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Full Length Research Paper

A study on the mechanism of resistance to streptomycin in *Xanthomonas oryzae* pv*. oryzae*

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11 streptomycin-resistant mutants of *Xanthomonas oryzae* pv*. oryzae* were obtained by streptomycin selection. These mutants could grow at 100 µg ml^{-1} of streptomycin while the wild-type strain (PXO99) could not grow at 2 µg ml^{-1}. Specific primers based on the conserved region of *X. oryzae* pv*. oryzae* were designed and used to amplify the gene encoding ribosomal protein S12 (*rps*L). Sequencing indicated that the *rps*L gene has 375 bp encoding 125 amino acid residues. One pair of specific primers with restriction enzyme site of *EcoR I* enzyme was designed and used to amplify the *rps*L gene from streptomycin-resistant strains and the wild-type sensitive strain of *X. oryzae* pv. *oryzae*. In all resistant strains, a mutation in which AAG was substituted for AGG (Lys→Arg) occurred either at codon 43 or 88. The mutations situated at codon 43 or 88 in *rps*L gene of streptomycin-resistant *X. oryzae* pv*. oryzae* strains could be detected by PCR-RFLP. Two plasmids, pUFRPS and pUFRPX, were constructed by ligating the *rps*L gene into the cosmid pUFR034. The plasmids pUFRPS and pUFRPX containing the Lys→Arg mutation of the *rps*L gene conferred streptomycin resistance were transformed into the sensitive wild-type strain by electroporation. Both transformants, PS1 and PS2, could grow at 50 µg ml^{-1} of streptomycin. The results strongly suggest that the mutation in *rps*L could result in resistance of *X. oryzae pv. oryzae* to streptomycin.

Key words: *Xanthomonas oryzae* pv*. oryzae*, streptomycin, ribosomal protein S12 (*rps*L), resistance, functional complementation, molecular diagnosis by PCR-RFLP.

INTRODUCTION

The antibiotic streptomycin was discovered in the 1950s (Sundin and Bender, 1993) and was first used to control bacterial pathogens of human diseases and then used to control bacterial pathogens of plants, including those that cause rice diseases (Xie and Wang, 1991; Zhu et al., 1992). Streptomycin had been used in China for the control of citrus bacterial canker disease (caused by *Xanthomonas campestris* pv. *citri*), bacterial soft rot of Chinese cabbage (caused by *Erwinia carotovora* subsp. *carotovora*), tobacco wildfire (caused by *Pseudomonas syringae* pv. *tabaci*), bacterial blight of rice (caused by *Xanthomonas oryzae* pv. *oryzae*), and leaf streak of rice. The antibiotic has now been used in agriculture for approximately 50 years (Xue, 2002).

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Recently, some streptomycin-resistant plant-pathogenic bacteria were reported in China. Of the 43 field strains of *P.syringae* pv. *tabaci* in Yun-nan province, 25 showed a medium or high-level of resistance to streptomycin (Li, 2007). Three streptomycin-resistance mechanisms have been described in plant-pathogenic bacteria. The first mechanism involves the reduced binding of streptomycin to ribosomes. Streptomycin interacts directly with the 30S ribosome subunit comprising several polypeptides including the ribosomal protein S12 and a 16S rRNA domain, thereby interfering with protein biosynthesis (Finken et al., 1993). Molecular analysis of highly resistant strains of *Erwinia amylovora* and *Mycobacterium tuberculosis* indicated that resistance arose from mutations in the *rps*L gene, which altered the ribosomal protein S12 (Chiou and Jones, 1995; Dobner et al., 1997). The second mechanism involves the *str*A–*str*B genes, which encode two streptomycin-modifying enzymes, aminogly-coside-3-phosphotransferase and aminoglycoside-6-phosphotransferase (Sundin, 2000; Sundin, 2002). The highly conserved *str*A–*str*B genes

have been described in many pathogenic bacteria including *E. amylovora* (Chiou and Jones, 1991; McManus and Jones, 1994), *P. syringae pv. papulans* (Jones and Norelli, 1991), *P. syringae pv. syringae* (Sundin and Bender, 1993), and *Salmonella typhimurium* (Madsen et al., 2000). The third mechanism involves the *aad*A gene, which had also been found to be associated with streptomycin resistance in many clinical bacteria (Poole, 2004; Li and Nikaido, 2004; Weldhagen, 2004) and *S. typhimurium* (Madsen et al., 2000).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) relies on PCR producing considerable amounts of amplicons of known nucleic acid sequence 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 endo-nucleases) recognize specific short palindromic sequences of four to eight nucleotides. They hydrolyze (cleave) the phosphodiester backbone of both DNA strands at these specific recognition sites (Al Dahouk et al., 2005). In this paper, we report the cloning of *rps*L from *X. oryzae pv. oryzae* and demonstrate that expression of a mutant *rps*L allele in a wild-type strain resulted in resistance to streptomycin in *X. oryzae pv. oryzae*.

MATERIALS AND METHODS

Antibiotics, bacterial strains, and culture conditions

Streptomycin sulphate was supplied by Amresco Company (Solon, OH, USA) and was dissolved in ddH_2O to obtain a stock solution (50000 $\mu g \cdot mL^{-1}$). PXO99, a wild-type strain of *X. oryzae* pv. *oryzicola* that is sensitive to streptomycin, was maintained on nutrient agar (NA) at 28°C. The NA contained 5 g polypeptone, 1 g yeast powder, 3 g beef extract, 15 g sucrose, and 17 g agar per litre. The pH was adjusted to 7.0 with 10 M NaOH. Nutrient broth (NB) contained the same ingredients as NA but lacked agar.

Induction of mutants

The "streptomycin-selection" method was performed as described by Wang et al. (2000). PXO99 was grown in 25 ml of NB containing 10 μgml^{-1} streptomycin at 28°C for 24 to 36 h. Then, 100 µl of the bacterial suspension was transferred into another 25 ml volume of NB containing 20 μgml^{-1} streptomycin and grown at 28°C for 24 to 36 h. This process was repeated with NB containing 50, 60, 70, 80, 90, and 100 μgml^{-1} streptomycin. The bacterial culture that could grow at the highest concentration was transferred to NB at the same concentration and grown at 28°C for 24 to 36 h. This process was repeated five times to ensure that the resistance was stable. The sensitivity of the bacterial strains to streptomycin was assayed as described subsequently (Table 1).

The median effective concentration (EC_{50}) of streptomycin

The median effective streptomycin concentration (EC_{50}) for the wild-type isolate and the 11 resistant strains of *X. oryzae pv. oryzae* was determined by bacterial growth inhibition (Li et al., 2006). The bacteria were grown in NB at 28°C to late logarithmic growth phase, and the suspension was diluted to approximately 10^7 CFU ml^{-1}. Aliquots (100 µl) of the suspension were added to 25 ml of NB in 50ml Erlenmeyer flasks containing various concentrations of streptomycin (0, 0.5, 1, 2, 4, and 8 μgml^{-1} for PXO99 and 0, 10, 20, 40, 80, and 100 μgml^{-1} for the mutants), and flasks were placed on an orbital shaker (28°C, 170 rpm). When the concentration of bacterial suspension in the control flask increased to about 10^8 CFU ml^{-1}, the optical densities of the bacterial suspensions in all flasks were measured with a nephelometer (WCY-WOG, Baoli, Beijing, China). Each treatment was repeated three times. Optical density values were regressed on streptomycin concentration, and the regression equation was used to determine the EC_{50}.

The minimum inhibitory concentration (MIC) of streptomycin

MIC was determined by the agar dilution method with some modifications (McManus and Jones, 1994). A bacterial suspension in the late logarithmic growth phase was diluted to approximately 10^8 CFU/ml, and 1.5 µl of the suspension was pipetted onto NA plates containing serial concentrations of streptomycin. For the resistant mutants, the streptomycin concentrations were 50, 100, 200, 400, and 800 μgml^{-1}. For PXO99, the concentrations were 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12, and 10.24 μgml^{-1}. The plates were incubated at 28°C for 72 h before they were examined for bacterial growth. In this MIC method, 1.5 µl of a bacterial suspension in the late logarithmic growth phase was pipetted onto plates without dilution.

Pathogenicity of mutants

Rice (*Oryzae sativa* ssp. indica) cultivar Shanyou63 was grown in the greenhouse and used in the pathogenicity assays. At the maximum tillering stage, three leaves of each of two plants were inoculated with a bacterial suspension of 10^8 CFU/ml of different *X. oryzae* pv.*oryzae* isolates. Inoculation was done by the leaf clipping method described by Kauffman et al. (1973), and the length of lesions on the inoculated leaf was measured after 15 days.

DNA isolation

DNA was isolated from the wild-type isolate and resistant strains as described by Ausubel et al. (1987). After the bacteria were grown in NB at 28°C for 24 h, 1.5 ml volume of bacterial suspension was centrifuged at 12 000 × *g* for 10 min. The pellet was resuspended in 567 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Proteinase K and sodium dodecyl sulfate (SDS) were added to final concentrations of 100 μgml^{-1} and 5 gL^{-1}, respectively. After incubation for 1 h at 37°C, sodium chloride and hexadecyl-trimethylammonium bromide (CTAB) were added to each preparation to final concentrations of 0.7 M and 10 gL^{-1}, 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 earlier. DNA was precipitated by adding a 0.6 volume of isopropanol and incubating at –20°C for 30 min. Samples were centrifuged at 12000 × *g* for 20 min, and the pellets were washed with 1 ml of 70% ethanol and centrifuged again. The DNA was dried at room temperature, and the pellet was then redissolved in 50 µl of sterilized ultrapure water and stored at –20°C.

PCR amplification and sequencing

A pair of oligonucleotide primers was designed (5'CGGACGAGG

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant genotype or characteristic	*rps*L PCR-RFLP *Mob II* pattern[a]
X. oryzae* pv. *oryzae		
YJ-p-1	Mutant derived from PXO99; Resistant to streptomycin	B
YJ-p-2	Mutant derived from PXO99; Resistant to streptomycin	B
YJ-p-3	Mutant derived from PXO99; Resistant to streptomycin	A
YJ-p-4	Mutant derived from PXO99; Resistant to streptomycin	A
YJ-p-5	Mutant derived from PXO99; Resistant to streptomycin	B
YJ-p-6	Mutant derived from PXO99; Resistant to streptomycin	B
YJ-p-7	Mutant derived from PXO99; Resistant to streptomycin	A
YJ-p-8	Mutant derived from PXO99; Resistant to streptomycin	B
YJ-p-9	Mutant derived from PXO99; Resistant to streptomycin	A
YJ-p-10	Mutant derived from PXO99; Resistant to streptomycin	B
YJ-p-11	Mutant derived from PXO99; Resistant to streptomycin	B
PXO99	Laboratory wild-type; sensitive to streptomycin	B
PS1	PXO99 with pUFRPS; resistant to streptomycin; Km^r	
PS2	PXO99 with pUFRPX; resistant to streptomycin; Km^r	
Plasmids		
pUFR034	IncW, Km^r, Mob^+, mob (p) $LacZa^+$, Par^+, cos	
pUFRPS	pUFR034 with an inserted 700-bp PCR fragment containing a mutation at codon 88 of *rps*L this study.	
pUFRPX	pUFR034 with an inserted 700-bp PCR fragment containing a mutation at codon 43 of *rps*L.	

[a] See Figure 3 for restriction patterns A and B.

AGTAAGCG3' and ATGAAGCGGGCAATGGT3') to amplify the *rps*L gene from *X. oryzae pv. oryzae.* DNA was amplified in a total reaction volume of 25 µl on an automatic thermal controller. The reaction mixture contained 2.5 µl of 10× buffer, 2 µl of dNTP, 1.5 µl of $MgCl_2$, 20 pmol of each primer, and 2.5 U of *Taq* polymerase. The PCR procedures were as follows: pre-denatuation at 94°C for 5 min, followed by 30 cycles at 94°C for 40 s, 63°C for 100 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. Amplified DNAs were detected by electrophoresis in 1.5% agarose gels. PCR products were recovered and sequenced by Liangzhong Bioengineering Company, Ltd. (Shanghai, China). Primers were synthesized by Shanghai Bioengineering Company (Shanghai, China). The sequence of *rps*L was obtained using the ORF Finder program.

Construction of recombinant plasmid

A pair of primers (rpsLF:5'CCTGAATTCGGACGTCGCAAGGCCT CGGGTC3' and rpsLR:5'GTCGAATTCTTAGCTCTTCGGGCGCT TGGC3') was designed to amplify *rps*L from strains YJ-P-1 and YJ-P-3. DNA was amplified in a total reaction volume of 25 µl on an automatic thermal controller. The reaction mixture contained 2.5 µl of 10× buffer, 2 µl of dNTP, 1.5 µl of $MgCl_2$, 20 pmol of each primer, and 2.5 U of *Taq* polymerase. The PCR procedure was as follows: Pre-denatuation at 94°C for 5 min; followed by 30 cycles at 94°C for 40 s, 65°C for 100 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR product was purified as described earlier. The PCR product was digested with *EcoR1* and then inserted into the *EcoR1* site of the vector pUFR034 (DeFeyter et al., 1990). The recombinant plasmid pUFRPS contained the Lys→Arg at codon 88 of *rps*L gene. pUFRPX contained the Lys→Arg at codon 43 of *rps*L gene.

Electroporation

PXO99 was transformed by electroporation (Mathre, 1980). The electroporation procedure was adapted for the transformation of *X. oryzae pv. oryzae* with the following modifications to optimize transformation. Cells were grown in NB at 28°C to late log phase and harvested by centrifugation (3000 x *g*, 5 min, 4°C). Pelleted cells were re-suspended and washed three times with ice-cold sterile 10% glycerol and finally re-suspended in 10% glycerol to a cell density of 10^{10} CFU ml^{-1}. The cell preparations were stored at -70°C. Electroporation was performed on 100 µl of cell suspensions in cuvettes and with a BioRad Gene Pulser (BioRad, California, USA) set at 2.5 kV, 200 Ω, and 25 µF. The time constants observed for electroporation ranged between 4.8 and 5.2 ms. Transformation efficiency was calculated as the number of transformants per µg of cosmid DNA from pUFR034. We routinely obtained 7×10^5 transformants per µg of DNA with this procedure.

RESULTS

Resistant mutants

11 streptomycin-resistant mutants (YJ-p-1, YJ-p-2, YJ-p-

Table 2. Sensitivity of resistant mutants and their parent strain of *X. oryzae pv.* oryzae to streptomycin.

Isolate or strain	EC_{50} (µg ml^{-1})	MIC (µg ml^{-1})	Relative resistance index[z]	Lesion length (cm)
PXO99	0.08	0.22		12.8ab [s]
YJ-p-1	18.64	>600	233.00	10.9b
YJ-p-2	25.40	>600	317.50	9.8b
YJ-p-3	22.16	>600	277.00	14.0a
YJ-p-4	19.11	>600	238.88	13.2ab
YJ-p-5	6.22	>600	77.75	9.9b
YJ-p-6	21.62	>600	270.25	12.4ab
YJ-p-7	8.98	>600	112.25	14.3a
YJ-p-8	6.01	>600	75.13	12.5ab
YJ-p-9	17.55	>600	219.38	10.1b
YJ-p-10	18.13	>600	226.63	9.6b
YJ-p-11	13.14	>600	164.25	11.8b

[z]EC_{50} of the resistant strain/EC_{50} of the wild type; [s]values in columns followed by similar letters were not significantly different according to Fisher's protected LSD test (p=0.05).

3, YJ-p-4, YJ-p-5, YJ-p-6, YJ-p-7, YJ-p-8, YJ-p-9, YJ-p-10 and YJ-p-11) were obtained by streptomycin selection. All mutants exhibited a high level of streptomycin resistance (Table 2). Unlike the wild-type isolate PXO99, these mutants could grow on NA containing 100 µgml^{-1} streptomycin. The MIC values of streptomycin for all resistant strains exceeded 600 µgml^{-1}, while the MIC value for PXO99 was 0.22 µgml^{-1}. The EC_{50} values of the resistant strains on NA ranged from 6.01 to 25.40 µgml^{-1} (Table 2), while the EC_{50} value of PXO99 was 0.08 µgml^{-1}. All the mutants exhibited the highest resistance to streptomycin with relative resistance index of 75.13 to 277.00. The lesion length produced by these mutants was not significantly reduced.

PCR amplification of *rps*L in *X. oryzae* pv. *oryzae*

PCR with primer pair of rpsLF and rpsLR generated a fragment that was ligated into pGEM-T and subsequently sequenced. An ORF of 375 bp was obtained with initiation codon ATG and termination codon TGA, and with a G+C of 61.36%. Blasting of the NCBI database indicated that the ORF had 97% similarity in nucleotide sequence and 100% similarity in deduced amino acid sequence with *X. oryzae* pv. *oryzicola* strain BLS256.

Mutation in *rps*L

By sequence comparison of PXO99 and resistant mutants, a mutation of Lys (AAG) to Arg (AGG) at the 43rd or 88th amino acid residue was found in all the streptomycin-resistant mutants (Table 3). Streptomycin-resistant mutants with the two different mutation types had the same MIC levels (Table 2). These results suggest that the two mutations in *rps*L could be involved in streptomycin resistance in *X. oryzae* pv. *oryzae* as previously reported for *M. tuberculosis* (Dobner et al., 1997).

Construction of the recombinant cosmids pUFRPS and pUFRPX

To verify that the sequence described previously was indeed conferred the resistance to streptomycin, complementation assays were performed. An intact *rps*L fragment (about 700 bp) containing the promoter was amplified by primer pair rpsLF and rpsLR from YJ-P-1 and YJ-P-3. The obtained *rps*L fragment was ligated into pUFR034 and introduced into PXO99 by electroporation, and the recombined cosmids pUFRPS (containing a mutation at codon 88 of *rps*L) and pUFRPX (containing a mutation at codon 43 of *rps*L) were screened. Out of 165 transformants that were stable in the absence of kanamycin selection, 64 transformants were identical to the parental wild-type strain PXO99 in their growth and colony characteristics in culture except that they could grow on NA supplemented with >50 µgml^{-1} of streptomycin (Figure 1). The results confirm that plasmids pUFRPS and pUFRPX complemented PXO99 for resistance to streptomycin, and that the single amino acid substitution in *rps*L at codon 43 or 88 confers streptomycin resistance to *X. oryzae* pv. *oryzae*.

RFLP analyses

The rpsL codon 43 in *X. oryzae* pv. oryzae has a *Mbo*II restriction enzyme site. Mutation at codon 43 of rpsL in streptomycin-resistant strains will cause loss of the *Mbo*II site. Results of the RFLP analyses of the rpsL genes are shown in Figures 2 and 3. Two RFLP patterns were

Table 3. Mutations in the deduced amino acid sequence of *rps*L in PXO99 and resistant mutants of *X. oryzae* pv. *Oryzae*.

Strain	Mutated site	Codon	Amino acid	Phenotype
PXO99	---	AAG	Lys	S
YJ -p-1	88	AGG	Arg	R
YJ -p-2	88	AGG	Arg	R
YJ -p-3	43	AGG	Arg	R
YJ -p-4	43	AGG	Arg	R
YJ -p-5	88	AGG	Arg	R
YJ-p-1	88	AGG	Arg	R
YJ-p-2	43	AGG	Arg	R
YJ-p-3	88	AGG	Arg	R
YJ-p-4	43	AGG	Arg	R
YJ-p-5	88	AGG	Arg	R
YJ-p-6	88	AGG	Arg	R

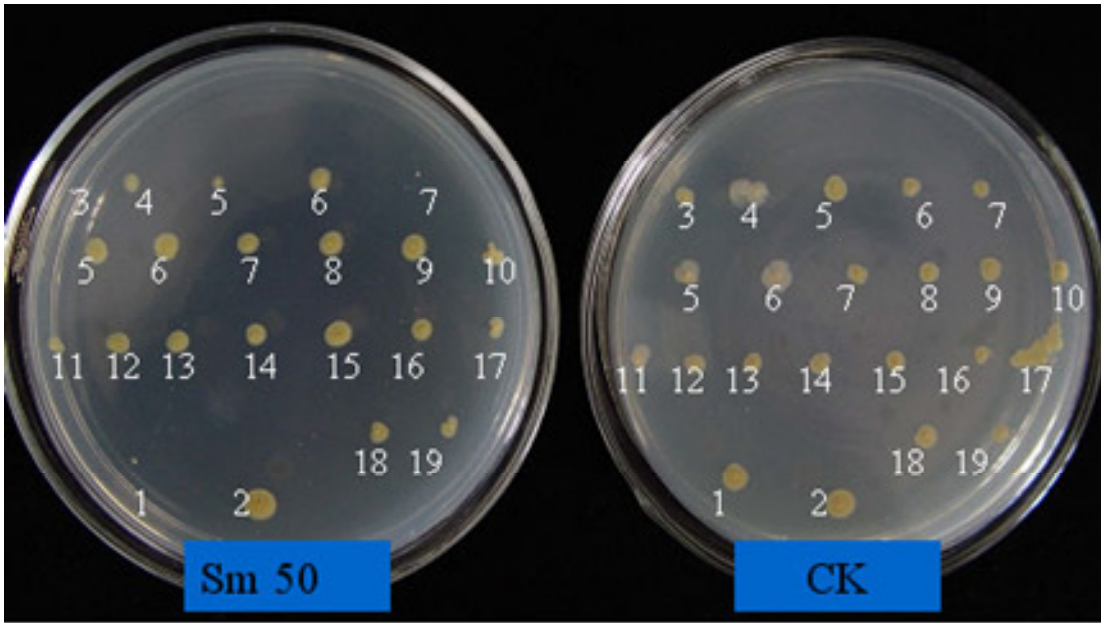

Figure 1. Growth of transformants and wild-type strain PXO99 on a medium (NA) without streptomycin (CK) or with 50 μgml^{-1} of streptomycin (Sm 50). 1, PXO99 (wild-type strain); 2:, YJ-P-1 (streptomycin-resistant strain); 3-10, PS1 (transformants containing pUFRPS); 11-19, PS2 (transformants containing pUFRPX).

Figure 2. Diagram of the portion of genomic DNA amplified by the primers *rps*LnF and *rps*LnR and then 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).

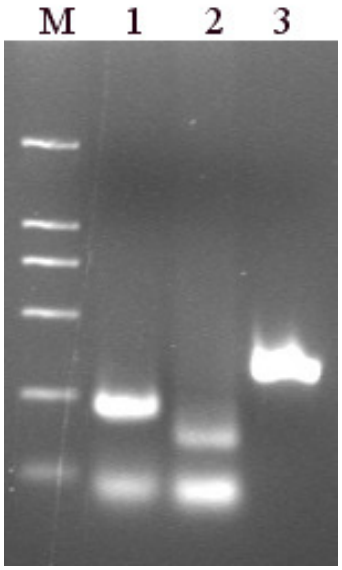

Figure 3. MboII restriction patterns of the PCR-amplified rpsL genes. Lane 1, digested PCR product with MboII (pattern A in Table 1); Lane 2, digested PCR product with MboII (pattern B in Table 1); Lane 3, undigested PCR product; M, DL2000.

observed. Pattern A suggested that the 304 bp product was digested by *Mbo*I into two fragments (201 and 103 bp) if a mutation at codon 43 existed. Pattern B showed that the 304 bp fragment was digested into three fragments (146, 103 and 55 bp) if no mutation occurred at codon 43. A 55 bp fragment was so short that it frequently went out in 2% agarose gels (Figures 2 and 3). In the 11 streptomycin–resistant strains of *X. oryzae* pv*. oryzae*, four strains belonged to Pattern A, and seven strains belonged to Pattern B. Streptomycin-sensitive strain PXO99 belonged to Pattern B (Table 2 and Figure 3).

DISCUSSION

Streptomycin, an aminocyclitol glycoside antibiotic, is commonly used as a first-line treatment against tuberculosis. Its mode of action is presumably the inhibition of protein synthesis resulting from interference with translational proofreading. More specifically, streptomycin binds to the 30S ribosomal subunit, thereby interfering with polypeptide synthesis and thus inhibiting translation (Sundin, 2002). Previous research showed that chromosomally acquired resistance to streptomycin was frequently 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 *str*A–*str*B genes (Sundin, 2000, 2002) and *aad*A gene (Poole, 2004; Li and Nikaido, 2004; Weldhagen, 2004) are also involved in streptomycin resistance. In this research, 11 streptomycin-resistant mutants were obtained by induction in laboratory. The EC_{50} and MIC values of the mutants were much higher than those of the wild-type strain PXO99, and the resistant index of the mutants was as high as 277. Interestingly, these resistant mutants did not exhibit reduced pathogenicity, suggesting no relationship between the resistance and pathogenicity.

In this study, the mechanism of the streptomycin resistance in *X. oryzae* pv. *oryzae* was associated with *rps*L gene. By sequence comparison of PXO99 and resistant mutants, it showed that the resistance was associated with a point mutation in this gene resulting in substitution of Arg by Lys at codon 43 or 88. The same point mutations were reported for streptomycin-resistant strains of *E. amylovora* (Chiou and Jones, 1995), *M. tuberculosis* (Dobner et al., 1997), and *Thermus thermophilus* (Gregory et al., 2001).

In a previous study of field isolates of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in southern China (Xu et al., 2010), a high level of streptomycin resistance was detected in 0.75% of the isolates. In that study, no point mutations in *rps*L and *rrs* genes were detected, indicating that streptomycin resistance in the field might be caused by other mechanisms than those detected in this study of laboratory-induced streptomycin resistant strains of *X. oryzae* pv. *oryzicola*. The transformation experiments in this study confirmed that the single mutation in the *rps*L gene was responsible for the high level of resistance to streptomycin in *X. oryzae* pv. *oryzae*. Moreover, our results showed that the streptomycin resistance was generated by streptomycin selection, due to a point mutation in the *rps*L gene at codon 43 or 88. Obtaining a better understanding of streptomycin resistance in *X. oryzae* pv. *oryae* will require the study of other molecular changes that might cause streptomycin resistance in this bacterium. Work to monitor the field resistance to streptomycin in *X. oryzae* pv. *oryae* is still on in our laboratory.

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