Trials for Rapid Identification of Phytopathogenic Bacteria by HPLC and the Direct Colony TLC

Matsuyama, Nobuaki
Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University

https://doi.org/10.5109/24096

出版情報：九州大学大学院農学研究院紀要. 40 (1/2), pp.87-91, 1995-12. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., 40 (1·2), 87-91 (1995)

Trials for Rapid Identification of Phytopathogenic Bacteria by HPLC and the Direct Colony TLC

Nobuaki Matsuyama

Laboratory of Plant Pathology, Faculty of Agriculture,
Kyushu University, Fukuoka 812-81, Japan
(Received July 17,1995)

A novel technique with HPLC for rapid identification of phytopathogenic bacteria was devised and its usefulness was estimated comparing with the direct colony TLC. One loopful bacteria was suspended in 0.5 ml of chloroform-methanol (2 : 1, v/v) in a small glass-vial and kept for 1 hr in room temperature. After the evaporation of the solvent, 1 ml of SO% aqueous methanol solution was added and the extract was dissolved. This solution was passed through a membrane filter and 20μl of it was subjected to HPLC. The diversity of chromatograms obtained for two genera of bacteria, *Clavibacter* and *Erwinia*, was quite obvious and the distinct differences were also observed at species level in *Erwinia*.

INTRODUCTION

The identification of phytopathogenic bacteria has been conducted by morphological, physiological, serological and pathological tests. These tests, however, are time-consuming and need experiences for analyzing results. In 1986, the direct colony thin layer chromatography was invented (Matsuyama *et al.*, 1986) and firstly applied for rapid identification of phytopathogenic bacteria (Matsuyama 1993a, Matsuyama et al., 1993b, c, d, Matsuyama and Furuya, 1993e). In these experiments, it was clarified that the differentiation of the phytopathogenic bacteria at generic level was available among *Clavibacter, Agrobacterium* and others. Further, in genus *Erwinia,* the profiles of *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* were clearly different. Such differences at species level were also observed among some species of genus *Pseudomonas.* These results indicated practical usefulness of this easy method for rapid identification of phytopathogenic bacteria. In the case of identification at subspecies or pathovar level, however, higher resolution ability in chromatography will be required. Therefore, the introduction of HPLC technique and invention of a novel procedure for more rapid and reliable identification of bacteria was conducted.

MATERIALS AND METHODS

Phytopathogenic bacteria used

Clavibacter michiganensis subsp. *michiganensis (N6601, N6204, N6206, N6207,* 5215), *C. m.* subsp. *sepedonicus* (I), *Erwinia carotovora* subsp. *carotovora (473-1,* 493-1, EH8519), *E. chrysanthemi* pv. *chrysanthemi* (Ichihara l-l, Ku8601, E8301), *E.*

This study was supported in part by the Grant-in-aid from the Ministry of Education, Science and Culture of Japan (No. 06454064).

ch. pv. *dianthicola* (Dianthi le, Dianthi 2n Sl) *E. ch.* pv. *zeae* (ALE8292p, NCPPB 377[T], R7, R8) were used in this experiment. Details of each isolate have been presented in the former reports (Kori *et al.*, 1992, Matsuyama *et al.*, 1993b, c, d, Matsuyama and Furuya, 1993e).

Culture

Each bacterial strain was cultured on the slant of King B medium (Eiken Chemical Co.) at 30°C for 3 days. The ingredient of the medium was as follows : 20.0g Peptone, 1.5g K_2HPO_4, 1.5g $MgSO_4 \cdot 7H_2O$, 15.0g agar, 1 l of 1% glycerol solution, pH 7.2.

Direct colony TLC method

One loopful bacterial cells was pasted directly on the origin of TLC plate (Merck Co.) and dried completely. This plate was developed with chloroform-methanol (2:1, v/v) for 10 min and dried. After scraping off the bacterial cells, this plate was developed to the same direction with chloroform-methanol-water (60 : 25 : 4, v/v/v). The spots of aminolipids were detected by ninhydrin and photocopied. The details have been presented in other papers (Matsuyama *et al.*, 1993a, b, c, d, Matsuyama and Furuya, 1993e).

Rapid identification method by HPLC

One loopful bacterial cells was suspended in 0.5 ml of chloroform-methanol (2 : 1, v/v) in a small glass-vial and capped tightly. Then it was kept for 1 hr in room temperature. After the evaporation of the solvent in a weighing glass-vessel (ca. 3cm in diameter), 1 ml of 80% aqueous methanol solution was added and the extract was dissolved. This solution was passed through a disposable membrane filter tip (Advantec Co., 0.2μm, PTFE) and 20μl of it was subjected to HPLC (Shimadzu LC-system : LC-9A, SPD-10A, Chromatopac C-R5A, DGU-10A) equipped with reversed-phase column (Shimpak HRC-ODS). The elution was carried out with 80% aqueous methanol solution at 1ml/min and the detection was conducted at 210 nm.

RESULTS AND DISCUSSION

As can be seen in Fig.1, the TLC chromatograms of genus *Clavibacter*, gram positive bacteria and genus *Erwinia*, gram negative bacteria, were quite different. The spot at Rf 0.63 which appears in the case of gram negative bacteria was absent in *Clavibacter michiganensis* subspecies used. This distinct difference was always observed regardless of use of young or aged bacterial cells (Matsuyama *et al., 1993c).* As reported previously, the chromatograms were different at generic level or sometimes at species level. The benchmark of each genus or species was stable and appeared on TLC chromatogram at high degree of reproducibility. When the benchmarks existed at similar Rf values, however, their distinction will not be easy. Therefore, the use of other complementary and easy chromatographic procedures has been required.

A novel technique for rapid identification of the phytopathogenic bacteria with HPLC was invented firstly by the author and was contributed in this report. This method

resembles with the direct colony TLC (Matsuyama et al., 1986, 1987, Matsuyama *et al.*, 1993a, b, c, d, Matsuyama and Furuya, 1993e) in principle. In the TLC method the extraction of lipids was carried out on a silica gel plate. While in this novel method by HPLC, the extraction was conducted in a small glass-vial with chloroform-methanol solution (2: 1, v/v). The extract was dissolved in a small volume of 80% aqueous methanol solution, subjected to HPLC and eluted with 80% aqueous methanol solution. The peaks on the chromatograms obtained will represent partly phospholipids from bacterial cells. In this system, the detection of peaks was conducted by UV (210nm) detector, and hence size of each peak will not always reflect the quantity of each lipid.

Distinct differences for HPLC profiles around Rt 2min were observed between genera *Clavibacter* and *Erwinia*. Further, obvious diversity at species level was also detected

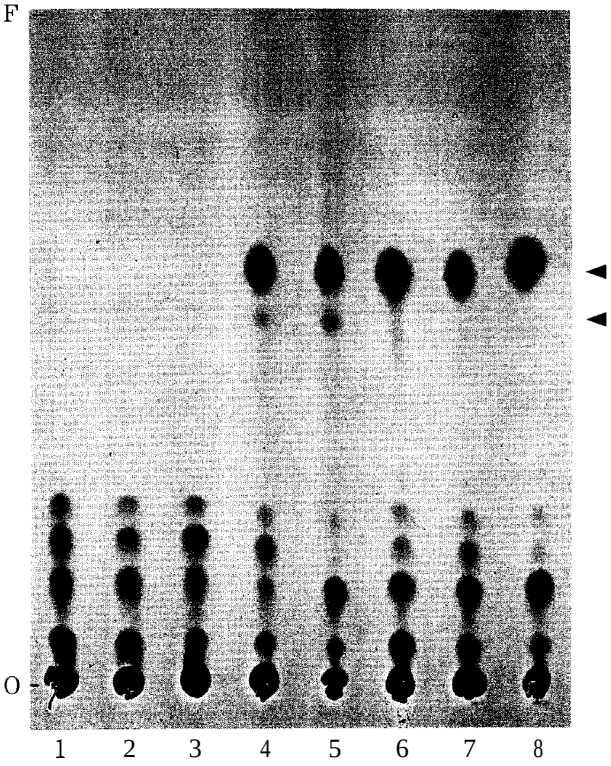

Fig. 1. TLC chromatogram of lipids from phytopathogenic bacteria.
1: *Clavibacter michiganensis* subsp. *michiganensis* N6206
2: 〃 〃 〃 〃 N6204
3: 〃 〃 〃 *sepedonicus* I
4: *Erwinia carotovoru* subsp. *carotovoru* 493-l
5: 〃 〃 〃 〃 473-l
6: 〃 *chrysanthemi* pv. *chrysanthemi* Ichihara 1-1
7: 〃 〃 pv. 〃 E8301 L1
8: 〃 〃 pv. *zeae* ALE8292p
Arrow heads indicate the benchmarks of Gram-negative bacteria (Rf 0.63) and *Erwinia carotovora* subsp.*carotovora* (Rf 0.55). 0 : Origin, F : Front

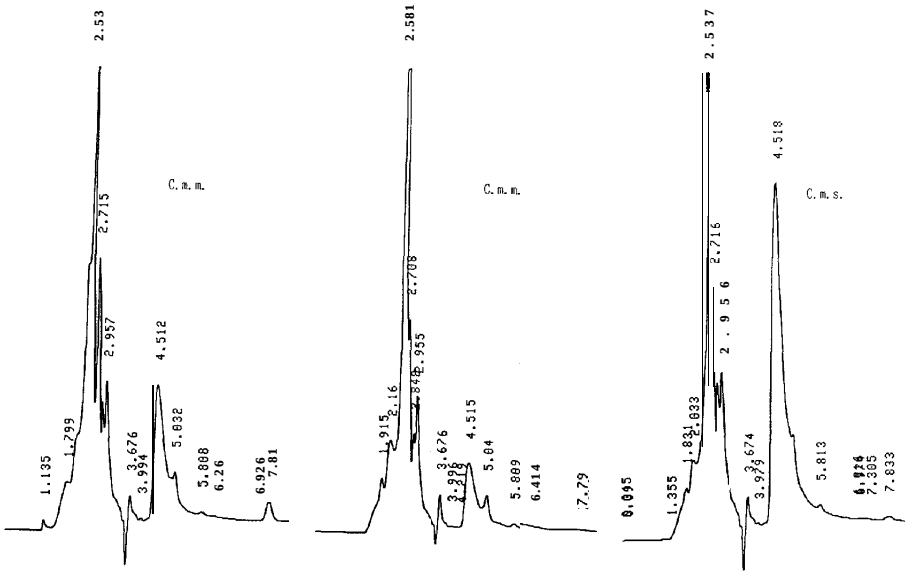

Fig. 2. HPLC chromatograms of *Clavibacter michiganensis* subspecies.
Left (C. m. m.) : *Clavibacter michiganensis* subsp. *michiganensis* N6206
Center (C. m. m.) : 〃 〃 〃 〃 N6204
Right (C. m. s.) : 〃 〃 〃 *sepedonicus* I

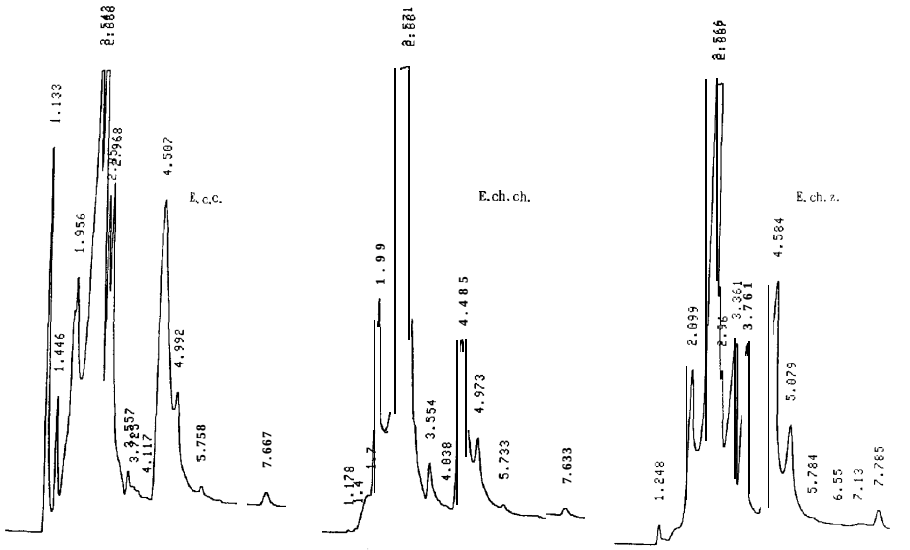

Fig. 3. HPLC chromatograms of *Erwinia* species.
Left (E. c. c.) : *Erwinia carotovora* subsp. *carotovora* 473-1
Center (E. ch. ch.): 〃 *chrysanthemi* pv. *chrysanthemi* Ichihara 1-1
Right (E. ch. z.) : 〃 〃 pv. *zeae* ALE8292p

between *Erwinia carotovora* subsp. *carotovora* and *E.* chrysanthemi like TLC chromatogram (Fig. 1, 2, 3). Several peaks appeared prior to Rt 2min will be benchmarks of *E. carotovoru* subsp. *carotovora* and these were absent on the chromatogram of *E. chrysanthemi.* Since the correspondence of each peak on HPLC chromatogram and each spot on TLC chromatogram has not been investigated, it is uncertain if the peak at ca. Rt 1.4min will correspond with the benchmark spot of *E. carotovora* subsp. *carotovora* at Rf 0.55 (Fig.1). Although rapid identification of bacteria at species level by this HPLC system is available with some exceptions,improvement of the procedures with other kind of columns and solvent systems will be required. This is the subject of future study.

ACKNOWLEDGEMENT

The author is grateful to Dr. N. Furuya for his kind supply of the phytopathogenic bacteria.

REFERENCES

Kori, Y., N. Furuya, K. Tsuno and N. Matsuyama 1992 Differentiation of *Erwinia chrysanthemi* and *E. carotovora* by the cellular fatty acid analysis. *J. Fac. Agn, Kyushu Univ.,* **37**(2): 173-178

Matsuyama, N. 1993a Rapid identification of phytopathogenic bacteria by the direct colony thin-layer chromatography. *Proc. 6th International Congress of Plant Pathology, Montreal, Canada,* p. 44

Matsuyama, N., Ismail Hossain Mian, Abdul Mannan Akanda and N. Furuya 1993b Application of the direct colony TLC method for identification of phytopathogenic bacteria. *J. Fac. Agr., Kyushu Univ.,* **37**(3,4): 281-285

Matsuyama, N., Ismail Hossain Mian, Abdul Mannan Akanda and N. Furuya 1993c Rapid identification of phytopathogenic bacteria by the direct colony thin-layer chromatography (1). *Proc. Assoc. PI. Prot. Kyushu, 39:* 60-64

Matsuyama, N., Ismail Hossain Mian, Abdul Mannan Akanda and N. Furuya 1993d. Comparative studies on thin-layer chromatograms of lipids from various phytopathogenic bacteria. *Ann. Phytopath. Soc. Japan, 59: 528-534*

Matsuyama, N. and N. Furuya 1993e Application of the direct colony TLC for identification of phytopathogenic bacteria (II) Chromatographic profile of *Erwinia* and *Pseudomonas* spp. *J. Fac. Agr., Kyushu Univ.,* **38**(1, 2): 89-95

Matsuyama N. and N. Furuya 1995 Trials of a rapid identification of phytopathogenic bacteria by HPLC I. Use of the reversed-phase column. *Ann. Phytopath. Soc. Japan, 61:* in press.

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

Matsuyama, T., M. Sogawa and I. Yano 1987 Direct colony thin-layer chromatography and rapid characterization of *Serratia marcescens* mutants deffective in production of wetting agents. *Appl. Environ. Microbiol, 53: 1186-1188*