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Application of the Direct Colony TLC Method for Identification of Phytopathogenic Bacteria

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Direct colony thin-layer chromatography was applied for the identification of the phytopathogenic bacteria. This method involves two steps such as the extraction of lipids from bacterial cells and their development. Chloroform-methanol (2:1, v/v) and chloroform-methanol-5M ammonia (60:25:4, v/v) were used as the solvents for the first and second developments, respectively. Omission of the first step and the direct development with the second solvent gave worse results with tailing. Inter-genus differences, at least, were observed among the chromatograms. Striking difference of chromatogram was observed between *Clavibacter* spp. (Gram positive) and others (Gram negative). The chromatograms of *Erwinia* spp. were characteristic and quite different from those of *Xanthomonas* spp. and *Agrobacterium tumefaciens*. Although some difficulties like the cases of *Pseudomonas* spp. are still remaining, the practical usefulness of this method for a rapid identification of phytopathogenic bacteria was certainly verified.

INTRODUCTION

The identification of phytopathogenic bacteria has been conducted by physiological, pathological and serological tests. While, the analytical data on bacterial cell membrane have been used for the identification and classification of the bacteria. Especially, the usefulness of the membrane lipid analysis has been emphasized (Ikemoto et al., 1978, Suzuki and Komagata, 1983, De Boer and Sasser, 1986, Chase et ***al.*** 1992, Kori et ***al., 1992).*** However, these methods take time for the preparation of the samples and are not convenient in the case of identification of many isolates.

A rapid and easy identification method by silica-gel thin layer chromatography (TLC) was invented by Matsuyama, T. et *al.* (1987) for the identification of *Serratia* spp. This direct colony TLC method involves two steps such as the extraction of lipids from the bacterial cells and the development of the extracts on a same TLC plate. Application of this method for the rapid identification of phytopathogenic bacteria was conducted. The abstract has been presented elsewhere (Matsuyama et ***al., 1992)*** and the details will be contributed in this report.

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MATERIALS AND METHODS

Bacteria: The various phytopathogenic bacteria of different sources were used for this experiment (Table 1).

Culture: The culture of the bacteria was conducted at 25°C for 3, 5, 15 days on the slant of the modified potato semi-synthetic agar medium (PSA): 39 g PDA medium (OXOID^R, UNIPATH Ltd.), 2.0g $Na_2HPO_4 \cdot 12H_2O$, 0.5g $Ca(NO_3)_2 \cdot 4H_2O$, 5g peptone, 1 l distilled water, pH 7.0. In this medium, sucrose was substituted with glucose and this part was different from the original PSA medium.

Thin layer chromatography: One loopful bacterial cells was taken from the colony of each isolate and spotted on silica gel TLC plate (Si 60, 0.25mm, Merck Co.), directly. Spots were separated 1.5cm each other and aparted 1.0cm from the bottom of the edge of TLC plate. The plate was developed at first with chloroform-methanol (CM, 2:1, v/v) for ca. 10min until the solvent front will reach 6cm-line from the origin. The plate was dried completely at room temperature, scraped off the bacterial cells and developed with the second solvent, chloroform-methanol-5M ammonia (CMA, 60:25:4, v/v) for ca. 1 hour.

After development, the plate was dried well, sprayed with ninhydrin solution

Table 1. List of phytopathogenic bacteria used in this experiment.

Phytopathogenic bacteria	Sources
Clavibacter michiganensis subsp. *sepedonicum* **1**	NIAES
Clavibacter michiganensis subsp. *sepedonicum* 17-4	NIAES
Clavibacter michiganensis subsp. ***michiganensis N6601***	NIAES
Clavibacter michiganensis subsp. ***michiganensis*** N6204	NIAES
Erwinia ***chysanthemi*** pv. ***chysanthemi*** Ku8601 L1	AKU
Erwinia ***carotovora*** subsp. ***carotovora 489-4***	LSPPM
Erwinia ***carotovora*** subsp. ***carotovora*** EH8519	NIAES
Xanthomonas *campestris* pv. *citri* N6102-1	NIAES
Xanthomonas oyzae pv. o *ryzae* Q7463	KNAES
Xanthomonas *oryzae* pv. ***oyzae*** T7174 SNR (12)	HNAES(TKAES)
Xanthomonas oyzae pv. ***oyzae*** Q7781	KNAES
Agrobacterium ***tumefaciens*** l-3a	AKU
Agrobacterium ***tumefaciens*** 1-SKRa	AKU
Pseudomonas *glumae* Kyu 82-34-2	KNAES
Pseudomonas *glumae* 2	KNAES
Pseudomonas solanacearum 8224	NRSVOT
Pseudomonas solanacearum 8109	NRSVOT
Pseudomonas solanacearum C319SR	KTES
Unidentified strain UI-PS6227	AKU

NIAES: National Institute of Agro-Environmental Sciences, Ibaraki, Japan
AKU : Faculty of Agriculture, Kyushu University, Fukuoka, Japan
HNAES: Hokuriku National Agricultural Experiment Station, Niigata, Japan
TKAES: Tokai-Kinki Agricultural Experiment Station, Japan
KNAES: Kyushu National Agricultural Experiment Station, Kumamoto, Japan
LSPPM: Laboratory of Seed and Post-Harvest Disease, Plant Pathology and Microbiology Division, Department of Agriculture, Thailand
NRSVOT: National Research Station of Vegetable, Ornamental and Tea, Iwate, Japan
KTES : Kagoshima Tobacco Experimental Station, Kagoshima, Japan

(Ninhydrin spray, Tokyo Kasei Co.) and kept at 100°C for 10 min. After the recording of the chromatogram, 50% sulfuric acid solution was sprayed and the plate was kept at 130-150°C for 15~30min.

The solvent, chloroform-methanol-water (CMW, 60:25:4, v/v) was also useful for the second development substituting for CMA solutions.

RESULTS AND DISCUSSION

In the case of the identification and a systematic classification of phytopathogenic bacteria, numerous tests on the structures and functions of bacteria have been required. These tests are quite time-consuming and need lots of facilities. Therefore more rapid and easy ways have been requested.

In 1987, Matsuyama et *al.* firstly reported the direct colony TLC method which is quite unique and practically useful.

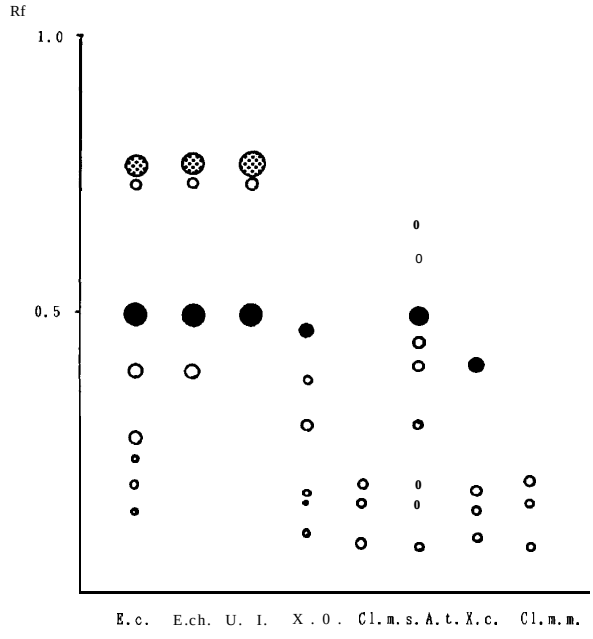

Fig. 1. Diagrammatic representation of the results obtained by the direct colony thin-layer chromatography.
E. c.:*Erwinia* ***carotovora*** subsp. ***carotovora 489-4***
E. ch.: ***Erwinia chrysanthemi*** pv. ***chrysanthemi Ku8601*** L1
U. I.: Unidentified strain UI-PS6227
X. o.: ***Xanthomonas*** *oryzae* pv. *oryzae* Q7463
Cl. m. s.: ***Clavibacter michiganensis*** subsp. *sepedonicum* 1
A. t.: *Agrobacterium* ***tumefaciens*** l-3a
X. c.: ***Xanthomonas*** *campestris* pv. *citri* N6102-1
Cl. m. m.: ***Clavibacter michiganensis*** subsp. ***michiganensis N6601***

As shown in Fig. 1, the chromatogram of each bacterium is characteristic. Striking difference was observed between Gram positive bacteria, *Clavibacter michiganensis* subspecies, and Gram negative bacteria. This result, however, was expected because of the distinct diversity between Gram positive and negative bacteria in various characteristics. Among Gram negative bacteria, the chromatograms of *Erwinia* spp. were distinct from others. The spots of Rf 0.79 and 0.75 were characteristic to the *Erwinia* spp. and the spot at ca. Rf 0.5 was larger and clearly different from that of *Xanthomonas* spp. The chromatogram of the unidentified isolate (Fig. 1, 3rd lane from the left) showed the characteristics of *Erwinia* spp. The electron microscopic observation also supported the identification by this TLC method.

Characteristic spot at Rf 0.78 was not easily detectable when CMW solvent was used for CMA. Ammonia in the solvent could relate with this result. The properties of the substances which are detectable with ninhydrin are unknown now. The possibility of aminolipids which have been stressed recently as the benchmarks for distinction of bacteria will be clarified in future.

Well reappearance of the results was obtained if the conditions of the experiment were kept uniformly. The chromatograms of the bacteria cultured for 3-15 days were almost same and no distinct differences were observed. Omission of the first step with CM solvent and direct development with CMA gave worse results with tailing.

The chromatography of some isolates of *Pseudomonas* spp. gave sometimes worse results and the spots were irregular-shaped. These trends seemed to be fixed character for some *Pseudomonas* isolates. Some extra-cellular substances could disturb the smooth extraction of lipids with the CM solvent. However, these problems were sometimes solved by using King B medium for PSA medium.

In this experiment, inter-genus distinction, at least, was succeeded and the inter-species or inter-pathovars distinction will be tried in near future by using many isolates of various origins. Although this direct colony TLC method could not be always applicable to the identification of *Pseudomonas* spp., this method will be useful mean for a rapid identification of phytopathogenic bacteria.

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