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Generation of PCR-based DNA Fragments for Specific Detection of *Streptomyces saraceticus* N45

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ABSTRACT

Streptomyces saraceticus strain N45, a saprophytic Gram-positive bacteria, has been shown to harbor high chitinase activity. Due to its potential use in biological control, the cloning of chitinase genes and the development of methods to quickly and precisely detect its presence have become necessary. In this study, PCR-based random amplified polymorphic DNA (RAPD) and PCR strategies were used to amplify random DNA fragments from the genome of *S. saraceticus* N45. Three amplified DNA fragments, 417, 523 and 655 bp in length, were further isolated, subcloned and sequenced. Nest primers were designed from terminal ends of these three fragments and used for further PCR reactions. A single specific band was produced from the genomic DNA of *S. saraceticus* N45 for each nest primer pair. These three single bands were *S. saraceticus* N45 specific and were not amplified from other species of *Streptomyces* or bacteria, such as *Ralstonia solanacearum*, *Agrobacterium tumefaciens*, *E. coli*, *Bacillus subtilis* and *Xanthomonas campestris* pv. *campestris*. Through detection of the coexistence of these three fragments in PCR reaction using DNA or bacterial cells directly, the presence of *S. saraceticus* N45 can be confirmed. Further Southern analysis indicated that these three DNA fragments were specifically present in the *S. saraceticus* N45 genome in a single copy manner, and therefore, that they can potentially be used as markers for identification of *S. saraceticus* N45.

Key Words: biological control, chitinase, random amplified polymorphic DNA (RAPD), *Streptomyces saraceticus*

I. Introduction

Biological control has been developed as an alternative to replacing chemicals to control various plant diseases (Jatala, 1986; Crawford *et al*., 1993; Sitrit *et al*., 1993; Schirmbock *et al*., 1994; Dowling and O'Gara, 1994; Larena and Melgarejo, 1996). Using this strategy, the microorganisms themselves or the antibiotics or degrading enzymes they produce can be used directly against various plant pathogens. Saprophytic Gram-positive bacteria in the genus *Streptomyces* have been shown to have characteristics which make them useful as biocontrol agents against soil-borne plant fungal pathogens (Whaley and Boyle, 1967; Tahvonen, 1982; Chattopadhyay and Nandi, 1982; Turhan, 1981; Mohamed, 1982; Crawford *et al*., 1993). These characteristics include the production of enzymes, such as chitinase, which degrade the fungal cell wall directly (McCarthy and Williams, l992; Gupta *et al*., 1995; Carrillo and Gomez Molina, 1998). The identification and characterization of strains of *Streptomyces* containing high chitinase activity as well as further isolation of these chitinase genes have, thus, become important and have been investigated extensively during the past few years. Many chitinase genes have been cloned and charactcrized from different species of *Streptomyces*, such as *S. coelicolor* (Saito *et al*., 1999), *S. griseus* (Ohno *et al*., 1996); *S. thermoviolaceus* (Tsujibo *et al*., 1993), *S. olivaceoviridis* (Blaak *et al*., 1993), *S. lividans* (Fujii and Miyashita, 1993; Miyashita and Fujii, 1993) and *S. plicatus* (Robbins *et al*., 1992).

For release and further manipulation of these strains so that they can be used as biological controls, the development of strategies for detecting the presence of these strains is important. DNA fingerprinting is an ideal method and has been widely used for specific identification of many organisms, including bacteria (Fani *et al*., 1993; Louws *et al*., 1999). The PCR-based random amplified polymorphic DNA (RAPD) method, one of the most successful methods of DNA fingerprinting, can be used to generate distinctively amplified patterns for different tested species (Akopyanz *et al*., 1992; Powers and Harris, 1993; Herder *et al*., 1994; Scott and McManus, 1994; Hartung *et al*., 1993; Manulis *et al*., 1994; Smith *et al*.,

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1994; Permaul *et al*., l 996; Kutchma *et al*., 1998). Specific amplified polymorphic DNA fragments can be isolated for use as probes to directly detect the existence of the organisms. In addition, the terminal ends of these DNA fragments can be designed to serve as primers to specifically amplify the same fragments from the organisms quickly.

S. saraceticus, different from many other species of *Streptomyces*, has not been studied very much in recent years to determine its molecular characteristics or antibiotic activity. In this report, a Taiwanese strain of *S. saraceticus* N45, which exhibits high chitinase activity and has been used to inhibit the hatching of eggs of southern root-knot nematode in Taiwan (Lee, 1995), was characterized. In the process of cloning chitinase genes from this strain, DNA fingerprinting was first successfully developed here to distinguish this strain from other bacteria, including various species of *Streptomyces*.

II. Materials and Methods

1. Bacteria and Growth Conditions

N45, a strain of *S. saraceticus* which exhibits high chitinase activity, was isolated in Taiwan (Lee, 1995). Four species of *Streptomyces*, *S. omiyaensis*, *S. parvulus*, *S. plicatus* and *S. coerulescens*, were purchased from FIRDI (Food Industry Research and Development Institute, Hsinchu, Taiwan). The strain of *Ralstonia solanacearum*, Ps95, was found to be fully virulent in tomatoes and was kindly provided by Drs. S.T. Hsu and K.C. Tzeng (Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan). *Xanthomonas campestris* pv. *campestris* and *Bacillus subtilis* were kindly supplied by Dr. W.M. Leu (Graduate Institute of Agricultural Biotechnology, National Chung Hsing University, Taichung, Taiwan) and Dr. B.Y. Chang (Graduate Institute of Biochemistry, National Chung Hsing University, Taichung, Taiwan), respectively. *Streptomyces* spp. were grown on YM agar medium (4 g yeast extract, 10 g malt extract, 4 g dextrose, 20 g agar, dissolved in 1 L of water, pH 7.3) at 28°C. For analysis of chitinase activity, *Streptomyces* spp. were grown on CM agar minimal medium (0.7 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, 0.001 g $ZnSO_4$, 15 g agar, dissolved in 1 L of water, pH 7.0) containing chitin (Sigma, St. Louis, MO, U.S.A.) as substrate. *R. solanacearum* Ps95 was grown on a tetrazolium chloride selective agar medium (Kelman, 1954; Yang and Ho, 1998) at 28°C. *E. coli* (DH10B) was grown on LB medium at 37°C whereas *Agrobacterium tumefaciens* (LBA4404), *X campestris* pv. *campestris* and *B. subtilis* were grown on LB medium at 28°C.

2. DNA Isolation

A modified version of the procedure of Kutchma *et al*. (1998) was used for bacteria DNA isolation. Mycelia or spores from species of *Streptomyces* were spun down and resuspended in 1 ml Tris-EDTA (TE) buffer after growing in YM liquid medium for 5 days at 28°C. TE buffer was removed after centrifugation (16000× g for 4 min at room temperature), and the pellet was resuspended in 500 μl TE containing 1 mg/ml lysozyme. After incubation at 37°C for 2 hours, 75 μl of 10% sodium dodecyl sulfate (SDS) and 125 μl of 5 M NaCl were added to this mixture. The samples were centrifuged (16000× g for 6 min at room temperature) after they were incubated in dry ice/ethanol (–70°C) for 3 min, in a 65°C water bath for 3 min and on ice for 10 min. RNase (with a final concentration of 200 μg/ml) was added to the supernatant, and the mixture was incubated at 37°C for 15 min. Proteinase K (with a final concentration of 50 μg/ml) was then added, and the mixture was incubated at 37°C for 30 min. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, and upper aqueous phase was recovered after centrifugation (16000× g for 4 min at room temperature). The DNA was precipitated by mixing the aqueous phase with 100% ethanol overnight. The DNA was pelleted by means of centrifugation (16000× g for 10 min at 4°C). After washing with 70% ethanol, the DNA was dried and dissolved in 100 μl of TE buffer. For DNA isolation of other bacteria (*E. coli*, *R. solanacearum*, *A. tumefaciens*, *X. campestris* pv. *campestris* and *B. subtilis*), cells were grown to the log phase, and then the procedure described above was employed. DNA was isolated from *Arabidopsis* ecotype S96, and then a modified version of the CTAB (Hexadecyl trimethyl-ammonium bromide) method (Yang *et al*., 1995) was performed.

3. PCR Amplification and Cloning

Genomic DNA from bacteria or plants was used to perform PCR reaction. About 50 ng of genomic DNA was used as a template for PCR amplification. The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl, 0.01% gelatin, 200 μM of each dNTP, and 0.1 μM of each oligonucleotide primer in a volume of 50 μl. The RAPD analysis was programmed for 5 cycles of 1 min at 95°C (denaturing step), of 1 min at 37°C (annealing step), and of 2 min at 72°C (extension step); followed by 30 cycles of 1 min at 95°C, of 1 min at 55°C, and of 2 min at 72°C; and finishing with extension for 10 min at 72°C. For regular PCR reaction, 35 cycles were performed, and the denaturing step was performed at 95°C for 1 min, the annealing step was performed at 55°C for 1 min and the extension step was performed at 72°C for 2 min. 10 μl of the amplifications were analyzed on 1% agarose gel. The selected RAPD and PCR fragments amplified from *S. saraceticus* N45 were cloned into PGEM-T Easy Vector (Promega, St. Louis, MO, U.S.A). Recombinant clones were screened for the desired insertion size, followed by DNA sequencing. Nest primers were designed for each recombinant clone and used for PCR amplification. For PCR using nest primers, the denaturing step was conducted at 95°C for 1 min, the annealing step was conducted at 65°C for 1 min

and the extension step was performed at 72°C for 2 min. 10 μl of each amplification was analyzed on 1.5 or 2% agarose gel. For PCR using bacterial cells directly, the cells were mixed with PCR reagents, and the mixture was used directly in PCR reaction. When nest primers were used for this reaction, the PCR condition was as described above. The number of bacteria used in each PCR reaction was determined by means of dilution-plating. The sequences of the oligonucleotide primer L1T used in RAPD analysis, the S2 and AS2 primers specific for the *S. plicatus* chitinase 63 (*chi63*) gene (Robbins *et al*., 1992), and nest primers used in the subsequent PCR are listed in Table 1.

4. DNA Gel Blot Analysis

For Southern hybridization, 2 μg genomic DNA from different species was digested with appropriate restriction enzymes, size fractionated in 1% agarose gels and transferred to Hybond N+ membranes (Amersham International, Buckinghamshire, U.K.). For highly stringent hybridization, the membranes were prehybridized for 30 min and hybridized with ^{32}P-dCTP-labeled DNA probes using multiprime DNA labelling systems (Amersham International) overnight at 65°C in the same solution (0.25 M Na_2HPO_4, pH 7.2, 7% SDS), and then washed twice in both solution I (20 mM Na_2HPO_4, pH 7.2, 5% SDS) and solution II (20 mM Na_2HPO_4, pH 7.2, 1% SDS) at 65°C for 30 min per wash. The blots were then air dried, covered with plastic wrap, and autoradiographed. The DNA probes (470, 350 and 510 bp fragments) specific for three PCR fragments were amplified using nest primers . The *S. plicatus* chitinase 63 (*chi63*) gene specific probe (460 bp fragment) was amplified by means of PCR.

III. Results

1. *S. saraceticus* N45 Contains High Chitinase Activity

S. saraceticus N45 was originally isolated from the rhizoshere of plants in the field in central Taiwan (Lee, 1995). This strain grows vigorously as small colonies and produces mycelium and spores typically observed in species of S*treptomyces* on solid medium (Fig. 1(A)). Similar to some species of *Streptomyces*, *S. saraceticus* N45 harbors strong chitinase activity. Clear zones produced by *S. saraceticus* N45 (Fig. 1(B)) after growing on CM agar medium containing chitin as the sole carbon source were comparable to those produced by *S. parvulus* (Fig. 1(C)) or *S. plicatus* (Fig. 1(D)). This result indicated the potential use of this strain as a biocontrol agent in controlling fungal diseases. In an attempt to clone chitinase genes from *S. saraceticus* N45, specific primers were derived from the chitinase 63 (*chi63*) gene of *S. plicatus* (Robbins *et al*., 1992) and the chitinase C (*chiC*) gene of *S. lividans* (Fujii and Miyashita, 1993), and were used for PCR amplification (Table 1). A 460 bp DNA fragment was amplified not only from the genomic DNA of *S. plicatus* as expected, but also from the genomic DNA of *S. parvulus* (Fig. 2). DNA sequence indicated that they shared high sequence homology (92% identity), and that the 5' end of their DNA sequences was similar to that of the *chi63* gene. For *S. saraceticus* N45, only an unexpected 655 bp DNA fragment was amplified (Fig. 2). Sequence comparison indicated that it is not the *chi63* gene from *S. plicatus* or the *chiC* gene from *S. lividans* since it shared no homology with these two genes. It also showed no obvious homology to any genes or DNA sequences reported. To answer the question of whether the *chi63* gene homologue was present in the genome of *S. saraceticus* N45, Southern blot analysis was performed using a 460 bp amplified *chi63* specific DNA fragment as a probe. As shown in Fig. 3, this probe detected signals in all five species of S*treptomyces*, indicating the presence of the *chi63* gene sequence in their genome. The observed size polymorphisms suggested diversity of this *chi63* gene among different species of *Streptomyces*. In addition, a much weaker signal was observed in *S. saraceticus* N45 (Fig. 3, lanes 1 and 2) than in other species of *Streptomyces* (Fig. 3, lanes 3 and 10). This result suggested that the low sequence homology of the *chi63* gene between *S. saraceticus* N45 and *S. plicatus* or *S. parvulus*

Table 1. Oligonucleotide Primers Used for PCR Analysis

Primer pairs		Origin of primer	Sequence (5' to 3')	PCR product	Reference
For PAPD-PCR				several bands	
L1T	(sense)	PAPD primer	GTTGGCGGGTGTCGGGGCTGGCTT		this study
For clone-655				655 bp	
S2	(sense)	*chi63/chiC* primer	AACGACTATCCAGCGGAAATTCTGA		Fujii and Miyashita (1992)
AS2	(antisense)	*chi63/chiC* primer	CGTCCGGTACGGCTGGGTGCGGGCA		Robbins *et al*. (1992)
For clone-655 nest				510 bp	
N655-1	(sense)	nest primer	ACTGGACCACGCCGAGTCTGCCGGA		this study
N655-2	(antisense)	nest primer	AGAAGGCATACGGCGTCTGAGTGAG		this study
For clone-523 nest				470 bp	
N523-1	(sense)	nest primer	CACTGTGATTCCCCGCTGTTCCCCG		this study
N523-2	(antisense)	nest primer	TAACTTGGTCACTCGGAAACCTTCA		this study
For clone-417 nest				350 bp	
N417-1	(sense)	nest primer	TCGGGTCGTCGTGACCGTACGCG		this study
N417-2	(antisense)	nest primer	TCCACATCTCCGAGCAGTTCGAG		this study

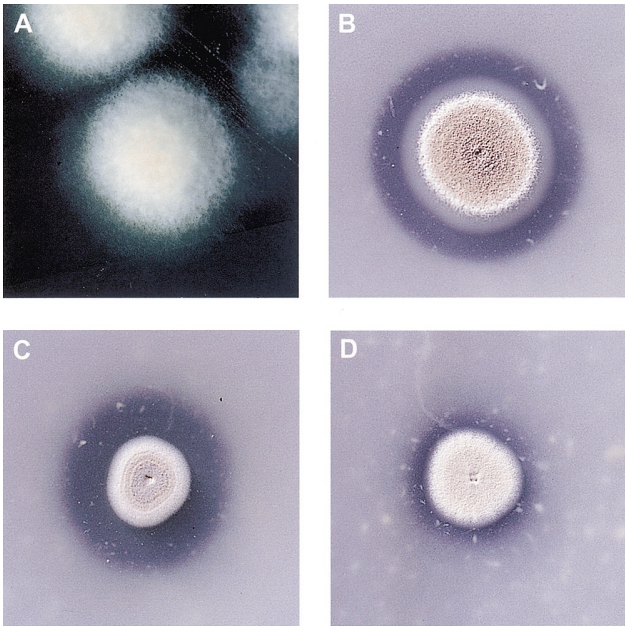

Fig. 1. The detection of chitinase activity from *S. saraceticus* N45. *S. saraceticus* N45 (A) were grown on YM agar medium without any chitin at 28°C for 7 days. Mycelium were observed on this solid medium. *S. saraceticus* N45 (B), *S. parvulus* (C) and *S. plicatus* (D) were grown on CM agar medium containing chitin at 28°C for 5 days. Clear zones were observed around the colonies, indicating the ability for these three species to degrade chitin.

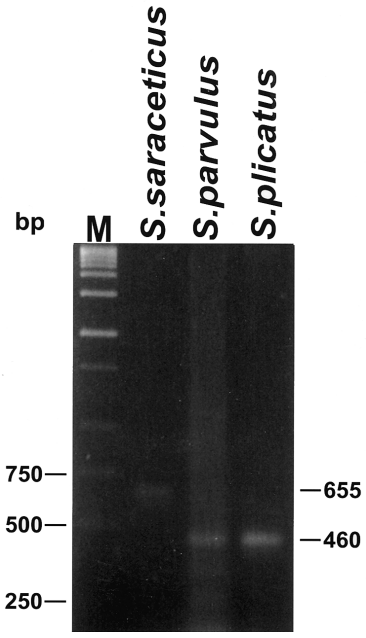

Fig. 2. PCR amplification of *chi63* gene specific DNA fragments from *S. plicatus*, *S. parvulus* and *S. saraceticus* N45. Primers S2 and AS2 were specific primers derived from *S. plicatus chi63* and *S. lividans chiC* genes. The amplified products were analyzed on 1.5% agarose gel. A 460 bp DNA fragment was amplified from both *S. parvulus* and *S. plicatus*. For *S. saraceticus* N45, a 655 bp DNA fragment was amplified. M, 1-kb ladder DNA markers.

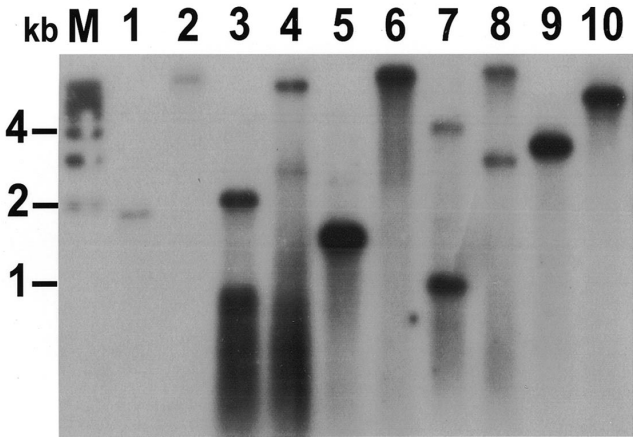

Fig. 3. The Chitinase 63 (*chi63*) gene is highly conserved in the genus of *Streptomyces*. Two μg genomic DNA from 5 different species of *Streptomyces* were digested with either *Sal*I or *Xho*I and hybridized with a ^{32}P-labeled 460 bp DNA fragment specific for *chi63* gene for highly stringent hybridization. M, 1-kb ladder DNA markers. Lanes 1 and 2, *S. saraceticus* N45; lanes 3 and 4, *S. parvulus*; lanes 5 and 6, *S. plicatus*; lanes 7 and 8, *S. omiyaensis*; and lanes 9 and 10, *S. coerulescens*. Lanes 1, 3, 5, 7, 9, *Sal*I digested DNA; lanes 2, 4, 6, 8, 10, *Xho*I digested DNA.

may be responsible for the difficulty in amplifying a *chi63* fragment from *S. saraceticus* N45 in PCR reaction. Although it was not the sequence for the *chi63* gene, DNA from this 655 bp PCR fragment was cloned and named clone-655. Nest primers were then designed for clone-655 (Table 1) and used for further detection of *S. saraceticus* N45 by means of PCR amplification.

2. RAPD Pattern for *S. saraceticus* N45 Is Distinct from Other Bacteria

In order to isolate more DNA fragments for specific detection of *S. saraceticus* N45, the RAPD-PCR strategy was used. A randomly synthesized 24-mer primer L1T was chosen to amplify random DNA fragments through PCR reaction in this experiment (Table 1). As shown in Fig. 4, random DNA fragments were amplified from total genomic DNA of all the species tested. As expected, the RAPD patterns were clearly different among the species, and each species generated unique patterns of DNA fragments amplified with the L1T primer. The results revealed that the pattern of *S. saraceticus* N45 (lane 1) is not only distinct from those of other species, such as *E. coli* (lane 6) and *A. tumefaciens* (lane 7), but also has nothing in common at all with other species of *Streptomyces* (lanes 2 to 5). Fewer bands were produced in species of *Streptomyces* than in other species of bacteria. For example, only two major bands were produced in *S. saraceticus* N45 (lane 1), and only 4 and 3 major bands were produced in *S. plicatus* (lane 2) and *S. coerulescens* (lane 5), respectively, whereas more than 10 bands were observed in

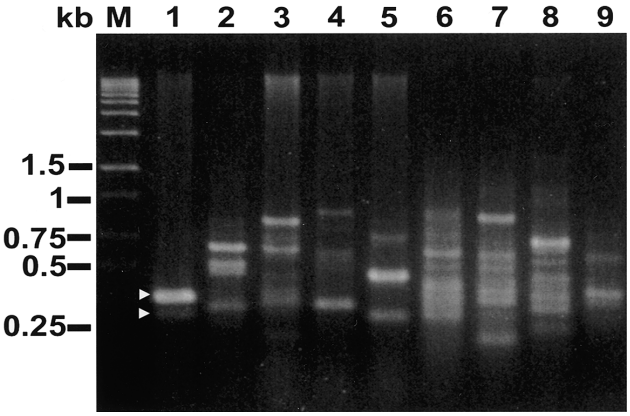

Fig. 4. Patterns of DNA fragments generated using the RAPD primer L1T from different bacteria and plant species. Total genomic DNAs were used as templates for amplification. The amplified products were analyzed on 1% agarose gel. M, 1-kb ladder DNA markers. Lanes 1 to 5: *S. saraceticus* N45, *S. plicalus*, *S. parvulus*, *S. omiyaensis*, and *S. coerulescens*, respectively. Lane 6, *E. coli* (DH10B); lane 7, *A. tumefaciens* (LBA4404); lane 8, *R. solanacearum* (Ps95); lane 9, *A. thaliana* (S96). The RAPD patterns are clearly different in various species, and the pattern of *S. saraceticus* N45 was distinct from those of other species of *Streptomyces*. Two bands (417 and 523 bp) indicated by arrows in lane 1 were used to further characterize *S. saraceticus* N45.

E. coli (lane 6), *A. tumefaciens* (lane 7) and *R. solanacearum* (lane 8). Two major bands (417 and 523 bp) produced in *S. saraceticus* N45 were further characterized. DNA from these two bands were cloned and sequenced, and named clone-417 and clone-523, respectively. DNA sequences for both clones showed no obvious homology to any gene or sequence reported. Nest primers were then designed for each clone and used for further detection of *S. saraceticus* N45 by means of PCR amplification.

3. Nest Primers Generated PCR Fragments Specific for *S. saraceticus* N45

Based on DNA sequences, specific nest primers were derived from both terminal ends of clone-655, clone-417 and clone-523 (Table 1), and used for PCR reaction. As shown in Fig. 5(A), an expected 510 bp DNA fragment was amplified specifically from the *S. saraceticus* N45 genome using primers N655-1 and N655-2, which are specific nest primers for clone-655. No bands were amplified from other species of *Streptomyces* nor from other bacteria or plant species tested. Primers N523-1 and N523-2, which are specific nest primers designed for clone-523 (Table 1) were able to amplify a 470 bp DNA fragment (Fig. 5(B)). This fragment was specific for *S. saraceticus* N45 and was not amplified from all the other species tested. Similar to the two fragments described above, a 350 bp DNA fragment was also amplified specifically from *S. saraceticus* N45 genome (Fig. 5(C)) using primers N417-1 and N417-2, which are nest primers for clone-417 (Table 1). No bands were amplified from other species tested. To examine the feasibility for these three nest primer pairs in the detection of *S. saraceticus* N45, DNA isolated from either broth-grown or agar-grown mycelia and spores were used as templates in PCR reaction. As shown in Fig. 5(D), DNA bands of expected sizes were amplified from both broth-grown and agar-grown *S. saraceticus* N45 for all three nest primer pairs (lanes l to 6). To examine the coexistence of these three bands, two or three nest primer pairs were used in the same PCR reactions. The results indicated that two or three expected bands were amplified by any combination of two nest primer pairs or mixture of all three nest primer pairs, respectively (Fig. 5(D), lanes 7 to 10). To test the sensitivity for these three nest primer pairs, a series of dilutions of *S. saraceticus* N45 cells were used directly in PCR reaction. As shown in Fig. 5(E), three expected bands were amplified by approximately as few as 50 cells of *S. saraceticus* N45 (lane 3) using the mixture of all three nest primer pairs. These three nest primer pairs, therefore, can potentially be used for specific detection of *S. saraceticus* N45 by means of PCR reactions.

4. Southern Analysis Revealed Specific Detection of *S. saraceticus* N45 Using PCR Fragments as Probes

To further characterize these three PCR fragments, Southern blot analysis was performed. In this experiment, PCR products amplified using nest primers derived from the three clones (Fig. 5) were ^{32}P-labeled and used as probes. As shown in Fig. 6(A), two clear bands (1 kb and 0.5 kb) were specifically observed in *Sal*I digested *S. saraceticus* N45 genomic DNA (lane 1) when clone-655 DNA was used as a probe. This result indicated that the clone-655 sequence is present as a single copy in the *S. saraceticus* N45 genome. No signals were detected in other species of *Streptomyces* or in other bacteria or plant species tested. Clone-417 revealed a strong band (> 12 kb) in *Bam*HI digested *S. saraceticus* N45 genomic DNA (Fig. 6(B), lane 1). This result indicated that DNA sequence in clone-417 is also present as a single copy in the genome. Similar to the result obtained from clone-655, the probe derived from clone-417 revealed no signals at all in other species of *Streptomyces* or in other bacteria and plant species tested (Fig. 6(B), lanes 2 – 9). The probe derived from clone-523 revealed a strong 12 kb band and two relatively weak bands (4 and 0.5 kb) in *Bam*HI digested *S. saraceticus* N45 genomic DNA (Fig. 6(C), lane 1), indicating that its DNA sequence exists as a single copy or as a few copies in the genome. This probe also revealed signals in some but not all other species of *Streptomyces*. For example, two bands (12 and 4 kb) were also observed in *S. plicatus* (lane 2) and *S. coerulescens* (lane 5) although in relatively weaker intensity, whereas no signals were detected in *S. parvulus* (lane 3) or *S. omiyaensis* (lane 4). The results also showed that the probe derived from clone-523 did not generate any signals in other bacteria or plant species tested. These data indicate that clone-

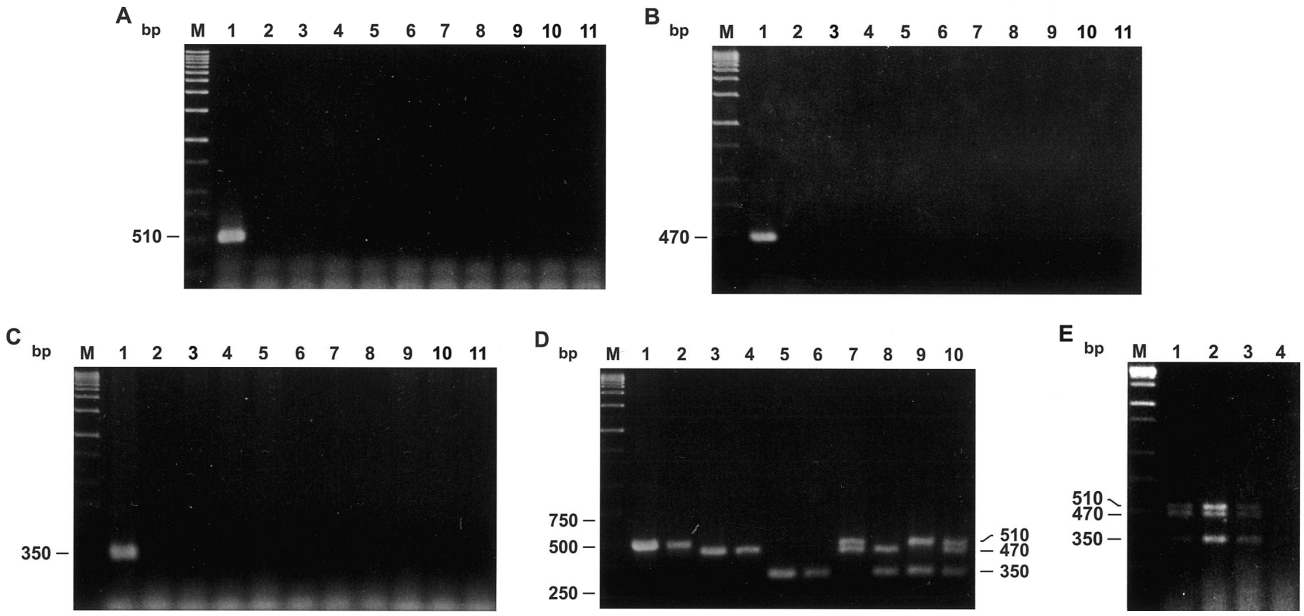

Fig. 5. Specific bands were amplified from genomic DNA of *S. saraceticus* N45 using nest primers derived from both ends of three different clones. (A) Primers N655-1 and N655-2 were specific nest primers derived from a 655 bp PCR fragment generated from *S. saraceticus* N45 (Fig. 2). A 510 bp DNA fragment was amplified specifically from the *S. saraceticus* N45 genome. No products were amplified from other species tested. (B) Primers N523-1 and N523-2 were specific nest primers derived from a 523 bp RAPD fragment generated from *S. saraceticus* N45 (Fig. 4). A 470 bp DNA fragment was amplified specifically from the *S. saraceticus* N45 genome. No products were amplified from other species tested. (C) Primers N417-1 and N417-2 were specific nest primers derived from a 417 bp RAPD fragment generated from *S. saraceticus* N45 (Fig. 4). A 350 bp DNA fragment was amplified specifically from the *S. saraceticus* N45 genome. No products were amplified from other species tested. (D) DNA bands of expected sizes were amplified from both broth-grown or agar-grown *S. saraceticus* N45 for all three primer pairs (lanes 1 to 6). In addition, two or three bands coexisted after PCR reactions conducted using combinations of two or three primer pairs (lanes 7 to 10). (E) Three bands coexisted after PCR reactions conducted using combinations of three primer pairs and approximately 5×10^3 (lane l), 5×10^2 (lane 2) or 5×10^1 (lane 3) cells of *S. saraceticus* N45 directly. No products could be amplified when the number of cells was 5×10^0 (lane 4).

Notes: (1) The PCR products were analyzed on 1.5% (for A, B, and C) or 2% (for D and E) agarose gel. (2) For (A) to (C), M, 1-kb ladder DNA markers. Lanes 1 to 5: *S. saraceticus* N45, *S. plicatus*, *S. parvulus*, *S. omiyaensis* and S. *coerulescens*, respectively. Lane 6, *E. coli* (DH10B); lane 7, *A. tumefaciens* (LBA4404); lane 8, *R. solanacearum* (Ps95); lane 9, *B. subtilis*; lane 10, *X. campestris* pv. *campestris*; and lane 11, *A. thaliana* (S96). (3) For (D), M, 1-kb ladder DNA markers. Lanes 1 and 2, primers N655-1 and N655-2 were used; lanes 3 and 4, primers N523-1 and N523-2 were used; lanes 5 and 6, primers N417-1 and N417-2 were used; lane 7, primers N655-1, N655-2, N523-1 and N523-2 were used; lane 8, primers N523-1, N523-2, N417-1 and N417-2 were used; lane 9, primers N655-l, N655-2, N417-1 and N417-2 were used; lane 10, all six primers were used. Lanes 1, 3, 5, 7, 8, 9 and 10: DNA from broth-grown *S. saraceticus* N45. Lanes 2, 4, and 6: DNA from agar-grown *S. saraceticus* N45.

655 and clone-417 are more specific than clone-523 for the detection of *S. saraceticus* N45 by means of Southern analysis.

IV. Discussion

The strain of *S. saraceticus* N45 degraded chitin efficiently. In an attempt to clone chitinase genes from this strain for use in biological control in the future, we investigated the molecular characteristics of this strain. Three DNA fragments, generated using PCR-based strategies, were identified as being able to specifically detect *S. saraceticus* N45 in a sensitive and rapid manner. Among these three, two fragments (clone-417 and clone-523) isolated from RAPD-PCR showed no obvious sequence homology to any known sequences and are, therefore, not likely to be able to encode any functional genes. Nest primer pairs derived from these two fragments amplified single band from *S. saraceticus* N45 only, indicated that they were *S. saraceticus* N45 specific. This specificity was further confirmed by means of Southern analysis. These two pairs of nest primers and their amplified products can, therefore, be used as diagnostic primers and probes, respectively. Since the primer L1T used in this RAPD analysis was randomly synthesized oligonucleotide, the result obtained here clearly demonstrates that RAPD is a simple and fast way to generate specific DNA fingerprinting for a given species.

The third DNA fragment (clone-655) was accidentally generated in this study using *chi63/chiC* specific primers. Although it was not the expected partial sequence of *chi63* and *chiC* genes or another known sequence, it was successfully developed as a diagnostic marker to distinguish *S. saraceticus* N45 from other species of *Streptomyces*. Since all three specific PCR fragments can be amplified from various DNA sources (broth- and agar-grown bacteria) of *S. saraceticus* N45, and since the coexistence of these three PCR fragments can be detected in a single PCR reaction in the presence of only

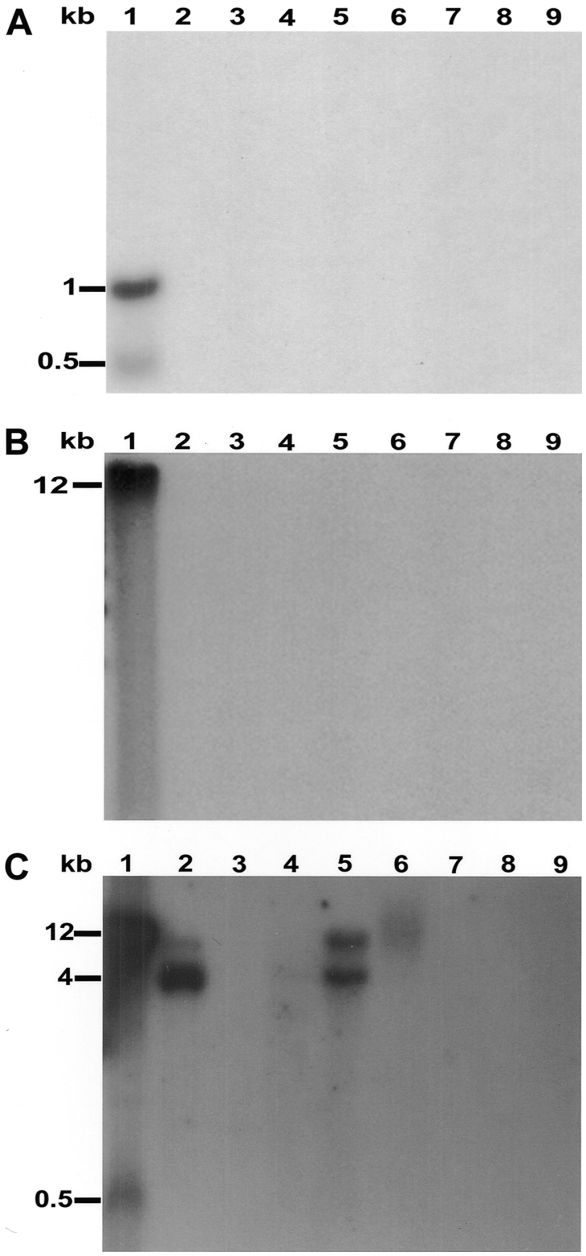

Fig. 6. Southern analysis of three different DNA fragments amplified by means of RAPD-PCR or PCR. (A) The probe derived from clone-655 specifically detected two bands (1 and 0.5 kb) in *Sal*I digested *S. saraceticus* N45 genomic DNA (lane 1). No signals were detected for other species of *Streptomyces*, other bacteria, or plant species. (B) The probe derived from clone-417 specifically detected a strong band (> 12 kb) in *Bam*HI digested *S. saraceticus* N45 genomic DNA (lane 1). No signals were detected for other species of *Streptomyce*s, other bacteria, or plant species. (C) The probe derived from clone-523 specifically detected three bands (12, 4 and 0.5 kb) in *Bam*HI digested *S. saraceticus* N45 genomic DNA (lane 1). Weak signals at various positions were detected in some species of *Streptomyces*, whereas no signals were detected in other bacteria or plant species tested. Lanes 1 to 5: *S. saraceticus* N45, *S. plicatus*, *S. parvulus*, *S. omiyaensis* and *S. coerulescens*, respectively. Lane 6, *E. coli* (DH10B); lane 7, *A. tumefaciens* (LBA4404); lane 8, *R. solanacearurn* (Ps95); lane 9, *A. thaliana* (S96).

50 cells of *S. saraceticus* N45, these three nest primer pairs are, therefore, considered very reliable for use as markers for the identification of *S. saraceticus* N45.

The inability to amplify a *chi63*-specific fragment from *S. saraceticus* N45 possibly reflects low sequence homology of the *chi63* gene between *S. saraceticus* N45 and *S. plicatus* in which the primers were designed. This assumption was supported by Southern analysis, in which the signal for the *chi63* gene was much weaker in *S. saraceticus* N45 than in other species of *Streptomyces* (Fig. 3). This observation suggests that screening of the genomic DNA library from *S. saraceticus* N45 to clone the homologue of the *chi63* gene may be a method that is better than the PCR-based cloning strategies.

The strengths and the patterns of the signals obtained from Southern analysis may reveal the possible phylogenetic relationships among the five species of *Streptomyces* tested in this study. The weakest signal for the *chi63* gene was detected in *S. saraceticus* N45, whereas signals of relatively higher intensity were observed in the other four species (Fig. 3). This result reveals that these four species may be closer to each other than to *S. saraceticus* N45. This assumption was supported by the Southern analysis, in which no signals were detected in the other four species when the probes from two of the three PCR clones were used (clone-655 and clone-417) (Figs. 6(A) and (B)). When the probe from clone-523 was used, similar signals and patterns for *S. saraceticus* N45 were detected in *S. plicatus* and *S. coerulescens*, but not in *S. parvulus* or *S. omiyaensis* (Fig. 6(C)). These data suggest that *S. plicatus* and *S. coerulescens* may be relatively closer to *S. saraceticus* N45. This assumption was supported by the patterns obtained through Southern analysis for the *chi63* gene, in which only one major band was detected in *S. saraceticus* N45, *S. plicatus* and *S. coerulescens*, whereas two major bands were observed in *S. parvulus* and *S. omiyaensis* in both enzyme digestions (Fig. 3). To further investigate the phylogenetic relationships among these species of *Streptomyces* in detail, generation and analysis of more PCR-based DNA fragments is needed, and efforts are underway in pursuit of this goal.

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References

Akopyanz, N., Bukanov, N.O., Westblom, T.U., Kresovich, S. and Berg, D. E. (1992) DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acid Res*., **20**: 5137-5142.

Blaak, H., Schnellmann, J., Walter, S., Henrissat, B. and Schrempf, H. (1993) Characteristics of an exochitinase from *Streptomyces olivaceoviridis*, its corresponding gene, putative protein domains and relationship to other chitinases. *Eur. J. Biochem*., **214**:659-669.

Carrillo, L. and Gomez Molina, S.E. (1998) Chitinase production by a strain of *Streptomyces griseoruber* isolated from rhizosphere of sugar cane. *Rev. Argent. Microbiol*., **30**:73-78.

Chattopadhyay, S.K. and Nandi, B. (1982) Inhibition of *Helminthosporium ozyzae* and *Alternaria solani* by *streptomyces longisporus* Waksman. *Plant Soil*, **69**:171-175.

Crawford, D.L., Lynch, J.M., Whipps, J.M. and Ousley, M.A. (1993) lsolation and characterization of actinomycete antagonists of fungal root pathogen. *Appl. Environ. Microbiol*., **59**:3899-3905.

Dowling, D.N. and O'Gara, F. (1994) Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. *TIBTECH*. **12**:133-141.

Fani, R., Damiani, G., Di Serio, C., Gallori, E., Friioni, A. and Bazzicalupo, M. (1993) Use of random amplified polymorphic DNA (RAPD) for generating specific DNA probes for microorganisms. *Mol. Ecol*., **2**:243-250.

Fujii, T. and Miyashita, K. (1993) Multiple domain structure in a chitinase gene (*chiC*) *of Streptomyces lividans*. *J. Gen. Microbiol*., **139**:677-686.

Gupta, R., Saxena, R.K., Chaturvedi, P. and Virdi, J.S. (1995) Chitinase production by *Streptomyces viridificans*: its potential in fungal cell wall lysis. *J. Appl. Bacteriol*., **74**:378-383.

Hartung, J.S., Daniel, J.F. and Pruvost, O.P. (1993) Detection of *Xanthornonas campestris* pv. *citri* by the polymerase chain reaction method. *Appl. Environ. Microbiol*., **59**:1143-1148.

Herder, S., Bellec, C., Meredith, S.E. and Cuny, G. (1994) Genomic fingerprinting of *Onchocerc*a species using random amplified polymorphic DNA. *Trop. Med. Parasitol*., **45**:199-202.

Jatala, P. (1986) Biological control of plant-parasitic nematodes. *Annu. Rev. Phytopathol*., **24**:453-589.

Kelman, A. (1954) The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. *Phytopathology*, **44**:693-695.

Kutchma, A.J., Roberts, M.A., Knaebel, D.B. and Crawford, D.L. (1998) Small-scale isolation of genomic DNA from *Streptomyces* mycelia or spores. *Biotechniques*, **24**:452-457.

Larena, I. and Melgarejo, P. (1996) Biological control of *Monilinia laxa* and *Fusarium oxysporum* f. sp. lycopersici by a lytic enzyme-producing penicillium purpurogenum. *Biol. Control*, **6**(3):361-367.

Lee, M.D. (1995) *Application of Chitinolytic Actinomycetes for Controlling Southern Root-Knot Nematode*. M.S. Thesis. Department of Plant Pathology. National Chung Hsing University, Taichung, Taiwan, R.O.C.

Louws, F.J., Rademaker, J.L.W. and de Bruijn, F.J. (1999) The three DS of PCR-Based genomic analysis of phytobacteria: diversity, detection, and disease diagnosis. *Annu. Rev. Phytopathol*., **37**:81-125.

Manulis, S., Valinsky, L., Lichter, A. and Gabriel, D.W. (1994) Sensitive and specific detection of *Xanthomonas eampestris* pv. *pelargonii* with DNA primers and probes identified by random amplified polymorphic DNA analysis. *Appl. Environ. Microbiol*., **60**:4094-4099.

McCarthy, A.J. and Williams, S.T. (1992) Actinomycetes as agents of biodegradation in the environment – a review. *Gene*, **115**:189-192.

Miyashita, K. and Fujii, T. (1993) Nucleotide sequence and analysis of a gene (*chiA*) for a chitinase from *Streptomyces lividans 66*. *Biosci. Biotechnol. Biochem*., **57**(10):1691-1698.

Mohamed, Z.K. (1982) Physiological and antagonistic activities of *Streptomycetes* in rhizoshere of some plants. *Egypt. J. Phytopathol*., **14**:121-128.

Ohno, T., Aemand, S., Hata, T., Nikaidou, N., Henrissat, B., Mitsutomi, M. and Watanabe, T. (1996) A modular family 19 chitinase found in the prokaryotic organism *Streptomyces griseus* HUT 6037. *J. Bacteriol*., **178**: 5065-5070.

Permaul, K., Pillay, D. and Pillay, B. (1996) Random-amplified polymorphic DNA (RAPD) analysis shows intraspecies difference among *Xanthomonas albilineans* strains. *Lett. Appl. Microbiol*., **23**:307-311.

Powers, T.O. and Harris, T.S. (1993) A polymerase chain reaction method for identification of five major *Meloidogyne* species. *J. Nematol*., **25**:1-6.

Robbins, P.W., Overbye, K., Albright, C., Benfield, B. and Pero, J. (1992) Cloning and high-level expression of chitinase-encoding gene of *Streptomyces plicatus. Gene*, **111**:69-76.

Saito, A., Fujii, T., Yoneyama, T., Redenbach, M., Ohno, T., Watanable, T. and Miyashita, K. (1999) High-multiplicity of chitinase genes in *Streptomyces coelicolor* A3(2). *Biosci. Biotechnol. Biochem*., **63**(4):710-718.

Schirmbock, M., Lorito, M., Wang, Y.L., Hayes, C.K., Arisan-Atac, I., Scala, F., Harman, G.E. and Kubicek, C.P. (1994) Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. *Appl. Environ. Microbiol*., **60**:4364-4370.

Scott, J.C. and McManus, D.P. (1994) The random amplification of polymorphic DNA can discriminate species and strains of *Echinococcus. Trop. Med. Parasitol*., **45**:1-4.

Sitrit, Y., Barak, Z., Kapulnik, Y., Oppenheim, A.B. and Chet, I. (1993) Expression of *Serratia marcesens* chitinase gene in *Rhizobium meliloti* during symbiosis on Alfalfa roots. *Molecular Plant-Microbe Interaction*, **6**:293-298.

Smith, J.J., Scott-Craig, J.S., Leadbetter, J.R., Bush, G.L., Roberts, D.L. and Fulbright, D.W. (1994) Characterization of random amplified polymorphic DNA (RAPD) products from *Xanthomonas campestris* and some common on the use of RAPD products in phylogenetic analysis. *Mol. Phylogenet. Evol*., **3**:134-145.

Tahvonen, R. (1982) Preliminary experiments into the use of *Streptomyces* spp. isolated from peat in the biological control of soil and seed-borne disease in peat culture. *J. Sci. Agric. Soc. Finland*, **54**:357-369.

Tsujibo, H., Endo, H., Minoura, K., Miyamoto, K. and Inamori, Y. (1993) Cloning and sequence analysis of gene encoding a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520. *Gene*, **134**:113-117.

Turhan, G. (1981) A new race of *Streptomyces ochra-ceischleroticus* in the biological control of some soil-borne plant pathogens. II. *In vivo* studies on the possibility of using C/2-9 against some important disease. *J. Plant Dis. Prot*., **88**:422-434.

Whaley, J.W. and Boyle, A.M. (1967) Antibiotic production by *Streptomyces* species from the rhizosphere of desert plant. *Phytopathology*, **5**7: 347-351.

Yang, C.H., Cheng, L.J. and Sung, Z.R. (1995) Genetic regulation of shoot development in *Arabidopsis*: the role of *EMF* genes. *Dev. Biol*., **169**: 421-435.

Yang, C.H. and Ho, G.D. (1998) Resistance and susceptibility of *Arabidopsis thaliana* to bacterial wilt caused by *Ralstonia solanacearum. Phytopathology,* **88**:330-334.