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Avirulence gene and insertion element-based RFLP as well as RAPD markers reveal high levels of genomic polymorphism in the rice pathogen *Xanthomonas oryzae* pv. *oryzae*

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Abstract

Genetic polymorphism within the genomes of bacterial pathogens determines their evolutionary potential during long-term interaction with their hosts. To investigate the level of genetic variation in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causative agent of rice bacterial blight disease, three DNA marker systems, including (i) restriction fragment length polymorphism (RFLP) of the *avrBs3/PthA* family genes (*avrXa27*), (ii) RFLP of insertion (IS) elements and (iii) random amplified polymorphic DNA (RAPD) markers, were used to detect polymorphism among 32 *Xoo* strains that differed in their virulence patterns. All these strains contained multiple *avrXa27* homologs that were variable in copy number and genomic location. RFLP of six IS elements revealed that these mobile sequences were abundant in *Xoo* genomes, with 150 of the total of 165 discernable markers being variable. Thirty-eight decamer primers of RAPD amplified a total of 691 bands, with 100% of them being variable. In addition, analysis of molecular variance (AMOVA) of data from RFLP analysis of IS elements and from RAPD analysis showed that most of the genetic variation residues were within *Xoo* populations, rather than between populations. Although all three DNA marker systems supported that substantial variation was maintained in *Xoo* genomes, Mantel tests did not identify significant correlation between the similarity coefficients calculated from them. The results of the present study indicated that *Xoo* genomes contain a high level of genetic polymorphism, which greatly facilitates the evolution of this important pathogen during interaction with its host rice plant.

Keywords: *Xanthomonas oryzae* pv. *oryzae*; Genetic polymorphism; RFLP; *AvrBs3/PthA* gene family; Insertion element; RAPD

Introduction

Bacterial pathogens and their hosts have co-evolved a relationship of reciprocal, "arms race"-like adaptation that has been observed in natural and experimental

Abbreviations: Xoo, *Xanthomonas oryzae* pv. *oryzae*; PVB, percentage of variable bands

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populations [7,8]. During this process, maintaining a high level of genomic polymorphism provides a solid genetic basis for bacterial pathogens to develop virulent capability to overcome host defense systems more efficiently, or to establish successful infection in novel hosts [40]. Many genetic factors, including accumulation of point mutations, gene insertion/deletion, fission/fusion, duplication and large-scale genomic rearrangement, contribute to high levels of genomic plasticity among bacterial populations [6,18,33,48]. In particular, horizontal gene transfer, a process through which genomes acquire novel DNA segments from distantly related organisms by transformation, conjugation or transduction, is a dominant genetic process that increases bacterial genetic variability [6,41]. Therefore, investigating the level of genomic polymorphism is a prerequisite to help us understand the evolutionary mechanism and potential of pathogenic bacteria [24].

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is a rod-shaped, obligately aerobic, Gram-negative bacterium. It is the causal agent of bacterial blight, the most destructive bacterial disease of rice (*Oryza sativa*). *Xoo* can infect rice by entering plant tissues through wounds, hydathodes, stomata or contaminated seeds, causing disease symptoms of water soaked to yellowish stripes on leaf blades, and eventually resulting in death of the infected leaves [30]. This disease was first reported in Japan in 1884 and it has now been identified in all rice-growing regions worldwide, causing 20–30% agricultural yield loss every year [30]. To prevent this disease, the most effective and economical strategy is breeding disease-resistant rice cultivars [30]. However, in agricultural practice, the ability of rice cultivars to resist infection is always broken down within a few years, which is usually caused by the rapid evolution of *Xoo* [39]. For example, in China, a survey on *Xoo* pathotypes revealed that along with extensive application of hybrid rice cultivars containing resistance genes *Xa3* and *Xa4*, the percentage of sampled *Xoo* strains that can overcome resistance of these cultivars has quickly increased from 40% to 65% within several years [45]. Consequently, it has been predicted that *Xoo* strains maintain a high level of genetic polymorphism that facilitates their rapid evolution.

In recent years, several studies have used molecular fingerprinting to detect genetic variation in *Xoo* strains [3,19,29]. However, most of these studies were limited to using only a few DNA markers to screen *Xoo* genomes, which can result in a biased estimation of the overall level of genetic diversity. Recently, complete genomic sequences of two *Xoo* strains (KACC10331 and MAFF 311018) have been deposited in public databases [20,31] and another (PXO99) is almost completely sequenced (our unpublished data). Availability of these genomic sequences greatly facilitates the selection of appropriate DNA markers that give unbiased estimates of genomic polymorphism.

Using this new genomic information, we analyzed the variability of 32 *Xoo* strains with three multi-locus methods of DNA fingerprinting, including restriction fragment length polymorphism (RFLP) analysis of the *avrBs3* /*PthA*-family avirulence gene *avrXa27*, RFLP analysis of insertional sequence (IS) elements and random amplified polymorphic DNA (RAPD). The *Xoo* strains were collected from the major rice distribution area, especially from Asia (Table 1). The first DNA markers, homologs of *avrBs3*/*PthA*, represent a dominant avirulence gene family encoded by *Xanthomonas*. Protein products of these genes play important roles in pathogenicity, because they are usually injected into host cells by the bacterial type III secretion system and function as effectors to suppress the host's innate immunity system [13]. Genes belonging to the *avrBs3*/*PthA* family contain a characteristic 102-bp sequence which is often repeated approximately 5.5–28.5 times [47]. Consequently, these genes can be used as specific multi-locus DNA markers to check genetic polymorphism of avirulence genes. The second DNA markers, IS elements, are mobile DNA sequences carrying only transposition-related genes, with a length of 0.7–2 kb. Bacterial genomes usually contain multiple copies of IS elements which belong to different families. These IS elements have been used as DNA markers to study the genetic variation, genomic rearrangement and genome plasticity of bacteria [22]. The third DNA marker, RAPD, is a PCR-based technique that uses single primers with an arbitrary nucleotide sequence to amplify anonymous PCR fragments from genomic template DNA [43]. However, although this technique is sensitive to reaction conditions, once stable conditions are established in the laboratory, RAPD has several advantages. These include the relatively unbiased sampling of the targeted genomes, the simultaneous generation of a large number of markers, its simplicity of use, and the requirement for only a small amount of material [5]. The present study aimed to assess genetic polymorphism in the genomes of *Xoo* using these three genetic marker systems, which will give insight into understanding the rapid evolution of this important bacterial pathogen.

Materials and methods

Bacterial strains, culture media and isolation of genomic DNA

Thirty-two *Xoo* strains collected from nine major rice-growing countries, mainly from Asia, were used in this study (Table 1). For DNA extraction, *Xoo* strains were grown overnight on a rotary shaker in PS medium