pv. *gladioli* ATCC10248^T
10. *B. cepacia* ATCC 25416^T

F: Solvent front, O: Origin
Double arrow heads indicate Rf 0.42–0.52

DISCUSSION

Though lipid profile has not been used routinely in bacterial identification, extensive studies have shown their potential as taxonomic markers. Matsuyama *et al.* (1986, 1987) invented direct colony TLC for lipid analysis and used this method for identification of *Serratia* spp. The comparison of lipid profiles for rapid identification of phytopathogenic bacteria was performed by using the direct colony TLC (Matsuyama *et al.*, 1993a,b,c; Matsuyama and Furuya, 1993; Matsuyama, 1995a, b).

Since drying of the bacterial cells pasted on TLC plate was time consuming, modification of the direct colony TLC method was conducted. Bacterial lipid was extracted in a glass vial for only 15 min with chloroform-methanol solution (2:1, v/v). Hence, the first development in the direct colony TLC for lipid extraction was avoided in the present method.

Among the strains used, chromatographic differences at species level were obviously observed and the species were distinguished on the basis of three benchmark lipid spots (S_1, S_2 and S_3) appeared on TLC plate under the common spot at Rf 0.76 (Fig. 1, 2), and this result completely agreed with the former result (Matsuyama, 1995b). However, 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 (Matsuyama and Furuya, 1993; Matsuyama, 1995b). 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. This result also justified the proposal of transferring of *B. caryophylli, B. cepacia, B. gladioli, B. glumae, B. plantarii* and *B. vandii* into a new genus *Burkholderia* (Yabuuchi *et al.*, 1992; Urakami *et al.*, 1994). On the other hand, chromatograms of *B. andropogonis* and *R. solanacearum* were roughly similar. But lipid spots at Rf 0.42–0.52 were absent in *R. solanacearum*. It also differed from other rRNA homology group II pseudomonads on the basis of these lipid spots. The lipid profile of *H. rubrisubalbicans* was quite unique. An unique profile of *H. rubrisubalbicans* was formerly reported (Matsuyama, 1995b) and the same result was confirmed also in the present solvent system. Stead (1992) reported that *H. rubrisubalbicans* and *R. solanacearum* could be differentiated from other rRNA homology group II pseudomonads on the basis of hydroxy fatty acid composition. These results were supported by the findings of our present lipid-TLC.

Thus, this simple lipid-TLC method is very practical and convenient for rapid identification and classification of phytopathogenic bacteria by comparison of lipid profiles.

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