Diverse Members of the AvrBs3/PthA Family of Type III Effectors Are Major Virulence Determinants in Bacterial Blight Disease of Rice

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AvrXa7 is a member of the *avrBs3/pthA* gene family and the only known type III secretion system effector gene from *Xanthomonas oryzae* pv. *oryzae* with a major contribution to bacterial growth and lesion formation in bacterial blight disease of rice. We examined the general requirement for effectors of the AvrBs3/PthA family in bacterial blight of rice by identifying effectors from diverse strains of the pathogen. Inactivation of single effector genes in representative strains from Japan, Korea, and the Philippines resulted in severely limited growth in plants. Five strains harbored one gene of the *avrBs3/pthA* family, while one strain had two genes with the equivalent virulence activity of *avrXa7*. Sequence analysis revealed three genes with unique repeat arrangements in comparison to *avrXa7*. Comparison of the repetitive regions revealed a potential motif for the group that was also present in the repetitive region of *avrBs3*. However, the repetitive region of *avrBs3* could not support virulence activity but, in combination with the C-terminal coding region of *avrXa7*, triggered a *Xa7*-dependent avirulence reaction. The results revealed diverse members of the *avrBs3/pthA* gene family with virulence activity in *X. oryzae* pv. *oryzae* and supported the hypothesis that bacterial blight disease of rice is highly dependent on a single class of type III effectors. The results also indicated that *avrXa7* avirulence specificity is separable from virulence activity.

Many gram-negative pathogenic bacteria are highly dependent on the type III secretion systems (TTSS) for virulence (Staskawicz et al. 2001). The TTSS pathways mediate the secretion of proteins (substrate virulence effectors) into the extracellular milieu and into the host cells themselves, wherein the effectors facilitate virulence (Buttner et al. 2002). Ablation of the TTSS in plant pathogen systems often results in the loss of pathogenesis or severe reduction of virulence. Substrate effectors of individual strains, on the other hand, are highly variable (Deng et al. 2003; Guttman et al. 2002; Salanoubat et al. 2002). Loss of individual effector genes often results in modest or no observable effects on virulence (White et al. 2000). The apparent modest role of individual effectors may be attributable to multiple factors, including effector redundancy, the incremental requirements for multiple effectors, or inappropriate testing environment. Nevertheless, type III effectors have a critical function in the ecology of the disease-causing species.

AvrBs2 was the first identified bacterial TTSS effector with virulence and avirulence activities and remains an important example of TTSS effector function (Kearney and Staskawicz 1990). The protein has avirulence activity when the strain harboring the gene for AvrBs2 is inoculated on pepper plants containing the *Bs2* gene for resistance. Loss of *avrBs2* in *Xanthomonas campestris* pv. *vesicatoria* results in an approximate fivefold loss in bacterial growth in the pepper plants (Gassmann et al. 2000). Genes for AvrBs2 homologs are present in many species and pathovars of *Xanthomonas*, suggesting that AvrBs2 has an important function in the general physiology of the various pathogens. A variety of type III effector genes have since been shown associated with virulence in plant pathogenic bacteria (Chang et al. 2004).

A few individual effector genes have dramatic effects on the virulence of the strains of phytopathogenic bacteria that harbor them. Principle among these are *pthA* (pathogenicity A) of *Xanthomonas axonopodis* pv. *citri* and *avrXa7* of *X. oryzae* pv. *oryzae*, both of which are required for full virulence of the respective strains on their host plants (Bai et al. 2000; Swarup et al. 1991; Yang et al. 2000). Loss of either *pthA* or *avrXa7* from their respective strains resulted in severe reductions in symptoms and the ability of the pathogen to multiply within the host tissue. Related genes in *X. campestris* pv. *malvacearum* have been shown to have a cumulative yet similar impact in strain XcmH1005, where sequential elimination of multiple copies of *avrBs3/pthA* genes was required before significant loss of virulence occurs (Yang et al. 1996). Additional copies of the family in *X. oryzae* pv. *oryzae* are also known to have additional yet minor contributions to virulence in strain PXO86, the original source of *avrXa7* (Bai et al. 2000).

PthA, *avrb6*, and *avrXa7* are members of the *avrBs3/pthA* gene family, the members of which are found in many *Xanthomonas* species and, recently, within the genome of *Ralstonia solanacearum* GMI1000 (Bonas et al. 1989; Salanoubat et al. 2002). Members of the *avrBs3/pthA* family are remarkably similar, differing primarily in the number and apparent nature of near-identical 102-base-pair direct repeats in the central portion of the coding sequence, which is referred to here as the repetitive region (White et al. 2000). The repetitive regions contribute to function and specificity during the elicitation of resistance and, in the absence of resistance, virulence on the respective host species (Herbers et al. 1992; Yang et al. 1994).

X. oryzae pv. *oryzae* is the causal organism of bacterial blight of rice. Evidence from field studies on strains with *Xa7*-dependent avirulence activity indicated that the predominant strains also have a dependency on *avrXa7* for virulence (Vera Cruz et al. 2000). At the same time, many strains of *X. oryzae*

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pv. *oryzae* have no *Xa7*-mediated avirulence activity, and the prevalence of related virulence effectors within *X. oryzae* pv. *oryzae* populations is unknown. Strains without *Xa7*-dependent avirulence activity may suppress the activity due to *avrXa7*, have another virulence gene of the same family without *Xa7*-mediated avirulence activity, or rely on unrelated effectors. We, therefore, examined the prevalence of *avrXa7* and related genes in the ability of diverse strains of *X. oryzae* pv. *oryzae* to cause disease. *X. oryzae* pv. *oryzicola*, which is a closely related pathogen and causes a nonvascular disease of rice called bacterial leaf streak, could possibly exchange effector genes with *X. oryzae* pv. *oryzae* in nature and represents a potential source of virulence effector genes. Therefore, an attempt was also made to identify virulence effectors from strain BLS303 of *X. oryzae* pv. *oryzicola* by complementation of a low virulence mutant of *X. oryzae* pv. *oryzae*.

RESULTS

Homologs of *avrXa7* are critical virulence factors in diverse strains of *X. oryzae* pv. *oryzae*.

Two approaches were taken to determine whether strains of *X. oryzae* pv. *oryzae* possessed additional virulence effectors

Fig. 1. Map of *avrXa7*. The gene is 4,341 bp in length. Functional regions indicated are the three nuclear localization signal motifs (NLS1, 2, 3) and an acidic activation domain (AD) in the C-terminal coding region. The EZ<KAN2> insertion used in mutagenesis is indicated above repetitive region. Code for restriction enzyme cleavage sites: B = *Bam*HI; P = *Pst*I; S = *Sph*I.

of the *AvrBs3/PthA* family. The first approach generated mutations in *avrBs3/pthA*-related genes in selected strains by homologous recombination or so-called knock-out mutagenesis, using a mutant copy of the *avrXa7* (Fig. 1). If a strain was dependent on a single effector of the gene family, then some kanamycin-resistant strains would suffer an appreciable loss of virulence. The lack of low virulence recombinants could be due to multiple yet equally competent virulence effector genes, multiple effector genes that contribute incrementally to virulence, or simply the lack of major effector genes of the *avrBs3/pthA* type in a particular strain. The mutant strains were examined by Southern hybridization to assess the number of rearrangements among the gene family members and to determine the number of recombination events associated with the antibiotic resistance marker.

A variety of strains were selected on the basis of lineage and diverse geographic origin and included JXO1^A, PXO61, PXO71, PXO99^A, and KXO85^A (Table 1). The target strains were analyzed for *avrBs3/avrPthA* gene content by Southern hybridization prior to genetic analyses. Genomic DNA was digested with *Sph*I, which cleaves on either side of the repeat coding region of most *avrBs3/pthA* genes, including *avrXa7*, and was probed with the central *Sph*I fragment of *avrXa7*. Hybridization signal revealed multiple fragments in each genome (Fig. 2). The observed bands are presumed to represent the internal *Sph*I fragments of gene family members in each genome, which differ in size according to the number of repeat units, although some bands may represent truncated genes within each strain. Assuming each band represented an intact gene, each strain of *X. oryzae* pv. *oryzae* was estimated to harbor approximately 14 to 16 copies. The copy number of genes in *X. oryzae* pv. *oryzicola* BLS303 was estimated at 18 copies. Thus, all of the strains could potentially carry one or more closely related copies of a virulence effector gene of the *avrBs3/pthA* family.

Mutagenesis was performed on the selected strains (with the exception of BLS303) by introducing the central 4.2-kb *Bam*HI fragment of *avrXa7* containing a gene for resistance to kanamycin in the eighth repeat (Fig. 1; discussed below). Isolates of PXO99^A, JXO1^A, and KXO85^A (30, 6, and 10 respectively) were selected as resistant to kanamycin after transfor-

Table 1. Bacterial strains and plasmids used in this study

Designation	Genotypes or relevant characteristics	Source or reference
Plasmids		
pUC19	Resistance to carbenicillin (Cb ^r)	Yanisch-Perron et al. 1985
pHM1	Broad host range resistance to spectinomycin, polylinker, <i>cos</i> site	Hopkins et al. 1992
pZWavrXa7	<i>avrXa7</i> in pBluescript II KS +, unique <i>Hind</i> III site after <i>avrXa7</i> coding region	Yang et al. 2000
pBY1	4.1-kb <i>Bam</i> HI fragment of <i>avrXa7</i> in pUC19, EZ:TN [Kan-2] in 8th repeat of <i>avrXa7</i>	This study
pZWpthXo1	<i>Sph</i> I fragment of <i>pthXo1</i> in pZWavrXa7	This study
pZWpthXo2	<i>Sph</i> I fragment of <i>pthXo2</i> in pZWavrXa7	This study
pZWpthXo3	<i>Sph</i> I fragment of <i>pthXo3</i> in pZWavrXa7	This study
pZWavrBs3-7HS	<i>Hinc</i> II/ <i>Sal</i> I fragment of <i>avrXa7</i> in pZWavrBs3	This study
<i>Escherichia coli</i>		
DH5α	F ⁺ <i>recA</i> , ϕ 80 <i>dlacZ</i> , Δ <i>M15</i>	Stratagene, La Jolla, CA, U.S.A.
XL1-Blue MRF ⁺	F ⁺ <i>proAB lacI</i> ^q Δ <i>M15</i> Tn10 (Tet ^r)	Stratagene
C2110	Nal ^r Rif ^r (<i>polA1</i> , <i>rha</i> , <i>his</i>)	Leong et al. 1982
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>		
PXO99 ^A	Philippine race 6; azacytidine resistant clone of PXO99	Hopkins et al. 1992
JXO1 ^A	Japanese race 1	J. Leach, Kansas State University
KXO85 ^A	Korean strain CK89021 (KAXCC10331)	J. Leach
PXO61	Philippine race 1	J. Leach
PXO71	Philippine race 4	J. Leach
PXO86	Philippine race 2, original source of <i>avrXa7</i>	Hopkins et al. 1992
PXO99 ^A ME2	PXO99 with pBY1 inserted into <i>pthXo1</i>	This study
JXO1 ^A ME5	JXO1A with pBY1 inserted into <i>pthXo2</i>	This study
KXO85 ^A ME3	PXO99 with pBY1 inserted into <i>avrXa7</i> -2	This study
<i>X. oryzae</i> pv. <i>oryzicola</i>		
BLS303	Philippines, bacterial leaf streak pathogen of rice	J. Leach

mation. Although mutagenesis of PXO61 and PXO71 was attempted, no kanamycin-resistant colonies were obtained, suggesting that restriction/modification systems may be preventing efficient DNA transfer in these two strains. No further

attempts were made to generate mutants in PXO61 and PXO71. Five recombinant isolates of PXO99^A displayed reduced virulence, while one and two reduced-virulence mutants were identified from JXO1^A and KXO85^A, respectively.

The genomic DNA from recombinants was analyzed by Southern hybridization using the *Sph*I fragment of *avrXa7* and, separately, antibiotic resistance gene of the transposon element as probes. The results for PXO99^A are shown as an example of the analyses. PXO99^A recombinants had a variety of polymorphisms associated with the *avrBs3/pthA* gene family (Fig. 3A). Low virulence in PXO99^A recombinants was associated with the change of a doublet band to a singlet band of approximately 2.9 kb (Fig. 3A). Additional and multiple polymorphisms were detected in some strains. For example, PXO99^AME2 and PXO99^AME42 both suffered the loss of a 1.9-kb fragment. However, PXO99^AME42 was fully virulent and possessed the doublet band at 2.9 kb (Fig. 3A). Upon probing with the fragment from the recombination marker, most strains were found to have a single band indicative of a single transposon (data not shown). The marker was associated with a 3.6-kb polymorphic fragment in PXO99^AME2 (Fig. 3A, marked by an asterisk).

Recombinants from JXO1^A and KXO85^A were similarly analyzed, and in each case, a common fragment was identified in reduced virulence strains (data not shown). Representative recombinants (PXO99^AME2, JXO1^AME5, and KXO85^AME3) with a single transposon, as evidenced by hybridization analysis

Fig. 2. Southern analysis of *avrBs3/pthA* genes in strains and pathovars of *Xanthomonas oryzae* pv. *oryzae*. DNA from strains was digested with *Sph*I and was probed with the 3.1-kb *Sph*I fragment of *avrXa7*. M represents molecular size markers. **A**, Lane 1, PXO61; lane 2, PXO71; lane 3, PXO86; lane 4, PXO99^A; lane 5, JXO1^A; lane 6, KXO85^A. **B**, Lane 1, *X. oryzae* pv. *oryzicola* BLS303. Numbers at each side indicate size of standards in kb.

Fig. 3. **A**, Southern hybridization analysis of genomic DNA from kanamycin-resistant isolates of PXO99^A. DNA was digested with *Sph*I and was probed with the *Sph*I fragment of *avrXa7*. The positions of the size markers are indicated at left. Strain names are indicated at top. Strain names followed with “T” designate strains with low virulence (ME2 and ME12). The solid arrows indicate band in ME2 of lower intensity relative to wild type and common to low-virulence strains and the band immediately below the 2.9-kb band, which does not change in intensity relative to wild type. The open arrow at right indicates an example of polymorphism not associated with low-virulence phenotype. Asterisks indicate bands associated with antibiotic resistance gene, which was used as a recombination marker. **B**, Southern hybridization analysis of genomic DNA from low-virulence strains PXO99^AME2, JXO1^AME5, and KXO85^AME3. Genomic DNA from each strain was digested with *Sph*I and was probed with a polymerase chain reaction fragment of the recombination marker. Strain names are indicated at the top. Size marker bands are indicated at left and were detected by spiking the probe with labeled 1-kb ladder DNA. Arrows at right indicate individual bands from each mutant. The density differences between PXO99^AME2, JXO1^AME5, and KXO85^AME3 are due to uneven DNA loading of genomic DNA.

(Fig. 3B) and reduced virulence, were selected for study. Inoculation of parental strains PXO99^A, JXO1^A, and KXO85^A into 14-day-old rice leaves resulted in typical water-soaked symptoms within the inoculated area within 3 days (Fig. 4, leaves 1, 3, and 5, respectively). The water-soaked tissue, which has a glassy or wet appearance, appeared as dark area within the background of the lighter uninfected leaf tissue. PXO99^AME2, JXO1^AME5, and KXO85^AME3 each caused symptoms with reduced water-soaked symptoms at the inoculation site (Fig. 4, leaves 2, 4, and 6, respectively). KXO85^A is the only strain of the study with *Xa7*-dependent avirulence activity. KXO85^A was isolated in South Korea and represented a different lineage than PXO86, the original source of *avrXa7*. KXO85^AME3 concomitantly lost the ability to elicit a *Xa7*-dependent hypersensitive response, indicating that a dual active homolog to *avrXa7* was present in the strain (data not shown).

Although bacterial numbers were not measured for all mutants, bacterial numbers were previously shown to be strictly correlated to lesion length (Bai et al. 2000; Yang et al. 2000). Bacterial populations were measured for the PXO99^A and PXO99^AME2. The populations for PXO99^A and PXO99^AME2 at day 10, the final day of lesion-length measurements, were $6.1 \times 10^8 \pm 0.7$ and $9.5 \times 10^6 \pm 0.3$ CFU per leaf.

Fig. 4. Phenotype of virulence mutants of *Xanthomonas oryzae* pv. *oryzae* on rice leaves. Each strain was inoculated by needleless syringe on 14-day-old seedlings of cultivar IR24. Leaves were photographed four days after inoculation. Leaf 1, PXO99^A; leaf 2, PXO99^AME2; leaf 3, JXO1^A; leaf 4, JXO1^AME5; leaf 5, KXO85^A; and leaf 6, KXO85^AME3.

Reduced virulence mutants were complemented by single virulence effector genes from the *avrBs3/pthA* family.

The hybridization data indicated PXO99^AME2, JXO1^AME5, and KXO85^AME3 had each suffered the loss of a single gene of the *avrBs3/pthA* gene family with virulence activity. Complementation tests were used to identify the respective effector gene from PXO99^A, JXO1^A, and KXO85^A from cosmid libraries of the *avrXa7*-related genes from each strain in the broad-host-range vector pHM1. Each library was transferred initially to PXO99^AME2, which is the best recipient for transfer of clones with large inserts, and the cosmids with candidate effector genes were identified by syringe-inoculation of the transformants (Table 2). Syringe-inoculation, while not quantitative, was used for rapid screening of candidate genes during cosmid screening and subcloning. Single cosmids often contained two or three *avrBs3/pthA* genes. After identification of cosmid clones harboring complementing genes, *Bam*HI fragments containing single candidate genes were identified by hybridization analyses and were subcloned. Each candidate gene was partially sequenced, using primers from sequences immediately upstream and downstream from the repetitive region (discussed below). The partial sequencing analysis revealed five to six repeats from each end of the repetitive region and allowed distinguishing of potentially different genes of the same size. One candidate effector gene was identified from PXO99^A, JXO1^A, and KXO85^A. The genes from PXO99^A and JXO1^A were named *pthXo1* and *pthXo2*, respectively (Table 2). The gene from KXO85^A also conferred *Xa7*-dependent avirulence activity and was named *avrXa7-2*.

Table 2. Cosmid clones of *avrBs3/pthA* gene family members

Strain library	Cosmids		Identified virulence gene
	With <i>avrBs3/pthA</i> gene ^a	With virulence activity	
PXO99 ^A	28	3	<i>pthXo1</i>
JXO1 ^A	32	4	<i>pthXo2</i>
KXO85 ^A	25	3	<i>avrXa7-2</i>
PXO61	26	3	<i>pthXo3</i>
PXO71	27	4	<i>pthXo1-2</i> , <i>pthXo2-2</i>
BLS303	35	0	none

^a Cosmids were selected by hybridization to *avrXa7* probe and often contained multiple genes of the same gene family.

Fig. 5. Complementation analyses of low virulence mutants of *Xanthomonas oryzae* pv. *oryzae*. The virulence effector gene from each parental strain was introduced into the respective mutant, and A, through C, the resulting transformants were tested for virulence using the leaf-clip inoculation method on rice cultivar IR24. D, IRBB7 (*Xa7*) leaves were leaf-clip inoculated by PXO86 (containing *avrXa7*), PXO99^A, and PXO99^AME2 for comparison of incompatible and compatible reactions. Strains were as follows. Column 1, PXO99^A; 2, PXO99^AME2; 3, PXO99^AME2 (pZWpthXo1); 4, JXO1^A; 5, JXO1^AME5; 6, JXO1^AME5 (pZWpthXo2); 7, KXO85^A; 8, KXO85^AME3; and 9, KXO85^AME3 (pZWavrXa7-2); 10, PXO86; 11, PXO99^AME2; and 12, PXO99^A. Lesion lengths were measured 10 days after inoculation of 20-day-old seedlings.

The candidate genes were next tested for complementation of the respective low-virulence mutant of the parental strain. Upon transfer of the virulence effector gene to the respective mutant, each strain recovered in virulence, as indicated by lesion-length assays (Fig. 5). The plasmid copy of each respective virulence effector gene did not fully restore virulence to that of the parental strain, as measured by lesion length. PXO99^A is the most virulent strain, based on lesion length assays, and reintroduction of *pthXo1* restored virulence to 86% of the parental strain. Similarly, *pthXo2* and *avrXa7-2* restored virulence to 78 and 83% of their respective parental strains. The lack of full complementation was also observed in previous studies with *avrXa7*, which restored virulence to PXO86mx53 to 86% of the parental strain PXO86 (Bai et al. 2000; Yang et al. 2000), and the reasons are unknown.

The reduction in lesion length by PXO99^AME2 due to the loss of *pthXo1* was also compared with reduction in lesion length due to the resistance reaction in the *avrXa7/Xa7* gene combination in strain PXO86 on the rice line IRBB7 (Fig. 5D, column 10). The lesion lengths due to inoculation with PXO99^AME2 were shorter than lesion length due to the elicitation of resistance (Fig. 5D, column 11).

The virulence effectors of the AvrBs3/PthA family are interchangeable.

The ability of the different genes to contribute to the virulence of a single strain was tested by cross complementation

of the loss of *pthXo1* in PXO99^AME2. Prior to cross-complementation analyses, an attempt was made to identify additional related effector genes from libraries of PXO61 and PXO71, in which no reduced-virulence mutants were obtained, and one strain of *X. oryzae* pv. *oryzicola*, BLS303. A single candidate gene was identified in PXO61 and two candidate genes were recovered from PXO71 by cross complementation of virulence in PXO99^AME2 (Table 2). No complementing clones were identified in a library of *avrBs3/pthA* homologs from BLS303. Two distinct clones from PXO71 were identified that complemented PXO99^AME2. However, initial sequencing of the *SphI* fragments, size similarity, and subsequent sequencing analysis led to the conclusion that the genes from PXO71 were similar to the genes from PXO99^A and JXO1^A, respectively, and were named *pthXo1-2* and *pthXo2-2*. A single unique gene was identified in PXO61 and was named *pthXo3*.

The three distinct virulence effectors (*avrXa7*, *pthXo2*, and *pthXo3*) were transferred individually to PXO99^AME2, and their effects were compared with complementation with *pthXo1*. Each transformant was tested on IR24 leaves for virulence by using syringe- and clip-inoculation methods. All transformants caused strong water-soaking symptoms like wild-type strain PXO99^A (data not shown). Lesion length measurements indicated that each gene complemented the loss of *pthXo1* from PXO99^AME2 to an equal degree (Fig. 6).

Virulence effectors of the AvrBs3/PthA family in *X. oryzae* pv. *oryzae* have diverse repetitive regions.

The central *SphI* fragment of each complementing gene was sequenced, revealing that *pthXo1* from PXO99^A, *pthXo2* from JXO1^A, and *pthXo3* from PXO61 were novel genes in terms of the number and arrangement of the predicted repeats in their respective repetitive regions, when compared with *avrXa7* and other family members (Fig. 7). The two genes from PXO71 were nearly identical to *pthXo1* and *pthXo2*, respectively, at the nucleotide level, and the gene from KXO85^A was nearly identical to *avrXa7* (data not shown). *pthXo1* had 23.5 repeat units compared with the 25.5 repeats of *avrXa7*. *pthXo2* had 21.5 repeats, and *pthXo3* had 28.5 repeats. Although each gene was distinct, *avrXa7* and *pthXo3* have the most similar features. Each contained an 18-bp duplication in a similar repeat (in repeats 13 and 15, respectively) and share the same first three repeats, the same last three repeats, and an internal five repeat pattern in their repetitive domains (Fig. 7, underlined repeat units). The sequences of the *SphI* fragments of *pthXo1*, *pthXo2*, *pthXo3* and the *BamHI* fragment of *avrXa7-2* were deposited in GenBank under accession numbers AY495676, AY495677, AY495678, and AY495675, respectively.

Fig. 6. Virulence complementation by different *pth* genes in PXO99^AME2. Lesion lengths were measured 10 days after inoculation of IR24 with PXO99^A (column 1) and PXO99^AME2 containing pHM1 (column 2), *pthXo1* (column 3), *pthXo2* (column 4), *pthXo3* (column 5), and *avrXa7* (column 6). Lesion length is indicated at left. Columns with the same letter indicate no significant differences at *P* < 0.05 by post-analysis of variance pairwise comparisons with the Tukey honestly significant difference test.

REPEAT	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
PROTEIN																													
AvrXa7	NI	HG	NI	NI	NS	HD	NN	HD	HD	HD	NS	N*	N*	HD	HD	NS	NS	NN	NN	NI	NG	NN	NI	N*	NS	N*			
PthXo1	NN	HD	NI	HG	HD	NG	N*	HD	HD	NI	NG	NG	NI	HD	NG	NN	NG	NI	NI	NI	NI	N*	ND	N*					
PthXo2	NI	HG	NI	NN	NN	NI	NN	HD	NI	HD	NS	NS	NS	HD	NN	HD	NG	HD	HD	HD	NG	NG							
PthXo3	NI	HG	NI	HG	NI	NI	NI	HD	NN	HD	HD	HD	NG	HD	N*	NI	HD	HD	NN	NS	NI	NN	NN	NG	NN	HD	N*	NS	N*
AvrXa10	NI	HG	NI	HG	NI	NI	NN	HD	NI	HD	NN	HG	NS	NG	HD	NG													
PthA	NI	NG	NI	NI	NG	HD	NG	HD	NG	NG	NG	NG	NS	HD	HD	NG	NG												
AvrBs3	HD	NG	NS	NG	NI	NI	NI	HD	HD	NG	NS	NS	HD	HD	HD	NG	HD	NG											
AvrBs4	NI	NG	NI	NI	NG	NG	NI	NS	NG	NI	NS	NG	HD	HD	NS	HD	NG	NG											
Avrb6	HD	NI	NG	HD	HD	NI	HD	NI	NS	HD	HD	HD	NN	NG															

Fig. 7. Alignment of repetitive regions of selected AvrBs3/PthA proteins as represented by 12th and 13th amino acids of each repeat. The single amino acid code was used for each amino residue. An asterisk (*) represents the missing 13th amino acid. R indicates a six-amino-acid-residue insertion in repeat (AvrXa7 and PthXo3). Similar arrangements of repeats shared between AvrXa7 and PthXo3 as identified by amino acid 12 and 13 are underlined. Bold-lettered residues indicate the eight-repeat pattern in all proteins with virulence activity on rice. PthXo1 has two overlapping eight-repeat arrangements.

AvrBs3 contains a repetitive region with *Xa7*-dependent avirulence activity without virulence activity.

The identification of three new distinct virulence effectors from *X. oryzae* pv. *oryzae* established that virulence activity could occur without *Xa7*-dependent avirulence activity and that virulence activity was provided by a variety of repeat configurations. Common among the virulence effectors was an eight-repeat pattern in which the first repeat was histidine (H) and aspartate (D) at codons 12 and 13 and the eighth repeat had asparagines (N) and glycine (G) (Fig. 7, the first and eighth repeat variable residues in bold). The six repeats in between the first and eighth were variable. The same pattern occurred in the repetitive regions of AvrBs3 and AvrB6 (Fig. 7). To test the hypothesis that a repetitive region with this pattern could function as a virulence effector in *X. oryzae* pv. *oryzae*, the 0.4 kb of the C-terminal coding region of *avrBs3* was replaced with the corresponding fragment of *avrXa7* (discussed below). The hybrid gene, named *avrBs3-7HS*, was introduced into strain PXO99^AME2, and the resulting strain was tested for virulence on rice. Syringe-inoculation (data not shown) and lesion-length measurements revealed that the strain with the hybrid gene did not complement the loss of *pthXo1* in PXO99^AME2 for virulence on the susceptible rice varieties IR24 (Fig. 8) and Nipponbare (data not shown). Interestingly, PXO99^AME2 with *avrBs3-7HS* elicited hypersensitive responses in rice carrying *Xa7* and in pepper plants with the *Bs3* resistance gene (Fig. 9). The results indicated that the eight-repeat pattern alone was not sufficient for virulence and, at the same time, revealed a gene with *Xa7*-dependent avirulence activity without corresponding virulence activity.

DISCUSSION

Involvement of *avrBs3/pthA* homologs in the pathogenicity of *X. oryzae* pv. *oryzae*.

Relatively few type III effector genes have been characterized from a single species or pathovar and within a single disease complex. Prior to this study, *avrXa7* from the Philippine strain PXO86 was the only *avrBs3/pthA* gene from *X. oryzae* pv. *oryzae* with a major contribution to virulence. Here, four

genes of the *avrBs3/pthA* gene family with virulence activity in rice, heretofore referred to as *pth* genes, were identified in five *X. oryzae* pv. *oryzae* strains. The genes, along with the original *avrXa7*, are considered here to be major virulence effector genes. Based on lesion-length measurements in a laboratory setting, the loss of these effectors in the different strains of *X. oryzae* pv. *oryzae* caused a severe loss of their ability to grow in the host. The loss was similar in degree to the reduction in growth caused by the elicitation of *Xa7*-dependent resistance, and the mutant strains, other environmental factors being equal, are predicted to be at a competitive disadvantage in the natural environment to strains with a major effector gene. Six strains of *X. oryzae* pv. *oryzae*, including PXO86, now have been shown to have one or two major *pth* genes, and only one major *pth* gene was identified in five of the strains. Mutations in four strains provided evidence that the strains were highly dependent on a single effector gene of the *avrBs3/pthA* gene family. Race 3 and race 9 strains from the Philippines also harbor *avrXa7*, and field studies suggest that *avrXa7* may be an important virulence factor in these strains (Vera Cruz et al. 2000). The results of this study, therefore, support the hypothesis that many, possibly all, strains of *X. oryzae* pv. *oryzae* require *pth* genes for full virulence.

Experiments to examine in greater detail the diversity of extant *pth* genes in *X. oryzae* pv. *oryzae* populations would require genetic analyses of the type performed here on more strains. However, evidence provided here indicated that at least some of the *pth* genes are widespread. An allele of *avrXa7* and *pthXo2* were identified as major *pth* genes in Korean and Japanese strains, respectively. *pthXo2* was also found in PXO71. PXO71, PXO86, PXO61, and PXO99^A are strains from the Philippines representing races 1, 2, 4, and 6 of the pathogen, respectively, and also represent genetically diverse lineages as determined by restriction fragment length polymorphism analyses (Adhikari et al. 1995; Leach et al. 1992). Although from the Philippines, lineage analyses indicated PXO99^A was an unusual strain and was related to strains from throughout India, Indonesia, Nepal, and Malaysia (Adhikari et al. 1995; cluster 5). The genetic relationship of PXO99^A to a large cluster of strains found throughout the rice-growing regions of Asia may

Fig. 8. Virulence activity analysis of a hybrid *avrBs3-7HS* in *Xanthomonas oryzae* pv. *oryzae*. The *HincII/SalI* fragment of *avrBs3* containing the C-terminal coding region was exchanged with the corresponding fragment from *avrXa7*. The hybrid gene was introduced in PXO99^AME2, and the transformant was tested by leaf-clip inoculation on IR24 by needleless syringe on 14-day-old seedlings. Lane 1, PXO99^A; lane 2, PXO99^AME2 (pHM1); and lane 3, PXO99^AME2 (pZWavrBs3-7HS).

Fig. 9. Avirulence leaf assays of hybrid *avrBs3-7HS* in *Xanthomonas oryzae* pv. *oryzae*. **A**, Rice leaves inoculated with PXO99^AME2 with *avrBs3* on IRBB7 (leaf 1), PXO99^AME2 with *avrBs3-7HS* on IRBB7 and IR24 (leaves 2 and 3), respectively, and PXO99^AME2 with *avrXa7* on IRBB7 (leaf 4). **B**, PXO99^AME2 with the genes as indicated on ECW-30R(*Bs3*). PXO99^AME2 does not elicit a hypersensitive reaction on ECW without *Bs3* (data not shown).

portend that *pthXo1* is also widely distributed (Adhikari et al. 1995). The other strains have been clustered into two large groups of strains found primarily in the Philippines (Adhikari et al. 1995; clusters 2 and 4). PXO61 contained *pthXo3*. A mutation was not obtained in *pthXo3*, and it is unknown whether *pthXo3* is the only major *pth* gene in PXO61. Strain migration and horizontal gene transfer among lineages may be responsible for the presence of a diverse yet limited number of effectors in *X. oryzae* pv. *oryzae*. At the same time, laboratory experiments with *avrXa7* provided evidence that additional novel versions can readily arise by rearrangement of the repetitive region within strains and populations (unpublished data). More *pth* genes seem likely to be identified in field strains of the rice pathogen in the future.

Despite the requirement for one or two major *pth* genes, all strains of *X. oryzae* pv. *oryzae* harbor multiple members of the *avrBs3/pthA* gene family. The presence of multiple copies may, as proposed by Yang and Gabriel (1995), facilitate the rapid generation of new *pth* genes in the event of host recognition or avoidance of the extant *pth* genes. The high gene-copy number may reflect a high degree of adaptive pressure on the pathogen populations due to the extensive cultivation and selection, either intentional or unintentional, of rice in environments highly conducive to bacterial disease. Interestingly, a relatively high number of copies of the *avrBs3/pthA* gene family also occurs in strains of *X. campestris* pv. *malvacearum*, the causal agent of bacterial blight of cotton (Yang et al. 1996). Cotton has also been an intensively managed crop for centuries, often under conditions favorable for bacterial disease. A similar situation may exist for strains of *X. oryzae* pv. *oryzicola*, which, if strain BLS303 is indicative of other strains, also harbor many copies of the gene family. At the same time, the other gene family members within a strain of *X. oryzae* pv. *oryzae* may have partial virulence activity, which here we refer to as minor virulence activity, although the term is not intended to imply the genes have no ecological consequences. Several minor genes of the *avrBs3/pthA* family in strain PXO86 were found to have a small effect on virulence, which could not be complemented by *avrXa7* (Bai et al. 2000). Strain XcmH1005 of *X. campestris* pv. *malvacearum* has 10 *avrBs3/pthA* genes, and full water-soaking symptoms on cotton required five of the 10 genes (De Feyter et al. 1991). Thus, multiple copies of the *avrBs3/pthA* gene family in individual strains may serve both as a reservoir of potential recombinatorial alleles and as additional virulence effectors.

Virulence effectors of the AvrBs3/PthA family in *X. oryzae* pv. *oryzae* have diverse repetitive regions.

The identification of new *pth* genes in the *X. oryzae* pv. *oryzae*-rice disease complex may also yield new insight into the structural requirements of the repetitive region for host specialization and *R* gene-mediated resistance. In this regard, we classified the repeats based on the variable amino acid residues at the 12th and 13th positions of each repeat, as a convenient method to distinguish repeat units and the different members of the *avrBs3/pthA* gene family. There is, in fact, little evidence that the variable region specifically is critical to protein function in either avirulence or virulence. However, previous studies with *avrBs3* and other gene family members have established that repeat rearrangements led to loss or changes in specificity of *R* gene-mediated resistance and host-specific virulence activities, indicating that some feature of individual repeats are involved (Herbers et al. 1992; Yang and Gabriel 1995; Zhu et al. 1998). Three distinct *pth* genes in terms of the repeat organization were identified here, bringing to four the number of naturally occurring *pth* genes with virulence specificity for rice. Additional members of the gene family in PXO86 may provide ad-

ditional virulence activities, although they are modest in comparison to the major genes (<10% the activity of *avrXa7*). Only *avrXa7* of the major virulence effector genes of *X. oryzae* pv. *oryzae* has been associated with an *R* gene-specific resistance in rice. One common pattern among the genes with virulence activity was a HD repeat followed by a NG repeat seven repeats later. Replacement with the corresponding region from *avrBs3*, which has the same pattern, failed to support the idea that the pattern alone was critical for virulence activity. Serendipitously, the new hybrid gene was discovered to elicit a *Xa7*-dependent reaction in rice and a *Bs3*-dependent reaction in pepper. This gene is the first *avrBs3/pthA* gene with no virulence activity while retaining *Xa7*-dependent recognition, and the finding showed, at least theoretically, that *Xa7* recognition is possible in the absence of virulence activity.

The potential for the general involvement of *avrBs3/pthA* effector genes in other bacterial disease complexes.

The dependence of the bacterial blight disease complex of rice on members of the *avrBs3/pthA* gene family may be representative of other disease complexes. Evidences from studies on citrus canker disease and the causal agent *X. axonopodis* pv. *citri* also indicate that the disease is highly dependent on *avrBs3/pthA* virulence effectors. Mutations in *pthA* severely reduced the disease symptoms and the ability for the bacteria to multiply in the host (Swarup et al. 1991). Genetic analysis has only been reported for a single strain, and *pthA* is the only well-characterized *pth* gene from *X. axonopodis* pv. *citri*. Yet *pthA*-related genes have been detected in other strains of *X. axonopodis* pv. *citri* (Cubero et al. 2002; da Silva et al. 2002). Recent preliminary reports indicate that related pathogens cause similar diseases of citrus, and additional *pth* genes (specifically, *pthB*, *pthC*, and *pthW*) have been identified (Brunings et al. 2003). Bacterial blight of cotton, also known as angular leaf spot, may also be highly dependent on the *avrBs3/pthA* effectors (Yang et al. 1996). Any involvement of the *avrBs3/pthA* genes in *X. oryzae* pv. *oryzicola* remains unknown, and a *pth* gene was not recovered from BLS303 that could compensate the loss of *pthXo1* in strain PXO99^ΔME2. *AvrBs3*, the first gene of the family to be identified, is the only *pth* gene with virulence activity from *X. campestris* pv. *vesicatoria* (Bonas et al. 1989). Therefore, mutational analyses of the *avrBs3/pthA* genes from other *Xanthomonas* strains similar to the approaches here are warranted.

Implications for plant adaptation and crop improvement.

One motivation for deciphering the mechanisms of effector function is for application to the design of better protective measures. Virulence effectors of bacterial plant pathogens are attractive targets for intervention, due to the possible fitness cost upon loss or alteration of the effector in avoidance of host recognition. Durable resistance may result if *R* genes are selected based on recognition of conserved motifs or functions required for virulence (Lauge et al. 1998; Tai et al. 1999). Alternatively, approaches might be possible that modify the virulence-targeted pathways of the host, rendering pathogen effectors ineffective. In either case, interference in effector function may prevent or delay adaptation of virulent pathogen populations, since simple loss of the elicitor from the pathogen population may result in a concomitant reduction of virulence (Leach et al. 2001). The effectiveness of interfering with virulence effectors depends on the prevalence, plasticity, and virulence contribution of individual effectors in the population. The results of this study provided both positive and negative aspects in regard to the deployment of *Xa7*. The variety of *pth* genes described in this study indicates that deployment of re-

sistance specific to any individual effector, such as *avrXa7*, is likely to be ineffective due to the availability of other virulence effector genes of the same type but without *Xa7* recognition. At the same time, strains of *X. oryzae* pv. *oryzae* in general appear to be highly dependent on a single type of effector, and therefore, strategies targeting the *pth* genes have potential for broad resistance of rice to bacterial blight. Alternatively, alterations of the host pathways or molecular complexes that are affected by *pth* gene products may provide immunity to many or all strains of the pathogen. Improved strategies should come with increased understanding of effector function and host recognition mechanisms.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA manipulations.

Strains of *Escherichia coli* and *X. oryzae* pv. *oryzae* and plasmids used in this study are listed in Table 1. Standard culture and DNA techniques were used for *E. coli* and recombinant DNA manipulations (Ausubel et al. 1988). *X. oryzae* pv. *oryzae* was grown in either nutrient broth (Difco Laboratories, Detroit) or tryptone sucrose agar (TSA) medium (tryptone, 10 g/liter; sucrose, 10 g/liter; glutamic acid, 1 g/liter; Difco Bacto agar, 15 g/liter) at 28°C. Isolates of *X. oryzae* pv. *oryzae* strains PXO99^A, JXO1^A, and KXO85^A were selected as presumed restriction modification mutants by growing on the TSA plates containing 200 mM of 5-azacytidine (Hopkins et al. 1992). Plasmids were introduced into *E. coli* and *X. oryzae* pv. *oryzae* by electroporation. Antibiotics used in this study were carbenicillin, 100 µg/ml; cephalixin, 10 µg/ml; kanamycin, 50 µg/ml; and spectinomycin, 100 µg/ml.

Plant materials and virulence assays.

Rice line IR24 and near-isogenic rice line IRBB7 containing the resistance gene *Xa7* were used for virulence and avirulence tests, respectively (Ogawa et al. 1987). Virulence assays were performed on 14- and 20-day-old rice plants by needleless syringe infiltration (Reimers et al. 1991) and leaf-clip inoculation, respectively, in growth chambers set at 28°C with 85% humidity for a 12-h day and 12-h night cycle (Kauffman et al. 1973). Symptoms were scored by measuring lesion lengths for clip inoculation and by observation of water-soaking after syringe infiltration, at the intervals indicated in the text. One-way analysis of variance (ANOVA) statistical analyses were performed on all measurements. The Tukey honest significant difference test was used for post-ANOVA pair-wise tests for significance, set at 5% ($P < 0.5$). Pepper varieties ECW-30R (Bs3), a near isogenic line for the resistance gene *Bs3* and ECW were used for avirulence assays of *avrBs3* and *avrBs3-7HS*.

Southern hybridization analysis.

Standard procedures for Southern blots were utilized (Ausubel et al. 1988). Genomic DNA from *X. oryzae* pv. *oryzae* strains was digested with *SphI* and was fractionated on a 1.25% agarose gel. Filters were probed with a ³²P-labeled 3.1-kb *SphI* fragment of *avrXa7*. Size markers were visualized by adding labeled size markers.

Transposon and marker-exchange mutagenesis.

The central *BamHI* fragment of *avrXa7* was cloned into pUC19. EZ::TN<KAN-2> was inserted in the repeat region of *avrXa7* in pUC19, using in vitro transposition (Goryshin et al. 1998) according to the supplier (Epicentre Technologies, Madison, WI, U.S.A.), to generate pBY1. pBY1 was electroporated into *X. oryzae* pv. *oryzae*, and the transformed cells were plated on TSA medium containing kanamycin. Clones with resistance to kanamycin were tested on IR24 rice plants for virulence.

Genomic DNA library construction and screening.

Genomic DNA of *X. oryzae* pv. *oryzae* strains was partially digested with *Sau3AI*, and DNA fragments larger than 20 kb were purified from 0.8% agarose gels by electro-elution. The DNA was then ligated into *BamHI*-digested cosmid vector pHM1 and was packaged into lambda phages, and the phages were transduced into XL1-blue MRF' cells, according to the supplier (MaxPlax; Epicentre Technologies). Colonies were selected on Luria Bertani agar for resistance to spectinomycin. Genomic libraries had titers of about 10⁶ and average inserts of about 30 kb (data not shown). The libraries were screened using a ³²P-labeled 3.1-kb *SphI* fragment of *avrXa7* as a probe. Candidate clones were introduced into each respective mutant of PXO99^A, JXO1^A, and KXO85^A by electroporation.

Complementation tests and gene characterization.

Respective virulence mutants of PXO99^A, JXO1^A, and KXO85^A were used as the recipients of positive clones obtained from genomic libraries of PXO99^A, JXO1^A, and KXO85^A, respectively. Transformants were inoculated on IR24 by leaf-clip inoculation and needleless syringe infiltration. Clones that restored mutant strains to virulence were further analyzed. DNA from the complementing cosmids was digested with *BamHI* and was analyzed by Southern hybridization. Positive *BamHI* fragments were extracted from gels and were subcloned into pBluescriptKS+ (Stratagene, La Jolla, CA, U.S.A.). The fragments were partially sequenced, using primers *Avr1stSph-F* (GCAGTGCATGCATGGCGCAATGCACTG) and *AvrHincII-R* (CGCCGGATCAGGGCGAGATAACTGGG). Unique genes based on the partial sequencing results were carried forward, and the *SphI* fragments with the repetitive regions were sequenced in their entirety. The fragment ends were sequenced from both ends, using the T3 and T7 primers, and internal sequence was performed by in vitro transposition tagging and sequencing, using outwardly directed primers derived from the right and left ends of the EZ::TN<KAN-2>. The *SphI* fragments of candidate *SphI* fragments were exchanged with the *BamHI* fragment of *avrXa7* in pZWavrXa7 (Table 1) (Yang et al. 2000), were linearized with *HindIII*, and were ligated into the broad-host-range vector pHM1. The ligated DNA was electroporated into *E. coli* C2110, and the transformed cells were plated on Luria agar plates containing spectinomycin and carbenicillin. Plasmid configuration was verified by restriction enzyme analysis, and plasmids were electroporated into the respective virulence mutants. The DNA sequencing was performed at the sequencing facilities of Kansas State University.

Construction of *avrBs3-7HS*.

The plasmid pZWavrXa7 was digested with *HincII* and *Sall*, the latter was previously introduced immediately downstream of the second *SphI* site in the C-terminal of *avrXa7* and *avrBs3* upon addition of a FLAG epitope (Zhu et al. 1998), and the gene fragment was gel-purified. The fragment was exchanged with the *HincII/Sall* fragment of *avrBs3* to create *avrBs3-7HS* from pZWavrBs3, which contains all N-terminal, the repetitive region, and 44 additional C-terminal proximal coding sequences of *avrBs3* and the approximately 400-bp *HincII/Sall* fragment of the C-terminal coding region of *avrXa7*. The resulting plasmid was cloned in pHM1 and was introduced into PXO99^AME2 by electroporation.

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