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Restriction-modification system and genetic variability of *Xanthomonas oryzae* pv. *oryzae*

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Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* is one of the most important diseases of rice. Host resistance, which relies on single, dominant resistance genes, is the only reliable method to control the disease at present. Pathogenic variation of the bacteria has been shown to follow the deployment of resistance genes in commercial cultivars. Information on the factors and the mechanisms for genetic variation of this pathogen is limited. Further, we have no clear evidence of whether population variability is due to sexual recombination or to variation introduced by mutations or intragenic recombination in a clonally maintained population.

The gene encoding the *Xor*II methylase (M·*Xor*II) was cloned from *Xanthomonas oryzae* pv. *oryzae* and characterized in *E. coli*. The M·*Xor*II activity was located on a 3.1 kb BamHI-BstXI fragment which contained two open reading frames (ORFs) of 1272 nucleotides (424 amino acids) and 408 nucleotides (136 amino acids)(Fig. 1). Ten polypeptide domains conserved in other ^{m5}cytosine methylases were identified in the deduced amino acid sequence of the 1272 ORF. Plasmid DNA isolated from *E. coli* harboring the *Xor*II methylase gene was not protected completely from digestion by R·*Xor*II or the isoschizomer R·*Pvu*I until the *Xor*II methylase gene was placed under the control of the *lac*Z promoter of the cloning vector. *E. coli* Mrr$^+$ strains were transformed poorly by plasmids containing the *Xor*II methylase gene, indicating the presence of at least one ^{m}CG in the recognition sequence for M·*Xor*II (CGATCG). The 408 nucleotide ORF was 36% identical at the amino acid level to sequences of the *E. coli dcm vsr* gene, which is required for very short patch repair. *X. oryzae* pv. *oryzae* genomic DNA that is resistant to digestion by *Pvu*I and *Xor*II hybridizes with a 7.0 kb fragment containing the *Xor*II methylase gene and vsr homolog, whereas DNA from strains that lack M·*Xor*II activity do not hybridize with the fragment. The *Xor*II methylase

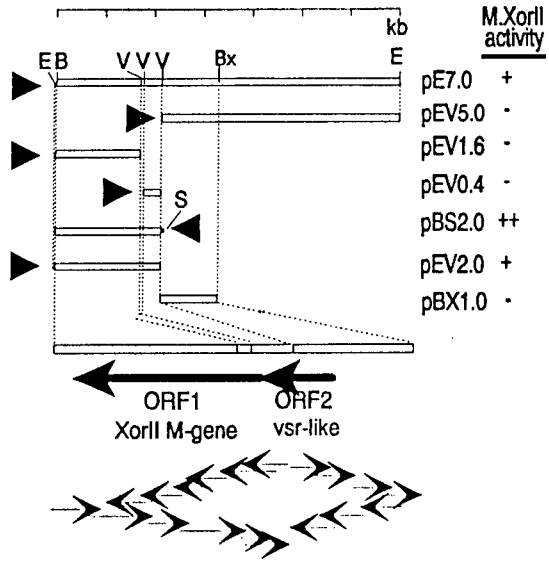

Figure 1. Restriction map of the pE7.0 and DNA sequencing strategy for the *xor* II M. the open box is the cloned *X. oryzae* pv. *oryzae* fragment, and the solid box at the end of each clone are vector pUFRO27 or pBluescript SK^+ sequences. The solid triangle shows the position and orientation of the *lac* Z promoter of pBluescript II. The tailed arrows show the fragment sequenced using T3, T7 or synthetic oligonucleotide primers. "+" or "++" indicate partial or complete resistance of plasmid DNA against digestion with *Pvu* I. "−" indicates digestion with *Pvu* I. E, *Eco*RI; V, *Eco*RV; B, *Bam*HI; S, *Sal* I; and Bx, *Bst*XI.

gene encodes a ^{m5}cytosine methylase based on amino acid sequence similarity to^{m5} cytosine methylases identified from other bacteria. Such a high degree of similarity indicates the gene was introduced to *X. oryzae* pv. *oryzae* from other prokaryotes through horizontal gene transfer mediated by either sexual conjugation, transformation, or phage-mediated transduction. The high similarity of the very short patch repair endonuclease gene (*vsr*) in amino acid sequence to other *vsr* genes found in different bacteria such as *E. coli, Bacillus subtilis, Haemophilus,* and *Arthrobacter* supports the idea of horizontal transfer of this gene to *X. oryzae* pv. *oryzae*.

The presence or absence of two DNA restriction modification (R-M) systems, *Xor*I and *Xor*II, was assessed in 184 strains of *X. oryzae* pv. *oryzae* collected from different major rice growing countries in Asia. All four possible phenotypes ($Xor I^+/Xor II^+$, $Xor I^+/Xor II^-$, $Xor I^-/Xor II^+$ and $Xor I^-/Xor II^-$) were detected in the population in a ratio of 1:2:2:2. The $Xor I^+/Xor II^+$ and $Xor I^-/Xor II^+$ phenotypes were predominantly observed in strains from southeast Asia (Philippines, Malaysia, and Indonesia), whereas strains with the phenotypes $Xor I^-/Xor II^-$ and $Xor I^+/Xor II^-$ were distributed in south (India and Nepal) and northeast Asia (China, Korea,

Japan), respectively. Based on the geographic distribution of the *Xor*I and *Xor*II R-M systems, we suggest that the *Xor*I modification system originated in northeast Asia and was later introduced to southeast Asia, while the *Xor*II system originated in southeast Asia and was moved from there to northeast and south Asia. Genetic diversity within each phenotypic grouping was high, and ranged from 0.88 for the *Xor*I$^+$/*Xor*II$^+$ group to 0.95 for the *Xor*I$^-$/*Xor*II$^-$ group. Genomic DNA from strains of *X. oryzae* pv. *oryzae* that were resistant to digestion with endonuclease *Xor*II also hybridized with a 7.0 kb clone that contained the *Xor*II R-M system, whereas strains which were digested by *Xor*II did not hybridize with the clone. Size polymorphisms were observed in fragments that hybridized with the 7.0 kb clone. DNA of most *X. oryzae* pv. *oryzae* strains from the Philippines, and all strains from Indonesia and Korea had identical hybridization patterns. Generally, a single RFLP pattern in *Xor*IIM$^+$ strains was found within a country, which indicates clonal maintenance of the *Xor*II R-M locus(Fig. 2). DNA of bacteriophage isolated from *Xor*II$^+$ *X. oryzae* pv. *oryzae* strains did not hybridize with the *Xor*II R-M clone. DNA from other species of Xanthomonas did not contain the *Xor*II modification genes, suggesting the system originated after xanthomonads had differentiated

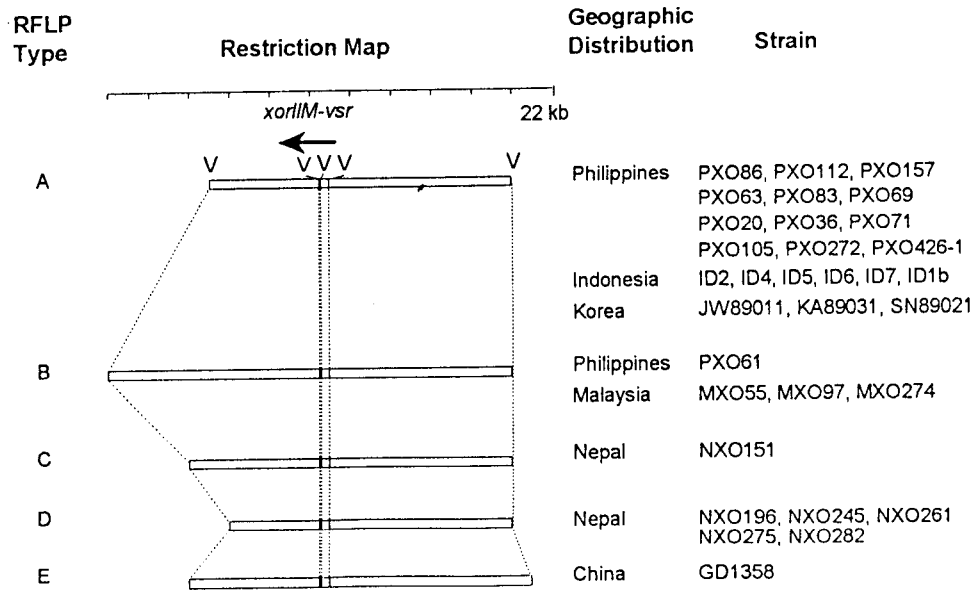

Figure 2. Map of randomly selected *Xor*IIM$^+$ *X. oryzae* pv. *oryzae* strains from different countries based on restriction fragment length polymorphism analysis. Fragment size differences of less than 0.5 kb in regions flanking the *Xor*II R−M genes were seen in some strains grouped in RFLP type D. *xor*IIM, *Xor*II methylase gene; vsr, very short patch repair endonuclease gene. V, *Eco*RV.

into species and pathovars. The absence of *Xor*I or *Xor*II systems in many strains could suggest that the evolution might be a fairly recent event. However, the presence of subpopulations with different R-M systems within one community and the existence of the same R-M systems in different countries suggest that the evolution of the R-M systems might occur long ago.

Restriction fragment length polymorphism (RFLP) data produced by analysis with a cloned avirulence gene and other repetitive elements from *X. oryzae* pv. *oryzae* have shown evidence of a strong selection effect of host resistance on genetic variation of this pathogen. Since the transformation and conjugation frequencies were affected by the R-M systems, effect of the R-M systems on genetic variation of the multilocus markers was anticipated. However, significant effects of R-M systems on genetic diversity were not observed, probably due to the strong selection pressure of host resistance and the highly variable characters of the multilocus markers. Because identical RFLP patterns in the genome around the *Xor*II R-M genes were observed in geographically different strains, we suggest that the R-M systems are clonally maintained and fairly stable. Classification of the *X. oryzae* pv. *oryzae* into biological groups based on R-M systems may increase the clarity of genetic variation illustrated by multilocus markers. This might also elucidate the effect of R-M systems on genetic variation.

X. oryzae pv. *oryzae* is a pathogen of such importance that it is on the quarrantine lists of most rice-producing countries. Based on extensive germ plasm exchange between countries, geographic movement of the pathogen is likely to occur. Recently, movement of this pathogen by man was suggested from lineage analysis based on the RFLP produced by multicopy markers. However, we do not know when and how the pathogen moved. The distribution of *Xor*II modification system in different countries with identical RFLP pattern around the *Xor*II R-M locus, which was found in strains collected during decades, clearly indicates geographic movement of this pathogen and stable maintenance of the *Xor*II R-M locus. The presence of different RFLP patterns found in strains collected from some countries was assumed to be from mutational or intra-recombinational event, which are followed by build-up of the mutant population in the community. If this is true, it seems to be time consuming process and therefore, suggests that geographic migration might have occurred long time ago. Although we do not have data on the mechanism of movement from the origin, one possible way for the geographic movement might have been through gradual natural infection, since until recently long distance germ plasm exchange did not occur. Certain strains, for example, those of the Philippine race 6, however, seem to have migrated fairly recently. Ex-

tinction of that population from the new habitat (the Philippines) is very interesting and it may have been due to infection by virulent phage in the habitat. The role of bacteriophage in the evolution of this pathogen is not known.