Detection of a mutation at codon 43 of the *rps*L gene in *Xanthomonas oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* by PCR-RFLP

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ABSTRACT. The aim of this study was to develop a method to detect a point mutation in the ribosomal S12 protein (*rps*L) gene in streptomycin-resistant strains of *Xanthomonas oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae*. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was developed to detect a point mutation in codon 43 of the *rps*L gene in *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae*. The 304-bp PCR product from the *rps*L gene was digested by *Mbo*II to form two fragments (201 and 103 bp) if there was a mutation at codon 43, or three fragments (146, 103, and 55 bp) if there was no mutation. Compared with the results from nucleotide sequencing, the PCR-RFLP method was accurate in detecting the point mutation at codon 43 of the *rps*L gene in streptomycin-resistant strains of *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae*. These results indicate that the

PCR-RFLP is a simple, rapid and reliable method for detecting the point mutation at codon 43 of the *rps*L gene.

Key words: Molecular diagnosis of PCR-RFLP; Point mutation; Ribosomal protein S12 (*rps*L) gene; *X. oryzae* pv. *oryzae*; *Xanthomonas oryzae* pv. *oryzicola*

INTRODUCTION

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae*, and bacterial leaf streak, caused by *X. oryzae pv. oryzicola,* are two of the most serious bacterial diseases of rice in many rice-growing regions of the world, including southern China (Strange et al., 2005; Jeung et al., 2006; Nino-Liu et al., 2006; Kang et al., 2008). Each of these diseases can cause a loss of at least 10% yield in susceptible rice varieties under appropriate climatic conditions (Chen et al., 2004; Lai et al., 2004; Kang et al., 2008; Salzberg et al., 2008).

The antibiotic streptomycin was discovered in the 1950s (Sundin et al., 1993) and was first used to control bacterial diseases in humans and then in agriculture to control bacterial diseases in crops such as rice (Yong et al., 2004). Streptomycin has also been used in China to control citrus bacterial canker disease (caused by *X. campestris* pv. *citri*), bacterial soft rot of Chinese cabbage (caused by *Erwinia carotovora* subsp *carotovora*), tobacco wildfire (caused by *Pseudomonas syringae* pv. *tabaci*), and bacterial blight and bacterial leaf streak of rice. Chromosomally acquired resistance to streptomycin is often due to mutations or other molecular changes in the genes encoding ribosomal protein S12 (*rps*L) and 16S rRNA (*rrs*) (Wu et al., 2006). The s*trA-strB* genes (Sundin et al., 2000, 2002) and *aadA* gene (Li et al., 2004; Poole et al., 2004; Weldhagen et al., 2004) are also involved in streptomycin resistance. In our previous research, laboratory-induced streptomycin resistance in *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* was found following the mutation of Lys (AAG) to Arg (AGG) at the 43rd or 88th amino acid residue in *rps*L.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) relies on the production of considerable amounts of amplicons of a known nucleic acid sequence by PCR and subsequent restriction-enzyme digestion of the amplified DNA products. The generated fragments are separated by gel electrophoresis according to their sizes. Restriction enzymes (DNA-dependent endonucleases) recognize specific short palindromic sequences of 4-8 nucleotides. They hydrolyze (cleave) the phosphodiester backbone of both DNA strands at these specific recognition sites (Al Dahouk et al., 2005).

In this study, we analyzed the *rps*L gene from mutant (streptomycin-resistant) and wild-type strains of *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae*, and developed a specific molecular test for mutations at codon 43 in *rps*L.

MATERIAL AND METHODS

Strains

The 23 strains used in this study are listed in Table 1. These strains included the streptomycin-sensitive strains RS105 and PXO99, and the streptomycin-resistant mutants from W-YNB9-1, SCB4-3, ZJ173, and PXO99. All strains were maintained on nutrient agar (NA) at 28°C.

NA contained 5 g polypeptone, 1 g yeast powder, 3 g beef extract, 15 g sucrose, and 17 g agar per liter. The pH was adjusted to 7.0 with 10 M sodium hydroxide. Nutrient broth (NB) medium was the NA medium without agar.

DNA isolation

DNA isolation was performed as described by Yong et al. (2011). After the bacterial suspension was cultured at 28°C overnight, 1.5 mL of the suspension was centrifuged at 12,000 *g* for 10 min. The pellet was resuspended in 567 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Proteinase K and sodium dodecyl sulfate (SDS) were added at final concentrations of 100 µg/mL and 5 g/L, respectively. After incubation for 1 h at 37°C, sodium chloride and hexadecyltrimethylammonium bromide (CTAB) were added to each preparation at final concentrations of 0.7 M and 10 g/L, respectively. The preparations were incubated at 65°C for 10 min, and DNA was extracted with chloroform + isoamylalcohol (24 + 1 by volume). Samples were shaken for 10 min and centrifuged at 12,000 *g* for 20 min. DNA was re-extracted with phenol + chloroform + isoamylalcohol (25 + 24 + 1 by volume) and centrifuged as described. DNA was precipitated by adding 0.6X volume of isopropanol and incubating at -20°C for 30 min. Samples were centrifuged at 12,000 *g* for 20 min, and the pellets were washed with 1 mL 70% ethanol and centrifuged. The DNA was dried at room temperature, and the pellet was then dissolved in sterilized ultrapure water (50 µL) and stored at -20°C.

PCR amplification

A pair of oligonucleotide primers was designed (*rps*LnF-5'-ATCAACTGATGACGACGATCA ATCA-3' and *rps*LnR-5'-CGGACGGTGTGATAACGCACAC-3') to amplify a 304-bp fragment of *rps*L from the genomic DNA of mutants of *X. oryzae* pv**.** *oryzicola* strain BLS256. PCR was performed in a total reaction volume of 25 µL on an automatic thermal controller. The reaction mixture contained 2.5 µL 10X buffer, 2 µL dNTPs, 1.5 µL $MgCl_2$, 20 pmol each primer, and 2.5 U *Taq* polymerase. The PCR procedure was as follows: pre-denaturation at 94°C for 5 min; followed by 30 cycles of 94°C for 40 s, 63°C for 100 s, and 72°C for 60 s; and a final extension at 72°C for 10 min. Amplified DNA fragments were detected by electrophoresis on 1.5% agarose gels.

PCR products were purified and sequenced by Liangzhong Bioengineering Company, Ltd. (Shanghai, China). Primers were synthesized by Shanghai Bioengineering Company (Shanghai, China).

PCR-RFLP

For rapid detection of the mutation at codon 43 of *rps*L, the PCR products were cleaved by the restriction enzyme *Mbo*II. The reaction mixture contained 5 µL PCR product, 1 µL *Mbo*II (10 U/µL), 1 µL reaction buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.5), and 3 µL deionized distilled water. The reaction was carried out at 37°C for 3 h, and the products were detected by electrophoresis on 2% agarose gels.

DNA sequencing

A pair of oligonucleotide primers was designed (5'-CGGACGAGGAGTAAGCG-3' and 5'-AT GAAGC G GGCAATGGT-3') to amplify the whole *rps*L sequence from BLS256. PCR products from

all 23 strains were purified and sequenced by Liangzhong Bioengineering Company, Ltd. (Shanghai, China). Primers were synthesized by Shanghai Bioengineering Company (Shanghai, China).

RESULTS

RFLP analysis

PCR with primers specific for *rps*LnF and *rps*LnR generated a fragment that was 30 4 bp from RS105, PXO99 and its mutants (Figure 1). The *rps*L codon 43 in wild-type strains of *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* contained a *Mbo*II restriction enzyme site. The mutation at codon 43 of *rps*L in streptomycin-resistant strains results in the loss of the *Mbo*II site at that codon.

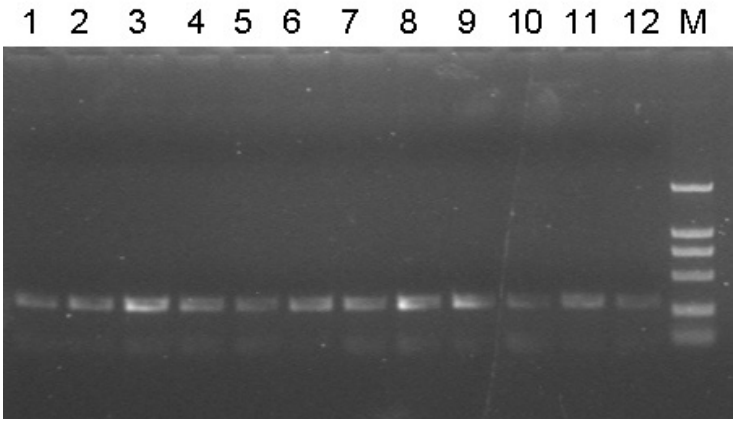

Figure 1. PCR fragments of *rps*L from RS105, PXO99 and its mutants amplified using the primers *rps*LnF and *rps*LnR. *Lanes 1-6* = UV-w-1, UV-w-2, UV-w-3, YJ-w-1, YJ-w-2, and RS105. *Lanes 7-12* = UV-z-1, YJ-z-1, UV-p-1, UV-p-2, YJ-p-1, and PXO99; *lane M* = DL2000.

The results of RFLP analyses of the *rps*L genes are shown in Figure 2. Two RFLP patterns were observed. In pattern A, the 304-bp product was digested by *Mbo*II, which generated two fragments (201 and 103 bp), indicating the presence of a mutation at codon 43. In pattern B, the 304-bp product was digested into three fragments (146, 103, and 55 bp), indicating the absence of a mutation at codon 43. The 55-bp fragment (the middle fragment) was so short that it often moved completely through the 2% agarose gels before the gels were stained (Figures 2 and 3).

Figure 2. Genomic DNA amplified by the primers *rps*LnF and *rps*LnR and cleaved by *Mbo*II. Arrowheads indicate the locations of the primers used for PCR. *Mbo*II restriction sites are indicated. C, the mutation at codon 43; D, the mutation at codon 88 or no mutation (wild-type strain).

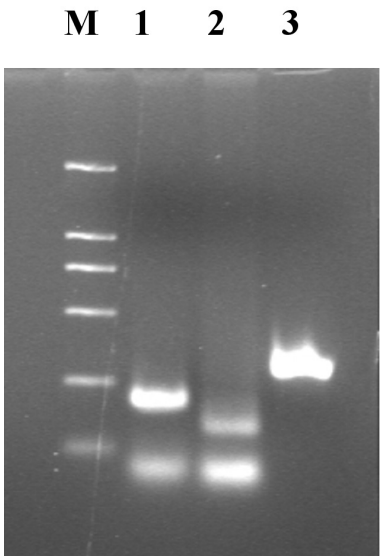

Figure 3. *Mbo*I restriction patterns of the PCR-amplified *rps*L gene. *Lane 1* = PCR product digested with *Mbo*I (pattern A in Table 1). *Lane 2* = digested PCR product with *Mbo*I (pattern B in Table 1). *Lane 3* = undigested PCR product; *lane M* = DL2000.

Of the 11 streptomycin-resistant strains of *X. oryzae* pv. *oryzicola*, four produced pattern A and seven produced pattern B (Figure 4 and Table 1). Of the 10 streptomycin-resistant strains of *X. oryzae* pv. *oryzae*, four produced pattern A and six produced pattern B (Figure 5 and Table 1). Streptomycin-sensitive strains RS105 and PXO99 produced pattern B (Figures 4, 5, and Table 1).

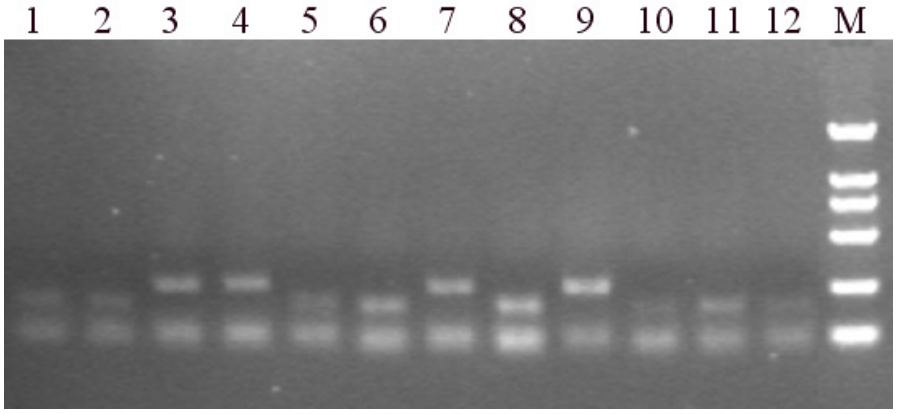

Figure 4. Detection of mutations at codon 43 of the *rps*L gene in *X. oryzae* pv. *oryzicola* by RFLP analysis. Two patterns of digestion of the 304-bp amplified fragment of the *rps*L gene by *Mbo*I are shown. *Lanes 1-12* = UV-w-1, UV-w-2, UV-w-3, YJ-w-1, YJ-w-2, YJ-w-3, YJ-w-4, UV-S-1, UV-S-2, YJ-S-1, YJ-S-2, and RS105, respectively; *lane M* = DL2000.

Table 1. Bacterial strains used.

Strain	Source of mutant	Response to streptomycin[a]	*rps*L PCR-RFLP *Mob*II pattern[b]
X. oryzae pv. *oryzicola*			
UV-w-1	W-YNB9-1	R	B
UV-w-2	W-YNB9-1	R	B
UV-w-3	W-YNB9-1	R	A
YJ-w-1	W-YNB9-1	R	A
YJ-w-2	W-YNB9-1	R	B
YJ-w-3	W-YNB9-1	R	B
YJ-w-4	W-YNB9-1	R	A
UV-s-1	SCB4-3	R	B
UV-s-2	SCB4-3	R	A
YJ-s-1	SCB4-3	R	B
YJ-s-2	SCB4-3	R	B
RS105	Laboratory wild-type	S	B
X. oryzae pv. oryzae			
UV-z-1	ZJ173	R	A
UV-z-2	ZJ173	R	B
UV-z-3	ZJ173	R	A
YJ-z-1	ZJ173	R	B
YJ-z-2	ZJ173	R	A
YJ-z-3	ZJ173	R	B
UV-p-1	PXO99	R	B
UV-p-2	PXO99	R	B
YJ-p-1	PXO99	R	B
YJ-p-2	PXO99	R	A
PXO99	Laboratory wild-type	S	B

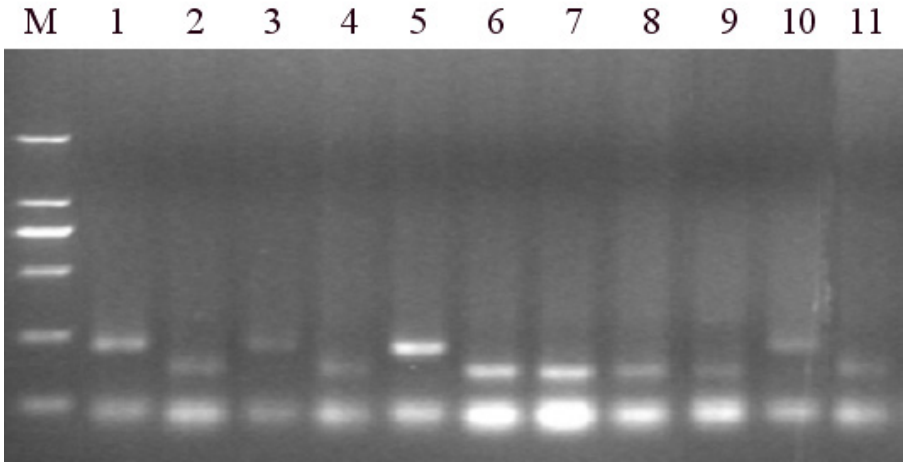

Figure 5. Detection of the mutation at codon 43 of the *rps*L gene in *X. oryzae* pv. oryzae by RFLP analysis. The two patterns of digestion of the 304-bp amplified fragment of the *rps*L gene by *Mbo*II are shown. *Lanes 1-11* = UV-z-1, UV-z-2, UV-z-3, YJ-z-1, YJ-z-2, YJ-z-3, UV-p-1, UV-p-2, YJ-p-1, YJ-p-2, and PXO99, respectively; *lane M* = DL2000.

DNA sequencing

DNA sequence analysis showed that the *rps*L amino acid sequences of *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* were 97% identical. An open read fragment (ORF) of 375 bp was obtained with the initiation codon ATG and the termination codon TGA, and the (G+C) content

was 61.36%. All streptomycin-resistant strains had a single-point mutation in the *rps*L gene. Out of the 21 streptomycin-resistant strains, eight were mutated at codon 43 and 13 were mutated at codon 88. AAG (Lys) at codon 43 or codon 88 was replaced by AGG (Arg), which resulted in the replacement of one amino acid t (Figure 6). The distribution of *rps*L mutations did not show a marked equilibrium between resistant mutants in that the mutation was more common at codon 88 than at codon 43, which was the same as previously reported for *Mycobacterium tuberculosis* (Dobner et al., 1997). The DNA sequencing data were completely consistent with the results of the RFLP analyses for these mutated strains.

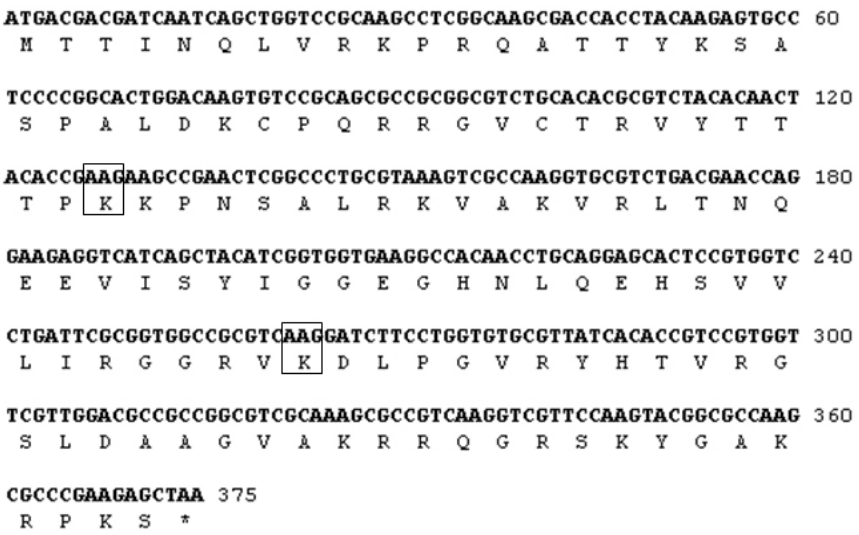

Figure 6. Nucleotide and deduced amino acid sequences of rpsL of *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* showing Lys (AAG) mutated to Arg (AGG) at the 43rd or 88th amino acid residue. These two mutations could be involved in streptomycin-resistance in *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae*.

DISCUSSION

Streptomycin is an aminocyclitol glycoside antibiotic that is commonly used in the first-line treatment of tuberculosis. This antibiotic interferes with translational proofreading and thus inhibits protein synthesis. More specifically, streptomycin binds to the 30S ribosomal subunit, thereby interfering with polypeptide synthesis and inhibiting translation (Dobner et al., 1997). Chromosomally acquired resistance to streptomycin has frequently been shown to be due to mutation or other molecular changes in the genes encoding ribosomal protein S12 (*rps*L) and 16S rRNA (*rrs*) (Wu et al., 2006). The strA-strB (Sundin et al., 2000, 2002) and aadA gene (Li et al., 2004; Poole et al, 2004; Weldhagen et al., 2004) can also confer streptomycin resistance.

In our previous research, the *rps*L gene was cloned and ligated into the vector pUFR034. The plasmid containing the Lys→Arg mutation at the 43rd or 88th codon of the *rps*L gene can confer streptomycin resistance to the sensitive wild-type strain by electroporation. This suggests that the streptomycin-resistance phenotype is caused by mutations in *rps*L. Sequence comparison showed that a point mutation in this gene resulted in substitution of Arg by Lys at position 43 or 88 in streptomycin-resistant mutants. The same mutation was also reported in streptomycin-resistant

strains of *E. amylovora* (Chiou et al., 1995), *M. tuberculosis* (Dobner et al., 1997), and *Thermus thermophilus* (Gregory et al., 2001). In the present study, the mutation frequency at codon 43 was 38.1% whereas that at codon 88 was 61.9%. Conspicuously, the distribution of *rps*L mutations did not show a marked equilibrium in resistant mutants in that the point mutation at codon 88 was more frequent than that at codon 43, which is also true for genomic mutations in *M. tuberculosis* (Dobner et al., 1997).

The current study describes a new PCR-RFLP technique that can be used to detect streptomycin-resistant strains of *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae.* This method is less time-consuming than the conventional fungicide-susceptibility testing technique that is used to discriminate the mutation leading to streptomycin resistance. Although DNA sequencing is the most reliable method for determining the exact nature of the mutation, this method requires specialist equipment and techniques and is therefore unsuitable, or at least difficult, for routine use in the laboratory. The current study confirmed that the mutation at codon 43 of *rps*L, which is associated with streptomycin resistance in *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* could be rapidly identified by a simple, low-cost PCR-RFLP method (Katsukawa et al., 1997). This method, however, could not detect mutations at codon 88 of *rps*L, and developing a PCR-RFLP method or other method to rapidly and easily detect mutations at this codon requires additional research.

Conflicts of interest

The authors declare they have no conflict of interest.

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