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Detection of *Acidovorax avenae* ssp. *avenae* in Rice Seeds Using BIO-PCR

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Abstract

The bacterium, *Acidovorax avenae* ssp. *avenae* causes several important plant diseases including bacterial stripe of rice, bacterial stalk rot of corn, bacterial leaf blight of oats, and red stripe of sugarcane and millet. Although the organism is seed-borne in rice, no reliable seed assay is available. A semiselective liquid medium based on D-sorbitol and L-pyroglutamic acid (SP medium), and two sets of polymerase chain reaction (PCR) primers were designed for use in a BIO-PCR assay for detection of *A. avenae* ssp. *avenae* in rice seeds. External primer set, Aaaf3 and Aaar2, and internal primer set, Aaaf5 and Aaar2, designed from a 619 bp fragment of the internal transcribed spacer region of the 16S–23S rDNA of *A. avenae* ssp. *avenae* strain CAa4 were specific at the subspecies level. A nested-PCR assay produced the expected DNA product from 58 rice strains tested but not with DNA from 27 strains of *A. avenae* ssp. *avenae* from corn and other hosts. Furthermore, the primers failed to amplify a PCR product from two strains of *A. avenae* ssp. *cattleyae*, 10 strains of *A. avenae* ssp. *citrulli*, or 64 other bacteria. When *A. avenae* ssp. *avenae* was enriched prior to PCR by incubating washings of naturally contaminated rice seeds for 12 h in SP liquid medium, populations of the pathogen increased over 1000-fold, whereas populations of saprophytic bacteria remained stable. Populations of the target bacterium routinely reached PCR-detectable levels after seed samples containing as few as 1–2 target cells and up to 10^4 cfu of other bacteria were soaked overnight in phosphate buffer and then enriched for 12 h in SP liquid medium. Assays of 64 naturally infected rice seed samples resulted in 53 being positive by BIO-PCR and 34 by classical PCR. The BIO-PCR assay provides a sensitive, reliable tool for the specific detection of *A. avenae* ssp. *avenae* in rice seeds.

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Introduction

Acidovorax avenae ssp. *avenae* (=*Pseudomonas avenae*) is the causal agent of several important plant diseases including bacterial stripe of rice (Goto, 1964; Shakya et al., 1985; Kadota, 1996), bacterial stalk rot of corn (Rosen, 1926; Summer and Schaad, 1977), bacterial leaf blight of oats (Manns, 1909), red stripe of sugarcane and millet (Martin and Wismer, 1989), and brown stripe of foxtail (Rosen, 1922). The organism was originally described by Manns (1909) as the cause of blade blight of oats in Ohio in 1909 and named *P. avenae*. A similar non-fluorescent pseudomonad described several years later on foxtail (Rosen, 1922) and corn (Rosen, 1926) as *P. alboprecipitans* was shown to be synonymous with *P. avenae* (Schaad et al., 1975). Furthermore, the same organism has been referred to as *P. setariae* and *P. panici* on rice (Goto, 1964) and as *P. rubrilineans* on sugarcane (Martin and Wismer, 1989). The latter three non-fluorescent pseudomonads were later reclassified as *P. avenae* based on numerical and genomic analysis (Hu et al., 1991). A recent proposal places all the above organisms in the new 'acidovorans' DNA–rRNA homology group as *A. avenae* ssp. *avenae* (Willems et al., 1992). This group contains many pathogens previously classified in the non-fluorescent pseudomonad group, including *P. avenae* (Hu et al., 1991), *P. cattleyae* (Savulescu, 1947), and *P. pseudoalcaligenes* ssp. *citrulli* (Schaad et al., 1978), which have been reclassified as *A. avenae* ssp. *avenae*, *cattleyae*, and *citrulli*, respectively, based upon the results of DNA–DNA hybridization, DNA–rRNA hybridization, polyacrylamide gel electrophoresis of whole-cell proteins and a numerical analysis of carbon assimilation tests (Willems et al., 1992). The natural host range of *A. avenae* ssp. *avenae* includes

several monocotyledonous plants but strains from rice and millet apparently infect only rice and millet, respectively (Nishiyama et al., 1979; Kadota, 1996).

Acidovorax avenae ssp. *avenae* is a widely distributed (Shakya et al., 1985) seed-borne pathogen of rice (Goto, 1964; Shakya and Chung, 1983) and can be recovered from seeds of some lots using general agar media (W. Y. Song, unpublished data). However, because *A. avenae* ssp. *avenae* is frequently overgrown by other seed-borne saprophytic bacteria, its recovery and identification is often difficult. Since the pathogen does not always produce distinct symptoms under field conditions, the disease is very difficult to diagnose. In such cases, disease diagnosis must rely on isolation of the pathogen on semiselective agar media and pathogenicity tests. However, isolation is difficult because many ubiquitous bacteria such as *P. fulva*, *P. corrugata* and *P. fluorescens* are often present in/on rice seeds (Cottyn et al., 1996; W. Y. Song, personal observations). Pathogenicity tests are very time-consuming and expensive because rice seedlings are difficult to grow and symptoms on seedlings are often non-discriminating. Several methods to detect *A. avenae* ssp. *avenae* have been proposed including growing-on tests (Shakya and Chang, 1983; Kihupi et al., 1996), isolation on semiselective agar media (Zeigler and Alvarez, 1989; Kadota, 1996), and serology (Shakya and Chung, 1983; Kadota, 1996), but none have become widely used.

Plant disease diagnosis has been improved greatly by polymerase chain reaction (PCR)-based detection methods (Henson and French, 1993). Primers have been described for many plant pathogenic bacteria (Schaad et al., 2001) including *A. avenae* at the species level (Song et al., 1997). However, the *A. avenae*-specific primers react with all subspecies of *A. avenae* and are not specific to strains pathogenic only to rice. Based on preliminary characterization of the 16S–23S ribosomal DNA intergenic transcribed spacer region (ITS) of *A. avenae* (Kim and Song, 1996), PCR primers were designed for the specific identification of all rice strains of *A. avenae* ssp. *avenae* (Song et al., 2001). Although PCR can be a very useful method for detecting bacteria in pure culture, the technique has limitations with natural seed or soil samples because of inhibition of PCR by numerous compounds and cells of other bacteria present in plant and soil extracts. Inhibitors can be reduced by extraction of DNA, however, this can reduce assay sensitivity and it requires the use of toxic chemicals. Also, classical PCR can result in false positives from detection of dead cells and/or free DNA. One way to avoid PCR inhibitors while increasing assay sensitivity is to enrich the target bacteria on agar or in liquid media prior to PCR, a technique termed BIO-PCR (Schaad et al., 1995). By using a short, 15–24 h enrichment, as few as 10 cfu/ml of target bacteria can be detected.

We describe a BIO-PCR procedure using a new semiselective liquid medium and PCR primers for a nested-PCR technique for the specific detection of *A. avenae* ssp. *avenae* in rice seeds.

Materials and Methods

Bacterial strains and identification

The sources of bacterial strains used in this study are shown in Table 1. Bacteria were maintained on yeast extract-dextrose-calcium carbonate (YDC) (Schaad, 1988) or King et al's medium B (KB) (King et al., 1954) and stored at −80°C. For short-term storage, cultures were stored on YDC slants at 22–24°C. To confirm the identification of *A. avenae*, strains were checked for colony morphology on YDC agar, poly-β-hydroxybutyrate accumulation on nile blue medium (Pierce and Schroth, 1994) and PCR amplification using *A. avenae*-specific primers, Oaf1 and Oar1 (Song et al., 1997). Final identity of all rice strains of *A. avenae* ssp. *avenae* was confirmed by inoculating rice seedlings at the 3- or 4-leaf stage, as described (Schaad et al., 1975), with slight modification. Briefly, rice strains grown overnight in nutrient broth-yeast extract (NBY) (Vidaver, 1967) liquid medium were diluted 100-fold in phosphate-buffered saline (PBS) (20 mM sodium phosphate, 0.85% NaCl, pH 7.6) to a concentration of approximately 10^8 cfu/ml. The whorl of 5–10 seedlings per strain was then inoculated using a 1 ml syringe and a 26-gauge needle. After 24 h in a dew chamber at 30°C and 100% relative humidity (RH), all plants were moved to a growth chamber (12 h day/12 h night) at 30°C and 60% RH. Control plants were inoculated with PBS in the same manner. Symptoms were recorded 5–7 days after inoculation.

Development of a semiselective liquid medium for BIO-PCR

The key to developing a reliable BIO-PCR enrichment technique is to use a medium that allows specific and rapid growth of the target bacterium and suppresses the growth of non-target bacteria. This can be difficult with rice because most rice seeds contain large numbers of bacteria, which may inhibit growth of *A. avenae* ssp. *avenae* (W. Y. Song, personal observations). Since no reliable agar or liquid media were available, we developed a new semiselective liquid medium. Presumptive utilization of carbon and nitrogen compounds for the specific growth of *A. avenae* ssp. *avenae* was determine by using Biolog GN Microplates (Biolog, Hayward, CA, USA) with eight rice strains (CAa4–6, MAFF 301502–4, and ATCC 19882) and one corn (ATCC 19860) strain. The most promising carbon and nitrogen compounds were then selected and tested by comparing the specificity and recovery efficiency by dilution-plating techniques. From these quantitative results a basal liquid medium containing 2.0 g D-sorbitol, 0.2 g L-pyroglutamic acid, 0.5 g of K_2HPO_4, and 3.0 g of Na_2HPO_4 per litre was selected and used to evaluate several inhibitors for reducing growth of rice-associated bacteria, without reducing growth of *A. avenae* ssp. *avenae*. The growth of the above-mentioned strains of *A. avenae* ssp. *avenae* along with 24 other known bacterial pathogens of rice,

Table 1
Origin and source of strains of species and subspecies of *Acidovorax avenae* used in this study and results of PCR with primer sets, Aaaf3/Aaar2 and Aaaf5/Aaar2

Strains (*n*)	Origin	Host	Source[a]	Amplification with[b]	
				Aaaf3/r2	Aaaf5/r2
Acidovorax avenae ssp. *avenae* (87)					
COA1–3, CAa1–12, 30–43 (29)	Korea	Rice	1	+	+
19882 (*Pseudomonas setariae*)	Japan	Rice	2	+	+
301502–11, 311056–58 (13)	Japan	Rice	3	+	+
39459–63 (5)	Indonesia	Rice	4	+	+
Nepal	Nepal	Rice	4	+	+
39123a	Niger	Rice	4	+	+
40560–66, 40582–A1 (8)	Tanzania	Rice	4	+	+
19860	USA	Corn	2	–	–
3107	Brazil	Corn	5	–	–
19307 (*P. rubrilineans*)	Reunion	Sugarcane	2	–	–
931 (*P. rubrilineans*)	Australia	Sugarcane	5	–	–
3111	Brazil	*Canna* sp.	5	–	–
3403PAv	USA	Vaseygrass	6	–	–
301036	Japan	Teoshinte	3	–	–
3431PAv	Nigeria	Millet	6	–	–
CAa31, 32Wg (2)	Korea	Wheatgrass	1	–	–
301027	Japan	Wheatgrass	3	–	–
CAa23, 24Rg (2)	Korea	Rescuegrass	1	–	–
301030	Japan	Rescuegrass	3	–	–
301141	Japan	Finger millet	3	–	–
CAa301Cg–311Cg (11)	Korea	Finger millet	1	–	–
301609	Japan	Dallis grass	3	–	–
Acidovorax avenae ssp. *cattleyae*					
301576	Japan	*Phalaenopsis* sp.	3	301576	301576
33619	USA	Orchid	2	–	–
Acidovorax avenae ssp. *citrulli* (10)					
29625	USA	Watermelon	2	–	–
HIB, H17, B164, 1214, WWG, Mie3, 2576	USA	Watermelon	7	–	–
9217, 8408	USA	Watermelon	8	–	–
Acidovorax facilis					
11228	USA	Soil	2	–	–
Acidovorax konjaci					
33996	Japan	Konjac	2	–	–
301465	Japan	Konjac	3	–	–
Varivorax paraxoxus					
17713	USA	Soil	2	–	–
Other rice pathogenic species (24)[c]		Rice	1, 2, 3, 4, 9, 10, 11, 12	–	–
Other non-rice species (11)[d]			2, 3, 10	–	–
Seed-extracted bacteria (29)					
Non-fluorescent *Pseudomonas* spp. (8)	Korea	Rice	12	–	–
Fluorescent *Pseudomonas* spp. (6)	Korea	Rice	12	–	–
Erwinia spp. (3)	Korea	Rice	12	–	–
Xanthomonas-like bacteria (7)	Korea	Rice	12	–	–
Unidentified strains (5)	Korea	Rice	12	–	–

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[b]Symbols: +, presence of 262 bp band in first polymerase chain reaction (PCR) step with Aaaf3/r2 primers and 241 bp band in second semi-nested-PCR step with Aaaf5/r2 primers; –, no visible band in ethidium bromide stained agarose gel.
[c]Source of species and strains of other rice pathogenic bacteria: *Burkholderia glumae* COG1-3 (1), 301095 and 301099 (3) and 33617 (2); *B. plantarii* 301723 (3); *Pseudomonas fuscovaginae* 301177 (3) and Fed 1259-3 (9); *P. marginalis* pv. *marginalis* 301173 (3); *P. oryzicola* PO101 (10); *P. syringae* pv. *syringae* Chil31-3 (9); *Xanthomonas oryzae* pv. *oryzae* CXO105, 211 and 315 (1) and 35933 (2); *X. oryzae* pv. *oryzicola* BLS-345 (11) and 49072 (2); *Pantoea anantis* 2101 (12); and *P. herbicola* CEh1 (1) and 23375 (2).
[d]Source of species and strains of other bacteria: *B. andropogonis* 23061 (2); *B. caryophylli* 25418 (2); *B. cepacia* 25416 (2); *B. gladioli* pv. *allicola* PA7 and PA16 (10) and 19302 (2); *B. gladioli* pv. *gladioli* 10248 and 25417 (2); *P. fluorescens* 13525 (2); *P. syringae* pv. *aptata* 301008 (3); and *P. syringae* pv. *syringae* 19310 (2).

11 other plant pathogenic bacteria and 29 saprophytes isolated from rice was determined in the new basal liquid medium designated sorbitol, pyroglutamic acid (SP) medium in comparison with growth in liquid NBY medium by dilution plating onto KB agar. In addition, inhibition of the growth of seed-borne saprophytes was determined by dilution plating of 2 h washings from several rice seed lots. Approximately 1 ml

aliquots were removed, diluted to 10^{-2} and 100 μl plated onto each of three plates of KB, YDC and the new SP basal medium.

To select inhibitors, two strains of *A. avenae* ssp. *avenae* from rice, CAa4 and MAFF 301506, and the type strain from corn, ATCC 19860, were grown in liquid NBY medium on a rotary shaker for 24 h at 28°C. After adjusting the concentration of bacteria to approximately 1×10^5 cfu/ml, the suspension was sprayed onto the surface of the basal SP agar medium using a chromatographic sprayer (Sigma Aldrich Co., St Louis, MO, USA). After 20 min, 20 μl-absorbable paper discs (Sigma-Aldrich Co., St Louis, MO, USA) were dipped into each antibiotic or other inhibitor solution, placed onto the agar surface with sterile forceps, and gently pressed down to ensure contact. The following antibiotics: ampicillin, bacitracin, carbenicillin-Na_2, cephalexin, chloramphenicol, dithioerythritol, erythromycin, kanamycin, kasugamycin, neomycin sulphate, novobiocin, nobomycin, phenithicillin, polymyxin B sulphate, trimesoprim, tetracycline and vancomycin and chemicals: certimide, 5-fluorouracil, LiCl, nalidixic acid, naringenin, nitrofurantoin, nystatin, rifampin, sodium selenite, tellurite, tergitol NP7, 2,3,5-triphenyl tetrazolium chloride and tyrothrica were screened. After incubating the plates at 28°C for 48 h, the diameter of the inhibition zones was measured and recorded. Those compounds that failed to inhibit the growth of *A. avenae* ssp. *avenae* were tested further for optimal concentration for *A. avenae* ssp. *avenae* growth and recovery and for their ability to inhibit saprophytes by adding each compound separately to the basal SP liquid medium. For the selection of optimal concentration, 100 ml of liquid SP medium containing each candidate inhibitor at each concentration (10, 25, 50, 100, 150 and 200 mg/l) was seeded with a 1.0-ml suspension [over-night liquid NBY culture adjusted to 0.1 optical density (OD; A_{600}) and diluted 10^{-2}] containing approximately 1×10^6 cfu of each of the above-mentioned eight rice strains or with 1.0 ml of undiluted rice seed extract (see below) and incubated on a rotary shaker at 28°C. After 24 or 48 h, 1 ml samples were removed, diluted to 10^{-2}, 10^{-3}, and 10^{-4} and each dilution plated onto each of three NBY agar plates to determine an increase or decrease in growth, as above.

The recovery of *A. avenae* ssp. *avenae* and reduction of saprophytic bacteria from rice seed extracts were then determined using the final semiselective SP liquid medium in comparison with NBY liquid medium, as described above. To identify presumptive colonies of *A. avenae* ssp. *avenae* recovered from this medium, a portion of the colony was transferred to 1 ml of water with a toothpick and PCR conducted with 1 μl of the suspension using species-specific primers, Oaf1 and Oar1, as described previously (Song et al., 1997).

Sequencing and selection of oligonucleotides

Based on expected variations in sequences, primers were designed from ITS region sequences of the 16S and 23S rDNA (Arnheim and Ehrlich, 1992; Dolzani et al., 1994; Gurtler and Barrie, 1995; Kim and Song, 1996) of *A. avenae* ssp. *avenae* strain CAa4. Preliminary screening for specificity was made using one strain from corn (ATCC 19860) and two strains from forage grasses, CAa24Rg (rescuegrass) and CAa32Wg (wheatgrass). PCR was performed as described (Kim and Song, 1996) using genomic DNA and universal primers, R16-1 and R23-2R (Nakagawa et al., 1994) that amplify the entire ITS region between 16S and 23S. The amplified DNA was electrophoresed in a 2% Metaphore gel (FMA BioProducts, Rockland, MD, USA) and the resulting target DNA band was excised and purified by using QIAquick gel extraction kit following the supplier's recommendations (Qiagen, Valencia, CA, USA). The sequencing of the purified full-length 950-bp product was performed by the dideoxy-chain termination method (Sanger et al., 1977) using the Dye Terminator Cycle Sequencing Core Kit (Applied Biosytems, Foster City, CA, USA) following the manufacturer's protocol on a ABI PRISM 310 Genetic Analyzer (Applied Biosytems). The size of the ITS fragments of *A. avenae* ssp. *avenae* strains CAa4, ATCC 19860, and CAa24Rg and CAa32Wg were 619, 620, 618 and 618 bp and the GC contents were 49.1%, 48.9%, 49.5% and 49.5%, respectively. The sequences were aligned using DNASIS (Hitachi, Life Science, Minamidal, Japan) and corrected manually; each had a single $tRNA^{Ala}$ gene of 73 bp. By comparing the ITS sequences of *A. avenae* ssp. *avenae* obtained in this study to those found by BLAST searching, candidate primers for PCR were selected using the program OLIGO (National Biosciences, Hamel, MN, USA). The following primers sequences were selected: Aaaf3 (SEQ 10), 5′-GTC ATC CTC CAC CAA CCA AG-3′; Aaaf5 (SEQ 11), 5′-TGC CCT GCG GTA GGG CG-3′; Aaar2 (SEQ 12), 5′-AGA ACA ATT CGT CAT TAC TGA AC-3′; and Aaar5 (SEQ 16), 5′-AAT TTT TGT TGC CGA CGG CAC G-3′ (Song et al., 2001).

Primers, Aaaf3, Aaaf5 and Aaar2, from the ITS sequence of *A. avenae* were used in the nested-PCR assay, and Aaaf5 and Aaar5 were used for preparing the hybridization probe, designated as Aaafr5, for Southern blots.

Nucleotide sequence accession numbers

The sequence data for the ITS fragment of strains CAa4, ATCC19860, CAa24Rg and CAa32Wg, have been deposited in GenBank as accession numbers AY080996, AY080997, AT080998, and AY080999, respectively.

PCR assay

The first PCR step (external primers), performed with primers, Aaaf3 and Aaar2, amplified a 262 bp fragment, while the nested-PCR product obtained with primers, Aaaf5 and Aaar2, was 241 bp. The primers were synthesized by Life Technologies (Rockville, MD, USA) or Bioneer Corporation (Chungwon, Chungbuk, Korea).

Specificity of PCR primers and Southern hybridization

Cells of *Acidovorax* species and other known and unknown bacterial species from seeds were grown overnight in basal SP liquid medium and 1 ml aliquots heated at 95°C for 15 min to lyse the cells. Primers were screened for specificity in 25 μl reactions containing 2–5 μl of the lysed cell suspensions, 1.5 mM $MgC1_2$, 200 μM each of dNTPs, 4 pmole of each primers, 1X PCR reaction buffer II and 1.0 U of AmpliTaq Gold polymerase as provided by the manufacturer (Applied Biosystems). All amplifications were conducted in a Perkin Elmer 9600 thermocycler (Perkin Elmer Cetus, Norwalk, CT, USA). For external primers, Aaaf3 and Aaar2, amplifications were conducted using an initial DNA denaturation step of 94°C for 10 min; followed by 25 cycles at 94°C for 30 s; 53°C for 30 s; 72°C for 30 s and a final extension step of 72°C for 7 min. For nested-PCR, 1 μl of the PCR product generated using the external primers was used with the primers, Aaaf5 and Aaar2. The amplification was conducted as follows: denaturation at 94°C for 5 min followed by 3 cycles at: 94°C for 30 s; 60°C for 30 s; 72°C for 30 s, three cycles at: 94°C for 30 s; 48°C for 30 s; 72°C for 30 s, then 25 cycles at 94°C for 30 s; 53°C for 30 s; 72°C for 30 s and a final extension step as above. To detect a product, 5–10 μl of the PCR reaction was electrophoresed on 1.2% agarose gels and stained with ethidium bromide, as described (Maniatis et al., 1989). Cells of 87 strains of *A. avenae* ssp. *avenae*, 10 strains of *A. avenae* ssp. *citrulli*, two strains of *A. avenae* ssp. *cattleyae*, two strains of *A. konjaci*, single strain of *A. facilis* (type species of the genus), 24 strains of other known rice pathogenic bacteria, 11 strains of other species, and 29 strains of unknown non-pathogenic bacteria from rice seeds, were used to screen the primers for specificity (Table 1).

For Southern blot hybridization, primers, Aaaf5 and Aaar5, of strain CAa4 were used. Gel-eluted DNA fragments (25 ng) were labelled with [γ-^{32}P]dCTP using RadPrime DNA Labelling System (Invitrogen Life Technologies, Carlsbad, CA, USA) and purified using QIAquick Nucleotide Removal Kit (Qiagen, Hilden, Germany). The amplification products resulting from primers, Aaaf3/r2 or Aaaf5/r2, were electrophoresed and hybridized with the labelled probe, as described (Maniatis et al., 1989). The membrane was exposed to BAS-MR imaging plate for 1 h and scanned by using BAS Imaging Plate Scanner (BAS-2500, Fujifilm, Japan).

Extraction of bacteria from seed and enrichment in the semiselective SP liquid medium

Rice seed samples (Table 2) were assayed by a modified method of Kim and Song (1996). Briefly, 30-g of seed (approximately 1000 seeds) was added to each of 10, 250 ml flasks containing 50 ml of PBS containing 0.01% Tween 20 and incubated stationary at 4°C for 15 h to avoid a large increase in number of saprophytes. The samples were centrifuged at 10 000 × ***g*** for 15 min, suspended in the same volume of SP liquid medium, and incubated overnight at 28°C on a rotary shaker at 200 rpm. After incubating at 4°C for 45 min, the number of suspect colonies of *A. avenae* ssp. *avenae* and total bacteria from the incubated cell suspension were determined by dilution plating onto KB agar, a medium resulting in optimum recovery of *A. avenae* ssp. *avenae*. Two 100-μl aliquots of the supernatant were transferred to microcentrifuge tubes, centrifuged at 10 000 × ***g*** for 10 min, and the cell pellets stored at 4°C for PCR, as described below.

Sensitivity of BIO-PCR with pure cultures and spiked seeds

To select a rice seed lot free of *A. avenae* ssp. *avenae*, seed samples from several lots were soaked overnight in buffer, as described above. The extract was serially diluted 10-fold to 10^{-3} in 10 mM Tris-HCl buffer (pH 8.0) and the presence of *A. avenae* ssp. *avenae* was determined by plating 100 μl aliquots onto each of six plates of KB and YDC media. After 48 h at 28°C, a portion of any visible suspect colony was removed from three of the plates by a toothpick and transferred to a PCR tube. Each of three remaining plates was washed with 5 ml of water and the washings used for PCR. PCR-amplification was performed with Oaf1 and Oar1 primers, as described previously (Song et al., 1997). Several seed lots of cv. Dongjin (harvested in 2000) were found to be free of *A. avenae* ssp. *avenae* and used for sensitivity studies.

For inoculum, cells of *A. avenae* ssp. *avenae* CAa4 were grown overnight in NBY liquid medium. The culture was serially diluted 10-fold to 10^{-7} in PBS buffer and 100 μl of the 10^{-5}–10^{-9} dilutions was plated onto each of three plates of KB agar to determine the bacterial concentration. Those plates resulting in 1–2, 10–20 and 100–200 cfu/100 μl were used for the recovery determinations. Each dilution was evaluated by PCR, also. For PCR, two 1-ml aliquots of each dilution were stored in microcentrifuge tubes at −20°C for later use as positive controls.

Extracts were prepared from the *A. avenae* ssp. *avenae*-free seed samples of cv. Dongjin seed lot by following the same seed extraction method described above. After overnight extraction, the 10 ml samples were seeded with approximately 1–2, 10–20 or 100–200 cfu *A. avenae* ssp. *avenae* cells/ml of seed extract. An unseeded sample was included as a negative control. The mixtures were incubated without shaking for 45 min at 4°C to allow for most of the coarse particles to settle out. Doubling (generation) times (Stanier et al., 1963) were used to determine the growth rates of the target bacterium and total numbers of bacteria in liquid SP medium. The colony counts were determined by dilution plating onto KB and semiselective SP agar media.

For control treatments without enrichment, two 1-ml aliquots of the supernatant containing different number of *A. avenae* ssp. *avenae* cells prepared as above were transferred to microcentrifuge tubes and centrifuged at 10 000 × ***g*** for 10 min. The resulting

Table 2
Detection of *Acidovorax avenae* ssp. *avenae* (*Aaa*) in rice seed samples spiked with *Aaa* and in naturally contaminated seeds with and without enrichment using agar plating and nested-PCR

Seed	Number of *Aaa* cells added (cfu/ml)	Number of samples analysed	*Aaa* detection; number of positive samples[a]					
			Without enrichment		With enrichment in liquid			
					NBY		SP	
			Plating	PCR	Plating	PCR	Plating	PCR
Spiked seed samples[b]	100–200	5	4	3	3	3	5	5
	10–20	5	3	3	2	2	5	5
	1–2	5	0	0	0	2	2	5
	0	5	0	0	0	0	0	0
Naturally contaminated lots[c]								
1. Dongjin 93, 94	–	5	0	0	0	0	2	2
2. Dongjin 95, 96, 97	–	18	5	12	3	9	7	15
3. Dongjin 98, 99	–	12	4	7	2	5	7	9
4. Dongan 97, 98	–	8	3	4	0	4	5	6
5. Dongan 99	–	5	2	3	0	0	4	5
6. Dongjin 00	–	16	6	8	2	3	13	16
Total number of positive samples	–	64	20	34	7	21	38	53

Abbreviations: NBY, nutrient broth-yeast; SP, D-sorbitol and L-pyroglutamic acid; PCR, polymerase chain reaction.
[a]The number of samples in which *Aaa* was detected. Seeds were soaked at 4°C for 15 h in phosphate buffered saline (PBS), centrifuged, and the pellet suspended in liquid NBY or SP medium. After removing 1 ml for agar plating and standard direct PCR (non-enrichment), the remaining sample was enriched by incubated overnight at 28°C on a rotary shaker. Plating assays were performed by diluting samples 10-fold to 10^{-3} and plating 100 μl onto each of three plates of Kings et al's medium (KB) and SP agar. Suspect colonies were cloned and identified as described in Materials and Methods. Nested-PCR was performed using primer pairs, Aaaf3/r2 and Aaaf5/r2, as described. All results were based upon Southern blot hybridization.
[b]Samples of 1000 rice seeds (from a seed lot assayed and found to be free of *Aaa*) were soaked in 50 ml of PBS at 4°C without shaking. After 15–18 h, 1 ml of a suspension of *Aaa* adjusted to result in 100–200, 10–20, 1–2, and 0 (negative control) cfu/ml of extract was added.
[c]Seeds were collected from infected rice paddies as indicated and stored at 10–12°C and 40–50% relative humidity (RH). Samples of 1000 seeds from each lot were tested, as described above.

pellets were resuspended in 100 μl of 10 mM Tris-HCl buffer (pH 8.0) containing 0.2 mg of proteinase K (TP) per ml. The bacteria suspension was heated at 100°C for 10 min and used directly for PCR or stored at −20°C for subsequent PCR assays.

For analysis of enrichment, 10 ml suspensions in 50 ml conical centrifuge tubes were centrifuged as above, suspended in the same volume of SP liquid medium, and incubated overnight at 30°C with shaking. After stationary incubation for 45 min to allow the coarse particles to settle out, two 100-μl aliquots of the overnight culture were transferred to microcentrifuge tubes and centrifuged as above. The pellets were resuspended in TP buffer, heated at 100°C for 10 min, and used for PCR or stored at −20°C as described above. Samples without bacteria were included as negative controls. All PCR results were confirmed by Southern blot hybridization, as described above.

Assaying naturally contaminated seeds

Sixty-four seed samples [1000 seeds (30–60 g)] collected between 1997 and 2000 from plants near Chonbuk and stored at 10–12°C and 40–50% RH to avoid loss in seed and bacterial viability were used (Table 2). Standard dilution plating procedures using KB and YDC agar were used to enumerate total cells of *A. avenae* ssp. *avenae* and seed-borne bacteria in each sample. Presumptive identification of *A. avenae* ssp. *avenae* was determined by the presence of round, convex non-mucoid, tan-coloured colonies on YDC agar (Schaad et al., 1975).

The same extraction and enrichment procedures were applied as described above. The seeds were washed, as described, and enriched in semiselective SP liquid medium for 24 h. Samples were assayed by dilution plating onto semiselective SP agar medium, classical PCR and BIO-PCR, as described.

Results

Bacterial strains and identification

Colonies of all the 47 strains of *A. avenae* ssp. *avenae* tested, including those received as *P. rubrilineans* and *P. setariae*, were non-fluorescent on KB medium, round, convex, non-mucoid, and tan in colour on YDC agar medium. Colonies of *A. avenae* ssp. *citrulli*, *A. konjaci*, and *A. avenae* ssp. *cattleyae* were indistinguishable from *A. avenae* ssp. *avenae* based on morphology. All strains were positive for poly-2-hydroxybutyrate on nile blue medium. All of the *A. avenae* ssp. *avenae* strains were positive by PCR when tested with the species-specific *A. avenae* primers, Oaf1 and Oar1, and all caused stem necrosis and eventual rotting in rice seedlings.

Final semiselective liquid medium and bacterial recovery

To prepare SP medium, filter-sterilized stock solutions of ampicillin (50 mg/ml in 70% ethanol) and vancomycin (12.5 mg/ml in sterile deionized water) were added

to the basal liquid medium aseptically after autoclaving at a final concentration of 150.0 mg and 25.0 mg/l, respectively. Ampicillin, vancomycin and LiCl at 150 μg/ml, 25 μg/ml, and 5 μg/ml, respectively, suppressed the growth of most saprophytic bacteria present in rice seed extracts, as well as most other rice bacteria known to be pathogenic to rice. For SP agar medium, 15 g of Difco agar (Difco, Detroit, MA, USA) were added per litre. For direct isolation from seeds, Tween 80 (10 ml/l), victoria blue B (40 mg/l) and bromthymol blue (15 mg/l) were added to SP agar medium to improve colony differentiation of *A. avenae* ssp. *avenae* from bacteria found associated with rice seed. The pH of SP medium was adjusted to 7.4 before autoclaving.

SP liquid medium supported growth of all eight strains of *A. avenae* ssp. *avenae* tested; recovery efficiencies ranged from 85.9 to 106.1% (mean of 95.8%) in comparison with that of liquid NBY medium after 24 h incubation. Growth rates of *A. avenae* ssp. *avenae* in SP liquid medium were 0.32 and 0.35 generations per hour, after 12 and 24 h, respectively. Populations of *A. avenae* ssp. *avenae* increased nearly 15-fold from 1060 to 15 750 cfu/ml after 12 h and nearly 360-fold to 376 150 cfu/ml after 24 h. Populations of other seed-borne bacteria decreased from 4820–2110 cfu/ml (56.2%) after 12 h and to 37 cfu/ml (99.2%) after 24 h (Fig. 1).

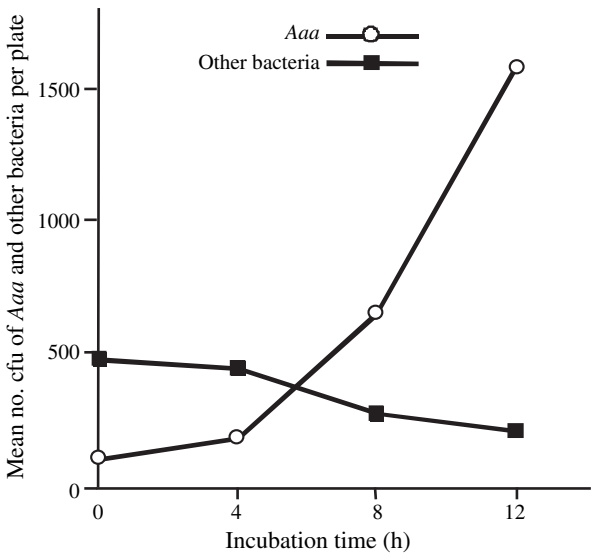

Fig. 1 Effect of D-sorbitol and L-pyroglutamic acid (SP) liquid medium on the selective growth of *Acidovorax avenae* ssp. *avenae* (*Aaa*) in the presence of other bacteria associated with rice seeds. After adjusting an overnight culture of *Aaa* to an absorbance of 0.1 at A_{600}, and diluting 10-fold to 10^{-3} [approximately 10^5 colony forming units (cfu)/ml], 100 μl together with 100 μl of a undiluted cell extract from rice seeds containing other bacteria were seeded into 5.0 ml of liquid SP medium at 28°C. Populations of *Aaa* and other bacteria were determined after incubating at 28°C without shaking for 0, 4, 8 and 12 h by removing and diluting a 100 μl sample 10-fold to 10^{-3} and plating 100 μl of 10^{-1}, 10^{-2}, and 10^{-3} onto each of three plates of KB and YDC agar plates. Only plates containing 20–200 colonies were used to determining cfus

Specificity of PCR primers and Southern analysis

External primers, Aaaf3 and Aaar2, resulted in a PCR product of 262 bp for all rice strains of *A. avenae* ssp. *avenae* (Table 1). None of the strains of *A. avenae* ssp. *avenae* originating from other hosts, strains of the phylogenetically related *A. avenae* ssp. *cattleyae*, *A. avenae* ssp. *citrulli*, and *A. facilis* or the 39 strains of other plant pathogenic bacteria, including *Burkholderia* sp. and *Xanthomonas* sp. produced a detectable PCR product. Nested primers, Aaaf5 and Aaar2, produced a 241-bp product with all rice strains of *A. avenae* ssp. *avenae*, but not with any of the other bacteria tested (Table 1). None of the 29 strains of unidentified seed-borne bacteria generated a product during the first or second round of PCR amplification (Table 1). Southern blot analysis confirmed the presence and identity of specific amplicons for the rice strains for both external and nested-PCR, and a lack of any amplicon for other bacteria.

Sensitivity of BIO-PCR with pure cultures and spiked rice seed

The mean percentage recovery of *A. avenae* ssp. *avenae* from spiked seeds enriched in semiselective SP liquid medium ranged from 78.5 to 120.7% in comparison with NBY liquid medium. Because of the rapid growth of saprophytic bacteria in NBY liquid medium after enrichment for 24 h, colonies of *A. avenae* ssp. *avenae* were not visible when the resulting growth was plated onto KB or YDC agar. In contrast, growth of saprophytic bacteria was reduced enough in semiselective SP liquid medium to allow visual detection of colonies of *A. avenae* ssp. *avenae* in most samples when plated onto KB or YDC agars. Without enrichment the threshold of detection with nested-PCR was 10–20 cfu/ml (Table 2, Fig. 2). In contrast, all samples that contained 1–2 cfu/ml were PCR-positive following enriched in SP liquid medium (Fig. 2). Agar plating and visual detection using enrichment in liquid SP detected as few as 1–2 cfu/ml, but only in two of the five samples (Table 2). All control samples (non-inoculated) were negative with or without enrichment.

Assay of naturally contaminated seed samples

Acidovorax avenae ssp. *avenae* was detected in 20 of 64 (31.2%) samples by direct agar plating of non-enriched samples and in 34 of 64 samples (53.1%) by classical PCR (Table 2). In contrast, the organism was detected in 53 of the 64 samples (82.8%) using BIO-PCR with the semiselective SP liquid medium (Table 2). Several representative colonies from the isolation plates were cloned, and pathogenicity to rice was confirmed for each. Populations recovered from each seed lot on SP agar medium ranged from 3×10^2 cfu/ml to 1.27×10^4/ml. Two of five of the older seed samples collected in 1993 and 1994 were positive by agar plating and by BIO-PCR using 12-h enrichment in semiselective SP liquid medium. None of the seed lots was positive by classical PCR.

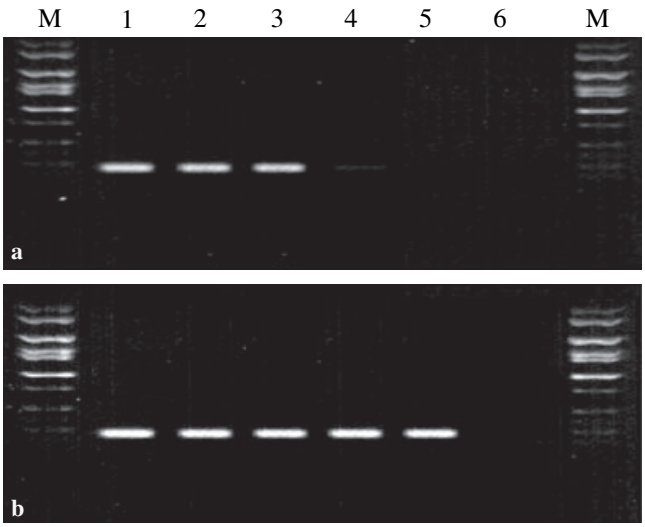

Fig. 2 Ethidium bromide stained 1.2% agarose gel showing sensitivity of a typical nested-polymerase chain reaction (PCR) assay in detecting *Acidovorax avenae* ssp. *avenae* in spiked seed samples without enrichment (a) and with enrichment in D-sorbitol and L-pyroglutamic acid (SP) liquid medium (b). The 224 bp products resulted from nested-PCR primers, Aaaf5/Aaar2. Seed extract samples were spiked with *A. avenae* ssp. *avenae* CAa4 as follows: lane 1, $1–2 \times 10^4$; lane 2, $1–2 \times 10^3$; lane 3, $1–2 \times 10^2$; lane 4, 10–20; and lane 5, 1–2 cfu/g of seeds. Lane 6 contained uninoculated seed extract and lane M, Hi-Lo DNA marker (Minnesota Molecular Lab, MN, USA)

Discussion

Rice seeds contaminated with *A. avenae* ssp. *avenae* are important sources of primary inoculum and a means of dissemination of the pathogen to new areas (Shakya and Chung, 1983; Kadota, 1996). Although methods including serology (Shakya and Chung, 1983; Kadota, 1996) and plant growing-on tests (Shakya and Chung, 1983) are available for detection of *A. avenae* ssp. *avenae* in rice seeds, none are specific or sensitive enough for reliable detection. Besides being relatively insensitive, serological techniques have the disadvantage of detecting dead or physiologically injured cells. In contrast, BIO-PCR results are based on viable cells. Furthermore, viable cultures for pathogenicity testing can be obtained from parallel agar plates in the BIO-PCR technique. Major disadvantages of greenhouse growing-on methods include time, expense and increased possibilities for cross-contamination. Moreover, similar symptoms caused by other bacterial pathogens of rice, such as *P. fuscovaginae*, *B. glumae* and *P. syringae* pv. *syringae*, can occur, making growing-on tests difficult to interpret.

Acidovorax avenae can be identified using a combination of phenotypic and pathogenicity tests, however, these methods are time-consuming and expensive. General plating and semiselective media have been useful for isolating *A. avenae* from diseased plants, but they lack the specificity needed for seed health testing. With the availability of pathogen-specific PCR primers, PCR has become a popular technique for identification of bacteria (Schaad et al., 2001). The ITS region between 16S and 23S rDNA has proven to be quite useful for designing specific PCR primers for identifying bacteria at the species level (Kostman et al., 1992; Navarro et al., 1992; Matar et al., 1993; Dolzani et al., 1994; Gurtler and Barrie, 1995; Leblond-Bourget et al., 1996; Song et al., 1997). These ITS regions are short stretches of DNA located between the 16S and 23S genes in prokaryotic rDNA loci (Neefs et al., 1990) that usually contain non-functional elements important for transcription and should be expected to exhibit sequence variation. Although the rice bacterium is classified together with strains from corn as a single species of *A. avenae* (Willems et al., 1992), our DNA/DNA reassociation assays, ITS sequencing and amplified fragment length polymorphism (AFLP) data show that the corn and rice strains should be classified as separate species (E. Postnikova, L. E. Claflin, I. Agarkova, A. Sechler, B. A. Ramundo, A. K. Vidaver and N. W. Schaad, unpublished data).

For BIO-PCR to be successful, a medium is needed that allows sufficient growth of the target bacterium to allow detection by PCR (generally 1×10^3 cells/ml or greater) before being overgrown by other bacteria (Schaad et al., 1995). Our preliminary evaluation of previously described semiselective agar media for isolation of *A. avenae* ssp. *avenae* from rice seeds (Summer and Schaad, 1977; Zeigler and Alvarez, 1989; Kadota, 1996) showed that the media either resulted in poor recovery of *A. avenae* ssp. *avenae* or failed to adequately inhibit the growth of saprophytic bacteria. Sorbitol-neutral red agar (SNR), developed for isolating *P. avenae* from corn plants and soil (Summer and Schaad, 1977) and *P. avenae*-selective medium (PASM), developed for assaying rice seeds (Kadota, 1996), worked for direct isolation with some limited success. We confirmed the value of using D-sorbitol as the sole carbon source in SNR medium (Summer and Schaad, 1977) for isolating corn strains of *A. avenae* ssp. *avenae*, but not rice strains. Furthermore, the medium allowed large numbers of saprophytic bacteria on rice seeds to grow. The rice strains tested in this study grew much slower on PASM than on semiselective SNR or SP agars (data not shown) and neither of these agar media worked well with BIO-PCR assays in preliminary tests. The number of cells of *A. avenae* ssp. *avenae* extracted from seeds and plated onto agar media are often underestimated because other bacteria severely inhibit the growth of *A. avenae* ssp. *avenae* (W. Y. Song, personal observation). However, SP liquid medium differentially suppresses other bacteria. Using SP liquid medium and a BIO-PCR protocol greatly improved the detection of *A. avenae* ssp. *avenae* in rice seeds. The increased rate of detection was, most likely, due to a combination of increased growth of the target bacterium, *A. avenae* ssp. *avenae*, and decreased growth of non-target bacteria. The combination of LiCl, ampicillin and vancomycin used in SP liquid medium allowed growth of *A. avenae* ssp. *avenae* while inhibiting most antagonistic rice seed microflora. Furthermore, the addition of Tween 80, victoria blue

B and bromthymol blue in SP agar medium allowed for differentiation of *A. avenae* ssp. *avenae* from most other rice seed bacteria.

Standard agar plating assays of rice seeds harvested from infected plants showed that few seeds were contaminated with *A. avenae* ssp. *avenae* and the level of contamination among seed lots varied widely. Colony counts of *A. avenae* ssp. *avenae* on KB or YDC ranged from 0.27 to 1.28% of the total recovered bacteria. The major advantage of our BIO-PCR liquid enrichment technique over classical PCR is an increase in the number of cells of *A. avenae* ssp. *avenae* and a reduction in the number of saprophytes. BIO-PCR, using SP liquid medium, should prove useful for detecting low numbers of the pathogen in seeds and also for monitoring epiphytic populations of *A. avenae* ssp. *avenae* on plants. The simple composition of SP medium makes it useful as a liquid or agar-based medium.

Use of SP liquid medium in a direct (without extraction of DNA) BIO-PCR assay allows for rapid, sensitive detection of *A. avenae* ssp. *avenae* in extracts of rice seed. If high numbers of other microflora are encountered after enrichment, a DNA extraction step could be included to reduce possible inhibition of PCR. The addition of a nested-PCR increases the sensitivity. These results agree with the use of nested-PCR for greater sensitivity for detection of a single copy target such as the phaseolotoxin gene sequence of *P. syringae* pv. *phaseolicola* (Prosen et al., 1993). Specificity is increased because any non-specific amplicons produced by the initial amplification using the external primers should not function as target DNA for subsequent amplification using the nested primers (Arnheim and Ehrlich, 1992). Specificity of the PCR amplification products was confirmed by Southern blot hybridization.

Sensitivity of this newly described liquid BIO-PCR technique is extremely high and is similar to the 1–2 cfu/ml sensitivity reported for detecting *Clavibacter michiganensis* ssp. *sepedonicus* in naturally infected potato tubers by real-time BIO-PCR using a semiselective agar medium (Schaad et al., 1999). In both cases, the presence of large numbers of other microorganisms prevents easy isolation of the pathogen on semiselective agar media. Semiselective SP liquid medium greatly inhibits growth of most other bacteria tested from naturally contaminated seed samples. The enrichment time necessary to detect 1–2 cfu/ml by PCR is only 12 h. These results show that BIO-PCR can be applied to seeds with moderately high levels of background microflora and still detect low levels of *A. avenae* ssp. *avenae*. The results obtained from seed samples analysed without enrichment varied considerably, illustrating the need for an enrichment step to obtain reproducible results and a high degree of sensitivity.

Techniques to directly detect *A. avenae* ssp. *avenae* in environmental or seed samples without an enrichment step certainly would be faster. However, there have been major obstacles to the development of such methods for assaying seeds including PCR inhibitors, small number of target cells, and large numbers of non-target seed-borne bacteria (Schaad et al., 1999 and unpublished observations). Although sample dilution is a simple and useful method to reduce the effect of the inhibitors, there is a risk of underestimating the amount of *A. avenae* ssp. *avenae* contamination in the samples. Furthermore, ubiquitous saprophytic bacteria, often dominant in rice seed samples, overgrow the target bacterium, and can inhibit the PCR reaction. BIO-PCR using a semiselective liquid medium and nested-PCR overcomes these problems and provides a highly sensitive assay for detecting *A. avenae* ssp. *avenae* in rice seeds. The potential risk of the dissemination of *A. avenae* ssp. *avenae* in seeds in the international exchange of germplasm of rice is of serious concern. BIO-PCR may be a valuable tool for detecting *A. avenae* ssp. *avenae* in seed lots, monitoring natural bacterial spread, tracking the pathogen in field studies and detecting waterborne or airborne cells caught on filters. The seed assay can be completed within 2–3 days, which is shorter than the 10–14 days required for traditional culturing and subsequent bacterial identification. The newly described assay could be completed in 16–18 h, including sampling and a 12 h enrichment, by using real-time PCR and portable rapid cycling PCR platforms (Schaad et al., 2002). Real-time PCR is quicker than classical PCR and reduces chances for contamination, however, the equipment is more costly than that needed for classical PCR and therefore is not available in many laboratories.

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