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Differentiation of Major Rice-Seedborne Bacteria by PCR-Amplified Polymorphism of Spacer Region Between 16S and 23S Ribosomal DNA

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PCR로 증폭된 16S와 23S rDNA 사이 Spacer 부위의 다형성에 의한 주요 벼종자전염성 세균의 구별

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Abstract : Polymorphisms of ribosomal DNA (rDNA) spacer region between 16S and 23S rDNA amplified by polymerase chain reaction (PCR) with a single set of primers R16-1 and R23-2R were applied for the differentiation of rice seedborne bacteria (fifty-one strains of *Pseudomonas avenae, P. glumae, P. fuscovaginae, P. syringae* pv. *syringae, Xanthomonas oryzae* pv. *oryzae, X. oryzae* pv. *oryzicola* and *Erwinia herbicola* isolated from rice seeds). The amplified products ranged about 820~950 bp in size, were specific to the bacterial species, and had differentiable polymorphisms. The amplified products specific to *Pseudomonas* species were 950 bp for *P. avenae*, 850 bp for *P. glumae*, 770 bp for *P. fuscovaginae*, and 1,240 bp, 1,100 bp and 820 bp for *P. syringae* pv. *syringae*. There was no intraspecies diversity in the polymorphism among the Korean isolates of *P. glumae* and *P. avenae*. In *Xanthomonas* species, a single unifying feature of polymorphism was produced, regardless of the isolates in a species with the primary bands of 860 bp in *X. oryzae* pv. *oryzae* and 880 bp in *X. oryzae* pv. *oryzicola* and additional secondary products at 890 bp, 440 bp and 370 bp in *X. oryzae* pv. *oryzicola*. All of the Korean strains except CXO211 belonged to type a, while four strains including only one Korean strain were designated as type b. Amplification of the 16S~23S spacer region of *E. herbicola* strains produced multiple bands, their band profiles were homogeneous, and there were no intraspecies variations among the strains. On the basis of the results, the differentiation of PCR-amplified spacer polymorphisms by using the R16-1 and 23-2R primers to 16S and 23S rDNA may be used for the rapid differentiation of seedborne bacteria of rice.

***Key words* : PCR, rice seedborne bacteria, rDNA spacer region, differentiation.**

Recently many plant bacteria such as *Pseudomonas, Xanthomonas,* and *Erwinia* species have been associated with symptoms in or on rice seeds, and regarded as seedborne pathogens (5, 8, 27). Especially four *Pseudomonas* spp. (*P. avenae, P. fuscovaginae, P. glumae* and *P. syringae* pv. *syringae*) and two *Xanthomonas* spp. (*X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*) have been well characterized and recognized as important seedborne bacterial pathogens in rice (5, 7, 8, 11, 14, 17, 25, 28, 29, 32). Other pathogens, which were reported to be related to rice seed-infection, were not examined in detail in their occurrence, pathogenicity and physiological characteristics (8, 33).

In Korea, *P. avenae* (26), *P. glumae* (4), *X. oryzae* pv. *oryzae* (6), and *E. herbicola* (5) were reported as seed-associated bacterial pathogens, but their occurrences and incidences in rice seeds have not been extensively studied yet partly because there had been no elaborate and simple methods for the detection and differentiation of the seedborne bacterial pathogens *in*

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situ. In a traditional method, seedborne pathogens are isolated on selective media and identified on the basis of phenotypical characteristics such as the color of older colonies and growing ability on the selective media, which is laborious and time-consuming, and needs specialty on the taxonomy of the isolated organisms for the species identification. Moreover, the morphological and biochemical characteristics among the four *Pseudomonas* spp. (33) and between the two *Xanthomonas* spp. (8) mentioned above are not obviously different, and thus special skill and experience are required to differentiate these species only by the observation of phenotypical characteristics. Also phenotypical markers may be affected by environmental conditions, making it difficult to accurately identify pathogens because of phenotypical variations. Thus, a simple and rapid method to detect and identify the seedborne bacteria is needed to screen their occurrences on rice in a large scale.

Recently, several techniques in molecular biology, such as DNA hybridization and restriction fragment length polymorphism (RFLP), have been applied for differentiating genetic characteristics of inter- and intraspecies; for example, the assessment of genetic diversity in *X. oryzae* pv. *oryzae* (1, 18). Polymerase chain reaction (PCR) with paired primers derived and constructed from characterized sequences (10, 18) is often applied for these techniques to amplify the genetic characteristics. The amplification of the ribosomal DNA (rDNA) spacer region between 16S and 23S rRNA genes by PCR was developed for DNA polymorphism assay (2, 13, 21). No prior sequence information is required for this method, which has been used to differentiate bacteria with close relationships and particularly to differentiate isolates below the species level (13, 21). Furthermore, PCR makes it possible to analyze a small amount of genetic material and handle a large number of specimens in a short time.

In this study, rDNA spacer region between 16S and 23S rRNA genes was amplified and was applied to major seedborne bacterial strains from Korea, which had been confirmed their genera or species by some recommended tests, to develop a simple and rapid method useful for differentiating and identifying the seedborne bacterial pathogens in rice such as *P. avenae, P. glumae, E. herbicola*, and *X. oryzae* pv. *oryzae*.

MATERIALS AND METHODS

Isolation of rice-seedborne bacteria. Rice seeds with symptoms of seed-discoloration that were suspected to be infected with the discoloration-related bacteria were collected from Chonbuk province, and used for the isolation of seedborne bacteria. The rice seeds were surface-disinfested in 0.1% (v/v) NaOCl for 3 min, rinsed in sterile deionized water, and soaked in sterile phosphate-buffered saline (PBS) solution (0.05 M phosphate buffer, pH 7.2, containing 0.85% NaCl and 0.01% Tween 20) at 4°C for 2 hrs, either individually or in a batch of 400~500 seeds.

For the bacterial isolation from individual seeds, a seed and the buffer were placed in a 0.5-ml microcentrifuge tube and shaken at 120 rpm, and the bottom of the tube was punctured with an 18-gauge needle. The 0.5-ml tube was placed into an 1.5-ml tube, secured with parafilm, and centrifuged at 10,000 g for 20 min. The supernatant was discarded, and the pellet in the 1.5-ml tube was suspended with 100 μl PBS. For the bacterial isolation from each batch of seeds, 400~500 seeds were soaked in PBS for 2 hrs. After removing the seeds, the remaining buffer suspension was centrifuged at 10,000 g for 20 min, and the pellet was resuspended in 1 ml of PBS and diluted to 10^{-3} or 10^{-4} with PBS. The samples from the individual seeds and the batches of seeds (100 μl for each) were directly plated onto Kim *et al*'s semiselective media, XCO and PG agar (15, 16), potato semi-synthetic agar (PSA) or King's B (KB) agar to isolate medium-specific bacterial strains.

Bacterial strains. Identification of the bacterial strains isolated from rice seeds was based on colony morphology, Gram stain, and recommended key tests for the differentiation of discoloration-related seedborne *Pseudomonas* spp. (33) and *E. herbicola* (5), and of seedborne *Xanthomonas* spp. (31). In this study, a total of 29 strains (14 *X. oryzae* pv. *oryzae* strains, 4 *Xanthomonas*-like organisms, 3 *P. avenae*, 3 *P. glumae* and 5 *E. herbicola* strains) were isolated and identified (Table 1). Other bacterial strains used in this study are also listed in Table 1, which are reference and type strains of *P. avenae* and *P. glumae*; one *Xanthomonas* species and one *E. herbicola* strain that are recognized as rice pathogens; reference strains of *P. fuscovaginae, P. syringae* pv. *syringae*, and *X. oryzae* pv. *oryzicola*; and unidentified bacteria isolated from rice seeds. All bacterial cultures were maintained on KB and/or PSA slants, unless otherwise noted.

Extraction of bacterial DNA. Total DNA was ex-

Table 1. Bacterial strains used in this study

Species and strain	Origin and source[a]	
Xanthomonas oryzae pv. *oryzae*		
CXO101	Chonbuk, Korea	This study
CXO103	Chonbuk, Korea	HCES, D. G. Lee
CXO104	Chonbuk, Korea	This study
CXO105	Chonbuk, Korea	This study
CXO106	Chonbuk, Korea	This study
CXO107	Chonbuk, Korea	This study
CXO110	Chonbuk, Korea	This study
CXO112	Chonbuk, Korea	This study
CXO211	Chonbuk, Korea	This study
CXO214	Chonbuk, Korea	This study
CXO215	Chonbuk, Korea	HCES, D. G. Lee
CXO316	Chonbuk, Korea	This study
CXO317	Chonbuk, Korea	This study
CXO319	Chonbuk, Korea	HCES, D. G. Lee
CXO321	Chonbuk, Korea	This study
CXO322	Chonbuk, Korea	This study
CXO323	Chonbuk, Korea	This study
X1-8	Texas, U.S.A.	D. A. Roth
NX-42	China	J. H. McBeath
B-792	Philippines	J. H. McBeath
#2(2-19)	Japan	N. W. Schaad
Xanthomonas-like organism		
X1-1	Texas, U.S.A.	D. A. Roth (Gonzalez)
X1-9	Texas, U.S.A.	D. A. Roth (Gonzalez)
NP-1	China	N. Thaveechai
NP-3	California, U.S.A.	J. H. McBeath
CUXL324	Chonbuk, Korea	This study
CUXL335	Chonbuk, Korea	This study
CUXL336	Chonbuk, Korea	This study
CUXL337	Chonbuk, Korea	This study
X. oryzae pv. *oryzicola*		
P501	Philippines	N. W. Schaad
P502	Philippines	N. W. Schaad
Pseudomonas avenae		
COA1	Chonbuk, Korea	This study
COG2	Chonbuk, Korea	This study
COA3	Chonbuk, Korea	This study
ATCC19860	ATCC	
Fed1240-2, Vai	Colombia	F. Correa
P. glumae		
COG1	Chonbuk, Korea	This study
COG2	Chonbuk, Korea	This study
COG3	Chonbuk, Korea	This study
K8239	Chungnam, Korea	J. E. Choi
ATCC33617	ATCC	
Sab1673-10	Colombia	F. Correa

Table 1. Continued

Species and strain	Origin and source[a]	
Erwinia herbicola		
CEh1	Chonbuk, Korea	This study
CEh2	Chonbuk, Korea	This study
CEh3	Chonbuk, Korea	This study
CEh4	Chonbuk, Korea	This study
CEh5	Chonbuk, Korea	This study
Eh8701	Chonbuk, Korea	K. C. Kim
P. fuscovaginae		
Fed1259-3 1R VM	Colombia	F. Correa
P. syringae pv. *syringae*		
Chi131-3	Chile	F. Correa

[a] ATCC : American Type Culture Collection; HCES : Honam Crop Experiment Station, Iri, Korea; D. A. Roth : Deaprtment of Plant, Soil and Insect Science, University of Wyoming; J. H. McBeath : Agricultural and Forestry Experiment Station, University of Alaska Fairbanks, Alaska 9902; N. Thaveechai : Department of Plant Pathology, Kasetsart University, Bankok 10903, Thailand; N. W. Schaad : USDA, Foreign Disease Weed Science Research, Fort Detrick Building 1301, Frederick, Maryland 21702, U.S.A.; F. Correa : Ciat, AA6713, Cal., Colombia, S. A.; J. E. Choi : Chungnam National University; K. C. Kim : Chonnam National University.

tracted from multiplicated cells in Luria-Bertani broth at 26°C for 24 hours. The cells were pelleted and washed with PBS by centrifugation. To make the pellet sensitive to lysis mixture, freeze-thaw steps were used several times in dry ice-ethanol bath and 68°C water bath and DNA was extracted using CTAB/phenol extraction method of Prosen *et al.* (22). Extracted genomic DNA was precipitated by adding 5 μg of yeast tRNA, washed with 70% ethanol, and freeze-dried in SpeedVac concentrator. Dried DNA was redissolved in 20 μl Tris-EDTA (TE) buffer (pH 7.4). DNA concentrations were determined spectrophotometrically and diluted to a concentration of 20 ng/μl prior to use for PCR amplification.

PCR amplification and detection of amplified products. DNA amplification was carried out in a final volume of 50 μl containing 25 mM KCl; 0.75 mM $MgCl_2$; 0.1 mM (each) dATP, dCTP, dGTP and dTTP (Boehringer Mannheim, Germany); 0.25 μM of primer; 1.3 U of *Taq* DNA polymerase (Amersham, USA); and 50 ng of template DNA. The primers used in this study were G1 & L1 (13), R16-1 & R23-2R and R16-2 & R23-1R (21) to select optimal ones that can amplify and differentiate all of related bacterial strains. Amplification was performed in PTC-100 Thermocycler (MJ Research Inc.). Two primers, R16-1 & R23-2R, with sequences CTTGTACACACCGCCCGTCA and TCCGGGTACTTAGATGTTTC, respectively, were chosen to differentiate test strains. After denaturation at 94°C for 5 min, the polymerase was added into amplification mixture at 82°C, and amplification was carried out 25 cycles as follows; 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and after the 25th cycle an additional 7-min incubation at 72°C.

Amplified products were separated by electrophoresis in 4% polyacrylamide gel in Tris-borate-EDTA electrophoresis buffer at 50 V, stained with ethidium bromide solution (0.5 μg/ml), and photographed under UV light. An 1-kb ladder (Amersham, USA) was used as molecular weight markers to compare and determine the size of PCR products.

RESULTS

Identification of bacterial strains isolated from rice seeds. The bacterial strains isolated from discolored seeds were identified as *P. avenae, P. glumae, E. herbicola*, and *X. oryzae* pv. *oryzae* (Table 1), of which the morphological and physiological characteristics were mostly consistent with those reported by Zeigler and Alvarez (33) for *P. avenae* and *P. glumae*, Choi *et al.* (5) for *E. herbicola*, and Vera Cruz *et al.* (31) for *X. oryzae* pv. *oryzae*, and were as follows. Three strains of *P. avenae* (COA1, COA2, and COA3); positive in oxidase, nitrate reduction and starch hydrolysis but negative in the production of lecithinase

and in the utilization of L-arginine, three *P. glumae* strains (COG1, COG2, and COG3); positive in the production of lecithinase and the utilization of L-arginine but negative in oxidase and nitrate reduction, five strains of *E. herbicola*; positive in catalase, nitrate reduction, acetoin production and growth in 5% NaCl but negative in oxidase, indole test and urase test, and 17 strains of *X. oryzae* pv. *oryzae*; negative in acetoin production, peptonization of litmus milk, phenylalanine deaminase and utilization of L-alanine but positive in sensitivity to 0.001% (w/v) cupric nitrate (data not shown). Also four unknown strains belonging to *Xanthomonas* species were isolated from rice seeds.

Selection of primers for amplification of the spacer region between 16S and 23S rRNA genes. Three sets of universal primers, G1 & L1 (13), R16-1 & R23-2R and R16-2 & R23-1R (21), were tested to compare their abilities to generate differentiable spacer polymorphism from genomic DNA of the selected isolates of *Pseudomonas* spp., *E. herbicola* and *Xanthomonas* spp. from rice seeds. Of these primers, a primer set of R16-1 & R23-2R allowed differential polymorphisms that could discriminate one from the other bacterial species (Fig. 1). The amplified products ranged about 820~950 bp in size, and were specific to the bacterial species and differentiable among the polymorphisms. In *Pseudomonas* species, the sizes of amplified products were similar among species; *P. avenae* (950 bp), *P. glumae* (850 bp), *P. fuscovaginae* (770 bp), and *P. syringae* pv. *syringae* (1,240 bp, 1,100 bp and 820 bp). In *Xanthomonas* species, the primary bands of 860 bp in *X. oryzae* pv. *oryzae* and 880 bp in *X. oryzae* pv. *oryzicola* appeared similar in size; however, additional secondary products at 890 bp, 440 bp and 370 bp were shown in *X. oryzae* pv. *oryzicola*. By using this primer set, reproducible and consistent bands were amplified from the tested strains, and even closely related *Pseudomonas* or *Xanthomonas* strains could be distinguished from one another (Fig. 1).

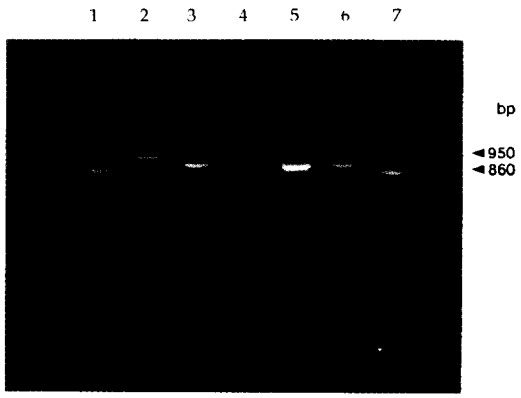

Fig. 1. Polymorphic patterns of PCR amplification products generated by using the R16-1 and R23-2R primers for the following bacterial genomic samples: Lane 1 : *X. oryzae* pv. *oryzae* CXO101; lane 2 : *P. avenae* COA1; lane 3 : *P. glumae* COG1; lane 4 : *E. herbicola* CEh1; lane 5 : *P. fuscovaginae* Fed1259-3; lane 6 : *P. syringae* pv. *syringae* Chi131-3; lane 7 : *X. oryzae* pv. *oryzicola* P501. The unnumbered lanes contained a DNA ladder having the following sizes; 2,000, 1,500, 1,000, 700, 500, 400, 300, 200, 100, and 50 bp.

Polymorphism of *Xanthomonas* species. Amplification of the 16S~23S spacer region of *Xanthomonas* species produced a single unifying feature of polymorphism, regardless of the isolates in a species (Fig. 2, Table 2). The primary bands were similar among the *Xanthomonas* species tested, ranging from 840 bp to 880 bp in size. However, secondary bands, which appeared commonly and were reproducible, were relatively diverse among the *Xanthomonas* species and among the isolates of *X. oryzae* pv. *oryzicola*.

All of the *X. oryzae* pv. *oryzae* strains produced the same primary band of 860 bp, but a weak secondary band of 430 bp or 460 bp, of which the polymorphism was designated as type a or type b (Table 2). All of the Korean strains except CXO211 belonged to type a, while four strains including only one Korean strain were designated as type b which seemed to be less common than type a in Korea. *Xanthomonas*-like strains showed a primary band at 840 bp and one weak band of 550 bp (type c in Fig. 2, Table 2) or one weak band of 650 bp (type d in Fig. 2, Table 2), and *X. oryzae* pv. *oryzicola* strains showed a primary product at 880 bp and three weak bands of 890 bp, 440 bp and 370 bp (type e in Fig. 2, Table 2).

Polymorphism of seedborne *Pseudomonas* and *Erwinia* species. Four strains of *P. avenae*, COA1, COA2, COA3 and ATCC19860, showed the same polymorphic patterns; one primary band of 950 bp and two secondary bands of 600 bp and 1,400 bp, while Fed1240-2 showed a primary band at 950 bp and a secondary band at 890 bp (Fig. 3). There was no intraspecies diversity in the polymorphism among the Korean isolates.

All the strains of *P. glumae* except Sab1673-10 pro-

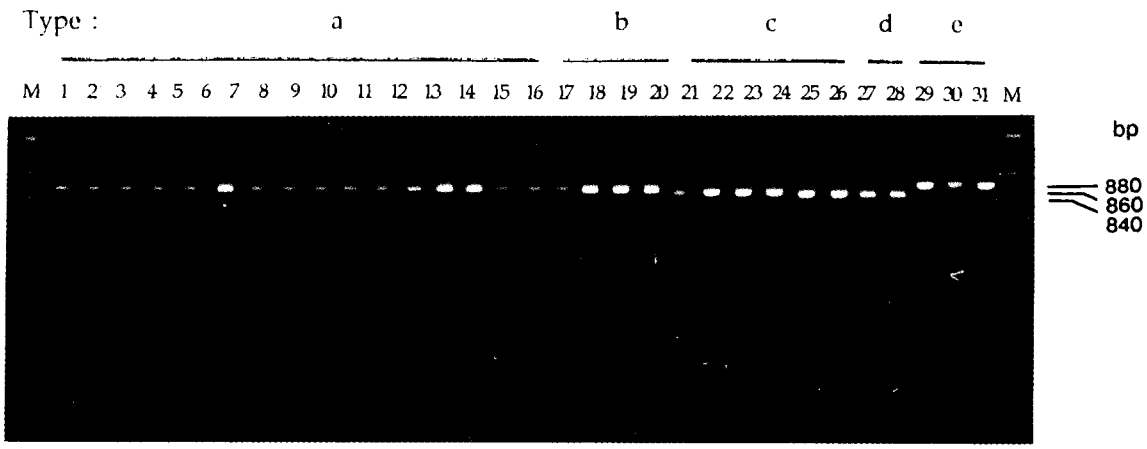

Fig. 2. Polymorphic patterns of PCR-amplified fragment of ribosomal DNA spacer regions of *Xanthomonas* spp. with R16-1 and R23-2R primers: Lanes 1 to 16 : *X. oryzae* pv. *oryzae* CXO101, 103, 104, 106, 107, 110, 112, 214, 215, 216, 316, 317, 319, 321, 322, and 323; lanes 17 to 20 : *X. oryzae* pv. *oryzae* CXO211, X1-8, B-792, and #2(2-19); lanes 21 to 26 : *Xanthomonas*-like bacteria NP-1, NP-9, CUXL324, 335, 336 and 337; lanes 27 and 28 : *Xanthomonas*-like bacteria X1-1 and X1-9; lanes 29 to 31 : *X. oryzae* pv. *oryzicola* P501, P502 and P504. The type assignments (type a to e) are given in Table 2. Lane M : DNA ladder having the following sizes; 2,000, 1,500, 1,000, 700, 500, 400, 300, 200, 100 and 50 bp.

Table 2. Summary of polymorphic patterns and sizes of spacer amplification products by *Xanthomonas oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and nonpathogenic *Xanthomonas*-like bacteria from rice seeds

Polymorphic pattern[a]	Species & strain (pathotype)	Primary band (bp)	Secondary bands (bp)
	X. oryzae pv. *oryzae*		
Type a	CXO101(1), CXO103(1), CXO104(1), CXO105(1), CXO106(1), CXO107(1), CXO110(1), CXO112(1), CXO214(2), CXO215(2), CXO316(3), CXO317(3), CXO319(3), CXO321(3), CXO322(3), CXO323(3)	860	430
Type b	CXO211, X1-8, B-792, #2(2-19)	860	460
	Xanthomonas-like bacteria		
Type c	NP-1, NP-9, CUXL324, CUXL335, CUXL336, CUXL337	840	550
Type d	X1-1, X1-9	840	650
	X. oryzae pv. *oryzicola*		
Type e	P501, P502, P504	880	890, 440, 370

[a] Typical patterns of PCR-amplified profiles from each strain appeared in Fig. 2.

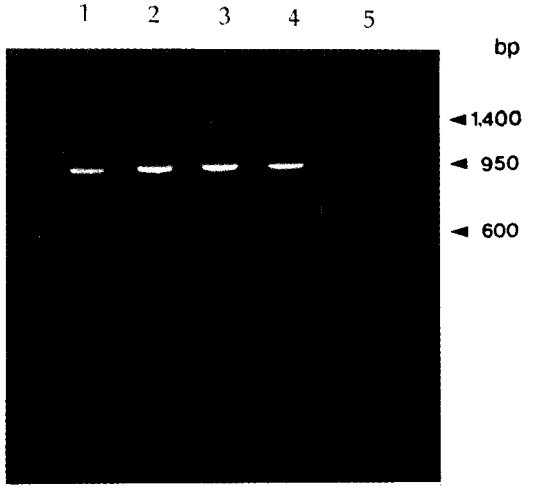

Fig. 3. Polymorphic patterns of *P. avenae* for the following bacterial genomic DNA samples with the primers of R16-1 and R23-2R; Lanes 1 to 4 : COA1, COA2, COA3 and ATCC19860, respectively; lane 5 : Fed1240-2. The unnumbered lanes contained DNA ladder having the following sizes; 2,000, 1,500, 1,000, 700, 500, 400, 300, 200, 100, and 50 bp.

duced the same primary band of 850 bp and two week bands at 730 bp and 760 bp (Fig. 4). Sab1673-10 was lack of the 850 bp band, but instead it produced a different primary band of 790 bp which was specific only

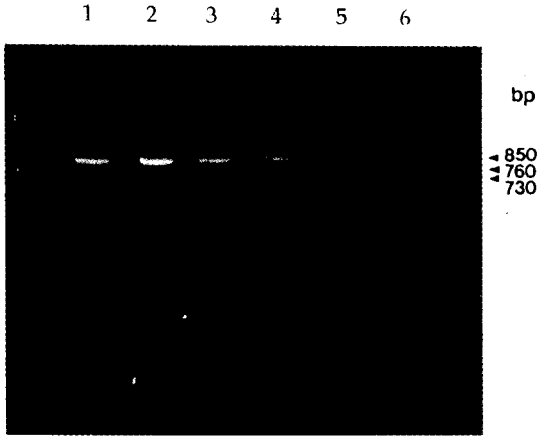

Fig. 4. Polymorphic patterns of *P. glumae* for the following bacterial genomic DNA samples by R16-1 and R23-2R primers; Lanes 1 to 5 : COG1, COG2, COG 3, K8239 and ATCC33617, respectively; lane 6 : Sab 1673-10. The unnumbered lanes contained DNA ladder having the following sizes; 2,000, 1,500, 1,000, 700, 500, 400, 300, 200, 100 and 50 bp.

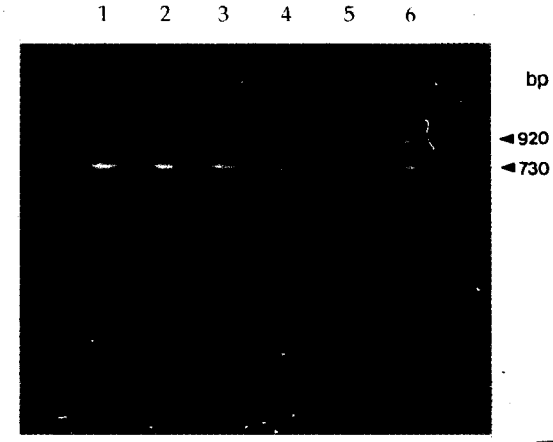

Fig. 5. Polymorphic patterns of *E. herbicola* for the following bacterial genomic samples: Lanes 1 to 6 : CEh 1, CEh2, CEh3, CEh4, CEh5 and Eh8701, respectively. The unnumbered lanes contained DNA ladder having the following sizes; 2,000, 1,500, 1,000, 700, 500, 400, 300, 200, 100 and 50 bp.

to this Columbia strain. The polymorphism of the Korean strains did not vary, and was identical to that of the standard strain of *P. glumae* (ATCC33617).

Amplification of the 16S~23S spacer region of *E. herbicola* strains produced multiple bands that could not be distinguished as primary and secondary bands. However, their band profiles were homogeneous, and there were no intraspecies variations among the strains. They showed two unique primary bands at 730 bp and 920 bp which were common to all the strains as shown in Fig. 5.

DISCUSSION

Amplification of rDNA spacer region by PCR with R16-1 and R23-2R primers could clearly differentiate four major rice-seedborne bacterial species reported in Korea, *P. avenae, P. glumae, E. herbicola* and *X. oryzae* pv. *oryzae*. There were few variations in the polymorphism of amplified ribosomal spacer regions among the tested Korean strains in a species. Although the primary products were similar in size, they were mostly typical to specific bacterial species. Also secondary products helped to provide unique polymorphisms to differentiate the bacterial species, which suggests that the polymorphism can be used as a reliable marker to differentiate one species from other species. Especially, only *X. oryzae* pv. *oryzae* has been analysed by using PCR methods among the seed-related pathogens so far. A ligation-mediated PCR showed 6 types of polymorphisms consisting of 1 to 7 amplified fragments (10). However, there has been no clear-cut polymorphism pattern produced by a stable single set of primers to detect all *X. oryzae* pv. *oryzae* strains so far. In our study, by using R16-1 and R23-2R primers, polymorphisms unique to the pathogen were produced, suggesting that the PCR method may be used as the assay of seed contamination specifically for the bacterial species. DNA spacer polymorphism by PCR amplification has been used to distinguish isolates below the species level of *Listeria, Staphylococcus*, and *Salmonella* species (13) and *Lactobacillus* strains (21). However, the spacer region polymorphism may not be generalized to give a criterion for the species identification because the sample size of bacterial strains tested in this study was not large enough to cover the whole intraspecies variations.

Korean seedborne bacteria, *P. avenae, P. glumae, E. herbicola*, and *X. oryzae* pv. *oryzae*, had characteristic primary products of rDNA spacer region: *P. avenae*, 950 bp; *P. glumae*, 850 bp; *E. herbicola*, 920 and 730 bp; and *X. oryzae* pv. *oryzae*, 860 bp, which are consistent with the sequencing data for 16S~23S ribosomal spacer regions (13, 21). In two Columbia strains, *P. avenae* Fed1240-2 and *P. glumae* Sab1673-

10, different primary products were produced by the amplification of this spacer region, suggesting that the polymorphism can be affected by a kind of regional differentiation of the species as reported in RFLP analysis showing genetic diversity of *X. oryzae* pv. *oryzae* (1).

In *Xanthomonas* spp., five types of polymorphic patterns were generated, two for *X. oryzae* pv. *oryzae*, two for nonpathogenic *Xanthomonas*-like strains, and one for *X. oryzae* pv. *oryzicola*. The primary products were similar among the polymorphic patterns, while weak secondary amplification products were diverse enough to differentiate the patterns, ranging from 370~890 bp in size. The source and nature of these weak bands are not well understood; however, they were consistent and reproducible, and thus they may be useful for providing additional information on the species identification besides the primary amplification products.

Variations in the pathogenicity and virulence of *X. oryzae* pv. *oryzae* have been reported in many studies, and thus its pathotypes have been generally accepted in many countries (3, 19, 20, 23, 24). In Korea, three pathotypes of *X. oryzae* pv. *oryzae*, K1, K2, and K3, were reported as major ones appearing in vast areas (19). We used, therefore, these three pathotypes and examined the variations of ribosomal DNA spacer in relation to the pathotypes. All of the tested *X. oryzae* pv. *oryzae* strains showed the same primary band at about 860 bp, but varied in the secondary band, dividing the polymorphic patterns into two types, type a and type b. All of the tested Korean strains except CXO211 were included in only type a, which was not consistent with the Korean pathotypes. This result was similar to that of a report in which morphological and physiological characteristics were not related to the pathotypes of *X. oryzae* pv. *oryzae* populations (23). Also, in RFLP and virulence analyses, all Korean strains were included in only one cluster (cluster 5) among five clusters from Asian collections of *X. oryzae* pv. *oryzae* (1).

A modified blotter test and a roll towel method have been used as simple detection methods for *P. avenae* (25) and for *X. oryzae* pv. *oryzae* (27), respectively, by directly confirming symptoms developed on rice seedlings. In addition to these methods, there are some methods with bacteriophages (9) and semiselective media (8, 15, 16, 30) for indirect simplified seed assays. Traditionally seedborne bacteria have been detected and identified on selective media developed on the basis of the phenotypic characteristics of the bacteria. The above simple detection methods, however, have difficulties in the bacterial identification; similar symptoms of grains produced by *Pseudomonas* spp. (33), heterogeneity of bacterial colonies (30) and similar phenotypes among *Xanthomonas* spp. (8), overgrowth of *E. herbicola* on *Pseudomonas* spp. (33), colony variations of *X. oryzae* pv. oryzae (12), etc. Low population density of bacteria in seeds, variation of strains in *X. oryzae* pv. *oryzae* and similar cellular metabolism between coexisting pathogens and saprophytes in a seed lot are other problems encountered with the use of selective media. For these methods, long experiences and profound knowledge on the bacterial taxonomy are needed for the exact identification of specific bacteria. On the other hand, detection and identification of bacteria using the polymorphism of rDNA spacer region with PCR-amplification is rapid and required no expert experiences. Also a large number of samples can be tested simultaneously. As shown in this study, this method was successfully used to differentiate the four seedborne bacterial pathogens of rice reported in Korea. Under accurate PCR conditions, this spacer PCR method was shown to be reproducible and less labor-intensive and to yield more-objective results than morphological observations. We thus suggest that the spacer PCR technique is a rapid and reliable tool to differentiate the seedborne bacterial pathogens and can be particularly used in the seed health test.

요 약

한 쌍의 R16-1과 R23-2R primer를 이용한 PCR에 의해 증폭된 16S와 23S rDNA 사이의 rDNA spacer 부위의 다형성들이 *Pseudomonas avenae, P. glumae, P. fuscovaginae, P. syringae* pv. *syringae, Xanthomonas oryzae* pv. *oryzae, X. oryzae* pv. *oryzicola* 및 *Erwinia herbicola* 등 벼 종자전염성 51개 균주의 구분을 위하여 적용되었다. 증폭산물은 820~950 bp의 크기였으며, 각각의 종에 특이적이었고 구분이 가능하였다. *Pseudomonas* species의 증폭산물은 *P. avenae*는 950 bp, *P. glumae*는 850 bp, *P. fuscovaginae*는 770 bp 및 *P. syringae* pv. *syringae*는 1,240, 1,100 및 820 bp로 특이적이었다. *P. avenae*와 *P. glumae*의 국내균주들은 다형성에 있어 종내 변이는 없었다. *X. oryzae* pv. *oryzae*의 860 bp와 *X. oryzae* pv. *oryzicola*의 880 bp의 1차 산물 및 *X. oryzae* pv. *oryzicola*의 890, 440 및 370 bp의 이차산물에서 *Xanthomonas* species의 종내에서

균주에 관련없이 단일화된 다형성을 보였다. CXO 211을 제외한 모든 국내 균주는 a형에 속한 반면 하나의 국내 균주를 포함하여 4개 균주는 b형이었다. *E. herbicola*의 spacer 부위 증폭은 여러 개의 band를 보였으며, 증폭상은 각각 동일하였고, strain간의 종내 변이는 없었다. 본 실험 결과에 의하여 16S와 23S rDNA에 R16-1과 R23-2R primer를 이용하여 PCR 증폭된 spacer 다형성의 구별은 종자전염성 세균의 신속한 구별에 이용될 수 있을 것이다.

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