

Rapid Extraction-HPLC as a Tool for Presumptive Identification of *Burkholderia gladioli*, *B. glumae* and *B. plantarii*, Causal Agents of Various Rice Diseases

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Rapid Extraction-HPLC as a Tool for Presumptive Identification of *Burkholderia gladioli*, *B. glumae* and *B. plantarii*, Causal Agents of Various Rice Diseases

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Rapid extraction-HPLC was performed to estimate its usefulness for presumptive identification of *Burkholderia gladioli*, *B. glumae* and *B. plantarii*. On HPLC chromatograms of 12 isolates of *B. gladioli*, a distinct peak appeared at Rt 6.2 min without exception. This peak was absent on the chromatograms for 18 isolates of *B. glumae* and 5 isolates of *B. plantarii* tested. Benchmark peak at Rt 3.2 min was observed on the chromatogram of *B. plantarii*. Together with the direct colony TLC method, this simplified HPLC analysis will be useful for identification of phytopathogenic bacteria.

INTRODUCTION

In general, the morphological, biochemical, serological and pathological tests are indispensable for the identification of phytopathogenic bacteria (Shaad, N. W., 2nd ed., 1988). However, it is time-consuming and needs much experience. Therefore, development of rapid and novel methods, even though presumptive, has long been required. Previously, the authors reported the usefulness of the direct colony TLC method which was originally invented for rapid identification of lipids (Matsuyama *et al.*, 1986, 1987). This method was successfully applied for rapid identification of phytopathogenic bacteria (Matsuyama *et al.*, 1993a, b, c, d; Matsuyama and Furuya, 1993e; Matsuyama, 1995a, b). High reproducibility for results, easiness and rapidity for performance indicated clearly its practical usefulness of this method for rapid diagnosis.

However, in the cases of *Burkholderia gladioli*, *B. glumae* and *B. plantarii*, causal agents of rice diseases (Ura *et al.*, 1996) and recognized as being closely related, TLC chromatograms are similar and not so easy to discriminate. To differentiate these species the rapid extraction-HPLC analysis which was invented by one of the authors (Matsuyama, 1995) was applied. A part of results was abstracted (Matsuyama *et al.*, 1996) and detail was presented in this report.

MATERIALS AND METHODS

Bacterial strains

Twelve isolates of *Burkholderia gladioli* (*Pseudomonas gladioli*), 18 isolates of *B. glumae* (*P. glumae*) and 5 isolates of *B. plantarii* (*P. plantarii*) were used in this experiment. The type cultures of these three species were obtained from ATCC, MAFF,

JCM and Dr. Azegami. Sources and origins were presented in Table 1.

Table 1. Phytopathogenic bacteria tested.

Strains	Origins	Sources
<i>Burkholderia gladioli</i>		
pv. <i>gladioli</i>		
ATCC 10248 [†]	Gladiolus	ATCC
MAFF 301064	Freesia	NIAR
MAFF 301580	Dendrobium	"
MAFF 302515	Tulip	"
MAFF 302537	Onion	"
MAFF 302544	Rice	"
1064	Freesia	NIAS
1065	"	"
E-14	Rice	AKU
<i>B. gladioli</i> pv. unidentified		
MAFF 302409	Adzuki bean	NIAR
MAFF 302418	Green gram	"
MAFF 302424	Cymbidium	"
<i>B. glumae</i>		
MAFF 301169 [†]	Rice grain	NIAR
Ku8104	"	AKU
Ku8105	"	"
Ku8112	"	"
Ku8114	"	"
8012	"	KNAES
8015	"	"
8020	"	"
N7401	Rice seedling	NIAS
N7501	"	"
N7503	"	"
N7504	"	"
N7505	"	"
2	Rice grain	KNAES
III	"	"
805	Unknown	NIAS
AZ8224	Rice grain	Dr. Azegami
AZ84448	"	"
<i>B. plantarii</i>		
AZ8201 [†]	Rice	Dr. Azegami
JCM5492 [†]	"	JCM
MAFF301723 [†]	"	Dr. Azegami
MAFF302484	"	NIAR
MAFF302387	"	"
<i>Erwinia carotovora</i>		
subsp. <i>carotovora</i>		
ATCC15713 [†]	Potato	ATCC

Abbreviations: ATCC; American Type Culture Collection, USA. NIAR; National Institute of Agricultural Resources, Tsukuba 305, Japan. NIAS; National Institute of Agricultural Sciences, Tokyo, Japan (This institute was consolidated to NIAR and National Institute of Agro-Environmental Sciences, NIAES). KNAES; Kyushu National Agricultural Experiment Station, Kumamoto 861-11, Japan. AKU; Faculty of Agriculture, Kyushu University, Fukuoka 812-81, Japan. JCM: Japan Collection of Microorganisms (RIKEN), Wako, Saitama 351-01, Japan.

Type Cultures of *Burkholderia gladioli*, *B. glumae* and *B. plantarii* represented as [†] at the shoulder of strain's numbers.

Direct colony TLC

Isolates were cultured on a slant of King's B medium (Eiken Co.) at 30°C for 3 days. One loop bacterial cells was applied directly on the origin of silica gel G (Si60) TLC plate (Merck Co.). After complete drying in an automatic desiccator and/or with a hair drier, the TLC plate was developed in a development glass chamber for 10 min with a chloroform-methanol solution (2:1, v/v) in an incubator at 25°C. The plate was taken out, dried completely and the bacterial cells were scraped off. The plate was developed in the same direction with a chloroform-methanol-water solution (60:25:4, v/v/v) in an incubator at 25°C for 1.5 hr. After development the plate was dried and the spots were detected by spraying with ninhydrin (Ninhydrin spray, Kanto Chem. Co.) and heating at 100°C for 10 min. The chromatogram obtained was recorded by a sensitively adjusted photocopy (Canon FC-3 II) or by a computer (Photoshop 3.0J, Adobe Co.).

Extraction of whole-cellular bacterial lipids

One loop bacterial cells which were cultured at 30°C for 3 days on slants of King's B medium (Eiken Co.) was applied on the bottom of a small glass-vial and 0.5 ml of a chloroform-methanol solution (2:1, v/v) was added. Bacterial cells were suspended in the solution by stirring with the loop and kept for 60 min at room temperature. The suspension was transferred into a weighing vessel (3 cm in diameter) and the solvent evaporated. One ml of 80% aqueous methanol solution was added and the extract was dissolved by stirring and rubbing, carefully, the inner-surface of vessel with a tip of plastic hypodermic-syringe (1 ml, Termo Co.). Suspensions were passed through a 0.2 µm filter (PTFE, Advantec Co.).

HPLC analysis

Twenty µl of the filtrate were applied to a HPLC (Shimadzu LC system, LC-9A, SPD10A, Chromatopac C-R5A, DGU-10A) equipped with a reversed-phase column (Totally porous, spherical particle, silica gel; 5 µm particle diameter, 120 Å pore diameter, octadecyl, Shim-pack HRC-ODS 6x150 mm). Elution was conducted with 80% aqueous methanol solution at a flow rate of 1 ml/min. Peaks were detected with a UV detector at 210 nm. Experiments were repeated five times, and the results were confirmed.

RESULTS AND DISCUSSION

The direct colony TLC is quite useful for rapid differentiation of various phytopathogenic bacteria within 2.5 hrs (Matsuyama *et al.*, 1993a, b, c, d; Matsuyama and Furuya, 1993e; Matsuyama, 1995a, b; Matsuyama *et al.*, 1996) except for the cases of interspecific differentiation of *Xanthomonas* and differentiation of *Pseudomonas syringae* pathovars.

As can be seen in Figs. 1, 2, the chromatographic profiles are different at generic and sometimes at specific level. In the case of the new genus *Burkholderia* (*Pseudomonas*, rRNA homology group II), the TLC chromatograms were distinctly different for most of the species (Matsuyama, 1995b). However, the TLC chromatograms of *B. gladioli*, *B. glumae* and *B. plantarii*, which are causal agents of various rice diseases (Kurita and Tabei, 1967; Uematsu *et al.*, 1976; Azegami *et al.*, 1983; Yasunaga *et al.*, 1986; Ura *et al.*,

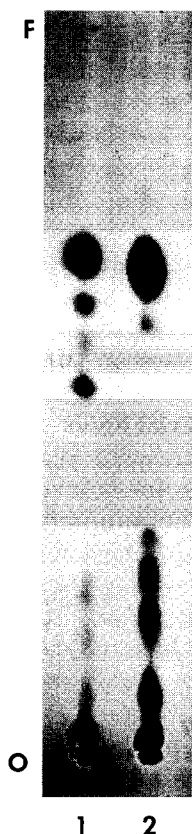


Fig. 1. Comparison of chromatograms of the direct colony TLC for *Burkholderia gladioli* pv. *gladioli* and *Erwinia carotovora* subsp. *carotovora*.
 Lane 1; *Burkholderia gladioli* pv. *gladioli* (ATCC10248^T), Lane 2; *Erwinia carotovora* subsp. *carotovora* (ATCC 15713^T).
 O; origin, F; front

1996), are similar (Fig. 2). Recently, one of authors (Matsuyama, 1995a) presented a novel identification method using HPLC. This technique is similar with the direct colony TLC for extraction of lipids, in principle. In the direct colony TLC, extraction of lipids is carried out on the silica gel (Si60) TLC plate. In the case of the rapid extraction-HPLC, this lipid extraction is conducted in chloroform-methanol solution (2:1, v/v) in a capped small glass-vial. The separation of extracted lipids was conducted with a reversed-phase HPLC and polar solvent system. Distinct differences were observed among *B. gladioli*, *B. glumae* and *B. plantarii* for HPLC chromatograms. The benchmark peak appeared at retention time (Rt) 6.2 min on the chromatogram of *B. gladioli* (Fig. 3). Whereas no peak was detected at the corresponding time in the cases of *B. glumae* and *B. plantarii* (Figs. 4, 5). This diversity among species was observed without any exception. On the chromatogram of *B. plantarii* benchmark peak appeared at Rt 3.2 min and absent on those of other two species (Figs. 3, 4, 5). This marker peak of *B. plantarii* was more

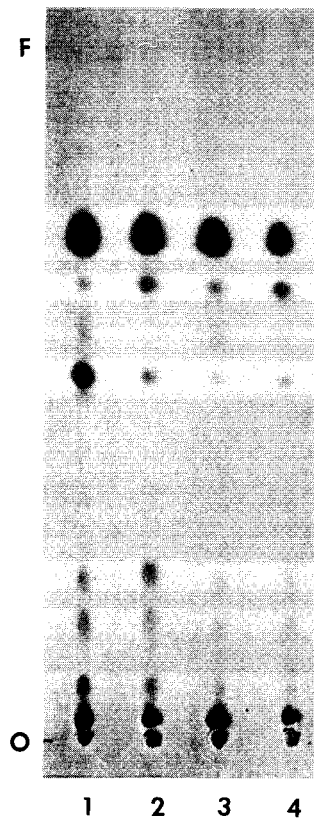


Fig. 2. Comparison of chromatograms of the direct colony TLC for *B. plantarii*, *B. glumae* and *B. gladioli* pv. *gladioli*.
 Lane 1; *B. plantarii* (JCM5492^T), Lane 2; *B. glumae* (MAFF301169^T),
 Lane 3; *B. gladioli* pv. *gladioli* (MAFF301580), Lane 4; *B. gladioli* pv. *gladioli*
 (ATCC10248^T)
 O; origin, F; front

distinctly detected when bacteria was cultured on YPDA (Yeast extract-Peptide-Dextrose-Agar) and AFG (glucose and Fe amended Ayers medium). The substance(s) appeared at Rt 3.2 min on the chromatogram of *B. plantarii* could be relative of tropolone (Azegami *et al.*, 1987). The identification of the substances appeared at Rt 6.2 and 3.2 min is the subject for future study. In the rapid extraction-HPLC, like the direct colony TLC, lipid extraction was performed under mild condition and each benchmark substance could be extracted at the intact state. This method is easy to perform and the result is obtained within 100 min. Together with the direct colony TLC analysis, this rapid extraction-HPLC analysis has practical use for rapid diagnosis of phytopathogenic diseases.

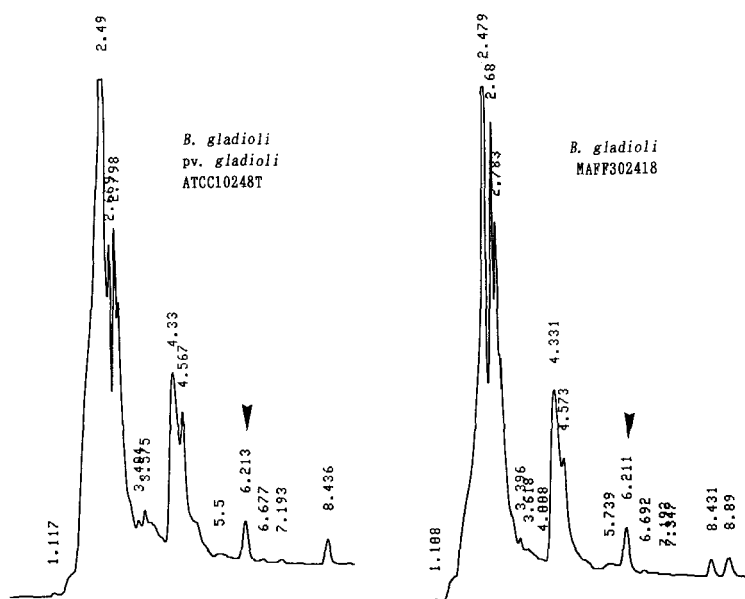


Fig. 3. Rapid extraction-HPLC chromatograms of *B. gladioli*. Arrow heads indicate the benchmark peak at Rt 6.2 min. This benchmark appeared on the chromatograms of *B. gladioli* without exception.
Left; ATCC10248^T, Right; MAFF302418

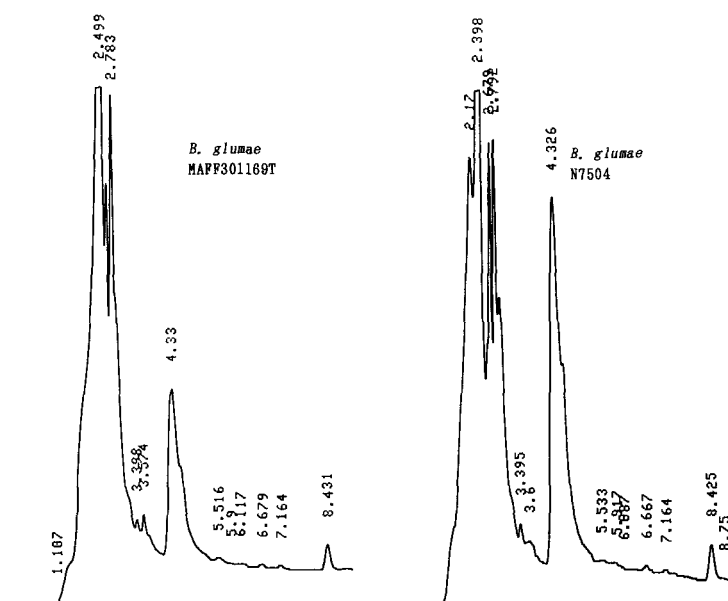


Fig. 4. Rapid extraction-HPLC chromatograms of *B. glumae*.
Left; MAFF301169^T, Right; N7504

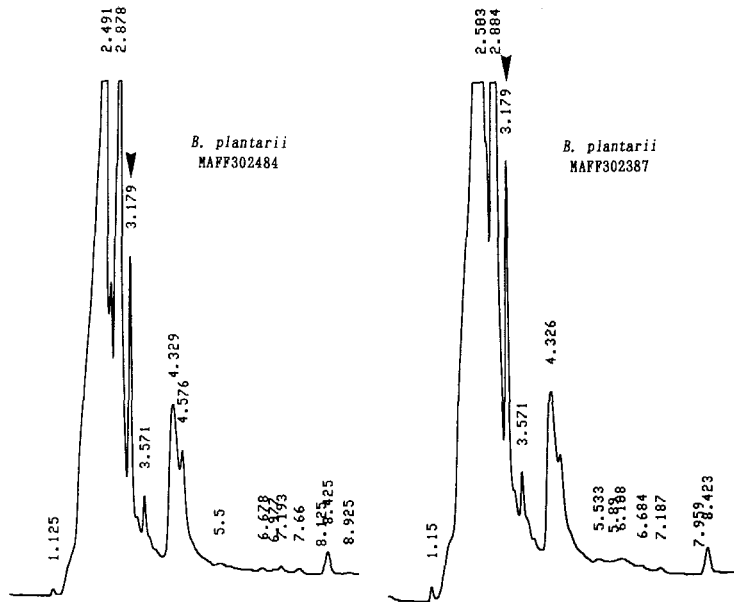


Fig. 5. Rapid extraction-HPLC chromatograms of *B. plantarii*. Arrow heads indicate the benchmark peak at Rt 3.2 min. Left; MAFF302484, Right; MAFF302387

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