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

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Presumptive Identification of Several Phytopathogenic Bacteria by Novel Diagnostic Tests

Nobuaki Matsuyama

Laboratory of Plant Pathology, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan

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Novel diagnostic techniques for presumptive identification of phytopathogenic bacteria were tested. Bacterial cells of *Erwinia* spp. except for *E. herbicola* pv. *milletiae* were smoothly and homogeneously suspended in chloroform-methanol solution (CM, 2:1, v/v). Whereas the suspensions of other kinds of bacteria were not homogeneous. In particular, cells of *Burkholderia gladioli* agglutinated and were finally suspended as a fibrous state. Whitish-blue, purple, yellow, red or pink fluorescence for some bacteria were observed at UV irradiation (365nm) to these suspensions. Furthermore, direct UV irradiation to cultured King's B slants of *B. plantarii* and *B. vandii* generated purple fluorescence. Bluish color of CM solution which contains 0.6% $CoCl_2$ rapidly changed to pinkish at suspending cells of *B. gladioli* without exception. In other cases, the color of suspension was purple or blue, and bluish precipitates were always observed. These tests will be useful for presumptive identification of bacteria together with the direct colony TLC, the modified TLC and the rapid extraction HPLC methods.

INTRODUCTION

Identification of phytopathogenic bacteria is time-consuming and its improvement has long been requested. In medical field, several kits like API 20NE (France) have been used for rapid identification of certain bacteria. Application of these kits for diagnosis of plant disease has also been attempted (Nishiyama, 1996).

Surprising reports that several phytopathogenic bacteria are also pathogenic to human being (Ederer and Matsen, 1972; Randall, 1980; Ross *et al.*, 1995; Baldani *et al.*, 1996) impressed on us the importance of rapid diagnosis.

The direct colony TLC method which was invented for rapid identification of lipids (Matsuyama *et al.*, 1986, 1987) was successfully applied for rapid identification of phytopathogenic bacteria (Matsuyama *et al.*, 1993a,b,c,d; Matsuyama and Furuya, 1993e; Matsuyama, 1995a, b). This TLC method was developed to the rapid extraction- HPLC technique (Matsuyama, 1995a). In this HPLC method, extraction of bacterial lipids was performed with chloroform-methanol solution (CM, 2:1, v/v) in a small glass-vial by suspending bacterial cells. In this extraction, states of bacterial cells of some bacteria were characteristic. Such intergeneric or -specific differences were also observed at UV (365 nm) irradiation and at $CoCl_2$ amendment to the suspension. These characteristics will be useful for presumptive identification together with other diagnostic tests. The abstracts were presented elsewhere (Matsuyama, 1996, 1997) and details will be contributed in this report.

MATERIALS AND METHODS

Bacterial strains

Totally 57 isolates for 6 genera and 14 species were tested. Origins and sources of the bacterial strains were presented in Table 1.

Table 1. Bacterial strains tested in this experiment.

Bacterial strains	Isolate	Origin	Source
Clavibacter michiganensis			
〃 subsp. *sepedonicus*	1	Potato	NIAS
〃 subsp. *michiganensis*	N6601	Tomato	〃
〃 〃	N6204	〃	〃
〃 〃	N6206	〃	〃
〃 〃	N6207	〃	〃
〃 species unidentified	5215	Wasabi	AKU
Erwinia carotovora			
〃 subsp. *carotovora*	ATCC15713^T	Potato	ATCC
〃 〃	473-1	Chinese Cabbage	LSPPM
〃 〃	493-1	Potato	〃
〃 subsp. *atroseptica*	ATCC33260^T	Potato	ATCC
E. chrysanthemi			
〃 pv. *chrysanthemi*	Ichihara 1-1	Pear	TAU
〃 〃	Ku8601 L1	〃	AKU
〃 〃	E8301	Chrysanthemum	SZU
〃 pv. *zeae*	NCPPB377^T	Corn	NCPPB
〃 〃	ALE8292p	Welsh onion	SZU
〃 〃	R7	Rice	〃
〃 pv. *dianthicola*	Dianth 1e	Carnation	SZU
〃 〃	Dianth 2n	〃	〃
E. herbicola pv. *milletiae*	1	Japanese wistaria	NIAS
Ralstonia solanacearum (*Pseudomonas*)	ATCC11696^T	Tomato	ATCC
〃	6211	〃	AKU
〃	C319-SR	Tobacco	KTES
Herbaspirillum (*Pseudomonas*) *rubrisubalbicans*	MAFF301626	Sugarcane	NIAR
〃	MAFF301628	〃	〃
Burkholderia cepacia (*Pseudomonas*)	ATCC25416^T	Onion	ATCC
〃	356-5	〃	NARC
B. gladioli pv. *gladioli*	ATCC10248^T	Gladiolus	ATCC
〃	NIAS1064	Freesia	NIAS
〃	NIAS1065	〃	〃
〃	MAFF301580	Dendrobium	NIAR
〃	MAFF302424	Cymbidium	〃
〃	MAFF302515	Tulip	〃
〃	MAFF302537	Onion	〃
〃	MAFF302544	Rice	〃
〃 pv. unidentified	MAFF302409	Adzuki bean	〃
〃 〃	MAFF302418	Green gram	〃
〃 〃	MAFF302424	Cymbidium	〃
B. gladioli pv. *alliicola*	ATCC19302^T	Onion	ATCC

Table 1. Continued.

Bacterial strains	Isolate	Origin	Source
B. glumae	MAFF301169^T	Rice grain	NIAR
〃	2	〃	KNAES
〃	Kyu82-34-2	〃	〃
〃	AZ8224	〃	K.Azegami
〃	AZ84448	〃	〃
〃	Ku8104	〃	AKU
〃	Ku8112	〃	〃
〃	8020	〃	KNAES
〃	N7503	Rice seedling	NIAS
〃	N7504	〃	〃
B. plantarii	MAFF301723^T	Rice seedling	K.Azegami
〃	MAFF302387	〃	NIAR
〃	MAFF302484	〃	〃
B. vandii	JCM7957^T	Vanda	JCM
B. caryophylli	NIAS1192	Carnation	NIAS
〃	NIAS1406	〃	〃
B. andropogonis	MAFF301129	Tulip	NIAR
Pseudomonas syringae	ATCC19310^T	Syringa	ATCC
〃 pv. *syringae*	1	〃	NIAS

NIAS: National Institute Agricultural Sciences, Tokyo, Japan. This Institute was reconstructed partly to NIAES (National Institute of Agro-Environmental Sciences, Tsukuba, Japan).
NARC: National Agricultural Research Center, Tsukuba, Japan.
TAU: Tokyo University of Agriculture, Setagaya, Japan.
SZU: Shizuoka University, Ohtani, Shizuoka, Japan.
AKU: Fac. Agriculture, Kyushu University, Fukuoka Japan.
KNAES: Kyushu National Agricultural Experiment Station, Nishigoshi, Kumamoto, Japan.
KTES: Kagoshima Tobacco Experiment Station, Kagoshima, Japan.
JCM: Japan Collection of Microorganisms, Wako, Saitama, Japan.
NCPPB: National Collection of Plant Pathogenic Bacteria, England.
ATCC: American Type Culture Collection, USA.
LSPPM: Laboratory of Seed and Post-harvest Disease, Plant Pathology and Microbiology Division, Department of Agriculture, Thailand.
NIAR: National Institute of Agricultural Resources, Tsukuba, Japan.

Culture

Each isolate was cultured on slant(10 ml) of King's B medium (Eiken Co.) at 25 °C for 3 to 10 days.

Observation of the state of bacterial cells in chloroform-methanol solution

One loopful bacterial cells was pasted on bottom of small glass-vial. After addition of 0.5 ml chloroform-methanol (CM, 2:1, v/v), cells were suspended by stirring and rubbing pasted cells on inner-surface of the vial with a loop. To avoid evaporation of the solvent the vial was capped tightly and state of bacterial cells in CM solution was observed. Relatively old culture, 6～10 days-old, is preferable for this test.

Fluorescing at UV-irradiation

Bacterial suspension in capped vial was UV-irradiated at 365 nm and appearance of fluorescence was recorded. Young culture, 3～5 days-old, is recommendable for this test.

Irradiation was also performed to cultured King's B slant.

Color change of $CoCl_2$ containing CM solution

Six hundred mg of $CoCl_2 \cdot 6H_2O$ was dissolved in 100 ml of CM solution. Bacterial cells were suspended in 0.5 ml of this bluish solution and color change of the solution and color of the precipitates were recorded. For highly reproducible results it is advisable to use 6～10 days-old culture.

RESULTS AND DISCUSSION

In performance of the rapid extraction-HPLC, bacterial cells were suspended in 0.5 ml of chloroform-methanol solution (CM, 2:1, v/v) in a small glass-vial by stirring with a loop. In this process, some bacterial strains showed characteristic properties which will be useful as tools for presumptive identification.

State of bacterial cells in CM solution

At careful observation on the state of bacterial cells in the suspension, novel diagnostic characters were found. Bacterial cells of *Erwinia* spp. were smoothly and uniformly suspended. The difference between *Erwinia* and other bacteria was clearly observed when 10 days-old cells were suspended (Table 2). Among species of *Erwinia*, *Erwinia herbicola* pv. *milletiae* was different from others. Bacterial cells of *B. gladioli* firstly agglutinated in CM solution and were finally suspended as a fibrous state (Matsuyama, 1997). This phenomenon could relate with the existence of some kind of polymers. This phenomenon was observed for *B. gladioli* without exception.

Fluorescing

It has been well-documented that *Pseudomonas fluorescens* and a part of *P. syringae* generate fluorescence at UV-irradiation. Surprisingly, other kinds of fluorescence were observed at irradiation of UV (365 nm) to bacterial suspension with CM solution for some species of *Burkholderia* (*Pseudomonas* spp., rRNA homology group II) and *Herbaspirillum rubrisubalbicans* (*P. rubrisubalbicans*). Suspension of *H. rubrisubalbicans* fluoresced in whitish-blue color. Fluorescence of *B. plantarii* and *B. vandii* was purple. This fluorescence can detect within 5 min after making suspension. This purplish fluorescence was also observed at direct UV-irradiation to cultured KB slant. The appearance of this purple fluorescence agreed with the ability of tropolone production of each isolate (data were not shown). Therefore, it indicates that the substance generated purple fluorescence could be the relative of tropolone.

Pinkish fluorescence appeared mainly on precipitated bacterial cells in CM suspension of *B. gladioli*, *B. cepacia* and *B. caryophylli* at UV-irradiation and rarely in *B. glumae*.

Distinction of *B. gladioli* by $CoCl_2$ amended CM solution

Novel technique for identification of *B. gladioli* was invented. Bluish color of $CoCl_2$ amended CM solution changed to pink when the cells of *B. gladioli* were suspended. Whitish precipitates, mainly be bacterial cells, were observed. Whereas in the cases of other kinds of bacteria the color changed to purple or blue and dark-blue sediments were always precipitated (Table 2).

Since the color of $CoCl_2$ changes from blue to pink with water, the phenomenon stated above will indicate the existence of substance which has water-hold capacity like

polysaccharide in *B. gladioli*.

Previously, it was indicated that the causal agent of a new rice disease, leaf sheath browning, is *B. glumae* (Yasunaga *et al.*, 1986). Recently, the authors clarified that *B. gladioli* is also a causal bacterium of this disease (Ura *et al.*,1996). Since the differentiation of *B. glumae* and *B. gladioli* by the direct colony TLC was difficult, the authors recommended application of the rapid extraction-HPLC for it (Matsuyama *et al.*,1997). Together with the HPLC method, simple and easy techniques presented in this report will be useful for differentiation of these closely related bacterial strains.

Table 2. Novel diagnostic tests differentiating genus or species of phytopathogenic bacteria.

Bacterial strains (Number of isolates)	State of bacterial cells in CM solution	Fluorescence generated in CM solution at UV(365 nm) irradiation	Color of suspension with $CoCl_2$ amended CM solution	Color of precipitates in $CoCl_2$ amended CM solution
Clavibacter michiganensis				
subsp. *sepedonicus* (n=1)	Granular [a]	Yellow	Purple	Dark-blue
subsp. *michiganensis* (n=3)	〃	〃	〃	〃
subsp. unidentified (n=1)	〃	〃	〃	〃
Erwinia carotovora				
subsp. *carotovora* (n=3)	Homogeneous [b]	〃	〃	〃
subsp. *atroseptica* (n=1)	〃		〃	〃
E. chrysanthemi				
pv. *chrysanthemi* (n=3)	〃	〃	〃	〃
pv. *zeae* (n=3)	〃	〃	〃	〃
pv. *dianthicola* (n=2)	〃	〃	〃	〃
E. herbicola				
pv. *milletiae* (n=1)	Granular	〃	〃	〃
Ralstonia solanacearum (n=3)	〃	〃	〃	〃
Herbaspirillum				
rubrisubalbicans (n=2)	〃	Whitish-blue	〃	〃
Burkholderia cepacia (n=2)	〃	Pink	〃	〃
B. gladioli				
pv. *gladioli* (n=8)	Fibrous	Pink to Red	Pink	White
pv. unidentified (n=3)	〃	〃	〃	〃
pv. *alliicola* (n=1)	〃	〃	〃	〃
B. glumae (n=10)	Granular	White to Faint blue	Purple	Dark-blue
B. plantarii (n=3)	〃	Purple [c]	〃	〃
B. vandii (n=1)	〃	〃	〃	〃
B. caryophylli (n=2)	〃	Orange	〃	〃
B. andropogonis (n=1)	〃	White	〃	〃
Pseudomonas syringae (n=2)	〃	Yellow	〃	〃

Fluorescence was mainly observed on bacterial cells precipitated. Whereas whitish-blue fluorescence in the case of *H. rubrisubalbicans* and purple fluorescence in the cases of *B. plantarii* and *B. vandii* were observed in supernatants,respectively.

$CoCl_2 \cdot 6H_2O$ was dissolved in CM solution so as to be 0.6%. One loopful bacterial cells was suspended in 0.5 ml of this solution in small glass-vial.

a); State of bacterial cells in chloroform-methanol solution (CM, 2:1, v/v). Cells were partly suspended and tiny masses of the cells were still remained.

b); Cells were suspended quite smoothly and uniformly in CM solution.

c); Purple fluorescence was also observed at UV (365 nm) irradiation to tubes of cultured King's B slant, directly.

Striking reports that *Herbaspirillum* (*Pseudomonas*) *rubrisubalbicans*, *B. cepacia* and *B. gladioli* are pathogenic not only to plant but also to human being (Ederer and Matsen, 1972; Randall, 1980; Ross, *et al.*, 1995; Baldani *et al.*, 1996) stressed on necessity of such researches for rapid diagnosis.

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